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1 # Oral treatments of *Echinococcus multilocularis*-infected mice with
2 # the anti-malarial drug mefloquine that potentially interacts with
3 # parasite ferritin and cystatin

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19 **Keywords: mefloquine; ferritin; cystatin; nicotinamide-phosphoribosyl-transferase;**
20 **chemotherapy; alveolar echinococcosis; mouse model.**

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source: https://doi.org/10.7892/boris.82042 | downloaded: 2.12.2024

22 **Abstract**

23 In this study, we investigated the effects of oral treatments of *E. multilocularis* infected mice
24 with the anti-malarial drug mefloquine, and identified proteins that bind to mefloquine in
25 parasite extracts and human cells by affinity chromatography. In a pilot experiment,
26 mefloquine treatment was applied 5 days per week and intensified by stepwise increasing the
27 dosage from 12.5mg/kg to 200mg/kg during 4 weeks followed by treatments of 100mg/kg
28 during the last 7 weeks. This resulted in a highly significant reduction of parasite weight in
29 mefloquine-treated mice compared to mock-treated mice, but the reduction was significantly
30 less efficacious as compared to the standard treatment regimen with albendazole. In a second
31 experiment, mefloquine was orally applied in three different treatment groups at dosages of
32 25, 50 or 100mg/kg, but only twice a week, for a period of 12 weeks. Treatment at 100mg/kg
33 had a profound impact on the parasite, similar to albendazole treatment at 200mg/kg/day (5
34 days/week for 12 weeks). No adverse side effects were noted. In order to identify proteins in
35 *E. multilocularis* metacestodes that physically interact with mefloquine, we performed affinity
36 chromatography of metacestode extracts on mefloquine coupled to epoxy-activated sepharose,
37 followed by SDS-PAGE and in-gel digestion/LC-MS/MS. This resulted in the identification
38 of *E. multilocularis* ferritin and cystatin as mefloquine-binding proteins. In contrast, when
39 human cells were exposed to mefloquine-affinity chromatography, nicotinamide
40 phosphoribosyl transferase was identified as a mefloquine-binding protein. This shows that
41 mefloquine interacts with different proteins in parasites and human cells.

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46 **INTRODUCTION**

47 The parasite *Echinococcus multilocularis* is an endoparasitic flatworm of the family
48 Taeniidae. The life cycle of *E. multilocularis* is based on a predator-prey relationship. The
49 definitive hosts are wild carnivores such as the red fox (*Vulpes vulpes*) and the arctic fox
50 (*Alopex lagopus*), but the tapeworm also infects, and develops within the intestine of,
51 domestic dogs and cats, increasing the infection pressure for humans [1, 2]. The definitive
52 hosts shed eggs, which contain a first larval stage, the oncosphere. When taken up orally,
53 oncospheres hatch as they reach the intestine, penetrate the intestinal wall and use the blood
54 and lymphatic system for dissemination. They typically invade the liver, where they develop
55 into the the metacestode stage, which causes human alveolar echinococcosis (AE). AE is
56 distributed in the Northern hemisphere, the endemic areas stretching from Northern America
57 through Central and Eastern Europe to Central and East Asia including Northern parts of
58 Japan [1]. The increase in the urban fox populations in Central Europe, together with the high
59 prevalence rate of *E. multilocularis* in foxes, has resulted in an increased environmental
60 contamination with *Echinococcus* eggs, and as a consequence, this has led to an increased risk
61 of transmission to humans [2].

62 Human AE manifests itself by tumor-like, infiltrative growth of metacestodes mainly in the
63 liver, but other organs might also be affected. AE is often compared with a slow-growing
64 liver cancer and if untreated the disease is usually lethal. The current strategy for treatment
65 consists of surgical measures complemented by chemotherapy with mebendazole (MBZ) or
66 albendazole (ABZ)). In inoperable cases, chemotherapy has proven to inhibit parasite
67 proliferation acting parasitostatic, but benzimidazoles act rarely curative, resulting in life-long
68 duration of treatment, high costs and elevated risk of side effects [3]. However,
69 benzimidazoles have prolonged the average life expectancy of European patients at diagnosis
70 from 3 to 20 years [3]. Nevertheless, alternative options for chemotherapy with parasiticidal
71 activity are needed [4].

