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In vitro evaluation of antimicrobial agents on *Acanthamoeba* sp. and evidence of a natural resilience to amphotericin B

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ABSTRACT

The free-living amoeba (FLA) *Acanthamoeba* sp. is an opportunistic pathogen that can cause amoebic keratitis (AK) or granulomatous amoebic encephalitis (GAE). While current treatments of AK are long with some relapses, no consensus therapy has been developed for GAE remaining lethal in 90% of the cases. In this context, efficient antiacanthamoebal drugs have to be identified. In this work, 15 drugs used in the treatment of AK or GAE or in other parasitic diseases were evaluated for their *in vitro* activity on *A. castellanii*. Hexamidine, voriconazole and clotrimazole exhibited the highest activities with IC_{50} values at 0.05 μM, 0.40 μM and 0.80 μM, respectively, while rifampicin, metronidazole and cotrimoxazole were inactive. Among 15 drug associations evaluated, no synergistic effect was observed, and one antagonism was determined between hexamidine and chlorhexidine. Interestingly, amphotericin B was the only drug presenting an increase of IC_{50} as a function of treatment duration. The amoebae susceptibility to amphotericin B cultured in the presence of 250 μM of the drug was similar to the one of a naive control, revealing that no resistant strain could be selected. However, the amoebae susceptibility always returned to an initial level at each passage. This natural and non-acquired adaptation to amphotericin B, qualified as resilience, was observed in several strains of *A. castellanii* and *A. polyphaga*. Using a pharmacological approach with effectors of different cellular mechanisms or transports, and an ultrastructural analysis of amphotericin B-treated amoebae, the involvement of several mitochondria-dependent pathways as well as multidrug resistant transporters was determined in amphotericin B resilience. Based on the observations from this study, the relevance of using amphotericin B in GAE treatments may be reconsidered, while the use of some other drugs, such as rifampicin or cotrimoxazole, is not relative to intrinsic antiacanthamoebal activity.

1. Introduction

Free-living amoebae (FLA) are ubiquitous and opportunistic protozoa widely distributed in nature. They can be found in natural and artificial environments such as soil, dust, rivers, or swimming pools, cooling towers of nuclear power plants or drinking water distribution networks (Rodriguez-Zaragoza, 1994; Martinez and Visvesvara, 1997; Schuster and Visvesvara, 2004). These amoebae present two main forms in their life cycle: the vegetative and motile form, called trophozoite, and the latent and non-motile form, called cyst, the latter being responsible of FLA dissemination in nature due to its capacity to resist to hostile environmental conditions. Remarkably, cysts of *Acanthamoeba* sp. have been described to resist to disinfection treatments such as chlorine, radiations, or hydrogen peroxide (Aksozek et al., 2002; Storey et al., 2004). Besides their ability to host diverse pathogenic microorganisms such as viruses, bacteria or fungi and to protect them from environmental conditions (Guimaraes et al., 2016), some FLA, such as *Acanthamoeba* sp., *Naegleria fowleri* or *Balamuthia mandrillaris*, are opportunistic pathogens and can cause severe ocular or cerebral pathologies (Visvesvara et al., 2007). For example, FLA from the genus *Acanthamoeba* can either cause *Acanthamoeba* Keratitis (AK), a painful and sight-threatening corneal infection which can lead to blindness without treatment, or Granulomatous Amoebic Encephalitis (GAE), a severe cerebral infection affecting mostly immunocompromised patients (Schuster and Visvesvara, 2004; Siddiqui et al., 2016).

Currently, the treatment of AK involves membrane-acting

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agents, such as chlorhexidine or polyhexamethylene biguanide, in combination with a diamidine (propamidine or hexamidine) for a period of up to one year, with infection recurrence in 10% of cases (Siddiqui et al., 2016; Carrijo-Carvalho et al., 2017). However, GAE remains lethal in more than 90% of cases, even after treatment with various combinations of drugs involving amphotericin B, rifampicin, cotrimoxazole, miltefosine, pentamidine, flucytosine, pyrimethamine and cotrimoxazole combination but also sterol targeting agents such as ketoconazole, voriconazole or clotrimazole (Schuster and Visvesvara, 2004; Siddiqui et al., 2016; Ong et al., 2017). Among these drugs, amphotericin B is one of the most commonly used for the treatment of GAE infections (Siddiqui et al., 2016; Ong et al., 2017). This antifungal drug has been described to bind preferentially to ergosterol, one of the major sterols in the plasma membrane of *Acanthamoeba* sp., leading to pore formation, ion leakage and cell death (Smith and Korn, 1968; Yang et al., 2013). Besides this homeostatic effect, amphotericin B has also been shown to induce an apoptosis-like mechanism in fungal cells as well as in parasites from the genus *Leishmania* sp., involving a loss of mitochondrial membrane potential and a rise of reactive oxygen species (ROS) (Lee et al., 2002; Mousavi and Robson, 2004; Cohen, 2010). In *Acanthamoeba castellanii*, the disruption of mitochondrial energetics and membrane potential has also been associated with oxidative stress (Trocha and Stobienia, 2007). However, the mode of action of amphotericin B in *Acanthamoeba* sp. remains currently not elucidated. In a first part of this study, the activity of antimicrobial drugs used in the treatment of AK, GAE or other parasitic diseases were evaluated *in vitro* against the FLA *Acanthamoaeba castellanii* at different times of incubation. Among them, amphotericin B was the only one showing a drastic change of activity as a function of time. The susceptibility of *Acanthamoeba* sp. for this polyene drug was further investigated in the present study.

2. Materials and methods

2.1. Chemicals

All chemicals, including amphotericin B (Fungizone®), were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), except for miltefosine, uranyle acetate and epoxy resin (Low Viscosity Premix Kit Medium) which were provided by Zentaris laboratories (Frankfurt, Germany), Merck (Fontenay-sous-Bois, France) and Agar Scientific (Oxford instruments, Gometz-la-Ville, France), respectively.

2.2. Acanthamoeba cultures

Two *Acanthamoeba castellanii* strains (ATCC 30010 strain and CCAP 1534/3 strain) isolated from environment, and two *Acanthamoeba polyphaga* strains (ATCC 50371 strain and CCAP 1501/3G strain) isolated from human eye infection were maintained twice a week at 27 °C in the dark without shaking in PYG medium (ATCC medium 712) containing 2% (w/v) proteose peptone, 0.1% (w/v) yeast extract, 400 μM $CaCl_2$, 4 mM $MgSO_4$, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, 50 μM $(NH_4)_2Fe(SO_4)_2$, 100 mM glucose.

2.3. In vitro evaluation of anti-acanthamoebal activities

All compounds were evaluated on *A. castellanii* and *A. polyphaga* using a resazurin assay adapted from McBride et al. (2005). Briefly, two-fold serial dilutions of the different compounds were performed from a maximal concentration of 100 μM–0.05 μM in 100 μl PYG medium in a 96-well plate (TPP, Dutscher, Brumath, France). *Acanthamoeba* strains were then added to each well at 5×10^4 amoebae/mL and the plates were further incubated at 27 °C for 3, 4 and 5 days in the dark without shaking. 20 μL of a resazurin solution at 1 mM were then added to each well, and the plates were incubated 8 h at 27 °C. The conversion of resazurin into resorufin was monitored by measuring OD_{570nm} (resorufin) and OD_{600nm} (resazurin; Labsystems Multiskan MS). The anti-acanthamoebal activity was expressed as IC_{50} (concentration of drug inhibiting 50% of amoebae growth in comparison to the control culture). The results were presented as the mean ± SD of independent experiments (n = 6).

