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Activity of Auranofin against Multiple Genotypes of *Naegleria fowleri* and its Synergistic Effect with Amphotericin B *in vitro*

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Abstract

Primary amebic meningoencephalitis, caused by brain infection with a free-living ameba *Naegleria fowleri*, leads to an extensive inflammation of brain and death within 3–7 days after symptoms begin. Treatment of primary amebic meningoencephalitis relies on amphotericin B in combination with other drugs, but use of amphotericin B is associated with severe adverse effects. Despite a fatality rate of over 97%, economic incentive to invest in development of antiamebic drugs by the pharmaceutical industry is lacking. Development of safe and rapidly acting drugs remains a critical unmet need to avert future deaths. Since FDA-approved anti-inflammatory and anti-arthritic drug auranofin is a known inhibitor of selenoprotein synthesis and thioredoxin reductase and the genome of *N. fowleri* encodes genes for both selenocysteine biosynthesis and thioredoxin reductases, we tested the effect of auranofin against *N. fowleri* strains of different genotypes from USA, Europe and Australia. Auranofin was equipotent against all tested strains with an EC_{50} of 1–2 μM. Our growth inhibition study at different time points demonstrated that auranofin is fast-acting and ~90% growth inhibition was achieved within 16 hours of drug exposure. A short exposure of *N. fowleri* to auranofin led to the accumulation of intracellular reactive oxygen species. This is consistent with auranofin's role in inhibiting antioxidant pathways. Further, combination of auranofin and amphotericin B led to 95% of growth inhibition with 2- to 9-fold dose reduction for amphotericin B and 3- to 20-fold dose reduction for auranofin. Auranofin has the potential to be repurposed for the treatment of primary amebic meningoencephalitis.

Graphical Abstract

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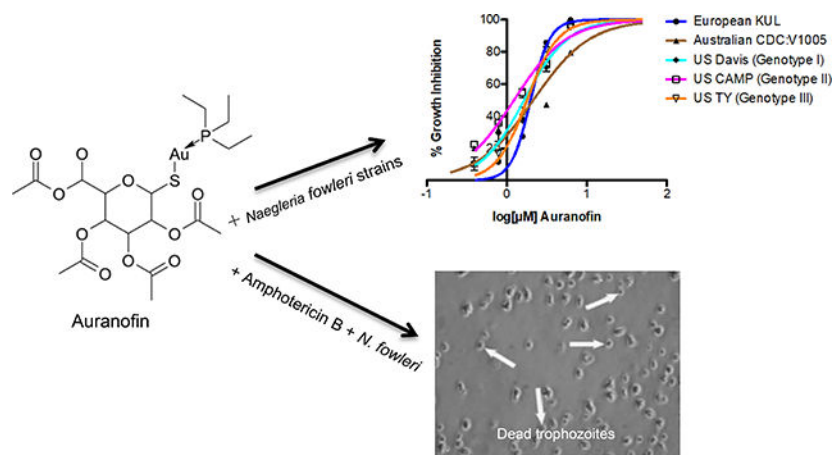
Keywords

free-living ameba; Naegleria; drug; primary amebic meningoencephalitis; auranofin; amphotericin B

INTRODUCTION

Naegleria, commonly found in water resources such as swimming pools having inadequate levels of chlorine, lakes and rivers, feed mostly on bacteria, but can also act as an opportunistic pathogen causing infections of the central nervous system (CNS). *Naegleria fowleri* causes severe primary amebic meningoencephalitis (PAM) and occurs disproportionately among children less than 13 years old [1] with recent recreational fresh water exposure. Only 4 people out of 145 known infected individuals in the United States from 1962 to 2019 have survived [2]. *N. fowleri* has been listed by the National Institute of Allergy and Infectious Diseases (NIAID) as a category B priority biodefense pathogen due to their low infectious dose and potential for dissemination through compromised water supplies in the United States. Through contaminated water, *N. fowleri* enters nostrils and then migrates via the olfactory nerves, through the cribriform plate into olfactory bulb of the brain. The time from initial exposure to onset of illness is usually 5–7 days but may be as short as 24 hours. Initial symptoms include sudden onset of bifrontal or bitemporal headaches, high fever, nuchal rigidity, anorexia, vomiting, irritability and restlessness. Other symptoms such as photophobia, neurological abnormalities, including altered mental status, lethargy, dizziness, ataxia, cranial nerve palsy, hallucinations, delirium, coma may occur late in the clinical course, leading to death in 3 to 7 days [3].

