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## *In vitro* activity of 1*H*-phenalen-1-one derivatives against *Acanthamoeba castellanii* Neff and their mechanisms of cell death



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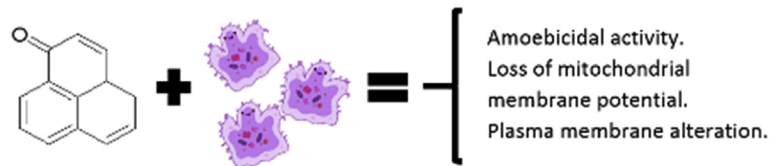
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### HIGHLIGHTS

- Phenalenones are potential anti-amoebic agents.
- Some phenalenones can induce a strong decrease in mitochondrial potential.
- One phenalenone could trigger apoptosis and affect the cytoplasmic membrane.

### GRAPHICAL ABSTRACT



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### ABSTRACT

*Acanthamoeba* is an opportunistic pathogen which is the causal agent of a sight-threatening ulceration of the cornea known as *Acanthamoeba* keratitis (AK) and, more rarely, an infection of the central nervous system called “granulomatous amoebic encephalitis” (GAE). The symptoms of AK are non-specific, and so it can be misdiagnosed as a viral, bacterial, or fungal keratitis. Furthermore, current therapeutic measures against AK are arduous, and show limited efficacy against the cyst stage of *Acanthamoeba*. 1*H*-Phenalen-1-one (PH) containing compounds have been isolated from plants and fungi, where they play a crucial role in the defense mechanism of plants. Natural as well as synthetic PHs exhibit a diverse range of biological activities against fungi, protozoan parasites or human cancer cells. New synthetic PHs have been tested in this study and they show a potential activity against this protozoa.

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## 1. Introduction

Free-living amoebae (FLA) of the genus *Acanthamoeba* are the causative agents of several opportunistic infections in humans, such as a sight-threatening ulceration of the cornea known as

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*Acanthamoeba* keratitis (AK), the usually fatal granulomatous amoebic.

Encephalitis (GAE), and also disseminated infections (mostly cutaneous and nasopharyngeal) (Marciano-Cabral and Cabral, 2003). Currently, the genus *Acanthamoeba* is classified into 19 different genotypes (T1 to T19) based on rRNA gene sequence analysis (Stothard et al., 1998, 1999; Horn et al., 1999; Booton et al., 2002; Gast, 2001; Hewett et al., 2003; Nuprasert et al., 2010; Corsaro and Venditti, 2010; Qvarnstrom et al., 2013; Magnet et al., 2014). The T4 genotype is the most common genotype related to amoebic infections, and also it is the most commonly isolated genotype from the environment (Siddiqui and Khan, 2012; Khan, 2006). Nevertheless, its association with pathogenicity is not explained by its apparent abundance alone (Maciver et al., 2013). *Acanthamoeba* species exist in a trophozoite stage (the metabolically active stage) and a cyst stage that is characterized by the presence of a highly resistant double cyst wall (Schuster and Visvesvara, 2004). Recurrent amoebic infections are a complication of the presence of *Acanthamoeba* cysts, as they are able to survive the currently available treatments (Aksozek et al., 2002). Diamidines (propamidine and hexamidine) and biguanides (chlorhexidine and polyhexamethylene biguanide) have been tested against *Acanthamoeba* trophozoites and cysts (Lorenzo-Morales et al., 2013; Lee et al., 2007) with successful outcomes. However, it has been reported that about 5% of patients with AK present persistent inflammation due to viable *Acanthamoeba* in the cornea, even after prolonged treatment with these molecules (Pérez-Santonja et al., 2003).

Necrosis is characterized by a series of morphological changes, such as increased cellular volume and rupture of the cytoplasmic membrane early in the process, which results in the release of the cellular content and induction of an inflammatory response in the host (Proskuryakov et al., 2003). Therefore, it is important to avoid the use of drugs which could induce a necrotic process in *Acanthamoeba* therapy, since these necrotic cells could be the cause of an inflammation process in the cornea or other infected organs.

