

RESEARCH ARTICLE

Auranofin is active against *Histoplasma capsulatum* and reduces the expression of virulence-related genes

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Abstract

Background

Auranofin is an approved anti-rheumatic drug that has a broad-range inhibitory action against several microorganisms, including human pathogenic fungi. The auranofin activity against *Histoplasma capsulatum*, the dimorphic fungus that causes histoplasmosis, has not been properly addressed. Since there are few therapeutic options for this life-threatening systemic mycosis, this study evaluated the effects of auranofin on *H. capsulatum* growth and expression of virulence factors.

Methodology/Principal findings

Minimal inhibitory and fungicidal concentrations (MIC and MFC, respectively) of auranofin against 15 *H. capsulatum* strains with distinct genetic backgrounds were determined using the yeast form of the fungus and a microdilution protocol. Auranofin activity was also assessed on a macrophage model of infection and on a *Tenebrio molitor* invertebrate animal model. Expression of virulence-related genes was compared between auranofin treated and untreated *H. capsulatum* yeast cells using a quantitative PCR assay. Auranofin affected the growth of different strains of *H. capsulatum*, with MIC and MFC values ranging from 1.25 to 5.0 µM and from 2.5 to >10 µM, respectively. Auranofin was able to kill intracellular *H. capsulatum* yeast cells and conferred protection against the fungus in the experimental animal model of infection. Moreover, the expression of catalase A, HSP70, superoxide dismutase, thioredoxin reductase, serine proteinase, cytochrome C peroxidase, histone 2B,

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formamidase, metallopeptidase, Y20 and YPS3 proteins were reduced after six hours of auranofin treatment. **CONCLUSIONS/SIGNIFICANCE:** Auranofin is fungicidal against *H. capsulatum* and reduces the expression of several virulence-related genes, which makes this anti-rheumatic drug a good candidate for new medicines against histoplasmosis.

Author summary

Histoplasmosis is a serious disease caused by the fungus *Histoplasma capsulatum*, which can be life-threatening and has limited treatment options. In this study, we investigated the potential of auranofin, a drug originally approved for treating rheumatoid arthritis, to combat *H. capsulatum*. We tested auranofin against different strains of the fungus and found that it effectively inhibited fungal growth. This drug was also effective in killing the fungus within cells from the immune system and provided protection in an experimental animal model. Furthermore, auranofin reduced the expression of several genes associated with the fungus's ability to cause disease. These genes are involved in critical functions such as detoxifying harmful substances and maintaining cellular structures. By suppressing these genes, auranofin hampers the fungus's ability to survive and cause infection. These findings suggest that auranofin could be repurposed as a treatment for histoplasmosis, offering a new avenue for therapy against this challenging infection. This research is significant because it highlights the potential of existing drugs to treat other diseases, broadening our arsenal of available treatments and contributing to better healthcare solutions.

1 Introduction

Auranofin is a gold-containing orally administered approved drug used for more than 40 years to treat rheumatoid arthritis. However, since the introduction of biological therapies for this disease, such as the chimeric monoclonal antibody infliximab, the original use of auranofin has remarkably decreased. Due to the auranofin safety, guaranteed by its four decades of use with low contraindications, this drug became an interesting target for drug repurposing [1]. In fact, studies, in different stages of clinical research, are in development to repurpose auranofin to several diseases, infectious or not [2–4].

Several studies have demonstrated the broad-range antimicrobial activity of auranofin against several bacteria [5], protozoa [3,6], viruses [7] and fungi [8]. The list of auranofin-susceptible fungi includes certain *Candida* species, *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Aspergillus fumigatus*, *Paecilomyces variotii*, *Scedosporium apiospermum*, *Lomentospora prolificans*, *Saccharomyces cerevisiae* and several chromoblastomycosis agents such as *Fonsecaea pedrosoi* and *Cladophialophora carrionii* [8–10]. Despite the great auranofin antifungal properties, its activity against some important human pathogenic fungi remains unelucidated.

Histoplasma capsulatum is the etiological agent of histoplasmosis, a systemic mycosis that is often related to outbreaks, usually reported in caves, mines, chicken coops and abandoned places [11]. This mycosis can affect immunocompetent people, usually as a chronic pulmonary disease [12], and immunosuppressed patients, especially people living with HIV/AIDS, who develop a life-threatening disseminated disease [13]. The infection starts with the inhalation of mycelial propagules, especially microconidia, which convert to the yeast form inside or outside phagocytes present in the lungs. These phagocytes may be vehicles for fungal dissemination

into the host, since the *H. capsulatum* yeast form is highly adapted to mammalian hosts [14]. Histoplasmosis is a prevalent opportunistic infection among people living with HIV in the Latin America region, as indicated by various studies [15–17].

A proper management of histoplasmosis depends on rapid diagnosis and specific treatment [18]. Currently, liposomal amphotericin B is the first-choice induction therapy for disseminated and moderate-to-severe histoplasmosis, followed by a consolidation regimen using itraconazole. Itraconazole alone is also the alternative for mild infections [19]. These two antifungal drugs present a series of adverse effects and drug interactions [20,21], which is a serious problem in critically ill immunosuppressed patients who receive several medications.

The antifungal pipeline includes three potential new antifungal drugs with antifungal activity against *H. capsulatum*: fosmanogepix, a drug that affects trafficking and anchoring manno-proteins to the fungal cell membrane and wall; ibrexafungerp, a triterpenoid that inhibits glucan synthase; and olorofim, an orotomide that targets pyrimidine synthesis. However, none of them have undergone specific clinical trials for histoplasmosis [22]. In this scenario, drug repurposing is an interesting alternative, since drugs with proven administration safety may skip some stages of clinical research [23]. As auranofin administration is safe [1] and it demonstrated antifungal activity against many fungi [24], we studied whether this anti-rheumatic drug can affect *H. capsulatum* growth and expression of genes that enhance fungal virulence during infection.

