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Breaking muscle: neurotoxic and myotoxic effects of Central American snake venoms and the relative efficacies of antivenom and varespladib

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Abstract

Background The snake genera *Atropoides*, *Cerrophidion*, and *Metlapilcoatlus* form a clade of neotropical pit vipers distributed across Mexico and Central America. This study evaluated the myotoxic and neurotoxic effects of nine species of *Atropoides*, *Cerrophidion*, and *Metlapilcoatlus*, and the neutralising efficacy of the ICP antivenom from Costa Rica against these effects, in the chick biventer cervicis nerve-muscle preparation. Given the prominence of PLA_2s within the venom proteomes of these species, we also aimed to determine the neutralising potency of the PLA_2 inhibitor, varespladib.

Results All venoms showed myotoxic and potential neurotoxic effects, with differential intra-genera and inter-genera potency. This variation was also seen in the antivenom ability to neutralise the muscle damaging pathophysiological effects observed. Variation was also seen in the relative response to the PLA_2 inhibitor varespladib. While the myotoxic effects of *M. mexicanus* and *M. nummifer* venoms were effectively neutralised by varespladib, indicating myotoxicity is PLA_2 mediated, those of *C. godmani* and *M. olmec* venoms were not, revealing that the myotoxicity is driven by non-PLA_2 toxin types.

Conclusions This study characterises the myotoxic and neurotoxic venom activity, as well as neutralisation of venom effects from the *Atropoides*, *Cerrophidion*, and *Metlapilcoatlus* clade of American crotalids. Our findings contribute significant clinical and evolutionary knowledge to a clade of poorly researched snakes. In addition, these results provide a platform for future research into the reciprocal interaction between ecological niche specialisation and venom evolution, as well as highlighting the need to test purified toxins to accurately evaluate the potential effects observed in these venoms.

Keywords Venom, Antivenom, Varespladib, Enzyme inhibitor, Evolution

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Background

The *Atropoides*/*Cerrophidion*/*Metlapilcoatlus* genera form a clade of morphologically and ecologically diverse neotropical pit vipers widely distributed across Central America and Mexico [1]. The jumping pit vipers were previously all placed within the *Atropoides* genera until genetically driven revisions split the snakes into *Atropoides* and the newly created *Metlapilcoatlus* genus [2]. *Metlapilcoatlus* currently contains six species, all of which are stout bodied snakes with similar appearance to *Atropoides*. Both genera are found in tropical and subtropical habitats at low elevation. In contrast, *Cerrophidion*, known as the montane pit vipers, contains five moderately stout snakes that inhabit high elevation cloud forest between 1300 and 3500 m in elevation [1].

Given these species wide distribution, relative abundance, and proximity to anthropogenic activity, these snakes may contribute a considerable number of bites that remain unrecognised and/or underreported. While species-specific reports of envenoming by *Atropoides*/*Cerrophidion*/*Metlapilcoatlus* are limited, the known clinical features are similar to the highly medically significant bites by snakes from the *Bothrops* genus [3, 4]. Outcomes of envenoming by *Bothrops* species range from mild to severe oedema, muscle damage, pain, swelling, systemic bleeding, and haemorrhage [5, 6].

Proteomic investigations within Crotalinae show the most abundant toxins within venoms are phospholipase A_2 (PLA_2), snake venom serine proteases (SVSP), and Zn^{2+}-dependent metalloproteinases (SVMP) [7]. While these studies remain limited for the *Metlapilcoatlus* species, there have been few characterisations of the venom proteome of *A. picadoi*, *M. mexicanus*, and *M. nummifer*, as well as the majority of *Cerrophidion* spp. that have been reported [8–11]. Despite the close phylogenetic relationship of these taxa, the dominant toxin family differs significantly between venoms. For example, the major toxin family present within *M. mexicanus* and *M. nummifer* venoms are PLA_2, ranging from 36.5 to 31% respectively [8, 12]. Predominance of these toxins is consistent with the PLA_2 associated myonecrosis reported in previous murine studies [8, 11–13]. In contrast, studies have shown that *A. picadoi* and *Cerrophidion* spp. share a similar venom profile, which is largely dominated by SVMPs. However, despite these similarities in overall proteomic composition, they show distinct variation in their central venom effects. *Cerrophidion* venom predominantly cause a myotoxic effect driven by PLA_2s, despite the majority of the venom proteome comprised of SVMP toxins [10, 14]. While the venom of *A. picadoi* induces potent haemorrhagic effects, moderate myotoxicity is also observed to a lesser extent [15, 16]. Significant variation was also observed in coagulotoxicity caused by these venoms, with anticoagulant activity due to enzyme inhibition being documented for *C. godmani* and *C. wilsoni*, while thrombin-like mode of anticoagulation was observed for *A. picadoi* and *C. tzotzilorum* venoms [17].

Rosales-Garcia et al. [9] identified a PLA_2 (transcripts) homologous to crotoxin, the toxin responsible for causing flaccid paralysis following *Crotalus* envenoming, in *C. godmani* venom. However, this toxin (transcript) was only present in a single specimen from the southern population and required further testing to determine any functional neurotoxic properties as relative concentration in the venom proteome was not ascertained. In addition, *Cerrophidion* and *Metlapilcoatlus* species were shown to possess venom that targets artificial post-synaptic acetylcholine receptors in a binding assay [18]. A large degree of variation in venom composition may be fundamental to the corresponding differences in clinical signs and symptoms following envenoming. As a result of this, it is difficult to predict the clinical course following envenoming from snake species that have been less studied. Therefore, understanding the myotoxic and, potentially, neurotoxic effects will enrich our knowledge of these venoms and supplement the sparse clinical descriptions of envenoming by these species.