Induction of programmed cell death (PCD) in parasites for drug development is a novel possibility that has been explored in great detail in several unicellular parasitic protozoa (Deponce, 2008) but has not been evaluated so far against the *Acanthamoeba* genus. PCD is a very complex type of genetically controlled death. Moreover, the morphological features that define this type of death occur in different stages. First, cell dehydration causes changes in cellular shape and size. Another characteristic event of this process is the condensation of nuclear chromatin. Importantly, the structural integrity and most of the functions of the cell membrane remain intact at least in the initial stages of the process. After this, certain changes start to appear, such as a lack of phospholipid asymmetry and exposure of phosphatidylserine (PS) on the cell exterior, which labels these cells as a target for phagocytic cells (Kerr et al., 1972; Fadok et al., 1998).

PCD in unicellular organisms has been reported so far in yeast, *Dictyostelium discoideum*, *Peridinium gatouense*, *Euglena gracilis*, *Tetrahymena thermophila*, trypanosomatids like *Trypanosoma* and *Leishmania*, *Plasmodium*, *Blastocystis hominis*, and *Entamoeba histolytica* (Deponce, 2008; Scheuerlein et al., 1995; Nguewa et al., 2004; Wanderley et al., 2005; Villalba et al., 2007). In these organisms, these apoptotic processes occur as a phenomenon that presumably benefits the rest of the population in some way. At present, the literature on the existence of apoptosis in *Acanthamoeba* is limited. Evidence for PCD in *Acanthamoeba* has been observed under different conditions as: infected with *Salmonella* (Feng et al., 2009); or after treatment with maslinic acid, caffeine, doxorubicin (Martín-Navarro et al., 2017; Baig et al., 2017; Hajaji et al., 2017), although the specific pathways and the molecules

involved are still unknown.

Natural and synthetic 1*H*-phenalen-1-one containing compounds exhibit a diverse range of biological activities such as antifungal (Quiñones et al., 2000; Lazzaro et al., 2004; Luque-Ortega et al., 2004; Otálvaro et al., 2007; Hidalgo et al., 2009), antiprotozoal (Luque-Ortega et al., 2004; Rosquete et al., 2010; Gutiérrez et al., 2013) and cytotoxic activity against human cancer cells (Mochida et al., 2006). The screening of a collection of new synthetic 1*H*-phenalen-1-one analogs has identified three active compounds (Fig. 1) against *Acanthamoeba castellanii* Neff whose mechanisms of cell death we have investigated.

## 2. Materials and methods

### 2.1. Cultures

*Acanthamoeba castellanii* Neff (ATCC 30010), a type strain from the American Type Culture Collection was used in this study. This strain was axenically grown in PYG medium (0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract and 1.5% (w/v) glucose) containing 40 µg gentamicin ml<sup>-1</sup> (Biochrom AG, Cultek, Granollers, Barcelona, Spain) previous it use for the assays. For the cytotoxicity assays the MCF-7, an epithelial cancer cell line derived from breast adenocarcinoma, was cultured in DMEM supplemented with 10% foetal bovine serum at 37 °C and 5% CO<sub>2</sub> atmosphere.

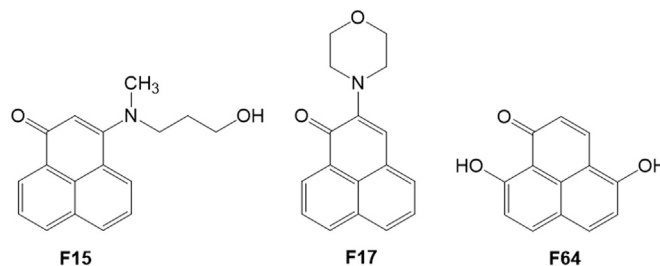
### 2.2. Chemicals

All compounds tested were synthesized (unpublished results). Stock solutions of these drugs were prepared in DMSO, protected from the light, at -20 °C till required.