The evaluation of drug interactions through their combination was performed as follows. From the determination of the IC_{50} of two compounds (A and B) at 3 days of treatment, the Fractional Inhibitory Concentration Index (FICI) was determined after the same period of treatment, according to the protocol previously described (Odds, 2003), to characterize the type of drugs interaction, namely antagonism, additivity or synergism. Briefly, the top concentration was firstly calculated for compound A and compound B as follows:

$$[\text{Compound A}]_{top} = 4IC_{50}^{\text{Compound A}} \quad \text{and} \quad [\text{Compound B}]_{top} = 4IC_{50}^{\text{Compound B}}$$

Compounds A and B were then mixed at different ratios of concentrations as follows:

- Association 1 was composed of 80% $[\text{Compound A}]_{top}$ and 20% $[\text{Compound B}]_{top}$
- Association 2 was composed of 60% $[\text{Compound A}]_{top}$ and 40% $[\text{Compound B}]_{top}$
- Association 3 was composed of 40% $[\text{Compound A}]_{top}$ and 60% $[\text{Compound B}]_{top}$
- Association 4 was composed of 20% $[\text{Compound A}]_{top}$ and 80% $[\text{Compound B}]_{top}$

Fractional Inhibitory Concentrations (FICs) were then calculated for each of the four drug associations according to the following equations:

$$FIC_1^A = \frac{IC_{50}^{A(\text{Assoc } 1)}}{IC_{50}^A} \quad \text{and} \quad FIC_1^B = \frac{IC_{50}^{B(\text{Assoc } 1)}}{IC_{50}^B}$$

Then, $\sum$FIC was calculated for each association according to the following equation:

$$\sum FIC_1 = FIC_1^A + FIC_1^B$$

The Fractional Inhibitory Concentrations Index (FICI) was then determined as the mean of the ΣFIC for the four drug associations as follows:

$$FICI = \frac{\sum FIC_1 + \sum FIC_2 + \sum FIC_3 + \sum FIC_4}{4}$$

Accordingly, drug combinations displayed a synergistic, additive or antagonist effect if FICI $\leq$ 0.5, 0.5 < FICI $\leq$ 4, or FICI >4, respectively (Odds, 2003). The results were presented as the mean ± SD of independent experiments (n = 4).

In order to analyze the effect of amphotericin B pre-incubation in the culture medium on the evolution of the polyene drug activity, 250 μM amphotericin B was incubated in PYG for 7 days in the dark at 27 °C, prior to add amoebae at 5×10^4 amoebae/mL and to determine the IC_{50} of amphotericin B at 3, 4 and 5 days of treatment, as described above. The results were presented as the mean ± SD of independent experiments (n = 3).

To study the effect of *Acanthamoeba* pre-incubation with amphotericin B on the evolution of the polyene drug activity, *A. castellanii* (ATCC 30010 strain) were cultured in the presence of increasing concentrations of amphotericin B until reaching a maximum of 250 μM, and were further maintained weekly at this concentration for 3 months before analyzing their growth and their susceptibility for the drug. The excess of amphotericin B was removed by washing amoebae 3 times in PYG medium without amphotericin B prior to determine the anti-acanthamoebal activity of the drug at 3, 4 and 5 days of treatment, as described above. The results were presented as the mean ± SD of independent experiments (n = 3).

Growth curves were generated by counting amoebae under light microscope using a Malassez chamber at regular intervals of time, as mentioned in the text, for a period of ten days. The results were presented as the mean ± SD of independent experiments (n = 3). The amoebae cultured in the presence of 250 μM amphotericin B for 3 months were named “AmB” in this work.

2.4. Analysis of Acanthamoeba mechanism of resilience for amphotericin B

Acanthamoeba cultures (ATCC 30010 strain) in log phase were firstly incubated at 1×10^5 amoebae/mL for 4 h at 27 °C in 15 mL of PYG medium containing KCN, glibenclamide, valinomycin, calcimycin, BAPTA-AM, valspodar, latrunculin B, vinblastine, wortmannin or dichlorobenzonitrile at twice the concentrations described in Table S1. These concentrations were determined to inhibit $\leq$25% of amoebal growth after 3 days of treatment at 27 °C (Table S1). Two fold dilutions of amphotericin B were then performed from a maximal concentration of 500 μM in 100 μl PYG medium in a 96-well plate and 100 μL of *Acanthamoeba* subcultures containing the drugs, to avoid any potential reversibility of action, was added to each well containing amphotericin B. The anti-acanthamoebal activity was further determined at 3, 4 and 5 days of treatment, as described above. The results are presented as the mean ± SD of independent experiments (n = 3).

2.5. Transmission electron microscopy

Acanthamoeba castellanii (ATCC 30010 strain) in log phase were treated with 250 μM amphotericin B for 3, 7 or 10 days, before centrifugation at 3000 g for 30 min at room temperature. Cell pellets were then fixed for 2 h at room temperature by replacing the supernatant by a fixative buffer containing 3% (v/v) glutaraldehyde, 1% (v/v) para-formaldehyde, 0.1 M sodium cacodylate pH 7.5. Cells were washed with 0.1 M sodium cacodylate buffer pH 7.5, incubated for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate pH 7.5 and then rinsed 3 times in 0.1 M sodium cacodylate pH 7.5. Pellets were further centrifuged in 2% low melting point agarose to obtain concentrated pellets in 1 mm^3 cubes. Dehydration in graded acetone series (50-70-90-100%) and embedding in graded series (50-100-100%) of epoxy resin (Low Viscosity Premix Kit Medium) mixed with acetone were processed manually. Blocks were then polymerized for 20 h at 60 °C and ultrathin sections (90 nm) were cut with an ultramicrotome UC6 (Leica Microsystems, Germany) and collected on formvar carbon-coated copper grids. Ultrathin sections were stained with uranyl acetate 2%, washed 3 times with water and treated by Reynolds lead citrate in order to increase membrane contrast (Reynolds, 1963). Grids were washed 3 times for 5 min with water before observation with a JEOL JEM-1400 transmission electron microscope operating at 80 kV. Images were acquired using a postcolumn high-resolution (11 megapixels) high-speed camera (SC1000 Orius; Gatan, France) with Digital Micrograph software (Gatan, v2.32.888.0), and further processed using ImageJ software (v. 1.47).

3. Results

In a first part of this work, 15 compounds previously used for the treatment of AK, GAE, or other parasitic diseases, were evaluated for their *in vitro* activity on *A. castellanii* (Table 1). Among them, the most active compound after 3 days of treatment was hexamidine with an IC_{50} at 0.04 μM. With the same treatment duration, voriconazole and clotrimazole displayed also promising activities with IC_{50} of 0.32 μM and 0.77 μM, respectively, and interesting activities with IC_{50} at the micromolar range or under 10 μM were obtained with chlorhexidine, pentamidine, flucytosine, amphotericin B, propamidine, miltefosine and ketoconazole. All the other molecules analyzed displayed an IC_{50} value superior to 10 μM. Indeed, paromomycin, a drug commonly used in the treatment of leishmaniasis (Sundar and Chakravarty, 2013), but also in the treatment of intestinal amoebiasis (Kikuchi et al., 2013), showed a modest IC_{50} value of 16.28 μM.

The three most active compounds, namely hexamidine, voriconazole, and clotrimazole, were then evaluated on *A. castellanii* in association with most of the drugs presenting an IC_{50} under 10 μM in order to determine the interaction effect of drug combinations, hoping for a synergistic effect (Table 2). Apart from the FICI obtained with the association hexamidine-chlorhexidine which was at 4.84 corresponding to an antagonistic effect, all the FICI were between 0.5 and 4 reflecting only an additive effect, and thus, an absence of any synergistic effect with the drug combinations analyzed (Odds, 2003).

In addition, the activity of all the 15 compounds was also evaluated after 4 and 5 days of treatment. Interestingly, the activities of

Table 1
In vitro activities of antimicrobial drugs on *Acanthamoeba castellanii*.

Drug	IC_{50} (μM) ± SD at 3 days	IC_{50} (μM) ± SD at 4 days	IC_{50} (μM) ± SD at 5 days
Hexamidine	0.04 ± 0.02	0.04 ± 0.01	0.06 ± 0.01
Voriconazole	0.32 ± 0.21	0.40 ± 0.14	0.52 ± 0.23
Clotrimazole	0.77 ± 0.04	0.74 ± 0.55	0.86 ± 0.40
Chlorhexidine	1.03 ± 0.14	1.10 ± 0.01	1.26 ± 0.38
Pentamidine	1.11 ± 0.10	1.05 ± 0.11	1.11 ± 0.01
Flucytosine	4.65 ± 0.13	7.65 ± 0.65	5.99 ± 0.78
Amphotericin B	**6.78 ± 1.24**	**65.98 ± 5.91**	**120.63 ± 8.76**
Propamidine	7.59 ± 3.25	8.96 ± 1.45	14.24 ± 3.38
Miltefosine	7.70 ± 2.64	11.22 ± 2.65	13.85 ± 5.59
Ketoconazole	7.98 ± 2.55	7.91 ± 3.78	11.58 ± 8.15
Paromomycin*	16.28 ± 3.09	18.84 ± 3.51	19.89 ± 1.60
Pyrimethamine*	49.60 ± 4.16	47.74 ± 3.92	61.02 ± 7.90
Rifampicin	>100	ND	ND
Metronidazole*	>100	ND	ND
Cotrimoxazole	>100	ND	ND

*These drugs have not, or rarely, been used in the treatment of *Acanthamoeba* infections. The *A. castellanii* ATCC 30010 strain was used in these evaluations. The results correspond to the mean of six independent experiments ±SD. ND = Not determined.
Amphotericin B appears in bold.