The polyvalent antivenom, PoliVal-ICP, manufactured by Instituto Clodomiro Picado, is used to treat envenoming by Viperidae in Central America [19–21]. The antivenom consists of equine antibodies through a process of hyperimmunisation against equal parts of venoms from *Bothrops asper*, *Crotalus simus*, and *Lachesis stenophrys*. Polyvalent antivenoms often display cross reactivity and are used against envenoming from species not used within the immunising mixture. The efficacy of the ICP antivenom has previously been tested against the lethal, haemorrhagic, proteolytic, and coagulotoxic effects of venoms from *Atropoides*, *Cerrophidion*, and *Metlapilcoatlus*, and provided effective neutralisation of inter-species venom effects [17, 22, 23]. However, venom phenotypes not captured within the immunising mixture can lead to poor neutralisation of venom effects. In vitro coagulation assays have shown poor neutralisation of the factor VII activating procoagulant effects of *Porthidium volcanicum* venom, a closely related pit viper species [17]. As it is often difficult to identify the offending snake beyond being a species of viperid, there is significant importance to understand not only species-specific clinical manifestations, but also effectiveness of available treatments [24]. Since there is no specific antivenom available against the venoms of *Atropoides*, *Cerrophidion*, or *Metlapilcoatlus*, there remains a reliance on effective cross neutralisation by non-species-specific antivenom.

Small molecule inhibitors have been gaining attention as potential alternatives or in use alongside traditional

antivenom strategies to neutralise venom effects. The PLA_2 inhibitor varespladib (LY31590) has shown preclinical efficacy against a variety of venoms from Elapidae and Viperidae species [25, 26]. Lewin et al. [27] demonstrated this broad efficacy against venoms with potent venom $sPLA_2$ inhibition from 28 snake species belonging to different families. In animal studies, varespladib also shows inhibitory action against the in vitro pre-synaptic neurotoxicity and myotoxicity in studies using skeletal neuromuscular preparations [28, 29]. Given the presence and activity of PLA_2 toxins described in the venoms of *Atropoides*, *Cerrophidion*, and *Metlapilcoatlus*, varespladib may be used as a pan-inhibitor to neutralise major symptoms following envenoming by these species.

This study aimed to expand on the current information available on these pit vipers and characterise the myotoxic properties of venoms from nine species within this clade, as well as elucidating any potential pre- or post-synaptic neurotoxic activity indicated by prior research. Since information on human envenoming by these species is scarce, we also tested the regionally relevant ICP antivenom to determine cross reactive efficacy between venom phenotypes. Since PLA_2s are a major component of the venom profiles of *Atropoides*, *Cerrophidion*, and *Metlapilcoatlus*, we also analysed the ability of varespladib to neutralise PLA_2 mediated activity present in the venoms.

Results

All venoms (30 µg/mL) caused significant inhibition of indirect twitches over a 3-h period. Venoms from *B. asper*, *C. godmani*, *C. petlalcalensis*, and *C. wilsoni* also significantly inhibited contractile responses to exogenous agonists ACh, CCh, and KCl, indicative of myotoxic activity (Fig, 1). While *C. tzotzilorum* caused inhibition of indirect twitches, this was comparably weaker compared to the effects of *B. asper*, *C. godmani*, *C. petlalcalensis*, and *C. wilsoni* venoms. Additionally, *C. tzotzilorum* venom did not significantly inhibit ACh, CCh, and KCl responses, indicating a weak pre-synaptic neurotoxic effect. The pre-addition of ICP antivenom (3 × recommended concentration) partially abolished the reduction of indirect twitches caused by *B. asper*, *C. godmani*, and *C. petlalcalensis* venoms. Additionally, antivenom was able to prevent the reduction in contractile responses to ACh, CCh, and KCl. In contrast, ICP antivenom was unable to prevent indirect twitch inhibition caused by *C. tzotzilorum* venom. Due to limited venom supplies, the efficacy of ICP antivenom against *C. wilsoni* venom was not tested. Rank potency of venom effects and efficacy of antivenom are indicated in Table 1.

Venoms from *A. picadoi*, *M. mexicanus*, *M. nummifer*, *M. occiduus*, and *M. olmec* all caused a significant reduction in indirect twitch height, indicating some pre-synaptic neurotoxic effects (Fig. 2). There were variable reductions in responses to ACh, CCh, and KCl; however, as the KCl response was significantly reduced by all venoms in this study, this indicates both a pre-synaptic neurotoxic and myotoxic effect of venoms. Only *M. olmec* produced a significant reduction in all agonists. Except for *M. olmec* venom, the pre-addition of ICP antivenom was unable to protect against twitch reduction caused by *Atropoides* and *Metlapilcoatlus* venoms. However, the presence of antivenom was able to significantly prevent the reductions in agonist responses of all venoms, indicating partial neutralisation of venom effects. While there was partial protection of the contractile response to KCl, this was still significantly different from the control response in each venom treatment. Rank potency of venom effects and efficacy of antivenom are indicated in Table 1.

To understand venom $sPLA_2$ induced activities and the neutralising potential of varespladib, varespladib (30 µg/mL) was preincubated with *M. mexicanus* and *M. nummifer* venoms (30 µg/mL) prevented inhibition of indirect twitches but was ineffective against *C. godmani* (30 µg/mL) venom (Fig. 3). The twitch inhibition caused by *M. olmec* (30 µg/mL) venom was partially delayed by the presence of varespladib; however, this effect was not statistically significant. Additionally, this is reflected in the agonist responses where reductions in agonist responses by *M. mexicanus* and *M. nummifer* venoms were prevented by varespladib, but *C. godmani* and *M. olmec* were not. Rank potency of venom effects and efficacy of varespladib are indicated in Table 1.