N. fowleri infection is not a notifiable or reportable disease in the US. Thus, it is possible that the infection is underreported because several states differ in their capacity to identify, investigate or report cases. For example, among all encephalitis deaths in the US between 1989 and 1998, 86.2% were due to unknown causes [4]. It is possible that lack of investigation and non-notification of PAM cases may be the reason for some of these encephalitis deaths with unknown causes. PAM kills over 97% of infected people and the high mortality rate and lack of effective therapy make PAM a particularly tragic infection for many families.

This high mortality is attributed to (i) delayed diagnosis, (ii) lack of safe and effective anti-*N. fowleri* drugs, and (iii) difficulty of delivering drugs to the brain.

The optimum treatment for PAM has not been established. To date, amphotericin B has been used, but it is not FDA-approved for this indication and no more than a dozen people worldwide have been successfully treated with amphotericin B, either alone or in combination with other drugs [5]. Recently, an investigational drug, miltefosine, has shown some promise in combination with other drugs and induced hypothermia and management of elevated intracranial pressure based on the principles of traumatic brain injury [6, 7]. However, one patient, who received miltefosine did not survive the infection. Thus, the discovery of new drugs to treat this deadly disease is a critical unmet need to prevent future deaths of children and young adults.

Metal-based compounds have been tested against different neglected tropical diseases. For example, silver polypyridylcomplexes were found active against *Leishmania Mexicana* [8] and platinum and palladium complexes showed potent activity against *Trypanosoma cruzi* [9, 10]. Palladium complexes exhibited better activity than the current drug metronidazole when tested against parasitic *Entamoeba histolytica* [11]. Gold complexes have been used for several years against rheumatoid arthritis [12] and gold-containing compounds were developed as lead compounds against *Trypanosoma*, *Leishmania* and *Plasmodium* [13,15]. Earlier we showed the amebicidal activity of a seleno-organic compound ebselen against *N. fowleri* [16]. We identified thioredoxin reductase in *N. fowleri* genome sequences. Mitochondrial thioredoxin reductase of *N. fowleri* contains selenocysteine [17], which enhances inhibitory effect of metal-containing drugs by facilitating metal release [18]. Since auranofin, an FDA-approved anti-arthritic drug is a known inhibitor of selenoprotein synthesis [19], we tested the activity of auranofin against different genotypes of *N. fowleri*. We then determined the killing effect of auranofin at different time points, tested the effect of auranofin on reactive oxygen species, and finally explored the effect of auranofin in combination with the current standard of care amphotericin B.

RESULTS AND DISCUSSION

In vitro activity of auranofin, amphotericin B and miltefosine against different genotypes of *N. fowleri*

Auranofin is an FDA-approved drug and has been in clinical use to treat rheumatoid arthritis since 1985 [20]. In a short communication published earlier [21], authors tested the activity of auranofin on two US strains HB-1 and Lee, which belonged to the same genotype I and only one strain (HB-1) was a human isolate [22]. Since drug susceptibility varies considerably from strains to strains of different genotypes of *N. fowleri* [23], it is imperative to test the effect of drugs against strains of different genotypes. We tested auranofin against five human strains of various genotypes originated from different geographic regions and compared its activity with that of standards of care, amphotericin B and miltefosine. Auranofin was equally potent against European KUL, Australian CDC:V1005, US genotype I Davis, US genotype II CAMP and US genotype III TY strains with EC_{50} ranging between 1–2 μM. Although auranofin is less potent than amphotericin B, amphotericin B is a highly toxic drug. On the

other hand, auranofin is about 15- to 60-fold more potent than CDC-recommended miltefosine (Table 1).

