



**HAL**  
open science

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

Tom Boissavy, Dante Rotili, Thomas Mouveaux, Emmanuel Roger, El  
Moukthar Aliouat, Christine Pierrot, Sergio Valente, Antonello Mai, Mathieu  
Gissot

► **To cite this version:**

Tom Boissavy, Dante Rotili, Thomas Mouveaux, Emmanuel Roger, El Moukthar Aliouat, et al..  
Hydroxamate-based compounds are potent inhibitors of *Toxoplasma gondii* HDAC biological activity.  
Antimicrobial Agents and Chemotherapy, In press, 10.1128/aac.00661-23 . hal-04252830

**HAL Id: hal-04252830**

**<https://hal.science/hal-04252830>**

Submitted on 21 Oct 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

3

4

5     Tom Boissavy <sup>1</sup>, Dante Rotili <sup>2</sup>, Thomas Mouveaux <sup>1</sup>, Emmanuel Roger <sup>1</sup>, El Moukhtar Aliouat <sup>1</sup>, Christine  
6     Pierrot <sup>1</sup>, Sergio Valente <sup>2</sup>, Antonello Mai <sup>2</sup> and Mathieu Gissot <sup>1,\*</sup>.

7

8             1. Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 9017 - CIIL - Center  
9                                    for Infection and Immunity of Lille, F-59000 Lille, France.

10            2. Dipartimento di Chimica e Tecnologie del Farmaco "Sapienza" Università di Roma, 00185  
11                                    Rome, Italy.

12

13     \* Corresponding author: [mathieu.gissot@pasteur-lille.fr](mailto:mathieu.gissot@pasteur-lille.fr)

14 **Abstract**

15 Toxoplasmosis is a critical health issue for immune-deficient individuals and the offspring of newly  
16 infected mothers. It is caused by a unicellular intracellular parasite called *Toxoplasma gondii* that is  
17 found worldwide. Although efficient drugs are commonly used to treat toxoplasmosis, serious adverse  
18 events are common. Therefore, new compounds with potent anti-*T. gondii* activity are needed to  
19 provide better suited treatments. We have tested compounds designed to target specifically histone  
20 deacetylase enzymes. Among the 55 compounds tested, we identified three compounds showing a  
21 concentration of drug required for 50% inhibition (IC<sub>50</sub>) in the low 100 nM range with a selectivity index  
22 of more than 100. These compounds are active at inhibiting the growth of the parasite *in vitro*, but  
23 also at preventing some of the consequences of the acute disease *in vivo*. Two of these hydroxamate  
24 based compound also induce a hyper-acetylation of the parasite histones while the parasitic acetylated  
25 tubulin level remains unchanged. These findings suggest that the enzymes regulating histone  
26 acetylation are potent therapeutic targets for the treatment of acute toxoplasmosis.

## 27 **Introduction**

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

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

48 To address the need for a better treatment, compounds targeting epigenetic mechanisms have been  
49 tested against *T. gondii* (6). Enzymes mediating epigenetic modifications of histones have been shown  
50 to be promising therapeutic targets (7). Indeed, the control of gene expression is essential to the  
51 survival of parasites and more acutely during growth phases (8, 9). Histone acetylation has been  
52 extensively investigated as a targeted epigenetic mechanism for identifying compounds that inhibit *T.*

53 *gondii* (10) and other parasites (11). Enzymes involved in the regulation of histone acetylation have  
54 been identified as promising therapeutic targets since several modulators of these enzymes were  
55 shown to bear an activity against *T. gondii* (6, 12–14). In particular, several inhibitors of histone  
56 deacetylases (HDAC) have been shown to suppress the tachyzoite growth *in vitro* (6, 12, 15–18)  
57 indicating that these enzymes are key for the survival of this fast-growing form of the parasite.  
58 Moreover, an hydroxamate-based HDAC inhibitor (HDACi) belonging to the uracil-based hydroxyamide  
59 (UBHA) class (19), MC1742, was also active *in vivo* to prevent the consequences of the acute disease  
60 in a mouse model of toxoplasmosis (6) and is active against other apicomplexan parasites as  
61 *Plasmodium falciparum*, the causative agent of malaria (20). MC1742 treatment of *T. gondii*  
62 tachyzoites resulted in elevated levels of parasite histone acetylation, significantly influencing gene  
63 expression (6). These findings strongly indicate that histone deacetylation plays a vital role in sustaining  
64 the specific gene expression program during the tachyzoite phase (6).

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

75 **Material and methods**

76 **Parasite strains and culture**

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

83 **Compounds.**

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

90 **Immunofluorescence analysis (IFAs)**

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

97 ***In vitro* anti-*T. gondii* activity**

98 IC<sub>50</sub> measurement of each compound was carried out using a luminometric assay using strains  
99 expressing luciferase (RH-LUC GFP or the PTG-LUC GFP) as previously described (6). Briefly, 10

100 compound concentrations were tested in triplicate from 2  $\mu$ M to 1 nM, since we aimed at identifying  
101 compounds that showed a better IC<sub>50</sub> than Pyrimethamine (660 nM as shown before (6)). Readings  
102 were performed after 24 or 40 hours for the type I or II strain, respectively. The parasite proliferation  
103 was measured by detecting the emission of light on a SPARK plate reader (TECAN). Graphpad Prism  
104 was used to plot a regression curve and calculate the IC<sub>50</sub> for each compound.

#### 105 **Measurements of cytotoxic activity**

106 HepG2 (liver hepatocellular carcinoma cells) and HFF (Human Foreskin Fibroblast) cells were used in  
107 this assay. Alamar Blue® assay is an oxidation-reduction indicator that reflects the compounds  
108 cytotoxicity. Cells were seeded in 96 wells plates and incubated with various concentrations (from 100  
109 nM to 100  $\mu$ M) of compounds in triplicates for 72h in 200  $\mu$ L total volume. Then, 20 $\mu$ L of Alamar Blue™  
110 HS cell viability reagent (Invitrogen) was added to the wells. After 24h of incubation, the samples'  
111 absorbance was measured using the SPARK plate reader (TECAN) at 570 nm and 600 nm. Wells without  
112 cells and with or without Alamar Blue™HS reagent were used to account for background. Cells  
113 incubated with various concentrations of solvent (DMSO; 0,8 % to 0,006 % (vol/vol).) were used as  
114 controls. Different concentrations (400  $\mu$ M to 3,125  $\mu$ M) of chloroquine, that has a known IC<sub>50</sub> on  
115 HFF cells (20), were used as an internal control. The measurements were analyzed as a dose-response  
116 curve to determine the IC<sub>50</sub>. Each experiment was performed in triplicate. The IC<sub>50</sub> were calculated  
117 using the online calculator available at <https://www.aatbio.com/tools/ic50-calculator>.

