Bioorganic Chemistry 99 (2020) 103791

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Identification of *N*-acyl quinolin-2(1*H*)-ones as new selective agents against clinical isolates of *Acanthamoeba* keratitis

María Reyes-Batlle[a], Mónica Blanco Freijo[b], Atteneri López-Arencibia[a,d], Jacob Lorenzo-Morales[a,d], Grant McNaughton-Smith[c], José E. Piñero[a,d,*], Teresa Abad-Grillo[b,*]

[a] *Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Laboratorio de Quimioterapias de Protozoos, Universidad de La Laguna, Tenerife, Spain*
[b] *Instituto Universitario de Bio-Orgánica 'Antonio González', Departamento de Química Orgánica, Universidad de La Laguna, Avda. Astrofísico Fco. Sánchez 2, 38206 La Laguna, Tenerife, Spain*
[c] *Centro Atlántico del Medicamento S.A (CEAMED S.A.), PCTT, La Laguna, Tenerife, Spain*
[d] *Departamento de Obstetricia, Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología, Universidad de La Laguna, Spain*

ARTICLE INFO

InChIKeys:
FIIZQHKGJMRJIL-VAWYXSNFSA-N
LISFMEBWQUVKPJ-UHFFFAOYSA-N
OKZVGSVWYGCZBL-UHFFFAOYSA-N
SYUWSRFFXSPVRJ-UHFFFAOYSA-N
HMKAGBNJQIPWAU-UHFFFAOYSA-N
WDTVDSANNWHEMZ-UHFFFAOYSA-N
LZZOAIIHTNQARH-UHFFFAOYSA-N
BMTFHDYRECNWRB-UHFFFAOYSA-N
QNSNRNGAWRFLOI-UHFFFAOYSA-N
KSALBPDBCBLHKN-UHFFFAOYSA-N
CCVMTVXRTQHYOE-UHFFFAOYSA-N
RPYGMZBSPGKWMR-UHFFFAOYSA-N*Keywords:*
Protozoa
Acanthamoeba
N-acyl quinolin-2(1*H*)-ones
Trophozoites
Cysts

ABSTRACT

A collection of *N*-substituted quinolin-2(1*H*)-ones were screened against a panel of clinically relevant protozoa (*Leishmania*, *Trypanosoma* and *Acanthamoeba*). Three quinolin-2(1*H*)-one compounds were identified as selective anti-A*canthamoeba* agents. Further assessment revealed that these compounds were active against both trophozoite and cyst forms of *A. castellanii* Neff, and caused protozoa death *via* apoptosis. The data presented herein identify *N*-acyl quinolin-2(1*H*)-ones as a promising new class of selective anti-A*canthamoeba* agents.

1. Introduction

Acanthamoeba genus belongs to the free-living amoebae protozoa group. They are found throughout the world and are widely distributed in the environment, as they are able to colonize different habitats such as water, soil and dust [1–3]. Pathogenic strains of *Acanthamoeba* can cause several human diseases such as granulomatous amoebic encephalitis (GAE), primary amoebic meningoencephalitis (PAM), *Acanthamoeba* keratitis (AK), and are associated with disseminated infections in the skin (cutaneous lesions), lungs, sinuses and kidneys [1,4]. GAE is a rare sub-acute necrotizing infection which usually occurs in chronically ill or debilitated patients, particularly those who are immunosuppressed or immune-deficient. The diagnosis of GAE is problematic because of non-specific symptoms (headache, low-grade fever, mood swings, lethargy and confusion) and the lack of a good reliable test, a brain biopsy being the most reliable method. Untreated GAE is often fatal. If caught early, brain surgery to remove the infected areas, is currently the most effective treatment. A small number of antibiotics/anti-fungal/anti-protozoal drugs have however demonstrated *in vitro* activity against *Acanthamoeba* (e.g. rifampicin, ketoconazole, miconazole, pentamidine, sulfadiazine, flucytosine, azithromycin, miltefosine, and caspofungin), and in limited cases there have been some positive results in patients [4,5].

Acanthamoeba keratitis (AK), is a sight-threatening ailment caused by a severe infection of the cornea by *Acanthamoeba*. Like GAE, the lack of awareness of AK, often results in a high level of misdiagnosis. The

* Corresponding authors.
E-mail addresses: jpinero@ull.edu.es (J.E. Piñero), tereabad@ull.es (T. Abad-Grillo).

https://doi.org/10.1016/j.bioorg.2020.103791
Received 14 February 2020; Received in revised form 19 March 2020; Accepted 22 March 2020
Available online 23 March 2020
0045-2068/ © 2020 Elsevier Inc. All rights reserved.

number of reported cases of AK worldwide is steadily increasing; of particular concern is the dramatic rise within those persons using contact lenses [6].

The *Acanthamoeba* life cycle consists of two distinct phases, trophozoites and cysts [7]. To date, evolutionary studies have led to the identification of 22 different genotypes (T1–T22) of *Acanthamoeba*, based on 18S rRNA gene sequencing [8–11]. Only some genotypes cause human infections [12], with the T4 genotype being the most pathogenic and the most common in the environment [11].

So far, there is no single agent sufficiently effective against AK, regardless of the genotype that caused it. This is due in part to the presence of *Acanthamoeba* cysts, which are often resistant to treatment, leading to recurrent amoebic infections [13]. The most commonly used anti-AK agents are functionalized biguanides such as: polyhexamethylene biguanide (PHMB) or combinations of chlorhexidine with aromatic diamidines [2a,7,14–18]. PHMB is effective at relatively low concentrations (0.02%), but only against trophozoites, while chlorhexidine, which is effective against both forms, requires a lengthy treatment regimen that can cause unwanted side effects [19–21]. Even so, about 5% of patients with AK present persistent inflammation due to viable *Acanthamoeba* in the cornea, even after prolonged treatment with these agents [21].

There is therefore a need to identify new anti-*acanthamoebic* drugs that are: (a) effective against both life phases of *Acanthamoeba*, (b) active against a variety of strains of *Acanthamoeba*, (c) less toxic than the current treatments.

As two anti-leishmainal drugs (pentamidine and miltefosine) have demonstrated *in vitro* activities against *Acanthamoeba*, we postulated that other compounds possessing anti-leishmainal activities may also be active against *Acanthamoeba*. Previous works from Messauodi [22a] and Khan [22b] had reported that small, functionalized quinolin-2(1*H*)-one derivatives (Fig. 1, compounds **1** and **2**) and 4-Arylamino-6-nitroquinazolines such as compound (**3**) (Fig. 1), possessed activities against Leishmainal protozoa. Furthermore, Khan and colleagues have recently shown that a series of 3-aryl quinanzolin-4(3*H*)-ones (Fig. 1, compound **4,** for example) possessed anti-AK activities, albeit at relatively high concentrations [23a], and were capable of decreasing the viability of *Acanthamoeba* strains that cause GAE and PAM [23b].

We therefore decided to synthesize and screen a collection of *N*-substituted quinolin-2(1*H*)-ones in order to assess their anti-protozoal activities (Fig. 2).

Herein we report the synthesis of a collection of *N*-substituted quinolin-2(1*H*)-ones, their anti-protozoal activities, and studies to elucidate the mode by which they cause protozoal death.

2. Results and discussion

2.1. Chemistry

The desired parent quinolin-2(1*H*)-one (**7**) was generated from aniline (**5**) via acylation with (2*E*)-3-phenylacryloyl chloride followed by an $AlCl_3$ promoted cyclization and dearylation sequence (Scheme 1) [24a,b].

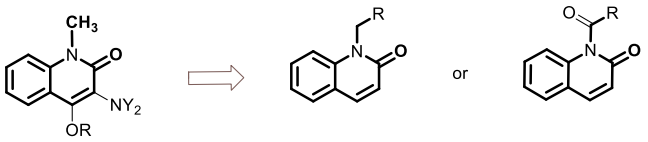


Fig. 2. Structural simplifications of the anti-leishmanial compounds **1** (R = Ts, Y = O) and **2** (R = H, Y = H): proposed *N*-alkyl and *N*-acylquinolin-2(1*H*)-one analogues.