Auranofin earlier showed efficacy against other protozoans including the diarrheagenic parasites *E. histolytica* [24], *Giardia lamblia* [25] and *Cryptosporidium parvum* [26], the sexually-transmitted parasite *Trichomonas vaginalis* [27], and intracellular parasites *Leishmania donovani* [28, 29], *Toxoplasma gondii* [30] and *T. cruzi* [31]. Recently, it was found to be effective against a free-living CNS-invasive ameba, *Balamuthia mandrillaris* [32]. Our study contributes in establishing auranofin as a broad-spectrum antiparasitic agent that is effective against multiple genotypes of *N. fowleri*.

Since auranofin is an FDA-approved drug, which is currently used for the treatment of rheumatoid arthritis, its toxicity against multiple human cell lines is well documented. The toxicity of auranofin differs depending on the mammalian cell types used. For example, it showed a CC_{50} of 8.2 μM when tested against primary human foreskin fibroblasts [30]. Auranofin demonstrated a CC_{50} of 4 μM against RAW264.7 macrophages [33]. The toxicity of auranofin to human keratinocyte was found to have a CC_{50} of 9.4 μM [34]. All these data show that auranofin has a selectivity indices ranging from 2 to 9, depending on the strain of *N. fowleri* and mammalian cell types examined. When tested against more relevant cell line for brain infection, auranofin did not show toxicity against human astrocytes at concentrations up to 5 μM [35]. Moreover, auranofin crosses the blood-brain barrier [35], which is a prerequisite for the antimicrobial drugs targeting brain infections. A 2 mg/kg dose administered in mice for 7 days, resulted in the brain concentration of about 5 μM [35], which is 2.5–5× of the EC_{50} of auranofin against European, Australian and three genotypes of US strains of *N. fowleri*. Recent studies with amphotericin B conjugated with nanoparticles not only enhanced growth inhibition of *N. fowleri* but also increased the delivery of the drug to brain [36, 37]. The employment of this strategy of loading auranofin into the nanoparticles may further improve the antiamebic effect and blood-brain barrier permeability of auranofin for the treatment of PAM.

Since *N. fowleri* infection causes extensive inflammation in the brain, the anti-inflammatory property of auranofin [20, 24] may also play a beneficial role by reducing the inflammation and normalizing intracranial pressure.

Effect of auranofin on growth inhibition at different time points

Since PAM has a rapid clinical course, it is important to identify a drug that is fast-acting. To establish how fast auranofin kills *N. fowleri*, we measured dose-response (EC_{50}) of auranofin at 2 h, 16 h, 24 h and 48 h of exposure. Growth inhibition curves generated at different time points (Figure 1) demonstrated ~90% growth inhibition at 12.5 μM of auranofin as early as 16 h post-exposure (EC_{50} of 6.3 μM) and 97% inhibition at the same concentration at 24 h post-exposure (EC_{50} of 3.1 μM).

The early pharmacokinetic studies of auranofin from long-term therapy after oral administration detected about 25% of the administered dose in plasma and the peak concentrations reached within 1–2 hour [38]. Recent phase I clinical trial in healthy individuals, who received 6 mg of auranofin orally daily for 7 days and were followed for

126 days, also showed that auranofin is safe and well-tolerated [39]. Once-daily dosing for 7 days led to a plasma gold concentration of 0.312 μg/ml or 1.58 μM [39], which is equal to the *in vitro* EC$_{50}$ of auranofin for *N. fowleri*. Mean terminal half-life of auranofin is approximately 35 days [39]. The FDA has approved clinical trials of auranofin at up to 21 mg/day for the treatment of relapsed chronic lymphocytic leukemia (CLL) after a daily total dose of 12 mg was found well-tolerated for at least 28 days (Clinical Trial registration no. NCT01419691). Population PK modeling and Monte Carlo simulation showed that the plasma gold concentration nearly doubled after 14 days of treatment with 9 mg/day of auranofin treatment [39]. The safety profile at higher dose and 28 days of treatment for the CLL suggests that higher plasma concentration of auranofin is achievable in the treatment of PAM. Relatively shorter treatment regimens required for the PAM patients than arthritis should minimize possible adverse events reported for auranofin [39].

