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In Vitro Activity of Squaramides and Acyclic Polyamine Derivatives against Trophozoites and Cysts of *Acanthamoeba castellanii*

M. J. Rosales[1*], M. Ximenis[2], A. Costa[2], C. Rotger[2], D. Romero[1], F. Olmo[1], E. Delgado[3], M. P. Clares[3], E. García-España[3], C. Marín[1], M. Sánchez[1]

[1]Departamento de Parasitología, Facultad de Ciencias, Universidad de Granada, Granada, Spain
[2]Universidad de las Islas Baleares, Palma de Mallorca, Spain
[3]Universidad de Valencia, Valencia, Spain
Email: *mjrosale@ugr.es

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Abstract

Pathogenic strains of *Acanthamoeba* cause keratitis (AK), granulomatous amoebic encephalitis (GAE), amoebic pneumonitis (AP), and skin infection in human and animals. The treatment of an *Acanthamoeba* infection is invariably very difficult and not always effective, and compounds that are amebicidic or amebistatic are frequently toxic and/or irritating for humans. Squaramides and polyamine derivatives have been demonstrated to have antitumor and antiprotozoal activity. The aim of this study was to investigate the activity of 5 squaramides and 5 acyclic polyamines against trophozoites and cysts of *A. castellanii* Neff. Amoebicidal activity against the trophozoites and cytotoxicity against Vero cells were evaluated with a colorimetric assay, using Alamar Blue®, and chlorhexidine digluconate was assayed as the reference drug. The squaramides 3 and 5 and the acyclic polyamine 6 appeared to be the most active against the trophozoites and their cytotoxicity was low, showing selectivity indexes of 28.3, 26, and 25.7, respectively, similar to the control drug, chlorhexidine digluconate (27.6). But only the squaramide 3 showed complete cysticidal activity at the concentrations of 100 and 200 μM, as the chlorhexidine digluconate. Further studies of the mechanism of action and *in vivo* assays are needed, but squaramide 3 could be used for developing novel therapeutic approaches against *Acanthamoeba* infections.

Keywords

Acanthamoeba, Squaramides, Acyclic Polyamines, Amoebicidal, Cysticidal

1. Introduction

Acanthamoeba is a free-living opportunistic protozoan parasite that is distributed in diverse environments including air, soil, freshwater, seawater, tap water, bottled mineral water, laboratory distilled water wash bottles, chlorinated swimming pools, and sewage. In addition, *Acanthamoeba* is known to among the most ubiquitous organisms that can be isolated from fish, reptiles, birds, and mammals [1] [2] [3] [4]. In fact, over 80% of immunocompetent individuals bear serum antibodies against *Acanthamoeba* antigens [5], clearly suggesting our common exposure to this parasite. Some *Acanthamoeba* species, such as *Acanthamoeba castellanii*, can cause amoebic keratitis (AK) in immunocompetent and immunocompromised individuals [6] [7], which is often associated with contact-lens wearers, as first reported by Naginton *et al.* [8]. On the other hand, immunocompromised individuals, including patients with AIDS, diabetes, lupus erythematosus or chemotherapy disorders, can develop granulomatous amoebic encephalitis (GAE) [9] [10]. However, this type of encephalitis is rarely observed in healthy people [11]. Amoebic pneumonitis (AP) as well as chronic lung and skin infection can be caused by *Acanthamoeba,* too. In addition, *Acanthamoeba* can serve as reservoirs for other pathogenic bacteria that are able to divide or simply survive within the cysts, such as *Pseudomonas, Legionella, Mycobacterium*, and *Francisella tularensis* [12] [13] [14].

Today, the therapy of these diseases is still problematic due to the lack of sufficiently effective drugs, resistance of the amoebas, variable efficacy between strains or species, and toxicity.

Therefore, there is a clear need for new anti-*Acanthamoeba* drugs. In recent years, many new treatments, natural and synthetic, have been tested with different degrees of success [15]-[24].

In this study, we have evaluated the effectiveness of several squaramides and acyclic polyamine compounds of new synthesis against trophozoites and cysts of *Acanthamoeba castellanii* Neff strain. Squaramide-based compounds have been demonstrated to be therapeutic, and we have described some series of oligomeric cyclosquaramides as kinase inhibitors with antitumor activity [25]. The antiparasitic activity of squaramides has been demonstrated, also, some showing antimalarial [26] and others antichagasic activity [27]. On the other hand, several macrocyclic scorpiand-like polyamines have proved effective against both the acute and chronic phases of Chagas disease [28].

2. Materials and Methods

2.1. Culture of *Acanthamoeba castellanii* Trophozoites and Encystment

Acanthamoeba castellanii Neff (ATCC 30010), obtained from The Center of Scientific Instrumentation of the University of Granada, was used in this study.

Trophozoites were axenically cultured in Falcon Flasks with Casitone Glucose Vitamins medium (CGV) supplemented with 10% inactivated fetal calf serum,

[29], at 28˚C.

Cyst forms were obtained as previously described, [30]. Briefly, *Acanthamoeba* trophozoites medium CGV was removed and 8% glucose in Roswell Park Memorial Institute medium (RPMI) 1640 was added. Amoebas were incubated at 30˚C for up to 48 h, followed by the addition of Sodium Dodecyl Sulfate (SDS) (0.5% final conc.). The trophozoites are SDS-sensitive and are immediately lysed upon addition of SDS, while cysts are SDS-resistant and remain intact [30].

2.2. Drugs Tested

The compounds tested were the squaramides and acyclic polyamines shown in Table 1. Squaramides 1 to 5 have a common side chain consisting of a N-(3-(dimetylaminopropyl)-N-methyl residue. This residue has proven critical for the design of squaramide-based antichagasic agents [27]. The second arm of the squaramide consisted of aliphatic chains with increasing lengths aimed at improving the lipophilicity of the squaramides. The amines tested are ethylenediamine derivatives with amine group mono- or dimethylated amine and the other one functionalized with a methyl pyridine substituent.