Deprotonation of (**7**) using sodium hydride in DMF, followed by the addition of alkyl electrophiles, generated the corresponding *N*-alkylated products (**8a-e**) in moderate to high yields. The electrophiles were chosen in order to produce products with calculated physiochemical properties that would favor good absorption/distribution properties, while varying their chemical nature (Table 1). Similarly, the addition of activated acyl groups (as either anhydrides or acid chlorides) to deprotonated (**7**) afforded the analogous *N*-acylquinolin-2(1*H*)-one derivatives (**9a-d**), while the reaction between **7** and cyanogen bromide afforded **9f** in good yield. Interestingly, when acetyl chloride was used as the electrophile instead of acetic anhydride, a small amount of the vinyl acetate **9e** (presumably via O-acylation of the intermediate **9c**) was generated.

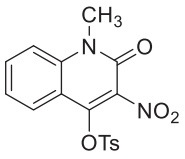
2.2. In vitro antiprotozoal activity

All of the quinolin-2(1*H*)-one-based compounds (**7**, **8a-e** and **9a-f)** were screened against clinically relevant forms of *Trypanosoma* (*T. cruzi*), *Leishmania* (*L. donovani* and *L. amazonensis*) and *Acanthamoeba* (*A. castellanii* Neff, a T4 genotype). Somewhat surprisingly, none of the compounds were active against *T. cruzi* or two strains of *Leishmania* (inhibitions values < 20% of control), even at concentrations as high as 50 μg/ml. Similarly, none of the *N*-alkyl quinolin-2(1*H*)-ones (**8a-e**) were active against *A. castellanii* Neff. Interestingly, the *N*-acylquinolin-2(1*H*)-ones derivatives (**9c** and **9d**), and the *N*-cyano compound (**9f**), did demonstrate activities against *A. castellanii* Neff (Table 2) with potencies similar to that observed for the anti-AK drug chlorhexidine.

Given their interesting anti-protozoan profile, we wished to investigate further their abilities to inhibit other clinically relevant strains of *Acanthamoeba* (Table 3). The compounds were therefore tested against a second clinically isolated T4 genotype (*A. polyphaga*), and two strains isolated from contact lenses [CLC-16 (T3) and CLC-51.l (T1)] [16]. Compound **9c** possessed similar IC_{50} values across the four strains tested (4.81–7.79 μg/mL), while **9f** was more potent against the two T4 clinical isolates, compared to the T3 and T1 strains isolated from contact lenses. Interestingly, while compound **9d** was the least active against the T4 isolates it was the most active against the CLC16 strain (T3), with an IC_{50} value similar to that produced by chlorhexidine.

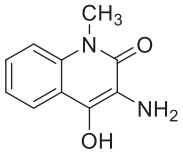
2.3. Anti-excystment assays

As already mentioned, the cystic phase can be more resistant to anti-



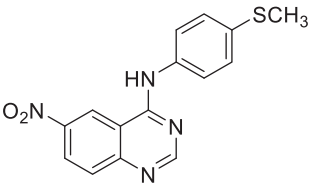
1

L. donovani, IC_{50} = 3 μM
T. b. brucei, MEC > 200 μM



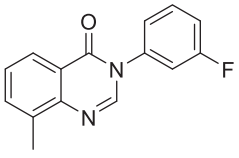
2

L. donovani, IC_{50} = 30 μM
T. b. brucei, MEC = 12.5 μM



3

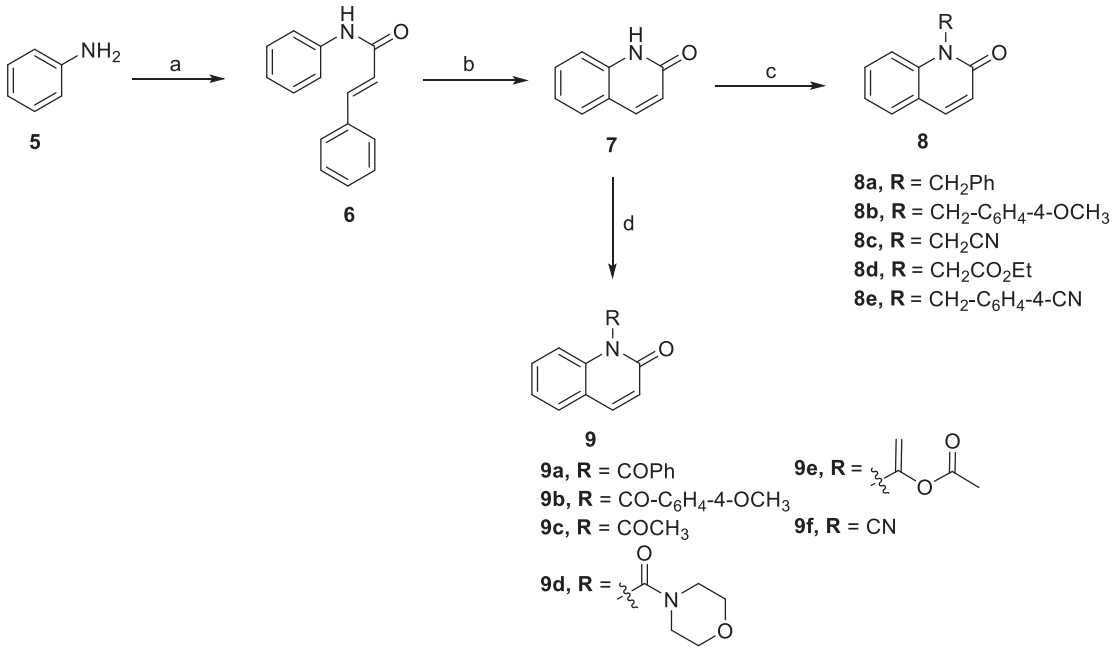
L. Major, IC_{50} = 1.9 μM



4

A. castellanii trophozoites
IC_{50} ~ 50 μg/ml

Fig. 1. Anti-protozoan quinolin-2(1*H*)-one derivatives **1** and **2.**



[a] Reagents and conditions: (a) (2*E*)-3-phenylacryloyl chloride, Py, toluene; (b) $AlCl_3$, PhCl, 90 °C, then 110 °C; (c) $R'CH_2X$ (X = Br, Cl), NaH, DMF, 0 °C-rt; (d) NaH, DMF, 0 °C-rt, then acid chloride, anhydride or BrCN

Scheme 1. Synthesis of *N*-alkyl and *N*-acylquinolin-2(1*H*)-one derivatives[a].

Table 1
Calculated and predicted physiochemical properties for compounds **7**, **8(a-e)** and **9(a-f)**. Average (range).

MW	LogP	tPSA	# of Lipinski volations[a]	Oral Bioavailability[a]
224.6 (145–279)	2.01 (0.84–3.15)	39.4 (20.3–49.9)	0 (all)	Good (all)

[a] Predicted using FAF-Drug4 web service [25].

Acanthamoeba therapy. Therefore, an assay was performed, using *A. castellanii* Neff cysts (as their potencies were relatively similar against this strain), to investigate whether these compounds could inhibit the emergence of the *Acanthamoeba* from this phase. Encouragingly, all three compounds (**9c**, **9d** and **9f**) inhibited the conversion of cysts into trophozoites, with IC_{50} values indistinguishable to that of chlorhexidine (Table 4).

2.4. Selectivity

To assess the specificity of these compounds, their cytotoxicity against murine macrophage cells (J774A.1), were measured [26]. The selectivity index (SI) was calculated as the ratio of the CC_{50} value for J774A.1 and the IC_{50} value for *A. castellanii* trophozoite stage (SI = CC_{50}/IC_{50}). Compound **9f** possessed an SI value (Table 5) marginally less than that found for the anti-AK drug chlorhexidine, while compounds **9c** and **9d** were found to have slightly higher SI values than chlorhexidine.

