AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Editor's Pick | Parasitology | Full-Length Text

Hydroxamate-based compounds are potent inhibitors of *Toxoplasma gondii* HDAC biological activity

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ABSTRACT Toxoplasmosis is a critical health issue for immune-deficient individuals and the offspring of newly infected mothers. It is caused by a unicellular intracellular parasite called *Toxoplasma gondii* that is found worldwide. Although efficient drugs are commonly used to treat toxoplasmosis, serious adverse events are common. Therefore, new compounds with potent anti-*T. gondii* activity are needed to provide better suited treatments. We have tested compounds designed to target specifically histone deacetylase enzymes. Among the 55 compounds tested, we identified three compounds showing a concentration of drug required for 50% inhibition (IC_{50}) in the low 100 nM range with a selectivity index of more than 100. These compounds are not only active at inhibiting the growth of the parasite *in vitro* but also at preventing some of the consequences of the acute disease *in vivo*. Two of these hydroxamate based compound also induce a hyper-acetylation of the parasite histones while the parasitic acetylated tubulin level remains unchanged. These findings suggest that the enzymes regulating histone acetylation are potent therapeutic targets for the treatment of acute toxoplasmosis.

KEYWORDS toxoplasma, HDAC, apicomplexa, toxoplasmosis, histone deacetylase inhibitors

Apicomplexa is a phylum of unicellular protozoan parasites that are obligate intracellular and highly transmissible. Among them are several human pathogen species, including *Plasmodium* spp., *Toxoplasma gondii,* and *Cryptosporidium* spp. Although toxoplasmosis generally remains symptomless in healthy individuals, it can lead to serious infections in the central nervous system among those with defective immune systems. Additionally, it may result in birth defects or even death in the offspring of newly infected mothers. The prevalence of *T. gondii* infection worldwide is significant, with estimates indicating that it affects between 30% and 70% of the human population, varying depending on geographical regions (1).

T. gondii has a complex life cycle and infection by oocysts or bradyzoites leads to the development of rapidly growing tachyzoites responsible for the clinical manifestations in humans (2). The acute phase of the disease is caused by tachyzoite proliferation, and the latent bradyzoites can persist in the host for prolonged periods, leading to the possibility of reactivation and causing encephalitis, especially in immunocompromised patients (3). The current treatment for toxoplasmosis involves pyrimethamine and sulfadiazine, which target the tachyzoite, but have adverse effects that limit their use. Undesirable side effects are frequently observed with this treatment regimen, with over 30% of patients experiencing adverse events leading to discontinuation of the treatment. Among these, bone marrow suppression is recognized as one of the most severe complications (4). Clindamycin, while being an alternative medication, carries comparable risks of side effects and is less effective in preventing relapse (5). Additionally, there are presently no

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The authors declare no conflict of interest.

Received 19 May 2023
Accepted 27 August 2023

Published 18 October 2023

treatments available that specifically target the bradyzoite, which is the dormant form of the parasite found in intermediate hosts, including humans.

To address the need for a better treatment, compounds targeting epigenetic mechanisms have been tested against *T. gondii* (6). Enzymes mediating epigenetic modifications of histones have been shown to be promising therapeutic targets (7). Indeed, the control of gene expression is essential to the survival of parasites and more acutely during growth phases (8, 9). Histone acetylation has been extensively investigated as a targeted epigenetic mechanism for identifying compounds that inhibit *T. gondii* (10) and other parasites (11). Enzymes involved in the regulation of histone acetylation have been identified as promising therapeutic targets since several modulators of these enzymes were shown to bear an activity against *T. gondii* (6, 12–14). In particular, several inhibitors of histone deacetylases (HDACs) have been shown to suppress the tachyzoite growth *in vitro* (6, 12, 15–18), indicating that these enzymes are key for the survival of this fast-growing form of the parasite. Moreover, a hydroxamate-based HDAC inhibitor (HDACi) belonging to the uracil-based hydroxyamide (UBHA) class (19), MC1742, was also active *in vivo* to prevent the consequences of the acute disease in a mouse model of toxoplasmosis (6) and against other apicomplexan parasites as *Plasmodium falciparum*, the causative agent of malaria (20). MC1742 treatment of *T. gondii* tachyzoites resulted in elevated levels of parasite histone acetylation, significantly influencing gene expression (6). These findings strongly indicate that histone deacetylation plays a vital role in sustaining the specific gene expression program during the tachyzoite phase (6).