Effect of auranofin on intracellular reactive oxygen species

Since auranofin is an inhibitor of selenoprotein synthesis [19] and thioredoxin reductase function [15, 18, 28, 40, 41], which is involved in protecting cells from damage caused by oxidative stress, we hypothesized that auranofin could exert potent activity against *N. fowleri* by targeting redox enzymes, thus inhibiting antioxidant pathways that maintain the intracellular redox homeostasis.

Since accumulation of intracellular reactive oxygen species is also associated with apoptosis [42, 43], there was a possibility that the treatment of cells with a concentration of drug that leads to cell death might mask the effect of auranofin as an inhibitor of the antioxidant pathways. Therefore, we undertook a short exposure of *N. fowleri* trophozoites with auranofin alone at a concentration that does not lead to cell death within specific time period. We compared the presence of intracellular reactive oxygen species between DMSO-treated control *N. fowleri* trophozoites and those treated with auranofin. After treatment with 3 μM auranofin alone or auranofin plus 300 μM hydrogen peroxide, H$_2$O$_2$, for 18 hours, we detected the presence of reactive oxygen species (Figure 2) with fluorescence generated by the oxidation of dichlorodihydrofluorescein added to the medium. This validates auranofin's role in inhibiting antioxidant pathways in *N. fowleri*.

Auranofin selectively inhibits dithiol function of the antioxidant enzymes such as thioredoxin reductase, thioredoxin-glutathione reductase, trypanothione reductase and glutathione peroxidase [15, 18, 28, 40, 41, 44]. The genome of *N. fowleri* encodes thioredoxin reductases and glutathione peroxidase. Whether the increased potency of auranofin against *N. fowleri* is due to more inhibition of *N. fowleri* thioredoxin reductase or glutathione peroxidase than human thioredoxin reductase or glutathione peroxidase, requires further investigation.

Combination of auranofin and amphotericin B

A successful treatment of PAM requires combination therapy. All treatment regimens that resulted in patient survival contained amphotericin B. Amphotericin B was used in combination with other drugs like rifampin, fluconazole, sulfadiazine, miconazole, sulfisoxazole, ketoconazole, dexamethasone, ornidazole and chloramphenicol to increase the

success of the treatment [45–52]. However, among these drugs only miconazole was reported to have an additive or synergistic effect with amphotericin B [50]. Tetracycline and minocycline were also found to have a synergistic effect when combined with amphotericin B [53]. Azithromycin showed synergistic effect with amphotericin B against *N. fowleri*, both *in vitro* and in a mouse model of PAM [54]. Yet no successful treatment with these combinations has been reported.

Pairing amphotericin B with a synergistic drug may reduce the dose required to achieve a maximum effect with minimum nephrotoxicity associated with amphotericin B. We determined the inhibitory effects of auranofin and amphotericin B at fixed concentration ratios. The dose-effect relationships between two drugs were assessed by classical isobolograms built to calculate Chou-Talalay combination indices (CI) and dose-reduction indices (DRI) as shown in Table 2, using CompuSyn software. The calculated parameters indicated synergy at different drug ratios. Thus, 95% growth inhibition with 2- to 9-fold dose reduction for amphotericin B and 3- to 20-fold dose reduction for auranofin was achieved.