Table 2
Activities of drug combinations on *Acanthamoeba castellanii*.

Combinations	FICI[a]
Hexamidine - Voriconazole	1.18
Hexamidine - Clotrimazole	1.04
Hexamidine - Chlorhexidine	4.84
Hexamidine - Pentamidine	1.29
Hexamidine - Amphotericin B	0.80
Hexamidine - Miltefosine	1.33
Voriconazole - Clotrimazole	1.47
Voriconazole - Chlorhexidine	1.23
Voriconazole - Pentamidine	1.34
Voriconazole - Amphotericin B	1.12
Voriconazole - Miltefosine	1.41
Clotrimazole - Chlorhexidine	1.29
Clotrimazole - Pentamidine	1.21
Clotrimazole - Amphotericin B	1.10
Clotrimazole - Miltefosine	1.47

These evaluations were performed on the *A. castellanii* ATCC 30010 strain. FICI were calculated from the anti-*Acanthamoeba* activities of drug combinations after 3 days of treatment. FICI $\leq$0.5 = synergistic effect; 0.5 < FICI $\leq$4 = additive effect; FICI >4 = antagonistic effect. Results correspond to the mean of four independent experiments ± SD.

[a] FICI = Fractional Inhibitory Concentration Index.

all compounds were stable with time, suggesting a sufficient time for drug effect, except for amphotericin B which displayed a marked increase of IC_{50} values between 3 and 5 days of treatment (Table 1). Furthermore, a similar evolution of amphotericin B IC_{50} with time was observed in one other *A. castellanii* strain (CCAP 1534/3) as well as with two strains of *A. polyphaga* (ATCC 50371 and CCAP 1501/3G; Table 3). In order to analyze the stability of drug activity, amphotericin B was incubated in culture medium for 7 days before evaluating the IC_{50} on *A. castellanii* at 3, 4 and 5 days. As shown in Table 4, the activity of pre-incubated amphotericin B was similar to the control at each incubation time. These results show that amphotericin B activity remained stable independently of a pre-incubation in culture medium. An attempt to select an amphotericin B-resistant strain was also performed by culturing *A. castellanii* with increasing concentrations of amphotericin B to a maximum of 250 μM. These amoebae were then maintained 3 months at this drug concentration before starting *in vitro* evaluations. Surprisingly, the susceptibility of these "AmB" amoebae to amphotericin B, either at 3, 4 or 5 days of treatment, was comparable to a naive control strain, indicating that the AmB strain was actually not resistant to amphotericin B (Table 5). However, according to their growth curve, a slower growth was observed for the AmB strain in medium containing 250 μM amphotericin B compared to the naive control in drug-free medium (Fig. 1). On the one hand, in the naive control strain, no lag phase was observed and the stationary phase was reached at the 5th day of culture, with a doubling time of around 22 h. On the other hand, the AmB strain displayed a lag phase of 2 days before starting the log phase and reaching the stationary phase at the 9th day of culture, with an extended doubling time of around 42 h. Nonetheless, the stationary phase was reached with a similar cell density in both cases at 2×10^6 amoebae/mL to 2.5×10^6 amoebae/mL. Interestingly, the AmB strain displayed a similar growth after each passage in the presence of the drug, without showing a tendancy to recover a growth curve related to the naive control strain. Furthermore, when the AmB strain was transferred to an amphotericin B-free medium, a growth curve similar to the naive control strain was observed, indicating the ability of these amoebae to recover the fitness of the control strain in only one passage in the absence of the drug (Fig. 1). Additionally, when a naive control strain was transferred to a medium containing 250 μM amphotericin B, amoebae proliferated slowly with a lag phase and a longer log phase, as for the AmB strain in the presence of the drug, from the first passage (Fig. 1). Altogether, these results show that, besides the incapacity to select an

Table 3
Evolution of Amphotericin B IC_{50} at 3, 4 and 5 days of treatment on different strains of *A. castellanii* and *A. polyphaga*.

Strains	IC_{50} (μM) ± SD at 3 days	IC_{50} (μM) ± SD at 4 days	IC_{50} (μM) ± SD at 5 days
A. castellanii ATCC 30010	5.94 ± 0.88	52.99 ± 9.27	>100
A. castellanii CCAP 1534/3	5.43 ± 0.25	45.10 ± 7.97	>100
A. polyphaga ATCC 50371	2.13 ± 0.19	45.90 ± 6.33	>100
A. polyphaga CCAP 1501/3G	7.15 ± 0.25	74.74 ± 6.95	>100

The results correspond to the mean of three independent experiments ± SD.

Table 4
Effect of Amphotericin B pre-incubation in culture medium on IC_{50} evolution at 3, 4 and 5 days of treatment on *Acanthamoeba castellanii*.

Drugs	IC_{50} (μM) ± SD at 3 days	IC_{50} (μM) ± SD at 4 days	IC_{50} (μM) ± SD at 5 days
Amphotericin B control[a]	6.83 ± 0.58	61.77 ± 13.81	>100
Pre-incubated Amphotericin B[b]	4.12 ± 0.11	46.50 ± 9.62	>100

These evaluations were performed on the *A. castellanii* ATCC 30010 strain. The results correspond to the mean of three independent experiments ± SD.

[a] Amphotericin B was directly used after dilution in culture medium for *A. castellanii* treatment.

[b] Amphotericin B was incubated for 7 days at 27 °C in culture medium before *A. castellanii* treatment.

Table 5
Effect of *Acanthamoeba castellanii* pre-incubation with Amphotericin B on IC_{50} evolution at 3, 4 and 5 days after treatment.

Strains	IC_{50} (μM) ± SD at 3 days	IC_{50} (μM) ± SD at 4 days	IC_{50} (μM) ± SD at 5 days
Control[a]	7.78 ± 0.88	61.75 ± 5.35	>100
AmB[b]	5.78 ± 0.24	70.21 ± 2.24	>100

These evaluations were performed on the *A. castellanii* ATCC 30010 strain. The results correspond to the mean of three independent experiments ± SD.

[a] Control = Naive *A. castellanii* cultured in absence of amphotericin B.

[b] AmB = *A. castellanii* pre-incubated with 250 μM amphotericin B in culture medium for 3 months before evaluation of amphotericin B activity.

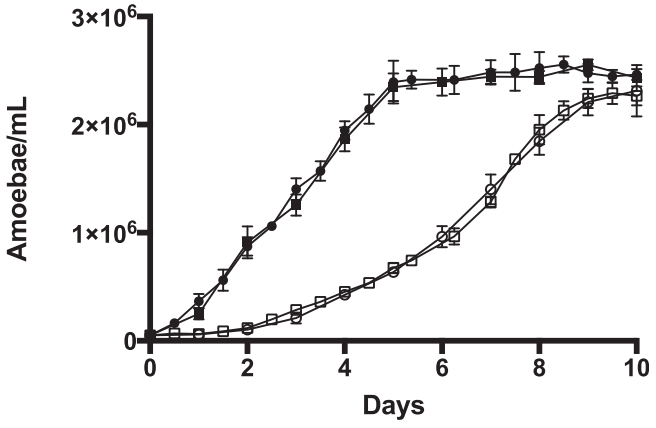

Fig. 1. Effect of amphotericin B treatment on *Acanthamoeba castellanii* growth curve. Growth curves of naive *A. castellanii* cultured in the presence (○) or absence (●) of 250 μM amphotericin B and of *A. castellanii* pre-incubated with 250 μM amphotericin B for 3 months cultured in the presence (□) or absence (■) of 250 μM amphotericin B. The curves were generated by counting amoebae under light microscope once (○, ■) or twice (●, □) a day using a Malassez chamber. This analysis was performed using the *A. castellanii* ATCC 30010 strain. The results correspond to the mean of three independent experiments ± SD.