2.5. Mechanisms of action studies

2.5.1. Plasma membrane permeability

A plasma membrane permeability study was undertaken in order to assess whether compounds **9c**, **9d** and **9f**, caused cell membrane instability. Treatment of trophozoites (*A. castellanii* Neff) with IC_{90} concentrations of the individual compounds, in the presence of SYTOX® Green (normally a non-penetrating fluorescent nuclear stain), identified that compound **9f** caused a time-dependent increase in membrane permeability (Fig. 3) [27,28]. In contrast, compounds **9c** and **9d** did not cause any internalization of the dye. This ability to increase membrane permeability may contribute to why the SI for **9f** is lower compared to those for **9c** and **9d**.

Table 2
Anti-amoebic activity of compounds **9c**, **9d** and **9f** against a panel of different protozoa.

Compound	*T. cruzi* (Epimastigotes)	*L. donovani* (Promastigotes)	*L. amazonensis* (Promastigotes)	*A. castellanii* (Neff)
	IC_{50} ± SD (μg/mL)			
9c	> 50	> 50	> 50	4.81 ± 0.21
9d	> 50	> 50	> 50	7.74 ± 1.09
9f	> 50	> 50	> 50	5.12 ± 0.42
Chlorhexidine	nd	nd	nd	1.53 ± 0.45
Benznidazole	0.83	nd	nd	nd
Miltefosine	nd	1.34 ± 0.12	2.65 ± 0.08	nd

A. castellanii (Neff, genotype T4); IC_{50} = Half maximal inhibitory concentration; each value indicates the mean of three independent experiments; nd = not determined. Chlorohexidine, Benznidazole and Miltefosine are the respective positive control drugs for *Acanthamoeba*, *Typanosoma* and *Leishmania* activities.

Table 3
Anti-amoebic activity of compounds **9c**, **9d** and **9f** against the four strains (trophozoite stage) and compared to reference drug chlorhexidine.

Compound	*A. castellanii* (Neff) (T4)		*A. polyphaga (T4)*	CLC-16 (T3)	CLC-51.l (T1)
	IC_{50} ± SD (μg/mL)	IC_{90} ± SD (μg/mL)	IC_{50} ± SD (μg/mL)		
9c	4.81 ± 0.21	12.86 ± 3.53	6.86 ± 0.66	7.79 ± 1.09	6.01 ± 0.66
9d	7.74 ± 1.09	19.5 ± 3.2	7.59 ± 0.25	4.17 ± 0.58	10.19 ± 0.76
9f	5.12 ± 0.42	13.6 ± 0.02	4.34 ± 0.69	16.02 ± 0.38	12.76 ± 0.80
Chlorhexidine	1.53 ± 0.45	8.15 ± 0.45	1.88 ± 0.495	3.04 ± 0.97	2.67 ± 0.38

A.castellanii (Neff, genotype T4); *A. polyphaga* (clinical isolate strain, genotype T4); *Acanthamoeba* spp. clinical isolate strains from contact lens cases: CLC-16 (genotype T3) and CLC-51.l (genotype T1); IC_{50} = Half maximal inhibitory concentration; CC_{50} = Half maximal cytotoxic concentration; each value indicates the mean of three independent experiments.

Table 4
Anti-excystment activity of compounds **9c**, **9d** and **9f**.

Compounds	*A. castellanii* Neff cysts IC_{50} ± SD (μg/mL)
9d	2.62 ± 0.27
9c	3.09 ± 0.46
9f	2.99 ± 0.80
Chlorhexidine	2.55 ± 0.36

IC_{50} = Half maximal inhibitory concentration; each value indicates the mean of three independent experiments.

Table 5
Cytotoxicity against J774A.1 (ATCC # TIB-67) and calculated SI of selected compounds.

Comp.	*A. castellanii* Neff IC_{50} ± SD (μg/mL)	J774A.1 macrophage CC_{50} ± SD (μg/mL)	SI
9d	7.74 ± 1.09	23.90 ± 2.84	3.1
9c	4.80 ± 0.21	16.80 ± 3.05	3.5
9f	5.12 ± 0.42	8.65 ± 1.40	1.7
Chlorhexidine	1.53 ± 0.45	3.74 ± 0.20	2.2

IC_{50} = Half maximal inhibitory concentration; CC_{50} = Half maximal cytotoxic concentration; SI = Selectivity index representing the ratio of CC_{50} (macrophage)/IC_{50}; Each value indicates the mean of three independent experiments.

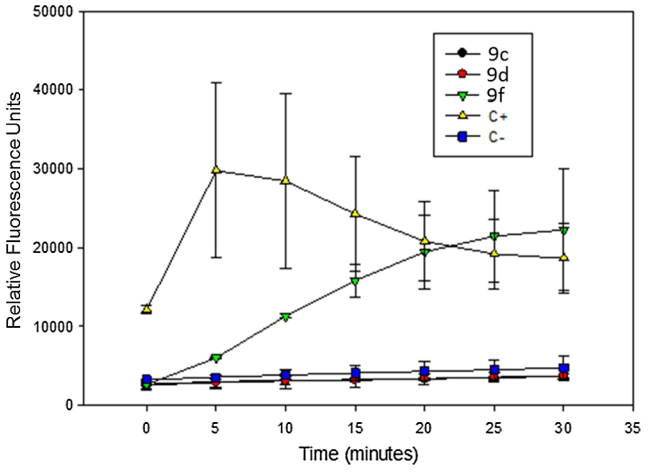


Fig. 3. Alteration of the plasma membrane permeability in *A. castellani* Neff produced by **9c**, **9d** and **9f** comparing vehicle (negative control, C−) and Triton X-100 (positive control C+). Error bars represent the standard deviation (SD). Each data point indicates the mean (N = 3) of duplicate independent experiments.

2.5.2. Effect on ATP levels

Treatment of trophozoites (*A. castellanii Neff*) with IC_{90} concentrations of **9c**, **9d** or **9f**, for 24 h caused substantial reductions in ATP levels (Fig. 4), suggesting a possible mitochondrial mode of action.

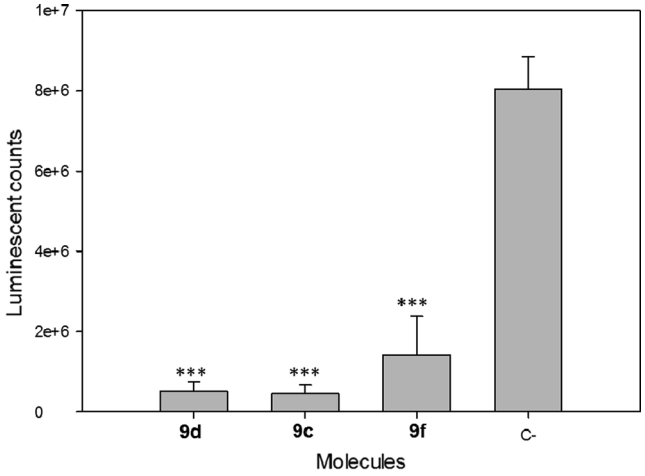


Fig. 4. Changes in *A. castellani* Neff ATP levels produce by the evaluated molecules. Error bars represent the standard deviation (SD). Each data point indicates the mean (N = 3) of duplicate experiments. (***: $p < 0.001$).

2.6. Mechanisms of protozoal death

Of particular concern for anti-AK treatment, is the mechanism by which protozoal death is attained. Compounds that produce necrosis (or non-programmed mechanisms) generally lead to unwanted increases in inflammatory processes in the infected organs [21].

2.6.1. Assay for apoptosis/necrosis determination

The level of apoptosis caused by the treatment of *A. castellanii Neff* protozoa with IC_{90} concentrations of either **9c** or **9d** for 24 h, was assessed by staining with Hoechst 33342 dye. In the same experiment, the level of necrosis was assessed by staining with propidium iodide (PI) [25,26]. Fluorescence microscopy, revealed that **9c** and **9d** caused a large increase in the number of cells containing condensed chromatin, while staining with propidium iodide was almost imperceptible (Fig. 5). Taken together these data indicated that **9c** and **9d** promote apoptosis rather than necrosis in these protozoa.