(See figure on next page.)

Fig. 1 Effects of venoms (30 µg/mL) from *B. asper*, *C. godmani*, *C. petlalcalensis*, *C. tzotzilorum*, and *C. wilsoni* in the presence and absence of ICP antivenom (3 × recommended concentration) on **A** indirect twitch height and **B** contractile responses. *Bothrops asper* (Costa Rica) was included as a positive control for the ICP antivenom experiment, as this venom is included in the immunising mixture. In all experiments, antivenom alone served as the control, except for *Cerrophidion wilsoni* venom, which was compared to a BSA vehicle (control) as this venom was not tested against antivenom due to a limited supply of venom stock. * indicates significant difference to the control ($p < 0.05$); # indicates venom alone is significantly different to venom in the presence of antivenom ($p < 0.05$). All venom and venom + antivenom experiments were performed in quadruplicate ($n = 4$), except for *C. wilsoni* which was performed in triplicate ($n = 3$)

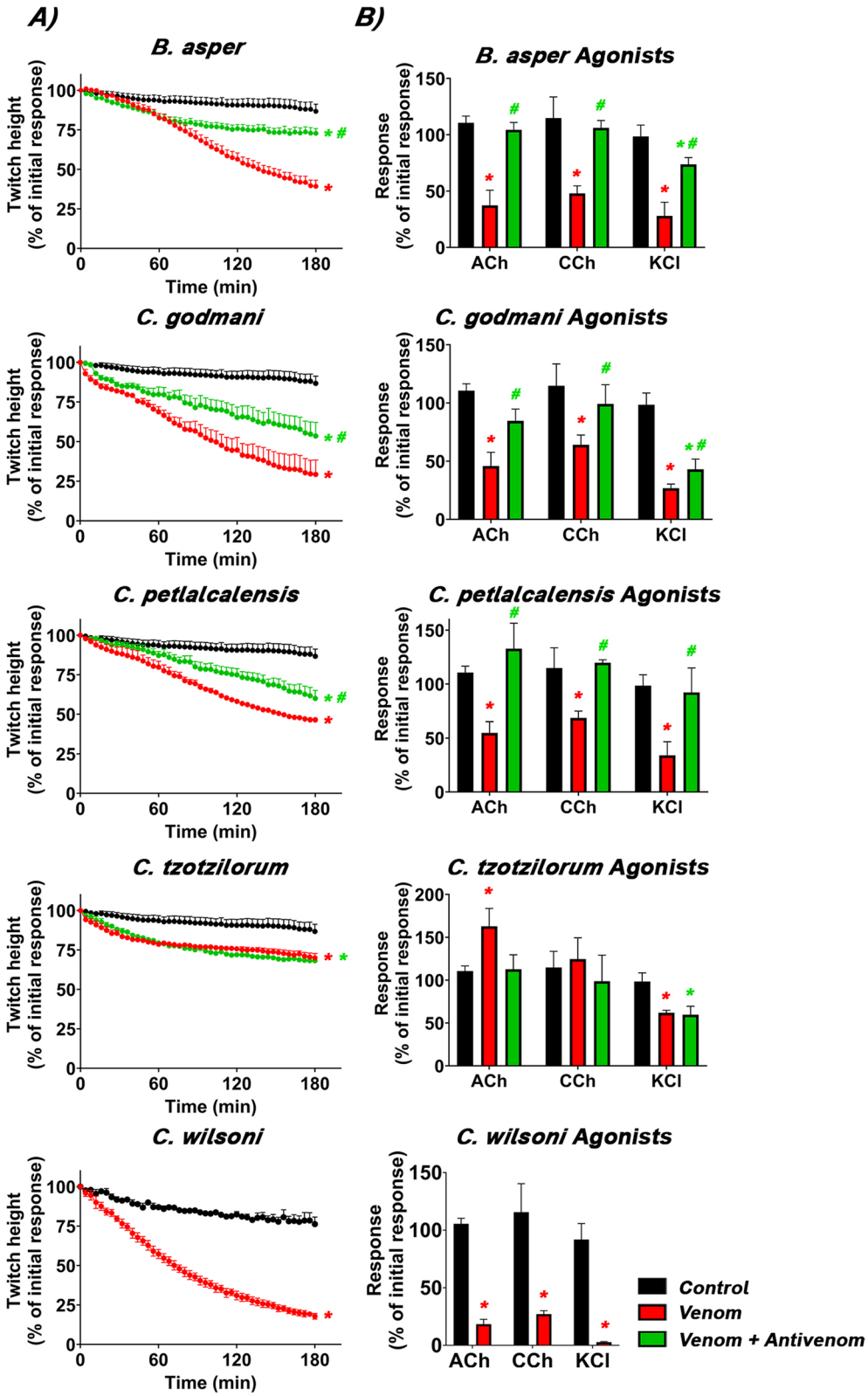

Fig. 1 (See legend on previous page.)

