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Melittin peptide kills *Trypanosoma cruzi* parasites by inducing different cell death pathways

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ABSTRACT

Antimicrobial peptides (AMPs) are components of the innate immune response that represent desirable alternatives to conventional pharmaceuticals, as they have a fast mode of action, a low likelihood of resistance development and can act in conjunction with existing drug regimens. AMPs exhibit strong inhibitory activity against both Gram-positive and Gram-negative bacteria, fungi, viruses, metazoans and other parasites, such as the protozoan *Leishmania*. Melittin is a naturally occurring AMP, which comprises 40–50% of the dry weight of *Apis mellifera* venom. Our group has recently shown that crude *A. mellifera* venom is lethal to *Trypanosoma cruzi*, the Chagas disease etiologic agent, and generates a variety of cell death phenotypes among treated parasites. Here, we demonstrate that the melittin affected all of *T. cruzi* developmental forms, including the intracellular amastigotes. The ultrastructural changes induced by melittin suggested the occurrence of different programmed cell death pathways, as was observed in *A. mellifera*-treated parasites. Autophagic cell death appeared to be the main death mechanism in epimastigotes. In contrast, melittin-treated trypomastigotes appeared to be dying via an apoptotic mechanism. Our findings confirm the great potential of AMPs, including melittin, as a potential source of new drugs for the treatment of neglected diseases, such as Chagas disease.

1. Introduction

Chagas disease is recognized by the World Health Organization (WHO) as one of the 13 most neglected tropical diseases in the world. This lifelong infection is caused by the protozoan parasite *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae) and was discovered in 1909 by the Brazilian physician Carlos Chagas (1879–1934) (Coura and Viñas, 2010). The geographical distribution of Chagas infection, including its reservoirs and vectors, extends from the Southern United States to Southern Argentina and Chile. According to estimates by the Pan American Health Organization and the WHO, 7.7 to 10 million people are chronically infected with *T. cruzi*, and 10,000 to 14,000 deaths per year are attributed to Chagas disease (Rassi et al., 2012).

The parasite is transmitted to man by the bite of the insect vector (Hemiptera: Reduviidae) and by non-vectorial mechanisms, such as blood transfusions, placental or birth canal transmission, organ transplants, the ingestion of

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contaminated food or liquid, the management of infected animals, and laboratory accidents (Moncayo and Silveira, 2009). Chagas disease has become a global illness due to the migration of people from Latin American endemic countries to non-endemic countries, including Canada, Spain, France, Japan and Australia (Coura and Viñas, 2010; Schmunis and Yadon, 2010). Beyond congenital transmission, these countries have little experience with Chagas disease with regards to blood donor surveillance and medical care for Chagas patients (Coura and Viñas, 2010; Schmunis and Yadon, 2010).

At present, there are only two effective drugs for the treatment of acute and early chronic phase Chagas patients: benznidazole and Nifurtimox. However, neither of these therapeutics meet the following World Health Organization criteria for an optimal drug such as (i) parasitological cure of the acute and chronic phases of the infection; (ii) effective with a single dose or with few doses; (iii) no side effects or teratogenic effects, among others (Coura, 2009). In fact, these two nitroheterocycle drugs are limited in that they are highly toxic and rarely beneficial during the chronic phase of the disease; moreover, these treatments only cure approximately 20% of all patients (Urbina and Docampo, 2003). These restrictions highlight the necessity for developing alternative synthetic or natural compounds that are effective for both the clinical treatment of Chagas disease and for the chemoprophylaxis of donated blood.