2 Material and methods

2.1 Strains

The reference strain *H. capsulatum* G217B (ATCC 26032) was used throughout the study. In addition, other 14 *H. capsulatum* strains, with different genotypes determined by MLST analyses in previous publications [25–28], were included in the *in vitro* antifungal susceptibility studies. All strains are maintained at the Pathogenic Fungal Collection of Fiocruz (WDCM 951). Fungal strains were maintained in the yeast-like form on ML-Gema agar medium [29] at 37°C.

2.2 *In vitro* antifungal tests

The minimal inhibitory concentration (MIC) of auranofin against the *H. capsulatum* strains was verified through the broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline [30], with a few modifications to test this dimorphic fungus and the antirheumatic drug, which are described herein: two-fold serial dilutions of a stock auranofin solution in dimethyl sulfoxide (2 mM) were made to obtain the final testing concentrations (range: 10 to 0.02 µM) in RPMI-1640 medium (Sigma-Aldrich Co., St. Louis, MO, USA), supplemented with 2% glucose (Neon Química, Susano, Brazil) and buffered (pH 7.2) with 3-N-morpholinopropanesulfonic acid (Sigma-Aldrich Co.). Fungal suspensions (1×10^6 yeast-like cells/mL) were prepared in sterile 0.9% saline solution after five days of growth. Fungal suspensions were then added to the wells of a flat-bottom 96-well plate (Corning, Glendale, AZ, USA) containing the different auranofin concentrations. For each strain, a drug-free and a cell-free control wells were included, as positive and negative growth controls, respectively. Plates were incubated at 35°C for five days. The MIC was determined as the lowest auranofin concentration that completely inhibited fungal growth (100% inhibition), as the EUCAST method suggest for amphotericin B. Additionally, to determine the minimal fungicidal concentration (MFC) as previously published for auranofin and fungal cells [9], 5 µL from each well without visual fungal growth from the plates used for MIC determinations and incubated at 37°C were plated on potato dextrose agar (Beckton, Dickinson

and Company, Sparks, MD, USA) and incubated for 21 days at 25°C. The lowest auranofin concentration without *H. capsulatum* growth after this incubation was defined as the MFC. Interpretations whether auranofin is fungicidal or fungistatic was as follows: if the MFC/MIC ratio was 1 or 2, it was considered fungicidal, otherwise, it was classified as fungistatic [31]. MIC and MFC determinations were performed in triplicate.

2.3 Synergism studies

The synergistic activity of auranofin with conventional antifungal agents was verified using the checkerboard method [32]. In this method, two drugs were loaded into a single 96-well plate, with varying concentrations of the auranofin-antifungal combination in each well. Initially, this experiment was conducted using the G217B reference strain to evaluate the synergistic effects of the combinations between auranofin and the antifungal drugs amphotericin B, itraconazole, and caspofungin (all from Sigma-Aldrich Co.). Subsequently, the experiment was replicated for the drug combinations that presented synergism using representative strains from different *H. capsulatum* phylogenetic species. The auranofin and antifungal dilutions were prepared following the methodology proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), starting from a 100-fold concentrated stock auranofin and antifungal solution [30]. The final concentrations of the auranofin and antifungal drug ranged from 0.156 to 10 µM and 0.0004 to 2 µM, respectively. The fungal inoculum and incubation conditions remained the same as those described for the *in vitro* antifungal tests. The interaction between the drugs was classified using the fractional inhibitory concentration index (FICI) [33]. The types of interactions between the drug combinations were classified as synergism if $FICI \leq 0.5$, indifference if $0.5 < FICI < 4$, and antagonism if $FICI \geq 4$ [32,33].

2.4 Morphology studies

The *H. capsulatum* G217B yeast-like cells were treated with a subinhibitory auranofin concentration (1/2 MIC) in HAM's F12 medium (Gibco, Grand Island, NY, USA) and incubated at 37°C for three days. A control culture without auranofin was made under the same conditions. Cells were harvested, washed three times with phosphate-buffered saline (PBS) and visualized with an AXIO Lab.A1 bright-field light microscope (Zeiss, Jena, Germany). The diameter of 100 cells of each condition was measured using the ImageJ 1.40 g software (National Institutes of Health, Bethesda, MD, USA). Three replicates were performed for each condition.

2.5 Regulation of gene expression

We treated *H. capsulatum* G217B yeast-like cells (1×10^6 cells/mL) with a subinhibitory concentration (1/2 MIC) of auranofin in HAM's F12 medium for three and six hours. The cells were harvested and subjected to total RNA extraction by mechanical cell rupture using a Bead-Beater (BioSpec, Bartlesville, OK, United States), 0.5 µm diameter glass beads and TRIzol (Invitrogen, Waltham, MA, USA), according to the manufacturer's protocol. Equal amounts of RNA (1 µg) of the RNAs were used to synthesize single stranded cDNAs (DNA complementary) using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) and oligo (dT) primer. Prior to PCR reactions, the cDNAs were diluted (1:5) by adding water. Quantitative PCR (qRT-PCR) was performed using the SYBR green PCR master mix (Applied Biosystems, Foster City, CA, United States), in the AriaMx real-time PCR system (Agilent Technologies, Santa Clara, CA, USA), with 10 pmol of each specific primer and 4 µL of template cDNA in a final volume of 20 µL. The genes encoding the virulence-related proteins catalase A (HCAG_05109), catalase B (HCAG_08064), heat shock protein (HSP) 70 (HCAG_01398), superoxide dismutase (HCAG_01543), thioredoxin reductase