### 2.3. Activity assays against the trophozoite stage of *Acanthamoeba* spp

The activity of the PHs was tested *in vitro* against the trophozoite stage of *Acanthamoeba castellanii* Neff using a colorimetric assay based on alamarBlue<sup>®</sup> reagent (Invitrogen, Life Technologies, Madrid, Spain) as previously described (McBride et al., 2005; Martín-Navarro et al., 2008).

Briefly, amoeba were counted, diluted (10<sup>4</sup>/well) and added to the wells in 96-well plates. After that, phenalenones were serially diluted in 150 µl PYG medium in a deepwell and were also added to the wells. Finally, 10% of alamarBlue<sup>®</sup> was added to the plates, and these were incubated at 27 °C. After 96 h, the plates were analysed using an EnSpire<sup>®</sup> Multimode Plate Reader (Perkin Elmer, Madrid, Spain) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Percentage of inhibition and 50% inhibitory concentrations (IC<sub>50</sub>) were calculated by no linear regression analysis with 95% confidence limits using Sigma Plot 12.0 statistical analysis software (Systat Software). All experiments were



**Fig. 1.** Chemical structures of the compounds assayed for antiamoebic activity: F15, 3-[(3-hydroxypropyl)(methyl)amino]-1*H*-phenalen-1-one; F17, 2-morpholino-1*H*-phenalen-1-one; F64, 4,9-dihydroxy-1*H*-phenalen-1-one.

performed three times each in duplicate, and the mean values were also calculated. A paired two-tailed *t*-test was used for analysis of the data. Values of  $P < 0.05$  were considered significant.

#### 2.4. Cytotoxicity assay

The MTT assay was performed to assess the cytotoxic effect. The reagent (tetrazolium salt) is a positively charged small molecule that undergoes NADPH-mediated conversion over to Formazan. MTT can enter viable cells and non-viable cells with ease. Upon conversion, the Formazan product precipitates inside cells near the cell surface and can be detected using a spectrophotometer. Briefly, the human cells were incubated with different concentrations of the PHs for 24 h in duplicate. After incubation, MTT was added, and later SDS was used to dissolve the formazan precipitate. To determine the cytotoxicity percentages, the average absorbance values of the duplicates were calculated and compared with negative and positive controls.

#### 2.5. Changes in the mitochondrial membrane potential ( $\Delta\Psi_m$ )

The  $\Delta\Psi_m$  was calculated using *JC-1 Mitochondrial Membrane Potential Assay Kit* (Cayman Chemical, Vitro, Madrid, Spain) as previously described (Martín-Navarro et al., 2015). The JC-1, a lipophilic cationic probe, accumulates in the mitochondrial matrix according to the membrane potential. In healthy cells the  $\Delta\Psi_m$  is normally high, JC-1 spontaneously forms complexes known as J-aggregates, showing intense red fluorescence (emission at 595 nm). In apoptotic or unhealthy cells with a low  $\Delta\Psi_m$ , JC-1 remains in its monomeric cytosolic form and shows only green fluorescence (emission at 535 nm). In brief, the trophozoites, after 24 h with different concentrations of PHs, were harvested and washed with buffer. The cells were then incubated at 26 °C for 30 min with JC-1 dye. Cells were then analysed by fluorescence measurement in black plates through spectrofluorometer using 490 nm as excitation wavelength. Data presented here are representative of three experiments. The ratio of the reading at 595 nm to the reading at 535 nm was considered as the relative  $\Delta\Psi_m$  value.

#### 2.6. Analysis of ATP levels

ATP level was measured using a Cell Titer-Glo<sup>®</sup> Luminescent Cell Viability Assay (Promega), which generates a proportional signal to

the ATP amount. Trophozoites were incubated with different concentrations of PHs for 24 h. Aliquots were taken and mixed with the kit reagent into white plates following the manufacturer's instructions for posterior measurement of the luminescence on a PerkinElmer spectrophotometer.

