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

3

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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 (IC₅₀) 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 (IC₅₀) 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% CO₂. 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 IC₅₀ 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 μ M to 1 nM, since we aimed at identifying
101 compounds that showed a better IC₅₀ 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 IC₅₀ 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 μ M) of compounds in triplicates for 72h in 200 μ L total volume. Then, 20 μ 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 μ M to 3,125 μ M) of chloroquine, that has a known IC₅₀ on
115 HFF cells (20), were used as an internal control. The measurements were analyzed as a dose-response
116 curve to determine the IC₅₀. Each experiment was performed in triplicate. The IC₅₀ 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 μ L) of the 76K strain. The next day, the mice were
130 injected intraperitoneally with different quantity of compounds (200 μ 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 μ 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 μ 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- μ 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 IC₅₀s 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 IC₅₀ 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 IC₅₀ higher than pyrimethamine (660 nM). We found 35
170 potential HDACi exhibiting a lower IC₅₀ *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 IC₅₀ than MC1742 and lower IC₅₀ 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 IC₅₀. 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 IC₅₀s of 15-156
177 nM (23). MC2590 also inhibits human HDAC isoforms HDAC4, HDAC5, HDAC7, HDAC9, HDAC11 with
178 IC₅₀s 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ *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 IC₅₀ 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 IC₅₀ 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.

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402 Bradyzoites Reveals their Metabolome and a Diminished Role of the Mitochondrial
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404

405 **Figure legends**

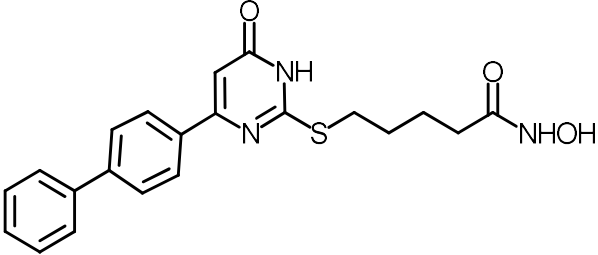
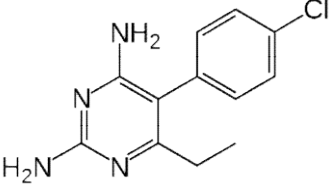
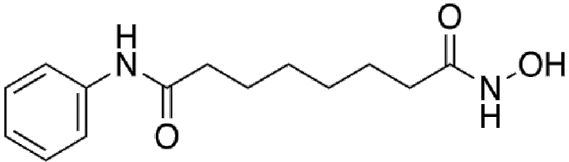
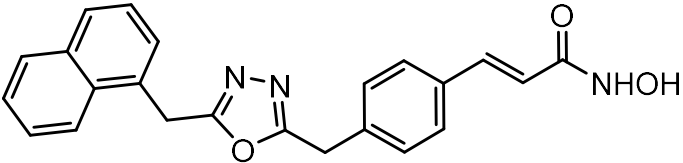
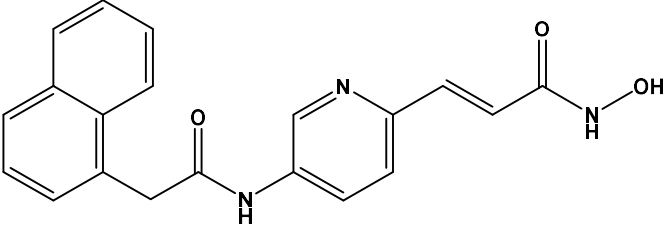
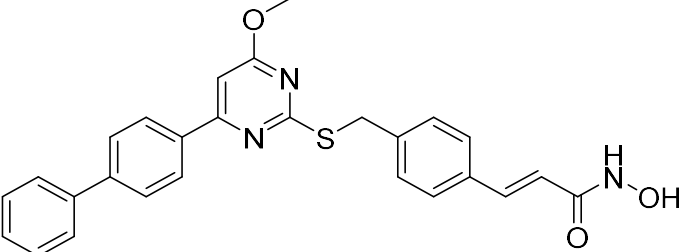
406 **Figure 1: Compounds potentially active against HDACs inhibit tachyzoite growth. (A)** Calculated IC₅₀
407 for each tested compound. Intracellular tachyzoite of the type II strain PTG-LUC GFP were treated for
408 40 hours at different concentrations (from 2 μM to 2 nM). The mean IC₅₀ for three biological replicates
409 is presented on the graph together with SD. Compounds were ranked based on their calculated IC₅₀.
410 The IC₅₀ 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 IC₅₀ 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: IC₅₀ of selected hydroxamate-based compounds and pyrimethamine.**