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

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

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

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

#### 134 **Primary neurons infections and bradyzoite in vitro targeting assay**

135 Neo-natal rat brain cells were collected from hippocampus and seeded as described in (22). After 14  
136 days of maturation, the primary brain cells were infected using 5000 parasites, of the PTG-LUC-GFP  
137 strain, per well of a 24 well plate at a MOI of 0.1. Fourteen days later, the infected primary cell culture  
138 was incubated with a compound ((MC2590, MC2059 or MC2125) concentration of to 3 times the  $IC_{50}$   
139 (as determined above against the tachyzoite stage) or an equivalent volume of DMSO. The culture was  
140 left for 7 more days and was then collected and lysed in 100  $\mu$ L of lysis buffer and processed as  
141 described before for luciferase detection.

#### 142 **Western-Blot**

143 Intracellular RH strain parasites were grown for 24 hours and then treated over-night (15 hours) with  
144 0.5  $\mu$ M of MC2590, MC2125, MC2059 or DMSO. Parasites were then purified by sequential syringe  
145 passage with 17-gauge and 26-gauge needles and filtration through a 3- $\mu$ m polycarbonate membrane  
146 filter. This allowed the collection of parasites free from the host-cell material. To prepare the total  
147 protein extracts, parasites were resuspended in 1X Laemmli buffer. These samples were then  
148 fractionated on a 15% SDS-polyacrylamide electrophoresis gel. The antibodies are listed in Table S5.  
149 Chemiluminescent detection of bands was carried out using Super Signal West Femto Maximum  
150 Sensitivity Substrate.



151 **Statistics and Reproducibility**

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

156 **Results**

157 ***Compounds potentially active against HDAC inhibit tachyzoite growth.***

158 HDACi have been shown to have a potent activity against *T. gondii*. In particular, hydroxamate-based  
159 HDACi were shown to have an activity *in vitro* and *in vivo* against the tachyzoite stage of this parasite  
160 (6). Therefore, we tested internal library of more than 50 hydroxamate-based compounds that  
161 potentially targeted HDACs for their anti-*T. gondii* activity (Figure 1A). These compounds have different  
162 structures but all have a backbone bearing an hydroxamic acid (Supplementary Table 1,2, 3 and 4). To  
163 do this, we first investigated their ability to inhibit the parasite proliferation in its tachyzoite form *in*  
164 *vitro*. We used a luciferase expressing type II parasite strain to take advantage of the large linear range  
165 of detection of luminescence. We compared the compounds calculated IC<sub>50</sub>s to the clinically relevant  
166 pyrimethamine, another hydroxamate based HDACi (suberoylanilide hydroxamic acid; SAHA) and the  
167 previously identified HDACi MC1742 (6). Among these compounds, 14 had an IC<sub>50</sub> higher than 1500  
168 nM and therefore could not be exactly measured using the dilution range used in this assay (Figure  
169 1A). Three other compounds showed an IC<sub>50</sub> higher than pyrimethamine (660 nM). We found 35  
170 potential HDACi exhibiting a lower IC<sub>50</sub> *in vitro* than pyrimethamine indicating the strong inhibitory  
171 potential of HDACi against the tachyzoite stage of *T. gondii* (Figure 1A and Supplementary Table 1).  
172 Three compounds (MC2125, MC2590 and MC2059) with a potential anti-HDAC activity were shown to  
173 have similar or lower IC<sub>50</sub> than MC1742 and lower IC<sub>50</sub> than SAHA, an hydroxamate-based HDACi that  
174 have been shown to inhibit parasite growth (Table 1). MC2125 is structurally similar of MC1742, but  
175 present a 6-time lower IC<sub>50</sub>. MC2590 is an hydroxamate-based compound that has been shown to  
176 potentially target human HDAC1-3, -6, -8, and -10 (class I/IIb-selective inhibitor) with IC<sub>50</sub>s of 15-156  
177 nM (23). MC2590 also inhibits human HDAC isoforms HDAC4, HDAC5, HDAC7, HDAC9, HDAC11 with  
178 IC<sub>50</sub>s of 1350-3890 nM (23). MC2059 is also an hydroxamate-based compound and has been shown to  
179 have an HDACi activity *in vitro* (24).

180 We confirmed the compounds anti-tachyzoite activity *in vitro* using IFA (Figure 1B). The mean parasite  
181 number per vacuole was assessed in presence of the compounds. When treated by solvent control

182 (DMSO), the parasites grew to a mean of 14 parasites per vacuole. The MC2125, MC2590 and MC2059  
183 treatment resulted in a strong decrease of the number of parasites per vacuole confirming that the  
184 compound can reach the parasite (and pass through the parasite vacuole) (Figure 1B). We also found  
185 that the parasites showed different phenotypes depending on which compound was used (Figure S1),  
186 but growth arrest due to defects in the cell cycle were apparent in all cases.

187 We measured the IC<sub>50</sub> of these compounds on type I or type II parasites and found similar results for  
188 both types, indicating the activity of the compounds was almost identical on different strains (Table  
189 2).

#### 190 ***Selected compounds present a good selectivity index.***

191 To ensure that the selected compounds did not show cytotoxicity at the tested concentration, we  
192 measured the IC<sub>50</sub> of these compounds against two cell types using the Alamar blue assay. The  
193 cytotoxicity of the compounds was measured on human foreskin fibroblasts (HFF) and on HepG2 cells,  
194 a hepatocellular carcinoma cell line. We were therefore able to calculate the selectivity index (SI) of  
195 these compounds and compare it to that of MC1742 (Table 2). Overall, MC2125, MC2590 and MC2059  
196 showed better SI than MC1742 with SI values above 200. However, HepG2 cells were more sensitive  
197 to these compounds and the calculated SI values were dropped to 40, 111 and 240 for MC2125,  
198 MC2059 and MC2590, respectively. Although MC2125 was the most efficient compound on tachyzoite  
199 growth, it was also more cytotoxic than the other two compounds (Table 2).

#### 200 ***MC2125 and MC2590 are a potent inhibitors of the parasite HDACs.***

201 MC1742 is an inhibitor of human class I/IIb HDACs (25) and *T. gondii* HDACs (6). To indirectly evaluate  
202 the HDACi activity of MC2125, MC2059, MC2590, and as a negative control, DMSO, we examined the  
203 comparative acetylation levels of various parasite histones following treatment. For that, we checked  
204 the relative acetylation level of Histone H4 (acH4 and H4K8ac) and Histone H3 (H3K9ac and H3K18ac).  
205 The level of methylation of Histone H3 at the lysine 4 position (H3K4me3) was also measured by  
206 Western-blot. MC2125 and MC2590 treatment induced a significant increase of acetylation of both

207 histone H3 and H4, while the level of H3K4me3 was unchanged (Figure 2). In contrast, MC2059 had no  
208 effect on the acetylation levels and therefore may not target the parasites HDACs (Figure 2 and S2).  
209 We also verified the level of acetylation of tubulin at the lysine 40 position by Western-blot and showed  
210 that the compound did not significantly influence the acetylation of this non-histone parasite protein.

