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Why myotoxin-containing snake venoms possess powerful nucleotidases?

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ABSTRACT

The venom of the snake *Bothrops asper* causes muscle necrosis, pain and inflammation. This venom contains myotoxins which cause an increase in intracellular Ca^{2+} concentration and release of K^+ and ATP from myotubes. ATP is a key danger molecule that triggers a variety of reactions, including activation of the innate immune response. Here, using ATP-luciferase bioluminescence imaging technique, we show for the first time *in vivo*, that the purified myotoxins induce rapid release of ATP, whilst the complete venom of *B. asper* does at a very small extent. This apparent contradiction is explained by the finding that the venom contains powerful nucleotidases that *in vivo* convert ATP into ADP, AMP and Adenosine. These findings indicate that high concentrations of adenosine are generated by the double action of the venom and provide the experimental basis to the suggestion that *in situ* generated adenosine plays an important role in envenomation via its hypotensive, paralyzing and anti-coagulant activities.

1. Introduction

Snake bite envenomation has a major impact on human health in tropical and sub-tropical regions of the world [1,2]. Snake venoms may cause death, but more frequently they cause permanent disabilities secondary to prominent tissue damage [3–6]. However, despite the large effect on public health, their importance is not sufficiently appreciated and the mechanisms of pathogenesis of the alterations which follow envenomation are not sufficiently understood. The majority of snake bite envenomations in Central and South America are caused by species of the genus *Bothrops*, including the very dangerous lance-head vipers *B. asper* and *B. atrox*. These venoms cause pain, hemorrhage, hypotension, and myonecrosis, together with inflammation. A major role in the pathophysiology of envenomations by *B. asper* is played by myotoxins which rapidly induce local myonecrosis, provoking a prominent inflammatory response and permanent tissue damage. We have previously studied in detail the effect of *B. asper* myotoxins on myotubes and *ex vivo* muscles and found that they cause Ca^{2+} entry and release of K^+ and ATP [7–10]. We have also shown that the released ATP diffuses around the site of toxin application and extends beyond the area of damage [10]. Indeed, ATP is known to exert a large variety of pharmacological effects [11–13]. Our experimental work was performed both with C2C12 cultured mouse myotubes and excised hind limb muscles. A consequent extension of these studies is that of assessing the venom effects *in vivo*. By imaging the luciferin emitted chemiluminescence, here we show that *B. asper* myotoxins injected into the hind limbs of mice also induce ATP release *in vivo*. However, on the contrary, little ATP was detected at the site of injection of the whole venom. This apparent contradiction led us to investigate the possible presence of adenine-nucleotidases in this venom, and here we report that these enzymes are present and highly active; not only this explains the ATP *in vivo* imaging data, but also provides an explanation for the evolutionary advantage of the presence of nucleotidases in all those snake venoms that also have myotoxins.

2. Materials and methods

2.1. Venoms and toxins

The venom of *B. asper* consisted of a pool obtained from more than 40 adult specimens collected in the Atlantic region of Costa Rica and kept at the serpentarium of Instituto Clodomiro Picado, University of Costa Rica; venom was lyophilized and stored at −20 °C. Myotoxins I and II were isolated from *B. asper* venom by ion-exchange chromatography on CM-Sephadex C-25 as described [14,15] followed by RP-HPLC on a C8 semi-preparative column (10 × 250 mm; Vydac) eluted at 2.0 ml/min with a 0–70% acetonitrile gradient containing 0.1% trifluoroacetic acid, during 30 min, on an Agilent 1200 instrument monitored at 215 nm. Homogeneity of the final preparations was assessed by analytical reverse-phase

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HPLC on a C4 column (4.6 × 150 mm) using a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (v/v). Venom samples were dissolved in 10 mM Hepes and 150 mM NaCl with 50% glycerol and sterilized by filtration through 0.22 μm GV Durapore® (Millipore).

2.2. Animals

Adult mice (C57 Bl6 strain) weighing 25–30 g were used. All experimental procedures involving animals were carried out in accordance with the Italian Animal Welfare Act and were approved by the local authority veterinary service.