For amoebicidal assays, squaramides and acyclic polyamines were prepared at different concentrations of 400, 200, 100, 50, 25, and 10 μM in CGV medium with 10% inactivated fetal bovine serum. The drugs were prepared at 200, 100, 50, and 10 μM concentrations in RPMI medium with 8% glucose for the studies of cysticidal activity. For cytotoxicity test on Vero cells the drugs were prepared at 800, 400, 200, 100, and 50 μM concentrations in RPMI with 10% inactivated fetal bovine serum.

Chlorhexidine digluconate (Sigma, Aldrich Ltd) was used as the control drug against *A. castellanii* and was prepared in the same way as the squaramides and acyclic polyamines.

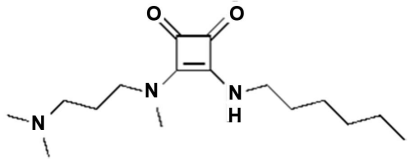
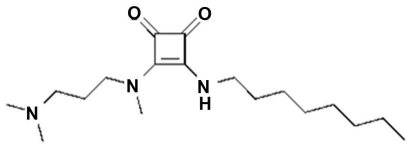
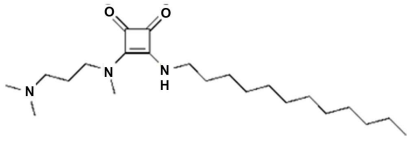
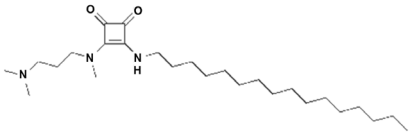
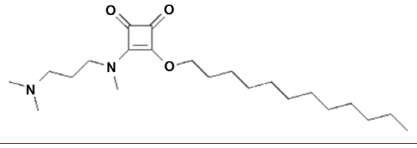
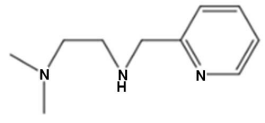
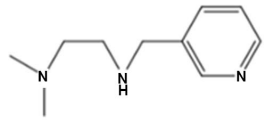
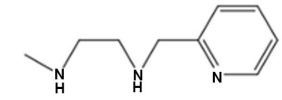
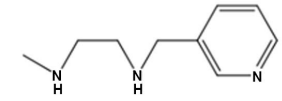
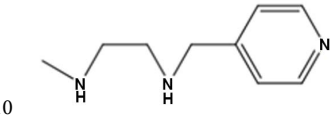
The solutions of the compounds were stored at −20˚C [27] [28]. These squaramides and acyclic polyamines are stable in this medium and can be stored without decomposition for long periods of time.

2.3. *In Vitro* Amoebicidal Assays

AlamarBlue Assay Reagent® was used in order to determine the *A. castellanii* anti-trophozoite activity of the squaramides and acyclic polyamines as previously described [15] [31] with some modifications.Assays were conducted using a 96-well microtiter plate (Sigma, Aldrich). Briefly, 1×10^4 trophozoites, in 100 μl of CGV medium with 10% inactivated fetal bovine serum, were seeded in the microtiter plates and were allowed to adhere for incubation 1 h at 28˚C.

After that, 100 μl of the solutions of the squaramides or acyclic polyamines prepared at 400, 200, 100, 50, 25, and 10 μM were added to each well. Alamar-Blue Assay Reagent® (Thermo Fisher Scientific) was placed into each well at 10% of the final volume, 20 μl. Test plates were incubated for 120 h at 28˚C in complete darkness to avoid the oxidation of the resazurin with the light. The plates

Table 1. Squaramides and Acyclic Polyamines tested against *Acanthamoeba castellanii.*

Chemical structure	Chemical name
Squaramides	
 1	3-((3-(dimethylamino)propyl)(methyl)amino)-4-(hexylamino)cyclobut-3-ene-1,2-dione
 2	3-((3-(dimethylamino)propyl)(methyl)amino)-4-(octylamino)cyclobut-3-ene-1,2-dione
 3	3-((3-(dimethylamino)propyl)(methyl)amino)-4-(dodecylamino)cyclobut-3-ene-1,2-dione
 4	3-((3-(dimethylamino)propyl)(methyl)amino)-4-(hexadecylamino)cyclobut-3-ene-1,2-dione
 5	3-((3-(dimethylamino)propyl)(methyl)amino)-4-(dodecyloxy)cyclobut-3-ene-1,2-dione
Acyclic Polyamines	
 6	1-(2-piridil)-5-metil-2,5-diazahexano
 7	1-(3-piridil)-5-metil-2,5-diazahexano
 8	1-(2-piridil)-2,5-diazahexano
 9	1-(3-piridil)-2,5-diazahexano
 10	1-(4-piridil)-2,5-diazahexano

were subsequently analyzed using a test wavelength of 570 nm and a reference wavelength of 630 nm with a microplate absorbance reader (Sunrise^{TM}, Tecan).

Chlorhexidine digluconate (Sigma, Aldrich Ltd) was used as reference drug

against *A. castellanii* trophozoites. This control drugs and other controls only with trophozoites, without drugs, were tested in the same way.

All the experiments were performed in quadruplicate and the concentration required to give 50% inhibition (IC_{50}) was calculated by linear regression analysis from Kc values at the concentrations used, (5, 12.5, 25, 50, 100, and 200 μM). Selective indexes, (IC_{50} Vero cells toxicity/IC_{50} activity on trophozoites forms of the parasite), were also calculated.

2.4. Cysticidal Activity Assays

Acanthamoeba castellanii cysts were obtained according to Cordingley *et al.* [30]. Cysts were washed twice with RPMI medium. Afterwards, 1×10^4 cyst were added to each well of a microtiter plate in 200 μl of the RPMI with 8% glucose and the compounds at 200, 100, 50, and 10 μM. In the same way was tested the control drug, chlorhexidine digluconate.