### 211 ***MC2590 is effective at preventing acute disease***

212 In order to assess the maximum dose that we could inject to mice without visual deleterious effects  
213 on mice behavior and health, we injected different concentration of compounds to mice and followed  
214 their body weight and behavior during 15 days (Figure S3). This experiment showed that injecting 60  
215 mg/kg of MC2059, 30mg/kg of MC2590 or 10mg/kg of MC2125 had no observable effect on mice  
216 weight (Figure S3A) and behavior (Figure S3B). However, increasing the concentration of MC2125 to  
217 30mg/kg was deleterious to mice (Figure S3).

218 To assess the ability of MC2125, MC2059 and MC2590 to prevent the outcome of the acute disease in  
219 a mouse model, we infected Balb/c mice with a lethal dose ( $10^5$  parasites) of *T. gondii* type II strain.  
220 We then recorded the survival of mice for 15 days (Figure 3A). In the control group (n=15), the survival  
221 rate was 0 %. Mice treated with MC2059 at either 30 mg/kg or 60 mg/kg did not survive the acute  
222 infection. In contrast, the mice treated with MC2125 did show a slight improvement in their ability to  
223 limit the consequences of the acute infection with 20 % of the mice surviving in the 10 mg/kg group  
224 (15 mice). Finally, MC2590 (30mg/kg) was the most efficient compound and increased the survival to  
225 60% of the mice (n=15) indicating that MC2590 was efficient at preventing the outcome of the acute  
226 disease in this mouse model (Figure 3A). However, none of these compounds was as efficient as  
227 pyrimethamine.

### 228 ***The tested compounds fail to reduce cyst burden in vitro.***

229 To test the effect of these molecules on the established chronic form of the parasite, we took  
230 advantage of the recently established primary brain cell model, that mimics *in vitro* the production of  
231 mature bradyzoite cysts, the latent form of the parasite (22). This new *in vitro* model is an efficient

232 alternative to the usage of mice to assess the compound ability to target established cysts. As observed  
233 previously for MC1742 (6), the HDACi tested here were not effective at eliminating the established  
234 cysts. Indeed, treatment with these compounds only marginally impacted the presence of bradyzoite  
235 cysts in the culture when compared to the DMSO treated culture and as measure by luminescence  
236 (Figure 3B).

## 237 Discussion

238 We explored the anti-*T. gondii* activity of compounds targeting HDACs. *T. gondii* encodes 5 potential  
239 HDACs (TgHDAC1 to 5) which are expressed at the tachyzoite and bradyzoite stage (22) and at least 4  
240 of them show negative fitness scores in CrispR/Cas9 screen targeting tachyzoites (26), indicating their  
241 importance at these stages of the parasite life cycle. These enzymes are implicated in the process of  
242 regulating the establishment and maintenance of gene expression profiles during the life cycle of the  
243 parasite, a mechanism that is likely crucial for adaptation to changing environments (8). The fact that  
244 these compounds might be crucial for the parasite's life cycle and the potential for repurposing them  
245 as drugs makes them appealing targets for further investigation. It has been shown that other HDACi  
246 have a potent activity against *T. gondii* and other apicomplexan parasites (20). Here, we tested more  
247 than 50 compounds potentially targeting parasite HDACs (Supplementary Table 1). Among these, 35  
248 compounds exhibited a lower IC<sub>50</sub> *in vitro* than pyrimethamine, the clinically relevant drug to treat  
249 toxoplasmosis. All of these compounds presented an hydroxamate group (Supplementary Table 1) that  
250 is known to inhibit metalloproteases or zinc hydrolases like HDACs (27). The high potency of these  
251 hydroxamate-based compounds against *T. gondii* tachyzoite growth suggests that this chemical group  
252 is a strong inhibitor of these enzymes in this parasite. Interestingly, the other -hydroxamate-based  
253 HDACi vorinostat (SAHA) is already approved by the FDA for the treatment of cancers (28) and has  
254 been shown to be active against *Cryptosporidium parvum* (29) *in vivo* and *T. gondii* (6, 12) *in vitro*,  
255 suggesting that it could be repurposed to treat parasitic diseases caused by apicomplexan parasites.  
256 Surprisingly, hydroxamate-based compounds were shown to have an IC<sub>50</sub> in the low nanomolar range  
257 against *P. falciparum*, but were unable to inhibit the growth of the *P. berghei* *in vivo* (20). This may be  
258 due to the poor pharmacokinetics properties of many hydroxamates (30). Establishing the  
259 pharmacokinetic parameters for these compounds could guide the design of further *in vivo*  
260 experiments. In contrast, MC1742 (6) and to a lesser extent MC2590 (this study) was able to limit the  
261 consequences of acute toxoplasmosis *in vivo*. Further research is warranted to improve these  
262 compounds activity and bioavailability.

263 Although, MC2590 and MC1742 were efficient at preventing the outcome of the acute disease, neither  
264 of these potential HDACi were able to eliminate the latent form of the parasite once established as it  
265 was shown *in vivo* for MC1742 (6) and *in vitro* for MC2590 (this study). It was speculated previously  
266 that MC1742 may have low availability and/or concentration of the molecule in the brain where  
267 bradyzoite cysts reside since this experiment was performed *in vivo* (6). However, using the newly *in*  
268 *vitro* model to produce bradyzoite cysts (22), we were able to show that the tested inhibitors had a  
269 poor activity against these encysted latent forms (Figure 3B). This suggests that using hydroxamate-  
270 based HDACi may not be of interest to target these forms. The bradyzoites are surrounded by a thick  
271 cyst wall that lies underneath the membrane of the parasitophorous vacuole and it is unclear how  
272 permeable this structure is. Indeed, our results suggest that hydroxamate compounds are not able to  
273 reach the bradyzoites. Alternatively, HDAC activity may not be essential at this latent stage of the life  
274 cycle. Only a handful of compounds were shown to reduce cyst burden (31, 32). Consequently, there  
275 is a need to develop novel *in vitro* systems enabling the testing compounds against bradyzoites. The  
276 exploration of organoids or *in vitro* cultures of primary brain or muscle cells holds the potential to  
277 unlock this new research pathway (22, 33).