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

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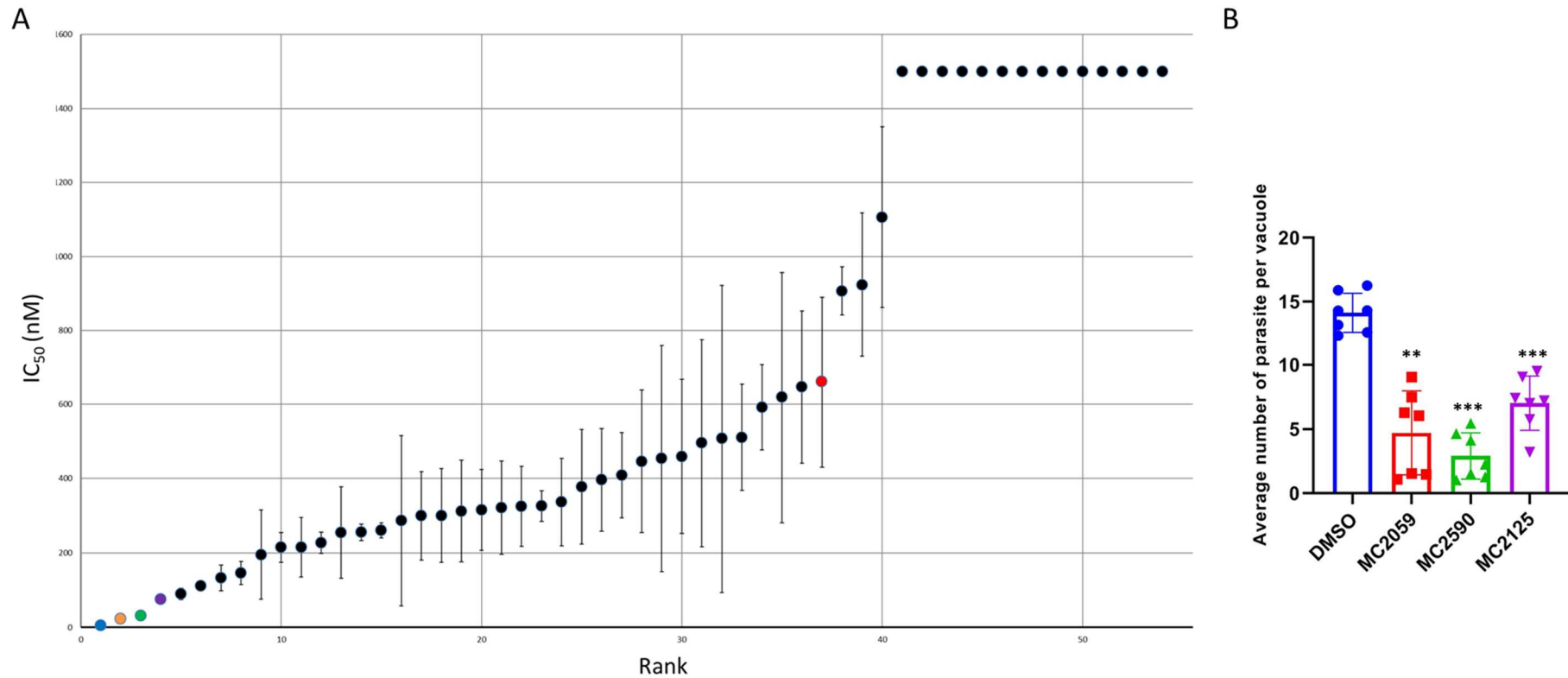
448 **Table 2: IC₅₀ of the selected compounds in *T.gondii* Type I and Type II strains and toxicity of the**
 449 **selected compounds in HFF and HepG2 cells.**

