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Synergistic combination of alkylphosphocholines with peptaibols in targeting *Leishmania infantum in vitro*

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ABSTRACT

Anti-leishmanial treatment increasingly encounters therapeutic limitations due to drug toxicity and development of resistance. The effort for new therapeutic strategies led us to work on combinations of chemically different compounds that could yield enhanced leishmanicidal effect. Peptaibols are a special type of antimicrobial peptides that are able to form ion channels in cell membranes and potentially affect cell viability. We assayed the antileishmanial activity of two well studied helical peptaibols, the 16-residue antiamoebin and the 20-residue alamethicin-analogue suzukacillin, and we evaluated the biological effect of their combination with the alkylphosphocholine miltefosine and its synthetic analogue TC52. The peptaibols tested exhibited only moderate antileishmanial activity, however their combination with miltefosine had a super-additive effect against the intracellular parasite (combination index 0.83 and 0.43 for antiamoebin and suzukacillin respectively). Drug combinations altered the redox stage of promastigotes, rapidly dissipated mitochondrial membrane potential and induced concatenation of mitochondrial network promoting spheroidal morphology. These results evidenced a potent and specific antileishmanial effect of the peptaibols/miltefosine combinations, achieved with significantly lower concentrations of the compounds compared to monotherapy. Furthermore, they revealed the importance of exploring novel classes of bioactive compounds such as peptaibols and demonstrated for the first time that they can act in synergy with currently used antileishmanial drugs to improve the therapeutic outcome.

1. Introduction

Leishmaniases are devastating human diseases of grossly underestimated public health impact. They are endemic in 88 countries worldwide with an estimate of 2 million new cases occurring annually (1.5 million cases of cutaneous leishmaniasis and 500.000 of visceral leishmaniasis) and about 12 million people currently infected (Desjeux et al., 1991). The responsible pathogens for these diseases belong to genus *Leishmania* and are unicellular parasites with digenetic life cycles: the extracellularly living promastigote that develops in the sandfly vector, and the intracellular amastigote that resides in the mammalian host cells. Human leishmaniases can manifest as cutaneous (CL), mucocutaneous (MCL) or visceral (VL), the latter being the most life threatening form if left untreated (Chappuis et al., 2007; David and Craft, 2009). VL can cause large-scale and tenacious epidemics, with high case–fatality rates. Children under the age of 15 years are the most severely affected group. Domestic dogs are the principal animal reservoir for the infection.

Although the *Leishmania* genome has been unraveled and the immunology of the disease is well characterized, an effective vaccine has not yet been discovered, rendering chemotherapy the only treatment. Besides the prohibitive cost for many endemic areas of the currently available drugs, their clinical efficacy is seriously jeopardized by severe side effects as well as the emergence of resistant parasites. All these factors point towards the necessity for the discovery of alternative treatments (Mishra et al., 2007).

The discovery of the antileishmanial activity of hexadecylphosphocholine (miltefosine, HePC), initially developed as an antitumor agent, constituted a major breakthrough in antileishmanial chemotherapy (Croft et al., 1987). HePC is effective against both VL and CL, is orally administered and displays good bioavailability (Croft et al., 2005; Croft, 2008). Moreover, it has been approved by the US FDA in

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2014 for the treatment of all forms of the disease. The cell membrane appears to be the primary site of HePC activity, due to interference with lipid metabolism and lipid-dependent signal transduction (Lux et al., 1996; Lira et al., 2001; Dorlo et al., 2012). The mitochondrion of trypanosomatids is a possible target of lipid compounds that induce an impairment of the energy production and modulation of the redox stage of the cell (Santa-Rita et al., 2004; Murray et al., 2014).

However, despite its advantages, HePC has a long half-life (100–200 h) and a low therapeutic ratio in humans, characteristics that encourage development of resistance. Moreover, teratogenesis (Herwaldt, 1999) and diminished efficacy of the compound when administered to HIV-coinfected patients (Sindermann et al., 2004) or against the wide range of CL syndromes, are important issues that have to be addressed. Finally, the use of HePC against kala-azar in Europe, prognosticate an increase in resistance and rapid obsolescence of this drug (Berman et al., 2006). One potential source of new therapeutic agents is the vast and diverse biological repertoire of antimicrobial peptides (AMPs). These 10–50 residues-long polypeptides constitute essential components of the innate immune systems of organisms from all Kingdoms (Hancock and Diamond, 2000). Recently, AMPs were used in clinical trials for systemic as well as topical treatment of bacterial infections (Ashby et al., 2014). They have been also proved effective antileishmanial agents (Gaidukov et al., 2003; Guerrero et al., 2004; Mangoni et al., 2005). Unlike antibiotics which target specific cellular activities, AMPs target cell membranes by altering fluidity or compromising the bilayer integrity by forming channels and facilitating the flux of cellular constituents (Teixeira et al., 2012). However the vulnerability of these peptides to either host- or parasite-derived proteases, raised some concerns regarding their adequacy as antileishmanial drugs (Kulkarni et al., 2006).

Of medical interest in this fast-growing field are a group of non-ribosomally synthesized AMPs termed peptaibols, representing a subgroup of bioactive peptides termed comprehensively peptaibiotics (https://peptaibiotics-database.boku.ac.at/django). The majority comprises linear peptides of 15–20 residues, defined as *N*-acetylated **pep**tides containing **Aib** (α-aminoisobuyric acid; 2-methylalanine) and a C-terminal bound 1,2-amino alcoh**ol** such as phenylalaninol. Due to their high content in sterically constrained Aib residues, they are incompatible as protease substrates (Bruckner et al., 1984). Peptaibols have attracted much attention due to their broad range of bioactivities which include growth inhibition of bacteria, fungi, protozoa and possibly helminthes (Szekeres et al., 2005). Furthermore, their insecticidal action on mosquito larvae has been reported (Matha et al., 1992).