278 Surprisingly, MC2059, a compound that has been shown to target human HDACs (24) was not able to  
279 induce an hyperacetylation of *T. gondii* histones or tubulin (Figure 2C). This indicates that this  
280 compound may have another target in the parasite. Hydroxamate-based compounds can inhibit  
281 metalloproteases or zinc hydrolases (27) and MC2059 may target these enzymatic activities.  
282 Alternatively, MC2059 may have an indirect effect on parasite growth through the inhibition of the  
283 human host HDAC activities. This may be also the case for MC2125 and MC2590, that have been proven  
284 to target the parasite HDACs, as the parasite is treated as well as the host. In the future, it would be of  
285 interest to identify the consequences of the compound treatment on the host and measure the gene  
286 expression perturbations induced by these HDACi at low concentration where no cytotoxic effect is  
287 visible on host cells. We have found that MC2125 had a cytotoxic effect on HepG2 cells at a 100 nM  
288 concentration, indicating that low concentration of these HDACi may have a direct consequence on

289 host cells, depending on their cellular type. Moreover, increasing the MC2125 dose to 30 mg/kg  
290 showed deleterious visible effects on mice, indicating that the dose could not be increased.

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

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

### 303 **Funding**

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

### 307 **Acknowledgments**

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

### 309 **Bibliography**

- 310 1. Montoya, J.G. and Liesenfeld, O. (2004) Toxoplasmosis. *Lancet Lond. Engl.*, **363**, 1965–1976.
- 311 2. Kim, K. and Weiss, L.M. (2004) *Toxoplasma gondii*: the model apicomplexan. *Int J Parasitol*, **34**, 423–  
312 32.



- 313 3. Munoz,M., Liesenfeld,O. and Heimesaat,M.M. (2011) Immunology of Toxoplasma gondii. *Immunol.*  
314 *Rev.*, **240**, 269–285.
- 315 4. Ben-Harari,R.R., Goodwin,E. and Casoy,J. (2017) Adverse Event Profile of Pyrimethamine-Based  
316 Therapy in Toxoplasmosis: A Systematic Review. *Drugs RD*, **17**, 523–544.
- 317 5. Katlama,C., De Wit,S., O’Doherty,E., Van Glabeke,M. and Clumeck,N. (1996) Pyrimethamine-  
318 clindamycin vs. pyrimethamine-sulfadiazine as acute and long-term therapy for toxoplasmic  
319 encephalitis in patients with AIDS. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, **22**, 268–  
320 275.
- 321 6. Mouveaux,T., Rotili,D., Boissavy,T., Roger,E., Pierrot,C., Mai,A. and Gissot,M. (2022) A potent  
322 HDAC inhibitor blocks Toxoplasma gondii tachyzoite growth and profoundly disrupts parasite  
323 gene expression. *Int. J. Antimicrob. Agents*, **59**, 106526.
- 324 7. Alday,P.H. and Doggett,J.S. (2017) Drugs in development for toxoplasmosis: advances, challenges,  
325 and current status. *Drug Des. Devel. Ther.*, **11**, 273–293.
- 326 8. Gissot,M., Kim,K., Schaap,D. and Ajioka,J.W. (2009) New eukaryotic systematics: a phylogenetic  
327 perspective of developmental gene expression in the Apicomplexa. *Int. J. Parasitol.*, **39**, 145–  
328 151.
- 329 9. Gissot,M. and Kim,K. (2008) How epigenomics contributes to the understanding of gene regulation  
330 in Toxoplasma gondii. *J. Eukaryot. Microbiol.*, **55**, 476–480.
- 331 10. Vanagas,L., Jeffers,V., Bogado,S.S., Dalmaso,M.C., Sullivan,W.J. and Angel,S.O. (2012)  
332 Toxoplasma histone acetylation remodelers as novel drug targets. *Expert Rev. Anti Infect.*  
333 *Ther.*, **10**, 1189–1201.
- 334 11. Fioravanti,R., Mautone,N., Rovere,A., Rotili,D. and Mai,A. (2020) Targeting histone  
335 acetylation/deacetylation in parasites: an update (2017-2020). *Curr. Opin. Chem. Biol.*, **57**,  
336 65–74.
- 337 12. Araujo-Silva,C.A., De Souza,W., Martins-Duarte,E.S. and Vommaro,R.C. (2021) HDAC inhibitors  
338 Tubastatin A and SAHA affect parasite cell division and are potential anti-Toxoplasma gondii  
339 chemotherapeutics. *Int. J. Parasitol. Drugs Drug Resist.*, **15**, 25–35.
- 340 13. Jeffers,V., Gao,H., Checkley,L.A., Liu,Y., Ferdig,M.T. and Sullivan,W.J. (2016) Garcinol Inhibits  
341 GCN5-Mediated Lysine Acetyltransferase Activity and Prevents Replication of the Parasite  
342 Toxoplasma gondii. *Antimicrob. Agents Chemother.*, **60**, 2164–2170.
- 343 14. Hailu,G.S., Robaa,D., Forgione,M., Sippl,W., Rotili,D. and Mai,A. (2017) Lysine Deacetylase  
344 Inhibitors in Parasites: Past, Present, and Future Perspectives. *J. Med. Chem.*, **60**, 4780–4804.
- 345 15. Zhang,Y., Zhang,Q., Li,H., Cong,H. and Qu,Y. (2022) In vitro and in vivo anti-Toxoplasma activities  
346 of HDAC inhibitor Panobinostat on experimental acute ocular toxoplasmosis. *Front. Cell.*  
347 *Infect. Microbiol.*, **12**, 1002817.
- 348 16. Loeuillet,C., Touquet,B., Oury,B., Eddaikra,N., Pons,J.L., Guichou,J.F., Labesse,G. and Sereno,D.  
349 (2018) Synthesis of aminophenylhydroxamate and aminobenzylhydroxamate derivatives and  
350 in vitro screening for antiparasitic and histone deacetylase inhibitory activity. *Int. J. Parasitol.*  
351 *Drugs Drug Resist.*, **8**, 59–66.