Table 1 Venom, venom + antivenom, and venom + varespladib effects on twitch height. Potency is expressed as time (min) taken to reach 75% inhibition (t_{75}) of the initial twitch response

Species	Venom	Venom + antivenom	Venom + varespladib
B. asper	78.5 ± 6.8	112 ± 23.9	–
A. picadoi	80 ± 27.3	132 ± 19.1	–
C. godmani	48 ± 4.2	87.5 ± 21.2	73.3 ± 10.4
C. petlalcalensis	66.5 ± 6.3	115 ± 16.8	–
C. tzotzilorum	115.5 ± 25.4	81.5 ± 4.6	–
C. wilsoni	36 ± 4	–	–
M. mexicanus	66 ± 14.4	80.66 ± 12.5	Neutralised
M. nummifer	75.5 ± 9.5	100 ± 12.9	Neutralised
M. occiduus	93 ± 21.2	56 ± 3.1	–
M. olmec	27 ± 2.5	72.66 ± 6.8	83.5 ± 9.6

– = not performed due to insufficient venom supply

Discussion

We examined the myotoxic and neurotoxic activity of *Atropoides picadoi*, four *Cerrophidion*, and four *Metlapilcoatlus* venoms and the efficacy of a nonspecific, regionally available polyvalent antivenom. Assays conducted using the isolated chick biventer cervicis nerve-muscle preparation indicated that all venoms from each clade demonstrated potent myotoxic effects and possible pre-synaptic neurotoxicity. Pre-synaptic neurotoxicity was more prominently seen in the venoms from *M. mexicanus* and *M. nummifer*, as the venom displayed the classic triphasic effects on twitch height, i.e. a decrease, followed by a transient increase, then inhibition of acetylcholine release [30]. Due to the direct action of myotoxins on skeletal muscle, the pre-synaptic neurotoxic effects of venoms can be masked by the co-presence of myotoxins as all contractile responses, including the response to KCl, will be affected. While proteomic studies of *M. nummifer* have not reported pre-synaptic neurotoxins, venoms with pre-synaptic neurotoxins typically exhibit low LD_{50} values [31–33], indicating potent venoms. Conversely, high doses of 6.9 µg/g have been reported for *M. nummifer* [11], suggesting that any pre-synaptic neurotoxin present may be in very low abundance. Moreover, clinical reports have not documented neurotoxic signs or symptoms in patients bitten by *M. nummifer*, but have been for *C. godmani* venom. As both pre-synaptic toxins and myotoxins are reportedly present within the venom proteome, and both activities are present, myotoxicity appears to take precedent in both the neuromuscular assay and envenomed patients [9]. It is also possible that *C. godmani* venom exhibits presence or absence of neurotoxic components depending on the individual from which the sample is obtained, as reported in transcriptomic studies of this species and in some studies of rattlesnakes [34, 35]. Previous studies using *Bothrops* venoms have shown that neuromuscular blockade in the chick biventer cervicis nerve-muscle preparation can occur over a range of concentrations (i.e. 50–200 µg/mL) and is associated with extensive muscle damage [36]. Additionally, a lower concentration of *Bothrops marajoensis* venom (i.e. 1 µg/mL) was shown to inhibit neurotransmission without observable myotoxicity (i.e. inhibiting responses to KCl), whereas a high concentration (20 µg/mL) inhibited both indirect twitches and agonist responses [37]. Considering the current study used a higher concentration of venom, it is likely that the effects of *Atropoides*, *Cerrophidion*, and *Metlapilcoatlus* venoms are due to a combination of pre-synaptic activity and myotoxic activity, supported by the attenuation of contractile responses to ACh, CCh, and KCl at 30 µg/mL. Comparatively, our findings suggest that these venoms have lesser myotoxic and perhaps neurotoxic potential compared to *Bothrops* venoms. In order to understand and distinguish the activity observed in this study, isolation of toxins and subsequent testing are necessary. For example, previous research into the activity of pre-synaptic toxins, such as paradoxin [38], has shown that decreasing calcium concentration in the physiological bathing medium accentuates their activity. In addition, further experiments evoking direct stimulation of the skeletal muscle preparation (myotoxicity studies) would be able to further differentiate the effects of the venom.

Previously, it was demonstrated that *Cerrophidion* and *Metlapilcoatlus* species possess venom that targets artificial post-synaptic acetylcholine receptors in a binding assay [17]. However, such post-synaptic effects were not observed in the current study. Instead, the decreases in responses to ACh and CCh were likely not due to any post-synaptic effect in these venoms, considering that there was a concomitant reduced response to KCl, which is a classic feature of myotoxic venoms. It has been well described that post-synaptic inhibition involves binding to acetylcholine binding sites on skeletal muscle nicotinic receptors [29] and hence, abolishes contractile responses to nicotinic agonists ACh and CCh with no effects on KCl responses in the chick biventer cervicis nerve-muscle preparation. The discrepancy between the artificial binding assay and ex vivo preparations could be due to a lack of functional activity of post-synaptic neurotoxins in *Cerrophidion* and *Metlapilcoatlus* venoms. Indeed, three-finger toxins were previously detected in *M. mexicanus* venom in low amounts (< 0.1% of the total venom proteins) but were reportedly absent in the venom of *A. picadoi* [8], despite showing potent binding at non-mammalian targets, i.e. avian [18]. Such three-finger-like toxins have also been previously described in other viperid venoms, including *Lachesis muta* [39, 40]. This unexpected finding warrants further isolation and