The microtiter plate was incubated for 120 h at 28˚C. Finally, cysts were slightly washed and fresh CGV medium with 10% inactivated fetal bovine serum was added to each well, the microtiter plate was incubated at 28˚C, and the number of cysts and trophozoites were counted using a Neubauer chamber at different times of 24, 48, 72, 120, and 192 h. The presence of trophozoites was considered as indication of cyst viability.

Controls only with cysts, without drugs, were tested in the same way.

All the experiments were made in quadruplicate

2.5. Cytotoxicity Test on Vero Cells

The cytotoxicity induced by each compound was tested with Vero cells (ATCC CCL-81) growing in RPMI (Gibco) supplemented with 10% inactivated fetal bovine serum in a humidified 95% air and 5% CO_2 atmosphere at 37˚C. Vero cells, 10^4 in 100 μl of medium, were added to each well of a microtiter plate and incubated for 24 h at 37˚C in a humid atmosphere enriched with 5% CO_2. Next, 100 μl of the drug prepared at 800, 400, 200, 100, and 50 μM were added to each well. AlamarBlue Assay Reagent® (Thermo Fisher Scientific) was placed into each well at 10% of the final volume, 20 μl. Test plates were incubated for 120 h at 37˚C and were subsequently analyzed using a test wavelength of 570 nm and a reference wavelength of 630 nm with a microplate absorbance reader (Sunrise™, Tecan).

Vero cells were treated with chlorhexidine digluconate in the same way and a control with only Vero cells without treatment were done.

All the experiments were conducted in quadruplicate and the concentration required to give 50% inhibition (IC_{50}) was calculated by linear regression analysis from Kc values at the concentrations used (400, 200, 100, 50 and 25 μM).

3. Results and Discussion

Amoebic keratitis (AK) is normally treated with a combination of a diamidine

(hexamidine or propamidine isothionate) and a biguanide (chlorhexidine or polyhexamethylene biguanide) [32] [33]. However, drug resistance against AK treatment has been demonstrated [4] [32] [34] [35] [36] and at present, there is no completely effective treatment for granulomatous amoebic encephalitis GAE [20].

For a better treatment against *Acanthamoeba*, we assayed two groups of compounds: squaramides and acyclic polyamines (**Table 1**). Antiparasitic activity (antimalarial and anti-Chagas) has been demonstrated for some of them [26] [27] [28]. Some aminosquaramide compounds induced changes in the *T. cruzi* glycolysis cycle and caused cytoplasmic and mitochondrial alterations [27] [28].

We assayed the amoebicidal and cysticidal activity of 5 squaramides and 5 acyclic polyamines against *A. castellanii* Neff vs. the reference drug, chlorhexidine digluconate, a standard antiseptic used treating *Acanthamoeba* keratitis with demonstrated cysticidal activity [9] [34].

The cytotoxic activity of the compounds was assayed against mammalian Vero cells and the selective index (IC_{50} Vero cells toxicity/IC_{50} activity on trophozoites forms of the parasite) was calculated for each drugs.

AlamarBlue Assay Reagent® was used to determine the anti-trophozoite, of *A. castellanii*, and cytotoxic activity. This technique was used because it is easy, sensitive and cheap. Resazurin, which is the active ingredient of AlamarBlue® reagent, is a non-toxic, cell-permeable compound that is blue colored and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. Viable cells continuously convert resazurin to resorufin, intensifying the overall fluorescence and color of the media surrounding cells.

The *in vitro* trophocidal activity, cytotoxicity, and selectivity indexes are summarised in **Table 2**. The analysis of these results reveals that two squaramides, 3 and 5, and two acyclic polyamines, 6 and 7, were the most effective against trophozoites of *A. castellanii*. Squaramide 3 gave the lowest IC_{50} value, 3.5 ± 0.5 μM vs. 5.3 ± 3.1 μM of the chlorhexidine digluconate, and its selectivity index was higher than for the control drug (28.3 vs. 27.64). Squaramide 5 with IC_{50} of 11.4 ± 1.2 and selectivity index of 26 showed lower trophocidal activity than that of squaramide 3 and the control drug but was less cytotoxic. Both squaramides feature similar structures but different substitution patterns of the cyclobutendione moiety. Squaramide 3 is a N, N-bisubstituted compound while squaramide 5 is a N,O squaramide (sometimes referred as squaramide-esters). Thus, the substitution of a NH group by O markedly disturbs the trophocidal activity but, at the same time, drastically reduces the cytotoxicity of 5. It is worth noting the parallelism between squaramide 3 and 5 and miltefosine, MF, (**Figure 1**). Miltefosine is a medication used mainly to treat leishmaniasis [37] and free-living amoeba infections such as granulomatous amoebic encephalitis caused by *Acanthamoeba* spp. and *Balamuthia mandrillaris* and primary amoebic meningoencephalitis caused by *Naegleria fowleri* [38] [39]. In 2013, two children survived and recovered from primary amoebic meningoencephalitis

Table 2. *In vitro* activity, toxicity and selectivity index for squaramides and acyclic polyamines derivatives on trophozoites of *Acanthamoeba castellanii*.

Compounds	Activity IC_{50} (μM)[a]	Toxicity IC_{50} Vero cells (μM)[b]	Selectivity index[c]
Chlorhexidine digluconate	5.33 ± 3	147.34 ± 1.7	27.6
1	91.3 ± 4.1	100.2 ± 2.3	1.09
2	134.3 ± 1.2	156.7 ± 3.5	1.16
3	3.5 ± 0.5	99.3 ± 1.2	28.3
4	343.5 ± 4.6	100.5 ± 6.9	0.3
5	11.4 ± 1.2	296.3 ± 3.6	26
6	26.7 ± 0.5	685.4 ± 8.6	25.7
7	36.9 ± 0.7	327.53 ± 3.6	8.87
8	783.9 ± 1.2	256.4 ± 38.2	0.33
9	186.3 ± 0.8	846.8 ± 22.9	4.54
10	278 ± 0.4	321.9 ± 40.9	1.15