- 352 17. Jublot,D., Cavailès,P., Kamche,S., Francisco,D., Fontinha,D., Prudêncio,M., Guichou,J.-F.,  
353 Labesse,G., Sereno,D. and Loeuillet,C. (2022) A Histone Deacetylase (HDAC) Inhibitor with  
354 Pleiotropic In Vitro Anti-Toxoplasma and Anti-Plasmodium Activities Controls Acute and  
355 Chronic Toxoplasma Infection in Mice. *Int. J. Mol. Sci.*, **23**, 3254.
- 356 18. Bougdour,A., Maubon,D., Baldacci,P., Ortet,P., Bastien,O., Bouillon,A., Barale,J.-C., Pelloux,H.,  
357 Ménard,R. and Hakimi,M.-A. (2009) Drug inhibition of HDAC3 and epigenetic control of  
358 differentiation in Apicomplexa parasites. *J. Exp. Med.*, **206**, 953–966.
- 359 19. Mai,A., Massa,S., Rotili,D., Pezzi,R., Bottoni,P., Scatena,R., Meraner,J. and Brosch,G. (2005)  
360 Exploring the connection unit in the HDAC inhibitor pharmacophore model: novel uracil-  
361 based hydroxamates. *Bioorg. Med. Chem. Lett.*, **15**, 4656–4661.
- 362 20. Bouchut,A., Rotili,D., Pierrot,C., Valente,S., Lafitte,S., Schultz,J., Hoglund,U., Mazzone,R., Lucidi,A.,  
363 Fabrizi,G., *et al.* (2019) Identification of novel quinazoline derivatives as potent  
364 antiplasmodial agents. *Eur. J. Med. Chem.*, **161**, 277–291.
- 365 21. Saeij,J.P.J., Boyle,J.P., Grigg,M.E., Arrizabalaga,G. and Boothroyd,J.C. (2005) Bioluminescence  
366 imaging of *Toxoplasma gondii* infection in living mice reveals dramatic differences between  
367 strains. *Infect. Immun.*, **73**, 695–702.
- 368 22. Mouveaux,T., Roger,E., Gueye,A., Eysert,F., Huot,L., Grenier-Boley,B., Lambert,J.-C. and Gissot,M.  
369 (2021) Primary brain cell infection by *Toxoplasma gondii* reveals the extent and dynamics of  
370 parasite differentiation and its impact on neuron biology. *Open Biol.*, **11**, 210053.
- 371 23. Di Bello,E., Sian,V., Bontempi,G., Zwergel,C., Fioravanti,R., Noce,B., Castiello,C., Tomassi,S.,  
372 Corinti,D., Passeri,D., *et al.* (2023) Novel pyridine-containing histone deacetylase inhibitors  
373 strongly arrest proliferation, induce apoptosis and modulate miRNAs in cancer cells. *Eur. J.*  
374 *Med. Chem.*, **247**, 115022.
- 375 24. Di Bello,E., Noce,B., Fioravanti,R., Zwergel,C., Valente,S., Rotili,D., Fianco,G., Trisciuglio,D.,  
376 Mourão,M.M., Sales,P., *et al.* (2022) Effects of Structurally Different HDAC Inhibitors against  
377 *Trypanosoma cruzi*, *Leishmania*, and *Schistosoma mansoni*. *ACS Infect. Dis.*, **8**, 1356–1366.
- 378 25. Di Pompo,G., Salerno,M., Rotili,D., Valente,S., Zwergel,C., Avnet,S., Lattanzi,G., Baldini,N. and  
379 Mai,A. (2015) Novel histone deacetylase inhibitors induce growth arrest, apoptosis, and  
380 differentiation in sarcoma cancer stem cells. *J. Med. Chem.*, **58**, 4073–4079.
- 381 26. Sidik,S.M., Huet,D., Ganesan,S.M., Huynh,M.-H., Wang,T., Nasamu,A.S., Thiru,P., Saeij,J.P.J.,  
382 Carruthers,V.B., Niles,J.C., *et al.* (2016) A Genome-wide CRISPR Screen in *Toxoplasma*  
383 Identifies Essential Apicomplexan Genes. *Cell*, **166**, 1423-1435.e12.
- 384 27. Lou,B. and Yang,K. (2003) Molecular diversity of hydroxamic acids: part II. Potential therapeutic  
385 applications. *Mini Rev. Med. Chem.*, **3**, 609–620.
- 386 28. Marks,P.A. and Breslow,R. (2007) Dimethyl sulfoxide to vorinostat: development of this histone  
387 deacetylase inhibitor as an anticancer drug. *Nat. Biotechnol.*, **25**, 84–90.
- 388 29. Guo,F., Zhang,H., McNair,N.N., Mead,J.R. and Zhu,G. (2018) The Existing Drug Vorinostat as a  
389 New Lead Against Cryptosporidiosis by Targeting the Parasite Histone Deacetylases. *J. Infect.*  
390 *Dis.*, **217**, 1110–1117.

- 391 30. Flipo, M., Charton, J., Hocine, A., Dassonneville, S., Deprez, B. and Deprez-Poulain, R. (2009)  
392 Hydroxamates: Relationships between Structure and Plasma Stability. *J. Med. Chem.*, **52**,  
393 6790–6802.
- 394 31. Martynowicz, J., Doggett, J.S. and Sullivan, W.J. (2020) Efficacy of Guanabenz Combination Therapy  
395 against Chronic Toxoplasmosis across Multiple Mouse Strains. *Antimicrob. Agents*  
396 *Chemother.*, **64**.
- 397 32. Doggett, J.S., Schultz, T., Miller, A.J., Bruzual, I., Pou, S., Winter, R., Dodean, R., Zakharov, L.N.,  
398 Nilsen, A., Riscoe, M.K., *et al.* (2020) Orally Bioavailable Endochin-Like Quinolone Carbonate  
399 Ester Prodrug Reduces *Toxoplasma gondii* Brain Cysts. *Antimicrob. Agents Chemother.*, **64**.
- 400 33. Christiansen, C., Maus, D., Melerowicz, F., Scholz, J., Murillo-León, M., Steinfeldt, T., Hoffmann, T.,  
401 Seeber, F. and Blume, M. (2021) A Novel in vitro Model for Mature *Toxoplasma gondii*  
402 Bradyzoites Reveals their Metabolome and a Diminished Role of the Mitochondrial  
403 Tricarboxylic Acid Cycle. *bioRxiv*, 10.1101/2021.01.15.426845.