72 Mefloquine (MEF) is a synthetic analogue of quinine commonly used in the treatment and
73 prophylaxis of chloroquine-resistant *Plasmodium falciparum* malaria [5, 6]. The mechanism
74 of action of MEF against *Plasmodium* species has not been completely elucidated, but several
75 investigations indicated a disturbance of haemoglobin metabolism, followed by the formation
76 of an insoluble polymer, hemozoin, causing parasite death [5]. It is not known whether a
77 hemozoin-related mode of action is relevant for the anti-echinococcal activity of mefloquine
78 *in vitro*.
79 MEF also exhibited considerable efficacy against other helminths, such as *Schistosoma*
80 *mansoni*, *S. japonicum*, *Opisthorchis viverrini*, *Brugia patei* and *B. malayi* [6-8]. We have
81 earlier demonstrated the efficacy of MEF against AE in experimentally infected mice when
82 the drug was applied intraperitoneally, but not when applied orally [9]. In this study we
83 present two distinct oral MEF treatment protocols in *E. multilocularis* infected mice, and
84 show that MEF treatment, when applied orally as a suspension in honey at a dosage of
85 100mg/kg twice a week, exhibits anti-echinococcal activity comparable to ABZ applied orally
86 at 200mg/kg/day. We also show that (i) the iron-binding protein ferritin, and (ii) cystatin, a
87 potential immune-modulator in parasite infections, bind to MEF, and thus possibly could be
88 targeted by MEF in *E. multilocularis* metacestodes. In contrast, MEF-affinity chromatography
89 of the human cancer cell line Caco2 results in binding of the enzyme nicotinamide
90 phosphoribosyl transferase (NPT).
91
92 **MATERIALS AND METHODS**
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94 **Biochemicals and compounds.** If not stated otherwise, all culture media and reagents were
95 purchased from Gibco-BRL (Zürich, Switzerland) and biochemical reagents were from Sigma
96 (St. Louis, Mo, USA). MEF was kindly supplied by Mepha Pharma AG (Aesch BL,
97 Switzerland).

98

99 ***In vitro* culture.** The culture of *E. multilocularis* (isolate H95) was carried out as previously
100 described [10]. The human colon carcinoma cell line Caco2 was maintained as previously
101 described by Müller et al [11].

102

103 **Experimental infection of mice and treatments with MEF and ABZ.** Female Balb/c mice
104 (age 9 weeks; average body weight average of 25g) were housed in a temperature-controlled
105 daylight/night cycle room with food and water *ad libitum*. Experiments were carried out
106 according to the Swiss Federal Animal Welfare regulations (TschV, SR 455) under the
107 licence number BE103/11. Metacestode solid fraction was obtained by extensive washing of
108 *in vitro* cultured parasites with PBS, breaking the vesicles mechanically, followed by
109 centrifugation [10]. The animals were randomly divided into experimental groups, and were
110 then infected by intraperitoneal injection of 100µL of metacestode solid fraction in 100µL
111 PBS.

