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EXPERIMENTAL THERAPEUTICS

Amoebicidal Activity of Caffeine and Maslinic Acid by the Induction of Programmed Cell Death in *Acanthamoeba*

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ABSTRACT Free-living amoebae of the genus *Acanthamoeba* are the causal agents of a sight-threatening ulceration of the cornea called *Acanthamoeba* keratitis, as well as the rare but usually fatal disease granulomatous amoebic encephalitis. Although there are many therapeutic options for the treatment of *Acanthamoeba* infections, they are generally lengthy and/or have limited efficacy. For the best clinical outcome, treatments should target both the trophozoite and the cyst stages, as cysts are known to confer resistance to treatment. In this study, we document the activities of caffeine and maslinic acid against both the trophozoite and the cyst stages of three clinical strains of *Acanthamoeba*. These drugs were chosen because they are reported to inhibit glycogen phosphorylase, which is required for encystation. Maslinic acid is also reported to be an inhibitor of extracellular proteases, which may be relevant since the protease activities of *Acanthamoeba* species are correlated with their pathogenicity. We also provide evidence for the first time that both drugs exert their anti-amoebal effects through programmed cell death.

KEYWORDS *Acanthamoeba*, caffeine, maslinic acid, programmed cell death, PCD

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Members of the genus *Acanthamoeba* are ubiquitous protists and include the causative agents of several infections in humans such as a sight-threating ulceration of the cornea known as *Acanthamoeba* keratitis (AK), the usually fatal granulomatous amoebic encephalitis (GAE), and also a range of disseminated infections, usually, but not exclusively, limited to the skin (1–4).

The life cycle of *Acanthamoeba* alternates between two stages: the trophozoite, which is the active growing stage, and the cyst which is a dormant stage resorted to when conditions become incompatible with growth. The cyst has a highly resistant double wall. The outer wall is fibrous and composed mainly of protein while the inner wall contains more than 30% cellulose (2, 5, 6, 7). In AK, the cysts are responsible for recurrent amoebic infections, as it is able to survive many of the current treatments (8, 9) and differentiate back to amoebae on the cessation of treatment. Diamidines (proamidine and hexamidine) and biguanides (chlorhexidine and polyhexamethylene biguanide [PHMB]), have been found to be effective against *Acanthamoeba* trophozoites and cysts *in vitro* (2, 3, 4, 10). However, it has been reported that about 5% of patients with AK are troubled with inflammation due to the persistence of *Acanthamoeba* infection caused by cysts surviving in the cornea, even after prolonged treatment with these agents (11). There is an urgent need for more effective treatments and so

TABLE 1 The alamarBlue cell viability assay was used to determine IC_{50} and IC_{90} values after 96 h for caffeine and maslinic acid tested against the four strains of *Acanthamoeba*

Treatment	ICs (μM) for *Acanthamoeba* strains							
	AcNeff[a]		CLC-16		CLC-41.r		CLC-51.l	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Caffeine	8.86 ± 2.11	22.71 ± 0.93	42.94 ± 4.22	72.47 ± 5.07	24.30 ± 2.01	52.25 ± 6.22	17.35 ± 0.72	45.65 ± 2.05
Maslinic acid	47.60 ± 0.53	85.68 ± 4.38	22.04 ± 2.28	37.15 ± 3.99	78.68 ± 6.05	103.78 ± 6.00	52.83 ± 2.76	83.75 ± 4.19
Amphotericin B	39.65 ± 0.56	100.01 ± 9.52	27.41 ± 1.28	71.89 ± 3.40	59.95 ± 1.05	145.44 ± 3.84	67.30 ± 5.68	170.11 ± 13.72
Chlorhexidine	5.23 ± 0.55	16.14 ± 0.90	11.89 ± 3.80	34.46 ± 2.43	2.45 ± 0.20	28.46 ± 4.33	10.47 ± 1.48	28.21 ± 1.80

[a]AcNeff, *Acathamoeba* Neff type strain (ATCC 30010, genotype T4).

there is also a need to identify and validate new therapeutic targets in *Acanthamoeba*, mostly focusing on key proteins related to cellular viability and the pathogenic mechanisms. In this report we explore two such targets, secreted proteases and glycogen phosphorylase.

Acanthamoeba secretes three types of proteolytic enzymes: serine proteases, cysteine proteases, and metalloproteases, and these cause at least part of the organism's pathogenicity (12). RNA interference (RNAi) silencing experiments confirm this (13, 14), and it has been demonstrated that serine proteases play a role in the important processes of encystment and excystation (13, 15, 16).

Glycogen phosphorylase is active during encystation in breaking down glycogen to release glucose-1-phosphate, a precursor of the cellulose required to construct the inner cyst wall. We have shown that the inhibition of this enzyme by RNAi blocks the formation of cysts (14). However, since RNAi is not yet widely approved, a drug which performs the same function is preferable in the treatment of AK.