3. Conclusions

Screening of a collection of *N*-substituted quinolin-2(1*H*)-ones against a panel of clinically relevant forms of *Trypanosoma*, *Leishmania* and *Acanthamoeba* protozoa identified compounds **9c**, **9d** and **9f** as being selectively toxic towards *Acanthameoba*. Further studies revealed these compounds were active against other clinically important strains of *Acanthameoba*, including two strains isolated from contact lenses. Importantly, these compounds were active against both trophozoite and cyst forms of *A. castellanii* Neff. Mechanism of action studies indicated that **9c** and **9d** significantly lowered ATP levels, without increasing cell membrane permeability, and produced protozoa death *via* apoptosis. Diseases produced by *Acanthameoba* are often difficult to diagnose,

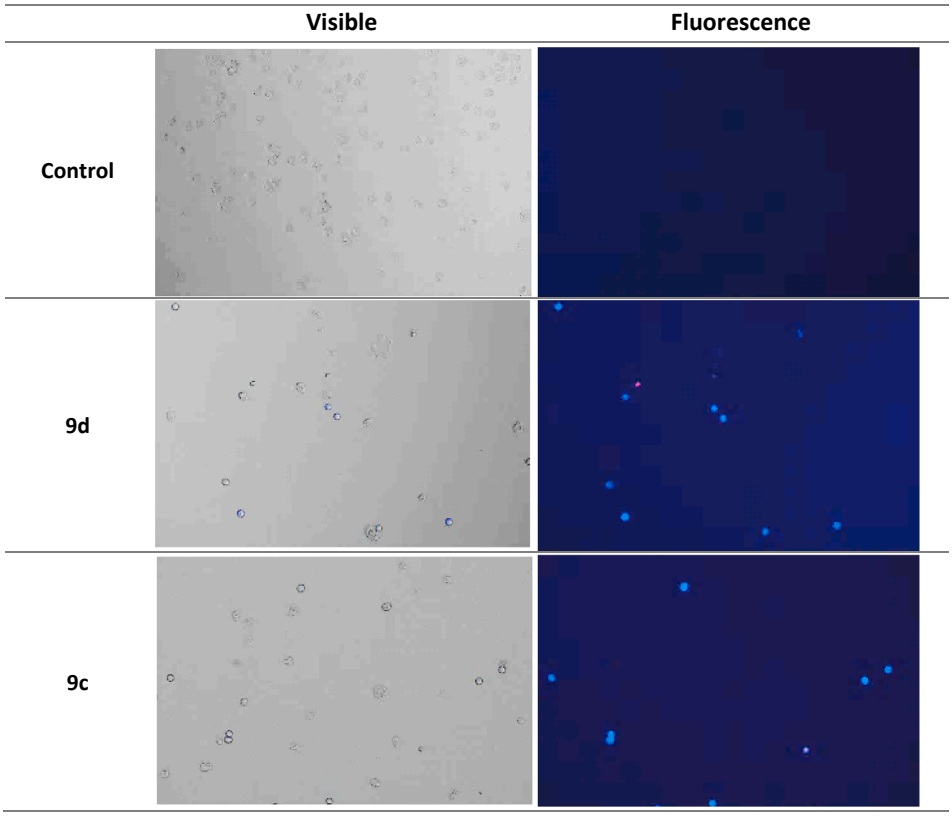


Fig. 5. Image based Live Cell Imaging Microscope EVOS FL Cell Imaging System. The effect of the quinolin-2(1*H*)-one derivatives (**9c** and **9d**) on chromatin condensation (apoptosis) and cell permeability (necrosis) using a Hoechst 33342/propidium iodide double stain.

which leads to delayed treatment and high mortality rates. Current treatments are not fully effective and new alternative treatments may help reduce these mortality rates. The data presented herein suggest that *N*-acyl substituted quinolin-2(1*H*)-ones such as **9c** and **9d** may serve as a new structural class in order to identify better anti-*Acanthamoeba* agents. Studies are in progress elucidate further the structure-activity relationship of these new active compounds, the results of which will be reported in due course.

4. Experimental section

4.1. General methods

All reagents and solvents were obtained from Aldrich Chemical Co. and used without further purification. Reactions with sensitive reagents were performed under inert atmosphere (argon or nitrogen) and organic solvents were dried by standard methods. Unless otherwise stated, solvents were removed under reduced pressure using a rotary evaporator at 40–60 °C. All reactions were monitored by analytical thin layer chromatography (TLC) on POLIGRAM® SIL G/UV_{254} silica gel coated plates (0.20 mm) from MACHEREY-NAGEL. Column chromatography was performed on silica gel 60 (0.063–0.20 mm) from MERCK. Preparative thin layer chromatography was carried out with GF silica gel plates (1 mm) with fluorescent indicator 254 nm (UNIPLATE). Compounds were visualized by ultraviolet light (254 nm). The purity of the final compounds was determined to be ≥95% by high pressure liquid chromatography (HPLC) using a Jasco PU-2080 intelligent HPLC pump (Jasco MD 2020 Plus multi-wavelength detector). ^{1}H and ^{13}C NMR spectra were recorded at room temperature (rt) on a Bruker Avance 400 or 500 MHz NMR spectrometer in the solvent indicated. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), integration, multiplicity and coupling constant (Hz), whereas ^{13}C NMR analyses are reported in terms of chemical shift. Melting points were determined using a Stuart Scientific SMP11 instrument. Low resolution (EIMS) and high-resolution mass spectrometry (HRMS) were performed on a Micromass AutoSpec magnetic *tri-sector* (*EBE* geometry) mass spectromter. Lyophilization was performed using a CHRIST ALPHA 2–4 lyophilizer.

4.2. Synthesis

4.2.1. (2E)-N,3-diphenylacrylamide (6)

To a solution of aniline **5** (6.0 mmol, 0.55 mL) and pyridine (6.0 mmol, 0.48 mL) in anhydrous toluene (5 mL) was added dropwise a solution of (2*E*)-3-phenylacryloyl chloride (1.0 g, 6.0 mmol) in anhydrous toluene. The reaction mixture was stirred for 2 h at room temperature. The precipitate formed was collected by filtration, washed with water and dried under reduced pressure at 50 °C. Product **6** (1.15 g, 86%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.16 (1H, s, NH), 7.74 (1H, d, J = 15.6 Hz, H-2), 7.67–7.66 (2H, m), 7.44–7.42 (2H, m), 7.33–7.30 (5H, m, Ar), 7.11 (1H, t, J = 7.2 Hz), 6.65 (1H, dd, J = 2.0, 15.6 Hz, H-3); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 164.6 (s, C-1), 142.4 (d), 138.2 (s), 134.7 (d), 130.0 (d), 129.2 (d, 2 × C), 128.9 (d, 2 × C), 128.1 (d, 2 × C), 124.6 (d, 2 × C), 121.1 (s), 120.3 (d); EIMS: *m/z* 224 $[M+1]^+$ (13), 223 $[M]^+$ (60), 130 (100), 103 (50), 93 (44), 77 (34); HRMS: calcd. for $C_{15}H_{13}NO$ 223.0997, found 223.1101.

4.2.2. Quinolin-2(1H)-one (7)

To a solution of **6** (1.0 g, 4.5 mmol) in anhydrous chlorobenzene (9 mL) was added $AlCl_3$ (3.0 g, 22.4 mmol) portion wise at room temperature. The reaction mixture was stirred at 90 °C for 3 h and then at 110 °C for 1 h. After cooling to room temperature, the precipitated product was collected by filtration, washed with water and dried under reduced pressure at 50 °C. Product **7** (600 mg, 92%) was obtained as a white solid. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ: 11.05 (1H, br s, NH), 7.89

(1H, d, J = 9.6 Hz, H-4), 7.66 (1H, d, J = 7.8 Hz, H-8), 7.52 (1H, dd, J = 7.4, 8.0 Hz), 7.41 (1H, d, J = 8.2 Hz, H-5), 7.20 (1H, dd, J = 7.4, 7.6 Hz), 6.53 (1H, d, J = 9.6 Hz, H-3); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ: 163.1 (s, C-2), 141.1 (d), 141.0 (s), 131.3 (d), 128.8 (d), 122.9 (d), 122.8 (d), 120.5 (s), 116.0 (d); EIMS: m/z 146 $[M^+ + 1]$ (14), 145 $[M]^+$ (100), 89 (20); HRMS: calcd. for C_9H_7NO 145.0528, found 145.0525.