2.3. In vivo ATP imaging

In vivo bioluminescent imaging was performed with an ultra low-noise, high sensitivity cooled CCD camera mounted on a light–tight imaging chamber (IVIS Lumina System, Caliper, Perkin Elmer). Tracking, monitoring and quantification of signals were controlled by the acquisition and analysis software Living Image. Mice were anesthetized with a continuous flux of isoflurane, positioned in the instrument chamber and injected with a 50 μl syringe fitted with a 29 gauge needle (Hamilton). For each mouse, the right leg was injected intramuscularly with a mixture composed of the reporter solution (luciferase–luciferine mix, Promega) and the toxin or the venom indicated, in a total volume of 20 μl. The contralateral leg was used as control, i.e. it was injected with the reporter solution plus the vehicle used to solubilize toxins. Two mice per each experimental run were monitored immediately after injections; luminescent images were obtained with constant exposure time periods of 5 min for a total time of 30 min; regions of interest were defined manually around the site of injection for determining total photon flux (photons per second).

2.4. Colorimetric assay of orthophosphate

ATPase, ADPase and 5′-nucleotidase activities were determined according to previously described methods [16–18], using a colorimetric assay for orthophosphate liberation from nucleotides. *B. asper* venom was diluted in incubation buffer (0.1 M glycine–NaOH, pH 8.9), in the range of 1.25–640 μg/ml. Samples (150 μl) of each venom dilution were added in duplicate to Eppendorf tubes. Incubation buffer alone was used as a blank. The same volume of 1 mM ATP (disodium salt, Sigma, USA), 1 mM ADP (sodium salt, Sigma, USA) or 1 mM AMP (sodium salt, Sigma, USA) dissolved in incubation buffer containing 3.8 mM $MgCl_2$, was subsequently added to the venom solutions and blanks and incubated for 15 min at 37 °C. Duplicates of orthophosphate solutions (KH_2PO_4, ranging from 1.88 to 30 nmol) were used for the construction of standard curves. The color reagent (81.1 mM ascorbic acid, 3 mM ammonium molybdate, and 0.5 M H_2SO_4) was added (700 μL for each tube) and then incubated at 37 °C for 1 h. Absorbance was determined at 820 nm using a Lambda 25 UV/V is spectrophotometer (Perkin Elmer) and values were plotted versus venom concentration. The amount of venom that liberated 15 nmol of orthophosphate was used for comparison. Specific activities of nucleotidases on ATP, ADP and AMP were calculated and expressed in μmol of orthophosphate/min/mg of venom. The mean values of three independent experiments were used.

3. Results

3.1. Injection of B. asper myotoxins causes ATP release in vivo

Lys-49 (Mt-II) and Asp-49 (Mt-I) myotoxins induce the release of ATP from cultured myotubes and excised muscle [10], which then displays its various functions [11–13]. It remained to be demonstrated whether this ATP release does occur *in vivo* as well. This type of analysis can be performed in animal models with the reporter system luciferase–luciferin. A previous work used the injection of stably transfected cells expressing the luciferase gene on their surface; in the presence of ATP, the administration of luciferin triggers a chemiluminescent reaction of luciferase, and the photons emitted can be detected by an appropriate imaging apparatus [19]. Other studies used different techniques, for example electroporation to administrate luciferase to cells [20]. In preliminary experiments, we found that the reporter system luciferase–luciferin adsorbs onto the muscle cells and reports efficiently changes in extracellular ATP levels. We took advantage of this finding and, consequently, injected the luciferase–luciferin mixture in the mouse hind legs together with toxins or venoms. We injected in the mouse hind limbs 50 μg of Mt-II myotoxin, an amount much lower than that present in *B. asper* venom: a single bite is estimated to inject milligrams of venom [21], and Lys-49 myotoxins comprise about 20% of venom proteins [22]. Fig. 1 shows that this amount is sufficient to induce a rapid release of ATP. The contralateral leg was injected with carrier solution and the differential effect is evident by simply examining the chemiluminescent signal (panel A of Fig. 1); the relative quantification is reported in panel B of Fig. 1. ATP runs down in about 10 min following dilution into the body fluids and owing to the activity of the ecto-ATPases present on cell surfaces. Panel C of Fig. 1 shows the effect of the other major myotoxin present in *B. asper* venom, the Asp-49 myotoxin Mt-I which acts via its PLA_2 activity and is less active than Mt-II on myotubes in culture [8,9]. Also Mt-I causes a rapid ATP release *in vivo*, but the extent of this release is lower than that caused by Mt-II, with a remarkable agreement of between the results obtained in cell cultures and the present results obtained *in vivo*. The absolute value of the extent of the effect varies from sample to sample but this is corrected by the internal ratio between one leg and the opposite one. Ratios are reported in panels B and D of Fig. 1. The injection of 100 μg of Mt-I produces an effect similar to that of 50 μg of Mt-II; in addition, the ATP release is less prolonged than in the case of Mt-II.