The use of amphotericin B is mainly limited due to its nephrotoxicity. When tested against three different epithelial kidney cell lines (Vero, MDCK-PTR9 and GMK), the CC_{50} of amphotericin B was about 5.4 μM [55, 56]. It showed a CC_{50} of 28.7 μM against human embryonic kidney cells (HEK293) [57]. Auranofin also did not show any cytotoxic effect on HEK293 when tested up to 5 μM concentration [58]. Considering the doses of amphotericin B and auranofin required to show synergism are much lower (Table 2) than the concentrations that demonstrate toxicity on kidney cells, we believe that the concentrations used in the combinations to achieve 95% growth inhibition of *N. fowleri* will not have any significant toxic effect on kidney cells. Since PAM is a brain infection and a combination of auranofin and amphotericin B may exert effect on brain cells, we considered the toxicity of auranofin and amphotericin B on human astrocytes, a type of brain cell. Both auranofin and amphotericin B did not exhibit any toxicity on human astrocytes at concentrations up to 5 μM [35] and 10.8 μM [59], respectively. Given that the combinations of auranofin and amphotericin B use lower concentrations to show synergistic activity against *N. fowleri* (Table 2) than the concentrations that demonstrate toxicity on astrocytes, it is unlikely that the doses used in the combination study will have toxic effect on human astrocytes. Although all the ratios that showed synergistic activity could be useful in the combination of auranofin and amphotericin B against *N. fowleri*, 1:1 ratio might be considered the best based on the use of low concentrations of both the drugs.

The drug combinations with highest synergy were analyzed microscopically for their effect on *N. fowleri* growth and morphology (Figure 3). The amphotericin B-auranofin pair, combined at concentrations of 0.6 μM and 4.5 μM, respectively, (CI=0.5), completely inhibited the trophozoite growth, had a detrimental effect on cell morphology and caused death of the majority of *N. fowleri* cells in 48 h of drug exposure; DMSO-treated control cells grew and appeared normal. These drug combination results give us an opportunity to reduce amphotericin concentration by an order of magnitude to achieve 95% of *N. fowleri* growth inhibition. This may allow minimizing the dose-limiting adverse effects of amphotericin B.

Future studies will involve confirmation of efficacy of auranofin, either alone or in combination with amphotericin B, in an animal model of PAM. Given that clinical trial for PAM is not possible, proving *in vivo* efficacy will allow repurposing of auranofin, either as a monotherapy or in combination with the standard of care amphotericin B, for the treatment of PAM.

METHODS

Maintenance of *N. fowleri*

Trophozoites of pathogenic *N. fowleri* European strain KUL (ATCC 30808) and different clinical strains were axenically cultured in Nelson's medium supplemented with 10% FBS at 37°C [60]. Clinical strains used in the study were Davis, CAMP, TY and CDC:V1005. These four clinical strains were acquired from Centers for Disease Control and Prevention (CDC), USA. US strains Davis, CAMP [61] and TY [62] belong to genotypes I, II and III, respectively. CDC:V1005 is an Australian strain. Experiments were conducted using trophozoites harvested at 48 hours when they were at the logarithmic phase of growth. Trophozoites were counted using a hemocytometer.

In vitro activity of auranofin, amphotericin B and miltefosine against different genotypes of *N. fowleri*

10 mM stock solutions of auranofin (Enzo Life Sciences) and amphotericin B (GoldBio) were prepared in 100% DMSO and stored at −20°C. 40 mM stock solution of miltefosine (Sigma) was prepared in sterile water. Sixteen concentrations of auranofin and amphotericin B and eight concentrations of miltefosine were tested against KUL, CAMP, Davis, TY and CDC:V1005 strains of *N. fowleri*. Briefly, 5 μL of 10 mM auranofin and amphotericin B was added in a 96-well clear bottom dilution plate. A two-fold serial dilution was then performed using 2.5 μL of compound and adding 2.5 μL of 100% DMSO, yielding a concentration range of 10 mM-0.0003 mM. From this dilution plate, 0.5 μL was transferred in triplicate into a 96-well screen plate followed by addition of 99.5 μL of trophozoites (10,000 amebae per well) into each well to yield a final 16-point concentration that ranged from 50 μM to 0.0015 μM in final 0.5% DMSO [16, 63]. Similarly, a final 8-point concentration spanning 200 μM to 1.5 μM was achieved from a 40 mM stock of miltefosine. 0.5% DMSO was used as a vehicle control and 50 μM amphotericin B was used as a positive control. Assay plates were incubated for 48 hours at 37°C and after 48 hours, plates were kept at room temperature for 30 minutes. Effects of auranofin, amphotericin B and miltefosine on cell viability were measured by adding 25 μL of CellTiter-Glo Luminescent Cell Viability Assay (Promega) in each well of the 96-well plates. CellTiter-Glo first induced cell lysis when plates were kept on an orbital shaker at room temperature for 10 minutes. The resulting luminescent signal was then stabilized after equilibrating the plates at room temperature for 10 minutes. Released ATP bioluminescence of the trophozoites was measured at room temperature using an EnVision Multilabel Reader (PerkinElmer, Waltham, MA).