#### 2.7. Phosphatidylserine externalization

Annexin V/propidium iodide (PI) double staining assay was performed using the *Tali<sup>®</sup> Apoptosis Kit - Annexin V Alexa Fluor<sup>®</sup> 488 & Propidium Iodide* according to the manufacturer's instructions (Life Technologies). Briefly, after being treated with the IC<sub>90</sub> of the tested molecules for 24 h, trophozoites were centrifuged at 1500 rpm for 10 min, washed twice with the Annexin Binding Buffer (ABB) and incubated with 5  $\mu$ l of annexin V for 20 min. After that, cells were centrifuged and resuspended in ABB containing 1  $\mu$ l of PI and incubated for 3 min at room temperature. Finally, 25  $\mu$ l of the stained cells were loaded into a *Tali<sup>®</sup> Cellular Analysis Slide* and were analysed in the *Tali<sup>®</sup> Image-Based Cytometer*. Data were collected using the *Tali<sup>®</sup> data acquisition and analysis software* (Life Technologies Corporation) (Martín-Navarro et al., 2015).

#### 2.8. Plasma membrane permeability

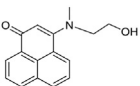
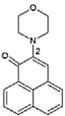
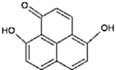
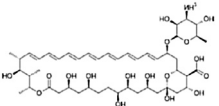
The SYTOX<sup>®</sup> Green assay was performed to detect membrane permeability alterations on the parasites. Briefly,  $2 \times 10^5$  trophozoites/ml were incubated with the SYTOX<sup>®</sup> Green at a final concentration of 1  $\mu$ M (Molecular Probes<sup>®</sup>) for 30 min in the dark at 26 °C. Subsequently, the protozoa were disposed to black plates and the tested compounds were added at the IC<sub>90</sub>. The increase in fluorescence due to binding of the fluorescent marker to the parasitic DNA was measured using an EnSpire<sup>®</sup> Multimode Plate Reader (Perkin Elmer, Madrid, Spain) with excitation wavelength of 504 nm and emission wavelength at 523 nm, and expressed as percentage relative to full permeabilized cells achieved by the addition of 0.1% Triton X-100 (Martín-Navarro et al., 2015).

### 3. Results and discussion

#### 3.1. Amoebicidal activity and cytotoxicity

After incubation of the amoeba with the compounds we observed a dose-dependent inhibition effect on the proliferation of

**Table 1**  
Inhibitory concentrations 50 of the PHs against *A. castellanii*. Percentages of inhibition of the PHs against human cells.

| Molecule       | Structure   | <i>Acanthamoeba castellanii</i> Neff<br>IC <sub>50</sub> ( $\mu$ M) | MCF-7<br>% Inhib $\pm$ DS (10 $\mu$ M) |
|----------------|---|---|--|
| F15            |  | 34,4 $\pm$ 2,39   | 17 $\pm$ 4                             |
| F17            |  | 25,4 $\pm$ 2,24   | 7 $\pm$ 1                              |
| F64            |  | 28,59 $\pm$ 3,97  | 16 $\pm$ 4                             |
| Amphotericin B |  | 39,65   | 17                                     |

the *A. castellanii* Neff tested strain for compounds F15, F17 and F64. The results against the trophozoite stage (Table 1) showed very low values of IC<sub>50</sub>. For instance, F17 showed an IC<sub>50</sub> of 25 μM against the trophozoite stage of *A. castellanii* Neff after the 96 h of treatment.

Once obtained the IC<sub>50</sub> values, we studied the cytotoxicity of these compounds against human breast cancer cell line. The results were expressed as percentage of cytotoxicity, and are shown in Table 1. As an example, compound F64 showed 16% of cytotoxicity when incubated at 10 μM against a human cell line.