404

#### 405 **Figure legends**

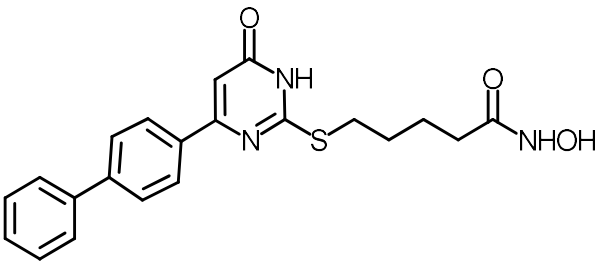
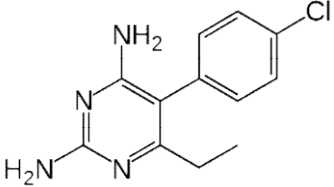
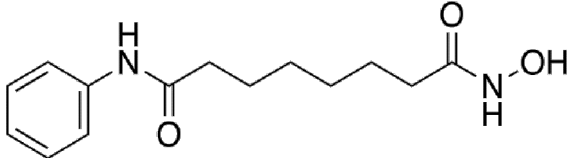
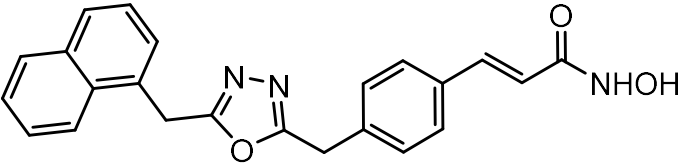
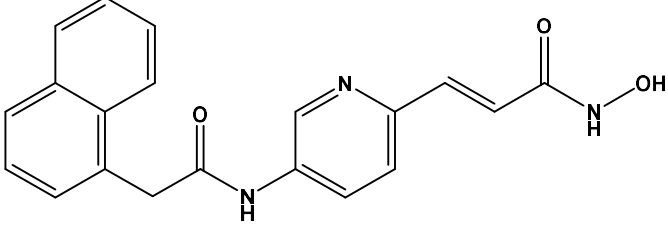
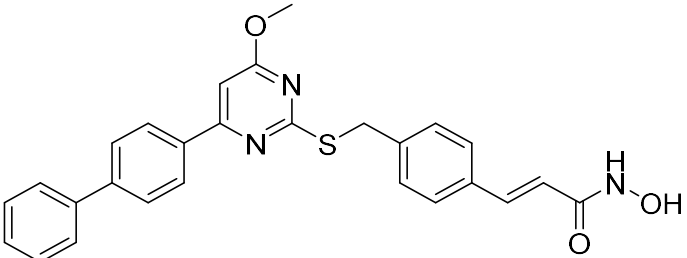
406 **Figure 1: Compounds potentially active against HDACs inhibit tachyzoite growth. (A)** Calculated IC<sub>50</sub>  
407 for each tested compound. Intracellular tachyzoite of the type II strain PTG-LUC GFP were treated for  
408 40 hours at different concentrations (from 2 μM to 2 nM). The mean IC<sub>50</sub> for three biological replicates  
409 is presented on the graph together with SD. Compounds were ranked based on their calculated IC<sub>50</sub>.  
410 The IC<sub>50</sub> values for Pyrimethamine (red), MC1742 (green), MC2059 (purple), MC2590 (orange) and  
411 MC2125 (blue) are highlighted in the graph. Mean ± SD (n=3 independent experiments). **(B)** Effect of  
412 the compounds on RH tachyzoites on intracellular growth. Tachyzoite intracellular growth was  
413 measured after 36 hours of exposure to different compounds at 2 times the IC<sub>50</sub> concentration. The  
414 average number of parasites per vacuole is used as a metric of parasite intracellular growth and  
415 measured by IFAs. Parasites treated with DMSO (blue bars) are used as a control. We measured the  
416 effect of MC2059 (red bars), MC2590 (green bars), MC2125 (purple bars) on intracellular growth. A  
417 Student's t-test was performed with the DMSO as control; two-tailed p-value; \*\*: p<0,01; \*\*\*:  
418 p<0,001; mean ± SD (n=6 independent experiments).

419 **Figure 2: Acetylation levels of *T. gondii* histones after treatment with MC2125, MC2590 and MC2059.**  
420 The purified RH parasite samples were treated either with DMSO or MC2125, MC2590 and MC2059  
421 (0,5 μM). **(A)**: Quantification of the signal for each indicated modified histone after treatment with  
422 DMSO (blue bars) or MC2125 (red bars). The signal was normalized to the signal given by the loading  
423 control (histone H3 Cterm, modified and unmodified). A Student's t-test was performed; two-tailed p-  
424 value; ns: p>0,05; \*\*: p<0,01; \*\*\*: p<0,001; mean ± SD (n=4 independent experiments). **(B)**  
425 Quantification of the signal for each indicated modified histone after treatment with DMSO (blue bars)

426 or MC2590 (green bars). The signal was normalized to the signal given by the loading control (histone  
427 H3 Cterm, modified and unmodified). A Student's t-test was performed; two-tailed p-value; ns:  $p > 0,05$ ;  
428 \*\*:  $p < 0,01$ ; \*\*\*:  $p < 0,001$ ; mean  $\pm$  SD (n=4 independent experiments). **(C)** Quantification of the signal  
429 for each indicated modified histone after treatment with DMSO (blue bars) or MC2059 (brown bars).  
430 The signal was normalized to the signal given by the loading control (histone H3 Cterm, modified and  
431 unmodified). A Student's t-test was performed; two-tailed p-value; ns:  $p > 0,05$ ; mean  $\pm$  SD (n=3  
432 independent experiments).

433 **Figure 3: Effect of the compounds on the outcome of the acute and latent toxoplasmosis. (A):** Effect  
434 of MC2059, MC2125 and MC2590 treatment on the outcome of the acute disease. One day after  
435 peritoneal injection of tachyzoites, mice were treated with different concentration of compounds once  
436 a day for 5 days. The percentage of survival is represented for each day during 15 days. Mice treated  
437 with DMSO (same regimen, blue line) are used as a negative control. Mice treated with Pyrimethamine  
438 (black line) are used as a positive control. The percentage of surviving mice after treatment with  
439 MC2059 (30mg/kg, light green or 60mg/kg, dark green), MC2590 (30mg/kg, brown) and MC2125  
440 (10mg/kg, red). A Log-rank (Mantel-Cox) test was performed; \*\*:  $p < 0,01$ ; \*\*\*\*:  $p < 0,0001$ ; mean  $\pm$  SD  
441 (n=15 mice per group). **(B):** Treatment of the bradyzoite (latent) form of the parasite with the different  
442 compounds. Latent bradyzoite cysts were obtained after 2 weeks of culture in primary brain cells and  
443 then treated with 3 times the  $IC_{50}$  for 7 days. The inhibition percentage was calculated when comparing  
444 the compound treated group to the control (DMSO treated cultures). Mean  $\pm$  SD (n=3 independent  
445 experiments).

446 **Table 1: IC<sub>50</sub> of selected hydroxamate-based compounds and pyrimethamine.**

Compounds	Structure	IC <sub>50</sub>
MC1742		30 ± 8 nM
Pyrimethamine		660 ± 230 nM
SAHA		247 ± 137 nM
MC2059		75 ± 9 nM
MC2590		23 ± 6 nM
MC2125		5 ± 1 nM