450 The IC₅₀ was determined in at least three independent experiments. Selectivity index was calculated
 451 based on the IC₅₀ of the Type II strain. ⁽¹⁾ MC1742 IC₅₀ in human fibroblasts (HFF cells) was determined
 452 in (20). ⁽²⁾ The X indicates values that were not available.

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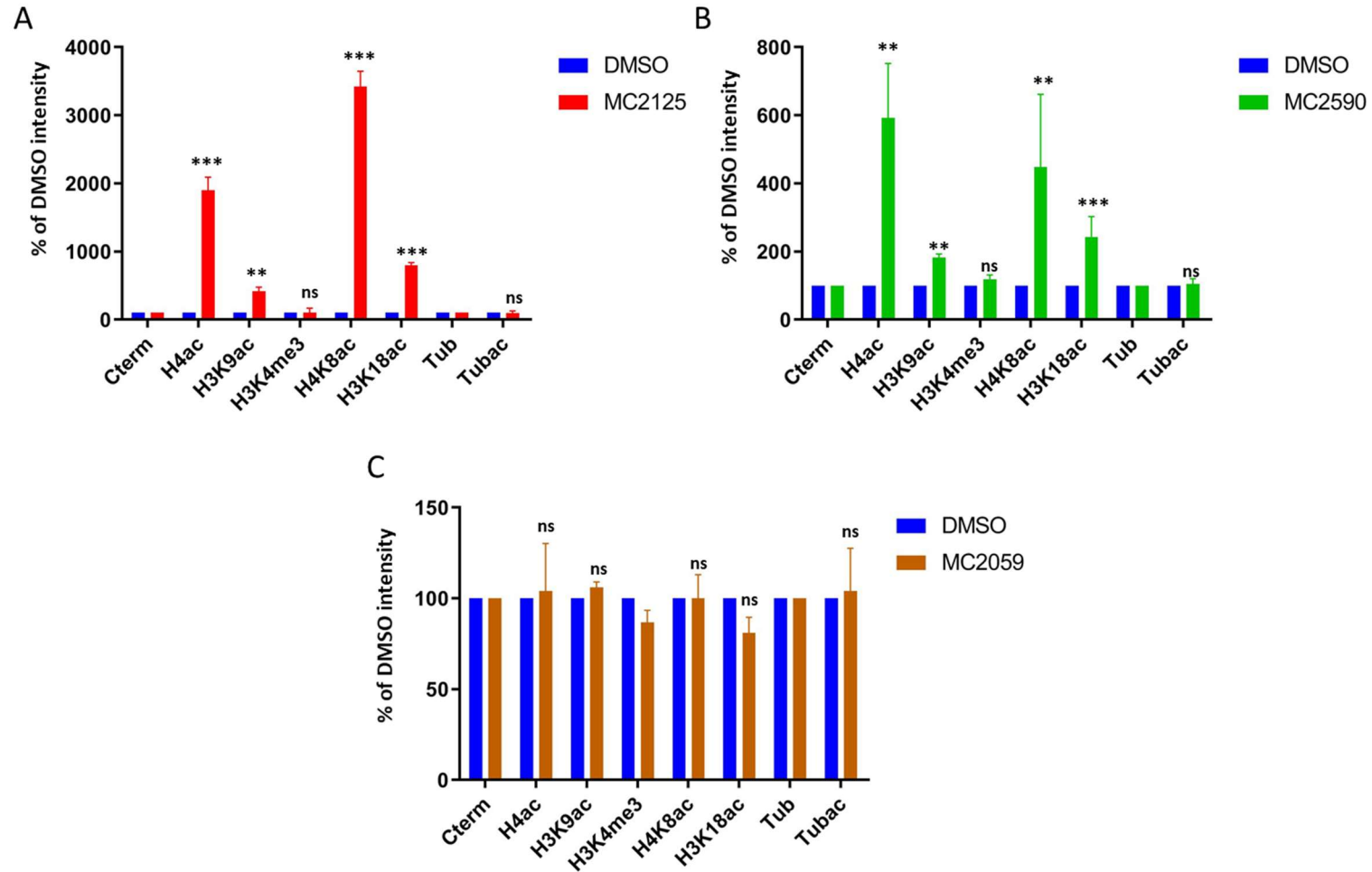
	MC1742	MC2059	MC2590	MC2125
Type I IC₅₀ (nM)	39 ± 7	89 ± 6	30 ± 6	10 ± 3
Type II IC₅₀ (nM)	30 ± 8	75 ± 9	23 ± 6	5 ± 1
HFF IC₅₀ (nM)	3000 ⁽¹⁾	15800 ± 1536	34553 ± 9386	16313 ± 2529
HepG2 IC₅₀ (nM)	χ ⁽²⁾	8364 ± 125	5531 ± 1879	198 ± 55
Selectivity index (IC₅₀ HFF/ IC₅₀ Type II)	100	211	1502	3263
Selectivity index (IC₅₀ HepG2/ IC₅₀ Type II)	χ ⁽²⁾	111	240	40