4.2.3. N-alkyl- and N-benzoylquinolin-2(1H)-one derivatives. General procedure

To a vigorously stirring solution of **5** in anhydrous DMF at 0 °C was added NaH (40%) portion wise. After 30–60 min the acylating or alkylating agent was added, and the reaction mixture was stirred for 1 h-5 days at room temperature and under argon, after which H_2O was added and the mixture was lyophilized·H_2O (10–15 mL) was added and the mixture was extracted with EtOAc (3 × 10 mL). The combined organics phases were washed with brine (10–15 mL), dried (Na_2SO_4), filtered and concentrated. Purification using column or preparative thin layer chromatography (hexane/EtOAc) afforded the desired products.

4.2.3.1. 1-Benzylquinolin-2(1H)-one ***(8a)***. From **5** (61.1 mg, 0.41 mmol), anhydrous DMF (1 mL), NaH 40% (22 mg, 0.92 mmol) and (bromomethyl)benzene (0.83 mmol, 98 μL); stirring for 2 h; column chromatography (hexane/EtOAc; 4:1 to 7:3); product **8a** (77 mg, 78%) was obtained as a colorless oil. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ: 7.90 (1H, d, J = 9.5 Hz, H-4), 7.68 (1H, d, J = 7.6 Hz), 7.46 (1H, dd, J = 7.4, 8.2 Hz), 7.38 (1H, d, J = 8.5 Hz), 7.31–7.26 (4H, m), 7.23–7.17 (2H, m), 6.70 (1H, d, J = 9.5 Hz, H-3), 5.58 (2H, s, H-1′); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ: 162.5 (s, C-2), 140.5 (s, C-8a), 140.4 (d), 138.1 (s), 131.4 (d), 129.8 (d), 129.5 (d, 2 × C), 127.8 (d), 127.5 (d, 2 × C), 122.8 (d), 122.2 (d), 121.7 (s), 115.9 (d), 45.8 (t, C-1′); EIMS: m/z 236 $[M+1]^+$ (22), 235 $[M]^+$, 100), 234 (43), 129 (44), 91 (57); HRMS: calcd. for $C_{16}H_{13}NO$ 235.0997, found 235.0996.

4.2.3.2. 1-(4-Methoxybenzyl)quinolin-2(1H)-one ***(8b)***. From **5** (61.1 mg, 0.41 mmol), anhydrous DMF (1 mL), NaH 40% (24 mg, 1 mmol) and 1-(chloromethyl)-4-methoxybenzene (0.83 mmol, 112 μL); stirring for 4 h; column chromatography (hexane/EtOAc, 4:1); product **8b** (79 mg, 72%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.72 (1H, d, J = 9.4 Hz, H-4), 7.55 (1H, d, J = 7.7 Hz), 7.43 (1H, dd, J = 7.4, 8.0 Hz), 7.30 (1H, d, J = 8.6 Hz), 7.19–7.16 (3H, m), 6.82 (2H, d, J = 8.6 Hz), 6.79 (1H, d, J = 9.6 Hz, H-3), 5.49 (2H, s, H-1′), 3.75 (3H, OCH_3); EIMS: m/z 266 $[M+1]^+$ (8), 265 $[M]^+$ (50), 130 (11), 121 (100), 69 (18). HRMS: calcd. for $C_{17}H_{15}NO_2$ 265.1103, found 265.1132.

4.2.3.3. (2-Oxoquinolin-1(2H)-yl)acetonitrile ***(8c)***. From **5** (0.36 mmol, 51.0 mg), anhydrous DMF (1.7 mL), NaH 40% (1.41 mmol, 33.8 mg) and bromoacetonitrile (1.584 mmol, 117.0 μL); stirring for 5 days; column chromatography (hexane/EtOAc, 9:1, 4:1); product **8c** (42 mg, 64%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.75 (1H, d, J = 9.6 Hz, H-4), 7.68–7.62 (2H, m), 7.37 (1H, d, J = 8.8 Hz), 7.34 (1H, t, J = 7.4 Hz), 6.72(1H, d, J = 9.5 Hz, H-3), 5.27 (2H, s, H-2′); EIMS: m/z 89 (12), 116 (10), 129 (26), 155 (20), 184 $[M]^+$ (100), 185 $[M+1]^+$ (13); HRMS: calcd. for $C_{11}H_8N_2O$ 184.0637, found 184.0643.

4.2.3.4. Ethyl 2-(2-oxoquinolin-1(2H)-yl)acetate ***(8d)***. From **5** (51.0 mg, 0.35 mmol), anhydrous DMF (1.7 mL), NaH 40% (34.0 mg, 1.40 mmol) and ethyl bromoacetate (0.70 mmol, 78.0 μL); stirring for 1 h 30 min; column chromatography (hexane/EtOAc, 9:1, 4:1); product **8d** (67.4 mg, 83%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.71 (1H, d, J = 9.5 Hz, H-4), 7.57 (1H, d, J = 7.8 Hz), 7.53 (1H, dd, J = 7.7, 8.1 Hz), 7.23 (1H, t, J = 7.5 Hz), 7.10 (1H, d, J = 8.5 Hz), 6.72 (1H, d, J = 9.5 Hz, H-3), 5.09 (2H, s, H-2′) 4.23 (2H, c, J = 7,1 Hz, H-1″), 1.25 (3H, t, J = 7,1 Hz, CH_3); EIMS: m/z 232 $[M]^+$ (14), 231 (M^+, 78), 185 (73), 158 (100), 130 (27), 129 (20), 128 (73), 77 (13); HRMS: calcd. for $C_{13}H_{13}NO_3$ 231.0895, found 231.0898.

4.2.3.5. 4-[(2-Oxoquinolin-1(2H)-yl)methyl]benzonitrile ***(8e)***. From **5** (70.0 mg, 0.48 mmol), anhydrous DMF (2.4 mL), NaH 40% (58.0 mg, 2.41 mmol) and 4-(bromometyl)benzonitrile (0.97 mmol, 189 mg); stirring for 1 h; column chromatography (hexane/EtOAc, 4:1, 1:1); product **8e** (88.0 mg, 70%) was obtained as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.63 (1H, d, J = 9.5 Hz, H-4), 7.58–7.55 (3H, m), 7.42 (1H, td, J = 1.5, 7.2 Hz), 7.29 (2H, d, J = 8.5 Hz), 7.20 (1H, td, J = 0.8, 8.0 Hz), 7.10 (1H, d, J = 8.5 Hz), 6.77 (1H, d, J = 9.4 Hz, H-3), 5.57 (2H, s, H-1′).

4.2.3.6. 1-Benzoylquinolin-2(1H)-one ***(9a)***. From **7** (51.0 mg, 0.35 mmol), anhydrous DMF (2.8 mL), 40% NaH (42.0 mg, 1.58 mmol) and benzoic anhydride (159 mg, 0.70 mmol); stirring for 40 min; preparative thin layer chromatography (hexane/EtOAc, 4:1); product **9a** (66.0 mg, 76%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.26 (3H, m), 8.04 (1H, d, J = 8.4 Hz), 7.83 (1H, d, J = 8.1 Hz), 7.71 (1H, t, J = 7.4 Hz), 7.63 (1H, t, J = 7.3 Hz), 7.52 (3H, m), 7.30 (1H, d, J = 8.7 Hz, H-3); EIMS: m/z 250 $[M+1]^+$ (2), 249 $[M]^+$ (12), 221 (48), 145 (14), 105 (100), 77 (32); HRMS: calcd. for $C_{16}H_{11}NO_2$ 249.0790, found 249.07691.