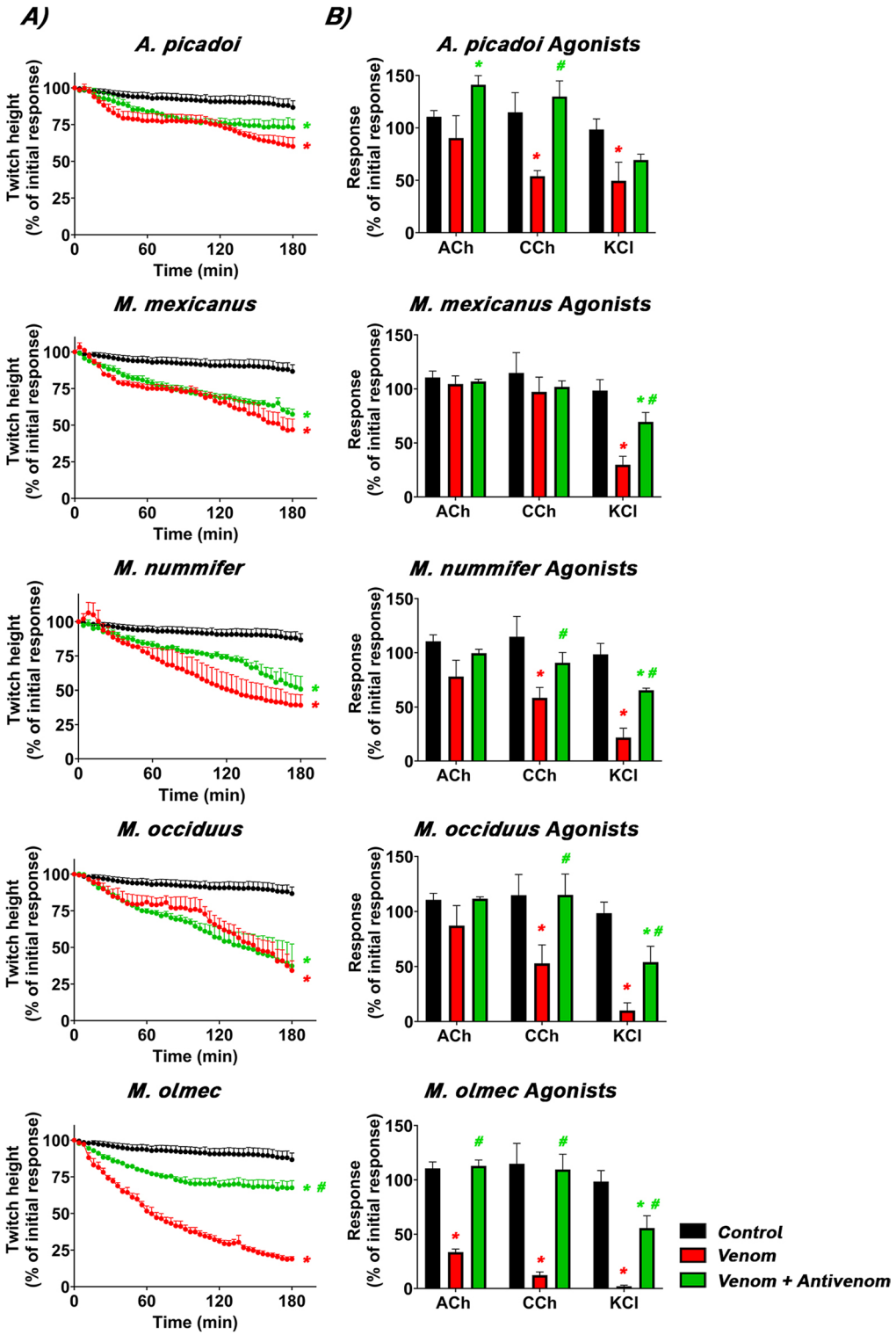

Fig. 2 Effects of venoms (30 µg/mL) from *A. picadoi*, *M. mexicanus*, *M. nummifer*, *M. occiduus*, and *M. olmec* in the presence and absence of ICP antivenom (3 × recommended concentration) on **A** indirect twitch height and **B** contractile responses. * indicates significant difference to the control ($p < 0.05$); # indicates venom is significantly different to venom + antivenom ($p < 0.05$). Venom alone experiments were performed in quadruplicate ($n = 4$); venom + antivenom experiments were performed in triplicate ($n = 3$)

