Acta Tropica 232 (2022) 106538

Contents lists available at ScienceDirect

Acta Tropica

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

5-Nitroindazole derivatives as potential therapeutic alternatives against *Acanthamoeba castellanii*

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ARTICLE INFO

Keywords:
Acanthamoeba castellanii
Amoebicidal
Chlorhexidine digluconate
5-nitroindazole derivatives

ABSTRACT

Amoebas of the genus *Acanthamoeba* are distributed worldwide, including species with a high pathogenic capacity for humans. In a similar way to what occurs with other parasitic protozoa, the available treatments show variable effectiveness in addition to high toxicity, which demands the development of new treatments. Positive results of 5-nitroindazole derivatives against several protozoa parasites suggest that these compounds may be a promising tool for the development of efficient antiparasitic drugs. In the present work we have evaluated the in vitro activity of ten 5-nitroindazole derivatives against *Acanthamoeba castellanii* trophozoites and cysts. To that end, AlamarBlue Assay Reagent® was used to determine the activity against trophozoites compared to the reference drug chlorhexidine digluconate. Cytotoxicity of the compounds was evaluated using Vero cells. The activity on cysts was evaluated by light microscopy and using a Neubauer chamber to quantifying cysts and presence of trophozoites, as an indication of cyst. Our results showed the effectiveness of the 5-nitroindazole derivatives tested against both trophozoites and cysts of *A. castellani* highlighting 5-nitroindazole derivative **8** which showed a 80% activity on cysts, which is higher than that of the reference drug. Moreover, 5-nitroindazole derivatives **8, 9** and **10** were more effective on trophozoites than the reference drug showing IC_{50} values lower than 5 μM. Taking together these results, these 5-nitroindazole derivatives specially compound **8**, might be a promising alternative for the development of more efficient treatments against *A. castellani* infection.

Introduction

Free-living amoebae are protozoa widely distributed in nature, with some species being pathogenic for humans specially the genera *Acanthamoeba* and *Naegleria*. These pathogens constitute an important public health problem since they have been isolated from many environments and surfaces such as soil, air, both fresh and salt water, contact lenses, ophthalmological solutions, dialysis units, surgical material, among others (Lacerda and Lira, 2021).

Amoebae belonging to the genus *Acanthamoeba* are the most common free-living amoebae which includes several species pathogenic for humans. In addition, these amoebas can be carriers of pathogenic bacteria such as *Legionella* spp., *Vibrio cholerae*, and *Escherichia coli* O157, increasing their pathogenic potential (Rayamajhee et al., 2021). *Acanthamoeba* presents two stages in its life cycle; a vegetative phase or trophozoite and a cyst highly resistant to adverse environmental conditions and the effects of disinfectants and antimicrobial agents (Lacerda and Lira, 2021) . This form of resistance can shift to the trophozoite stage when environmental conditions get back to optimal. Both trophozoites and cysts can initiate the pathology after entry into the host. Although infection by this amoebae is rare, ocular infection is the most common outcome of *Acanthamoeba* infection leading to inflammation of the cornea or keratitis, for which the use of contact lenses is a high risk factor (Shu and Ting, 2021). In immunocompromised patients the infection may lead to severe complications such as granulomatous encephalitis (Natalia et al., 2018).

Keratitis caused by *Acanthamoeba* is difficult to treat, largely due to early misdiagnosis, which affects the patient's prognosis. The recommended initial treatment consists of topical administration of biguanide such as polyhexamethylene biguanide (PHMB) 0.02–0.08% or

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https://doi.org/10.1016/j.actatropica.2022.106538
Received 16 March 2022; Received in revised form 20 May 2022; Accepted 22 May 2022
Available online 23 May 2022

chlorhexidine 0.02–0.06%, along with or without a topical diamidine, such as propamidine isethionate 0.1%. Unfortunately these treatments can lead to adverse effects due to the toxicity of biguanides and chlorhexidine gluconate (Hidalgo and Dominguez, 2001; Lee et al., 2007). Therefore, the development of new effective treatments without adverse effects for the patient is necessary.

Many effective compounds for the treatment of protozoan infections are nitro compounds that produce free radicals with effects against the parasite but also with detrimental effects for the patient, even leading to cancer development (Lorenzo-Morales et al., 2015). However, other compounds such as 5- nitroimidazoles (e.g. metronidazole) have antiparasitic activity without producing neoplastic effects in the patient (Lorenzo-Morales et al., 2015). In recent years there have been reports of studies where the action of 5-nitrozole derivatives has been tested against different parasitic protozoa, such as *Trypanosoma cruzi, T. brucei, Trichomonas vaginalis* and *Leishmania* spp. (Arán et al., 2005, 2012; Rodríguez et al., 2009; Fonseca-berzal et al., 2016, 2018; Martín-Escolano et al., 2018). Similarly to other groups, we have also recently applied the use of 5- nitroindazole derivatives with success against *T. cruzi* (Muro et al., 2014).