In this manuscript, we aimed at discovering new compounds that would show an anti-*T. gondii* activity both *in vitro* and *in vivo*. We investigated the anti-*T. gondii* activity of hydroxamate-based compounds that were designed to target HDAC activities. We tested more than 50 compounds and demonstrated that more than 30 compounds showed potent activity against the tachyzoite form of this parasite *in vitro*. Two compounds presented a concentration of drug required for 50% inhibition (IC_{50}) lower than that of other hydroxamate-based HDACi, including MC1742. Treatment with these two inhibitors strongly increased the level of parasite histone acetylation indicating that they were *bona fide* HDACi of the parasite. They were also shown to limit the consequences of acute toxoplasmosis in a mouse model of the disease indicating that hydroxamate HDACis are interesting candidates to further expand the therapeutic arsenal against *T. gondii*.

MATERIALS AND METHODS

Parasite strains and culture

Toxoplasma gondii tachyzoites of the type I [RH and RH-LUC GFP (21)] and type II [76K and PTG-LUC-GFP (21)] strains were propagated *in vitro* in human foreskin fibroblasts (HFF) using Dulbeccos's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 1% penicillin-streptomycin (complete media). Tachyzoites were grown in ventilated tissue culture flasks at 37°C and 5% CO_2. The luciferase gene is expressed under the control of the *tubulin* promoter and is therefore expressed in both tachyzoites and bradyzoites (21).

Compounds

The structures, source, references, and molecular weight for all HDACis used in the study are listed in Table S1 to S4. All compounds were originally dissolved in 100% dimethyl sulfoxide (DMSO) to a 20 mM stock solution. For *in vitro* experiments, the stock solutions were diluted in complete media to the required concentrations. For *in vivo* experiments, the stock solutions were diluted in PBS before injection. Solutions at the final concentration were prepared just before use. Pyrimethamine was resuspended in DMSO to a 7 mg/mL concentration.

Immunofluorescence analysis (IFA)

HFF cells were infected with *T. gondii* tachyzoites of the RH strain for 24 hours in presence of the compound or solvent and then fixed using 4% paraformaldehyde for 30 minutes. The coverslips were incubated with primary antibodies (Table S5), and then secondary antibodies coupled to Alexa Fluor 488 or Alexa Fluor 594. Confocal imaging was performed with a ZEISS LSM880 Confocal Microscope. All images were processed using Carl Zeiss ZEN software. Manual counting of the number of parasites per vacuole was carried out on at least 100 vacuoles for each biological replicate ($n = 3$).

In vitro anti-*T. gondii* activity

IC_{50} measurement of each compound was carried out using a luminometric assay using strains expressing luciferase (RH-LUC GFP or the PTG-LUC GFP) as previously described (6). Briefly, 10 compound concentrations were tested in triplicate from 2 µM to 1 nM, since we aimed at identifying compounds that showed a better IC_{50} than Pyrimethamine [660 nM as shown before (6)]. Readings were performed after 24 or 40 hours for the type I or II strain, respectively. The parasite proliferation was measured by detecting the emission of light on a SPARK plate reader (TECAN). Graphpad Prism was used to plot a regression curve and calculate the IC_{50} for each compound.

Measurements of cytotoxic activity

HepG2 (liver hepatocellular carcinoma cells) and HFF cells were used in this assay. Alamar Blue assay is an oxidation-reduction indicator that reflects the compounds cytotoxicity. Cells were seeded in 96-well plate and incubated with various concentrations (from 100 nM to 100 µM) of compounds in triplicates for 72 hours in 200 µL total volume. Then, 20 µL of Alamar Blue HS cell viability reagent (Invitrogen) was added to the wells. After 24 hours of incubation, the samples' absorbance was measured using the SPARK plate reader (TECAN) at 570 nm and 600 nm. Wells without cells and with or without Alamar Blue HS reagent were used to account for background. Cells incubated with various concentrations of solvent [DMSO; 0.8% to 0.006% (vol/vol)] were used as controls. Different concentrations (400 µM to 3,125 µM) of chloroquine, which has a known IC_{50} on HFF cells (20), were used as an internal control. The measurements were analyzed as a dose-response curve to determine the IC_{50}. Each experiment was performed in triplicate. The IC_{50} were calculated using the online calculator available at https://www.aatbio.com/tools/ic50-calculator.