455

456 **Figure 1: Compounds potentially active against HDACs inhibit tachyzoite growth.** (A) Calculated IC₅₀ 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 IC₅₀ for three biological replicates is
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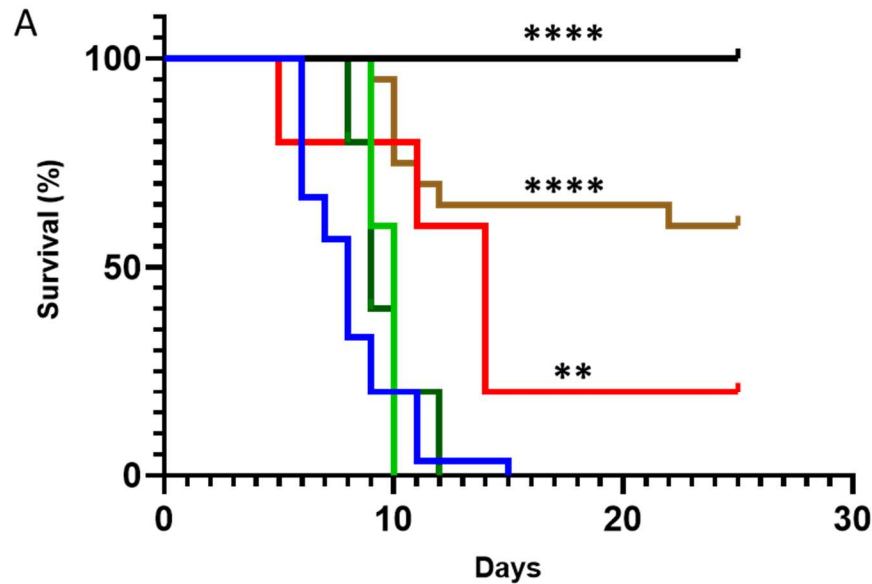
462 with DMSO (blue bars) are used as a control. We measured the effect of MC2059 (red bars), MC2590 (green bars), MC2125 (purple bars) on intracellular
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466 **Figure 2: Acetylation levels of *T. gondii* histones after treatment with MC2125, MC2590 and MC2059.** The purified RH parasite samples were treated either
 467 with DMSO or MC2125, MC2590 and MC2059 (0,5 μ 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
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