A. castellanii strain resistant to amphotericin B (Table 5), the adaptation of the AmB strain to the drug was reversible and, more importantly, not acquired, as opposed to an acquired phenomenon of resistance. As the amoebae returned to an initial state of susceptibility for amphotericin B at each new passage in a medium containing the drug, this phenomenon was qualified as "resilience". To our knowledge, this is the first time that such phenomenon was described for amphotericin B in *Acanthamoeba*.

Amphotericin B resilience was further investigated by treating amoebae with effectors altering specifically different cellular transports or pathways prior to evaluate antiacanthamoebal activity of amphotericin B at 3, 4 and 5 days of treatment. No change in IC_{50} evolution was observed when *A. castellanii* was treated with calcimycin, a calcium ionophore, or BAPTA-AM, an intracellular calcium chelator (Fig. 2A). The involvement of the cytoskeleton in amphotericin B resilience was also analyzed by inhibiting actin and tubulin polymerization with latrunculin B and vinblastine, respectively. These drugs were also ineffective to modify the amphotericin B IC_{50} increase from 3 to 5 days of treatment.

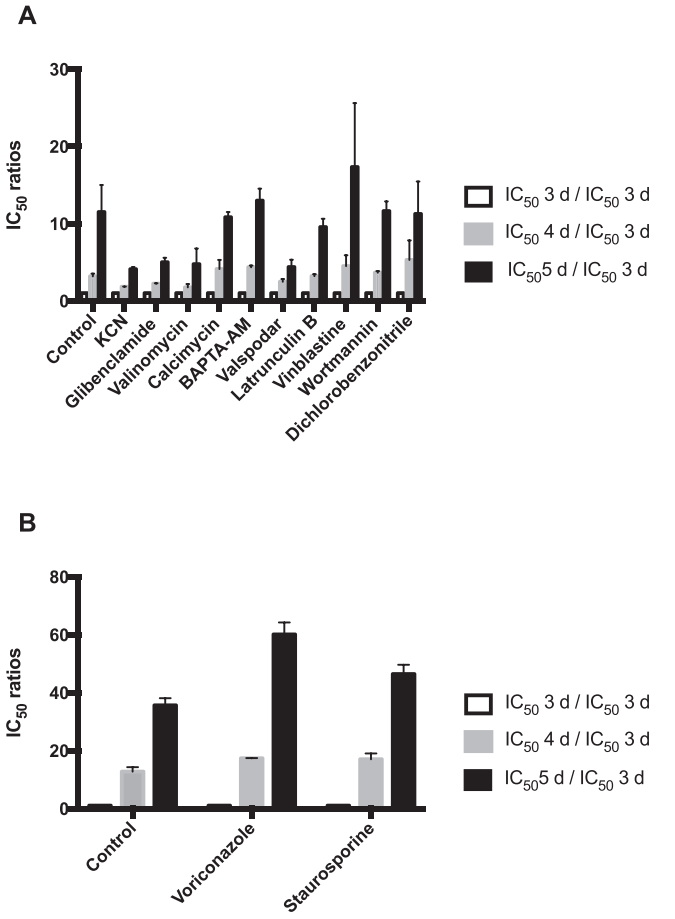

Fig. 2. Influence of effectors on the evolution of amphotericin B antiacanthamoebal activity.

Amphotericin B antiacanthamoebal activity was evaluated at 3, 4 and 5 days of treatment in the presence of 100 μM potassium cyanide (KCN), 100 μM glibenclamide, 20 μM valinomycin, 1 μM calcimycin, 30 μM BAPTA-AM, 10 μM valspodar, 1 μM latrunculin B, 25 μM vinblastine, 50 μM wortmannin or 10 μM dichlorobenzonitrile (A), or 0.1 μM voriconazole or 1 μM staurosporine (B). These drug concentrations were determined to inhibit ≤25% of parasite growth after 3 days of incubation (Table S1). Amoebae were pre-treated with drugs alone for 4 h at twice these concentrations prior to start the evaluation. The IC_{50} ratios correspond to the IC_{50} obtained at 3, 4 or 5 days normalized to the IC_{50} at 3 days. Thus, an increase in this ratio between 3 and 5 days of treatment reflects an increase of IC_{50}. These evaluations were performed using the *Acanthamoeba castellanii* ATCC 30010 strain. The results correspond to the mean of three independent experiments ± SD.

Although no obvious increase in cyst proportion was observed when amoebae were cultured in the presence of amphotericin B (data not shown), the involvement of encystment was analyzed by treating *Acanthamoeba* with wortmannin and dichlorobenzonitrile (DCB), previously described to inhibit this cellular process in *A. castellanii* (Dudley et al., 2007; Moon et al., 2015). As shown in Fig. 2A, no effect on amphotericin B antiacanthamoebal activity was observed with these drugs. However, a treatment of the amoebae by KCN, valinomycin and glibenclamide, an inhibitor of oxygen consumption in the respiratory chain, a potassium ionophore and a potassium channel blocker, respectively, lead to a loss of IC_{50} increase at 4 and 5 days of treatment (Fig. 2A). A similar result was obtained with valspodar, an inhibitor of P-glycoprotein (PgP) involved in drug efflux and encoded by the multidrug resistance gene MDR-1 (Boesch et al., 1991). Interestingly, the increase of amphotericin B IC_{50} from 3 to 5 days of treatment was amplified with staurosporine, a pro-apoptotic kinase inhibitor, and, to a higher extent, with voriconazole which has been previously described to induce apoptosis in *A. castellanii* (Fig. 2B) (Martin-Navarro et al., 2015).

An ultrastructural analysis was then performed on *A. castellanii* grown in the presence of 250 μM amphotericin B for 3, 7 and 10 days, corresponding to early- and late-log phase and stationary phase, respectively (Fig. 1). These amphotericin B-treated amoebae were compared to untreated control in log phase cultured for 3 days in drug-free medium (Figs. 1 and 3A–D). Both amphotericin B-treated and untreated amoebae displayed multiple mitochondria in their cytoplasm (Fig. 3A,E,I,M). In contrast to the untreated control where the mitochondria exhibited inner and outer membranes delimitating evenly the matrix with well defined tubular cristae (Fig. 3B–D), the mitochondria of the amphotericin B-treated amoebae displayed some membrane bulges with poorly defined tubular cristae, especially after 3 and 7 days of culture (Fig. 3F–H and J–L). When the amphotericin B-treated amoebae reached the stationary phase, after 10 days of culture, tubular cristae were more discernible, but not well delineated as for the control (Fig. 3N,O). Double membranes surrounding either partially or entirely mitochondria were also observed in these non-dividing amoebae (Fig. 3N,O). Furthermore, intracristae granules were more frequently observed in mitochondria of amphotericin B-treated amoebae compared to the control (Fig. 3D,F,J,N,O).

4. Discussion

As the treatment of FLA remains empirical and the *in vitro* activities of antiacanthamoebal drugs reported in the literature are variable depending on the time of treatment or the *Acanthamoeba* species used (Duma and Finley, 1976; Elder et al., 1994; Ondarza et al., 2006; Martin-Navarro et al., 2013; Carrijo-Carvalho et al., 2017; Nakaminami et al., 2017; Ortilles et al., 2017), the main objective of this study was to determine in our conditions the *in vitro* efficiency of clinically used antimicrobial agents on *A. castellanii* at different times of treatment in order to propose a rationale for their use in therapy. This approach was completed by a study deciphering the mechanism of action of amphotericin B showing a surprising evolution of antiacanthamoebal activity as a function of time.