Measurement of *in vivo* observable adverse effect level of the compounds

To evaluate if the compound would have deleterious visible effects on mice health and behavior, we treated five 6-week-old Balb/c mice intraperitoneally with either DMSO (a 7% solution in PBS) or the compounds (MC2590, MC2059, or MC2125) at different doses (10, 30, or 60 mg/kg) daily for 5 days. As a measure of their global health status, we measured their weight and followed signs of modification of their grooming and exploration behavior following the scoring table as described in Table S6. A global score was recorded for each mouse daily for 15 days.

In vivo anti *T. gondii* activity in a mouse model

The Institut Pasteur de Lille's ethical committee approved the animal housing and experimentation conditions in accordance with the French Council in Animal Care guidelines for the care and use of animals (protocol #11082–2017072816548341 v2). Six- to eight-week-old Balb/c mice were injected intraperitoneally with a 10^5 parasites dose (in 200 µL) of the 76K strain. The next day, the mice were injected intraperitoneally with different quantity of compounds (200 µL in a 7% DMSO solution) daily for 5 days. Pyrimethamine was administered in tap water using a 100 times dilution of the stock solution (7 mg/mL) diluted in tap water. Differences in survival curves were assessed by a log-rank (Mantel-Cox) test with a minimal number of 15 mice per group.

Primary neurons infections and bradyzoite *in vitro* targeting assay

Neo-natal rat brain cells were collected from hippocampus and seeded as described in reference (22). After 14 days of maturation, the primary brain cells were infected using 5,000 parasites, of the PTG-LUC-GFP strain, per well of a 24-well plate at a MOI of 0.1. Fourteen days later, the infected primary cell culture was incubated with a compound (MC2590, MC2059, or MC2125) concentration of three times the IC_{50} (as determined above against the tachyzoite stage) or an equivalent volume of DMSO. The culture was left for 7 more days, then collected and lysed in 100 µL of lysis buffer, and processed as described before for luciferase detection.

Western blot

Intracellular RH strain parasites were grown for 24 hours and then treated overnight (15 hours) with 0.5 µM of MC2590, MC2125, MC2059, or DMSO. Parasites were then purified by sequential syringe passage with 17-gauge and 26-gauge needles and filtration through a 3 µm polycarbonate membrane filter. This allowed the collection of parasites free from the host-cell material. To prepare the total protein extracts, parasites were resuspended in 1× Laemmli buffer. These samples were then fractionated on a 15% SDS-polyacrylamide electrophoresis gel. The antibodies are listed in Table S5. Chemiluminescent detection of bands was carried out using Super Signal West Femto Maximum Sensitivity Substrate.

Statistics and reproducibility

All data were analyzed with GraphPad Prism software version 8. Differences in the means were assessed by Student's *t*-test. Differences in survival curves were assessed by a log-rank (Mantel-Cox) test. In all cases, *P*-values were two-sided, and $P < 0.05$ was considered as significant. All experiments were repeated at least three times using biologically independent replicates.

RESULTS

Compounds potentially active against HDAC inhibit tachyzoite growth

HDACis have been shown to have a potent activity against *T. gondii*. In particular, hydroxamate-based HDACis were shown to have an activity *in vitro* and *in vivo* against the tachyzoite stage of this parasite (6). Therefore, we tested an internal library of more than 50 hydroxamate-based compounds that potentially targeted HDACs for their anti-*T. gondii* activity (Fig. 1A). These compounds have different structures but all have a backbone bearing an hydroxamic acid (Table S1 to S4). To do this, we first investigated their ability to inhibit the parasite proliferation in its tachyzoite form *in vitro*. We used a luciferase expressing type II parasite strain to take advantage of the large linear range of detection of luminescence. We compared the compounds calculated IC_{50}s to the clinically relevant pyrimethamine, another hydroxamate-based HDACi (suberoylanilide hydroxamic acid; SAHA) and the previously identified HDACi MC1742 (6). Among these compounds, 14 had an IC_{50} higher than 1,500 nM and therefore their IC_{50}s could not be exactly measured using the dilution range in this assay (Fig. 1A). Three other compounds showed an IC_{50} higher than pyrimethamine (660 nM). We found 35 potential HDACis exhibiting a lower IC_{50}*in vitro* than pyrimethamine indicating the strong inhibitory potential of HDACi against the tachyzoite stage of *T. gondii* (Fig. 1A; Table S1 to S4). Three compounds (MC2125, MC2590, and MC2059) with a potential anti-HDAC activity were shown to have a similar or lower IC_{50} than MC1742 and lower IC_{50} than SAHA, a hydroxamate-based HDACis that have been shown to inhibit parasite growth (Table 1). MC2125 is structurally similar to MC1742, but present a six-time lower IC_{50}. MC2590 is a hydroxamate-based compound that was shown to potentially target human HDAC1-3, -6,-8, and -10 (class I/IIb-selective inhibitor) with IC_{50}s of 15–156 nM (23). MC2590 also inhibits human HDAC isoforms HDAC4, HDAC5, HDAC7, HDAC9, and HDAC11 with IC_{50}s