4.2.3.7. 1-(4-Methoxybenzoyl)quinolin-2(1H)-one ***(9b)***. From **7** (50.0 mg, 0.34 mmol), anhydrous DMF (1.7 mL), 40% NaH (41.0 mg, 1.72 mmol) and 4-methoxybenzoyl chloride (118 mg, 0.70 mmol); stirring for 1 h 30 min; preparative thin layer chromatography (hexane/EtOAc, 7:3); product **9b** (69.9 mg, 73%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.22 (3H, m), 8.03 (1H, d, J = 8.4 Hz), 7.82 (1H, d, J = 8.1 Hz), 7.70 (1H, t, J = 7.5 Hz), 7.52 (1H, t, J = 7.4 Hz), 7.29 (1H, d, J = 8.7 Hz), 6.97 (2H, d, J = 8.6 Hz), 3.85 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 164.7 (s), 164.3 (s), 156.9 (s), 146.7 (s), 139.8 (d), 132.7 (d, 2 × C), 130.2 (d), 128.6 (d), 127.6 (d), 127.2 (s), 126.5 (d), 121.3 (s), 113.9 (d, 2 × C), 55.5 (c, CH_3); EIMS: m/z 280 $[M+1]^+$ (2), 279 $[M]^+$ (8), 152 (18), 145 (13), 135 (100); HRMS: calcd. for $C_{17}H_{13}NO_3$ 279.0895, found 279.0907; UV–Vis (EtOH) λ_{max}: 313, 265, 230, 203, 201 nm; IR ($CHCl_3$) ν_{max}: 3062, 2360, 1725, 1603, 1259, 1165, 846 cm^{-1}; mp: 82–84 °C.

4.2.3.8. 1-Acetylquinolin-2(1H)-one ***(9c)***. From **7** (55.0 mg, 0.38 mmol), anhydrous DMF (2 mL), 40% NaH (45.0 mg, 1.9 mmol) and acetic anhydride (0.76 mmol, 72.0 μL); stirring for 5 h 30 min; column chromatography (hexane/EtOAc, 9:1, 4:1); product **9c** (35 mg, 49%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.25 (1H, d, J = 8.7 Hz, H-4), 8.01 (1H, d, J = 8.4 Hz), 7.85 (1H, d, J = 8.1 Hz), 7.71 (1H, t, J = 7.6 Hz), 7.56 (1H, t, J = 7.4 Hz), 7.21 (1H, d, J = 8.7 Hz, H-3), 2.40 (3H, s, CH_3); EIMS: m/z 89 (7), 117 (24), 145 (100), 146 (10), 187 $[M]^+$ (8). HRMS: calcd. for $C_{11}H_9NO_2$ 187.0633, found 187.0631

4.2.3.9. 1-(Morfolin-4-ylcarbonyl)quinolin-2(1H)-one ***(9d)***. From **7** (50 mg, 0.35 mmol), anhydrous DMF (2 mL), 40% NaH (24.8 mg, 1.03 mmol) and morpholine-4-carbonyl chloride (1.04 mmol, 121.0 μL); stirring for 2 h; column chromatography (hexane/EtOAc, 4:1); product **9d** (25 mg, 28%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.23 (1H, d, J = 8.7 Hz, H-4), 8.01 (1H, d, J = 8.4 Hz), 7.84 (1H, d, J = 8.1 Hz), 7.72 (1H, t, J = 7.6 Hz), 7.54 (1H, t, J = 7.6 Hz), 7.25 (1H, d, J = 7.5 Hz), 3.78 (6H, br s), 3.62 (2H, br s). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 156.8 (s, C-2), 152.9 (s, C-1′), 146.6 (s), 139.9 (d), 130.3 (d), 128.6 (d), 127.6 (d), 127.1 (s), 126.5 (d), 115.8 (d), 66.7 (t), 66.6 (t), 45.2 (t), 44.2 (t); EIMS: m/z 258 $[M]^+$ (21), 146 (18), 145 (100), 114 (34), 70 (36); HRMS: calcd. for $C_{14}H_{14}N_2O_3$ 258.1004, found 258.1004; UV–Vis (EtOH) λ_{max}: 204, 230, 300, 313 nm; IR ($CHCl_3$) ν_{max}: 3587, 3064, 1719, 1196, cm^{-1}; mp: 104–106 °C.

4.2.3.10. 1-(2-Oxoquinolin-1(2H)-il)vinyl acetate ***(9e)***. From **7** (55.0 mg, 0.38 mmol), anhydrous DMF (2 mL), 40% NaH (27.0 mg, 1.14 mmol) and acetyl chloride (1.14 mmol, 81.0 μL); stirring for 3 days; preparative thin layer chromatography (hexane/EtOAc, 1:1); product **9e** (1.6 mg, 2%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) ***δ*****:** 7.71 (1H, d, J = 9.6 Hz, H-4), 7.60 (1H, d, J = 8.5 Hz), 7.52 (2H, m), 7.24 (1H, m), 6.67 (1H, d, J = 9.6 Hz, H-3), 5.71 (1H, d, J = 2.4 Hz, H-2), 5.28 (1H, d, J = 2.4 Hz, H-2′), 2.17 (3H, s, CH_3); EIMS: *m/z* 229 $[M]^+$ (1), 187 (14), 186 (38), 159 (18), 146 (15), 145 (100), 128 (11), 117 (24); HRMS: calcd. for $C_{13}H_{11}NO_3$ 229.0739, found 229.0728.

4.2.3.11. (2-Oxoquinolin-1-(2H)-yl)carbonitrile ***(9f)***. From **7** (48.0 mg, 0.33 mmol), anhydrous DMF (1.6 mL), 40% NaH (32 mg, 1.32 mmol) and cyanogen bromide (70 mg, 0.66 mmol); stirring for 3 h 30 min; column chromatography (CH_2Cl_2); product **9f (**46 mg, 55%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 7.76 (1H, d, J = 9.8 Hz, H-4), 7.66 (3H, m), 7.40 (1H, m), 6.62 (1H, d, J = 9.8 Hz, H-3); EIMS: *m/z* 171 $[M]^+$ (11), 170 $[M]^+$ (100), 142 (10), 141 (55), 115 (24); HRMS: calcd. for $C_{10}H_6N_2O$ 170.0480, found 170.0481.

5. Biology

5.1. General

In this study the following positive controls were used. Chlorhexidine: for acanthamoeba assays. Benznidazole: for trypanosome assay. Miltefosine: for leishmania assays. Triton X: as a pore-inducing agent in the plasma membrane permeability assay. Negative controls used vehicle, plus the same percentage of DMSO as added with the serially diluted compounds.

5.2. Acanthamoeba cultures

In this study four *Acanthamoeba* strains were used: (1) *A. castellanii* Neff genotype T4 (ATCC® 30010™) and three clinical isolates: (2) *A. polyphaga* (ATCC® 30461™) genotype T4 and two strains isolated from contact lens cases: (3) CLC16, *Acanthamoeba* spp. genotype T3 and (4) CLC51, *Acanthamoeba* spp. genotype T1 [16]. All of them were axenically grown in PYG medium (0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract and 1.5% (w/v) glucose) containing 20 μg/ml of gentamicin (Biochrom AG, Cultek, Granollers, Barcelona, Spain).

5.3. Macrophage culture

The murine macrophages J774A.1 (ATCC# TIB-67) cell line was cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum at 37 °C and 5% CO_2 atmosphere.

5.4. Anti-Acanthamoeba assay

The biological activity of the molecules against *Acanthamoeba* was evaluated using the alamarBlue® method as previously described [16] in the four strains we have described above. The first step was to seed the *Acanthamoeba* culture in triplicate on a 96-well microtiter plates (50 μL from a stock solution of 10^4 cells/ml). After the trophozoites were adhered to the bottom of the well, 50 μL of serial dilutions of the molecules were added to the 96 plate wells. Finally, the alamarBlue® Reagent (Life Technologies, Madrid, Spain) was placed into each well at an amount equal to 10% of the final volume. Then, plates containing alamarBlue® were incubated during 96 h at 28 °C with a soft agitation. After this incubation, the plates were analysed using an EnSpire® Multimode Plate Reader (Perkin Elmer, Madrid, Spain) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Percentage of inhibition of the molecules, 50% and 90% inhibitory concentrations (IC_{50}, IC_{90}), were calculated by no linear regression analysis with 95% confidence limits using Sigma Plot 12.0 statistical analysis software (Systat Software). All the experiments were carried out three times each in triplicate, calculating the mean values.