Data analysis and statistics

Growth inhibition percentage relative to maximum and minimum reference signal controls was calculated using the formula:

% inhibition = (1- [(experimental value − mean of maximum signal reference control)/(mean of minimum signal reference control − mean of maximum signal reference control)]) × 100

EC_{50} of auranofin, amphotericin B and miltefosine and 95% confidence intervals were determined using GraphPad Prism software 5.0.

Effect of auranofin on growth inhibition at different time points

To establish how fast auranofin kills *N. fowleri*, we measured the effect of auranofin on KUL strain at different time points. For this, KUL strain was incubated with different concentrations of auranofin, starting from 0.39 μM to 50 μM, in triplicate for 2 hours, 16 hours, 24 hours and 48 hours in 96-well microplates. Growth inhibition percentage and EC_{50} at different timepoints was determined by using the CellTiter-Glo Luminescent Cell Viability Assay following the same protocol as described above. Experiment was done in triplicate in three independent biological replicates.

Measurement of intracellular reactive oxygen species

To assess the effect of auranofin on antioxidant pathways of *N. fowleri*, 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (Sigma) was used. DCFDA is a cell-permeable molecule which is oxidized by the reactive oxygen species inside the cell forming the fluorescent dichlorofluorescein (DCF) [64]. Reactive oxygen species production assay was optimized for *N. fowleri* following earlier method [24]. Briefly, 100 μL (20,000 amebae) of *N. fowleri* KUL trophozoites were preincubated in a transparent, flat bottom 96-well plate (Fisher Scientific) with 0.5% DMSO, 3 μM auranofin in duplicate for 18 h. In another set, compound-treated and 0.5% DMSO-treated trophozoites were incubated with 300 μM H_2O_2 for 2 h. After 2 h, media were removed from the wells, washed and replaced with prewarmed Nelson's medium containing 0.4 mM of DCFDA. The plate was incubated at 37°C for 30 min in the dark. Each well was washed again and pictures of the fluorescent cells were taken with an Axio Vert.A1 inverted phase microscope (Zeiss) and Zen lite software (Zeiss).

Combination of auranofin and amphotericin B

To determine the effect of combination of auranofin and amphotericin B against *N. fowleri*, eight-point two-fold dilutions of auranofin and amphotericin B were made from 1.25 mM amphotericin B and 5 mM auranofin. 0.25 μL of each serially diluted drug was transferred into a solid bottom tissue culture 96-well plate (E&K Scientific) in a matrix-way. 99.5 μL of *N. fowleri* trophozoites (10,000 amebae) were added to yield a final volume of 100 μL per well reaching different ratios (1:1, 1:2, 1:4, 1:8, 1:16, 2:1, 4:1, 8:1) in the combination matrix formed by the mixtures of amphotericin B from 3.125 μM to 0.024 μM in rows and auranofin from 12.5 μM to 0.095 μM in columns. Identical concentrations of amphotericin B and auranofin were separately achieved in two columns to test the effect of each drug independently. 0.5% DMSO was used as a negative control and 50 μM amphotericin B as a

positive control. All the experiments were performed in triplicate. The assay plates were incubated for 48 h at 37°C. The plates were equilibrated to room temperature and the CellTiter-Glo® luminescent cell viability assay was used to quantify the growth inhibition percentage of each combination as well as the individual drugs. The growth inhibition percentage values were calculated and the effect of combination was analyzed using CompuSyn software following the Chou-Talalay method [65, 66]. The combination index (CI) values indicating either the additive (CI=1) or the antagonistic (CI>1) or the synergistic (CI<1) effect [67] were calculated from the CompuSyn software.