3.2. Injection of B. asper venom causes little ATP release in vivo

From these data, the fact that the two myotoxins are present together in *B. asper* venom and act synergistically in inducing Ca^{2+} entry from the medium into muscle cells [9], we were expecting that the injection of the venom would induce a large ATP release *in vivo*. On the contrary, the injection of 150 μg of BaV induced a very small ATP release as compared with that released by the isolated myotoxins (Fig. 2, panel A). As myotoxins comprise approximately 30% of total venom proteins [22], their content in the venom dose used here is lower with respect to the experiment with purified toxins, but higher venom doses could not be used because they caused a strong local damage which saturated the ATP reporter system and that did not allow for an appropriate measure. Another way to compare the effect of toxins and venom that also overcomes the variability among animals is to determine the internal ratio between the total photons emitted from the treated leg to the control leg. Panel B of Fig. 2 shows that both toxins generate an emission four times higher than the control, for the venom the ratio is only 1.3, thus confirming the lower effect induced by the venom.

3.3. B. asper venom contains powerful nucleotidases

A possible way to explain this apparent paradox is to assume that ATP released by the myotoxins is hydrolyzed as soon as it comes out from the damaged muscle cells. Indeed, it was recently

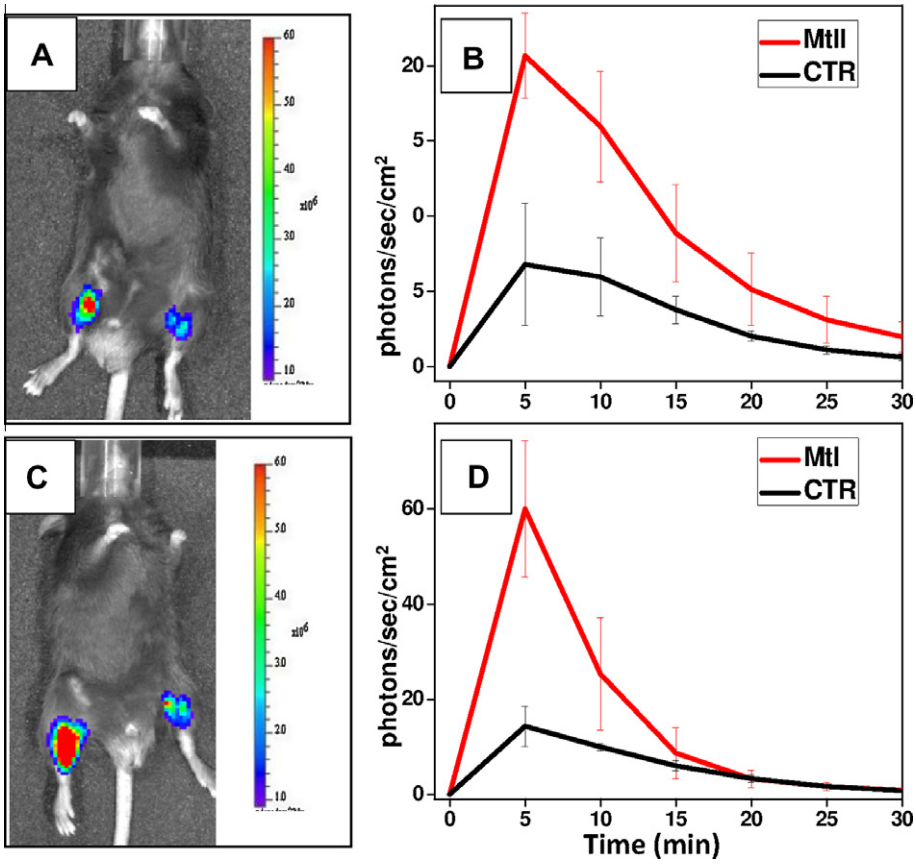
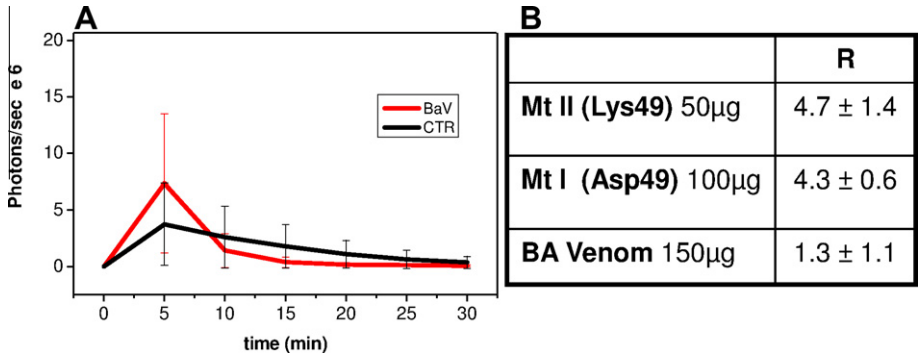