In the present study we have tested the activity of various 5-nitroindazole derivatives against *A. castellanii* trophozoites and cysts, comparing the activity of these new compounds to that of chlorhexidine digluconate as the reference drug. The results provided in this study show a greater effectiveness of these newly synthesized compounds compared to the reference drug, indicating that these 5-nitroindazole

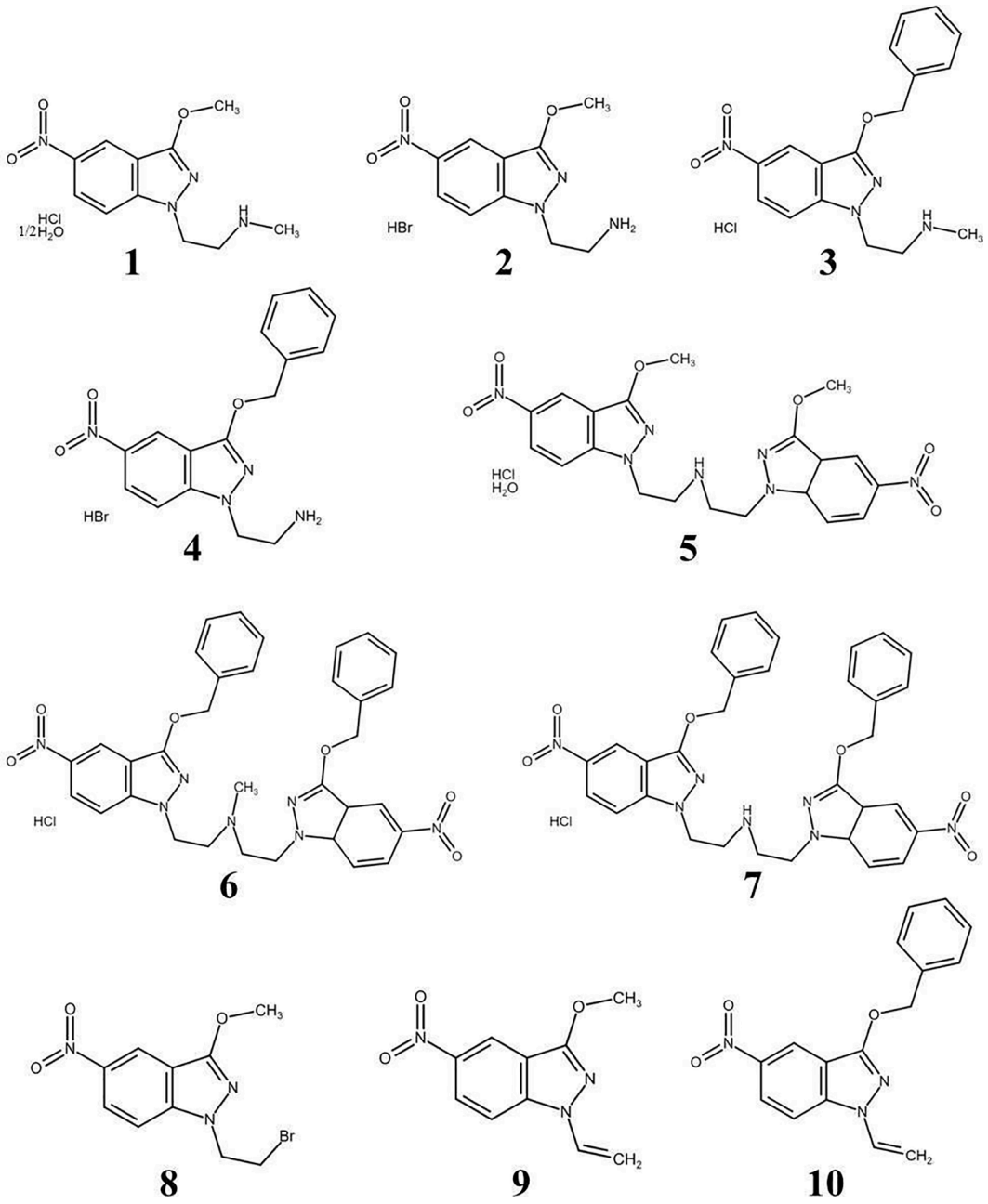

Fig. 1. 5-nitroindazole derivatives investigated in this study. 1, *N*-Methyl-2-(3-methoxy-5-nitro-1*H*-indazol-1-yl)ethylamine hydrochloride; 2, 2-(3-Methoxy-5-nitro-1*H*-indazol-1-yl)ethylamine hydrobromide; 3, *N*-Methyl-2-(3-benzyloxy-5-nitro-1*H*-indazol-1-yl)ethylamine hydrochloride; 4, 2-(3-Benzyloxy-5-nitro-1*H*-indazol-1-yl)ethylamine hydrobromide; 5, Bis[2-(3-*methoxy*-5-*nitro*-1*H*-*indazol*-1-*yl*)*ethyl*]amine hydrochloride; 6, *N*-Methylbis[2-(3-*benzyloxy*-5-*nitro*-1*H*-*indazol-yl*)*ethyl*]amine hydrochloride; 7, Bis[2-(3-*benzyloxy*-5-*nitro*-1*H*-*indazol*-1-*yl*)*ethyl*]amine hydrochloride; 8, 1-(2-Bromoethyl)−3-methoxy-5-nitro-1*H*-indazole; 9, 3-Methoxy-5-nitro-1-vinyl-1*H*-indazole; 10, 3-Benzyloxy-5-nitro-1-vinyl-1*H*-indazole.