Among the drugs evaluated for their *in vitro* anti-*Acanthamoeba* activity, the best activity was obtained with hexamidine, a diamidine containing an alkyl chain of 6 carbons, with a stable IC_{50} at 0.05 μM at 3, 4 and 5 days of treatment. Hexamidine is used as a first-line treatment for AK in combination with chlorhexidine, which also showed in our study an interesting IC_{50} in the micromolar range (Siddiqui et al., 2016; Carrijo-Carvalho et al., 2017). Two other diamidines presenting shorter alkyl chain of 3 and 5

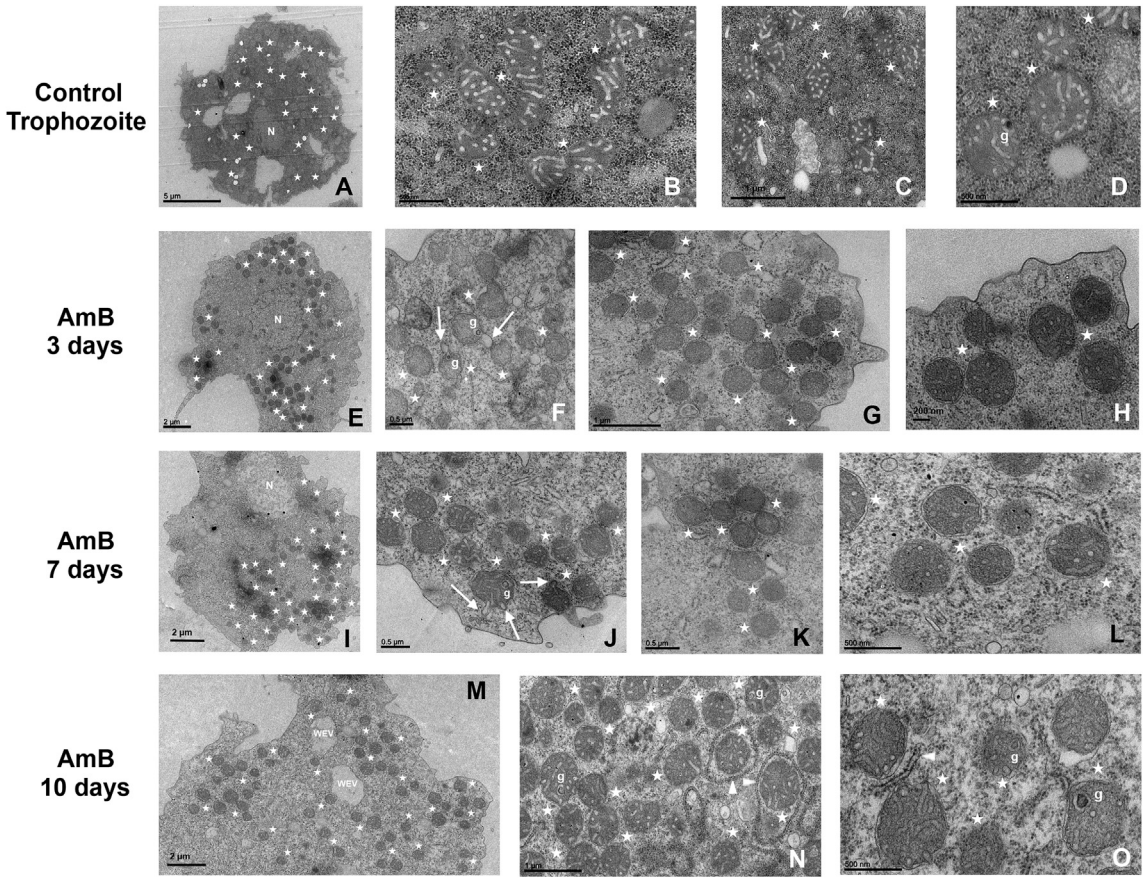

Fig. 3. Ultrastructural analysis of *Acanthamoeba castellanii* treated with amphotericin B.
A. castellanii cultured in absence of amphotericin B for a period of 3 days corresponding to the log phase (A-D), or in the presence of 250 μM amphotericin B for a period of 3 days (E-H), 7 days (I-L), or 10 days (M-O) corresponding to early-, late-log and stationary phase, respectively. Apart from the image K which is a higher magnification of image I, all images were collected from different cells at the following magnification: 1000× (A), 1200× (E), 1500× (I and M), 4000× (F), 5000× (B, C, J and N), 6000× (G and K), 8000× (H) and 10000× (D, L and O). These evaluations were performed using the *Acanthamoeba castellanii* ATCC 30010 strain. ★: mitochondria; N: Nucleus; WEV: Water-Expulsion Vesicle; g: intracristae granule; arrow: mitochondrial membrane swelling; arrowhead: double membrane surrounding mitochondria.

carbons, namely propamidine and pentamidine, respectively, were evaluated in this work. Propamidine is commonly used in the treatment of AK, while pentamidine has been occasionally employed in GAE therapy (Schuster and Visvesvara, 2004; Siddiqui et al., 2016; Carrijo-Carvalho et al., 2017; Ong et al., 2017). Higher IC_{50} values at around 10 μM and 1 μM, at either 3, 4 or 5 days of treatment, were obtained with propamidine and pentamidine compared to hexamidine, indicating an increase of anti-*Acanthamoeba* activity in relation with the length of diamidine alkyl chain. These results are in agreement with a previous study showing that the antiacanthamoebal activity of diamidines is proportional to the length of their alkyl chain (Perrine et al., 1995). The three azoles evaluated in this work, namely voriconazole, clotrimazole and ketoconazole, have been previously used for the treatment of *Acanthamoeba* infections (Schuster and Visvesvara, 2004; Ong et al., 2017). Among these azoles, voriconazole and clotrimazole showed the best antiacanthamoebal activities with IC_{50} values in the submicromolar range while ketoconazole IC_{50} values were at around 10 μM, independently of the treatment duration. These results are in agreement with a previous work showing that clotrimazole has the best *in vitro* antiacanthamoebal activity in comparison with other azoles such as bifonazole, ketoconazole, itraconazole and fluconazole (Schuster, 1993). Flucytosine and miltefosine are two other compounds that have been used in *Acanthamoeba* infections (Schuster and Visvesvara, 2004; Siddiqui et al., 2016; Ong et al., 2017) exhibiting interesting IC_{50} values between 4.5 μM and 8 μM and at around 10 μM, respectively. No significant variation of IC_{50} was observed with these drugs as a function of time. While some *Acanthamoeba* strains have been described to be capable of growing at flucytosine concentrations as high as 310 μM (Stevens and O'Dell, 1974), the antiacanthamoebal activity of miltefosine reported in the literature is in agreement with the IC_{50} values obtained in the present study (McBride et al., 2005). The difference observed with flucytosine between the study of Stevens and O'Dell (1974) and our work may be due to the use of distinct *Acanthamoeba* strains. Despite that paromomycin has been rarely used in the treatment of *Acanthamoeba* infections with unconvincing outcomes (Skarin et al., 1996), this drug displayed a modest *in vitro* antiacanthamoebal activity in our conditions with IC_{50} values between 16 μM and 20 μM at either 3, 4 or 5 days of treatment, which is in agreement with the IC_{50} values previously obtained with the same *A. castellanii* strain as well as with several *Acanthamoeba* isolates (Nakaminami et al., 2017). Furthermore, metronidazole, which has been also infrequently used in the treatment of AK or GAE (Horne et al., 1994; Gunawan et al., 2016), was inactive on *A. castellanii* in our study, although an IC_{50} value at 17.5 μM was obtained with this drug on another *Acanthamoeba* species, *A. polyphaga* (Ondarza et al., 2006). Surprisingly, in line with a previous work showing an absence of *in vitro* activity of rifampicin on *A. polyphaga* (Ondarza et al., 2006), rifampicin as well as cotrimoxazole, two drugs that are frequently used in GAE treatment (Schuster and Visvesvara, 2004; Siddiqui

et al., 2016; Ong et al., 2017), did not display an *in vitro* antiacanthamoebal activity in our conditions (IC_{50} > 100 μM) revealing a possible inappropriate utilization of these drugs in *Acanthamoeba* infections. Among the drug combinations analyzed in this work, no one displayed a synergistic effect, except for the association of hexamidine and chlorhexidine which exhibited an antagonistic effect. These results indicate that a particular care should be exercised when hexamidine and chlorhexidine are associated in the treatment of *Acanthamoeba* infections, especially as the first-line therapy for AK involves the combination of membrane-acting agents, such as polyhexamethylene biguanide or chlorhexidine, with a diamidine (propamidine, pentamidine or hexamidine; Siddiqui et al., 2016; Carrijo-Carvalho et al., 2017).