Fig. 1. Imaging of the ATP released after injection of *B. asper* myotoxins Mt-I and Mt-II. Panels (A and C) representative chemiluminescence image after 5 min from injection (pseudocolor); the right leg was injected with myotoxins (50 μg of *B. asper* Mt-II toxin, or 100 μg of *B. asper* Mt-I), while the contralateral leg was injected with the same volume of vehicle. Panels (B and D) quantification of emitted photons in the injected area; data are the mean of five independent experiments ± standard deviation.



	R
Mt II (Lys49) 50μg	4.7 ± 1.4
Mt I (Asp49) 100μg	4.3 ± 0.6
BA Venom 150μg	1.3 ± 1.1

Fig. 2. ATP released after injection of *B. asper* venom. Mice were injected with 150 μg of *B. asper* venom. (A) quantification of emitted photons in the injected area; red line: leg injected with *B. asper* venom; black line: contralateral leg injected with vehicle used as control. (B) comparison of ATP release induced by purified myotoxin and whole *B. asper* venom. The reported value *R* is the ratio between total emitted photons in the treated leg in respect to the control leg, in the first 5 min after injection. Data are the mean of five independent experiments ± standard deviation. For interpretation of color in Fig. 2, the reader is referred to the web version of this article.

reported that the venoms of other *Bothrops* species contain adenine-nucleotidase activities [23,24]. Therefore, we assayed *B. asper* venom for its ability to hydrolyze the phosphates bound to the sugar groups of ATP, ADP and AMP. Fig. 3 shows that *B. asper* venom not only hydrolyzes ATP, but also ADP and AMP. The major activities are ATPase and 5′-nucleotidase, leading to final formation of adenosine. The unescapable conclusion that is derived from the ensemble of the results presented here is that the ATP released *in vivo* by the myotoxins is rapidly hydrolyzed by the nucleotidases co-present in the venom, originating other molecular species that could, in turn, contribute to the pathophysiology of envenomation. This novel possibility is discussed hereafter.

4. Discussion

Snake venoms are complex mixtures of different components that range from proteins, acting as enzymes or membrane perturbing agents, to ligands and small molecules such as purines [25]. A successful approach for understanding venom composition and action has been the purification of various components and the study of their activity mainly using biochemical and cell biology methods. However, this approach did show the presence of nucleotidases in many snake venoms, but could not explain their function [24]. Despite the success of this reductionistic method, there is a growing consensus that it is necessary to study the individual