derivatives may be the basis for the development of more effective and less toxic drugs against infections caused by these amoebas.

Materials and methods

Compounds

The tested compounds were 5-nitroindazole derivatives (Fig. 1) that have already shown antiprotozoal activity against *Trypanosoma cruzi* (Martín-Escolano et al., 2018) and *Leishmania* spp. (Martín-Montes et al., 2019). Amines **1**–**4** were obtained from 1-(2-bromoethyl) derivative **8**, wich was in turn prepared by alkylation of 3-methoxy-5-nitroindazole with 1,2-dibromoethane. Treatment of compound **8** or the corresponding 3-O-benzyl derivative with ammonia or methylamine afforded compounds **1**–**4**. Further alkylation of compounds **2, 3** or **4** with an additional equivalent of alkyl bromide **8** or its 3-O-benzyl analogue afforded amines **5**–**7**. 1-Vinyl derivatives **9** and **10** were obtained as minor byproducts in these processes; nevertheless, the most convenient synthetic method is based on the dehydrohalogenation of compound **8** or its 3-O-benzyl analogue with potassium carbonate (Martín-Escolano et al., 2018).

Chlorhexidine digluconate (Sigma-Aldrich) was used as reference drug against *A. castellanii*.

Culture of A. castellanii trophozoites

Trophozoites of *A. castellanii* Neff (ATCC 30,010) were axenically cultured in Willaert's CGV (Casitone Glucose Vitamin) medium – 20 g/L Bacto Casitone, 5 g/L NaCl, 1 g/L glucose, 2 mg/L folic acid, 200 mg/L biotin, 500,000 u.i. penicillin, 50 mg/L streptomycin – supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) at 28 °C (Willaert, 1971). Passages were routinely performed every 5–7 days, depending on cell growth. Cultures were monitored via light microscopy examination for growth, morphology and contaminants.

Resazurin-based amoebicidal viability assays

The *A. castellanii* anti-trophozoite activity of compounds was evaluated using the AlamarBlue Cell Viability Reagent® as previously described (Martín-Escolano et al., 2021). In short, it was assessed using microtiter 96-well flat bottom plates by seeding 1×10^4 trophozoites/well in CGV medium supplemented with 10% heat-inactivated FCS. Cell concentration was determined quantitatively by the trypan blue dye exclusion method. Plates were incubated at 28 °C for 1 h to allow for trophozoites adhesion. After that, compounds and chlorhexidine digluconate (Sigma-Aldrich) were added in 200 μL/well volumes in CGV medium supplemented with 10% heat-inactivated FCS. Compounds and chlorhexidine digluconate were serially prepared to reach final concentrations ranging from 400 to 0.4 μM in plates. Positive (untreated trophozoites) and negative (containing only culture medium) growth controls were also included. 20 μL AlamarBlue Reagent® (Thermo Fisher Scientific) was then added into each well, and the plates were incubated at 28 °C for 120 h in complete darkness. Then, 20 μL of 20% (w/v) SDS was added to facilitate the solubilisation of the viability reagent and lysis of the parasites. Finally, the plates were incubated for further 20 min, and the cell viability was assessed by absorbance measurements at 570 nm (reference at 630 nm) using a Sunrise™ Tecan microplate reader.

Relative absorbance was converted into viability percentages, and these values were used to perform nonlinear regression analyses using GraphPad Prism 6 to determine the IC_{50} and IC_{90} values, i.e., the concentrations required to results in 50% and 90% inhibition. Each compound concentration was tested in triplicate in three separate determinations.

Representative images of *A. castellanii* trophozoites were taken using an inverted microscope (× 400) after 120 h of incubation. Cultures were incubated at 28 °C for 120 h with chlorhexidine digluconate and 5-nitroindazole derivatives **8**–**10** at 5 μM. Positive (untreated) growth controls were also included.

Cytotoxicity tests

The cytotoxicity induced by each compound and chlorhexidine digluconate was tested using mammalian Vero cells (ATCC CCL-81). Vero cells were cultured in RPMI medium supplemented with 10% heat-inactivated FCS in a humidified 95% air 5% CO_2 atmosphere at 37 °C.