112 The pilot experiment was performed with 15 mice, which were divided into 3 groups of 5
113 animals each. Compounds were formulated in 1% carboxymethylcellulose (CMC) at
114 concentrations indicated below. Starting at 6 weeks post infection, mice were treated by
115 gavage for a period of 11 weeks with either 100µL honey/CMC 1% (control group), 100µL
116 ABZ (200mg/kg in honey/CMC 1%; ABZ group) or 100µL MEF at different concentrations
117 (from 12.5mg/kg up to 200mg/kg in honey/CMC 1%; MEF group), basically 5 days per week
118 and according to the treatment regimen depicted in Supplementary Figure 1. During the
119 treatment, animals were carefully monitored and checked for clinical signs of impaired health
120 such as weight loss, ruffled coat, hunched back, and changes in behavior (inactivity,
121 nervousness), and the treatment regimen was adjusted accordingly. At the end of the
122 treatment period of 11 weeks, the animals were euthanized, and after necropsy the parasite
123 tissue was collected and the parasite weight measured. After pressing this material through a

124 metal tea strainer, 100µL of the resulting metacestode solution were re-injected into 2 mice
125 per group to observe whether the parasite was still viable and regrowing.
126 Experiment 2 was performed with 39 mice of 9 weeks age, which were divided into 4
127 treatment groups of 8 animals each, and a control group of 7 animals. Starting at 6 weeks
128 post-infection, mice were treated as follows: the control group received 100µL
129 honey/CMC1%; the ABZ group received ABZ (200mg/kg) emulsified in honey/CMC1%; the
130 MEF treatment groups were treated by gavage of MEF emulsified in honey/CMC 1% at
131 25mg/kg, 50mg/kg and 100mg/kg, respectively. The treatments were performed for a period
132 of 12 weeks on 5 days/week for the ABZ-treated group and twice weekly for the MEF-treated
133 groups. On those days where no MEF, but ABZ, was administered, mice from the MEF
134 groups received honey/CMC1%. At the end of the study the animals were euthanized, and
135 after necropsy the parasite tissue was collected and the parasite weight measured. The
136 parasiticidal activity was assessed by *in vitro* cultivation of resected parasite-material.
137 The data was analyzed by use of the software R version 3.0.1. Upon removal of Grubbs
138 outliers, data was analyzed by one-way ANOVA Bonferroni-adjusted *P* values calculated by
139 Pairwise T-Test. Data was visualized by boxplot in Microsoft Office Excel 2010.
140

141 **Coupling of MEF to epoxy-activated sepharose matrix.** MEF was coupled to epoxy-
142 activated sepharose matrix with a 12C spacer [11]. For this, 0.5g sepharose was extensively
143 washed with distilled water and sedimented, followed by a two-step wash with coupling
144 buffer (100mM $NaHCO_3$, pH 9.5). 20mg of MEF-HCl, solubilised in 1 ml DMSO, was added
145 to 1ml of the washed epoxy-sepharose matrix and incubated for 72h on a a slow horizontal
146 shaker at 37°C for 3 days in order to allow coupling to the epoxy-group. The resulting matrix
147 was then transferred to a chromatography column (Novagen, Merck, Darmstadt, Germany)
148 and washed with 20ml coupling buffer, followed by 1M ethanolamine pH 9.5 for 4h at room
149 temperature in the absence of light, in order to block residual groups. Subsequently, the