Caffeine and maslinic acid are reported to be glycogen phosphorylase inhibitors (17, 18). Maslinic acid is a natural triterpene isolated from the olive tree (*Olea europaea*) with multiple biological properties, such as antimicrobial and antiparasitic activity (19, 20, 21, 22). Maslinic acid has been reported to be a potent inhibitor of glycogen phosphorylase and extracellular proteases of parasites such as *Toxoplasma gondii* and serine proteases from *Cryptosporidium* (19, 23, 24, 25, 26).

RESULTS

Caffeine and maslinic acid were both amoebicidal and cysticidal. Caffeine and maslinic acid were both active against the trophozoite stage of different strains of *Acanthamoeba*. Caffeine had higher activity than maslinic acid (except against strain CLC-16) (Table 1). Although both products seemed to have a lower activity than chlorhexidine, their activity was still higher that of amphotericin B (Table 1).

When cells were grown in encystation medium and stained with Congo red in order to analyze by flow cytometry, control cells were clearly divided into 3 main populations: P3 trophozoites, P4 cysts stained with Congo red, and P5 precysts stained with Congo red (Fig. 1A). However, after treatments using caffeine and maslinic acid, trophozoites were unable to either encyst or form mature cysts (Fig. 1B and C).

The effects of caffeine and maslinic acid on cell proliferation from 24 to 72 h were checked. It was noted that both active principles decreased cell proliferation in a dose-dependent manner (Fig. 2). Furthermore, significant differences between the control and the 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}, respectively) were observed, except at 24 h when maslinic acid was used (Fig. 2B). No significant differences between both inhibitory concentrations were observed, which may serve to establish the IC_{50} as sufficient to eliminate the cell population.

Caffeine and maslinic acid showed low cytotoxicity to vertebrate cells. The results showed that caffeine (both IC_{50} and IC_{90}, C50 and C90, respectively, for *Acanthamoeba*) and maslinic acid IC_{50} (M50) were not cytotoxic toward HeLa or J774.A1 vertebrate cells. Caffeine and maslinic acid showed significantly low cytotoxicity compared to the reference drugs chlorhexidine and amphotericin B (Fig. 3).

