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Amoebicidal activity of caffeine and maslinic acid by the induction of Programmed Cell Death in *Acanthamoeba*.

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Running Head: Caffeine and maslinic acid induce PCD 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, and the rare but usually fatal 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, the treatments should target both the trophozoite and the cyst stages as the later are known to confer resistance to treatment. In this study we document the activity 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 activity of *Acanthamoeba* is correlated with their pathogenicity. We also provide evidence or the first time that both drugs exert their anti-amoebal effects through programmed cell death.

Keywords: *Acanthamoeba*, caffeine, maslinic acid, Programmed Cell Death.

41

42 **INTRODUCTION**

43 The genus *Acanthamoeba* are ubiquitous protists which are the causative agents of several
44 opportunistic infections in humans such as a sight-threating ulceration of the cornea
45 known as *Acanthamoeba* Keratitis (AK), the usually fatal Granulomatous Amoebic
46 Encephalitis (GAE) and also a range of disseminated infections, usually, but not
47 exclusively, limited to the skin (1-4).

48 The life cycle of *Acanthamoeba* alternates between two stages: the trophozoite, which is
49 the active growing stage, and the cyst which is a dormant stage resorted to when
50 conditions become incompatible with growth. The cyst has a highly resistant double wall.
51 The outer wall is fibrous and composed mainly of protein while the inner wall is of a
52 granular nature and contains more than 30% cellulose (2, 5, 6, 7). In AK, the cyst is
53 responsible for recurrent amoebic infections, as it is able to survive many of the current
54 treatments (8, 9) and differentiate back to amoebae on the cessation of treatment.
55 Diamidines (proamidine and hexamidine) and biguanides (chlorhexidine and
56 polyhexamethylene biguanide (PHMB)), have been found to be effective against
57 *Acanthamoeba* trophozoites and cysts *in vitro* (2, 3, 4, 10). However, it has been reported
58 that about 5% of patients with AK are troubled with inflammation due to the presence of
59 *Acanthamoeba* cyst surviving in the cornea, even after prolonged treatment with these
60 agents (11). There is an urgent need for more effective treatments and so there is also a
61 need to identify and validate new therapeutic targets against *Acanthamoeba* mostly
62 focusing on key proteins related to cellular viability and the pathogenic mechanisms. In
63 this report we explore two such targets, the secreted proteases and glycogen
64 phosphorylase.

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

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

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

80

81 **RESULTS**

82 **Caffeine and maslinic acid were both amoebicidal and cysticidal.** Caffeine and
83 maslinic acid were both active against the trophozoite stage of different strains of
84 *Acanthamoeba*. Caffeine has higher activity than maslinic acid (except for strain CLC-
85 16) (Table 1). Although both products seem to have a lower activity than chlorhexidine,
86 their activity is still higher that the other reference drug, amphotericin B (Table 1).
87 When cells were grown in encystation medium and stained with Congo Red in order to
88 analyse by flow cytometry, control cells were clearly divided in 3 main populations: P3
89 trophozoites; P4 cysts stained with Congo Red; P5 pre-cysts stained with Congo Red

(Figure 1A). However, after the treatments using caffeine and maslinic acid, trophozoites were unable to neither encyst nor form mature cysts (Figure 1B, 1C).

The effect of caffeine and maslinic acid on cell proliferation from 24 to 72 h was checked. It was noted that both active principles decreased the cell proliferation in a dose-dependent manner (Figure 2). Furthermore, significant differences between the control and the IC_{50} and IC_{90} were observed (except at 24h when maslinic acid was used (Figure 2B)) and no significant differences between both concentrations were observed, which may serve to establish the IC_{50} as the concentration 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 (Figure 3).

Caffeine and maslinic acid induce larger amount 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 higher amount of intracellular DNA was detected in all cases with significant differences between the detected DNA in the lysate and the supernatant. Just in the case of caffeine (Fig. 4A), significant differences between the used concentrations were also observed at 48h after the treatment.

Caffeine and maslinic acid induce PCD which can be observed with double stain assay. When double staining was performed, caffeine and maslinic acid caused nuclei staining with Hoechst 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 high cytoplasmic background stain (Fig. 5D) whereas cells displaying PCD presented bright blue nuclei due to karyopyknosis and chromatin condensation (Fig 5E-F). Dead cells were not able to exclude propidium iodide, a DNA binding dye, and so the remnant of the nuclei in these dead cells stained red (Fig 5G-I). These images show that both caffeine and maslinic acid caused PCD after 24 hours

Caffeine and maslinic acid cause plasma membrane permeability. Treated amoebae with caffeine and maslinic acid induced cellular membrane damage after 1 h of treatment. None of the tested products induced the same level of fluorescence observed in the positive control (Fig. 6D). Nevertheless, cellular membrane disruption was checked and confirmed using fluorescence microscopy in treated cells (Fig. 6A-C).