150 column was extensively washed with PBS and PBS/DMSO (1:2) in order to remove unbound
151 mefloquine. In additon to the MEF-sepharose column a mock-column was prepared
152 containing epoxy-sepharose treated identically as described above but in the absence of MEF.
153 The columns were stored in PBS containing 0.02% NaN_3 at 4°C.
154
155 **Tandem-affinity chromatography of metacestode extracts and Caco2 cell lysates on**
156 **epoxy-sepharose and MEF-epoxy-sepharose columns.** Metacestode extracts were prepared
157 from *in vitro* cultured parasites maintained under axenic conditions (i.e. in culture medium,
158 but without rat hepatoma cells) and without FCS for 72h. After extensive washing with PBS,
159 parasites were mechanically disrupted in PBS, centrifuged, and the pellet was resuspended in
160 PBS containing Triton X-100 (0.1 %), 100µL Halt protease inhibitor (Thermo Fisher
161 Scientific, Rockford, IL, USA) and 100 µL EDTA, and incubated at room temperature for 10
162 min. Following centrifugation at 4696 x g for 5min at room temperature. The supernatant
163 (12ml) was collected and used for tandem-affinity chromatography. Cell free extracts from
164 human Caco2 cells were prepared as described [11]. Extracts were first passed over the
165 sepharose column without compound (mock column) at a flow rate of 0.2 ml/min, and the
166 flow-through was collected, and then further loaded onto the MEF-sepharose matrix. Prior to
167 elution, columns were washed with 8 column volumes of PBS. Elutions of both columns were
168 done with PBS containing 10 mM MEF-HCl from a 100mM stock in DMSO. A second
169 elution step was performed by applying 4ml of a low pH-buffer (100 mM glycine, pH 2.9).
170 Fractions of 4ml volume each were collected and precipitated overnight with 80% acetone at -
171 20°C. The precipitates were solubilized in 30µl Laemmli buffer and separated by 10% SDS-
172 PAGE using a Hoefer minigel 250 apparatus (Amersham, GE Healthcare, Little Chalfont,
173 United Kingdom). Proteins were visualized by silver staining [12]. For mass spectrometry
174 (MS) analysis, colloidal Coomassie staining was applied overnight, and selected protein
175 bands were cut out with a clean scalpel, placed in Eppendorf tubes containing ethanol/distilled

176 water (1:4) and stored at 4°C. The in-gel digestion/LC-MS/MS analysis was performed by the
177 Mass Spectrometry and Proteomics Facility at the Department of Clinical Research of the
178 University of Bern. The sequences obtained were blasted against standard protein databases
179 for eukaryotes and against the database for *Echinococcus multilocularis*
180 (http://www.genedb.org/Homepage/Emultilocularis).
181
182 **Cytotoxicity assays.** Toxicity of MEF on Caco2 cells was determined and IC_{50} values were
183 calculated as previously described [11].
184
185
186 **RESULTS**
187
188 **Oral application of MEF acts against *E. multilocularis* metacestodes in experimentally**
189 **infected mice.** Initial experiments carried out earlier had shown that MEF treatment of *E.*
190 *multilocularis*-infected mice at25 mg/kg twice a week by oral route did not lead to a reduction
191 in parasite weight, while intraperitoneal application had a profound effect [9].
192 In a pilot experiment, we applied an intensified MEF treatment protocol characterized by a
193 stepwise increase of drug concentration (see Supplementary Figure 1). During an initial
194 period of 9 days, 12.5mg/kg of MEF was applied 5 times, followed by 2 days without
195 treatment and another 2 consecutive days of MEF treatment (see Supplementary Figure 1). No
196 adverse effects were noted, thus the dose was increased to 50mg/kg for another 3, 5 and 4
197 days treatments, all within a 16 days period (see Supplementary Figure 1). Again, no adverse
198 effects or behavioural changes were noted. The dose was increased to 100mg/kg for 2 days,
199 and then increased to 200mg/kg for another 2 days, which then resulted in rapid weight loss
200 (see Supplementary Figure 1) and ruffled coats in three of five mice, and one mouse died on
201 day 30. Subsequently the MEF dosage was kept at 100mg/kg, and was applied as such 31