The biological activity of peptaibols is attributed to their membrane modifying properties, and specifically to the formation of channels that result in leakage of cytoplasmic material, ultimately leading to cell death (Boheim, 1974; Balaram et al., 1992; Peltola et al., 2004; Shi et al., 2010). Furthermore, peptaibols seem to cause pathological changes to the ultrastructure of mitochondria (Reed and Lardy, 1975; Bruckner and Toniolo, 2013). It has been shown that the peptaibol AAM exhibits trypanocidal activity in a mouse model for trypanosomiasis (Kumar et al., 1991), effect attributed to the channel-forming capacity that dissipates the parasite mitochondrial membrane potential, or alters the parasite's plasma membrane integrity (Nagaraj et al., 2001).

In our previous studies on structure-activity relationships of alkylphospholipids we described miltefosine derivatives substituted by rings of various sizes in the lipid portion and/or the head group, which have exhibited improved antileishmanial activity and reduced toxicity with respect to the prototype approved drug HePC (Avlonitis et al., 2003; Calogeropoulou et al., 2008; Papanastasiou et al., 2010; Godinho et al., 2013).

In this report we describe the leishmanicidal effect of two peptaibols, antiamoebin (AAM) and suzukacillin (SZ). We determined their activity against the promastigote and intracellular amastigote form of the parasite as well as their cytotoxicity and hemolytic activity. We assessed their effect on the physiology of the mitochondrion in the promastigote by measuring Reactive Oxygen Species (ROS) production, mitochondrial membrane potential ($\Delta\Psi$m) alteration and recording morphological changes. Finally we evaluated the synergistic effect of combining suboptimal concentrations of these peptaibols with HePC, or with its less active synthetic triasolyl-substituted alkylphosphocholine (APC) analogue TC52, against the intracellular amastigote.

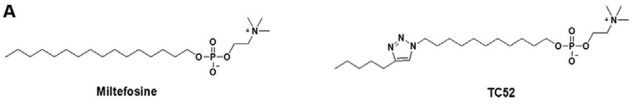
2. Materials and methods

2.1. Parasite and cell culture

A field strain of *L. infantum* was isolated from the spleen of an infected dog which was euthanized after advanced kala-azar disease diagnosis. Briefly, the spleen was cleaned by washing in sterile PBS (Invitrogen, Grand Island, NY, USA) and 70% ethanol (Sigma Aldrich Inc, St Louis, USA). Then a small piece was carefully dissected and homogenized in RPMI, supplemented with 10% FBS (decomplemented at 56 °C for 30 min) and 2% penicillin/streptomycin. The homogenized solution was placed in a Falcon flask (Nunc, Denmark, Roskild) and was incubated at 26 °C, until promastigotes of the parasite were visible. The human monocytic THP-1 cell line was also cultured in RPMI containing FBS and antibiotics as indicated above, and was maintained at 37 °C in a humidified atmosphere containing 5% CO_2. All cell culture media were obtained from Invitrogen and cell culture flasks and plates were purchased from Nunc and Costar (Cambridge, MA).

2.2. Ether phospholipids and peptides

HePC and the analogue TC52, a 1,2,3-triazolyl-substituted derivative ({2-[11-(4′-pentyl-(1′,2′,3′)triazol-1-yl)undecylphosphinyloxy] ethyl} N,N,N trimethylammonium inner salt) were synthesized in house (manuscript on synthesis and activity of the analogue, in preparation) (Fig. 1A). AAM was isolated from the fermentation broth of *Emericellopsis synnematicola* CBS 176.60 (Jaworski and Bruckner, 2000) and suzukacillin A (SZ-A) from *Trichoderma viride* C1 (Krause et al., 2006). Both peptides represent microheterogenous mixtures, distinguished by limited exchange of a single or few amino acids. However, major component of AAM preparation is antiamoebin I and of SZ-A is SZ-A4 (Fig. 1B). Stock solutions were prepared in DMSO (Sigma) and preserved at −20 °C.



B

Antiamoebin I

Ac-Phe[1]-Aib-Aib-Aib-*D*-Iva-Gly-Leu-Aib-Aib[9]-Hyp-Gln-*D*-Iva-Hyp[13]-Aib-Pro-Pheol[16]

Suzukacillin-A4

Ac-Aib[1]-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Lxx-Aib-Gly-Aib-Aib[13]-Pro-Vxx-Aib-Vxx-Gln-Gln-Pheol[20]

Alamethicin I

Ac-Aib[1]-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib[13]-Pro-Val-Aib-Aib-Glu-Gln-Pheol[20]

Fig. 1. A. Chemical structures of Miltefosine and its analogue TC52. **B.** Sequences of AAM I (major sequence) and SZ-A4 in comparison to alamethicin I (= alamethicin F30/3). Abbreviations: Ac, acetyl; Phe, phenylalanine; Ala, alanine; Aib, α-aminoisobutyric acid (2-methylalanine); Iva, isovaline (2-ethylalanine); Gly, glycine; Lxx, leucine (Leu) or isoleucine (Ile); Vxx, valine (Val) or isovaline (Iva); Hyp, *trans*-4-hydroxyproline; Pro, proline; Gln, glutamine; Glu, glutamic acid; Pheol, L-phenylalaninol. Chiral amino acids and Pheol are of the L-configuration with the exception of *D*-Iva.