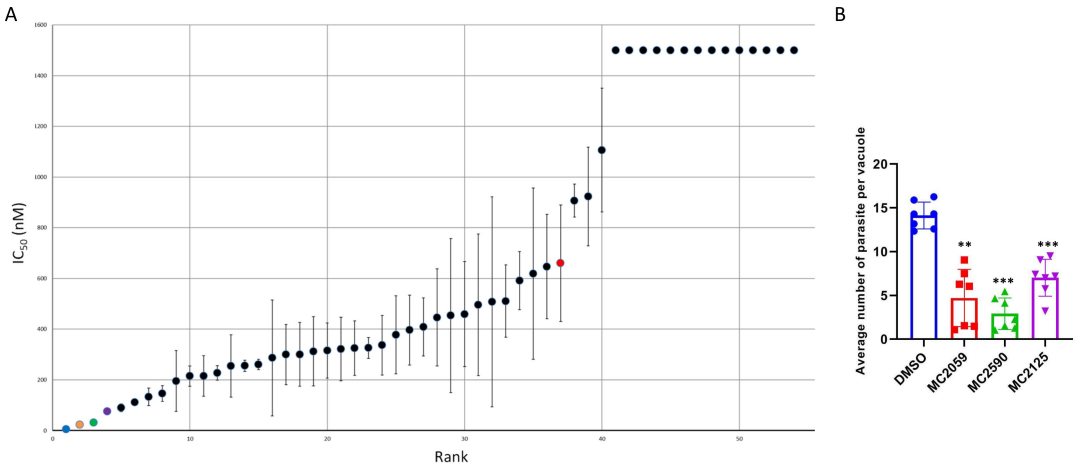


FIG 1 Compounds potentially active against HDACs inhibit tachyzoite growth. (A) Calculated IC_{50} for each tested compound. Intracellular tachyzoite of the type II strain PTG-LUC GFP was treated for 40 hours at different concentrations (from 2 µM to 2 nM). The mean IC_{50} for three biological replicates is presented on the graph together with SD. Compounds were ranked based on their calculated IC_{50}. The IC_{50} values for pyrimethamine (red), MC1742 (green), MC2059 (purple), MC2590 (orange), and MC2125 (blue) are highlighted in the graph. Mean ± SD (n = 3 independent experiments). (B) Effect of the compounds on RH tachyzoites on intracellular growth. Tachyzoite intracellular growth was measured after 36 hours of exposure to different compounds at two times the IC_{50} concentration. The average number of parasites per vacuole is used as a metric of parasite intracellular growth and measured by IFAs. Parasites treated with DMSO (blue bars) are used as a control. We measured the effect of MC2059 (red bars), MC2590 (green bars), and MC2125 (purple bars) on intracellular growth. A Student's t-test was performed with the DMSO as control; two-tailed P-value; $^{**}P < 0.01$; $^{***}P < 0.001$; mean ± SD (n = 6 independent experiments).

of 1350–3890 nM (23). MC2059 is also a hydroxamate-based compound and was shown to have an HDACi activity *in vitro* (24).

We confirmed the compounds anti-tachyzoite activity *in vitro* using IFA (Fig. 1B). The mean parasite number per vacuole was assessed in presence of the compounds. When treated by solvent control (DMSO), the parasites grew to a mean of 14 parasites per vacuole. The MC2125, MC2590, and MC2059 treatments resulted in a strong decrease of the number of parasites per vacuole confirming that the compound can reach the parasite (and pass through the parasite vacuole) (Fig. 1B). We also found that the parasites showed different phenotypes depending on which compound was used (Fig. S1), but growth arrest due to defects in the cell cycle was apparent in all cases.

We measured the IC_{50} of these compounds on type I or type II parasites and found similar results for both types, indicating the activity of the compounds was almost identical on different strains (Table 2).