202 times during the residual 48 days, during which the mice recovered body weight again and did
203 not show further side effects. No changes in body weight or any other adverse effects were
204 noted in ABZ treated mice. At the end of the 11 week experimental treatment, mice had
205 ingested a total of 4.0875g/kg of MEF per mouse or 10.8g/kg of ABZ (ratio MEF : ABZ =
206 1:2.45), respectively. Measurement of parasite weights at the end of the treatment period of
207 11 weeks showed there was a significantly beneficial effect of MEF treatment compared to
208 the non-treated control group (p=0.0016, Fig. 1A). However, the intensified MEF treatment
209 was not as efficacious as daily ABZ treatment, as the parasite weights in the MEF treatment
210 group were significantly higher compared to the ABZ treatment group (p = 0.0495) (Fig. 1A).
211 ABZ treatment led to a highly significant reduction of cyst weight (p=7.4E-05). Drug
212 treatments did not act parasiticidal, since reinfection with cyst material of two mice per group
213 resulted in parasite-regrowth in all cases (data not shown).
214 In experiment 2, MEF was applied to mice orally by feeding of a MEF-honey suspension, at
215 drug concentrations of 25 mg/kg, 50mg/kg or 100mg/kg only twice a week. As reference
216 control, mice were treated with ABZ (200mg/kg on 5 days per week). No adverse side effects,
217 no weight loss and no behavioural changes could be detected in any of the animals during the
218 time of drug treatment. During these 12 weeks, mice treated with 100mg/kg of MEF twice per
219 week ingested a total of 2.4g/kg of MEF, and mice treated with ABZ were given a total of 12
220 g/kg of ABZ (MEF : ABZ = 1:5). Measurement of the parasite burden at the end of the
221 treatment period of 12 weeks showed that oral MEF treatment at 100mg/kg twice a week was
222 as efficacious as daily ABZ treatment (200mg/kg/day) (Fig. 1B). The reduction in parasite
223 burden was a significant in the ABZ-treated group (p=0.018) and in the MEF (100mg/kg)
224 treated mice (p=0.018), while MEF treatment at lower doses (25 and 50mg/kg) showed a non-
225 significant reduction. None of these drug treatments acted parasiticidal, since *in vitro* culture
226 of parasite material from all the treatment groups resulted in regrowth of metacestode
227 vesicles.

228

229 **Epoxy-sepharose-immoblilized MEF binds *E. multilocularis* ferritin and cystatin.** In
230 order to identify proteins that interact with MEF, *E. multilocularis* metacestode fractions were
231 exposed to sepharose-bound MEF. The fractions eluted from the mock-sepharose and the
232 MEF-sepharose columns were analyzed by SDS-PAGE and silver staining (Fig. 2). After
233 loading *E. multilocularis* extract onto the mock-sepharose and subsequent MEF affinity
234 chromatography of the flow-through, the columns were washed with MEF-containing elution
235 buffer and subsequently with low pH buffer. As seen in Fig. 2, washing of the mock-
236 sepharose column with MEF-containing elution buffer did not result in removal of any
237 proteins. The elution with low pH-buffer revealed that considerable amounts of *E.*
238 *multilocularis* proteins bound to the sepharose matrix and were released again at low pH.
239 MEF-sepharose affinity chromatography of the mock-sepharose-flow-through fraction
240 yielded a major double-protein band of around 20kDa eluting with MEF-containing buffer.
241 Subsequent washing with low-pH buffer did not result in further release of proteins (Fig. 2).
242 The two bands of the MEF elution (indicated with arrows as 1 and 2, Fig. 2) were cut out and
243 analysed by in-gel digestion/LC-MS/MS. Both protein bands were identified as ferritin and
244 cystatin from *E. multilocularis* (Table 1; EmuJ_000382200.1 and EmuJ_000849600.1).

245

246 **Epoxy-sepharose-immoblilized MEF binds to human nicotinamide-phosphoribosyl-**
247 **transferase (NAPRT)**

248 Human cancer cells have been shown to be highly susceptible to antimalarials including MEF
249 [13]. The susceptibility of the human colon cancer cell line Caco2 against MEF was assessed
250 *in vitro*. The IC_{50} for non-confluent Caco2 cells was 0.7±0.1µM, and confluent cells exhibited
251 an IC_{50} of 3.6±0.7µM. MEF binding proteins in human cells were identified using the
252 identical approach as for *E. multilocularis* metacestodes, resulting in the elution of a single
253 major band of approximately 50kDa. Subsequent elutions with with low-pH buffer were