In our study, all drugs showed a stable IC_{50} as a function of time, except for amphotericin B which displayed an intriguing decrease of activity between 3 and 5 days of treatment. This drug, which has been frequently used for the treatment of GAE (Schuster and Visvesvara, 2004; Siddiqui et al., 2016; Carrijo-Carvalho et al., 2017), exhibited an IC_{50} value at 6.78 μM at 3 days of treatment. This result was in agreement with IC_{50} values previously reported at the same time of treatment on *A. polyphaga* (Ondarza et al., 2006). Another study showed higher IC_{50} values of amphotericin B, between 25 μM and 67 μM, on *Acanthamoba* sp. isolates after a treatment of 4 days, in line with our results (Martin-Navarro et al., 2013). The decrease of antiacanthamoebal activity of amphotericin B as a function of time was also observed in a previous study where the MIC_{100} (minimal concentration inhibiting 100% of amoebae growth) of amphotericin B was determined at 6.76 μM, 108 μM and above 108 μM at 2, 3 and 6 days of treatment, respectively (Mattana et al., 2004). However, these authors did not investigate this phenomenon further and observed this change of activity with other antimicrobial agents as well, such as acyclovir or polymyxin B. In our study, amphotericin B was the only drug displaying a change of antiacanthamoebal activity as a function of time. This phenomenon was not altered by incubating amphotericin B in culture medium prior to *in vitro* antiacanthamoebal evaluation and was observed with several strains of two different species of *Acanthamoeba* (*A. castellanii* and *A. polyphaga*), showing that the increase of amphotericin B IC_{50} as a function of time was not due to drug degradation in the medium and could be potentially generalized to the genus *Acanthamoeba*. Furthermore, no resistant strain could have been selected as the susceptibility of *Acanthamoeba castellanii* for amphotericin B at 3, 4 and 5 days of treatment was similar between naive control amoebae and *A. castellanii* cultured for 3 months in the presence of 250 μM amphotericin B. This phenomenon of adaptation to amphotericin B was thus qualified as resilience as the amoebae did not acquire a resistance phenotype, but rather returned to an initial state of susceptibility at each passage in the presence of the drug. Nonetheless, microbial communities under a pressing disturbance, in opposition to a pulse disturbance, have the tendancy to create a stable state of resistance rather than returning to an initial state of susceptibility and to develop resilience (Shade et al., 2012). Therefore, in our case, *Acanthamoeba* has surprisingly adopted a strategy of resilience rather than resistance against amphotericin B probably due to a more advantageous fitness cost.

An approach to understand the mechanism of action of amphotericin B was to use pharmacological tools having specific actions on a cell. The treatment of amoebae with a calcium ionophore (calcimycin), an intracellular calcium chelator (BAPTA-AM), or inhibitors of encystment (wortmannin and DCB), or actin and tubulin polymerization (latrunculin B and vinblastine, respectively), did not alter amphotericin B IC_{50} evolution with time. Although calcimycin and BAPTA-AM displayed an IC_{50} at 1.97 μM and 73.08 μM on *A. castellanii* after 3 days of treatment, respectively (Table S1), to our knowledge, the mode of action of these drugs on calcium channels or intracellular calcium in *Acanthamoeba* has not been investigated. Furthermore, the lack of action of vinblastine is consistent with a previous work showing that *Acanthamoeba* tubulin was resistant to this drug (Henriquez et al., 2008). Therefore, an absence of activity of calcimycin, BAPTA-AM or vinblastine on the increase of amphotericin B IC_{50} between 3 and 5 days of treatment does not exclude the involvement of calcium or tubulin in the phenomenon of amphotericin B resilience. In relation with the inefficiency of encystment inhibitors to impact the amphotericin B IC_{50} increase with time, no obvious expansion of cyst proportion was observed when amoebae were cultured in the presence of the drug. Furthermore, the action of wortmannin and DCB on the inhibition of encystment, and of latrunculin B on actin depolymerization have been previously described in *A. castellanii* (Dudley et al., 2007; Soto-Arrendondo et al., 2014; Moon et al., 2015), suggesting that encystment and actin polymerization are not involved in amphotericin B resilience.

Although potassium cyanide has been reported to generate a collapse of the mitochondrial membrane potential (Jarmuszkiewicz et al., 1998), it did not present an antiacanthamoebal activity in our conditions (Table S1). This lack of activity may be due to the existence of two branching pathways in the respiratory chain of *A. castellanii*, one classical cytochrome pathway and one cyanide-resistant non-electrogenic alternative oxidase pathway (Jarmuszkiewicz et al., 1998). In agreement with a previous study showing that valinomycin has only a limited and transient effect on membrane potential of *Acanthamoeba castellanii* (Dolowy, 1990), a negligeable antiacanthamoebal activity was obtained with this drug in our work (Table S1). Likewise, glibenclamide was inefficient on *A. castellanii* with an IC_{50} above 100 μM at 3 days of treatment. Nonetheless, glibenclamide and valinomycin have been described to act on the membrane potential of mitochondria isolated from *A. castellanii* (Kicinska et al., 2007). In the present study, KCN, glibenclamide, and valinomycin inhibited amphotericin B IC_{50} increase as a function of time, indicating that mitochondrial membrane potential is required in the phenomenon of amphotericin B resilience in *Acanthamoeba*. Furthermore, in mammalian cells, glibenclamide and valinomycin have been reported to block the multidrug resistance proteins MRP-1 and PgP, respectively, involved in drug efflux (Weaver et al., 1993; Conseil et al., 2005). Valspodar, another PgP inhibitor (Boesch et al., 1991), also prevented amphotericin B IC_{50} increase with time, showing that besides mitochondrial membrane potential, multidrug resistance transporters are also involved in amphotericin B resilience in *Acanthamoeba*. Consistently, amphotericin B resistance in the protozoan parasite *Leishmania* has been noticeably characterized by an increase of MDR-1 expression level, encoding for PgP (Purkait et al., 2012). A low level of ergosterol has also been associated with amphotericin B resistance in this kinetoplastid parasite as well as in fungi (Purkait et al., 2012; Cuenca-Estrella, 2014). Moreover, a disruption of mitochondrial function in *Candida albicans* has been described to result in a reduction of cellular ergosterol levels and an increase of amphotericin B resistance (Geraghty and Kavanagh, 2003). Therefore, the mechanism of amphotericin B resistance in *Leishmania* and in fungi involves several factors, such as mitochondrial function or multidrug resistance transporters, that are also used in the phenomenon of amphotericin B resilience in *Acanthamoeba.* Nonetheless, in the latter case, these factors would not be acquired from one passage to another.

The amplification of amphotericin B IC_{50} increase with time by using pro-apoptotic compounds, such as staurosporine and voriconazole (Martin-Navarro et al., 2015), indicate that an apoptosis-like pathway is involved in the phenomenon of amphotericin B resilience in *Acanthamoeba*. Programmed cell death has also been implicated in the mode of action of amphotericin B in fungi and in

Leishmania (Lee et al., 2002; Mousavi and Robson, 2004; Cohen, 2010). In particular, amphotericin B treatment in *C. albicans* has been shown to activate mitochondrial activity leading to an excess of ROS and subsequently cell death (Belenky et al., 2013). Two main apoptotic pathways are currently described in eukaryotic cells: the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway (Elmore, 2007). In this latter pathway, mitochondria are required as both a source and a target of oxidative stress, which is principally triggered by an overproduction of ROS, leading to a loss of mitochondrial membrane potential, and ultimately cell death. Nonetheless, *A. castellanii* mitochondria have been described to resist for a period of 5 min to oxidative stress induced by H_2O_2 at up to 25 mM (Jarmuszkiewicz et al., 2008). Based on our results, our hypothesis would be that an apoptosis-like pathway, requiring a functional mitochondrial membrane potential for initial activation, would be induced during amphotericin B resilience in *Acanthamoeba*. This pathway would be then redirected to allow amoebae survival and proliferation in the presence of amphotericin B. In agreement with our results, a previous work performed in *C. albicans* isolates has shown that despite an induction of early apoptosis markers following amphotericin B treatment, some cells were able to recover and to form colonies on plates, indicating the possibility to redirect the programmed cell death pathway induced in amphotericin B treated cells (Yang et al., 2010). Reversibility of apoptotic pathways has also been observed in other eukaryotic organisms (Elmore, 2007).