5.5. Anti-excystment activity

The *A. castellanii* Neff cysts were prepared as it has been described before [16,25] and following the defined assay protocol [28]. Mature cysts were harvested and washed using PYG medium and seeded in triplicate on a 96-well microtiter plates: 50 μL from a cyst solution of 10^4 cysts/ml and 50 μL from serial dilutions of the most active molecules studied. As a negative control we have used *A. castellanii* cysts incubated with PYG medium. At last, the alamarBlue Reagent® (Life Technologies, Madrid, Spain) was placed into each well at 10% and the plates were incubated for 168 h at 26 °C. The plates were analysed using an EnSpire® Multimode Plate Reader (Perkin Elmer, Madrid, Spain) just as in the trophozoite activity assay. All the experiments were carried out three times each in triplicate, calculating the mean values.

5.6. Cytotoxicity assay

To evaluate the cytotoxicity for the most active molecules we have used the alamarBlue® method as previously described [16]. Firstly, the macrophages J774.A1 (ATCC # TIB-67) were seeded in triplicate on a 96-well microtiter plates with 50 μL from a stock solution of 10^5 cells/ml. Once the macrophages were adhered to the bottom of the well, 50 μL of serial dilutions of the molecules were added to the wells. At last, the AlamarBlue Reagent® (Life Technologies, Madrid, Spain) was placed into each well at 10% and the plates were incubated for 24 h at 37 °C in presence of CO_2 at 5%. The plates were analysed using an EnSpire® Multimode Plate Reader (Perkin Elmer, Madrid, Spain) just as in the activity assay. The 50% cytotoxic concentration (CC_{50}) was calculated with the Sigma Plot 12.0 statistical analysis software (Systat Software) as was explained above. All the experiments were carried out three times each in triplicate, calculating the mean values.

5.7. Cell death mechanisms

5.7.1. Analysis of ATP levels

In order to evaluate the amount of ATP the cells were incubated with the molecules IC_{90} during 24 h. The ATP level was measured using a Cell Titer-Glo® Luminescent Cell Viability Assay (Promega), which generates a proportional signal to the ATP amount. Then, the incubation aliquots from the different treatments were taken and mixed with the kit reagent into white-wall 96-well microtiter plate (Nunc; Thermo Fisher Scientific Inc., Massachusetts, USA) following the manufacturer's instructions for posterior measurement of the luminescence on a PerkinElmer spectrophotometer. As a negative control we have used an *A. castellanii* culture incubated with PYG medium. A One Way Anova Analysis of variance was carried out in order to compare the negative control (cells incubated in presence of PYG medium) with the amoeba treated with the different molecules. The experiments were carried out twice, each in triplicate, calculating the mean values.

5.7.2. Plasma membrane permeability

SYTOX® Green nucleic acid stain (Invitrogen, Life Technologies SA, Madrid, Spain) is a high-affinity nucleic acid stain (maximum of absorption and emission at 504 and 523 nm, respectively) that makes cells with compromised plasma membranes bright green fluorescent [27]. The experiment was carried out by following the manufacturer's recommendations. Firstly, 10^5 cells/ml were seeded in a black-wall 96-well microtiter plate (Nunc; Thermo Fisher Scientific Inc., Massachusetts, USA) with the previously calculated IC_{90s} of each evaluated molecule. As negative and positive control, PYG medium and 2.5% of Triton X-100 [Sigma] respectively were added in order to analyse healthy and fully permeabilized cells accordingly. Measurement was

performed by using an EnSpire microplate reader (PerkinElmer) every 5 min for 3 h, after which samples of amoebae were removed for fluorescence microscopy analysis. A One Way Anova Analysis of variance was carried out in order to compare the negative control (cells incubated in presence of PYG medium) with the amoeba treated with the different molecules. The experiments were carried out three times each in duplicate, calculating the mean values.

5.7.3. Double stain assay for apoptosis/necrosis determination

A double stain apoptosis detection kit (Hoechst 33342/propidium iodide, Invitrogen by Thermo Fisher Scientific) and a Live Cell Imaging Microscope EVOS FL Cell Imaging System (Invitrogen) were used. The kit stains the condensed chromatin of apoptotic cells more brightly than the chromatin of normal cells, and the red-fluorescent PI dye is permeant only to dead cells. The experiment was carried out following the manufacturer. 10^5 cells were incubated in a 24-well plate for 24 h with the previously calculated IC_{90}. The double staining pattern allows the identification of three groups in a cellular population: live cells show only a low level of blue Hoechst 33342 fluorescence, apoptotic cells show a higher level of blue fluorescence, and dead cells show low-blue and high-red PI fluorescence, as this dye only penetrates dead cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

T. A. G. thanks the support by the Organic Chemistry Department, Universidad de La Laguna. MRB was funded RICET (RD16/0027/0001 of the program of Redes Temáticas de Investigación Cooperativa, FIS) and A. L. A. was funded by the Agustín de Betancourt Program.

Appendix A. Supplementary material

^{1}H NMR and ^{13}C NMR spectra and HPLC conditions, data table and spectra. Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.103791. These data include MOL files and InChiKeys of the most important compounds described in this article.

References

[1] F. Marciano-Cabral, G. Cabral, *Acanthamoeba* spp. as agents of disease in humans, Clin. Microbiol. (2003) 273–307, https://doi.org/10.1128/CMR.16.2.273-307.2003.

[2] (a) R. Siddiqui, N.A. Khan, Biology and pathogenesis of Acanthamoeba, Parasites Vectors 5 (6) (2012), https://doi.org/10.1186/1756-3305-5-6;
(b) J. Lorenzo-Morales, C.M. Martín-Navarro, A. López-Arencibia, F. Arnalich-Montiel, J.E. Piñero, B. Valladares, Acanthamoeba keratitis: an emerging disease gathering importance worldwide? Trends Parasitol. 29 (2013) 181–187, https://doi.org/10.1016/j.pt.2013.01.006.

[3] J. Lorenzo-Morales, J.F. Lindo, E. Martinez, D. Calder, E. Figueruelo, B. Valladares, A. Ortega-Rivas, Pathogenic *Acanthamoeba* strains from water sources in Jamaica, West Indies, Ann. Trop. Med. Parasitol. 99 (2005) 751–758, https://doi.org/10.1179/136485905X65215.

[4] K. Król-Turmińska, A. Olender, Human infections caused by free-living amoebae, Ann. Agric. Environ. Med. 24 (2) (2017) 254–260, https://doi.org/10.5604/12321966.1233568.

[5] G.S. Visvesvara, Free-living amebae as opportunistic agents of human disease, J. Neuroparasitol. 1 (2010), https://doi.org/10.4172/2314-7326.1000106.

[6] D. Verhelst, C. Koppen, J. Van Looveren, A. Meheus, M.J. Tassignon, the Belgian Keratitis Study Group. Contact lens-related corneal ulcers requiring hospitalization: a 7-year retrospective study in Belgium, Acta Ophthalmol. Scand. 84 (2006) 522–526, https://doi.org/10.1111/j.1600-0420.2006.00681.x.

[7] F.L. Schuster, G.S. Visvesvara, Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals, Int. J. Parasitol. 34 (2004) 1001–1027, https://doi.org/10.1016/j.ijpara.2004.06.004.

[8] D. Corsaro, J. Walochnik, M. Köhsler, M.B. Rott, Acanthamoeba misidentification and multiple labels: redefining genotypes T16, T19, and T20 and proposal for Acanthamoeba micheli sp nov. (genotype T19), Parasitol. Res. 114 (2015) 2481–2490, https://doi.org/10.1007/s00436-015-4445-8.