Cytotoxicity was evaluated using the AlamarBlue Cell Viability Reagent® as previously described (Martín-Escolano et al., 2021). Briefly, 1×10^4 Vero cells/well were seeded in microtiter 96-well flat bottom plates, and the plates were incubated in a humidified 95% air 5% CO_2 atmosphere at 37 °C for 24 h. After that, compounds and chlorhexidine digluconate were added in 200 μL/well volumes in RPMI medium supplemented with 10% heat-inactivated FCS. Compounds and chlorhexidine digluconate were serially prepared to reach final concentrations ranging from 800 to 25 μM in plates. Positive (untreated Vero cells) and negative (containing only culture medium) growth controls were also included. 20 μL AlamarBlue Reagent® (Thermo Fisher Scientific) was then added into each well, and the plates were incubated in a humidified 95% air 5% CO_2 atmosphere at 37 °C for 120 h in complete darkness. Finally, the same procedure as described for amoebicidal viability was followed to determine the IC_{50} values. Selective indexes, (IC_{50} Vero cells toxicity/IC_{50} activity on trophozoites forms of the parasite), were also calculated. Each compound concentration was tested in triplicate in three separate determinations.

Activity assays against cysts

Cysts forms were obtained as previously described by Cordingley et al. (1996) . Briefly, trophozoites were cultured in RPMI medium supplemented with 8% (w/v) glucose at 30 °C for 48 h, and sodium dodecyl sulfate (SDS) was added to reach a final concentration of 0.5% (w/v). Trophozoites that had not been encysted are SDS-sensitive and are immediately lysed, while cysts are SDS-resistant and remain intact. Finally, cysts were washed and collected by centrifugation.

The activity on cysts was evaluated using microtiter 96-well flat bottom plates by seeding 1×10^4 cyst/well in RPMI medium supplemented with 8% (w/v) glucose, and after addition of the compounds and chlorhexidine digluconate at a range concentration from 200 to 10 μM in 200 μl/well volumes. Positive (untreated cysts) and negative (containing only culture medium) growth controls were also included. Plates were incubated at 28 °C for 120 h. After that, cysts were slightly washed, fresh CGV medium supplemented with 10% heat-inactivated FCS was added, and plates were incubated for further 24 h, 48 h, 72 h, 120 h and 192 h at 28 °C. The number of cysts and trophozoites were counted at these different times using a Neubauer chamber, and the presence of trophozoites was considered as an indication of cyst viability (Martín-Escolano et al., 2021). Each compound concentration was tested in triplicate in three separate determinations.

Results

The anti-trophozoite activity, toxicity to mammalian Vero cells, and selectivity index of 5-nitroindazole derivatives are shown in Table 1. All 5-nitroindazole derivatives showed a dose-dependent activity on trophozoites, as well as chlorhexidine digluconate. 5-Nitroindazole derivatives **8, 9** and **10** were the most effective against *A. castellanii* trophozoites, even exhibiting higher efficacy than chlorhexidine digluconate. In particular, 5-Nitroindazole derivatives **8, 9** and **10** presented IC_{50} values of 2.6 ± 0.7, 4.7 ± 0.9 and 3.9 ± 0.6 μM (endpoint 120 h), respectively, compared to 5.2 ± 0.7 μM for chlorhexidine digluconate. In addition, the cytotoxicity of these 5-nitroindazole

Table 1
Anti- trophozoicidal activity, toxicity to mammalian Vero cells, and selectivity index of 5-nitroindazole derivatives.

Compound	Activity IC_{50} (μM)	Toxicity IC_{50} Vero cells (μM)	Selectivity index[a]
Chlorhexidine digluconate	5.2 ± 0.7	137.4 ± 11.3	26.4
1	123.7 ± 4.9	456.9 ± 28.9	3.7
2	156.1 ± 2.7	493.9 ± 41.7	3.2
3	363.4 ± 6.9	87.9 ± 2.7	0.2
4	204.8 ± 7.5	34.9 ± 1.6	0.2
5	256.0 ± 10.1	100.9 ± 3.2	0.4
6	324.2 ± 7.3	178.9 ± 0.6	0.6
7	215.3 ± 1.5	72.3 ± 2.7	0.3
8	2.6 ± 0.7	355.2 ± 6.1	136.6
9	4.7 ± 0.9	240.0 ± 8.4	51.1
10	3.9 ± 0.6	266.5 ± 10.7	68.3

Results are averages of three separate determinations. IC_{50}, concentration required to give 50% inhibition. [a]Selectivity index = IC_{50} Vero cells/IC_{50} activity on trophozoites.

derivatives was lower than the cytotoxicity of chlorhexidine digluconate, so the selectivity index was substantially higher for these 5-nitroindazole derivatives than for chlorhexidine digluconate (136.6, 51.1 and 68.3 versus 26.4, respectively).