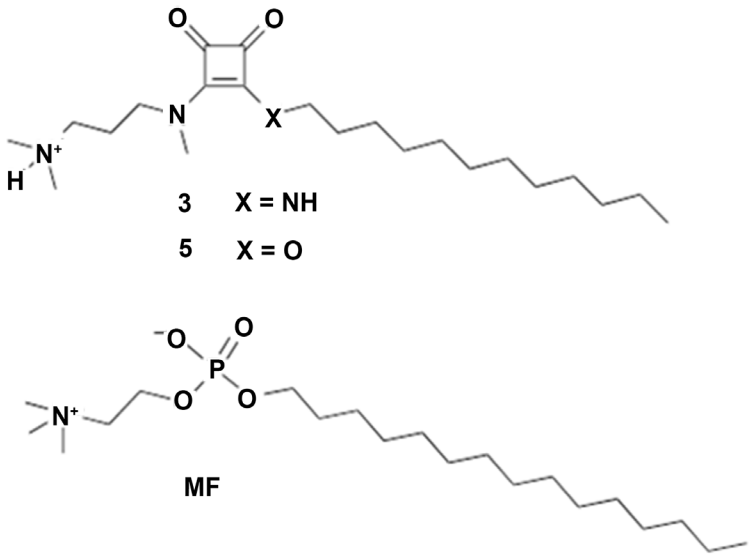


Figure 1. Molecular structures of squaramides 3 and 5 compared to that of the miltefosine (MF).

after treatment with miltefosine [39] [40]. In 2016, after treatment that included miltefosine, another child became the fourth person in the United States to survive *Naegleria fowleri* infection [41].

In the neutral or slightly acidic intracellular cytoplasmatic environment of the parasite, squaramide 3 and 5 are heavily protonated at the dimethyl amino group. On the other hand, the squaramide moiety is known to act as a successful masked phosphate group in bioisosteric replacement [42] [43].

Although all the acyclic polyamines (6-10) were less effective against the trophozoites than the squaramides, their cytotoxicity against Vero cells was very

low, reaching a selectivity index of 25.7 for polyamine 6. While polyamines 6 and 7 are isomers, differing only in the disposition of the nitrogen of the pyridine ring, their differences in amoebicidal activity and cytotoxicity was remarkable, with polyamine 7 showing a selective index of only 8.87. Notably, isomeric polyamines 8-10, which differ from 6-7 in the presence of only one methyl group instead of two at one of their ends, registered much lower amoebicidal activity, (**Table 1**).

The effect of the drugs against the trophozoites was observed by inverted microscopy. **Figure 2** shows the appearance of the trophozoites at the lowest concentration tested, 5 μM, after 120 h of treatment with chlorhexidine digluconate (**Figure 2(b)**) and with the most effective compounds: 3 (**Figure 2(c)**), 5 (**Figure 2(d)**), and 6 (**Figure 2(e)**). It was observed that the alterations were dose dependent, but at the lower concentration (5 μM) many trophozoites were detached and dead, appearing agglutinated and floating in the supernatant, especially with chlorhexidine digluconate (**Figure 2(b)**) and compound 3 (**Figure 2(c)**). Treated trophozoites showed structural alterations such as reduction in size, loss of acanthopodia and roundness vs. the control trophozoites (**Figure 2(a)**). Trophozoites treated with the highest concentration of compounds (200 μM) during 120 h are showed in **Figures 2(f)-(i)**. They were rounded, agglutinated, without internal content, dead and many cells debris from the destruction of the trophozoites appeared floating in the supernatant **Figures 2(f)-(h)**. **Figures 2(i)** shows trophozoites treated with polyamine 6 with similar appearance to that treated with 5 μM concentration.

After the study of the amoebicidal and cytoxic activity of the drugs, squaramides 3 and 5 and polyamine 6 were selected to be tested against *A. castellanii* cysts, which are less susceptible to the treatments than are trophozoites. Although in many recent publications on chemotherapy against *Acanthamoeba*, [15] [17] [18] [22] [24] [44], only the amoebicidal activity of the different products is assayed, it has become evident that it is necessary to know the cysticidal activity because trophozoites and cysts are present in the eyes, lungs, brain, and skin of the patients, and a good treatment against the trophozoite is not always effective against cysts, which are more resistant. **Figure 3** shows the cysticidal activity of the chlorhexidine digluconate (**Figure 3(a)**) and compounds 3, 5 and 6 (**Figures 3(b)-(d)**, respectively) versus a control of cysts without treatment.

The results showed that no cysts reverted to trophozoites with chlorhexidine digluconate and compound 3, (**Figure 3(a)** and **Figure 3(b)**), at 100 and 200 μM during of 192 h that the study lasted, and therefore they were presumed to be non-viable. When cysts were treated with 50 μM of these drugs, very few trophozoites (no more than 9%) could be seen. The cysts treated with compound 5 and 6 reverted with time to trophozoites at the concentrations assayed (**Figure 3(a)** and **Figure 3(d)**).

Chlorhexidine digluconate is used at 0.02% concentration in the initial therapy of *Acanthamoeba* keratitis. It acts by damaging the membrane of the