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ABBREVIATIONS

CNS   central nervous system

PAM   primary amebic meningoencephalitis

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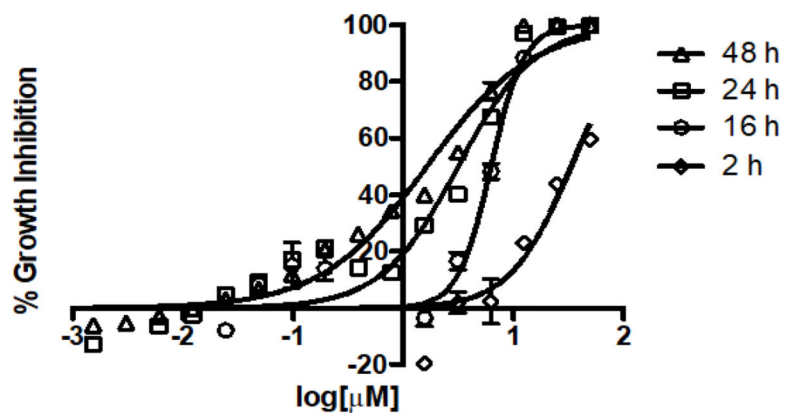
Figure 1.
Growth inhibition curves of auranofin at different time points against *N. fowleri*.
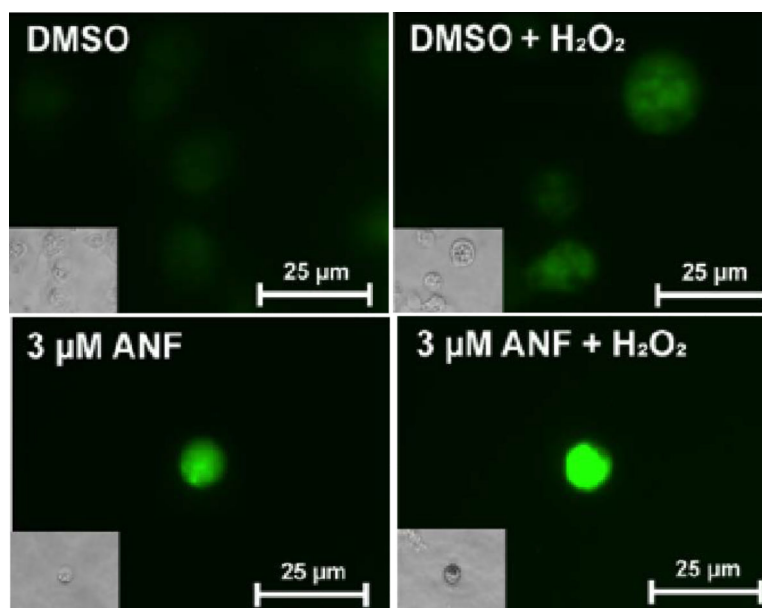
Figure 2.
Accumulation of intracellular reactive oxygen species in *N. fowleri* is enhanced by exposure to auranofin. Fluorescence imaging of *N. fowleri* detected reactive oxygen species within trophozoites following treatment with 3 μM of auranofin (ANF) for 18 hours or auranofin plus H_2O_2. Control trophozoites were treated with 0.5% DMSO alone and 0.5% DMSO plus 300 μM H_2O_2. Magnification, ×40.
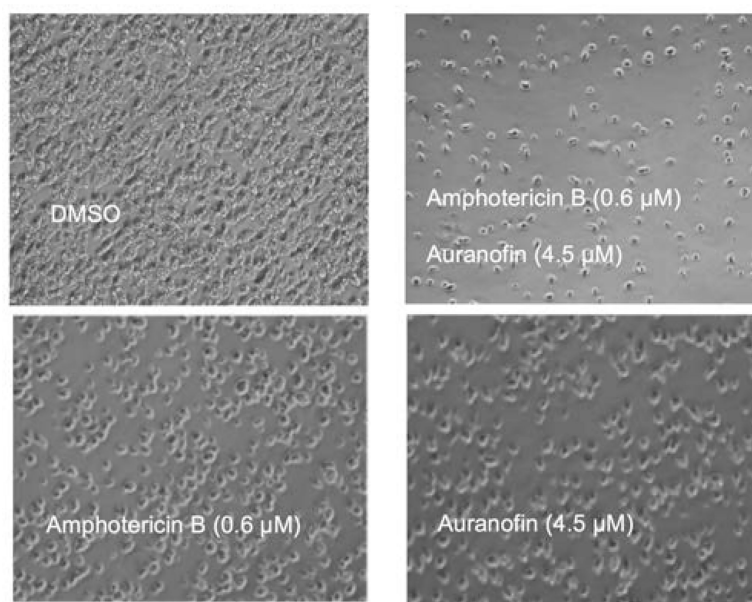
Figure 3.
Synergistic effect of drugs at low concentrations. The phase contrast microscope images show *N. fowleri* trophozoites treated for 48 hours with 0.5% DMSO, 0.6 μM amphotericin B, 4.5 μM of auranofin, and a combination of 0.6 μM of amphotericin B and 4.5 μM of auranofin. The auranofin-amphotericin B-treated *N. fowleri* cells visible in the microscope field are rounded, much smaller in size and not viable, whereas DMSO-, 0.6 μM amphotericin B- and 4.5 μM of auranofin-treated cells are irregularly shaped with visible cytoplasm. Magnification, ×20.