254 devoid of major bands (Fig. 3). The 50kDa band was subjected to analysis by mass
255 spectrometry and identified as the 491 amino acid protein nicotinamide-phosphoribosyl-
256 transferase (NAPRT; P43490).
257
258 **DISCUSSION**
259
260 As the pilot study showed, a stepwise increase of oral MEF input was significantly effective,
261 but had detrimental effects, resulting in a lower efficacy of MEF compared to ABZ, and
262 obvious adverse side effects once higher concentrations than 100mg/kg were applied.
263 The second study demonstrates that oral application of mefloquine at 100mg/kg twice a week
264 for 12 weeks in experimentally infected mice acts against *E. multilocularis* metacestodes in a
265 similar manner as oral application of albendazole (200mg/kg/ on 5 days per week).
266 Mefloquine applied at 25mg/kg or 50mg/kg was ineffective, confirming results from earlier
267 studies [9]. At the lower concentrations the bioavailability of MEF was probably too low to
268 elicit a therapeutically active serum level, while an increased MEF input resulted in serum
269 levels that induced toxicity. For comparison, high single oral MEF doses of 200 to 400mg/kg
270 were necessary to achieve significant worm burden reductions in *S. mansoni*- and *S.*
271 *japonicum*-infected mice. However, for extended treatments (as they would be anticipated in
272 AE patients), the application of MEF could be problematic. Daily doses above 12.5mg/kg
273 have been reported to cause epilepsy or impairment of motor performance, and increased
274 lethality was observed in mice at dosages of 30mg/kg and more [14-18]. Despite of the
275 reported adverse effects of MEF when applied in high doses [18], no adverse effects were
276 observed in the behavior or overall health of the animals throughout the treatment phase in
277 experiment 2, most likely due to the fact that treatments were restricted to twice a week. In
278 any case, this study shows the critical role of dosage to achieve anti-parasitic activity without
279 causing adverse effects. Further it has to be highlighted that unlike shown in a previous *in*

280 *vitro* study [9], *in vivo* MEF treatment did not kill the parasite completely. Full parasiticidal
281 activity, and especially toxic activity against *E. multilocularis* stem cells, would be necessary
282 to completely kill the parasite and avoid the necessity of life-long drug uptake [19].
283 Research on novel compounds for the treatment of secondary AE in mice should focus on
284 drugs or drug classes that are already marketed and/or in clinical development, and these are
285 preferably cancer drugs or broad-spectrum anti-parasitic compounds. In this respect, MEF is
286 commercially available, FDA-approved, and has been extensively characterized in terms of
287 bioavailability, pharmacokinetics, and toxicity. However, the adverse effects reported upon
288 the uptake of MEF are a problematic issue. The drug is known to cause neurotoxicity, and
289 neuropsychiatric effects have been reported. Thus, it would be important to further investigate
290 derivatives of MEF, and identify those compounds with similar anti-parasitic activity but with
291 an impaired ability to pass through the blood-brain barrier. In addition, it has been shown that
292 MEF is metabolized only slowly, with a plasma half-life in humans of 2-4 weeks, and
293 metabolization occurs in the liver. During long-term treatments, as they would be anticipated
294 in human patients suffering from AE, which represents mainly a liver-associated disease, this
295 could cause significant adverse effects.
296
297 The interaction of *E. multilocularis* ferritin and cystatin with MEF by affinity
298 chromatography could indicate a possible anti-echinococcal mechanism of action for the drug.
299 Both proteins were detected in two bands that migrated closely together on SDS-PAGE.
300 Ferritin is an important intracellular iron-storage protein, generally composed of 24 subunits
301 of around 20kDa and these 24 subunits are made up by ferritin heavy and light chains [20]. In
302 *E. multilocularis* there has only one gene been identified for ferritin. Invertebrate ferritins
303 show greater similarity towards the vertebrate heavy chains than towards the light chains, and
304 ferritin heavy and light chains have been identified in nematodes and trematodes [21]. Thus,
305 possibly the light chain homologue has not been identified in the *E. multilocularis* genome so