Fig. 2 shows the dose-response graphs for chlorhexidine digluconate and the most active 5-nitroindazole derivatives (**8, 9** and **10**), along with the IC_{50} and IC_{90} values for each of these compounds. IC_{90} values are important from a clinical point of view as they correspond to those concentrations that allow a total eradication of the parasite from patients. Here, chlorhexidine digluconate showed an IC_{90} value of 85.7 ± 7.1 μM compared to 98.1 ± 8.2, 151.5 ± 11.8 and 131.6 ± 16.9 μM for 5-nitroindazole derivatives **8, 9** and **10**, respectively. However, chlorhexidine digluconate showed IC_{90} values close to the toxicity values (Table 1), so we can highlight 5-nitroindazole derivative **8** as a potential compound for the treatment of *Acanthamoeba* infections.

It should be noted that the activity of chlorhexidine digluconate obtained in the present work is in agreement with the activities described in the literature, with IC_{50} and IC_{90} values ranging from 1 to ~ 30 μM and from ~ 50 to ~ 200 μM, respectively (Heredero-bermejo et al., 2015; Nomura et al., 2015; Moon et al., 2018; Padzik et al., 2018; Shing et al., 2020; Chen et al., 2021). The differences observed are due to 1) the different *A. castellanii* strains evaluated, 2) the number of cells seeded and the incubation time with the drug, and 3) the method used to measure the activity of the drug against *A. castellanii*.

The trophozoicidal activity was also observed by inverted microscopy. Fig. 3 shows the appearance of untreated trophozoites (Fig. 3A) vs trophozoites treated with chlorhexidine digluconate (Fig. 3B) and the most active 5-nitroindazole derivatives **8**–**10** (Fig. 3C–E) at 5 μM after 120 h of incubation. Untreated trophozoites showed a standard ameboid morphology, exhibiting contractile vacuoles and acanthopodia (Fig. 3A). Chlorhexidine digluconate-treated trophozoites (Fig. 3B) appeared agglutinated and floating in the supernatant, showing significant structural alterations such as loss of acanthopodia, roundness and reduction in size compared to untreated trophozoites. 5-Nitroindazole derivative **9**-treated trophozoites (Fig. 3D) showed a similar appearance to chlorhexidine digluconate-treated trophozoites. 5-Nitroindazole derivatives **8** and **10** ((Fig. 3C and E) caused detachment and death of (nearly) all trophozoites; no structural alterations could be observed as cells were lysed.

5-Nitroindazole derivatives **8, 9** and **10** were also tested against cysts, resistant forms that are less susceptible to treatment than trophozoites. Fig. 4 shows the activity on cysts of chlorhexidine digluconate (Fig. 4A) and 5-nitroindazole derivatives **8**–**10** (Fig. 4B–D, respectively). Cyst viability was studied by assessing the excystment during 192 h in non-encystment medium from previously untreated (control) and treated cysts for 120 h in encystment medium. Close to 100% of the untreated (control) cysts transformed into trophozoites after 192 h of incubation in non-encystment medium. These trophozoites were viable, showed acanthopodia and proliferated to establishing new cultures. Chlorhexidine digluconate-treated cysts did not transform into trophozoites at concentrations of 200, 100 and 50 μM, presuming they were no viable. In contrast, cysts treated at 10 μM chlorhexidine digluconate showed similar behavior to untreated (control) cysts after 192 h of incubation in non-encystment medium (Fig. 4A), and new transformed trophozoites proliferated to establishing new cultures. All 5-nitroindazole derivatives (Fig. 4B-D) showed higher activity on cysts than chlorhexidine digluconate at 10 μM. Here, 5-nitroindazole derivative **8** should be highlighted: no transformation to trophozoites was observed at concentrations of 200, 100 and 50 μM, and only 24.2% transformation was observed at 10 μM after 192 h of incubation (Fig. 4B).

Discussion

As the activities of amoebicidal treatments and drugs reported in the literature is often unsuccessful because of the limited drug efficacy, frequent adverse effects and increasing drug resistance (Martín-Navarro et al., 2015; Niyyati et al., 2016; Hajaji et al., 2017; Ortillés et al., 2017), the development of new compounds is strongly needed. Hence, research on potential and effective treatments against *Acanthamoeba* is gaining considerable interest in public health research. The main objective of this study was to determine the anti-*A. castellanii* activity of 10 5-nitroindazole derivatives against *A. castellanii* in order to compare their activities with those of the reference drug chlorhexidine digluconate, and