Amoebae treated with caffeine and maslinic acid showed signs of early PCD. *Acanthamoeba* Neff treated with the assayed active principles showed externalization of PS. Number of cells suffering PCD or death were counted and show a clearly different 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 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 hrs.

DISCUSSION

Caffeine and maslinic acid are glycogen phosphorylase inhibitors (17, 18) and maslinic acid inhibits the extracellular protease in a number of different parasites (19, 23, 24, 25, 26). Their activity 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 an anti-cyst activity had been 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 both on proteases and on the glycogen phosphorylase activity that is 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 induces apoptosis in various vertebrate cell types, for example caffeine induce PCD in neuroblastoma cells, pancreatic and lung adenocarcinoma (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 and maslinic acid activate PCD and markers 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, leading to DNA fragmentation, chromatin condensation and membrane disruption. Other members of the family of caspases are the metacaspases and

paracaspases, the latter have been found in plants, fungi and protozoa and their function is not limited to cell death, but have roles in sporulation, embryogenesis (38, 39, 40). In *Acanthamoeba*, a type-1 metacaspase has been identified. Its function is related to encystment (41), and activity relating to the osmoregulation processes has also been inferred (42). Our group has previously found caspase-3-like activity stimulated by statin drugs in *Acanthamoeba* by the same method (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 recognises and cleaves this motif belongs to a different protease family.

Treatment that induces necrosis in parasites produce an inflammatory response in the host (44), so it is important to avoid the use of necrotic drugs to reduce inflammation in delicate tissues such as the eye in the case of AK, or the brain in GAE. Maslinic acid is well tolerated in mice (45) and rat (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 fact that maslinic acid inhibits encystment, is toxic to cysts and trophozoites and the killing mechanism acts through PCD makes this an especially promising candidate for AK treatment.

MATERIAL 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

188 *Acanthamoeba* Neff (ATCC 30010, genotype T4) were used in this study. These strains
189 were grown axenically in PYG medium [0.75 % (w/v) proteose peptone, 0.75 % (w/v)
190 yeast extract and 1.5 % (w/v) glucose] containing 40 μg/ml gentamicin (Biochrom AG,
191 Cultek, Granollers, Barcelona, Spain) at room temperature.

192 **Chemicals.** Two drugs were selected for the different experiments: caffeine (Sigma-
193 Aldrich Chemistry Ltd.; Madrid, Spain) and maslinic acid (was kindly provided by
194 *Instituto de Biotecnología*, Department of Parasitology, University of Granada, Spain).
195 Their results were compared with chlorhexidine (chlorhexidine digluconate; Alfa Aesar)
196 and amphotericin B (Sigma-Aldrich Chemistry Ltd.; Madrid, Spain), used as reference
197 drugs.

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

200 **Cysticidal activity.** The effect of the assayed drugs against cyst was evaluated incubating
201 10^5 cells/ml of *Acanthamoeba* Neff with the previously calculated IC_{90} in Neff´s
202 Encystment Medium (induces encystation of amoebic strains) (NEM; 0.1 M KCl, 8 mM
203 $MgSO_4 \cdot 7H_2O$, 0.4 mM $CaCl_2 \cdot 2H_2O$, 1 mM $NaHCO_3$, 20 mM ammediol [2-amino-2-
204 methyl-1,3-propanediol] Sigma Aldrich Chemistry Ltd., Madrid, Spain, pH 8.8, at 25 ºC).
205 After 24, 48 and 72 h, samples were collected in flow cytometry tubes where they were
206 stained with the vital stain Congo Red (Fisher Scientific) at 10 μg/ml for 30 minutes. This
207 stain has a high affinity for cellulose, making it is useful to stain mature cysts.

208 Samples were analysed by flow cytometry using a BD FACS Canto™ II (Becton &
209 Dickinson) and the software BD FACS Diva. The different populations of cells were
210 separated accordingly to size and complexity, as follow: P1 total population; P2 cysts; P3
211 trophozoites; P4 cysts stained with Congo Red; P5 pre-cysts stained with Congo Red.

212 **Cell proliferation.** In order to study the effect of the tested active compounds on the
213 *Acanthamoeba* Neff cell proliferation, a Cell Proliferation ELISA, BrdU (colourimetric)
214 kit was used (Roche) following the manufacture's recommendations and as previously
215 described (49).

216 **Mammalian Cytotoxicity test.** The cytotoxicity produced by active compounds was
217 evaluated against cell lines from mammals: murine macrophages (ATCC TIB-67) and
218 HeLa cells (ATCC CCL-2). A Cytotoxicity Detection Kit (LDH) (Roche Applied
219 Science) was used following the manufacture's recommendations. Results were classified
220 based on a previously establish parameters. The active principles that have percentages
221 of cytotoxicity between 0-10%, were defined as being non- cytotoxic. Values between
222 10-25% as having low cytotoxicity, 25-40%, as having moderate cytotoxicity while
223 values >40%, have high cytotoxicity (14, 49).