(HCAG_07019), serine proteinase (HCAG_00635), cytochrome C peroxidase (HCAG_08658), Histone 2B (HCAG_03525), formamidase (HCAG_08831), metalloproteinase (HCAG_04252), Y20 protein (HCAG_04745), and yeast phase specific protein 3 (YPS 3) (Q00950) were selected for analysis. The constitutively expressed *glyceraldehyde 3-phosphate dehydrogenase* (HCAG_04910) gene [34–36] was selected to normalize the samples. Intron spanning primers were designed using the Primer 3 tool and are listed at the S1 Table. A cDNA aliquot from each sample diluted serially at 1:5 was mixed and used to generate a relative standard curve. All analyses were performed in triplicate. The relative expression levels of selected genes were obtained using the standard curve method for relative quantification [37]. Transcription levels of cells treated with auranofin were normalized in relation to transcription levels of the untreated control condition.

2.6 Intracellular antifungal activity

The intracellular activity of auranofin on the *H. capsulatum* reference strain was determined by counting colony-forming units (CFUs) recovered from macrophage infection. J774 1.6 macrophages (Rio de Janeiro Cell Bank–BCRJ/UFRJ, accession number 0273) were employed. Macrophages (less than 10 passages) were maintained in RPMI 1640 medium (Sigma-Aldrich) with 10% v/v fetal bovine serum and MEM non-essential amino acid solution (Sigma Aldrich), at 37°C in a CO₂ incubator (ThermoFisher Scientific, Waltham, MA, USA) until the cells were confluent. A total of 10⁶ J774 macrophages were plated per well of a 96-well plate in RPMI medium containing IFN- γ (1 U/mL) (Sigma Aldrich) and incubated for 24 h at 37°C and 5% CO₂. In parallel, *H. capsulatum* yeast cells were grown in HAM's F12 medium for 72 h. Next, 5 \times 10⁶ yeast-like cells were added per well, to give a yeast:macrophage cell ratio of 5:1. After two hours of yeast/macrophage interaction, non-phagocytosed/non-adhered yeast-like cells were removed after three washes with PBS [38]. In addition, auranofin was added to the wells at the following concentrations: ¼ MIC, MIC, and 4 \times MIC. Controls consisted in: (i) culture medium only (sterility control); (ii) macrophages only (macrophage control); (iii) *H. capsulatum* yeast-like cells only (planktonic control); and (iv) *H. capsulatum* yeast-like cells with macrophages (5:1 ratio) without auranofin (intracellular control). Cultures were incubated for 24 h at 37°C and 5% CO₂. After this incubation, macrophages were lysed with cold water and fungal cells were recovered. The number of viable *H. capsulatum* cells was determined based on CFUs counting. The lysates were cultured in ML-Gema agar medium and CFUs were determined after growth at 37°C for 10 days. Four technical replicates were performed for three biological replicates.

2.7 Invertebrate model for *in vivo* activity

For *in vivo* activity studies, we adapted a previously published invertebrate model [39], using *Tenebrio molitor* larvae of at least 1 cm, with regular motility, without dark spots or gray marks. Ten animals were inoculated in the last left proleg with 1 \times 10⁴ *H. capsulatum* yeast-like cells. Animals were treated with an injection of 10 μ L of a 5.7 mg/kg auranofin solution, two and 24 hours after fungal injection (auranofin group). Other animal groups consisted in: (i) non-injected larvae (sham group); (ii) traumatized with the syringe (trauma group); (iii) injected with sterile PBS (non-infected group); (iv) infected with *H. capsulatum* and treated with PBS (infected group); (v) infected with *H. capsulatum* and treated with itraconazole (Sigma-Aldrich) at the same doses and times (itraconazole group); and (vi) injected with PBS, instead of *H. capsulatum* cells and treated with auranofin (auranofin control group). Drug concentrations in the experiments were based a safe itraconazole dose to treat *Galleria mellonella* larvae, previously described [40]. Then, the animals were kept in Petri dishes at room

temperature and were fed ad libitum on bran flour. Mortality and phenotypic changes were recorded for 14 days. Larvae that convert to pupa were censored. This experiment was performed in three biological replicates.

2.8 Statistical analyses

The Prism 9.0 software (GraphPad software, La Jolla, CA, USA) was used for data analyses. The normality of the data was evaluated using the Shapiro-Wilk test to decide between parametric or non-parametric tests. The comparison of cellular diameter was performed using the Student's *t* test. qPCR data was evaluated using the Student's *t* test. Intracellular antifungal activity was evaluated with the Wilcoxon test. Survival curves were compared using the Log-rank (Mantel-Cox) test. A 0.05 significance level was adopted for all analyses.

3 Results

3.1 Auranofin has anti-*Histoplasma* activity

Table 1 presents MIC and MFC data for the *in vitro* auranofin susceptibility test of the 15 *H. capsulatum* strains. Median MIC value (MIC₅₀) was 2.5 μ M, while the MIC₉₀ was 5 μ M (range: 1.25–5.0 μ M / geometric mean: 2.74 μ M). Median MFC was 5.0 μ M (range: 2.5–>10 μ M). MFC:MIC ratio was equal or lower than two for nine strains (60%), indicating that auranofin was fungicidal against some *H. capsulatum* strains located into different genetic clades.

3.2 Auranofin presents synergism with amphotericin B against *H. capsulatum*

The initial checkerboard experiment conducted using the *H. capsulatum* G217B reference strain revealed indifferent interactions between auranofin and itraconazole or caspofungin (FICI values of 0.548 and 2.0, respectively). Conversely, the interaction between auranofin and

Table 1. Antifungal susceptibility data of auranofin against 15 *Histoplasma capsulatum* strains from distinct genetic clades.