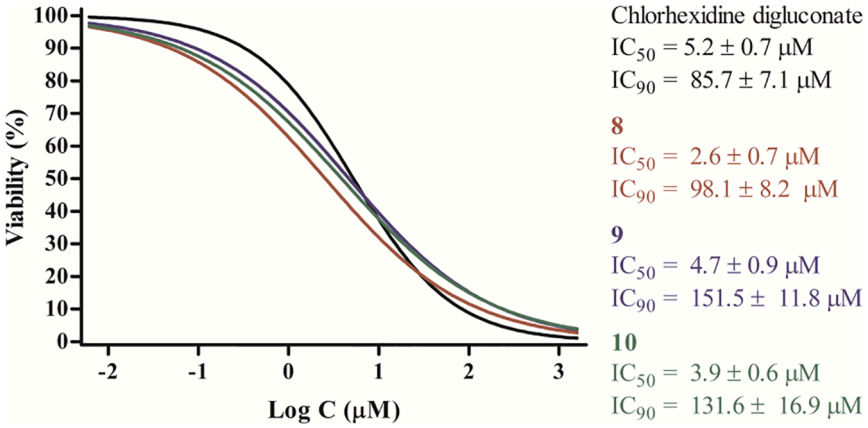

Fig. 2. Dose-response growth curves of *Acanthamoeba castellanii* trophozoites treated with (A) chlorhexidine digluconate and (B–D) 5-nitroindazole derivatives 8–10 at 120 h endpoint.

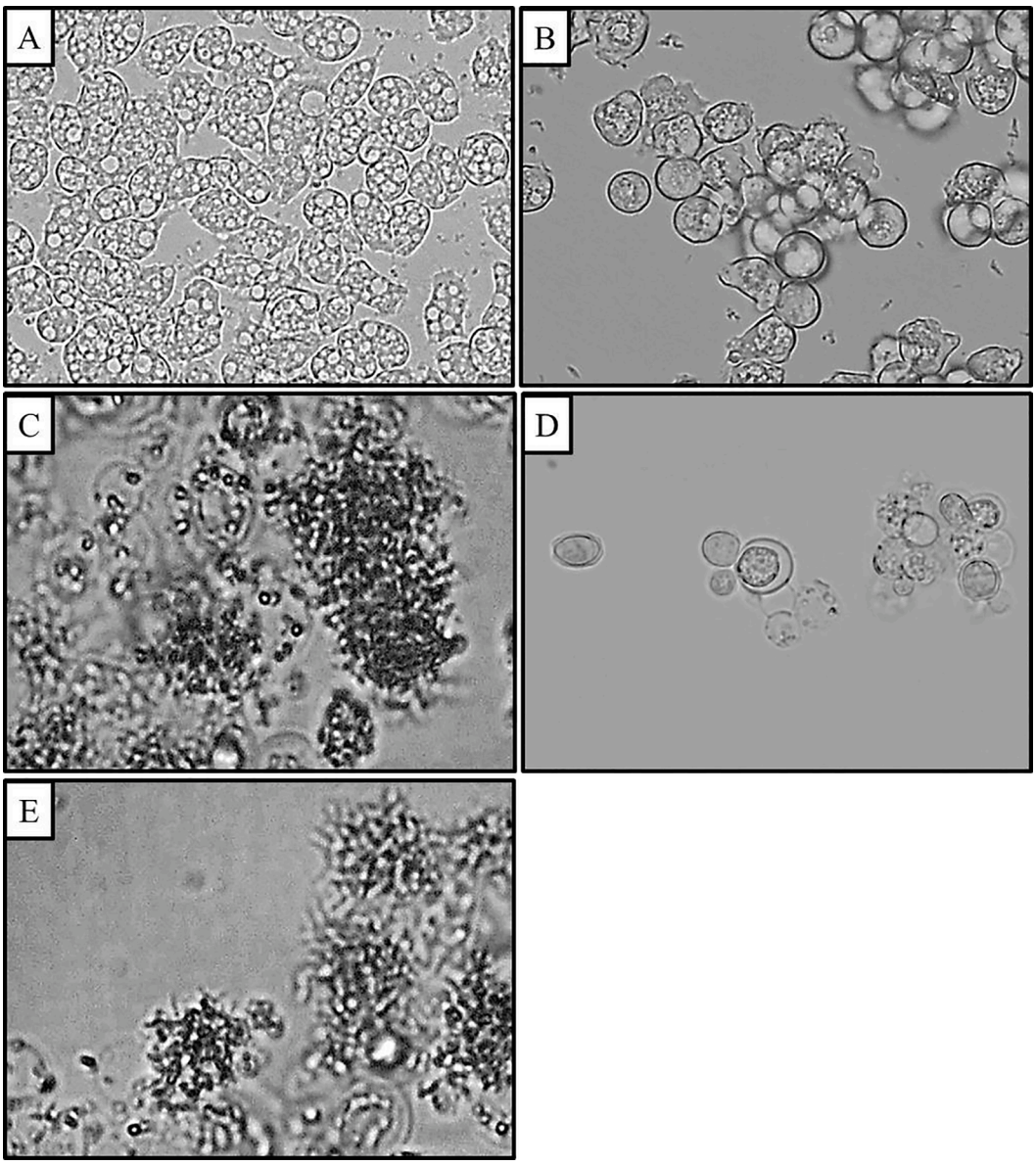

Fig. 3. Inverted microscopy pictures × 400 of *Acanthamoeba castellanii* trophozoites treated with compounds for 120 h at 5 μM. (A) Untreated trophozoites, (B) trophozoites treated with chlorhexidine digluconate, and (C-E) trophozoites treated with 5-nitroindazole derivatives 8–10.

to identify new anti-*Acanthamoeba* compounds as potential candidates for further development for the treatment of *Acanthamoeba* infections. It should be highlighted that these 5-nitroindazole derivatives have shown in vitro and in vivo efficacy against other protozoan parasites such as *T. cruzi, T. brucei, Leishmania* spp. and *Trichomonas vaginalis* (Rodríguez et al., 2009; Fonseca-berzal et al., 2014, 2016, 2018; Muro et al., 2014; Martín-Escolano et al., 2018; Martín-Montes et al., 2019).