306 far due to more pronounced sequence divergence. Further, *E. multilocularis* ferritin has a
307 predicted size of 19.8kDa that is in accordance with the apparent molecular weight of the
308 protein bands on the silver stained gel. Ferritin is responsible for the maintenance and
309 transport of iron in a non-toxic form In addition, ferritin heavy chain possesses ferroxidase
310 activity, converting Fe^{2+} to Fe^{3+}. Fe^{2+} can interact with hydrogen peroxide, resulting in highly
311 damaging hydroxyl radical formation [20]. Thus, we propose that there could be a link
312 between iron metabolism of the parasite and the mode of action of MEF in *E. multilocularis*
313 metacestodes. *Plasmodium* species use haemoglobin, an extracellular Fe-containing protein of
314 vertebrates, as a nutrient source. Interestingly, the mode of action of MEF in plasmodia is
315 linked to binding of the heme part of haemoglobin [5], which implies that the Fe-containing
316 residue is crucial both in plasmodia and *Echinococcus* for the action of MEF.
317 Cystatins are inhibitors of cysteine proteases of the C1 family. However, in the field of
318 parasitology, cystatins are given special attention, because they regulate the activity of
319 proteases involved in parasite migration, and they are immunomodulators of parasite
320 infections [22]. Interestingly, in the trematode *Schistosoma mansoni* cystatin is involved in
321 the regulation of haemoglobin degradation [23]. The *E. multilocularis* cystatin has a predicted
322 molecular weight of 30.9kDa. This is not in accordance with the apparent molecular weight of
323 the protein bands on gel of about 20kDa, thus the detected band could have resulted from
324 protein degradation.
325 One has to keep in mind, however, that MEF may bind to proteins that do not contain iron. In
326 *Schistosoma*, enolase has been identified as a MEF binding protein [24]. Here, we show that
327 human NAPRT binds to MEF. NAPRT, also known as visfatin, is a key enzyme in the NAD
328 salvage pathway and acts as an immunoregulatory cytokine [25]. By activating sirtuin,
329 NAPRT promotes cell longevity [26] and has neuroprotective effects [27]. Conversely,
330 inhibition of NAPRT induces apoptosis rendering this protein an interesting target for the
331 development of anti-tumor agents [28].

332 MEF is not the only anti-malarial exhibiting anti-echinococcal activity. For artemisinin
333 derivatives such as antimalarial peroxides it has been shown that peroxide-bond reduction by
334 hemoglobin-derived Fe^{2+} produces short-lived alkoxy radicals, which quickly rearrange to a
335 carbon-centred radical that alkylates heme, forming heme-drug adducts [29]. Similar to
336 mefloquine, these artemisinins also have a high activity against schistosomes, which like
337 plasmodia, digest haemoglobin, in this instance by ingestion of erythrocytes [30]. The anti-
338 schistosomal properties of MEF have also been established, and the presence of haemin was
339 shown to enhance the treatment efficacy [6, 30]. However, MEF-affinity chromatography of
340 *Schistosoma mansoni* schistosomula had revealed that the glycolytic enzyme enolase binds to
341 the drug [24], and this study had demonstrated a potential role of MEF as an inhibitor of
342 glycolysis in stages where heme degradation was not relevant.
343 In conclusion, MEF is effective in the treatment of AE when administered orally at high
344 doses, but only twice a week. Moreover, the drug presents the advantages of being
345 commercially available and currently used for the prophylaxis and treatment of malaria.
346 Despite much discussion, the mechanism of action of MEF is not yet clarified for
347 *Plasmodium* species. For *E. multilocularis,* we have identified ferritin and cystatin as two
348 MEF-binding proteins, which are clearly different from NAPRT pulled out from human cell
349 extracts, and their role as drug targets for MEF in these parasites will be further evaluated in
350 future studies.
351

352 **Acknowledgements**

353 We thank Myriam Siffert (University of Berne) for responsibly taking care of the animals and
354 Sophie Lagache from the Core Facility of Proteomics and Mass Spectrometry at the
355 University of Berne for careful analysis of protein samples.