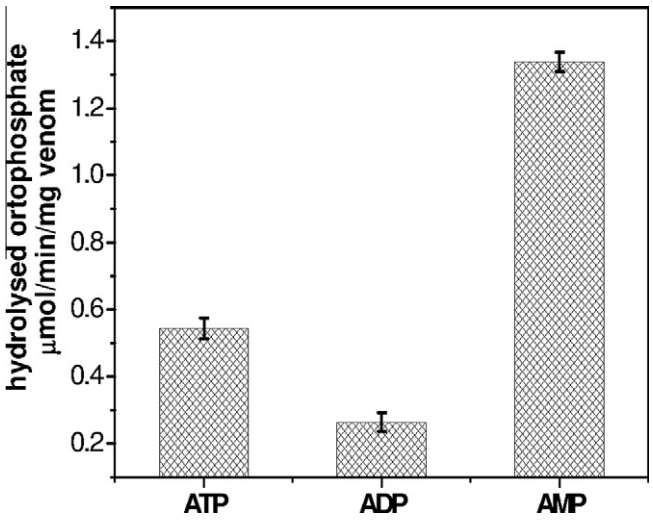


Fig. 3. Nucleotidase activities of *B. asper* venom. The venom hydrolytic activities on ATP, ADP and AMP are reported in panels A, B and C respectively, expressed as µmol of orthophosphate/min/mg of venom. The mean values of three independent experiments ± standard deviation are reported.

activities within the whole venom and *in vivo*, in order to understand and model the actual complexity of envenomation [4]. The present work attempts to follow this line. Major components of the venoms of the most dangerous Latin American snakes, those belonging to the genus *Bothrops*, are myotoxins which cause extensive myonecrosis [15,26].

A major finding reported here is that they cause release of ATP from muscles *in vivo* as they do *in vitro* [10], but a second and more important finding is that the crude *B. asper* venom induces, under the same conditions, a much lower release of ATP. This unexpected and "negative" result can be understood on the basis of the present finding that this venom possesses enzymes that are able to rapidly degrade ATP down to adenosine (Fig. 3). A detailed biochemical characterization of these enzymes is beyond the scope of the present work as the turn-over data are sufficient to make some biologically relevant estimation. The three activities: ATPase, ADPase and AMPase have different turn-over values with the ADP hydrolytic reaction being the slower one. This may indicate that there might be a transient accumulation of ADP which is well known for inducing platelet aggregation. However, from the data obtained with *ex vivo* muscles [10] we estimate that 50 µg of Lys-49 Mt-II myotoxin plus 50 µg of Asp-49 Mt-I myotoxin induce the release of about 4 nmol of ATP in 5 min; from Fig. 3 we estimate that 150 µg *B. asper* venom, which contains approximately 50 µg of myotoxins, is capable of hydrolyzing about 200 nmol of ATP to Adenosine, taking ADP hydrolysis as the rate limiting step. Therefore, the ATP hydrolyzing enzymatic activities present in the venom largely exceed the amount of ATP released by the myotoxins contained in the venom.

The presence of Adenosine in snake venoms was reported long ago [27,28] and it was suggested that it could contribute to the hypotensive effect of these venoms [[29] and references cited therein]. At variance, purines are present in many venoms but not in those of the subfamily *Crotalinae* [24] which include the *Crotalus* and *Bothrops* genera. On the other hand, these snakes possess ATP hydrolyzing enzymes [[18,23,24,30] and references cited therein; present work]. The evolutionary significance of the presence of ATP hydrolyzing enzymes in *Crotalinae* venoms was unexplained, though it was suggested that they could contribute to prey capture [23,24,29] without defining the possible mechanism of action, as there is no ATP in these venoms nor in circulating fluids [19,31].

The present work provides the first experimental evidence, *in vivo*, that the myotoxins present in the *B. asper* venom indeed release large amounts of ATP from muscles. In cultured myotubes this occurs via alteration of the sarcolemma permeability [9,10] and it is proposed that the same mechanism operates *in vivo*. In combination with the large ATP hydrolytic capacity of *B. asper* venom, hereby described, this indicates that a large amount of Adenosine can be generated within the injected tissue. With respect to the subduing of prey, it appears that the well know hypotensive and neuromuscular paralyzing effect of Adenosine may play an important role in the initial phase, just after bite and venom injection. In fact, Adenosine has multiple effects that lead to hypotension: (i) by acting on the heart, (ii) by causing vasodilatation, and (iii) by increased microvascular permeability directly and indirectly via the induction of the release of the content of mast cells granules [32–35]. An additional important activity of Adenosine is the one exerted on peripheral and central neurons with inhibi-