Strain	Genetic clade	Auranofin MIC ^a (μ M / μ g/mL)	Auranofin MFC ^b (μ M)	MFC/MIC ratio
G217B	NAm2	1.25 / 0.84	10 / 6.72	8
G184A	Panama	5 / 3.36	5 / 3.36	1
39942	Panama	1.25 / 0.84	5 / 3.36	4
CÃO 4	RJ	5 / 3.36	10 / 6.72	2
IGS 4/5	RJ	5 / 3.36	5 / 3.36	1
TI01	RJ	5 / 3.36	>10 / >6.72	>2
39439	RJ	1.25 / 0.84	2.5 / 1.68	2
IPEC 24_11	RJ	5 / 3.36	10 / 6.72	2
IPEC 28_11	RJ	2.5 / 1.68	>10 / >6.72	>4
CE 25/14	Northeast BR1	1.25 / 0.84	10 / 6.72	4
INI 02/16	LAmB1	5 / 3.36	10 / 6.72	2
IPEC 11_12	LAmB1	2.5 / 1.68	5 / 3.36	2
EH394H	LAmA1	5 / 3.36	5 / 3.36	1
EH53	LAmA	1.25 / 0.84	5 / 3.36	4
20231	Unknown1	2.5 / 1.68	5 / 3.36	2

^a MIC: Minimal inhibitory concentration

^b MFC: Minimal fungicidal concentration

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Table 2. Checkerboard assay data of auranofin and amphotericin B against *Histoplasma capsulatum* strains from distinct genetic clades.

Strain	Genetic clade	MIC ^a (μM)			FICI ^d	Interpretation
		AUR ^b	AMB ^c	AUR/AMB		
G217B	Nam2	1.25	0.125	0.156/0.008	0.1875	Synergism
G184A	Panama	5	0.03	0.625/0.008	0.375	Synergism
IGS 4/5	RJ	5	0.062	0.312/0.015	0.3125	Synergism
CE 25/14	Northeast BR1	2.5	0.03	0.312/0.008	0.375	Synergism
IPEC 11_12	LAmB	5	0.03	0.312/0.008	0.3125	Synergism
EH394H	LAmA1	5	0.015	5/0.008	1.5	Indifference

^a MIC: Minimal inhibitory concentration^b AUR: Auranofin^c AMB: Amphotericin B^d FICI: fractional inhibitory concentration index<https://doi.org/10.1371/journal.pntd.0012586.t002>

amphotericin B exhibited synergy for this strain (FICI = 0.176). Subsequently, we performed checkerboard assays with auranofin and amphotericin B on five additional strains from distinct genotypes. Table 2 describes the checkerboard results of auranofin and amphotericin B for these six strains. Notably, this interaction demonstrated synergy in five strains and indifference in one.

3.3 Auranofin changes *H. capsulatum* cellular size

Both auranofin-treated (Fig 1A) and -untreated (Fig 1B) *H. capsulatum* yeast cells presented a similar spherical to ovoid format. However, the diameter of *H. capsulatum* yeast cells treated with a subinhibitory concentration of auranofin showed a lower size when compared to the untreated control ($P < 0.05$, Fig 1C).

3.4 Auranofin regulates the expression of virulence-related genes

PCR efficiencies ranged between 90% and 110% for each of the primer pairs, indicating that all real time assays had similar good efficiencies, confirming the validation of the primers and the precision of experiments. These data are presented in S1 Table. Fig 2 presents the transcriptional levels of 12 genes related to fungal virulence. In general, the transcripts expression levels, except for catalase B, were significantly altered in at least one of the two time points evaluated. After three hours of auranofin interaction, the genes coding for HSP70, serine proteinase, histone 2B, formamidase, and YPS3 were down-regulated, while the genes encoding catalase A, superoxide dismutase, thioredoxin reductase, metalloproteinase, and Y20 protein were up-regulated. On the other hand, after six hours of auranofin exposure, the gene encoding thioredoxin reductase was up-regulated, while all other genes, except for the catalase B gene, which was not affected by auranofin, were down-regulated.

3.5 Auranofin is effective against intracellular *H. capsulatum* yeast cells

Fig 3 presents the intracellular anti-*Histoplasma* activity of auranofin using a macrophage model. A reduced intracellular *H. capsulatum* CFU counts was observed as compared to the planktonic control, that is, same culture medium, but no macrophages ($P < 0.0001$). Auranofin at subinhibitory (1.25 μM / 0.84 μg/mL) and inhibitory concentrations (5 μM / 3.36 μg/mL) reduced *H. capsulatum* CFU counts as compared with the planktonic ($P < 0.0001$) and intracellular controls, without auranofin supplementation ($P < 0.05$). Moreover, intracellular