When comparing all results with the reference drug Amphotericin B, we could observe that the activity as well as the cytotoxicity of the PHs are very similar to the reference drug Amphotericin B (Martín-Navarro et al., 2013). Even, compound F64 has lower IC<sub>50</sub> when compared to the reference drug, and their toxicity is almost the same against the MCF-7 cell line (Tavangar et al., 2017).

Previous studies have shown that the combined application of a lipid formulation of amphotericin B and voriconazole was effective

in eradicating a skin infection caused by *Acanthamoeba* (Walia et al., 2007). Also Amphotericin B has been used in the treatment of *Acanthamoeba* Encephalitis in combination with more drugs as fluconazole, and azithromycin among others (Azzam et al., 2015). Therefore, these three PHs are candidates to continue their study of mechanism of cell death in this protozoa, specially compound F64.

### 3.2. Mitochondrial alterations

The ATP levels of the trophozoites were not altered after the treatment with the selected PHs at the IC<sub>90</sub> after the 24 h of treatment (Fig. 2). On the other hand, the molecules F15 and F64 induced a strong decrease of the mitochondrial membrane potential when incubated with *Acanthamoeba castellanii* Neff trophozoite (Fig. 2) during 24 h, because the ratio between the monomers and the aggregates of JC-1 dye was reduced more than the half when compared to the negative control. One of the signals of apoptosis in eukaryotic cells is the loss of mitochondrial membrane potential,

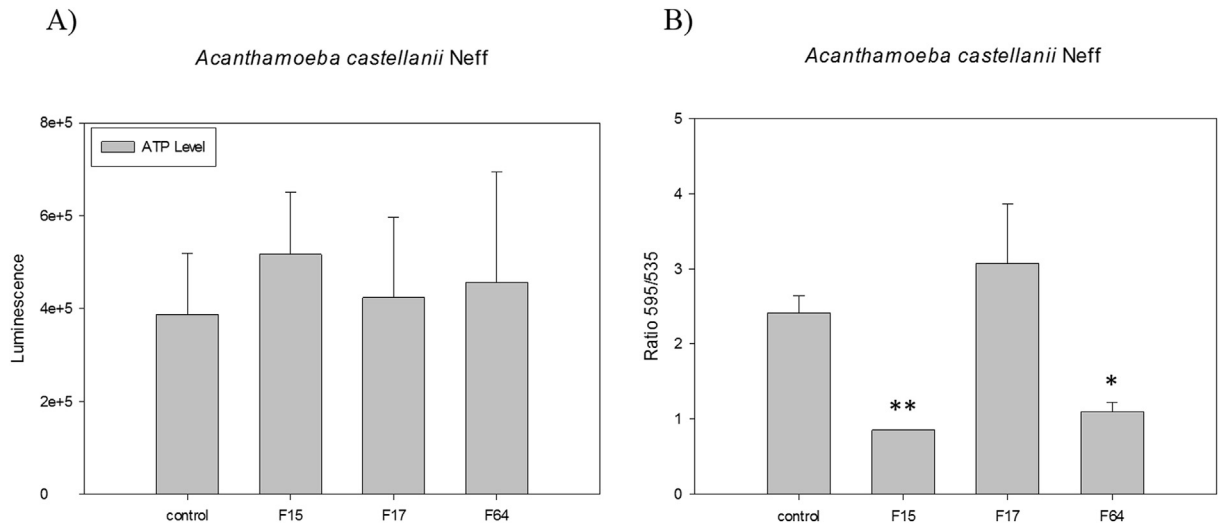


Fig. 2. A) Changes in the mitochondrial membrane potential ( $\Delta\Psi_m$ ) of *A. castellanii* Neff after 24 h of incubation with the IC<sub>90</sub> of PHs. B) ATP levels of *A. castellanii* Neff after 24 h of incubation with the IC<sub>90</sub> of PHs. Error bars represent the standard deviation (SD). Each data point indicates the mean of three measurements.