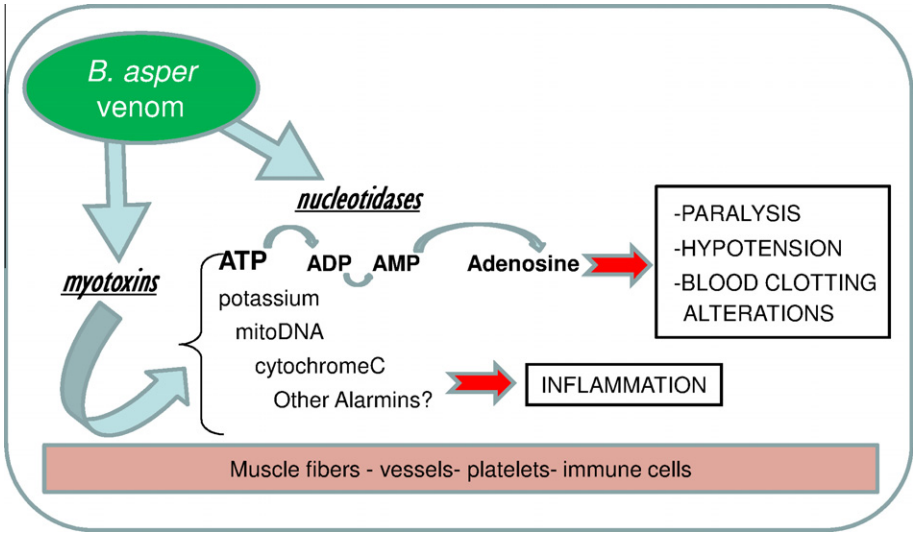


Fig. 4. Schematic representation of synergy between *B. asper* venom myotoxins and nucleotidases. When *B. asper* venom enters in contact to tissues, its enzymes combine their activities to produce Adenosine: myotoxins causing the ATP release and nucleotidases quickly hydrolyzing it. Adenosine then exerts a variety of effects, the most prominent ones being: (i) inhibition of neurotransmitter release with peripheral paralysis, (ii) hypotension via action on heart and increase of vasculature dimensions and permeability, and (iii) inhibition of platelet aggregation which favors hemorrhage. The figure also shows that the venom via its myotoxins causes the release of mitochondrial mediators (alarmins) of the innate immune reaction, as recently reported [41].

tion of neurotransmitter release [23,29,36,37] which might contribute, together with the strong pain sensation, to the immobilization of the prey; however it should be mentioned that in humans, that are much larger than the usual *Bothrops* prey, i.e. usually rodents, the paralyzing effect of the venom is lower. Immediately thereafter, a most dangerous consequence of *Bothrops* snake bite is the development of prominent local hemorrhage, which has been ascribed mainly to a set of metalloproteases present in the venom [38,39]. The present study adds to this aspect of pathogenesis, as blood clotting defects due to platelet dysfunction contribute to hemorrhage, and Adenosine is a powerful inhibitor of platelet aggregation, likely acting at A2A and A2B receptors [40].

The present findings unravel a novel aspect of the complex pathogenesis of envenomation, which is summarized in Fig. 4. This scheme may be extended to all snake venoms containing, at the same time, powerful myotoxins and ATP hydrolyzing enzymes and does explain this evolutionary conserved co-presence. After venom injection, the myotoxins alter the permeability of muscle fibers with entry of Ca^{2+}, release of K^+ and ATP and damage of mitochondria; ATP, together with mitochondrial alarmins and other pro-inflammatory mediators, induce inflammation [41]. Extracellular ATP is however rapidly hydrolyzed to Adenosine by a set of venom nucleotidases. Adenosine then exerts its multiple effects, resulting, among others, in hypotension, paralysis and alterations of blood clotting which favors local hemorrhage. From the data reported here, the evolutionary advantage of including in the same venom myotoxins and nucleotidases is evident.