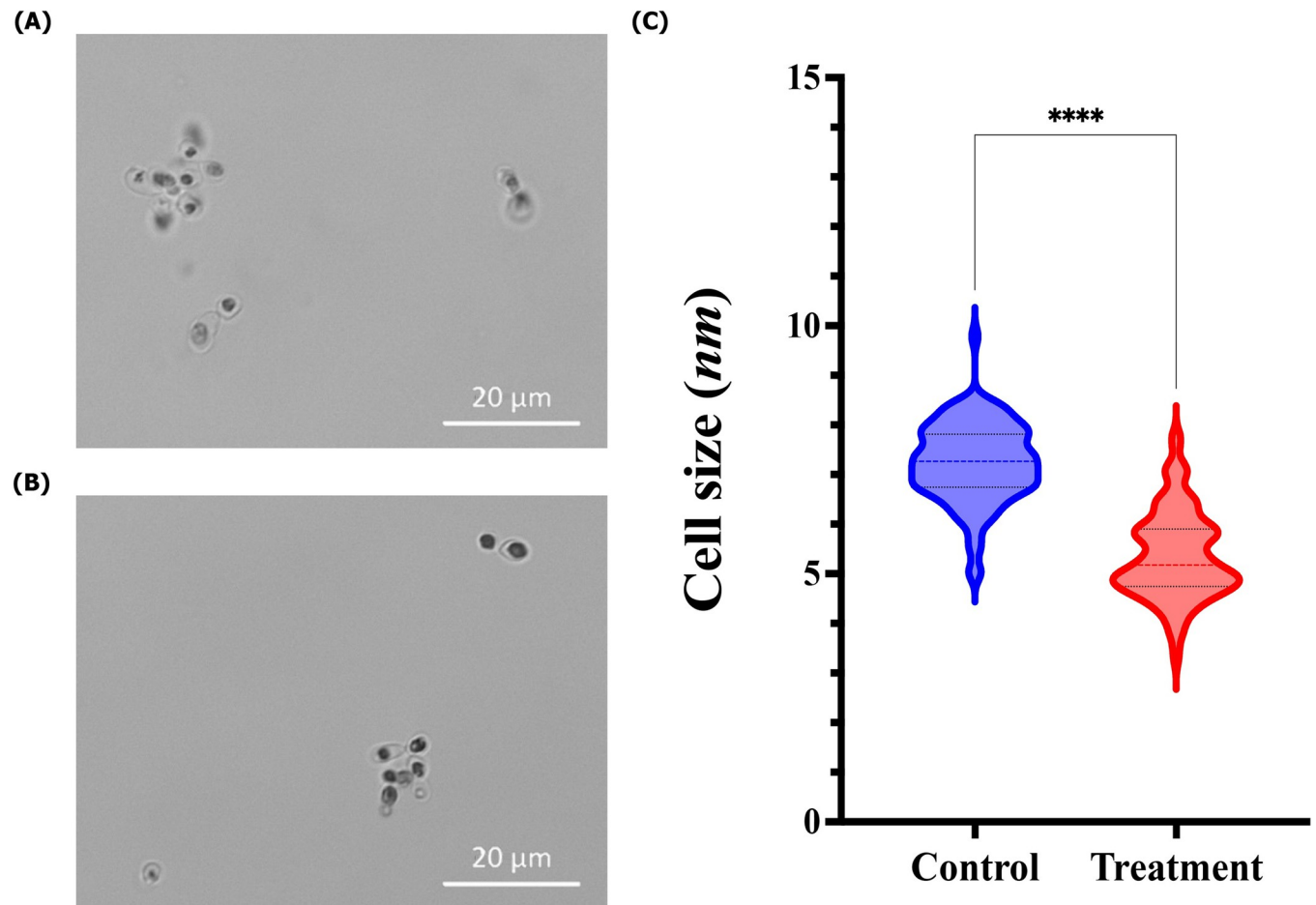


Fig 1. Influence of auranofin in the morphology of *Histoplasma capsulatum* G217B reference strain. (A) *H. capsulatum* yeast cells after 72 h incubation at 37°C in Ham's F-12 drug-free medium; (B) *H. capsulatum* yeast cells after treatment with auranofin (0.625 μ M) (Bars: 20 μ m); (C) Comparison of the diameter size of *H. capsulatum* yeast cells, treated or not with auranofin. Optical microscopy images are representative of three independent experiments. The color line inside the violins represent the median diameter size and the black lines the interquartile ranges of 100 measurements performed in triplicate. **** $P < 0.0001$, Student's t test.

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H. capsulatum CFU counts were lower than planktonic *H. capsulatum* in the presence of sub-inhibitory and inhibitory auranofin concentrations ($P < 0.05$). High auranofin concentration (20 μ M / 13.4 μ g/mL) also reduced *H. capsulatum* CFU counts compared to the planktonic ($P < 0.0001$) and intracellular control ($P < 0.05$), but CFU counts of intracellular and planktonic *H. capsulatum* cells at this drug concentration were similar ($P = 0.7077$).

3.6 Auranofin is effective in an experimental model of *H. capsulatum* infection

Fig 4 presents the survival curves of *T. molitor* larvae challenged with the reference *H. capsulatum* G217B strain. As expected, the sham, trauma, and non-infected groups presented more than 80% survival at the end of the experiment, as well as the auranofin control group that presented similar survival to the other control groups ($P = 0.4265$). Non-treated infected larvae presented 80% deaths, with a median survival of seven days. Animals treated with both itraconazole and auranofin were significantly protected, as compared to the infected group ($P =$