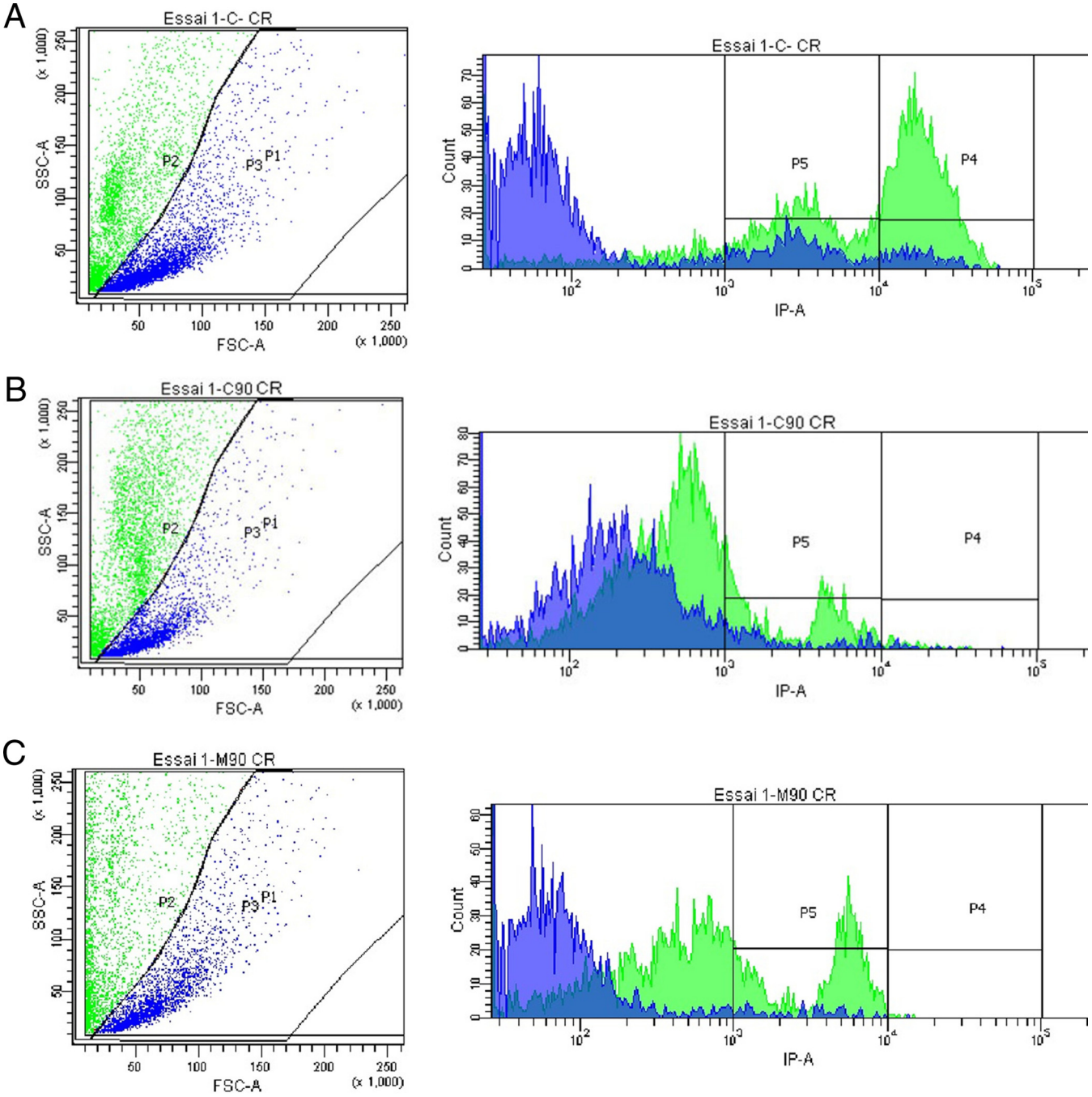

FIG 1 The *Acanthamoeba* Neff strain separated into subpopulations after 72 h, as follows: P1, total cell population, analyzed by the flow cytometer; P2, cysts; P3, trophozoites, analyzed according to size and complexity of cells; P4, cysts; P5, precysts, analyzed by fluorescence emitted by the Congo red staining. (A) Control. (B) Cells after treatment with caffeine IC_{90}. (C) Cells after treatment with maslinic acid IC_{90}.

Caffeine and maslinic acid induced larger amounts of DNA in the cell lysate than in the supernatant. When *Acanthamoeba* Neff was treated with the IC_{50} and IC_{90} of caffeine and maslinic acid, a larger amount of DNA was observed in the cell lysate compared to the detected levels in the supernatant (Fig. 4). Therefore, a larger amount of intracellular DNA was detected in all cases, with significant differences between the detected DNA in the lysate and the supernatant. In the case of caffeine (Fig. 4A), significant differences between the concentrations were also observed at 48 h after the treatment.

Caffeine and maslinic acid induced PCD, which was observed with double stain assay. When double staining was performed, caffeine and maslinic acid caused nuclei

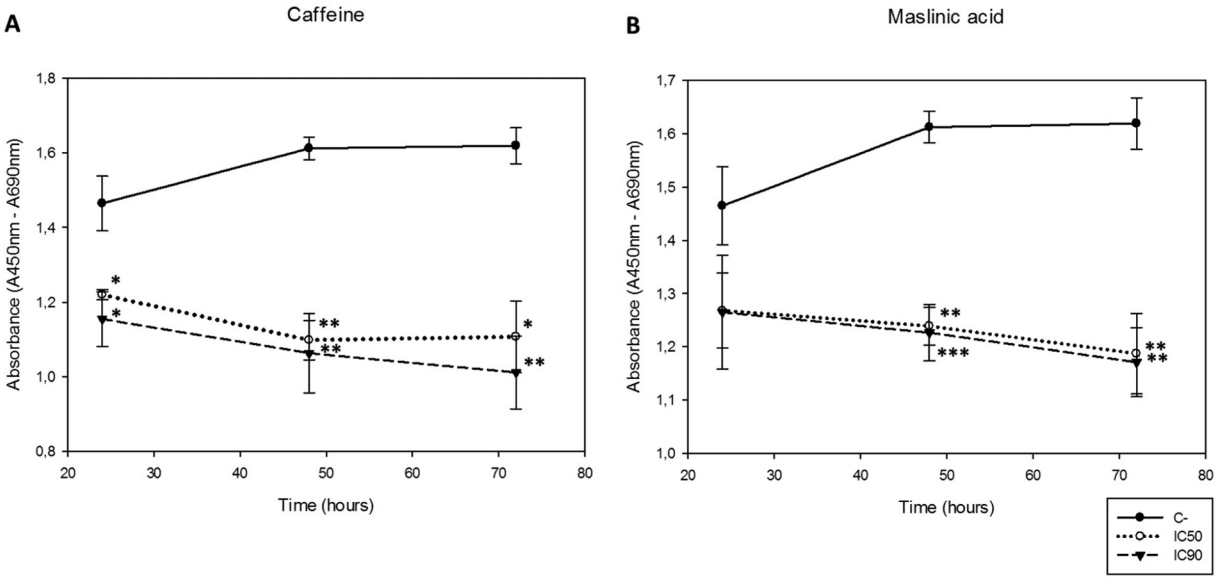

FIG 2 Observed cell proliferation after incubation of the *Acanthamoeba* Neff strain with caffeine and maslinic acid using the previously obtained IC_{50} and IC_{90} compared to the control. Results were analyzed at 24, 48, and 72 h. (A) Effects on cell proliferation when cells were treated with caffeine. (B) Effects on cell proliferation when cells were treated with maslinic acid. Statistical differences between both concentrations and the control were observed (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

staining with Hoechst stain, demonstrating the presence of condensed chromatin (Fig. 5). Moreover, the differences between the three cells population were clear and thus live cells were detected under fluorescence microscopy, as they showed faint blue nuclei against a dark cytoplasmic background stain (Fig. 5D), whereas cells displaying programmed cell death (PCD) presented bright blue nuclei due to karyopyknosis and chromatin condensation (Fig. 5E and F). Dead cells were not able to exclude propidium iodide, a DNA binding dye, and so the remnants of the nuclei in these dead cells stained red (Fig. 5G to I). These images show that both caffeine and maslinic acid caused PCD after 24 h.

