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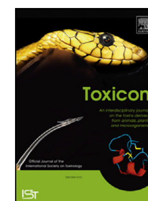

Anthelmintic effects of a cationic toxin from a South American rattlesnake venom

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ABSTRACT

Despite the unquestionable importance of the highly cationic feature of several small polypeptides with high content of positively charged amino acids for their biological activities, positively charged peptides do not necessarily have the capacity to cross the cell membranes. Interestingly, we found that crotamine, a positively charged amphiphilic peptide from the South American rattlesnake venom, has a unique cell-penetrating property with affinity for acidic vesicles, besides a well-characterized antimicrobial and antitumoral activities. In spite of a remarkable *in vitro* antifungal activity of crotamine against *Candida* spp., no significant effect of this peptide could be observed in the course of *Candida albicans* and *Candida krusei* infection on *Caenorhabditis elegans* asssed *in vivo*. These experiments, in which the nematode *C. elegans* was used as a living host, suggested, however, the potential anthelmintic activity of crotamine because of its uptake by the worms and accumulation in their acidic compartments. As described in the present work, this lysosomotropic property is consistent with a previously proposed mechanism of toxicity of crotamine on mammalian tumoral cell lines. This study also allowed us to propose the cationic peptides with lysosomotropic property, as crotamine, as a potential new class of anthelmentics with ability to overcome the challenging problems of drug resistance.

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1. Introduction

Crotamine is a small cationic peptide originally found in the venom of the South American rattlesnake *Crotalus durissus terrificus* (Gonçalves and Polson, 1947; Gonçalves and Vieira, 1950; Gonçalves and Arantes 1956). This polypeptide is composed by 42 amino acid residues, from which 11 are Arg/Lys residues, giving it a characteristic positive net charge with a pI of about 9.5. All these basic residues are exposed in the native crotamine surface, supported by the presence of three disulfide bonds that also confer a highly stable 3D structure for this molecule (Fadel et al., 2005; Nicastro et al., 2003). The presence of two acidic and several neutral residues also provides to crotamine an amphipatic structure, which was shown to confer several of its biological effects, including antimicrobial, antimalarial and antitumoral activities (Nascimento et al., 2007; Hayashi et al., 2008, 2012; Yamane et al., 2013; Costa et al., 2014; El Chamy Maluf et al., 2015). These activities are mostly dependent on the ability of crotamine to target acidic vesicles such as lysosomes (Nascimento et al., 2007; Hayashi et al., 2008).

Lysosomal vesicles are small organelles, whose acidic lumen has a pH around 5, which is lower than that of the cytosol (about pH 7.2). Nematode lysosomes harbor about 50–60 acidic hydrolases, which are responsible for intracellular digestion processes and also for apoptosis mechanisms (Voer et al., 2008; Hindle et al., 2011; Pierzyńska-Mach et al., 2014), as similarly described for mammalian lysosomes (Villamil et al., 2014; Appelqvist et al., 2013; Aits and Jäättelä, 2013). Clokey and Jacobson (1986) were the first to identify

Abbreviations: AO, acridine orange; Cy3, cyanine 3 dye; GFP, green fluorescent protein; NGM, nematode growth medium; LB, luria bertani; BHI, brain heart infusion; DIC, differential interference contrast; CHO, Chinese hamster ovary.

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lysosomes in *Caenorhabditis elegans* worms. Transgenic worms expressing a green fluorescent protein (GFP)-fused lysosomal reporter protein confirmed the presence of these acidic vesicles in adult worms (Clokey and Jacobson, 1986; Treusch et al., 2004), which can also be stained by lysosomal fluorescent markers, *e.g.* acridine orange (AO) or lysotracker (Voer et al., 2008).

Anthelmintic drugs have been used to control human and veterinary infections caused by worms such as trematoda, cestodes and nematodes. They act on targets such as the nicotinic acetylcholine receptor, the GABA receptor, glutamate-modulated chloride channels, voltage-dependent Ca^{+2} channels, β-tubulin, mitochondrial complexes I and II, and cathepsin B (Rana and Misra-Bhattacharya, 2013; Taylor et al., 2013; Holden-Dye and Walker, 2014). However, an important concern is the increasing drug resistance of the worms to the currently very limited range of commercially available anthelmintics (Matthews, 2014; Lanusse et al., 2014; Geary et al., 2010; Kaplan, 2004).

Studies using parasitic nematodes are considerably difficult due to the necessity of the presence of the host organism for parasite maintenance. For this reason, the free living nematode *C. elegans* has been widely employed as a model for new anthelmintic identification, albeit it is important to take into account the recognized differences in the physiology of the parasites and the free-living nematodes (Geary and Thompson, 2001; Holden-Dye and Walker, 2012; Taylor et al., 2013; Kumarasingha et al., 2014).

Consistent with the ability of crotamine to target acidic vesicles in other species (Nascimento et al., 2007; Hayashi et al., 2008; El Chamy Maluf et al., 2015), in the present work we found accumulation of the peptide in the lysosomes of the worms. These findings provide insights into the identification of a novel class of potential anthelmentics capable of overcoming the challenging drug resistance problems described for these parasites, due to its unique the mechanism of action based on lysosomotropic properties.