Table 1.

EC$_{50}$ values of auranofin against different strains of *N. fowleri*

Drugs	Strains	EC$_{50}$ (μM)		
		Mean	95% Lower CL	95% Upper CL
Auranofin	European KUL	2 ± 0.01	1.9	2.1
	Australian CDC:V1005	2.2 ± 0.03	1.9	2. 6
	US Davis (Genotype I)	1.6 ± 0.03	1.4	1.9
	US CAMP (Genotype II)	1.2 ± 0.02	1.1	1.4
	US TY (Genotype III)	1.8 ± 0.01	1.7	1.9
Amphotericin B	European KUL	0.09 ± 0.03	0.08	0.1
	Australian CDC:V1005	0.2 ± 0.05	0.1	0.2
	US Davis (Genotype I)	0.06 ± 0.05	0.05	0.08
	US CAMP (Genotype II)	0.06 ± 0.04	0.05	0.08
	US TY (Genotype III)	0.06 ± 0.02	0.05	0.06
Miltefosine	European KUL	54.5 ± 0.01	51.4	57.8
	Australian CDC:V1005	15.9 ± 0.09	10.3	24.7
	US Davis (Genotype I)	58.9 ± 0.07	41.3	83.9
	US CAMP (Genotype II)	21.8 ± 0.02	19.9	23.8
	US TY (Genotype III)	37.7 ± 0.02	33.1	43

CL = confidence limit

Table 2.

Summary of synergism assay with amphotericin B and auranofin, shown for 95% growth inhibition of *N. fowleri* trophozoites

Amphotericin B : Auranofin ratio	% Growth Inhibition	Combination Index (CI)	Dose Reduction Index (DRI)		Dose required to achieve 95% inhibition (μM)	
			Amphotericin B	Auranofin	Amphotericin B	Auranofin
1:16	95	0.5 ± 0.3	9.3 ± 3.2	2.8 ± 1.6	0.3 ± 0.1	5.2 ± 1.1
1:8	95	0.5 ± 0.1	5.2 ± 0.5	3.1 ± 1	0.6 ± 0.1	4.5 ± 0.5
1:4	95	0.7 ± 0.2	2.8 ± 0.4	3.3 ± 1	1.0 ± 0.1	4.1 ± 0.1
1:2	95	0.5 ± 0.1	3.0 ± 0.4	7.2 ± 2.5	0.9 ± 0.1	1.9 ± 0.2
1:1	95	0.5 ± 0.1	2.6 ± 0.1	12.2 ± 3.2	1.1 ± 0.1	1.1 ± 0.1
2:1	95	0.6 ± 0.1	2.0 ± 0.4	19.5 ± 7.4	1.4 ± 0.2	0.7 ± 0.1