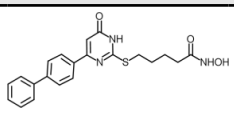
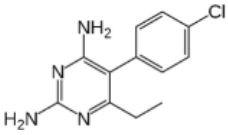
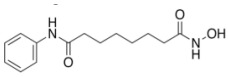
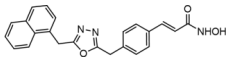
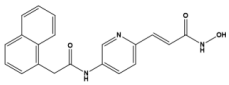
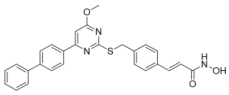
Selected compounds present a good selectivity index

To ensure that the selected compounds did not show cytotoxicity at the tested concentration, we measured the IC_{50} of these compounds against two cell types using the Alamar Blue assay. The cytotoxicity of the compounds was measured on HFF and on HepG2 cells, a hepatocellular carcinoma cell line. We were therefore able to calculate the selectivity index (SI) of these compounds and compare it to that of MC1742 (Table 2). Overall, MC2125, MC2590, and MC2059 showed better SI than MC1742 with SI values above 200. However, HepG2 cells were more sensitive to these compounds and the calculated SI values were dropped to 40, 111, and 240 for MC2125, MC2059, and MC2590, respectively. Although MC2125 was the most efficient compound on tachyzoite growth, it was also more cytotoxic than the other two compounds (Table 2).

MC2125 and MC2590 are potent inhibitors of the parasite HDACs

MC1742 is an inhibitor of human class I/IIb HDACs (25) and *T. gondii* HDACs (6). To indirectly evaluate the HDACi activity of MC2125, MC2059, MC2590, and as a negative control, DMSO, we examined the comparative acetylation levels of various parasite

TABLE 1 IC_{50} of selected hydroxamate-based compounds and pyrimethamine

Compounds	Structure	IC50
MC1742		30 ± 8 nM
Pyrimethamine		660 ± 230 nM
SAHA		247 ± 137 nM
MC2059		75 ± 9 nM
MC2590		23 ± 6 nM
MC2125		5 ± 1 nM

histones following treatment. For that, we checked the relative acetylation level of Histone H4 (acH4 and H4K8ac) and Histone H3 (H3K9ac and H3K18ac). The level of methylation of Histone H3 at the lysine 4 position (H3K4me3) was also measured by Western blot. MC2125 and MC2590 treatments induced a significant increase of acetylation of both Histone H3 and H4, while the level of H3K4me3 was unchanged (Fig. 2). In contrast, MC2059 had no effect on the acetylation levels and therefore may not target the parasites HDACs (Fig. 2; Fig. S2). We also verified the level of acetylation of tubulin at the lysine 40 position by Western blot and showed that the compound did not significantly influence the acetylation of this non-histone parasite protein.

MC2590 is effective at preventing acute disease

In order to assess the maximum dose that we could inject to mice without visual deleterious effects on mice behaviour and health, we injected different concentrations of compounds to mice and followed their body weight and behaviour during 15 days (Fig. S3). This experiment showed that injecting 60 mg/kg of MC2059, 30 mg/kg of MC2590, or 10 mg/kg of MC2125 had no observable effect on mice weight (Fig. S3A) and behaviour (Fig. S3B). However, increasing the concentration of MC2125 to 30 mg/kg was deleterious to mice (Fig. S3).

TABLE 2 IC_{50} of the selected compounds in *T.gondii* Type I and Type II strains and toxicity of the selected compounds in HFF and HepG2 cells[c]

	MC1742	MC2059	MC2590	MC2125
Type I IC_{50} (nM)	39 ± 7	89 ± 6	30 ± 6	10 ± 3
Type II IC_{50} (nM)	30 ± 8	75 ± 9	23 ± 6	5 ± 1
HFF IC_{50} (nM)	3,000[a]	15,800 ± 1536	34,553 ± 9,386	16,313 ± 2,529
HepG2 IC_{50} (nM)	X[b]	8,364 ± 125	5,531 ± 1,879	198 ± 55
Selectivity index (IC_{50} HHF/ IC_{50} Type II)	100	211	1,502	3,263
Selectivity index (IC_{50} HepG2/IC_{50} Type II)	X[b]	111	240	40

[a]MC1742 IC_{50} in human fibroblasts (HFF cells) was determined in (20).
[b]The X indicates values that were not available.
[c]The IC_{50} was determined in at least three independent experiments. Selectivity index was calculated based on the IC_{50} of the Type II strain.