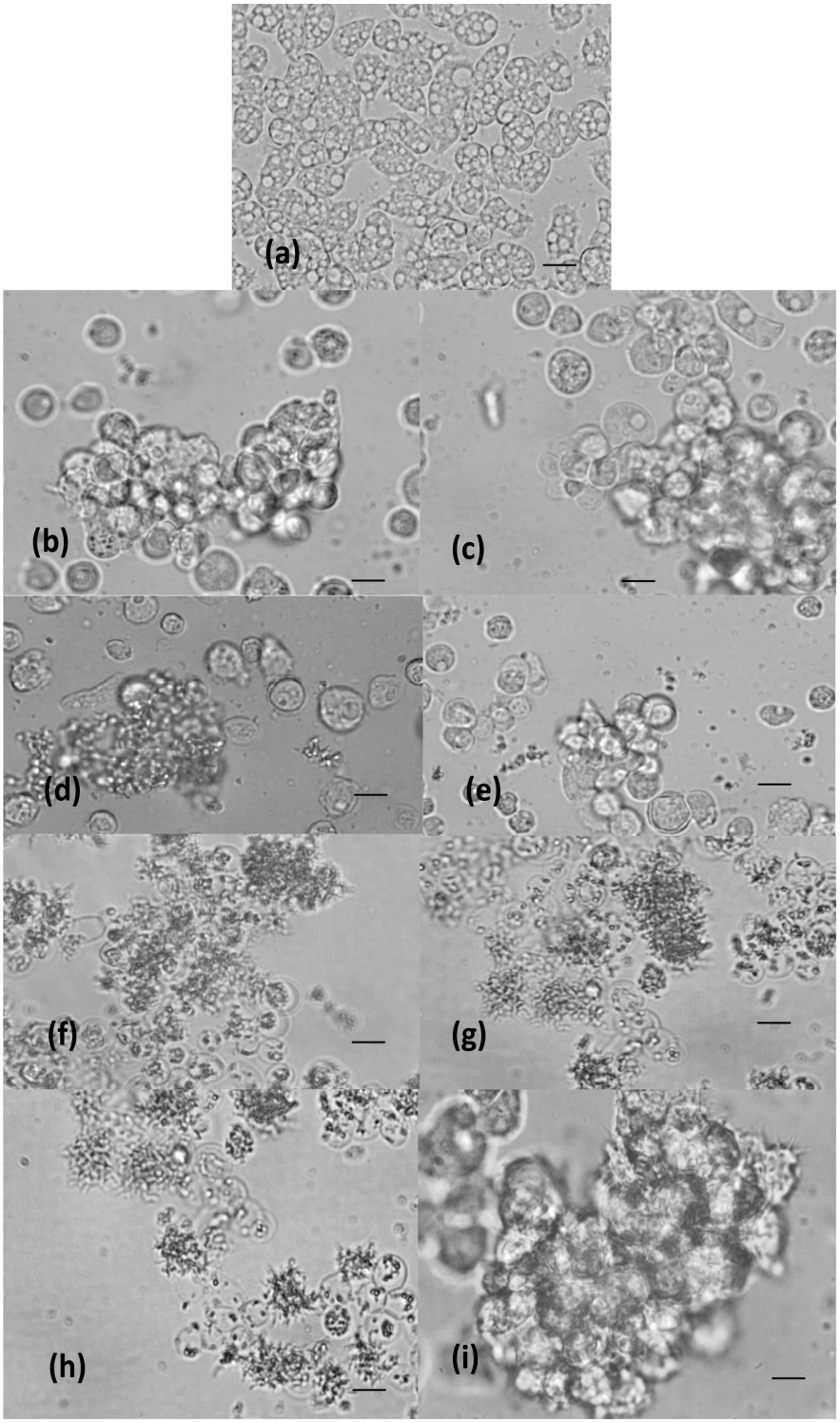


Figure 2. a) Trophozoites control of *Acanthamoeba castellanii* Neff. (b)-(e) Trophozoites treated with chlorhexidine digluconate (b) and compounds 3 (c), 5 (d), 6 (e) at concentration 5 μM during 120 h. They appeared detached, rounded, agglutinated and floating in the supernatant. (f)-(i) Trophozoites treated with chorhexidine digluconate (f) and compounds 3 (g), 5 (h), 6 (i) at concentration 200 μM during 120 h. Trophozoites in (f), (g) and (h) were dead and cell debris appeared floating in the supernatant. ×400. Scale bar = 10 μm.