Caffeine and maslinic acid caused plasma membrane permeability. Caffeine and maslinic acid induced cellular membrane damage in treated amoebae after 1 h of treatment. None of the tested products induced the same level of fluorescence

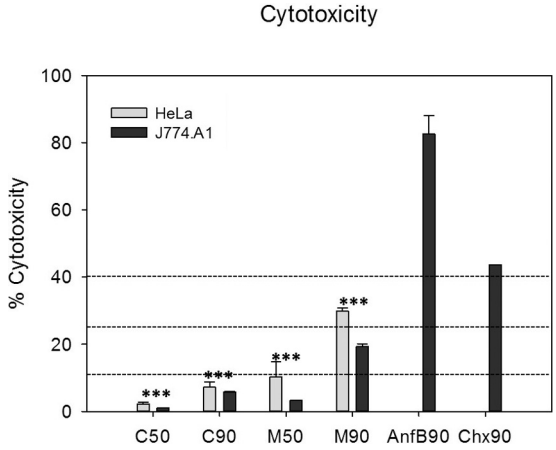

FIG 3 Cytotoxicity levels of the tested caffeine and maslinic acid against *Acanthamoeba* (IC_{50} and IC_{90}) were evaluated against two cell lines: HeLa and murine macrophages. Values lower than 10% correspond to a null cytotoxicity, so the results showed that caffeine IC_{50} (C50), caffeine IC_{90} (C90), and maslinic acid IC_{50} (M50) were not cytotoxic. Values between 10% to 25% correspond to a low cytotoxicity, which was the case for maslinic acid IC_{90} (M90). Statistical differences between cytotoxicity of the assayed drugs and that produced by the reference drugs chlorhexidine IC_{90} (Chx90) and amphotericin B (Anf90) were observed in both cell lines (***, $P < 0.001$).

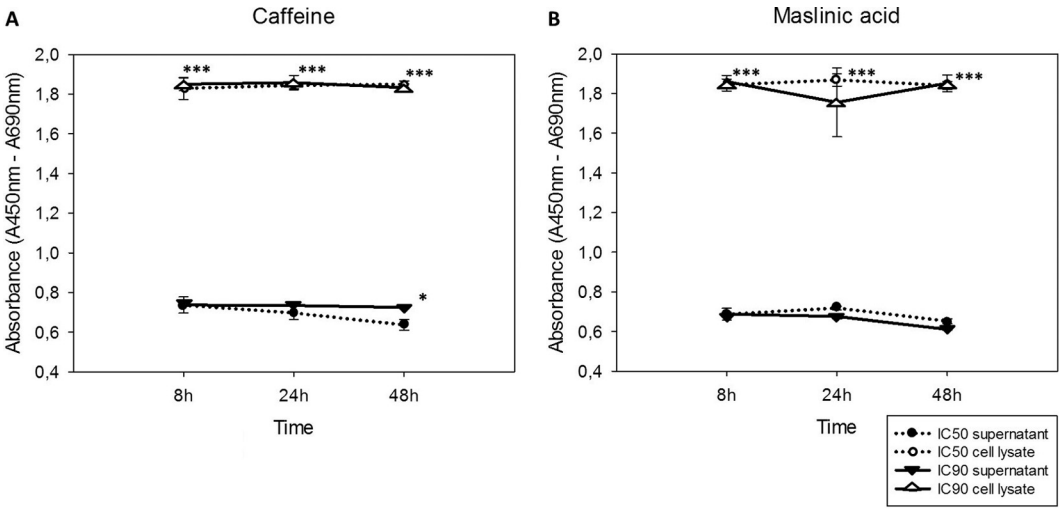

FIG 4 Amount of DNA detected over time in the culture supernatant and cell lysate (absorbance versus time). (A) Caffeine. (B) Maslinic acid. Statistical differences (***, $P < 0.001$; *, $P < 0.05$) are shown for result comparison between supernatant (filled symbols) and cell lysate (empty symbols) values.

observed in the positive control (Fig. 6D). Nevertheless, cellular membrane disruption was checked and confirmed using fluorescence microscopy in treated cells (Fig. 6A to C).