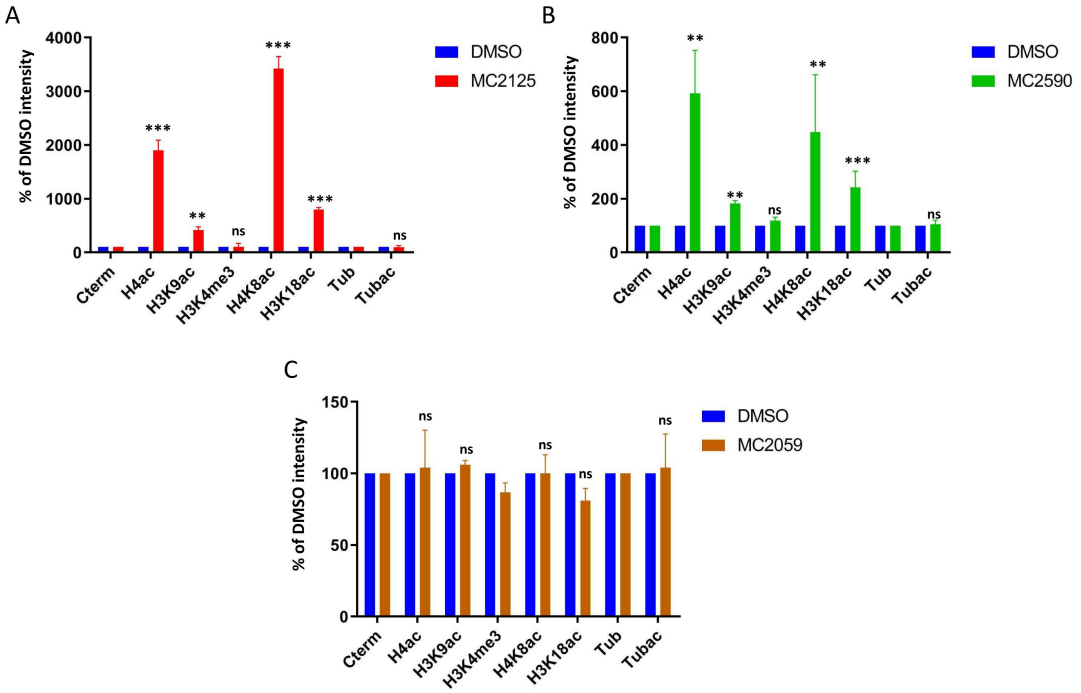
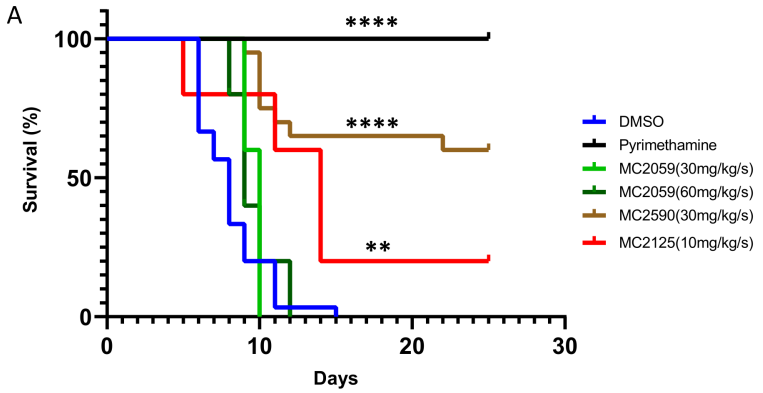


FIG 2 Acetylation levels of *T. gondii* histones after treatment with MC2125, MC2590, and MC2059. The purified RH parasite samples were treated either with DMSO or MC2125, MC2590, and MC2059 (0.5 µM). (A) Quantification of the signal for each indicated modified histone after treatment with DMSO (blue bars) or MC2125 (red bars). The signal was normalized to the signal given by the loading control (Histone H3 Cterm, modified and unmodified). A Student's *t*-test was performed; two-tailed *P*-value; ns: $P > 0.05$; **$P < 0.01$; ***$P < 0.001$; mean ± SD ($n = 4$ independent experiments). (B) Quantification of the signal for each indicated modified histone after treatment with DMSO (blue bars) or MC2590 (green bars). The signal was normalized to the signal given by the loading control (Histone H3 Cterm, modified and unmodified). A Student's *t*-test was performed; two-tailed *P*-value; ns: $P > 0.05$; **: $P < 0.01$; ***: $P < 0.001$; mean ± SD ($n = 4$ independent experiments). (C) Quantification of the signal for each indicated modified histone after treatment with DMSO (blue bars) or MC2059 (brown bars). The signal was normalized to the signal given by the loading control (Histone H3 Cterm, modified and unmodified). A Student's *t*-test was performed; two-tailed *P*-value; ns: $P > 0.05$; mean ± SD ($n = 3$ independent experiments).