447

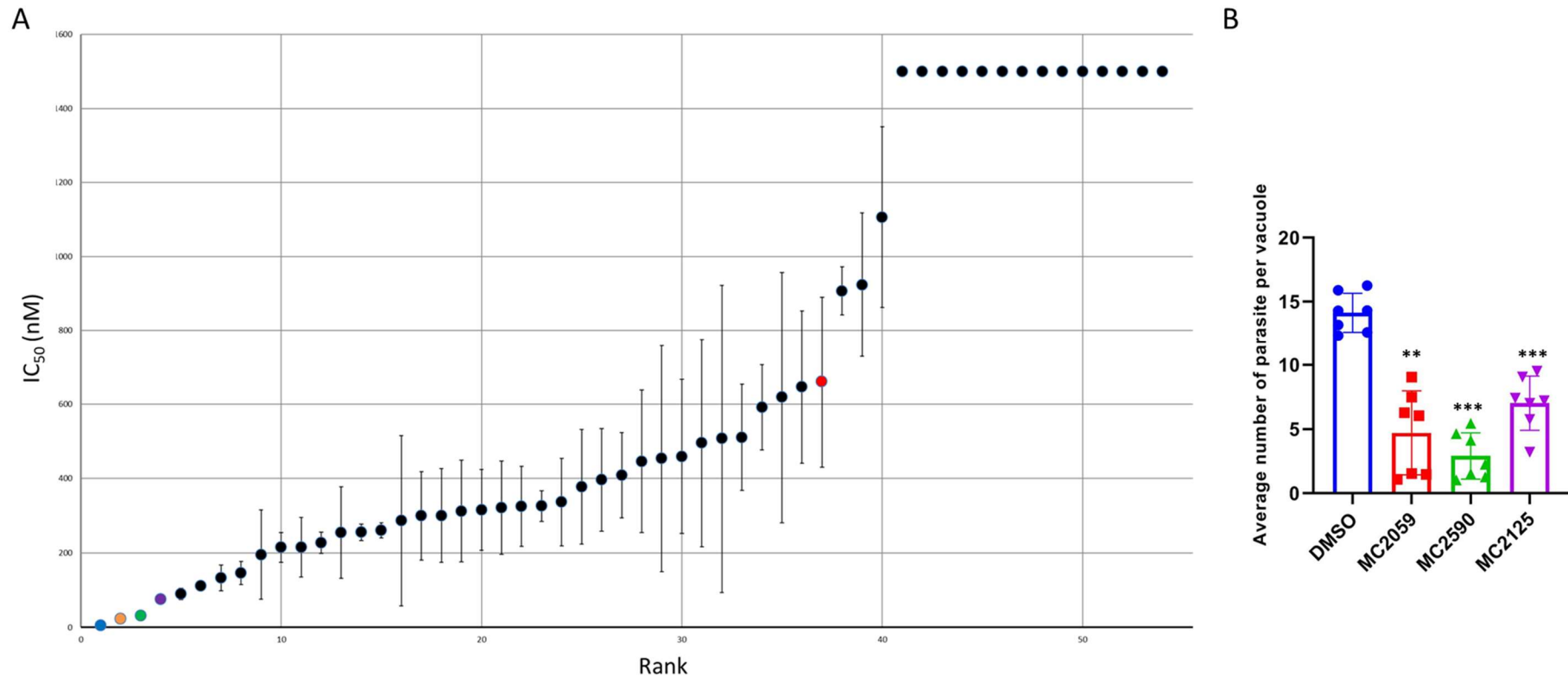
448 **Table 2: IC<sub>50</sub> of the selected compounds in *T.gondii* Type I and Type II strains and toxicity of the**  
 449 **selected compounds in HFF and HepG2 cells.**

450 The IC<sub>50</sub> was determined in at least three independent experiments. Selectivity index was calculated  
 451 based on the IC<sub>50</sub> of the Type II strain. <sup>(1)</sup> MC1742 IC<sub>50</sub> in human fibroblasts (HFF cells) was determined  
 452 in (20). <sup>(2)</sup> The X indicates values that were not available.

453

454

	<b>MC1742</b>	<b>MC2059</b>	<b>MC2590</b>	<b>MC2125</b>
<b>Type I IC<sub>50</sub> (nM)</b>	39 ± 7	89 ± 6	30 ± 6	10 ± 3
<b>Type II IC<sub>50</sub> (nM)</b>	30 ± 8	75 ± 9	23 ± 6	5 ± 1
<b>HFF IC<sub>50</sub> (nM)</b>	3000 <sup>(1)</sup>	15800 ± 1536	34553 ± 9386	16313 ± 2529
<b>HepG2 IC<sub>50</sub> (nM)</b>	χ <sup>(2)</sup>	8364 ± 125	5531 ± 1879	198 ± 55
<b>Selectivity index (IC<sub>50</sub> HFF/ IC<sub>50</sub> Type II)</b>	100	211	1502	3263
<b>Selectivity index (IC<sub>50</sub> HepG2/ IC<sub>50</sub> Type II)</b>	χ <sup>(2)</sup>	111	240	40