Amoebae treated with caffeine and maslinic acid showed signs of early PCD. *Acanthamoeba* Neff treated with the assayed active principles showed externalization of phosphatidylserine (PS). The number of cells suffering PCD or death were counted and showed a clear difference between the control and the treated cells (Fig. 7). Moreover, the statistical analyses showed significant differences in the percentage of detected PCD cells after treatment with all the assayed drugs with respect to the control. Therefore, early stages of apoptosis in the treated *Acanthamoeba* cells were demonstrated.

***Acanthamoeba* caspase-3-like activity was detected after treatment with caffeine and maslinic acid.** A significant caspase-3-like activity was detected in amoebae treated with caffeine or maslinic acid using a chromogenic probe attached to a substrate peptide. Caspase-3 like activity developed in the presence of both drugs, especially after 24 h (Fig. 8).

DISCUSSION

Caffeine and maslinic acid are glycogen phosphorylase inhibitors (17, 18) and maslinic acid inhibits the secreted proteases of a number of different parasites (19, 23, 24, 25, 26). The activity of these drugs had been successfully tested against different protozoa (19, 20, 23, 24, 25, 26) and maslinic acid has been found to be active against *Acanthamoeba* (22). In the present study, anti-*Acanthamoeba* activity of these drugs has been described using a range of *Acanthamoeba* strains. The fact that both drugs have anti-cyst activity was established by viability and proliferative assay and analyzed by flow cytometry in this study. The finding that both drugs blocked the development of cysts is compatible with their inhibitory effects on both proteases and the glycogen phosphorylase activity required for glucose release from glycogen to form the cellulose wall (6), but this does not explain caffeine's toxic effect on trophozoites. However, it is known that caffeine and maslinic acid induce apoptosis in various vertebrate cell types. For example, caffeine induce PCD in neuroblastoma cells and pancreatic and lung adenocarcinomas (27, 28, 29, 30) and maslinic acid induces apoptosis in metastatic cell lines and in colon cancer cells (31, 32). We could find no study in which either drug was reported to induce PCD in any protist.

PCD and PCD-like processes have been reported in a wide variety of protists (33), including *Acanthamoeba* (33, 34, 35, 36, 37). The present study has shown that caffeine