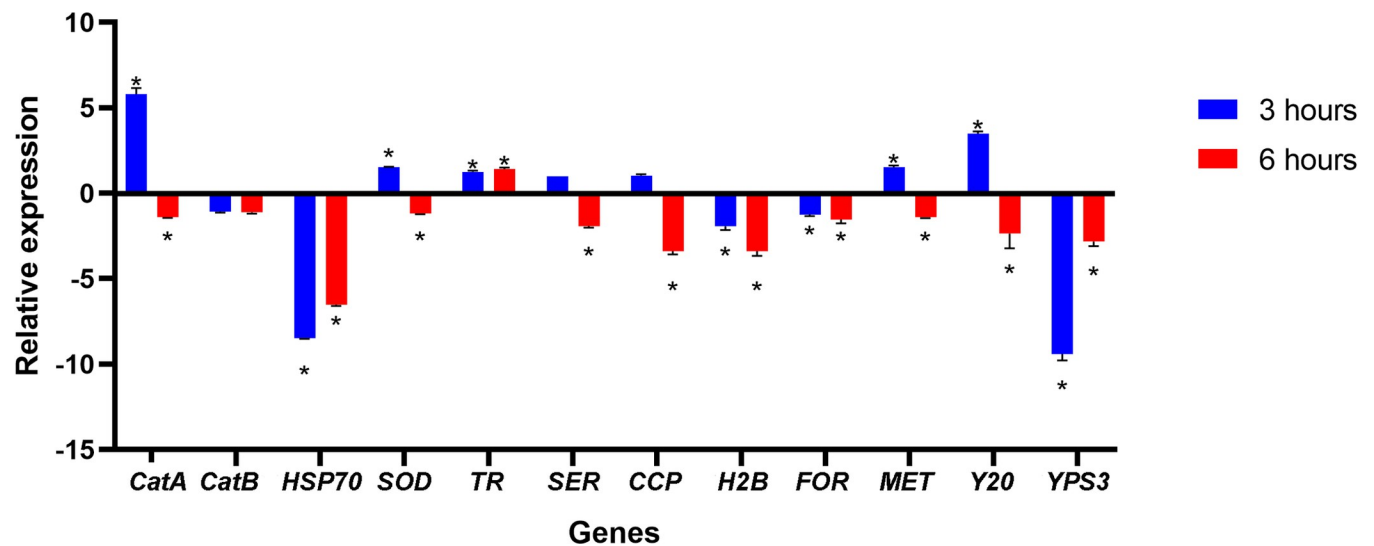


Fig 2. Influence of auranofin in the expression of some virulence-related genes by the *Histoplasma capsulatum* G217B reference strain. Results are presented as the mean and standard deviations derived from three independent experiments. The mean and standard deviations of the relative expression levels of each gene following three (blue bars) or six (red bars) hours of exposure to auranofin (0.625 μ M) are illustrated. CatA: catalase A; CatB: catalase B; HSP70: heat shock protein 70 kDa; SOD: superoxide dismutase; TR: thioredoxin reductase; SER: serine proteinase; CCP: cytochrome C peroxidase; H2B: histone 2B; FOR: formamidase; MET: metalloproteinase; Y20: Y20 protein; YPS3: yeast phase specific protein 3. * $P < 0.05$, Student's *t* test.

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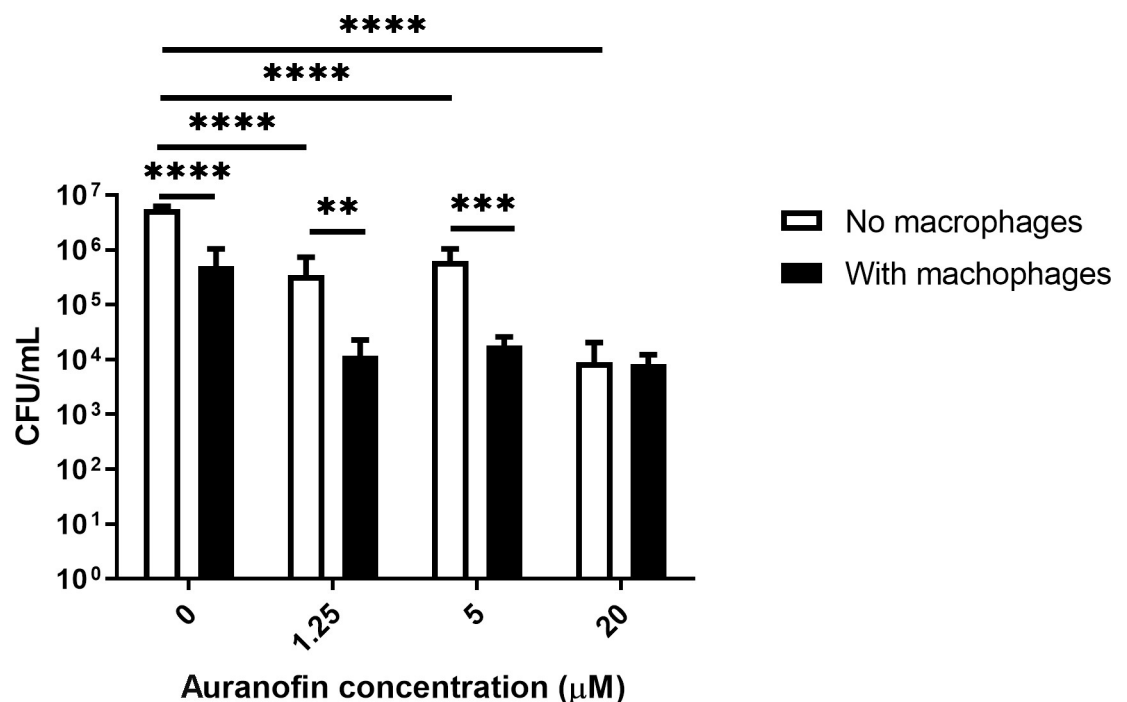


Fig 3. Antifungal activity of auranofin against intracellular *H. capsulatum* G217B reference strain. Results are presented as the mean and standard deviations derived from three independent experiments. The mean and standard deviations of the colony count units per milliliter (CFU/mL) of *H. capsulatum* planktonic (white bars) and intracellular (black bars) yeast cells after treatment with different auranofin concentrations (0, 0.125, 5, and 20 μ M) are illustrated. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, Wilcoxon test.