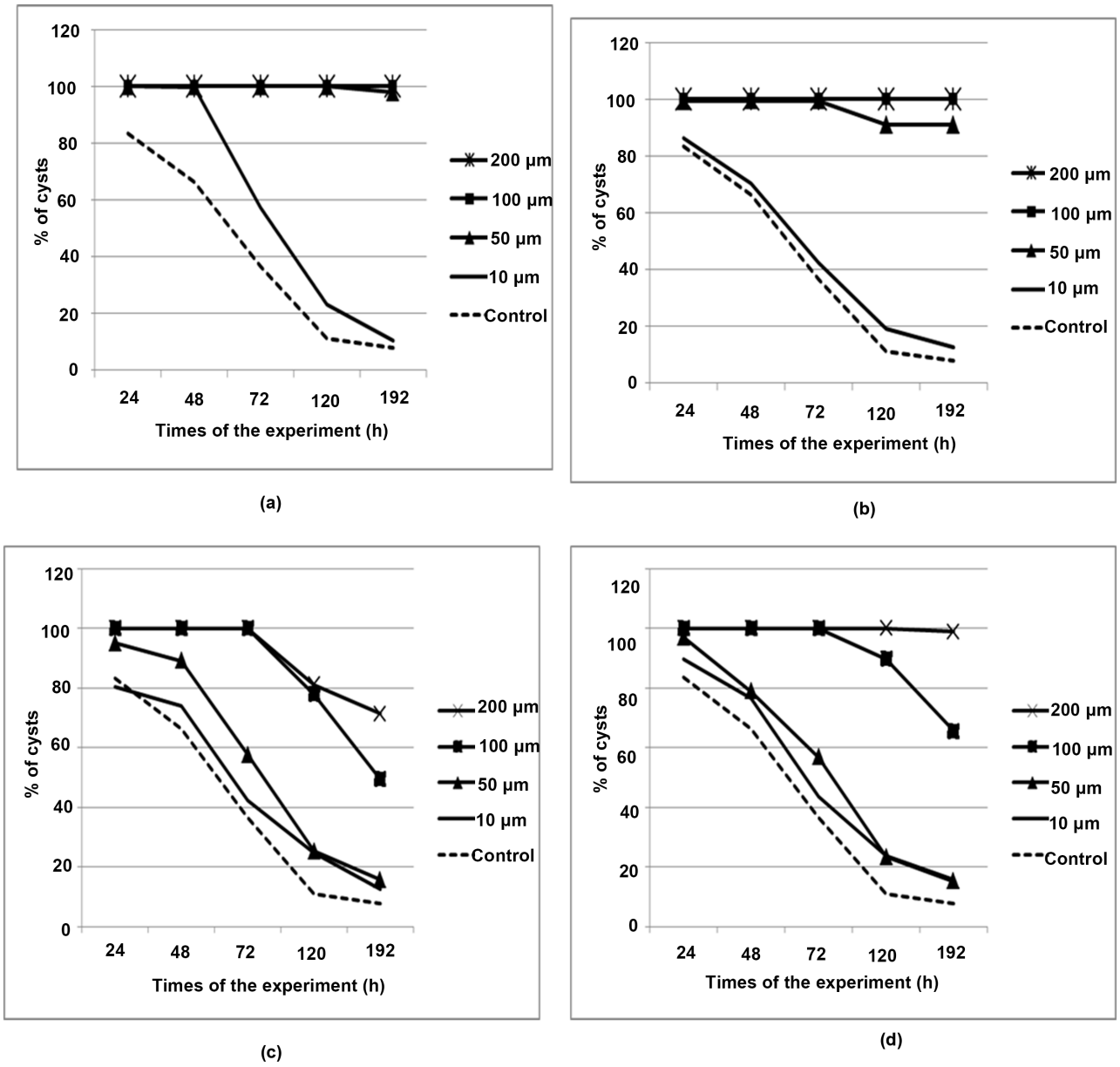


Figure 3. Percentage of cysts when they were treated with: (a) Chlorhexidine digluconate, (b) Squaramide 3, (c) Squaramide 5 and (d) Polyamine 6. No cysts reverted to trophozoites with chlorhexidine digluconate (a) and compound 3 (b) at 100 and 200 µM during of 192 h.

amoebas, with irreversible loss of calcium and cell electrolytes from the cytoplasm, causing cell lysis and death [32]. However, it is difficult to believe that a chemical destroying the membrane of the amoeba would not at the same time affect the plasma membranes of the ocular cells, and it has been demonstrated that with continued medical treatment, the iris and lens cells die, and cataracts develop [45]. Also, the cysticidal activity of chlorhexidine digluconate at 0.02% is only of the 80% [44]. In this study, cysticidal activity of chlorhexidine digluconate and squaramide 3 was similar, but squaramide 3 was more effective against the trophozoites and thus served as an alternative in the treatment of *Acantha-*

moeba infections, (AK, GAE, EP), according to *in vivo* tests. However, chlorhexidine digluconate has shown poor corneal penetration into the anterior chamber after topical administration [46].

Squaramides and acyclic polyamines were selected, placing emphasis on the molecular properties that determine drugability but also on the commercial availability and affordability of the starting reagents and the production process. These factors are coupled with a short synthesis and simple purification steps as well as low cost.

In conclusion, some of the squaramides and acyclic polyamines tested exert effective amoebicidal and/or cysticidal activity against *A. castellanii* Neff *in vitro*, comparable to control drug with less cytotoxicity, especially squaramide 3 with IC_{50} of 3.5 μM against trophozoites and cysticidal activity at concentrations of 100 and 200 μM. Further chemical modifications and *in vitro* as well as *in vivo* studies are needed to understand the molecular mechanisms of action and the alterations produced in the amoebas, as well as to assess their ocular toxicity in animal models.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] De Jonckheere, J.F. (1991) Ecology of *Acanthamoeba*. *Clinical Infectious Diseases*, **13**, S385-S387. https://doi.org/10.1093/clind/13.Supplement_5.S385

[2] Kilvington, S. and White, D.G. (1994) *Acanthamoeba*: Biology, Ecology and Human Disease. *Reviews in Medical Microbiology*, **5**, 12-20. https://doi.org/10.1097/00013542-199401000-00003

[3] Khan, N.A. and Paget, T.A. (2002) Molecular Tools for Speciation and Epidemiological Studies of *Acanthamoeba*. *Current Microbiology*, **44**, 444-449. https://doi.org/10.1007/s00284-001-0050-4

[4] Khan, N.A. (2009) *Acanthamoeba* Biology and Pathogenesis. Caister Academic Press, Chicago, 2-16.

[5] Chappell, C.L., Wright, J.A., Coletta, M. and Newsome, A.L. (2001) Standardized Method of Measuring *Acanthamoeba* Antibodies in Sera from Healthy Human Subjects. *Clinical and Diagnostic Laboratory Immunology*, **8**, 724-730.

[6] Mahgoub, M.A. (2010) *Acanthamoeba* Keratitis. *Parasitologists United Journal*, **3**, 9-18.

[7] Rusciano, G., Capriglione, P., Pesce, G., Del Prete, S., Cennamo, G., Di Cave, D.,

Cerulli, L. and Sasso, A. (2013) Raman Microspectroscopy Analysis in the Treatment of A*canthamoeba* Keratitis. *PLoS ONE*, **8**, e72127. https://doi.org/10.1371/journal.pone.0072127

[8] Naginton, J., Watson, P.G., Playfair, T.J., McGill, J., Jones, B.R. and Steele, A.D. (1974) Amoebic Infection of the Eye. *The Lancet*, **28**, 1537-1540. https://doi.org/10.1016/S0140-6736(74)90285-2

[9] Khan, N.A. (2006) *Acanthamoeba*: Biology and Increasing Importance in Human Health. *FEMS Microbiology Reviews*, **30**, 564-595. https://doi.org/10.1111/j.1574-6976.2006.00023.x

[10] Visvesvara, G.S., Moura, H. and Schuster, F.L. (2007) Pathogenic and Opportunistic Free-Living Amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri* and *Sappinia diploidea*. *FEMS Immunology & Medical Microbiology*, **50**, 1-26. https://doi.org/10.1111/j.1574-695X.2007.00232.x

[11] Sheng, W.H., Hung, C.C., Huang, H.H., Liang, S., Cheng, Y.J., Ji, D.D. and Chang, S.C. (2009) First Case of Granulomatous Amebic Encephalitis Caused by *Acanthamoeba castellanii* in Taiwan. *The American Journal of Tropical Medicine and Hygiene*, **81**, 277-279.