To assess the ability of MC2125, MC2059, and MC2590 to prevent the outcome of the acute disease in a mouse model, we infected Balb/c mice with a lethal dose (10^5 parasites) of *T. gondii* type II strain. We then recorded the survival of mice for 15 days (Fig. 3A). In the control group ($n = 15$), the survival rate was 0%. Mice treated with MC2059 at either 30 mg/kg or 60 mg/kg did not survive the acute infection. In contrast, the mice treated with MC2125 did show a slight improvement in their ability to limit the consequences of the acute infection with 20% of the mice surviving in the 10 mg/kg group (15 mice). Finally, MC2590 (30 mg/kg) was the most efficient compound and increased the survival to 60% of the mice ($n = 15$), indicating that MC2590 was efficient at preventing the outcome of the acute disease in this mouse model (Fig. 3A). However, none of these compounds was as efficient as pyrimethamine.

The tested compounds fail to reduce cyst burden *in vitro*

To test the effect of these molecules on the established chronic form of the parasite, we took advantage of the recently established primary brain cell model that mimics *in vitro* the production of mature bradyzoite cysts, the latent form of the parasite (22). This new *in vitro* model is an efficient alternative to the usage of mice to assess the compound ability to target established cysts. As observed previously for MC1742 (6), the HDACis tested here were not effective at eliminating the established cysts. Indeed, treatment with these compounds only marginally impacted the presence of bradyzoite cysts in the culture when compared to the DMSO treated culture and as measure by luminescence (Fig. 3B).



B

	MC2059	MC2590	MC2125
Inhibition (%)	**19 ± 13**	**14 ± 9**	**9 ± 3**

FIG 3 Effect of the compounds on the outcome of the acute and latent toxoplasmosis. (A) Effect of MC2059, MC2125, and MC2590 treatments on the outcome of the acute disease. One day after peritoneal injection of tachyzoites, mice were treated with different concentration of compounds once a day for 5 days. The percentage of survival is represented for each day during 15 days. Mice treated with DMSO (same regimen, blue line) are used as a negative control. Mice treated with Pyrimethamine (black line) are used as a positive control. The percentage of surviving mice after treatment with MC2059 (30 mg/kg, light green or 60 mg/kg, dark green), MC2590 (30 mg/kg, brown) and MC2125 (10 mg/kg, red). A log-rank (Mantel-Cox) test was performed; $^{**}P < 0.01$; $^{****}P < 0.0001$; mean ± SD (n = 15 mice per group). (B) Treatment of the bradyzoite (latent) form of the parasite with the different compounds. Latent bradyzoite cysts were obtained after 2 weeks of culture in primary brain cells and then treated with three times the IC_{50} for 7 days. The inhibition percentage was calculated when comparing the compound treated group to the control (DMSO treated cultures). Mean ± SD (n = 3 independent experiments).

DISCUSSION

We explored the anti-*T. gondii* activity of compounds targeting HDACs. *T. gondii* encodes five potential HDACs (TgHDAC1 to 5) which are expressed at the tachyzoite and bradyzoite stage (22) and at least four of them show negative fitness scores in CrispR/Cas9 screen targeting tachyzoites (26), indicating their importance at these stages of the parasite life cycle. These enzymes are implicated in the process of regulating the establishment and maintenance of gene expression profiles during the life cycle of the parasite, a mechanism that is likely crucial for adaptation to changing environments (8). The fact that these compounds might be crucial for the parasite's life cycle and the potential for repurposing them as drugs makes them appealing targets for further investigation. It has been shown that other HDACis have a potent activity against *T. gondii* and other apicomplexan parasites (20). Here, we tested more than 50 compounds potentially targeting parasite HDACs (Table S1 to S4). Among these, 35 compounds exhibited a lower IC_{50}*in vitro* than pyrimethamine, the clinically relevant drug to treat toxoplasmosis. All of these compounds presented a hydroxamate group (Table S1 to S4) that is known to inhibit metalloproteases or zinc hydrolases like HDACs (27). The high potency of these hydroxamate-based compounds against *T. gondii* tachyzoite growth suggests that this chemical group is a strong inhibitor of these enzymes in this parasite. Interestingly, the other hydroxamate-based HDACi vorinostat (SAHA) is already approved by the FDA for the treatment of cancers (28) and has been shown to be active against *Cryptosporidium parvum* (29) *in vivo* and *T. gondii* (6, 12) *in vitro*, suggesting that it could be repurposed to treat parasitic diseases caused by apicomplexan parasites. Surprisingly, hydroxamate-based compounds were shown to have an IC_{50} in the low nanomolar range against *P. falciparum* but were unable to inhibit the growth of the *P. berghei in vivo* (20). This may be due to the poor pharmacokinetics properties of many hydroxamates (30). Establishing the pharmacokinetic paramaters for these compounds could guide the design of further *in vivo* experiments. In contrast, MC1742 (6) and to a lesser extent MC2590 (this study) was able to limit the consequences of acute toxoplasmosis *in vivo*. Further research is warranted to improve these compounds activity and bioavailability.