<https://doi.org/10.1371/journal.pntd.0012586.g003>

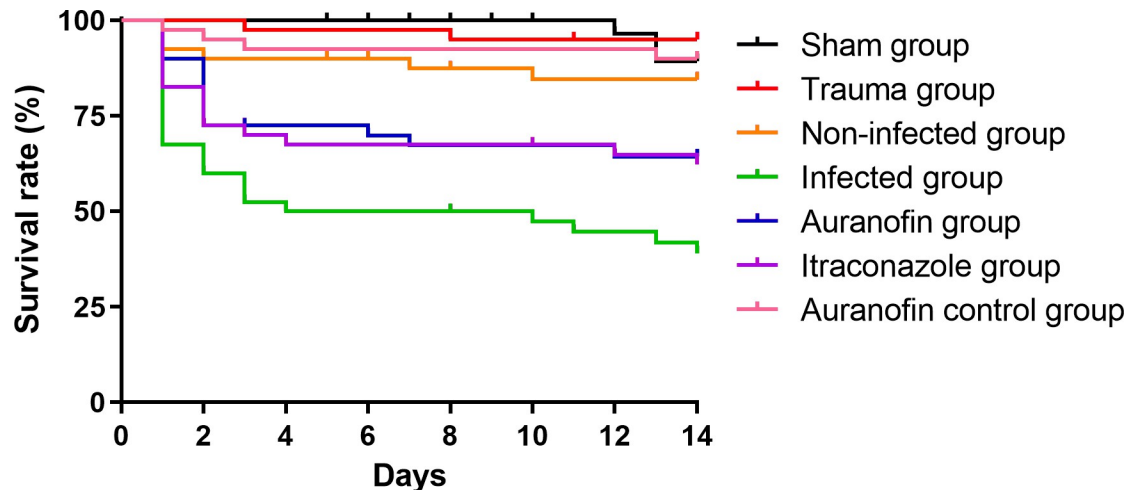


Fig 4. Antifungal activity of auranofin in the *Tenebrio molitor* infection model. Survival curves illustrate larvae infected with *Histoplasma capsulatum* G217B and treated with auranofin or itraconazole (5.7 mg/kg), represented by the blue and purple lines, respectively. Controls consisted in non-injected larvae (sham group, black line); larvae traumatized with the syringe (trauma group, red line); larvae injected with sterile PBS (non-infected group, orange line); infected with *H. capsulatum* and inoculated with PBS instead of auranofin or itraconazole (infected group, green line); and injected with PBS, instead of *H. capsulatum* cells and treated with auranofin 5.7 mg/kg (auranofin control group, pink line). Each curve represents a group of 30 larvae, monitored daily for survival for up to 14 days after infection. Larvae that converted to pupa were censored.

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0.0407 and 0.0227, respectively). Moreover, survival curves of animals treated with itraconazole and auranofin did not present significant differences ($P = 0.7969$).

4 Discussion

Currently, drug repurposing is a promising subject into the drug discovery field, as it identifies new therapeutic opportunities for old drugs, already approved to treat another disease [41]. This strategy has gained importance to discover new drugs for neglected, rare or emerging diseases [42]. One of the strategies described to advance into the treatment of disseminated histoplasmosis is the drug repurposing approach, which would benefit individuals with histoplasmosis and other comorbidities [43]. Here, we present auranofin as a good candidate for the treatment of this life-threatening systemic mycosis. Besides anti-*Histoplasma* activity, this anti-rheumatic drug is also effective against other diseases that can occur in patients with histoplasmosis, such as neurotoxoplasmosis [3], drug-resistant bacterial infections [44], several types of cancer [45], candidiasis, and cryptococcosis [46], which improves the attractiveness towards auranofin repurposing.

The first evidence for the auranofin repurposing potential for histoplasmosis was its good *in vitro* activity against *H. capsulatum* strains of different genetic genotypes, such as NAM2, LAMa, LAMB1, Panama, RJ, Northeast BR1, and Unknown1. The MIC herein described, 1.25 to 5 μM (0.85 to 3.4 $\mu\text{g/mL}$), are similar to those found for other fungi. For instance, auranofin MICs against *Scedosporium* spp. ranged from 1 to 8 $\mu\text{g/mL}$ [47], against black fungi that cause chromoblastomycosis from 1.25 to 2.5 μM [9], against *Candida* spp. from 0.25 to >16 $\mu\text{g/mL}$, and from 1 to 2 $\mu\text{g/mL}$ against both *Cryptococcus neoformans* and *Blastomyces dermatitidis* [8]. Moreover, it has been described that auranofin at 32 $\mu\text{g/mL}$ reduces the viability rate of mammalian cells by no more than 50%, indicating that auranofin toxicity is higher for *H. capsulatum* [48]. In addition to its isolated anti-*Histoplasma* activity, auranofin also demonstrated *in vitro* synergism with amphotericin B, a medication commonly employed in the treatment of histoplasmosis, among the majority of strains tested. This observation indicates a promising

prospect for future therapeutic interventions against this mycosis. It is important to emphasize that the low bioabsorption of auranofin, estimated to be around 25% [45], underscores the need for adjustments in the formulation of this drug to effectively translate the results of this study in clinical practice.

Good candidates for drug repurposing against mycotic diseases should have a fungicidal, rather than a fungistatic nature [49]. In the present study, auranofin was fungicidal against 53.8% of the *H. capsulatum* strains tested, which is another advantage of this drug in the context of histoplasmosis treatment. Auranofin has also showed a fungicidal profile against *Phialophora verrucosa* and *Exophiala dermatitidis* [9].