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References

[1] A. Kasturiratne, A.R. Wickremasinghe, N. de Silva, N.K. Gunawardena, A. Pathmeswaran, R. Premaratna, L. Savioli, D.G. Lalloo, H.J. de Silva, The global burden of snake bite: a literature analysis and modelling based on regional estimates of envenoming and deaths, PLoS Med. 5 (2008) e218.
[2] J.P. Chippaux, Snake-bites: appraisal of the global situation, Bull. World Health Organ. 76 (1998) 515–524.
[3] J.M. Gutiérrez, R.D.G. Theakston, D.A. Warrell, Confronting the neglected problem of snake bite envenoming: the need for a global partnership, PLoS Med. 3 (2006) e150.
[4] J.M. Gutiérrez, D. Williams, H.W. Fan, D.A. Warrell, Snake bite envenoming from a global perspective: towards an integrated approach, Toxicon 56 (2010) 1223–1235.
[5] World Health Organisation, Rabies and envenomings: a neglected public health issue, WHO Press, Geneva, (2007).
[6] D. Williams, J.M. Gutiérrez, R. Harrison, D.A. Warrell, J. White, K.D. Winkel, P. Gopalakrishnakone, The global snake bite initiative: an antidote for snake bite, Lancet 375 (2010) 89–91.
[7] M. Cintra-Francischinelli, P. Pizzo, Y. Angulo, J.M. Gutiérrez, C. Montecucco, B. Lomonte, The C-terminal region of a lys49 myotoxin mediates Ca^{2+} influx in C2C12 myotubes, Toxicon 55 (2010) 590–596.
[8] Y. Angulo, B. Lomonte, Biochemistry and toxicology of toxins purified from the venom of the snake *Bothrops asper*, Toxicon 54 (2009) 949–957.
[9] M. Cintra-Francischinelli, P. Pizzo, L. Rodrigues-Simioni, L.A. Ponce-Soto, O. Rossetto, B. Lomonte, J.M. Gutiérrez, T. Pozzan, C. Montecucco, Calcium imaging of muscle cells treated with snake myotoxins reveals toxin synergism and presence of acceptors, Cell. Mol. Life Sci. 66 (2009) 1718–1728.
[10] M. Cintra-Francischinelli, P. Caccin, A. Chiavegato, P. Pizzo, G. Carmignoto, Y. Angulo, B. Lomonte, J.M. Gutiérrez, C. Montecucco, *Bothrops* snake myotoxins induce a large efflux of ATP and potassium with spreading of cell damage and pain, Proc. Nat. Acad. Sci. USA 107 (2010) 14140–14145.
[11] G. Burnstock, Discovery of purinergic signalling, the initial resistance and current explosion of interest, Br. J. Pharmacol. 167 (2012) 238–255.
[12] G. Burnstock, U. Krügel, M.P. Abbracchio, P. Illes, Purinergic signalling: from normal behaviour to pathological brain function, Prog. Neurobiol. 95 (2011) 229–274.
[13] F. Di Virgilio, S. Ceruti, P. Bramanti, M.P. Abbracchio, Purinergic signalling in inflammation of the central nervous system, Trends Neurosci. 32 (2009) 79–87.
[14] B. Lomonte, J.M. Gutiérrez, A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo), Toxicon 27 (1989) 725–733.
[15] J.M. Gutiérrez, C.L. Ownby, G.V. Odell, Pathogenesis of myonecrosis induced by crude venom and a myotoxin of *Bothrops asper*, Exp. Mol. Pathol. 40 (1984) 367–379.
[16] B. Ames, Assay of inorganic phosphate, total phosphate and phosphatases, Methods in Enzymology, Vol. VIII: Complex Carbohydrates (1966) 115–118.
[17] E. Sulkowski, W. Bjork, M.S. Laskowski, A specific and nonspecific alkaline monophosphatase in the venom of *Bothrops atrox* and their occurrence in the purified venom phosphodiesterase, J. Biol. Chem. 238 (1963) 2477–2486.
[18] P.B.V. Sales, M.L. Santoro, Nucleotidase and dnase activities in brazilian snake venoms, Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 147 (2008) 85–95.
[19] P. Pellegatti, L. Raffaghello, G. Bianchi, F. Piccardi, V. Pistoia, F. Di Virgilio, Increased level of extracellular ATP at tumor sites: *in vivo* imaging with plasma membrane luciferase, PLoS ONE 3 (2008) e2599.
