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In vitro anti-*Acanthamoeba* synergistic effect of chlorhexidine and cationic carbosilane dendrimers against both trophozoite and cyst forms

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ABSTRACT

Acanthamoeba sp. are the causative agents of severe illnesses in humans such as *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE). Medical therapy is not yet well established. Treatments of AK last for several months and generate toxicity, resistances appear due to the cysts stage and recurrences can occur. In this study has been demonstrated that the combination of chlorhexidine digluconate (CLX) and carbosilane dendrimers containing ammonium or guanidine moieties has *in vitro* synergistic effect against *Acanthamoeba polyphaga*. This synergy provokes an important reduction in the minimal trophozoite amoebicidal concentration (MTAC) of CLX, which means a reduction of their toxic effects on human cells. Moreover, some CLX/dendrimer combinations show important activity against the cyst resistance stage.

1. Introduction

Amoebae fro`m *Acanthamoeba* genus are opportunistic pathogens of humans and animals (Radford et al., 2002; Schuster and Visvesvara, 2004) that have been isolated from multitude of habitats (Khan, 2015). As human pathogens, these protozoa are responsible for severe and painful illness (Khan, 2006; Kumar and Lloyd, 2002; Marciano-Cabral and Cabral, 2003). These organisms have two stages in their life cycle: the mobile and infective stage, trophozoite; and the resistant stage, cyst, which occurs under unfavorable environmental conditions and in some cases in the presence of certain concentrations of chemical compounds. Therefore the encystment stage causes the main problems in the treatment of amoeba infections because many biocides available are ineffective in killing them due to its high resistance (Kumar and Lloyd, 2002; Thomas et al., 2010). *Acanthamoeba* sp. are the causative agents of *Acanthamoeba* keratitis (AK), a painful disease of the eye specially associated with the wearing of contact lenses, and chronic granulomatous amoebic encephalitis (GAE), a fatal disease of the central nervous system in immunosuppressed patients (Khan, 2006; Kumar and Lloyd, 2002; Marciano-Cabral and Cabral, 2003). AK infections can be treated with two biguanides, polyhexamethylene biguanide (PHMB) or chlorhexidine digluconate (CLX), in combination with other agents. (Polat and Vural, 2012; Seal, 1996). However, treatments last for several months (6–12 months), are arduous (initially require hourly application during day and night) and can generate additional problems due to the toxicity of the drugs employed or the appearance of resistances by the cyst stage. Finally it must be mentioned that these treatments are not totally effective and studies to assess alternative agents are needed (Khan, 2006; Martín-Navarro et al., 2008; Schuster and Visvesvara, 2004).

An important advance in the treatment of AK has been the use of association therapy or combination therapy by employing different drugs (Clarke et al., 2012). Topical therapies usually involve a biguanide (PHMB or CLX at 0.02%, 200 ppm) in combination with a diamidine (propamidine or hexamidine at 0.1%, 100 ppm) initially at a high frequency. There have been

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different combinations reported such as PHMB-Propamidine (Duguid et al., 1997), PHBM-hexamidine, CLX-hexamidine or also a double biguanide action PHBM-CLX (Ferrari et al., 2011). Also adjuncts to biguanide and diamidine therapy have been reported like CLX-oral ketoconazole-topical steroids (Mathers, 2006), PHMB-propamidine-neosporin (Thebpatiphat et al., 2007), or miltefosine-PHMB (Polat et al., 2014). All these combinations have improved treatment outcome, nevertheless problems related to toxicity of the drugs and resistance are still present (Dart et al., 2009). Therefore, the importance of testing new drugs against these organisms is crucial.