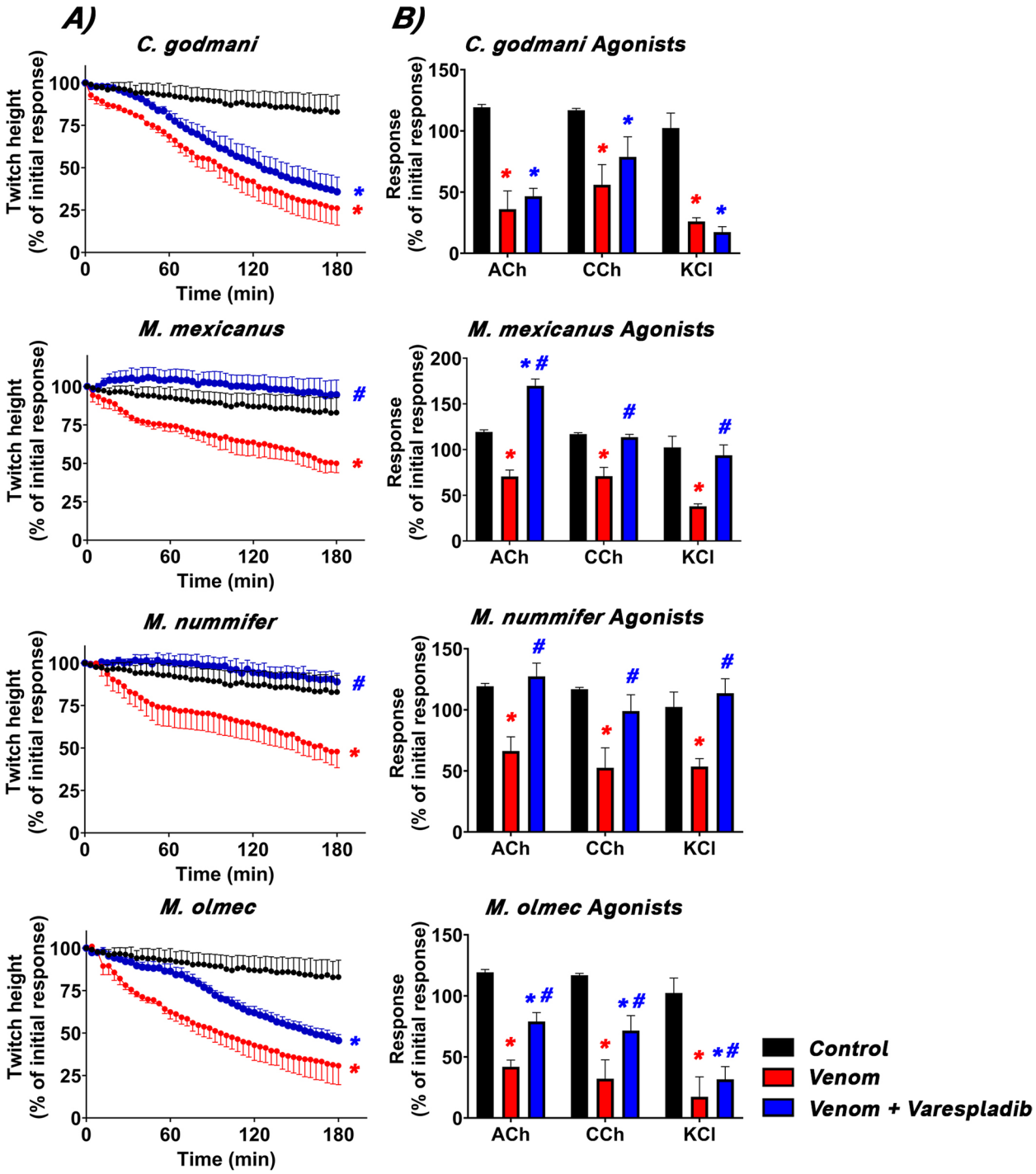

Fig. 3 Effects of venoms (30 µg/mL) from *C. godmani*, *M. mexicanus*, *M. nummifer*, and *M. olmec* on twitch height in the presence and absence of varespladib (30 µg/mL) on **A** indirect twitch height and **B** contractile responses. * indicates significant difference to the control ($p < 0.05$); # indicates venom alone is significantly different to venom + varespladib ($p < 0.05$). All venom alone and venom + varespladib experiments were performed in triplicate ($n = 3$)

characterisation of venom toxins within these clades to determine the toxin type responsible.

In the current study, antivenom and the PLA_2 inhibitor varespladib were highly variable in their relative capacity to neutralise the tissue damaging effects of the examined venoms. These variations include substantial intra-genera differential response. For some venoms, while the rate of decrease in twitch height was delayed, there was still

a consistent downward trend which indicates that the venom was only partially or unsustainably neutralised.

We tested the ICP polyvalent antivenom against *Bothrops asper*, since this is the species present within the immunising mixture most closely related to the study species. However, ICP antivenom was only able to partially neutralise the effects of *B. asper* venom. Rather than inefficacy, this is more likely to indicate that an insufficient concentration of antivenom was used. Specifically, PoliVal-ICP was shown previously to neutralise the venom of *B. asper* with high potency [41]. In the presence of antivenom, the protective effects against *A. picadoi* venom-induced twitch inhibition were somewhat delayed, as there was still some progressive decline in twitch height over time. This effect was similar to that of *C. godmani*, *C. petlalcalensis*, and *M. olmec* venoms. This indicates that there was both delayed and incomplete neutralisation of toxins, consistent with a lack of sufficient antivenom concentration rather than antivenom inefficacy. Additionally, the antivenoms were able to protect against reductions in ACh and CCh, but had variable effects on KCl. This likely correlates to the neutralisation of pre-synaptic neurotoxic effects and partial efficacy against the myotoxic effects of venom. In contrast, the venoms of *C. tzotzilorum*, *M. mexicanus*, *M. nummifer*, and *M. occiduus* were not neutralised. However, the antivenom was able to protect against reductions in agonist contractile responses. These results suggest the lack of cross reactivity of ICP antivenom to neutralise the neurotoxicity and/or myotoxicity of these venoms, and highlight a possible consequence of low antigenic similarities between these venoms and the immunogens in the antivenom. These results align with previous findings by Solano et al. [41], where the PoliVal-ICP antivenom was unable to cross-neutralise the lethality of *A. mexicanus* (now *M. mexicanus*). It was also demonstrated that PoliVal-ICP was able to neutralise the lethality of *A. picadoi* and *Cerrophidion sasai* venoms [41]. This was similar to our findings of partial neutralisation of *C. godmani*, *C. petlalcalensis*, and *A. picadoi* venoms. Further supporting the concept that there is less significant antigenic similarity between the main toxins in *B. asper*, *Atropoides*, *Cerrophidion*, and *Metlapilcoatlus* venoms, and the lower immunoreactivity against some of the toxins within these venoms may be due to the low immunogenicity of some venom components. In fact, it is more likely that the neutralisation of PLA_2 toxins requires high antivenom to venom ratios as they are of relatively low immunogenicity compared to the high molecular weight SVMPs, which are also highly immunogenic in horses [42]. Unfortunately, higher antivenom concentrations were not tested due to possible changes in osmolarity and hence, tissue viability.