[9] P.A. Fuerst, G.C. Booton, M. Crary, Phylogenetic analysis and the evolution of the 18S rRNA gene typing system of *Acanthamoeba*, J. Eukaryot Microbiol. 62 (2015) 69–84, https://doi.org/10.1111/jeu.12186.

[10] A.K. Tice, L.L. Shadwick, A.M. Fiore-Donno, S. Geisen, S. Kang, G.A. Schuler, F.W. Spiegel, K.A. Wilkinson, M. Bonkowski, K. Dumack, D.J. Lahr, E. Voelcker, S. Clauß, J. Zhang, M.W. Brown, Expansion of the molecular and morphological diversity of Acanthamoebidae (Centramoebida, Amoebozoa) and identification of a novel life cycle type within the group, Biol. Direct 11 (2016) 69, https://doi.org/10.1186/s13062-016-0171-0.

[11] A. Haniloo, A. Pezeshki, A. Mahmmodzadeh, E. Kadkhodamohammadi, Genotyping of *Acanthamoeba* spp. from water sources from Northwestern Iran, Acta Parasitol. 62 (4) (2017) 790–795, https://doi.org/10.1515/ap-2017-0095.

[12] M. Adamska, Molecular characterization of *Acanthamoeba* spp. occurring in water bodies and patients in Poland and redefinition of Polish T16 Genotype, J. Eukaryot Microbiol. 63 (2015) 262–270, https://doi.org/10.1111/jeu.12275.

[13] A. Aksozek, K. McClellan, K. Howard, J.Y. Niederkorn, H. Alizadeh, Resistance of *Acanthamoeba castellanii* cysts to physical, chemical, and radiological conditions, J. Parasitol. 88 (2002) 621–623, https://doi.org/10.1645/0022-3395(2002)088[0621:ROACCT]2.0.CO;2.

[14] D.F.P. Larkin, S. Kilvington, J.K.G. Dart, Treatment of *Acanthamoeba* keratitis with polyhexamethylene biguanide, Ophthalmology 99 (1992) 185–191, https://doi.org/10.1016/s0161-6420(92)31994-3.

[15] N. Lim, D. Goh, C. Bunce, W. Xing, G. Fraenkel, T.R. Poole, L. Ficker, Comparison of polyhexamethylene biguanide and chlorhexidine as monotherapy agents in the treatment of *Acanthamoeba* keratitis, Am. J. Ophthalmol. 145 (2008) 130–135, https://doi.org/10.1016/j.ajo.2007.08.040.

[16] C.M. Martín-Navarro, J. Lorenzo-Morales, M.G. Cabrera-Serra, F. Rancel, N.M. Coronado-Alvarez, J.E. Piñero, B. Valladares, The potential pathogenicity of chlorhexidine-sensitive *Acanthamoeba* strains isolated from contact lens cases from asymptomatic individuals in Tenerife, Canary Islands, Spain, J. Med. Microbiol. 57 (2008) 1399–1404, https://doi.org/10.1099/jmm.0.2008/003459-0.

[17] R. Kumar, D. Lloyd, Recent advances in the treatment of *Acanthamoeba* keratitis, Clin. Infect. Dis. 35 (2002) 434–441, https://doi.org/10.1086/341487.

[18] J.E. Lee, B.S. Oum, H.Y. Choi, H.S. Yu, J.S. Lee, Cysticidal effect on *Acanthamoeba* and toxicity on human keratocytes by polyhexamethylene biguanide and chlorhexidine, Cornea 26 (2007) 736–741, https://doi.org/10.1097/ICO.0b013e31805b7e8e.

[19] G. Ferrari, S. Matuska, P. Rama, Double-biguanide therapy for resistant *Acanthamoeba* keratitis, Case Reports Ophthalmol. 2 (2011) 338–342, https://doi.org/10.1159/000334270.

[20] M. Itahashi, S. Higaki, M. Fukuda, H. Mishima, Y. Shimomura, Utility of real-time polymerase chain reaction in diagnosing and treating *Acanthamoeba* keratitis, Cornea 30 (2011) 1233–1237, https://doi.org/10.1097/ICO.0b013e3182032196.

[21] J.J. Pérez-Santonja, S. Kilvington, R. Hughes, A. Tufail, M. Matheson, J.K. Dart, Persistently culture positive *Acanthamoeba* keratitis: in vivo resistance and in vitro sensitivity, Ophthalmology 110 (2003) 1593–1600, https://doi.org/10.1016/S0161-6420(03)00481-0.

[22] (a) D. Audisio, S. Messaoudi, S. Cojean, J.-F. Peyrat, J.-D. Brion, C. Bories, F. Huteau, P.M. Loiseau, M. Alami, Synthesis and antikinetoplastid activities of 3-substituted quinolinones derivatives, Eur. J. Med. Chem. 52 (2012) 44–50, https://doi.org/10.1016/j.ejmech.2012.03.003;
(b) S.M. Saad, N. Ghouri, S. Perveen, K.M. Khan, M.I. Choudhary, 4-Arylamino-6-nitroquinazolines: Synthesis and their activities against neglected disease Leishmaniasis, Eur. J. Med. Chem. (2015), https://doi.org/10.1016/j.ejmech.2015.11.016.

[23] (a) A. Anwar, M.S. Shahbaz, S.M. Saad, K.M. Khan, R. Siddiqui, N.A. Khan, Novel antiacanthamoebic compounds belonging to quinazolinones, Eur. J. Med. Chem. 182 (2019) 111575–111586, https://doi.org/10.1016/j.ejmech.2019.111575;
(b) M.R. Mungroo, M.S. Shahbaz, A. Anwar, S.M. Saad, K.M. Khan, N.A. Khan, R. Siddiqui, Aryl quinazolinone derivatives as novel therapeutic agents against brain-eating amoebae, ACS Chem. Neurosci. (2020 Jan 21), https://doi.org/10.1021/acschemneuro.9b00596.

[24] (a) T. Manimaran, T.K. Thiruvengadam, V.T. Ramakrishnan, Synthesis of coumarins (2-Oxo-2H-1-benzopyrans), thiacoumarins (2-Oxo-2H-1-benzothiopyrans, and carbostyrils (2-Oxo-1,2-dihydroquinolines), Synthesis (1975) 739–741, https://doi.org/10.1055/s-1975-23918;
(b) M.C. Elliott, S.W. Wordingham, Reversibility in lewis-acid promoted reactions of N-arylcinnamamides, Synlett 5 (2004) 898–990, https://doi.org/10.1055/s-2004-820024.

[25] FAF-Drugs4 (Free ADME-Tox Filtering Tool). http://fafdrugs4.mti.univ-paris-diderot.fr/index.html. For a description and reference for each parameter, see http://fafdrugs4.mti.univ-paris-diderot.fr/descriptors.html.

[26] The murine macrophage cell line J774A.1 is a surrogate “normal/healthy” cell line for specificity testing, that has been used extensively for anti-protozoal compounds. Furthermore, as murine models are typically the next development step for potential anti-AK and anti-GAE agents, their toxic effects in murine cell lines is warranted.

[27] I. Sifaoui, M. Reyes-Batlle, A. López-Arencibia, O. Chiboub, J. Rodríguez-Martín, P. Rocha-Cabrera, B. Valladares, J.E. Piñero, J. Lorenzo-Morales, Toxic effects of selected proprietary dry eye drops on *Acanthamoeba*, Sci. Rep. 8 (2018) 8520, https://doi.org/10.1038/s41598-018-26914-3.

[28] C.M. Martín-Navarro, A. López-Arencibia, I. Sifaoui, M. Reyes-Batlle, B. Valladares, E. Martínez-Carretero, J.E. Piñero, S.K. Maciver, J. Lorenzo-Morales, Statins and voriconazole induce programmed cell death in *Acanthamoeba castellanii*, Antimicrob. Agents Chemother. 59 (2015) 2817–2824, https://doi.org/10.1128/AAC.00066-15.