Antimicrobial peptides (AMPs), which are a component of innate immunity, are ancient evolutionary weapons. They have been isolated from virtually every kingdom and phylum, which attests to their role as a mechanism of the primitive immune response (Andreu and Rivas, 1998). They are a unique and diverse group of molecules, and they have been divided into subgroups on the basis of their amino acid composition and structure. AMPs are diverse in length, overall charge, and conformation, but a large majority of these molecules are cationic and amphipathic (Yeaman and Yount, 2003). They are defined as peptides of 12–50 amino acids in length, with a molecular mass of less than 10 kDa and a net positive charge ranging from +2 to +7 due to an excess of basic amino acids (arginine, lysine and histidine) over acidic amino acids (aspartate and glutamate). Generally, 50% or more of the AMP amino acids are hydrophobic, a fact reflected by the interaction of such peptides with bacterial membranes as part of their mechanism of action (Hancock and Diamond, 2000; Teixeira et al., 2012). AMPs display certain features that make them appealing as alternatives to conventional pharmaceuticals, including their fast mode of action, low likelihood of resistance development and ability to act in conjunction with existing drug regimens (Zasloff, 2002). AMPs show a high level of toxicity against both Gram-positive and Gram-negative bacteria, as well as fungi, viruses, metazoans, other parasites, and even cancer cells (Hoskin and Ramamoorthy, 2008; Zasloff, 2002). McGwire and Kulkarni (2010) and Harrington (2011) have described the AMPs and synthetic derivatives that are active against the related kinetoplasts *T. cruzi*, *Leishmania* spp., and African trypanosomes. The largest group of AMPs currently known consists of the linear cationic α-helical peptides; more than 300 members have been described thus far, and melittin is among the most represented AMPs (Yeaman and Yount, 2003).

Melittin is a naturally and cytolytic occurring AMP, which is a highly basic 26-residue peptide that is almost entirely hydrophobic but with a hydrophilic sequence (Lys-Arg-Lys-Arg) near the C-terminus; with a 2846.46 of molecular weight, and which comprises 40–50% of *Apis mellifera* venom dry weight (Habermann, 1972). It is water-soluble and its aggregation of monomeric melittin to a tetramer is promoted by high salt, high melittin concentration, and high pH (Raghuraman and Chattopadhyay, 2007). There is substantial evidence that melittin can permeabilize cell membranes by inducing pore formation and lyse prokaryotic and eukaryotic cells in a non-selective manner (Raghuraman and Chattopadhyay, 2007; Papo and Shai, 2003). This mechanism of action is responsible for the hemolytic, anti-microbial (Bechinger, 1997; Blondelle and Houghten, 1991; Chicharro et al., 2001; Díaz-Achirica et al., 1998; Lazarev et al., 2002; Luque-Ortega et al., 2003; Pérez-Cordero et al., 2011; Tosteson et al., 1985) and anti-tumor (Holle et al., 2009; Li et al., 2006; Winder et al., 1998) activities of melittin.

The melittin peptide has been shown to exhibit strong inhibitory activity against the protozoan parasite *Leishmania* (Akuffo et al., 1998; Pérez-Cordero et al., 2011). Interestingly, it has been shown that cecropin A–melittin hybrid peptides present remarkable leishmanicidal activity with minimal cytotoxic activity against host cells (Chicharro et al., 2001; Díaz-Achirica et al., 1998; Luque-Ortega et al., 2003) even *in vivo* (Alberola et al., 2004; Luque-Ortega et al., 2001). Thus far, only three studies have shown the lytic effects of melittin on *T. cruzi* epimastigotes and trypomastigotes (Azambuja et al., 1989; Jacobs et al., 2003; Fieck et al., 2010). However, none of these studies investigated the effects of mellitin on parasite morphology, including the cell death phenotype. Furthermore, only the study by Jacobs et al. (2003) considered the effects of melittin on host cells, where it was shown to be non-toxic to glioblastoma cells.

Recently, our group showed that *A. mellifera* crude venom could affect the viability and ultrastructure of all *T. cruzi* developmental forms, including the intracellular amastigotes, at concentrations that were approximately 100-fold lower than those required to cause toxicity in mammalian cells (Adade et al., 2012). Interestingly, the venom-treated parasites exhibited different programmed cell death pathways; autophagic cell death appeared to be the predominant death mechanism in epimastigotes, whereas venom-treated trypomastigotes appeared to undergo apoptotic cell death. In the present work, we (i) investigated our hypothesis that the melittin component of *A. mellifera* venom was responsible for parasite damage and for the different cell death profiles observed in epimastigotes and trypomastigotes and (ii) more carefully examined the effects of melittin on the growth of all *T. cruzi* developmental forms, including the intracellular amastigotes. Because melittin is considered to be unsuitable for therapeutic use due to its hemolytic activity and because previous reports have shown that this toxicity is decreased when melittin is used as a hybrid with another AMP, we (iii) tested the toxicity of the peptide against epithelial cells and