[20] J. Ju, S.E. Miller, E. Jackson, K. Cadwell, D. Piwnica-Worms, C.C. Weihl, Quantitation of selective autophagic protein aggregate degradation *in vitro* and *in vivo* using luciferase reporters, Autophagy 5 (2009) 511–519.
[21] R. Bolaños, Toxicity of Costa Rican snake venoms for the white mouse, Am. J. Trop. Med. Hyg. 21 (1972) 360–363.
[22] A. Alape-Girón, M. Flores-Díaz, L. Sanz, M. Madrigal, J. Escolano, M. Sasa, J.J. Calvete, Studies on the venom proteome of *Bothrops asper*: perspectives and applications, Toxicon 54 (2009) 938–948.
[23] B.L. Dhananjaya, C.J.M. D'Souza, The pharmacological role of nucleotidases in snake venoms, Cell Biochem. Funct. 28 (2010) 171–177.
[24] S.D. Aird, Taxonomic distribution and quantitative analysis of free purine and pyrimidine nucleosides in snake venoms, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 140 (2005) 109–126.
[25] J.J. Calvete, Proteomic tools against the neglected pathology of snake bite envenoming, Expert Rev. Proteomics 8 (2011) 739–758.
[26] J.M. Gutiérrez, T. Escalante, A. Rucavado, Experimental pathophysiology of systemic alterations induced by *Bothrops asper* snake venom, Toxicon 54 (2009) 976–987.
[27] H.M. Doery, Purine compounds in snake venoms, Nature 177 (1956) 381–382.
[28] F.G. Fisher, H. Dorfel, Adenosin imgift der puffotter bitis arietans (schlangengifte i), Z. Phys. Chem. 296 (1954) 232–238.
[29] S.D. Aird, Ophidian envenomation strategies and the role of purines, Toxicon 40 (2002) 335–393.
[30] N.H. Tan, G. Ponnudurai, A comparative study of the biological properties of some venom of snakes of the genus *Bothrops* (American lance-headed viper), Comp. Biochem. Physiol. B 100 (1991) 361–365.
[31] N. Dale, B.G. Frenguelli, Measurement of purine release with microelectrode biosensors, Purinergic Signal. 8 (2012) 27–40.
[32] M.G. Collis, The vasodilator role of adenosine, Pharmacol. Ther. 41 (1989) 143–162.
[33] R.A. Olsson, J.D. Pearson, Cardiovascular purinoceptors, Physiol. Rev. 70 (1990) 761–845.
[34] D.M. Gawlowski, W.N. Durán, Dose-related effects of adenosine and bradykinin on microvascular permselectivity to macromolecules in the hamster cheek pouch, Circ. Res. 58 (1986) 348–355.
[35] I. Biaggioni, Clinical and molecular pharmacologic characteristics of adenosine-induced vasodilation, Clin. Pharmacol. Ther. 75 (2004) 137–139.
[36] E.M. Silinsky, Adenosine decreases both presynaptic calcium currents and neurotransmitter release at the mouse neuromuscular junction, J. Physiol. 558 (2004) 389–401.
[37] E.M. Silinsky, On the mechanism by which adenosine receptor activation inhibits the release of acetylcholine from motor nerve endings, J. Physiol. 346 (1984) 243–256.
[38] J.M. Gutiérrez, A. Rucavado, T. Escalante, C. Díaz, Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage, Toxicon 45 (2005) 997–1011.
[39] T. Escalante, A. Rucavado, J.W. Fox, J.M. Gutiérrez, Key events in microvascular damage induced by snake venom hemorrhagic metalloproteinases, J. Proteomics 74 (2011) 1781–1794.
[40] H.A. Johnston-Cox, D. Yang, K. Ravid, Physiological implications of adenosine receptor-mediated platelet aggregation, J. Cell. Physiol. 226 (2011) 46–51.
[41] I. Zornetta, P. Caccin, J. Fernandez, B. Lomonte, J.M. Gutierrez, C. Montecucco, Envenomations by *Bothrops* and *Crotalus* snakes induce the release of mitochondrial alarmins, PLoS Negl. Trop. Dis. 6 (2012) e1526.