[12] Brown, M.R. and Barker, J. (1999) Unexplored Reservoirs of Pathogenic Bacteria: Protozoa and Biofilms. *Trends in Microbiology*, **7**, 46-50. https://doi.org/10.1016/S0966-842X(98)01425-5

[13] Abd, H.T., Johansson, I., Golovoliov, G., Sandstrom, A. and Forsman, M. (2003) Survival and Growth of *Francisella tularensis* in *Acanthamoeba castellanii*. *Applied and Environmental Microbiology*, **69**, 600-606. https://doi.org/10.1128/AEM.69.1.600-606.2003

[14] Greub, R.J. and Raoult, D. (2004) Microorganisms Resistant to Free-Living Amoebae. *Clinical Microbiology Reviews*, **17**, 413-433. https://doi.org/10.1128/CMR.17.2.413-433.2004

[15] Martin-Navarro, C.M, Lopez-Arencibia, A., Lorenzo-Morales, J., Oramas-Royo, S., Hernandez-Molina, R., Estevez-Braun, A., Ravelo, A.G., Valladares, B. and Pinero, J.E. (2010) *Acanthamoeba castellanii* Neff: *In Vitro* Activity against the Trophozoite Stage of a Natural Sesquiterpene and a Synthetic Cobalt(II)-Lapachol Complex. *Experimental Parasitology*, **126**,106-108. https://doi.org/10.1016/j.exppara.2009.12.015

[16] Martín-Navarro, C.M., López-Arencibia, A., Arnalich-Montiel, F., Valladares, B., Piñero, J.E. and Lorenzo-Morales, J. (2013) Evaluation of the *in Vitro* Activity of Commercially Available Moxifloxacin and Voriconazole Eye-Drops against Clinical Strains of *Acanthamoeba*. *Graefe's Archive for Clinical and Experimental Ophthalmology*, **251**, 2111-2117. https://doi.org/10.1007/s00417-013-2371-y

[17] Borase, H.B., Patil, C.D., Sauter, I.P., Rott, M.B. and Patil, S.V. (2013) Amoebicidal Activity of Phytosynthesized Silver Nanoparticles and Their *in Vitro* Cytotoxicity to Human Cells. *FEMS Microbiology Letters*, **345**, 127-131. https://doi.org/10.1111/1574-6968.12195

[18] Derda, M., Thiem, B., Budwianoski, J., Hada, E., Wojt, W.J. and Wojkkowiak-Giera, A. (2013) The Evaluation of Amebicidal Activity of *Eryngium Planum* Extracts. *Acta Poloniae Pharmaceutica Drug Research*, **70**, 1027-1034.

[19] Debnath, A., Tunac, J.B., Silva-Olivares, A., Galindo-Gómez, S., Shibayama, M. and McKerrow, J.H. (2014) *In Vitro* Efficacy of Corifungin against *Acanthamoeba castellanii* Trophozoites and Cysts. *Antimicrobial Agents and Chemotherapy*, **58**, 1523-1528. https://doi.org/10.1128/AAC.02254-13

[20] Kulsoom, H., Baig, A.M., Siddiqui, R. and Khan, N.A. (2014) Combined Drug

Therapy in the Management of Granulomatous Amoebic Encephalitis Due to *Acanthamoeba spp*. and *Balamuthia mandrillaris*. *Experimental Parasitology*, **145**, S115-S120. https://doi.org/10.1016/j.exppara.2014.03.025

[21] Lovieno, A., Miller, D., Ledee, D.R. and Alfonso, E.C. (2014) Cysticidal Activity of Antifungals against Different Genotypes of *Acanthamoeba*. *Antimicrobial Agents and Chemotherapy*, **58**, 5626-5628. https://doi.org/10.1128/AAC.02635-14

[22] Sifaoui, I., López-Arencibia, A., Ticona, J.C., Martín-Navarro, C.M., Reyes-Batlle, M., Mejri, M., Lorenzo-Morales, J., Jiménez, A.I., Valladares, B., Lopez-Bazzocchi, I., Abderabba, M. and Piñero, J. (2014) Bioassay Guided Isolation and Identification of Anti-*Acanthamoeba* Compounds from Tunisian Olive Leaf Extracts. *Experimental Parasitology*, **145**, 111-114. https://doi.org/10.1016/j.exppara.2014.02.018

[23] Heredero-Bermejo, I., Copa-Patiño, J.L., Soliveri, J., Fuentes-Paniagua, E., De la Mata, F.J., Gomez, R. and Perez-Serrano, J. (2015) Evaluation of the Activity of New Cationic Carbosilane Dendrimers on Trophozoites and Cysts of *Acanthamoeba polyphaga*. *Parasitology Research*, **114**, 473-486. https://doi.org/10.1007/s00436-014-4205-1

[24] Kuzma, L., Derda, M., Hadas, E. and Wysokinska, H. (2015) Abietane Diterpenoids from *Salvia sclarea* Transformed Roots as Growth Inhibitors of Pathogenic *Acanthamoeba* spp. *Parasitology Research*, **114**, 323-327. https://doi.org/10.1007/s00436-014-4211-3

[25] Villalonga, P., Fernandez de Mattos, S., Ramis, G., Obrador-Hevia, A., Sampedro, A., Rotger, C. and Costa, A. (2012) Cyclosquaramides as Kinase Inhibitors with Anticancer Activity. *ChemMedChem*, **7**, 1472-1480. https://doi.org/10.1002/cmdc.201200157

[26] Kumar, S.P., Gloria, P.M.C., Gonçalves, L.M., Gut, J., Rosenthal, J., Moreira, R. and Santos, M.M. (2012) Squaric Acid: A Valuable Scaffold for Developing Antimalarials. *Medicinal Chemistry Communications*, **3**, 489-493. https://doi.org/10.1039/c2md20011b