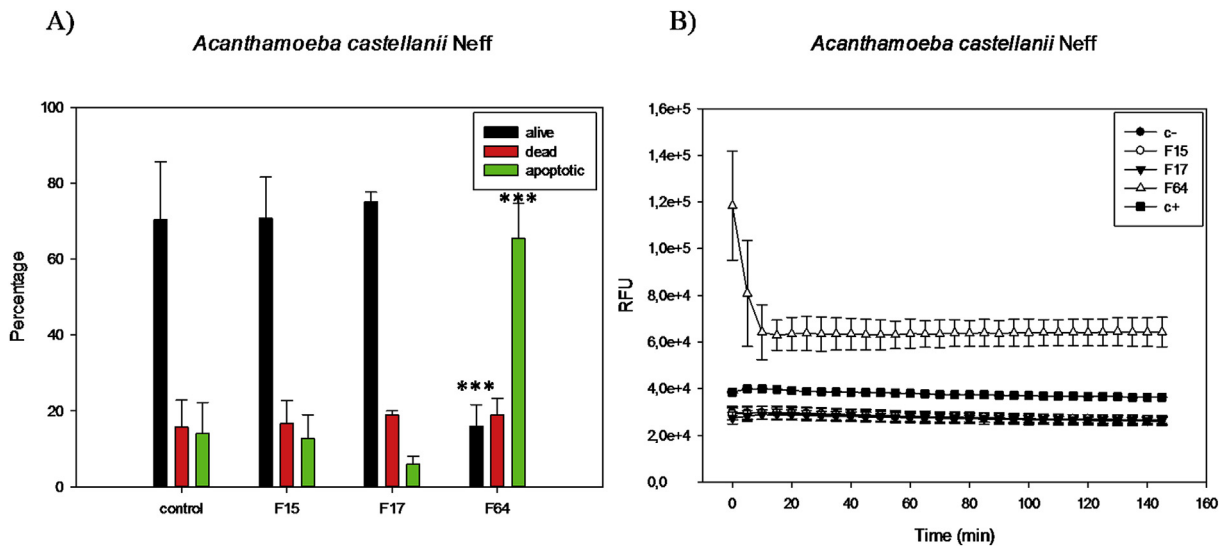


Fig. 3. A) Results of the phosphatidylserine exposure after 48 h of incubation of *A. castellanii* Neff with the IC<sub>90</sub> of PHs. Error bars represent the standard deviation (SD). Each data point indicates the mean of three measurements. B) Plasma membrane permeability assay results. Error bars represent the standard deviation (SD). Each data point indicates the mean of three measurements.

but as well the apoptotic process needs of energy, in form of ATP, to start and support all the mechanisms involved on this process.

### 3.3. Cytoplasmic membrane perturbations

Moreover, some PH derivative induced externalization of PS in *Acanthamoeba*. In this case F64 induced a strong increase in the PS exposure. This can be traduced in an apoptotic pathway were the PS change from the inner to the outer part of the cytoplasmic membrane (Fig. 3). PS exposure is a typical hallmark of apoptosis and thus, validates observations of the apoptotic process in *A. castellanii* after treatment with the phenalenone F64.

The compound F64 was also the only one to induce an increase of the cytoplasmic membrane permeability (Fig. 3), we could observe this effect after the incubation of the treated trophozoites with the SytoxGreen dye. This effect can be due to different causes: for example, the membrane blebbing that occurs later in apoptotic processes in eukaryotic cells; or maybe the molecule P64 has the target of its action on the membrane of the trophozoite.

## 4. Conclusions

In conclusion, substituted PHs were active against the *Acanthamoeba* strain tested in this study, and some of them could induce an apoptosis-like process on *Acanthamoeba castellanii* Neff, avoiding an unnecessary immune response, and considering PHs as good compounds for further studies.

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