Ultrastructural analysis of amphotericin B treated *Acanthamoeba* revealed a strong alteration of mitochondrial cristae after 3 and 7 days of treatment. Similar events of mitochondrial cristae alteration have been depicted in acanthamoebal cysts characterized by a lower respiration rate (Bowers and Korn, 1969). The shape of mitochondrial cristae has also been shown to be altered during apoptosis and to modulate the respiratory efficiency in mammalian cells (Cogliati et al., 2013). Our study indicate that apoptosis, as opposed to encystment, is involved in the phenomenon of amphotericin B resilience. Therefore, alteration of mitochondrial cristae would be induced in the apoptosis-like pathway activated following amphotericin B treatment, as well as in encystment, but it would not be sufficient for the achievement of this latter process. At 10 days of treatment, mitochondrial cristae were less altered but some double membranes, similar to autophagosomes, were observed surrounding mitochondria either totally or partially. This process of mitophagy, defined as a quality control mechanism eliminating damaged mitochondria, was only noticed in stationary phase amoebae and could be a consequence of mitochondrial alterations observed in the log phase. In agreement with these results, mitochondrial lesions can induce mitophagy in yeast (Priault et al., 2005). Moreover, a high degree of crosstalk has been identified between mitophagy and apoptosis (Hamacher-Brady and Brady, 2016), showing that the phenomenon of amphotericin B resilience is a complex mechanism involving several mitochondria-dependent pathways.

5. Conclusion

Among the 15 antimicrobial agents analyzed in this work, hexamidine, voriconazole, and clotrimazole exhibited the best *in vitro* antiacanthamoebal activities. No synergistic effect was observed with any of the drug associations evaluated, and an antagonism was determined for hexamidine-chlorhexidine. Interestingly, the adaptation of *Acanthamoeba* to amphotericin B always returned to an initial level of susceptibility, reflecting the establishment of a non-acquired phenomenon, qualified as resilience. The analysis of its mechanism revealed the involvement of mitochondria-dependent pathways and multidrug resistance transporters. Further works, including proteomics or metabolomics approaches, would allow to decipher these pathways in details and to identify the molecular mediators induced during the phenomenon of amphotericin B resilience in *Acanthamoeba*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2017.09.002.

References

Aksozek, A., McClellan, K., Howard, K., Niederkorn, J.Y., Alizadeh, H., 2002. Resistance of *Acanthamoeba castellanii* cysts to physical, chemical, and radiological conditions. J. Parasitol. 88, 621–623.

Belenky, P., Camacho, D., Collins, J.J., 2013. Fungicidal drugs induce a common oxidative-damage cellular death pathway. Cell Rep. 3, 350–358.

Boesch, D., Gavériaux, C., Jachez, B., Pourtier-Manzanedo, A., Bollinger, P., Loor, F., 1991. *In vivo* circumvention of P-glycoprotein-mediated multidrug resistance of tumor cells with SDZ PSC 833. Cancer Res. 51, 4226–4233.

Bowers, B., Korn, E.D., 1969. The fine structure of *Acanthamoba castellanii* (Neff strain). II. Encystment. J. Cell Biol. 41, 786–805.

Carrijo-Carvalho, L.C., Sant'ana, V.P., Foronda, A.S., de Freitas, D., de Sousa Carvalho, F.R., 2017. Therapeutic agents and biocides for ocular infections by free-living amoebae of *Acanthamoeba* genus. Surv. Ophtalmol. 62, 203–218.

Cogliati, S., Frezza, C., Soriano, M.E., Varanita, T., Quintana-Cabrera, R., Corrado, M., Cipolat, S., Costa, V., Casarin, A., Gomes, L.C., Perales-Clemente, E., Salviati, L., Fernandez-Silva, P., Enriquez, J.A., Scorrano, L., 2013. Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. Cell 155, 160–171.

Cohen, B.E., 2010. Amphotericin B membrane action: role of two types of ion channels in eliciting cell survival and lethal effects. J. Membr. Biol. 238, 1–20.

Conseil, G., Deeley, R.G., Cole, S.P., 2005. Role of two adjacent cytoplasmic tyrosine residues in MRP1 (ABCC1) transport activity and sensitivity to sulfonylureas. Biochem. Pharmacol. 69, 451–461.

Cuenca-Estrella, M., 2014. Antifungal drug resistance mechanisms in pathogenic fungi: from bench to bedside. Clin. Microbiol. Infect. 20, 54–59.

Dolowy, K., 1990. Depolarization of the cell membrane causes inhibition of cell locomotion and pinocytosis in *Acanthamoeba castellanii* and *Amoeba proteus*. Protoplasma 155, 210–220.

Dudley, R., Alsam, S., Khan, N.A., 2007. Cellulose biosynthesis pathway is a potential target in the improved treatment of *Acanthamoeba* keratitis. Appl. Microbiol. Biotechnol. 75, 133–140.

Duma, R.J., Finley, R., 1976. *In vitro* susceptibility of *Naegleria* and *Acanthamoeba* species to a variety of therapeutic agents. Antimicrob. Agents Chemother. 10, 370–376.

Elder, M.J., Kilvington, S., Dart, J.K., 1994. A clinicopathologic study of *in vitro* sensitivity testing and *Acanthamoeba* keratitis. Invest. ophtamol. Vis. Sci. 35, 1059–1064.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.

Geraghty, P., Kavanagh, K., 2003. Disruption of mitochondrial function in *Candida albicans* leads to reduced cellular ergosterol levels and elevated growth in the presence of amphotericin B. Arch. Microbiol. 179, 295–300.

Guimaraes, A.J., Gomes, K.X., Cortines, J.R., Peralta, J.M., Peralta, R.H., 2016. *Acanthamoeba spp.* as a universal host for pathogenic microorganisms: one bridge from environment to host virulence. Microbiol. Res. 193, 30–38.

Gunawan, P.I., Idarto, A., Saharso, D., 2016. *Acanthamoeba* infection in a drowning child. Ethiop. J. Health Sci. 26, 289–292.

Hamacher-Brady, A., Brady, N.R., 2016. Mitophagy programs: mechanisms and physiological implications of mitochondrial targeting by autophagy. Cell. Mol. Life Sci. 73, 775–795.

Henriquez, F.L., Ingram, P.R., Muench, S.P., Rice, D.W., Roberts, C.W., 2008. Molecular basis for resistance of *Acanthamoeba* tubulins to all major classes of antitubulin compounds. Antimicrob. Agents Chemother. 52, 1133–1135.

Horne, D.D., Frizell, M.E., Ingham, L., Jans, R.G., Gubash, S.M., Anand, C.M., Athar, M.A., 1994. *Acanthamoeba* keratitis: an emerging clinical problem. CMAJ 150, 923–925.