In this context, cationic polymers have been developed as possible agents with antimicrobial activity, low toxicity toward human cells (Carmona-Ribeiro and de Melo-Carrasco, 2013; Muñoz-Bonilla and Fernández-García, 2012; Xue et al., 2015) and their activity being related with the multivalency of the systems. Their main target is the plasmatic membrane in eukaryotic cells and the cytoplasmic membrane in bacteria, due to the cationic nature of the macromolecules (Chen and Cooper, 2002; McDonnell and Russell, 1999). One type of these macromolecules are dendrimers, which are highly branched monodisperse macromolecules with well-defined globular shape. The presence of high density of active cationic surface groups at their periphery have shown potential as antibacterial agents (Chen et al., 2000; Fuentes-Paniagua et al., 2016; Lazniewska et al., 2012; Mintzer et al., 2012) or as amoebicidal agents against *Acanthamoeba* trophozoites and cysts (Heredero-Bermejo et al., 2015, 2013).

The main purpose of this work is to reduce the problems associated to the CLX treatments, centred on their *in vitro* and *in vivo* toxicity (de Souza et al., 2007; Rahman et al., 1998) and the inability to eliminate the cyst forms completely (Mafra et al., 2013) within the biocompatibility threshold both in mono and combination therapy. With this aim, we have evaluated the combination of chlorhexidine digluconate (CLX) with cationic carbosilane dendrimers as new alternative against trophozoites and cysts of a pathogenic strain of *Acanthamoeba polyphaga*. The dendrimers employed are first generation derivatives, since low generation compounds have shown the best relationship between amoebicidal activity and toxicity (Fuentes-Paniagua et al., 2014; Heredero-Bermejo et al., 2015). With respect to peripheral functions of dendrimers, the groups chosen are ammonium, due to the reasons described above, and guanidine, as this last group is present in CLX.

2. Materials and methods

2.1. General considerations

Solvents (HPLC grade) were used as received. NMR spectra were recorded on a Varian Unity VXR-300 (300.13 (^{1}H), 75.47 (^{13}C) MHz) or on a Bruker AV400 (400.13 (^{1}H), 100.60 (^{13}C), 79.49 (^{29}Si) MHz). Chemical shifts (δ) are given in ppm. ^{1}H and ^{13}C resonances were measured relative to solvent peaks considering TMS = 0 ppm, meanwhile ^{15}N and ^{29}Si resonances were measured relative to external MeNo and TMS, respectively. When necessary, assignment of resonances was done from HSQC, HMBC, COSY and TOCSY NMR experiments. Elemental analyses were performed on a LECO CHNS-932. Mass Spectra were obtained from a Bruker Ultraflex III for MALDI-TOF in dithranol, an Agilent 6210 TOF LC/MS for ESI-TOF in MeOH/H_2O with $(NH_4)(HCO_2)$, and an AB Sciex QSTAR for ESI-POS in H_2O/MeOH. Compounds 1H-pyrazole-1-carboxamidine hydrochloride, diisopropylethylamine (DIPEA) and chlorhexidine digluconate (CLX) were obtained from commercial sources. Compound $G1O_3(S\text{-}NH_3Cl)_6$ (**1**) was synthesized as published (Fuentes-Paniagua et al., 2014).

2.2. Synthesis of $G1O_3(S\text{-}GU\text{-}Cl)_6$ (2) ($GU = NHC(NH_2)NH_2^+$)

A suspension of **1** (0.342 g, 0.270 mmol), 1H-pyrazole-1-carboxamidine hydrochloride (0.359 g, 2.450 mmol) and DIPEA (6.57 mmol) in EtOH was heated overnight at 55 °C in a teflon valved ampoule, under inert atmosphere. Next, volatiles are removed under vacuum. Afterward, the solution was washed with MeOH/acetone twice, rendering **2** as a pale yellow solid very hygroscopic (0.325, 83%). Also, the wax can be solved in water, dialyzed (MWCO = 500), and finally lyophilized to give **2** with similar yield.