Both *Cerrophidion* and *Metlapilcoatlus* genera had other species with venoms that were not neutralised to any significant level by antivenom (*C. tzotzilorum*, *M. mexicanus*, *M. nummifer*, *M. occiduus*). This indicates that while the venoms are proteomically similar within a genus [8–12] and have similar myotoxic actions, there are significant differences in sites on the toxins which are specifically recognised by the antivenom antibodies. This leads to the remarkable level of intra-genus responses seen here, exemplified by *Metlapilcoatlus*, which contained both the highest potency and was best neutralised of all the venoms tested (*M. olmec*) while the other species were not neutralised. This underscores the fundamental toxinological paradigm that antivenom efficacy cannot be predicted by taxonomy, venom protein composition, or functional activity, a trend which has been seen in a myriad of other venoms [43–46].

While the chick biventer cervicis nerve-muscle preparation provides valuable insights into the action of snake venoms and toxins at the skeletal neuromuscular junction, care needs to be taken when extrapolating data to predict the outcomes of human envenoming. While there is usually excellent correlation between the in vitro studies and clinical outcomes, there are examples of venoms/toxins producing potent in vitro neurotoxicity but having no neurotoxic effects in humans [47]. This discrepancy may be due to species differences, or the quantity of potency of individual toxins in snake venoms. However, while taxon selectivity of neurotoxins has been extensively documented (c.f. [48–51]), there has to-date not been any studies showing similar selectivity of myotoxicity. In addition, the myotoxic effects documented in this study are also consistent with the available human envenomation clinical reports for these species.

The PLA_2 inhibitor varespladib was also highly variable in its efficacy. Varespladib partially delayed the twitch inhibition caused by *C. godmani* and *M. olmec* venoms; however, this effect was not found to be statistically different. This partial effect is consistent with neutralisation of PLA_2 toxins but with other toxin types (such as SVMP) remaining active. As such, there was better neutralisation capacity of *C. godmani* and *M. olmec* venoms in the presence of antivenom, compared to varespladib alone, which supports the notion that myotoxicity in this venom is likely to be due to both PLA_2 and SVMP toxins. In contrast, a fascinating pattern emerged for both *M. mexicanus* and *M. nummifer* venoms which, consistent with the antivenom results, differed sharply from *M. olmec* venom. Activity from both venoms were neutralised by varespladib, which is consistent with prior research suggesting the potent myonecrosis described in *M. mexicanus* and *M. nummifer* venoms is driven by PLA_2 toxins [12, 13, 52].

The lack of protective ability of varespladib against *M. olmec* and *C. godmani* venoms may indicate PLA_2 toxins are not responsible for venom-induced myotoxicity. Proteomic characterisation studies regarding *M. olmec* venom are absent hence further studies are required to determine the main toxins responsible. In contrast, the composition of *C. godmani* venom was suggested to be more similar to that of *M. mexicanus*, with a high proportion of PLA_2 venom toxins (36.5%) compared to other toxins, such as SVMPs (18.2%) [10]. Hence, it is unclear why varespladib did not protect against venom-induced twitch inhibition. While varespladib has been shown to have broad enzymatic inhibition of diverse venoms, there remains a gap in our knowledge of its specific effects on non-enzymatic "$sPLA_2$-like" toxins commonly referred to basic $sPLA_2$s. Interestingly, varespladib was previously shown to be unable to fully neutralise the cytotoxic effects of *Crotalus vegrandis* venom, which was hypothesised to be a result of the crotoxin complex preventing varespladib from interacting with the PLA_2 [53], much work remains to be done to fully describe drug-venom interactions that impact upon efficacy. As such, future work requires fractionating the venoms and repeating these tests to ascertain the toxin types responsible, which was outside the scope of the current study due to insufficient venom supply.

Conclusions

This study aimed to characterise the myotoxic and neurotoxic activity from the poorly studied *Atropoides*, *Cerrophidion*, and *Metlapilcoatlus* clade of American crotalids. Potent myotoxicity has been described in *C. godmani*, *C. petlalcalensis*, *C. wilsoni*, *M. mexicanus*, *M. nummifer*, *M occiduus*, and *M. olmec* venoms. This finding is consistent with the abundance of PLA_2 toxins found within the proteome of these venoms, though with differing proportions and often not the dominant toxin. We report variable efficacy of the ICP antivenom where partial or poor neutralisation was shown against the venoms. The small molecule inhibitor, varespladib, showed effective neutralisation of *M. mexicanus* and *M. nummifer* venom effects, while *C. godmani* and *M. olmec* venoms remained poorly protected, indicating that not all these venom effects are mediated by enzymatically active PLA_2 toxins. This study has added to the growing body of knowledge surrounding poorly studied *Atropoides*, *Cerrophidion*, and *Metlapilcoatlus* snakes.

Methods

Venom and antivenom

Venom work was undertaken under University of Queensland Biosafety Approval #IBC134BSBS2015. Venom samples (pooled from four specimens per venom) were sourced from species: *Atropoides picadoi* (Costa Rica), *Bothrops asper* (Costa Rica), *Cerrophidion godmani* (Siltepec, Chiapas, Mexico), *Cerrophidion petlalcalensis* (San Andres Tenejapan, Veracruz, Mexico), *Cerrophidion tzotzilorum* (San Cristobal, Chiapas, Mexico), *Cerrophidion wilsoni* (Honduras), *Metlapilcoatlus mexicanus* (Chiapas, Mexico), *Metlapilcoatlus nummifer* (Veracruz, Mexico), *Metlapilcoatlus occiduus* (Mapastepec, Chiapas, Mexico), and *Metlapilcoatlus olmec* (Los Tuxtlas, Veracruz, Mexico). Lyophilised venoms were reconstituted in 0.05% BSA to a working stock concentration of 2 mg/mL and stored at − 20 °C until use. The ICP antivenom (batch #6800122POLF Expiry date: 01/2027) was made to manufacturer's instructions, where 10 mL neutralises no less than 30 mg *Bothrops asper*, 20 mg *Crotalus simus*, and 30 mg *Lachesis stenophrys* venoms.