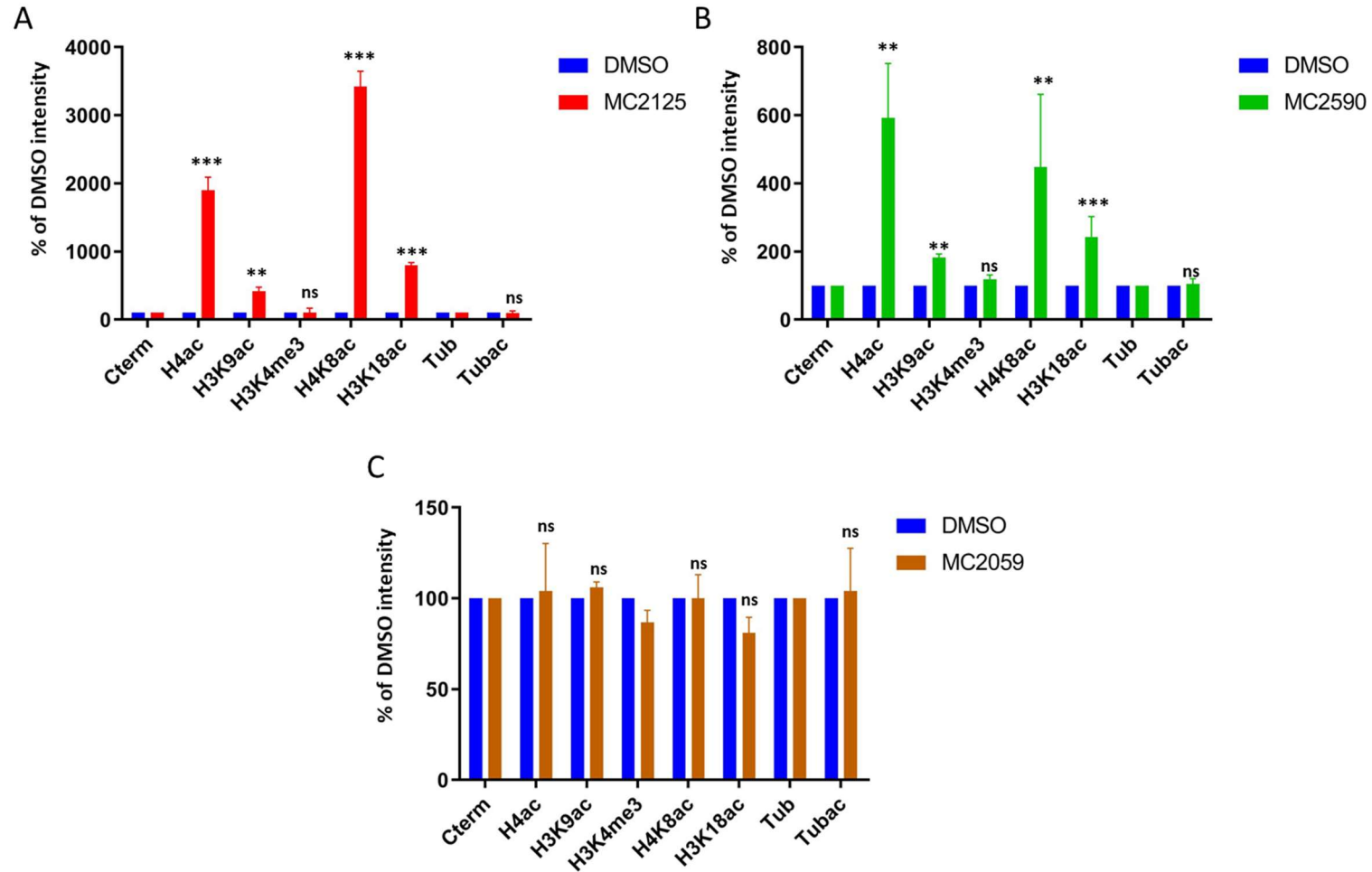


455

456 **Figure 1: Compounds potentially active against HDACs inhibit tachyzoite growth.** (A) Calculated IC<sub>50</sub> for each tested compound. Intracellular tachyzoite of  
 457 the type II strain PTG-LUC GFP were treated for 40 hours at different concentrations (from 2 μM to 2 nM). The mean IC<sub>50</sub> for three biological replicates is  
 458 presented on the graph together with SD. Compounds were ranked based on their calculated IC<sub>50</sub>. The IC<sub>50</sub> values for Pyrimethamine (red), MC1742 (green),  
 459 MC2059 (purple), MC2590 (orange) and MC2125 (blue) are highlighted in the graph. Mean ± SD (n=3 independent experiments). (B) Effect of the compounds  
 460 on RH tachyzoites on intracellular growth. Tachyzoite intracellular growth was measured after 36 hours of exposure to different compounds at 2 times the  
 461 IC<sub>50</sub> concentration. The average number of parasites per vacuole is used as a metric of parasite intracellular growth and measured by IFAs. Parasites treated

462 with DMSO (blue bars) are used as a control. We measured the effect of MC2059 (red bars), MC2590 (green bars), MC2125 (purple bars) on intracellular  
463 growth. A Student's t-test was performed with the DMSO as control; two-tailed p-value; \*\*:  $p < 0,01$ ; \*\*\*:  $p < 0,001$ ; mean  $\pm$  SD (n=6 independent experiments).

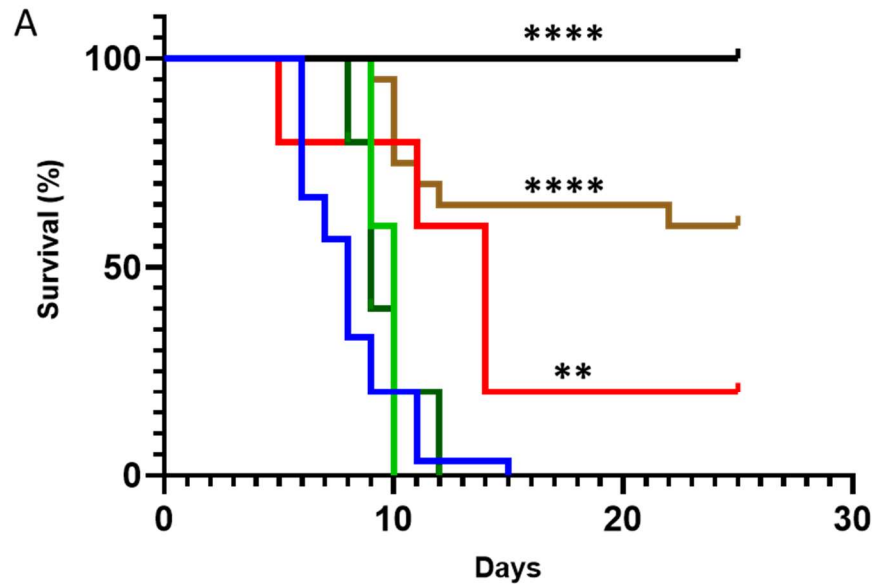




466 **Figure 2: Acetylation levels of *T. gondii* histones after treatment with MC2125, MC2590 and MC2059.** The purified RH parasite samples were treated either  
 467 with DMSO or MC2125, MC2590 and MC2059 (0,5  $\mu$ M). **(A):** Quantification of the signal for each indicated modified histone after treatment with DMSO (blue

468 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-  
469 test was performed; two-tailed p-value; ns:  $p > 0,05$ ; \*\*:  $p < 0,01$ ; \*\*\*:  $p < 0,001$ ; mean  $\pm$  SD (n=4 independent experiments). **(B)** Quantification of the signal for  
470 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  
471 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  $\pm$  SD  
472 (n=4 independent experiments). **(C)** Quantification of the signal for each indicated modified histone after treatment with DMSO (blue bars) or MC2059 (brown  
473 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;  
474 two-tailed p-value; ns:  $p > 0,05$ ; mean  $\pm$  SD (n=3 independent experiments).

475



B

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

476

477 **Figure 3: Effect of the compounds on the outcome of the acute and latent toxoplasmosis. (A):** Effect of MC2059, MC2125 and MC2590 treatment on the  
 478 outcome of the acute disease. One day after peritoneal injection of tachyzoites, mice were treated with different concentration of compounds once a day for  
 479 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  
 480 control. Mice treated with Pyrimethamine (black line) are used as a positive control. The percentage of surviving mice after treatment with MC2059 (30mg/kg,  
 481 light green or 60mg/kg, dark green), MC2590 (30mg/kg, brown) and MC2125 (10mg/kg, red). A Log-rank (Mantel-Cox) test was performed; \*\*:  $p < 0,01$ ; \*\*\*\*:  
 482  $p < 0,0001$ ; mean  $\pm$  SD (n=15 mice per group). **(B):** Treatment of the bradyzoite (latent) form of the parasite with the different compounds. Latent bradyzoite  
 483 cysts were obtained after 2 weeks of culture in primary brain cells and then treated with 3 times the  $IC_{50}$  for 7 days. The inhibition percentage was calculated  
 484 when comparing the compound treated group to the control (DMSO treated cultures). Mean  $\pm$  SD (n=3 independent experiments).

485