Jarmuszkiewicz, W., Sluse-Goffart, C.M., Hryniewiecka, L., Michejda, J., Sluse, F.E., 1998. Electron partitioning between the two branching quinol-oxidizing pathways in *Acanthamoeba castellanii* mitochondria during steady state 3

respiration. J. Biol. Chem. 273, 10174–10180.
Jarmuszkiewicz, W., Antos-Krzeminska, N., Drachal-Chrul, D., Matkovic, K., Nobik, W., Pienkowska, J., Swida, A., Woyda-Ploszczyca, A., Budzinska, M., 2008. Basic energetic parameters of *Acanthamoeba castellanii* mitochondria and their resistance to oxidative stress. Acta Biochim. Pol. 55, 349–355.
Kicinska, A., Swida, A., Bednarczyk, P., Koszela-Piotrowska, I., Choma, K., Dolowy, K., Szewczyk, A., Jarmuszkiewicz, W., 2007. ATP-sensitive potassium channel in mitochondria of the eukaryotic organism *Acanthamoeba castellanii*. J. Biol. Chem. 282, 17433–17441.
Kikuchi, T., Koga, M., Shimizu, S., Miura, T., Maruyama, H., Kimura, M., 2013. Efficacy and safety of paromomycin for treating amoebiasis in Japan. Parasitol. Int. 62, 497–501.
Lee, N., Bertholet, S., Debrabant, A., Muller, J., Duncan, R., Nakhasi, H.L., 2002. Programmed cell death in the unicellular protozoan parasite *Leishmania*. Cell Death Differ. 9, 53–64.
Martin-Navarro, C.M., Lopez-Arencibia, A., Arnalich-Montiel, F., Valladares, B., Pinero, J.E., Lorenzo-Morales, J., 2013. Evaluation of the *in vitro* activity of commercially available moxifloxacin and voriconazole eye-drops against clinical strains of *Acanthamoeba*. Graefes Arch. Clin. Exp. Ophtamol 251, 2111–2117.
Martin-Navarro, C.M., Lopez-Arencibia, A., Sifaoui, I., Reyes-Batlle, M., Valladares, B., Martinez-Carretero, E., Pinero, J.E., Maciver, S.K., Lorenzo-Morales, J., 2015. Statins and voriconazole induce programmed cell death in *Acanthamoeba castellanii*. Antimicrob. Agents Chemother. 59, 2817–2824.
Martinez, A.J., Visvesvara, G.S., 1997. Free-living, amphizoic and opportunistic amoebae. Brain Pathol. 7, 583–598.
Mattana, A., Biancu, G., Alberti, L., Accardo, A., Delogu, G., Fiori, P.L., Cappuccinelli, P., 2004. *In vitro* evaluation of the effectiveness of the macrolide rokitamycin and chlorpromazine against *Acanthamoeba castellanii*. Antimicrob. Agents Chemother. 48, 4520–4527.
McBride, J., Ingram, P.R., Henriquez, F.L., Roberts, C.W., 2005. Development of colorimetric mirotiter plate assay for assessment of antimicrobials against *Acanthamoeba*. J. Clin. Microbiol. 43, 629–634.
Moon, E.K., Hong, Y., Chung, D.I., Goo, Y.K., Kong, H.H., 2015. Potential value of cellulose synthesis inhibitors combined with PHMB in the treatment of *Acanthamoeba* keratitis. Cornea 24, 1593–1598.
Mousavi, S.A., Robson, G.D., 2004. Oxidative and amphotericin B–mediated cell death in the opportunistic pathogen *Aspergillus fumigatus* is associated with an apoptotic-like phenotype. Microbiology 150, 1937–1945.
Nakaminami, H., Tanuma, K., Enomoto, K., Yoshimura, Y., Onuki, T., Nihonyanagi, S., Hamada, Y., Nogushi, N., 2017. Evaluation of *in vitro* antimamoebic activity of antimicrobial agents against clinical *Acanthamoeba* isolates. J. Ocul. Pharmacol. Ther. (in press).
Odds, F.C., 2003. Synergy, antagonism, and what the chequerboard puts between them. J. Antimicrob. Chemother. 52, 1.
Ondarza, R.N., Iturbe, A., Hernandez, E., 2006. *In vitro* antiproliferative effect of neuroleptics, antimycotics and antibiotics on the human pathogens *Acanthamoeba polyphaga* and *Naegleria fowleri*. Arch. Med. Res. 37, 723–729.
Ong, T.Y.Y., Khan, N.A., Siddiqui, R., 2017. Brain-eating amoebae: predilection sites in the brain and disease outcome. J. Clin. Microbiol. 55, 1989–1997.
Ortilles, A., Belloc, J., Rubio, E., Fernandez, M.T., Benito, M., Cristobal, J.A., Calvo, B., Goni, P., 2017. *In vitro* development of an effective treatment for *Acanthamoeba* keratitis. Int. J. Antimicrob. Agents 50, 325–333.
Perrine, D., Chenu, J.P., Georges, P., Lancelot, J.C., Saturnino, C., Robba, M., 1995. Amoebicidal efficiencies of various diamidines against two strains of *Acanthamoeba polyphaga*. Antimicrob. Agents Chemother. 39, 339–342.
Priault, M., Salin, B., Schaeffer, J., Vallette, F.M., di Rago, J.P., Martinou, J.C., 2005. Impairing the bioenergetics status and the biogenesis of mitochondria triggers mitophagy in yeast. Cell Death Differ. 12, 1613–1621.
Purkait, B., Singh, R., Wasnik, K., Das, S., Kumar, A., Paine, M., Dikhit, M., Singh, D., Sardar, A.H., Ghosh, A.K., Das, P., 2012. Up-regulation of silent information regulator 2 (Sir2) is associated with amphotericin B resistance in clinical isolates of *Leishmania donovani*. J. Antimicrob. Chemother. 70, 1343–1356.
Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208–212.
Rodriguez-Zaragoza, S., 1994. Ecology of free-living amoebae. Crit. Rev. Microbiol. 20, 225–241.
Schuster, F.L., 1993. Comparative effect of selected azole compounds on trophic and cystic stages of *Acanthamoeba polyphaga*. J. Eukaryot. Microbiol. 40, 563–569.
Schuster, F.L., Visvesvara, G.S., 2004. Opportunistic amoebae: challenges in prophylaxis and treatment. Drug resist. updat. 7, 41–51.
Shade, A., Peter, H., Allison, S.D., Baho, D.L., Berga, M., Bürgmann, H., Huber, D.H., Langenheder, S., Lennon, J.T., Martiny, J.B., Matulich, K.L., Schmidt, T.M., Handelsman, J., 2012. Fundamentals in microbial community resistance and resilience. Front. Microbiol. 3, 417.
Siddiqui, R., Aqeel, Y., Khan, N.A., 2016. The development of drugs against *Acanthamoeba* infections. Antimicrob. Agents Chemother. 60, 6441–6450.
Skarin, A., Floren, I., Kiss, K., Miörner, H., Stenevi, U., 1996. *Acanthamoeba* keratitis in the south of Sweden. Acta ophtamol. Scand. 74, 593–597.
Smith, F.R., Korn, E.D., 1968. 7-Dehydrostigmasterol and ergosterol: the major sterol of an amoeba. J. Lipid. Res. 9, 405–408.
Soto-Arrendondo, K.J., Flores-Villavicencio, L.L., Serrano-Luna, J.J., Shibayama, M., Sabanero-Lopez, M., 2014. Biochemical and cellular mechanisms regulating *Acanthamoeba castellanii* adherence to host cells. Parasitology 141, 531–541.
Stevens, A.R., O'Dell, W.D., 1974. *In vitro* and *in vivo* activity of 5-fluorocytosine on *Acanthamoeba*. Antimicrob. Agents Chemother. 6, 282–289.
Storey, M.V., Winiecka-Krusnell, J., Ashbolt, N.J., Stenström, T.A., 2004. The efficacy of heat and chlorine treatment against thermotolerant Acanthamoebae and Legionellae. Scand. J. Infect. Dis. 36, 656–662.
Sundar, S., Chakravarty, J., 2013. Leishmaniasis: an update of current pharmacotherapy. Expert Opin. Pharmacother. 14, 53–63.
Trocha, L.K., Stobienia, O., 2007. Response of *Acanthamoeba castellanii* mitochondria to oxidative stress. Acta Biochim. Pol. 54, 797–803.
Visvesvara, G.S., Moura, H., Schuster, F.L., 2007. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri* and *Sappinia diploidea*. FEMS Immunol. Med. Microbiol. 50, 1–26.
Weaver, J.L., Szabo Jr., G., Pine, P.S., Gottesmann, M.M., Goldensberg, S., Aszalos, A., 1993. The effect of ion channel blockers, immunosuppressive agents, and other drugs on the activity of the multi-drug transporter. Int. J. Cancer 54, 456–461.
Yang, C., Gong, W., Lu, J., Zhu, X., Qi, Q., 2010. Antifungal drug susceptibility of oral *Candida albicans* isolates may be associated with apoptotic responses to amphotericin B. J. Oral Pathol. Med. 39, 182–187.
Yang, T.S., Ou, K.L., Peng, P.W., Liou, B.C., Wang, W.T., Huang, Y.C., Tsai, C.M., Su, C.H., 2013. Quantifying membrane permeability of amphotericin B ion channels in single living cells. Biochim. Biophys. Acta 1828, 1794–1801.