Drugs

The following chemicals and drugs were purchased from Sigma-Aldrich, St Louis, MO, USA: acetylcholine (ACh), carbamylcholine (CCh), d-tubocurarine (dTc), bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO). Potassium chloride (KCl) was purchased from Merck (Darmstadt, Germany). All chemicals were dissolved in MilliQ water, except varespladib, which was dissolved in DMSO to a final concentration of 10 mM.

Chick biventer cervicis nerve-muscle preparation

Male 5–10-day-old chicks (White Leghorn crossed with New Hampshire) were obtained from Wagner's Poultry, Coldstream, Victoria (Australia). Animals were then housed at Monash Animal Services with free access to food and drinking water until euthanasia by CO_2 inhalation. Two biventer tissues were dissected from each chick and vertically mounted on a wire holder under 1 g resting tension in 5 mL organ baths containing a physiological salt solution (118.4 mM of NaCl, 4.7 mM of KCl, 1.2 mM of $MgSO_4$, 1.2 mM of KH_2PO_4, 2.5 mM of $CaCl_2$, 25 mM of $NaHCO_3$, and 11.1 mM of glucose), where they were maintained at 34 °C and bubbled with carbogen (95% O_2 and 5% CO_2). The motor nerves of the tissue were stimulated with a supramaximal voltage of 10–15 V at a frequency of 0.1 Hz and a duration of 0.2 ms using an LE series electrical stimulator. The corresponding twitches were recorded using a PowerLab system (ADInstruments Pty Ltd, Bella Vista, Australia) via a Grass FT03 force transducer. The tissues were allowed to equilibrate for at least 20 min, or until a stable twitch height was achieved. Selective nerve stimulation was confirmed by the abolishment of twitches by the addition of dTc (10 μM). The preparation was then repeatedly washed with physiological solution to restore twitches. In the absence of electrical stimulation, contractile responses to exogenous ACh

(1 mM, 30 s), CCh (20 μM, 60 s), and KCl (40 mM, 30 s) were obtained. Between each addition, the previous agonist was washed out and allowed to return to baseline before the next addition. Following this, electrical stimulation was recommended for at least 20–30 min or until a steady twitch height was achieved. Venoms were added to the organ bath to make a final concentration of 30 μg/mL; this concentration is based on our prior work in which venoms which are potently myotoxic will show a strong effect. For neutralisation studies, antivenom was added to the organ bath 15 min before the addition of venom at 3× the recommended concentration according to manufacturer's instructions, in which the antivenom alone was shown to have no inhibitory effect on the twitches. Varespladib was added at a 1:1 ratio of the venom concentration with a pre-incubation period of 15 min prior to addition of the mixture into the organ bath. Experiments were terminated after 3 h if twitches were not abolished beforehand. At the end of the experiment, responses to exogenous agonists ACh, CCh, and KCl were repeated as previously stated.

All animal experiments used in this study were approved by the Animal Ethics Committee of Monash University (Number 26830, approval date 30 April 2021).

Statistics

Twitch height was measured every 4 min after the addition of venom and expressed as a percentage of the pre-venom twitch height. Post-venom contractile responses to exogenous agonists were expressed as a percentage of the corresponding initial pre-venom contractile response. Data are presented as mean ± standard error of mean (SEM) where n is the number of tissue preparations. Significance was determined using a two-way ANOVA followed by Tukey's post hoc multiple comparisons. All data and statistical analyses were performed using Prism 10.2.2 (GraphPad Software, San Diego, CA, USA, 2022). $p < 0.05$ was considered statistically significant for all analyses.

Abbreviations

ACh	Acetylcholine
CCh	Carbamylcholine
DMSO	Dimethyl sulfoxide
dTc	D-Tubocurarine
ICP	Instituto Clodomiro Picado
KCl	Potassium chloride
PLA_2	Phospholipase A_2
$sPLA_2$	Secretory phospholipase A_2
SVMP	Snake venom metalloproteinases
SVSP	Snake venom serine protease

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12915-024-02044-3.

Supplementary Material 1.

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Not applicable.

Authors' Contribution

LJ: writing—review and editing, writing—original draft, visualisation, validation, methodology, investigation, formal analysis, data curation, conceptualisation. ML: writing—review and editing, formal analysis, methodology, data curation. ENC: writing—review and editing, visualisation, investigation. VZ: writing—review and editing, resources. WCH: writing—review and editing, methodology, resources, supervision. ML: writing—review and editing, resources. BGF: writing—review and editing, supervision, resources, project administration, methodology, funding acquisition, conceptualisation. All authors read and approved the final manuscript.

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Data availability

Data is presented in the figures and raw data is in the supplementary file.

Declarations

Ethics approval

All animal experiments used in this study were approved by the Animal Ethics Committee of Monash University (Number 26830, approval date 30 April 2021).

Consent to participate

Not applicable.

Competing interests

ML was employed by the company Ophirex, which made enzyme-inhibitor products tested in this manuscript. However, the company had no input in experimental design or reviewing of results before publication. The other authors declare no conflicts of interest.

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