2. Materials and methods

2.1. Purification of native crotamine from snake venom

The crude venom of *C. durissus terrificus* was extracted from snakes kept in the serpentarium of the Faculdade de Medicina de Ribeirão Preto, São Paulo University - Ribeirão Preto (USP-RP). The crotamine was prepared and purified according to the procedure described by Hayashi et al. (2012), and was kindly provided by Dr. Eduardo B. Oliveira (authorization of access to genetic resources No. 010426/2010 COAPG/DABS/CNPq, term of concession No. 20100104268).

2.2. Caenorhabditis elegans

2.2.1. Cultivation and manipulation of C. elegans worms

Wild-type N2 Bristol hermaphrodites were kept in petri dishes containing nematode growth medium (NGM, composed by 3.0 g NaCl, 2.5 g bactopeptone, 17 g agar, autoclaved and supplemented with 24 mL phosphate buffer, 1 mL 1 M $CaCl_2$, 1 mL 1 M $MgSO_4$, and 1 mL 5 mg/mL cholesterol dissolved in ethanol) and fed with OP50-1 wild type *E. coli*. The bacteria was grown overnight at 37 °C in Luria Bertani (LB) medium containing 100 μg/μL streptomycin, concentrated 10-fold in the following day, and streaked onto the NGM plates, on which the worms were subsequently maintained.

2.2.2. Synchronization of worms

We used synchronized worms for our biological tests. To this end, plates containing pregnant hermaphrodites were washed with 3 mL of M9 medium (Na_2HPO_4 6.0 g, KH_2PO_4 3.0 g, NaCl 5.0 g, $MgSO_4$ 0.25 g for a final volume of 1 L) and the worms were transferred to a Falcon tube. The suspension of worms was added 500 μL of 5 M NaOH and 1 mL of 2.5% sodium hypochlorite. The suspension was mixed on vortex for 10 min until complete dissolution of the worms. The suspension containing the eggs was then centrifuged at 1800 × g for 1 min, and the pellet was washed with M9 medium, re-centrifuged and plated over NGM dishes containing OP50-1 bacteria.

2.2.3. C. elegans survival assay

Wild-type N2 Bristol hermaphrodites at L2 stage were transferred to 96-wells plate containing 98 μL of M9 medium (20% BHI broth, 80% M9 medium, 10 μg/mL colesterol, 90 μg/mL kanamicin) and increasing concentrations of native crotamine (5, 10, 20 and 200 μM) in 2 μL of sterile Milli-Q water 18Ω (control) were added per well. Ten worms were added to each well in triplicate, and worm survival was measured for the following 3 days. Survival analysis was performed with the aid of a platinum loop that was used to stimulate the worm. Lack of movement confirmed that the animal was dead.

2.2.4. Crotamine intake by C. elegans worms

As a first approach to investigate the main route through which crotamine was absorbed by the worms, we studied the colocalization of fluorescently labeled crotamine (Cy3-crotamine) and OP50 *E. coli* bacteria with constitutive expression of reporter Green Fluorescent Protein (OP50-GFP). Wild-type hermaphrodites were plated after synchronization (as described above) onto previously prepared dishes containing solid NGM and OP50-GFP bacteria. The worms were then monitored until they reached the L4 larval stage, and then, they were incubated in liquid M9 medium containing 20 μM of Cy3-crotamine for 24 h, at 20 °C. After incubation, worms were washed in M9 medium to remove the excess of labeled crotamine from the worm cuticle. The nematodes were then analyzed by Leica TCS SP8 confocal microscopy (Leica Microsystems, Wetzlar, Germany), using oil immersion objective of 20 ×, 40 × and 63 × (as detailed in the legends), at wavelengths λ_{EX} 548 nm and λ_{EM} 562 nm for Cy3-crotamine (red), and λ_{EX} 488 nm and λ_{EM} 530 nm for GFP (green).

2.2.5. Staining with acridine orange (AO)

In vivo labeling with AO was performed using wild type *C. elegans* worms, essentially as described in the protocol found in Methods in Cell Biology [www.wormbook.org, as described by Gumienny et al. (1999) and Lettre et al. (2014)], with some modifications. L4/young adult hermaphrodites were incubated for 6 h at 20 °C in the dark in 96-wells plates containing AO diluted in M9 medium (0.1 mg/mL). There were two groups: (1) the control group that was incubated only with the AO (0.1 mg/mL) and (2) the experimental group that was incubated with AO (0.1 mg/mL) and 20 μM of native crotamine. These worms were then analyzed by confocal microscopy and quantified by fluorimetry.

2.2.5.1. Imaging analysis of AO staining by confocal microscopy. For confocal microscopy analysis, the worms were prepared as described above and placed onto microscope slides with agarose pads as described in Methods in Cell Biology (www.wormbook.org), with some adaptations. The agarose pad was prepared with 2.5% agarose dissolved in M9 medium, and the slides were kept in a moist chamber until use.

The worms were then washed with M9 medium to remove the excess of AO and/or crotamine, and placed for a few minutes on a plate with NGM containing wild type OP50-1. These worms were finally immobilized with a drop of 0.75% sodium azide and placed onto agarose pads on glass slides. The slides were mounted with a coverslip and sealed with silicone before the imaging analysis using

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