Treatment of fungal cells with repurposed drugs may induce modifications in their cellular morphology [50]. For this reason, we investigated whether auranofin may induce this phenotypic change and it was observed that despite retaining the original shape, auranofin-treated cells were significantly smaller. This result was intriguing, as previous studies have described an alternative mechanism of action for auranofin in fungal cells involving the Mia40–Erv1 pathway. According to this model, auranofin induces a metabolic shift from respiration to fermentation without disrupting membrane potential or mitochondrial function [45]. However, yeast cells undergoing fermentation are typically larger than those undergoing respiration [51]. This observation suggests that either the Mia40–Erv1 pathway is not involved in auranofin's mechanism of action in *H. capsulatum*, or the relationship between cell size and metabolic state (fermentation versus respiration) in *H. capsulatum* differs from that observed in *S. cerevisiae*.

Cellular size is connected with virulence in some fungal species. For instance, smaller cells of *C. neoformans*, *Mucor circinelloides*, and *Paracoccidioides brasiliensis* are more likely to be phagocytosed and killed by macrophages [52–54]. To the best of our knowledge, there are no studies linking *H. capsulatum* virulence with its cellular size, but it is possible that a similar behavior occurs with this dimorphic fungus, thus auranofin may contribute to reduce *H. capsulatum* virulence. In our *in vitro* model of macrophage / *H. capsulatum* interaction, auranofin at inhibitory and subinhibitory concentrations enhanced macrophage killing of fungal yeast-like cells. This may have occurred by a direct action of auranofin in intracellular *H. capsulatum* cells or indirectly, by a reduction of yeast size, facilitating macrophage killing.

In fact, there are some studies reporting virulence reduction when pathogenic fungi are treated with compounds presenting antifungal activity [55–57]. To evaluate this issue, it was performed a qPCR assay targeting some genes associated with virulence in *H. capsulatum* or other fungal models. Two catalases were evaluated and only catalase A presented gene expression alterations in response to auranofin treatment. This is similar to what happens with transcript levels of this gene in response to cell morphology or oxidative stress [58]. Several genes related to the oxidative stress were down-regulated, especially after six hours of drug interaction. However, the thioredoxin reductase gene was the sole evaluated gene up-regulated on the two time points herein studied. In human cells, auranofin is a strong thioredoxin reductase enzymatic inhibitor [7]. The results of the current study do not allow us to conclude whether the same mechanism of action occurs in *H. capsulatum*. However, if this is indeed the case, the overexpression of this gene is likely a compensatory mechanism necessary because the existing enzymes are rendered non-functional as a result of auranofin action. In addition, the early up-regulation of catalase A, superoxide dismutase, and thioredoxin reductase suggests an oxidative stress mode of action of auranofin. Another interesting and strongly down-regulated gene was *YPS3*. This protein is located in cell surface and produced only by the pathogenic yeast-like form of the fungus, probably associated with fungal dissemination to some host organs, especially liver and spleen [59]. This is another encouraging result that drives the repurposing study of auranofin to treat histoplasmosis [46]. A similar mechanism may occur in *H. capsulatum* yeast cells, which remains to be elucidated.

Finally, we used an invertebrate model to test the *in vivo* activity of auranofin. In last years, these animal models have gained importance in several science fields as a response to the public concern about the use of mammalian animals in research studies, with good success in several areas of biology and medicine [60]. However, some limitations of their use in drug discovery are the differences among invertebrate and human proteins, the absence of an adaptive immune response, and the lack of drug metabolism in insects [61]. In this study, auranofin showed to be as protective as itraconazole in the *T. molitor* larvae model. This model has gained relevance in medical mycology, including the antifungal study field, because this insect, which is susceptible to a broad range of pathogenic fungi, has a complex innate immunity, comprising cellular and humoral components [62]. Again, the auranofin activity in this experiment may be a direct result of the antifungal action of auranofin on *H. capsulatum* cells or an indirect result of the reduction of *H. capsulatum* virulence arsenal. Quantifying fungal CFU in infected animals would help clarify this issue. However, *H. capsulatum* is a slow-growing fungus, and recovering CFU of this species from larvae is extremely challenging due to the presence of contaminant fungi in the larvae microbiota, which grow faster than *H. capsulatum*. Future studies with mammalian hosts should address these hypotheses.

The repositioning of auranofin as an anti-*Histoplasma* drug holds significant relevance for public health. Originally developed as a gold compound for rheumatoid arthritis, auranofin emerges as a promising candidate for treating histoplasmosis, a fungal infection that can be life-threatening, particularly in immunocompromised individuals. Its potential repurposing offers a cost-effective and readily available therapeutic option, which is crucial given the high costs and limited accessibility of current antifungal treatments. By providing a viable alternative, auranofin could enhance treatment accessibility, reduce healthcare burdens, and improve patient outcomes in regions where histoplasmosis is prevalent, ultimately contributing to a more effective management of this serious infectious disease.

In addition to the limitations related to the animal model used for the *in vivo* study discussed above, there are other weaknesses in the current work: (i) we were unable to include *H. capsulatum* strains representing all known genotypes; (ii) there may be other genes with differential expression after auranofin treatment that were not evaluated in this study; and (iii) for most strains, the auranofin MIC was higher than the maximum plasma gold concentration following a daily administration of 6 mg of auranofin for one week, which is 1.584 μM [1]. This indicates that some modifications to this medication are necessary to adapt it for use in histoplasmosis patients.

In conclusion, auranofin seems to be a promising candidate for a repurposed drug to treat histoplasmosis, due to its fungicidal nature against most *H. capsulatum* strains and its potential to reduce *H. capsulatum* virulence.

Supporting information

S1 Table. qPCR primers, slope values, and efficiency of the *Histoplasma capsulatum* G217B primers used in this study.
(XLSX)

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