Chlorhexidine digluconate is a standard antiseptic used for the treatment of *Acanthamoeba* keratitis. It acts damaging the membrane of the amoebas, causing cell lysis and death (Radford et al., 2002), as well as exhibiting activity on cysts (Schuster and Visvesvara, 2004; Khan, 2006). However, as a compound capable of destroying the amoeba membrane, a comparable adverse effect is caused on the plasma membranes on the patient́s iris and lens cells due to long-term treatment resulting in the development of cataracts (Ehlers and Hjortdal, 2004; El-Sayed et al., 2012). On the other hand, it has shown poor corneal penetration into the anterior chamber after topical application (Banich et al., 2003), and activity on cysts at clinical concentrations (0.02%) is only 80% (El-Sayed et al., 2012).

The amoebicidal activity of 5-nitroindazole derivatives is described for the first time in this article. Given the limited number of compounds tested, establishing a general relationship between chemical structure and amoebicidal activity is not possible. It is evident that indazole-derived amine salts **1**–**7**, designed to obtain compounds soluble in water and with improved pharmacokinetic properties, do not present significant activity. However, compounds **8**–**10**, neutral, lipophilic and sparingly soluble in water, are very efficient. The greater activity of 1-(2-bromoethyl) derivative **8** in relation to that of 1-vinyl derivatives **9** and **10** may be due to the alkylating properties of the former. Obviously, it is necessary to study more 5-nitroindazole derivatives, with varied substituents in the different positions of the ring, in order to have a more exact view of their effect on amoebicidal activity.

Here, 5-nitroindazole derivatives **8, 9** and **10** were more effective against trophozoites than the reference drug chlorhexidine digluconate, showing IC_{50} values lower than 5 μM. IC_{90} values are more relevant from a clinical viewpoint, and in this respect chlorhexidine digluconate showed the lowest IC_{90} value (85.7 ± 7.1 μM); however, 5-nitroindazole derivative **8** showed a very similar IC_{90} value (98.1 ± 8.2 μM) and its