Data for **2**: ^{1}H NMR (D2O): δ −0.03 (s, 9H, Si*Me*), 0.48 (m, 6H, O$(CH_2)_3CH_2$Si), 0.84 (m, 12H, SiCH_2CH_2S), 1.25 (m, 6H, O$(CH_2)_2CH_2$), 1.31 (m, 6H, OCH_2CH_2), 2.56 (m, 12H, CH_2S), 2.72 (m, 12H, SCH_2), 3.37 (m, 12H, CH_2NH), 3.74 (m, 12H, OCH_2), 5.93 (m, 3H, C_6H_3); ^{13}C NMR (D2O): δ −2.4 (Si*Me*), 10.8 (O$(CH_2)_3CH_2$Si), 17.2 (SiCH_2CH_2S), 18.3 (O$(CH_2)_2CH_2$), 24.2 (CH_2S), 27.3 (SCH_2), 29.8 (OCH_2CH_2), 39.1 (CH_2NH), 93.4 (CH, C_6H_3), 156.7 (NCN), 160.7 (C_{ipso}, C_6H_3), 15N NMR (D2O): δ −303.5; 29Si NMR (D2O): δ 2.8. Elemental analysis ($C_{51}H_{114}Cl_6N_{18}O_3S_6Si_3$, FW 1516,93): Teor (%): C, 40.38; H, 7.57; N, 16.62; S, 12.68. Obt (%):, 41.02; H, 7.11; N, 16.20; S, 12.29. MS (uma): $[M\text{-}4H\text{-}6Cl]^{2+}$ = 649.33; $[M\text{-}3H\text{-}6Cl]^{3+}$ = 433.22; $[M\text{-}2H\text{-}6Cl]^{4+}$ = 325.17.

2.3. Acanthamoeba *strain: trophozoites and cysts*

Acanthamoeba polyphaga 2961 (a clinical isolate kindly supplied by Dr. E. Hadas, Poznan University of Medical Sciences, Poland) was grown in 25-cm^2 flasks containing 5 ml of peptone–yeast extract–glucose medium supplemented with 2% Bactocasitone (PYG-B) (Heredero-Bermejo et al., 2012), and incubated at 32 °C.

Cysts were obtained by culturing 3–4 days trophozoites in non-nutrient Neff's Encystment Medium (NEM: 0.1 M KCl, 8 mM $MgSO_4{\cdot}7H_2O$, 0.4 mM $CaCl_2{\cdot}2H_2O$, 1 mM $NaHCO_3$, 20 mM ammediol [2-amino-2-methyl-1,3-propanediol; Sigma] pH 8.8 at 32 °C) as described previously (Heredero-Bermejo et al., 2015).

2.4. In vitro *drug assays*

Experiments for drug testing on trophozoites were performed in sterile 48-well microtiter plates (NUNC™) and 96-well microtiter plates for cysts. Amoebae from log-phase cultures were resuspended in PYG-B medium at a density of 4×10^5 trophozoites/ml and cysts were resuspended in NEM at a density of 10^5 cysts/ml. 200 mL of the calibrated trophozoite suspension or 100 μl of the cyst suspension were added to each well. Control wells containing trophozoites or cysts received 200 μl or 100 μl of distilled water instead of drug solutions, respectively. Plates were sealed with Parafilm® and incubated at 32 °C for 24 h. Assays were performed in triplicate and were repeated at least twice.

2.5. Trophocidal properties of the drugs

The viability of trophozoites treated for 24 h was assessed by direct counting using the 0.2% Congo red exclusion assay. (Heredero-Bermejo et al., 2015) Samples were placed in a Fuchs–Rosenthal manual counting chamber and trophozoites counted using optical microscopy (Carl Zeiss). The Minimum Trophozoite Amoebicidal Concentration (MTAC) was defined as the lowest concentration of test solution that produced a complete destruction of trophozoites (Elder et al., 1994).

2.6. Cysticidal properties of the drugs

Cyst viability was studied by assessing excystment of samples treated for 24 h. Wells were washed twice with Phosphate Buffered