Although, MC2590 and MC1742 were efficient at preventing the outcome of the acute disease, neither of these potential HDACi was able to eliminate the latent form of the parasite once established as it was shown *in vivo* for MC1742 (6) and *in vitro* for

MC2590 (this study). It was speculated previously that MC1742 may have low availability and/or concentration of the molecule in the brain where bradyzoite cysts reside since this experiment was performed *in vivo* (6). However, using the newly *in vitro* model to produce bradyzoite cysts (22), we were able to show that the tested inhibitors had a poor activity against these encysted latent forms (Fig. 3B). This suggests that using hydroxamate-based HDACi may not be of interest to target these forms. The bradyzoites are surrounded by a thick cyst wall that lies underneath the membrane of the parasitophorous vacuole and it is unclear how permeable this structure is. Indeed, our results suggest that hydroxamate compounds are not able to reach the bradyzoites. Alternatively, HDAC activity may not be essential at this latent stage of the life cycle. Only a handful of compounds were shown to reduce cyst burden (31, 32). Consequently, there is a need to develop novel *in vitro* systems enabling the testing compounds against bradyzoites. The exploration of organoids or *in vitro* cultures of primary brain or muscle cells holds the potential to unlock this new research pathway (22, 33).

Surprisingly, MC2059, a compound that has been shown to target human HDACs (24) was not able to induce an hyperacetylation of *T. gondii* histones or tubulin (Fig. 2C). This indicates that this compound may have another target in the parasite. Hydroxamate-based compounds can inhibit metalloproteases or zinc hydrolases (27) and MC2059 may target these enzymatic activities. Alternatively, MC2059 may have an indirect effect on parasite growth through the inhibition of the human host HDAC activities. This may be also the case for MC2125 and MC2590, which have been proven to target the parasite HDACs, as the parasite is treated as well as the host. In the future, it would be of interest to identify the consequences of the compound treatment on the host and measure the gene expression perturbations induced by these HDACi at low concentration where no cytotoxic effect is visible on host cells. We found that MC2125 had a cytotoxic effect on HepG2 cells at a 100 nM concentration, indicating that low concentration of these HDACis may have a direct consequence on host cells, depending on their cellular type. Moreover, increasing the MC2125 dose to 30 mg/kg showed deleterious visible effects on mice, indicating that the dose could not be increased.

Among the compounds tested in the study, MC2590 proved to be the most potent. It displayed significant efficacy in inhibiting growth *in vitro*, exhibiting an IC_{50} lower than pyrimethamine, other hydroxamate-based HDACi, and similar to that of MC1742. It also presents an attractive selectivity index and may target parasitic HDACs. Therefore, MC2590 is another example of a hydroxamate-based HDACi with a potent activity against this parasite and also highlights the importance of these enzymatic activities for the survival of *T. gondii* tachyzoites *in vitro*. MC2590 was also effective at preventing the outcome of the acute disease, although to a lesser extent than MC1742. These HDACis are therefore promising compounds to treat acute toxoplasmosis. However, they need to be chemically improved to match the *in vivo* activity of pyrimethamine.

In summary, we have discovered an HDACi that demonstrates strong anti-*T. gondii* activity both *in vitro* and *in vivo*, as evidenced by its efficacy in a mouse model of acute disease. MC2590 is a promising compound for future treatment of the acute phase of toxoplasmosis.

ACKNOWLEDGMENTS

The authors wish to thank the BioImaging Center Lille for access to instruments.

This work was supported by Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (INSERM), and a Region Haut de France together with iSITE grant (START-AIRR) to MG.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental file 1 (AAC00661-23-s0001.pdf). Fig. S1 to S3.
Supplemental file 2 (AAC00661-23-s0002.docx). Supplemental figure legends.
Supplemental file 3 (AAC00661-23-s0003.docx). Tables S1 to S4.
Supplemental file 4 (AAC00661-23-s0004.docx). Table S5.
Supplemental file 5 (AAC00661-23-s0005.docx). Table S6.

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