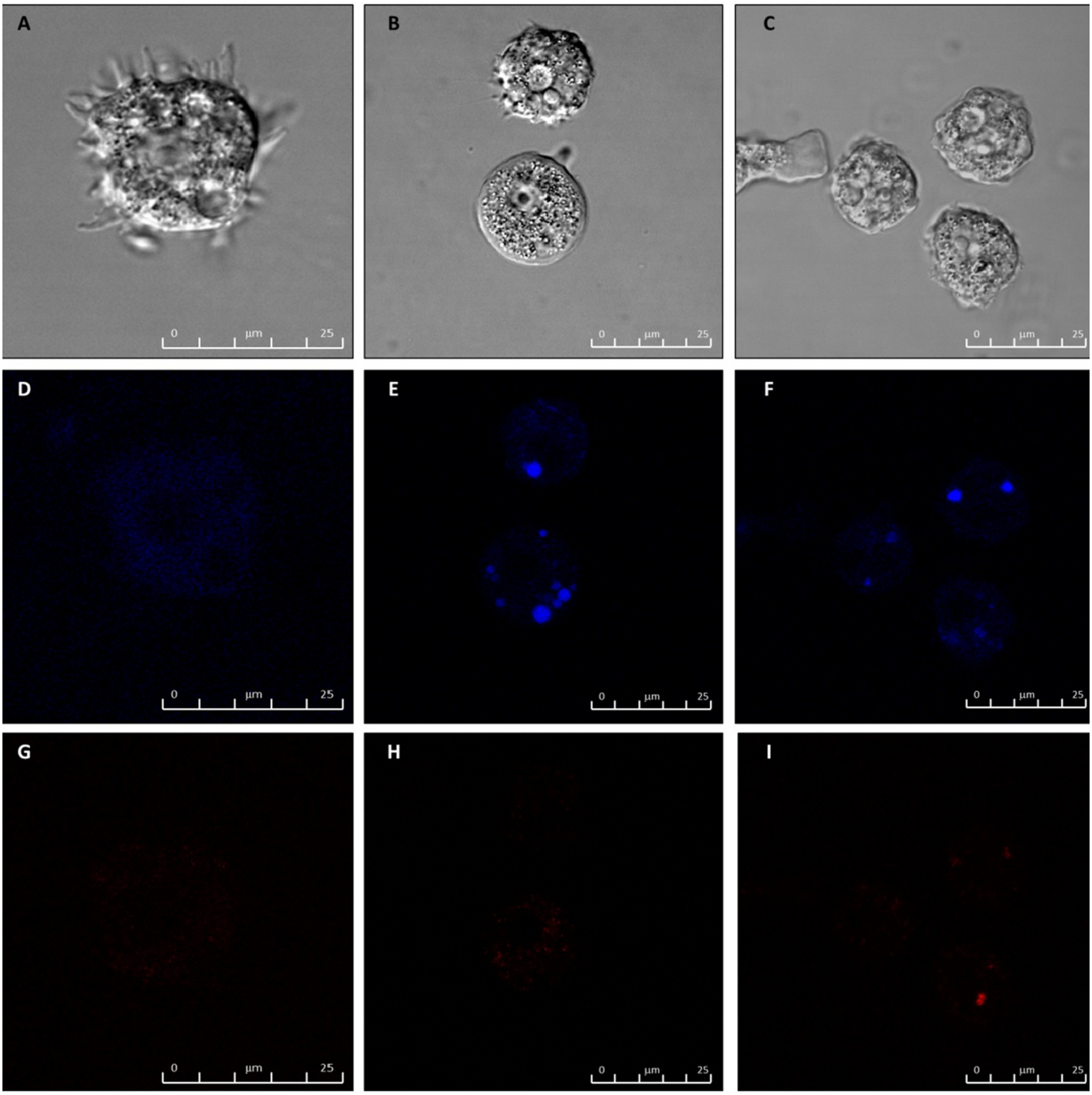

FIG 5 Hoechst staining is different in control cells, where uniformly faint blue nuclei are observed, and in treated cells (at 24 h), where the nuclei are bright blue. (A to C) Phase contrast where A is the control, B caffeine (IC_{50}), and C maslinic acid (IC_{50}). (D to F) Hoechst channel where D is the control, E caffeine, and F maslinic acid. (G to I) Propidium iodine channel where G is the control, H caffeine, and I maslinic acid. Bar, 25 μm.

and maslinic acid activate PCD and associated phenomena such as externalization of phosphatidylserine, chromatin condensation, and DNA fragmentation. We also found evidence for the involvement of a caspase-3-like enzyme since the well-known DEVD-*p*-nitroalanine caspase-3 substrate was cleaved in *Acanthamoeba*. Caspase-3 is an effector caspase that is responsible for DNA fragmentation, chromatin condensation, and membrane disruption. Other members of the family of caspases are the metacaspases and paracaspases. Paracaspases have been found in plants, fungi, and protozoa. Their function is not limited to cell death, but includes roles in sporulation and embryogenesis (38, 39, 40). In *Acanthamoeba*, a type-1 metacaspase has been identi-

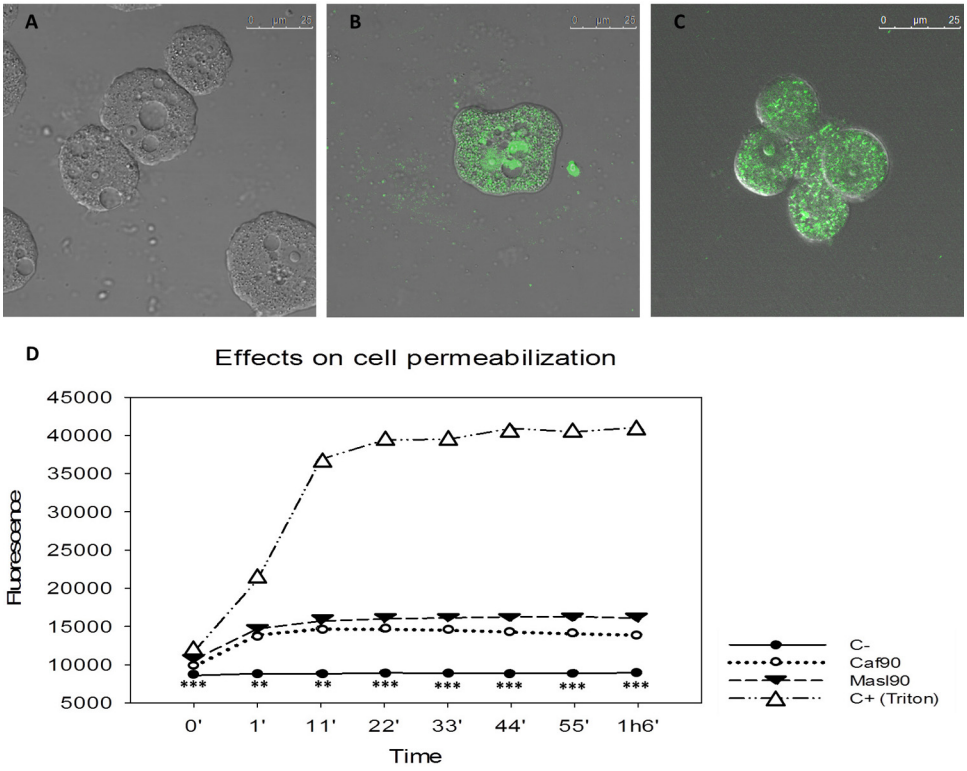

FIG 6 Permeabilization of the cellular membrane. Fluorescence from the SYTOX Green nucleic acid stain can be observed when cells were treated with the different treatments after 2 h. (A) Control. (B) Caffeine. (C) Maslinic acid. (D) Differences between the total permeabilization of control (with addition of triton) and drug-treated cells were apparent when fluorescence of the cells was measured. Statistical differences (***, $P < 0.001$; **, $P < 0.01$) are shown for results obtained between negative control and the different treatments. Bar, 25 μm.