[27] Olmo, F., Rotger, C., Ramírez-Macías, I., Martínez, L., Marín, C., Carreras, L., Urbanová, K., Vega, M., Chaves-Lemaur, G., Sampedro, A., Rosales, M.J., Sánchez-Moreno, M. and Costa, A. (2014) Synthesis and Biological Evaluation of NN'-Squaramides with High *in Vivo* Efficacy and Low Toxicity: Toward a Low-Cost Drug against Chagas Disease. *Journal of Medicinal Chemistry*, **57**, 987-999. https://doi.org/10.1021/jm4017015

[28] Sanchez-Moreno, M., Marín, C., Navarro, P., Lamarque, L., García-Espana, E., Miranda, C., Huertas, O., Olmo, F., Gomez-Contreras, F., Pitarch, J. and Arrebola, F. (2012) *In Vitro* and *in Vivo* Trypanosomycidal Activity of Pyrazole-Containing Macrocyclic and Macrobicyclic Polyamines: Their Action on Acute and Chronic Phases of Chagas Disease. *Journal of Medicinal Chemistry*, **55**, 4231-4243.

[29] Willaert, E. (1971) Isolement et culture *in Vitro* des amibes du genre Naegleria. *Annales de la Société Belge de Médecine Tropicale*, **51**, 701-708.

[30] Cordingley, J.S., Wills, R.A. and Villemez, C.L. (1996) Osmolarity Is an Independent Trigger of *Acanthamoeba castellanii* Differentiation. *Journal of Cellular Biochemistry*, **61**, 167-171. https://doi.org/10.1002/(SICI)1097-4644(19960501)61:2<167::AID-JCB1>3.0.CO;2-S

[31] McBride, J., Ingram, P.R. and Henríquez, F.L. (2005) Development of Colorimetric Microtiter Plate Assay for Assessment of Antimicrobials against *Acanthamoeba*. *Journal of Clinical Microbiology*, **43**, 629-634.

https://doi.org/10.1128/JCM.43.2.629-634.2005

[32] Seal, D.V. (2003) *Acanthamoeba* Keratitis Update-Incidence, Molecular Epidemiology and New Drugs for Treatment. *Eye*, **17**, 893-905. https://doi.org/10.1038/sj.eye.6700563

[33] Walochnik, J., Picher, O., Aspöck, C., Ullmann, M., Sommer, R. and Aspöck, H. (1999) Interactions of "Limax Amoebae" and Gram-Negative Bacteria: Experimental Studies and Review of Current Problems. *Tokai Journal of Experimental and Clinical Medicine*, **23**, 273-278.

[34] Schuster, F.L. and Visvevara, G.S. (2004) Opportunistic Amoebae: Challenges in Prophylaxis and Treatment. *Drug Resistance Updates*, **7**, 41-51. https://doi.org/10.1016/j.drup.2004.01.002

[35] Bang, S., Edell, E., Eghrari, A.O. and Gottsch, J.D. (2010) Treatment with Voriconazole in 3 Eyes with Resistant *Acanthamoeba* Keratitis. *American Journal of Ophthalmology*, **149**, 66-69. https://doi.org/10.1016/j.ajo.2009.08.004

[36] Ferrari, G., Matuska, A. and Rama, P. (2011) Double-Biguanide Therapy for Resistant *Acanthamoeba* Keratitis. *Case Reports in Ophthalmology*, **2**, 338-342. https://doi.org/10.1159/000334270

[37] Yao, S. (2014) FDA Approves Impavido to Treat Tropical Disease Leishmaniasis. FDA News Release. U.S. Food and Drug Administration.

[38] Cope, J.R. (2013) Investigational Drug Available Directly from CDC for the Treatment of Infections with Free-Living Amebae. *Morbidity and Mortality Weekly Report*, **62**, 666.

[39] CDC (2014) *Naegleria fowleri*—Primary Amebic Meningoencephalitis (PAM). Centers for Disease Control and Prevention.

[40] Gholipour, B. (2013) Brain-Eating Amoeba: How One Girl Survived.

[41] Goldschmidt, D. and Scutti, S. (2016) Rare Recovery: Florida Teen Survives Brain-Eating Amoeba.

[42] Sato, K., Seio, K. and Sekine, M.J. (2002) Squaryl Group as a New Mimic of Phosphate Group in Modified Oligodeoxynucleotides: Synthesis and Properties of New Oligodeoxynucleotide Analogues Containing an Internucleotidic Squaryldiamide Linkage. *American Chemical Society*, **124**, 12715-12724. https://doi.org/10.1021/ja027131f

[43] Sato, K., Tawarada, R., Seio, K. and Sekine, M. (2004) Synthesis and Structural Properties of New Oligodeoxynucleotide Analogues Containing a 2,5-Internucleotidic Squaryldiamide Linkage Capable of Formation of a Watson-Crick Base Pair with Adenine and a Wobble Base Pair with Guanine at the 3-Downstream Junction Site. *European Journal of Organic Chemistry*, 2142-2150. https://doi.org/10.1002/ejoc.200300682

[44] Nagwa, M.E., Khadiga, A.I., Sabah, A.A. and Hafez, M.H. (2012) *In Vitro* Amoebicidal Activity of Etanol Extracts of *Arachis hypogaea* L., *Curcuma longa* L. and *Pancratium maritimum* L. on *Acanthamoeba castellanii* cysts. *Parasitology Research*, **110**, 1985-1992. https://doi.org/10.1007/s00436-011-2727-3

[45] Ehlers, N. and Hjortdal, J. (2004) Are Cataract and Iris Atrophy Toxic Complications of Medical Treatment of *Acanthamoeba* Keratitis? *Acta Ophthalmologica Scandinavica*, **82**, 228-231. https://doi.org/10.1111/j.1600-0420.2004.00237.x

[46] Banich, A.M., Bu, P., Jacob, G., Fox, I., Zhang, X., Perlman, J.I. and Bouchar, C. (2003) Penetration of Chlorhexidine into the Rabbit Anterior Chamber Following Topical Administration. *Investigative Ophthalmology & Visual Science*, **44**, U317.