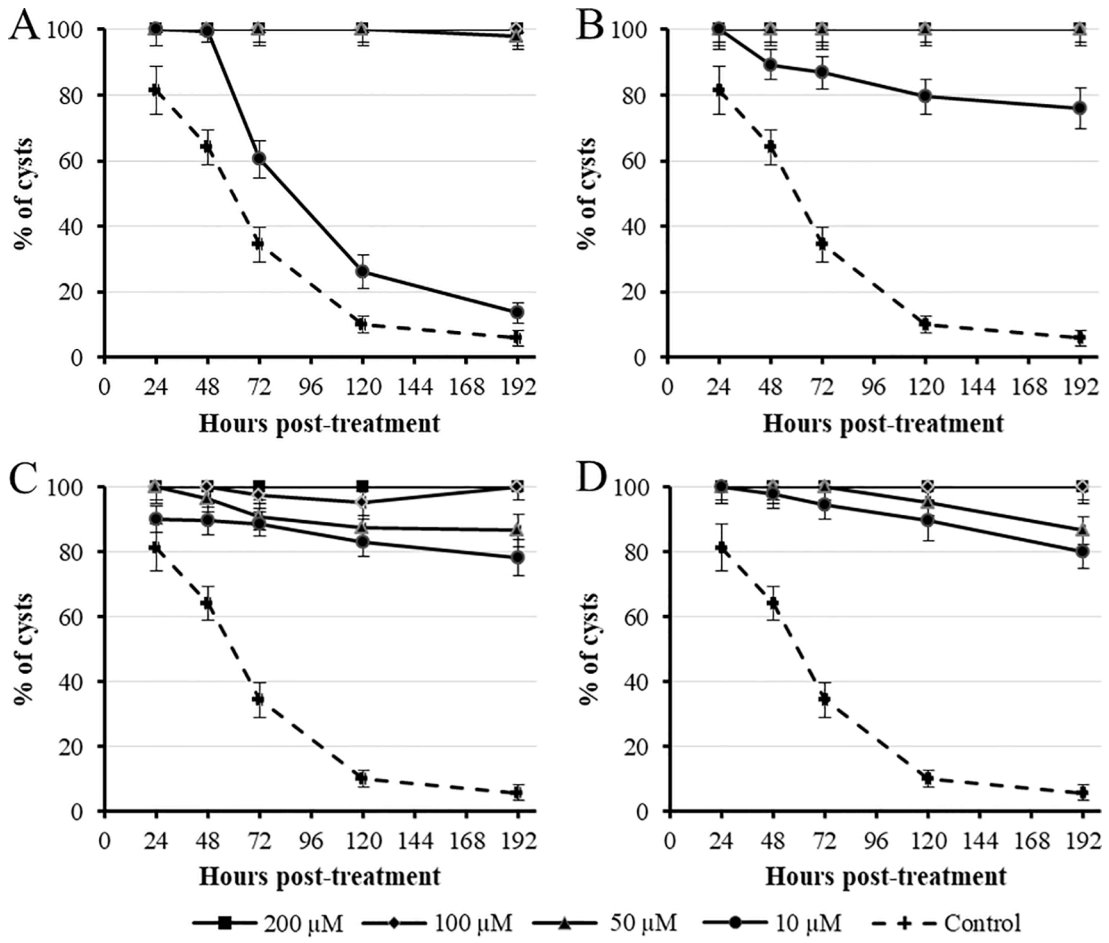

Fig. 4. Percentage of cysts after treatment with (A) the reference drug and (B–D) 5-nitroindazole derivatives 8–10 at concentrations of 200, 100, 50 and 10 µM for 120 h in encystment medium and subsequent incubation during 192 h in culture medium CGV with 10% heat-inactivated fetal calf serum. Non-viable cysts did not revert to trophozoites.

toxicity is much lower (see Table 1). This would allow 5-nitroindazole derivative **8** to be administered at higher concentrations with the aim of total elimination of the parasite from patients. Other clinically important point is activity on cysts as cysts are resistant forms that are less susceptible to treatments. Contrary to trophozoites, cysts are highly resistant to chemical injury and can be present in the eyes, brain, lungs and skin of patients; the development of new treatments is focused on compounds with activity on cysts. Here, 5-nitroindazole derivative **8** exhibited the highest activity on cysts, even higher than that of chlorhexidine digluconate. Indeed, only a concentration of 10 µM was needed to kill around 80% of the cysts.

5-Nitroindazole derivatives induce oxidative stress in protozoan parasites such as *T. cruzi* and *Leishmania* spp. (Muro et al., 2014; Fonseca-berzal et al., 2016; Martín-Escolano et al., 2018; Martín-Montes et al., 2019). On the other hand, it has also been proposed that the interference with mitochondrial enzymes involved in the catabolism of these parasites and mitochondrial alterations (Muro et al., 2014; Martín-Escolano et al., 2018) could contribute to the cidal activity of these 5-nitroindazole derivatives. Accumulation of succinate, pyruvate and malate by causing a blockade of the glycolytic pathway have been observed in treated protozoa (Martín-Escolano et al., 2018). Ultrastructural alterations such as morphological changes, large vacuolization, mitochondria damage and cytoskeleton disruption were also observed by transmission electron microscopy in treated protozoa (Muro et al., 2014). According to this, oxidative stress, metabolite and mitochondrial alterations causing energy deficient conditions such as mitochondrial dysfunction, could be the ultimate reasons of the anti-*A. castellanii* activity of these 5-nitroindazole derivatives. However, it is evident that the mode of action of 5-nitroindazole derivative **8** needs to be investigated more thoroughly. In order to continue our investigations, the synthesis of other 5-nitroindazole derivatives, including fluorescent derivatives, is planned.

Conclusions

In summary, 5-nitroindazole **8** was the most effective, with higher anti-*A. castellanii* activity and lower toxicity than the reference drug chlorhexidine digluconate. From all these data, it can be concluded that 5-nitroindazole **8** is a potential candidate for the development of an alternative and efficient drug treatment against *A. castellanii* infections. The cidal activity, stability and low-cost of synthesis make this compound a promising molecule for the development of an affordable therapy against *A. castellanii*. Futher chemical modifications and in vivo tests are needed to enhance its activity, as well as to evaluate its efficiency and tolerability in animal models.

CRediT authorship contribution statement

Rubén Martín-Escolano: Conceptualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Gregorio Pérez-Cordón:** Investigation, Data curation, Validation, Writing – review & editing. **Vicente J. Arán:** Investigation, Formal analysis, Validation. **Clotilde Marín:** Validation, Project administration, Funding acquisition. **Manuel Sánchez-Moreno:** Resources,

Validation, Project administration, Funding acquisition. **María José Rosales:** Conceptualization, Resources, Methodology, Validation, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflicts of interests.

Funding

This work was supported by the Spanish MINECO, FEDER funds of the E. U. [CTQ2013–14892 and 2010-CSD2010–00065]; the Unidad de Excelencia MDM [2015–0038]; and the Generalitat Valenciana (PROMETEO II 2015–002). R.M.-E. is grateful for the fellowship from the Alfonso Martín Escudero Foundation.

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