fied. Its function is related to encystment (41), and activity relating to the osmoregulation processes has also been inferred (42). Our group has previously used the same method and found caspase-3-like activity stimulated by statin drugs in *Acanthamoeba* (34), and others have reported that violacein induces caspase-3 activity in *Acanthamoeba* (43), but we can find no obvious caspase-3 homologs in the various *Acanthamoeba* genome databases. It is possible that the *Acanthamoeba* enzyme that recognizes and cleaves this motif belongs to a different protease family.

Treatment that induces necrosis in parasites produces an inflammatory response in the host (44), so it is important to avoid the use of necrotic drugs in order to reduce inflammation in delicate tissues such as the eye in the case of AK, or the brain in GAE.

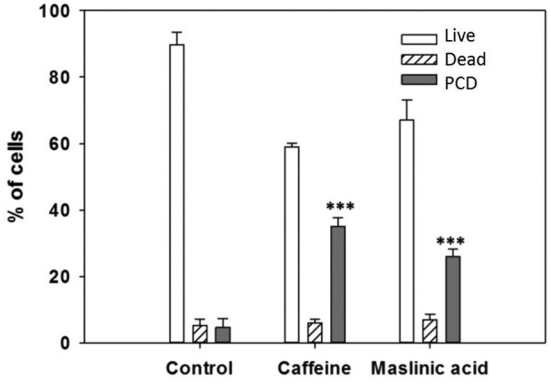

FIG 7 Histogram comparing cells and treatments. Results are represented in percentages and statistical differences (***, $P < 0.001$) are shown comparing apoptotic cells after treatments with the control.

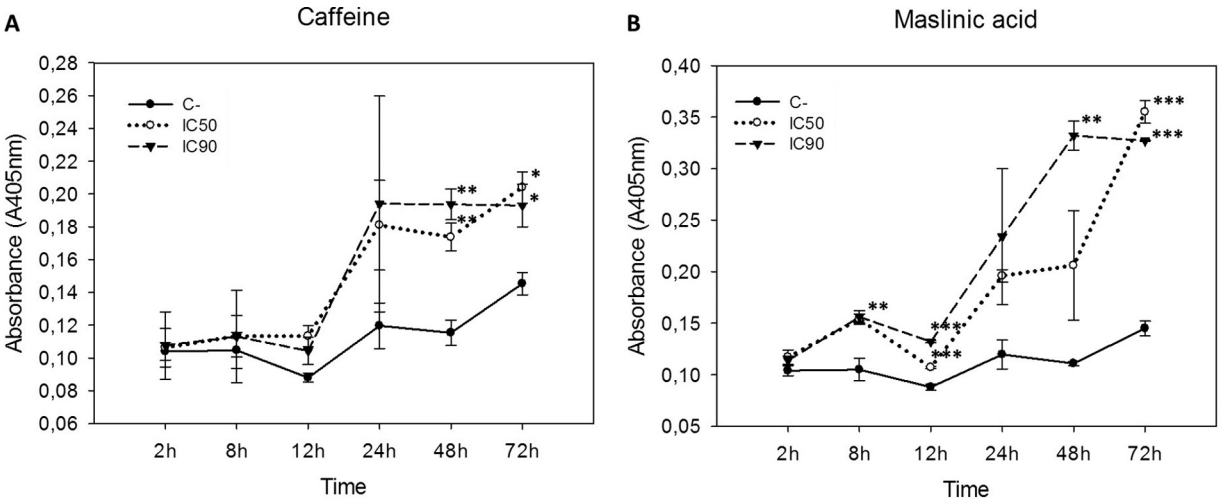

FIG 8 Caspase-like activity absorbance versus time (2 to 72 h). Statistical differences (***, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$) are shown comparing control with the different treatment concentrations. (A) Caffeine. (B) Maslinic acid.

Maslinic acid is well tolerated in mice (45) and rats (46) and we know that humans tolerate caffeine well, but it remains to be seen if either drug can be safely and comfortably introduced to the eye surface (or the brain) at the required concentration to be an effective treatment. The facts that maslinic acid inhibits encystment, is toxic to cysts and trophozoites, and the killing mechanism acts through PCD make maslinic acid an especially promising candidate for AK treatment.

MATERIALS AND METHODS

Acanthamoeba strains. Three clinical isolates (CLC-16, genotype T3; CLC-41.r, genotype T4; and CLC-51, genotype T1) obtained in a previous study in our laboratory (47) and the type strain *Acanthamoeba* Neff (ATCC 30010, genotype T4) were used in this study. These strains were grown axenically in peptone yeast glucose (PYG) medium (0.75% [wt/vol] proteose peptone, 0.75% [wt/vol] yeast extract, and 1.5% [wt/vol] glucose) containing 40 μg/ml gentamicin (Biochrom AG, Cultek, Granollers, Barcelona, Spain) at room temperature.