224 **Cellular DNA Fragmentation.** A cellular DNA fragmentation kit (Roche) was used.
225 This kit is an Enzyme-Linked ImmunoSorbent Assay (ELISA) for the detection of BrdU-
226 labeled DNA fragments in culture supernatants and cellular lysates. The procedure for
227 characterization of cell death consists in two parts: 1. Analysis of supernatant which will
228 contain DNA fragments at early stages of necrosis and late stages of apoptosis. 2. The
229 remaining cells are lysed in order to release apoptotic DNA fragments located in the
230 cytoplasm. The experiment was carried out following manufacturer recommendations
231 and as previously described (34).

232 **Double Stain assay for apoptosis determination.** A double stain apoptosis detection kit
233 (Hoechst 33342/PI) (Genscript, Piscataway, NJ, USA) and an inverted confocal
234 microscope (Leica DMI 4000B) was used. The experiment was carried out following
235 manufactures recommendations and as previously described (34). 10^5 cells/well were
236 incubated in a 24-well plate for 24h with the previously calculated IC_{50} (Table 1). The

double staining pattern allows the identification of three groups in a cellular population: live cells will show only a low level of blue Hoechst 33342 fluorescence; apoptotic cells will show a higher level of blue fluorescence, and dead cells will 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 rendering cells with compromised plasma membranes brightly green fluorescent.

Experiment was carried out following manufacturers´ recommendations and as a previously described (34). A positive control with 2.5% of triton X-100 (Sigma) was added in order to obtain fully permeabilized cells.

Caspase-like activity detection. A Caspase-3 Colorimetric Assay Kit (Genscript, Piscataway, NJ, USA) was used following manufacturers´ recommendations and as 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 and multiple Post-hoc analysis and Tukey's test using the Sigma Plot 12.0 software (Systat Software).

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439 81.

440 49.

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442 **FIGURE LEGENDS**

443 **Table 1.** The AlamarBlue® cell viability assay was used to determine IC_{50} and IC_{90} (μM)
444 values (after 96 hours) for caffeine and maslinic acid tested against the four strains of
445 *Acanthamoeba*.

446

447 **Figure 1.** *Acanthamoeba* Neff separated into sub-populations after 72h: P1, total cell
448 population, analysed by the flowcytometer; P2, cysts; P3, trophozoites, analysed
449 according to size and complexity of cells. P4, cysts; P5, precysts, analysed by
450 fluorescence emitted by the Congo Red staining. A. Control. B. Cells after treatment with
451 caffeine IC_{90}. C. Cells after treatment with maslinic acid IC_{90}.

452 **Figure 2.** Observed cell proliferation after incubation of *Acanthamoeba* Neff strain with
453 caffeine and maslinic acid using the previously obtained IC_{50} and IC_{90} compared to the
454 control. Results were analysed at 24, 48 and 72h. A. Effects on cell proliferation when
455 cells were treated with caffeine. B. Effects on cell proliferation when cells were treated
456 with maslinic acid. Statistically between both concentration and the control were
457 observed (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

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

466 **Figure 4.** Amount of DNA detected over time in the culture supernatant and cell lysate
467 (Absorbance Vs Time). A. Caffeine. B. Maslinic acid. Statistically differences (*** $p <$
468 0.001; * $p < 0.05$) are showed comparing results obtained between supernatants (filled
469 symbols) and cell lysate (empty symbols) values.

470 **Figure 5.** Hoechst staining is different in control cells, where uniformly faint blue nuclei
471 are observed, and in treated cells (at 24h), where the nuclei are bright blue. A-C Phase
472 contrast where A is the Control, B caffeine (IC_{50}) and C maslinic acid (IC_{50}). D-F Hoechst
473 channel where D is the Control, E caffeine and F maslinic acid. G-I Propidium Iodine
474 channel were G is the Control, H caffeine and I maslinic acid. Scale bars are 25μm.

475 **Figure 6.** Permeabilization of the cellular membrane. Fluorescence from the SYTOX®
476 Green nucleic acid stain can be observed when cells were treated with the different
477 treatments after 2 hours. A. Control. B. Caffeine. C. Maslinic acid. D. Differences
478 between the total permeabilization control (addition of triton) and the drug treated cells
479 were apparent when fluorescence of the cells was measured. Statistically differences (***
480 $p < 0.001$; ** $p < 0.01$) are showed comparing results obtained between negative control
481 and the different treatments. Scale bars are 25μm.

Figure 7. Histogram where cells and treatments are compared. Results are represented in percentages and statistically differences (*** $p < 0.001$) are showed comparing apoptotic cells after treatments with the control.

Figure 8. Caspase-like activity (2-72h) (Absorbance vs Time). Statistically differences (*** $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$) are showed comparing control with the different concentrations. A. Caffeine. B. Maslinic acid.