Chemicals. Two drugs were selected for the different experiments: caffeine (Sigma-Aldrich Chemistry Ltd.; Madrid, Spain) and maslinic acid (kindly provided by *Instituto de Biotecnología*, Department of Parasitology, University of Granada, Spain, and synthetized as previously described) (45). Results obtained with caffeine and maslinic acid were compared to results with chlorhexidine (chlorhexidine digluconate; Alfa Aesar) and amphotericin B (Sigma-Aldrich Chemistry Ltd.; Madrid, Spain), used as positive controls.

Activity assays. The anti-trophozoite activities of the assayed drugs were determined by the alamarBlue assay as previously described (47, 48, 49).

Cysticidal activity. The effects of drugs against cysts were evaluated by incubating *Acanthamoeba* Neff (10^5 cells/ml) with the previously calculated IC_{90} for each drug in Neff's encystment medium (which induces encystation) (NEM; 0.1 M KCl, 8 mM $MgSO_4{\cdot}7H_2O$, 0.4 mM $CaCl_2{\cdot}2H_2O$, 1 mM $NaHCO_3$, 20 mM ammediol [2-amino-2-methyl-1,3-propanediol]; Sigma-Aldrich Chemistry Ltd., Madrid, Spain) pH 8.8, at 25°C. After 24, 48, and 72 h, samples were collected in flow cytometry tubes, where they were stained with the vital stain Congo red (Fisher Scientific) at 10 μg/ml for 30 min. This stain has a high affinity for cellulose, making it useful to stain mature cysts.

Samples were analyzed by flow cytometry using a BD FACSCanto II (Becton & Dickinson) driven by the manufacturer's software, BD FACS Diva. The different populations of cells were separated accordingly to size and complexity, as follows: P1 total population; P2 cysts; P3 trophozoites; P4 cysts stained with Congo red; P5 precysts stained with Congo red.

Cell proliferation. In order to study the effect of the drugs on *Acanthamoeba* Neff cell proliferation, a Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche) was used following the manufacture's recommendations and as previously described (49).

Mammalian cytotoxicity test. The cytotoxicity produced by active compounds was evaluated against two mammalian cell lines, murine macrophages (ATCC TIB-67) and HeLa cells (ATCC CCL-2). A Cytotoxicity Detection kit (LDH) (Roche Applied Science) was used following the manufacture's recommendations. Results were classified based on previously established parameters. Drugs that have percentages of cytotoxicity between 0% to 10% were defined as being noncytotoxic, values between 10% to 25% as having low cytotoxicity, 25% to 40% as having moderate cytotoxicity, and values >40% having high cytotoxicity (14, 49).

Cellular DNA fragmentation. A cellular DNA fragmentation kit (Roche) was used. This kit is an enzyme-linked immunosorbent assay (ELISA) for the detection of bromodeoxyuridine (BrdU)-labeled

DNA fragments in culture supernatants and cellular lysates. The procedure for characterization of cell death consists of two parts: 1. Analysis of the supernatant, which contains DNA fragments at early stages of necrosis and late stages of apoptosis. 2. The remaining cells are lysed in order to release apoptotic DNA fragments located in the cytoplasm. The experiment was carried out following the manufacturer recommendations and as previously described (34).

Double stain assay for apoptosis determination. A double stain apoptosis detection kit (Hoechst 33342/PI) (Genscript, Piscataway, NJ, USA) and an inverted confocal microscope (Leica DMI 4000B) were used. The experiment was carried out following the manufacturer recommendations and as previously described (34). 10^5 cells/well were incubated in a 24-well plate for 24 h with the previously calculated IC_{50} (Table 1). 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 propidium iodide (PI) fluorescence, as this dye only penetrates dead cells.

Plasma membrane permeability. SYTOX Green nucleic acid stain (Invitrogen, Life Technologies SA, Madrid, Spain) is a high-affinity nucleic acid stain (absorption and emission maxima at 504 and 523 nm, respectively) that fluorescently stains permeable cells. The experiment was carried out following the manufacturer recommendations and as previously described (34). A positive control using 2.5% of Triton X-100 (Sigma) to fully permeabilize cells was included in the study.

Caspase-like activity detection. A Caspase-3 Colorimetric Assay kit (Genscript, Piscataway, NJ, USA) was used following manufacturer recommendations and as in a previously described study (34). The assay is based on the chromophore *p*-nitroalanine, which is coupled to a peptide containing the caspase-3 substrate DEVD. On completion, the optical density (at 405 nm) of the experiment is compared to controls to determine caspase-3 activity.

Statistical analysis. The obtained results were compared by one-way ANOVA, multiple *post hoc* analyses, and Tukey's test using the Sigma Plot 12.0 software (Systat Software).

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