356

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437 **Figure legends**

438

439 **Figure 1. Effects of oral treatments of *E. multilocularis* infected mice in terms of**
440 **recovered parasite weight.** In the pilot experiment (A), oral application of albendazole
441 (ABZ) was compared with mefloquine treatment (MEF) for a period of 11 weeks at dosages
442 as indicated in Supplementary Fig. 1. The control group received oral treatment with solvent
443 only (honey/CMC 1%). Note that the differences in parasite weights between the ABZ, MEF
444 and control group are all significantly different from each other. In experiment 2 (B) mice
445 were treated for a period of 12 weeks with either honey/CMC 1% (control), albendazole
446 (ABZ; 200 mg/kg daily on 5 days per week) or mefloquine (MEF; 25, 50 or 100 mg/kg twice
447 per week). Note the significantly reduced parasite weight in the ABZ and MEF (100mg/kg)
448 group, while MEF was ineffective at 25 and 50 mg/kg. For statistical analysis, Grubbs outliers
449 were removed, and further testing performed with ANOVA and Bonferroni-adjusted *P* values
450 calculated by Pairwise T-Test.

451

452 **Figure 2. SDS-PAGE visualization of proteins from soluble *E. multilocularis* metacestode**
453 **extract eluted after affinity chromatography with mefloquine bound to epoxy-activated**
454 **sepharose.** Silver stained gel showing fractions eluted from the unloaded epoxy-sepharose
455 column (Mock column) and the subsequent mefloquine column. Pre-mef = flow-through
456 fraction obtained prior to mefloquine elution; mef = fraction eluted with excess mefloquine;
457 pre-pH = fraction obtained prior to pH-shift elution; pH = fractions eluted with low-pH
458 buffer. The two bands eluted with excess mefloquine (indicated by arrows 1 and 2) were cut
459 out and subjected to LC-MS/MS analysis.

460

461 **Figure 3. SDS-PAGE visualization of proteins from soluble human colon cancer (Caco2)**
462 **cell extract eluted after affinity chromatography with mefloquine bound to epoxy-**
463 **activated sepharose.** Silver stained gel showing cell extract (CE) loaded onto the columns,
464 flow through (FT) and wash fraction (pre-mef = flow-through fraction obtained prior to
465 mefloquine elution. mef = fraction eluted with excess mefloquine; pH1 and pH2 = fractions
466 eluted with low-pH buffer. The band eluted with excess mefloquine (indicated by arrow) was
467 cut out and subjected to LC-MS/MS analysis.

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472 **Supplementary Figure 1.** For the pilot experiment, the treatment regimen (A) and the
473 development of mean body weights measured during treatments (B) are presented.
474 Albendazole (ABZ) dosage was kept continuously at 200 mg/kg per application, while the
475 dosages for mefloquine (MEF) varied. Monitoring of the mean body weight during the
476 treatments shows a marginal impact of ABZ, while for MEF the high dosage of 200 mg/kg
477 twice resulted in weight loss and subsequent death of one animal on day 30 (arrow). The
478 remaining mice recovered when MEF-treatment was continued at lower dosage (31 x 100
479 mg/kg during the next 48 days) and these mice finally achieved a similar body weight as
480 ABZ-treated mice.
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AC	Description	PMSS band 1	PMSS band 2	Protein score	Cov %	#PSMs	#Peptides
EmuJ_000382200.1	ferritin	3875.722	3655.734	1758.516	93.68	1111	81
EmuJ_000849600.1	proteinase inhibitor I25, cystatin	31.046	5.346	1367.22	77.09	386	64
EmuJ_000684200.1	Lipid transport protein, N terminal		5.922	60.982	2.21	7	7
EmuJ_000720500.1	ATP synthase subunit alpha, mitochondrial		5.269	17.903	4.2	2	2

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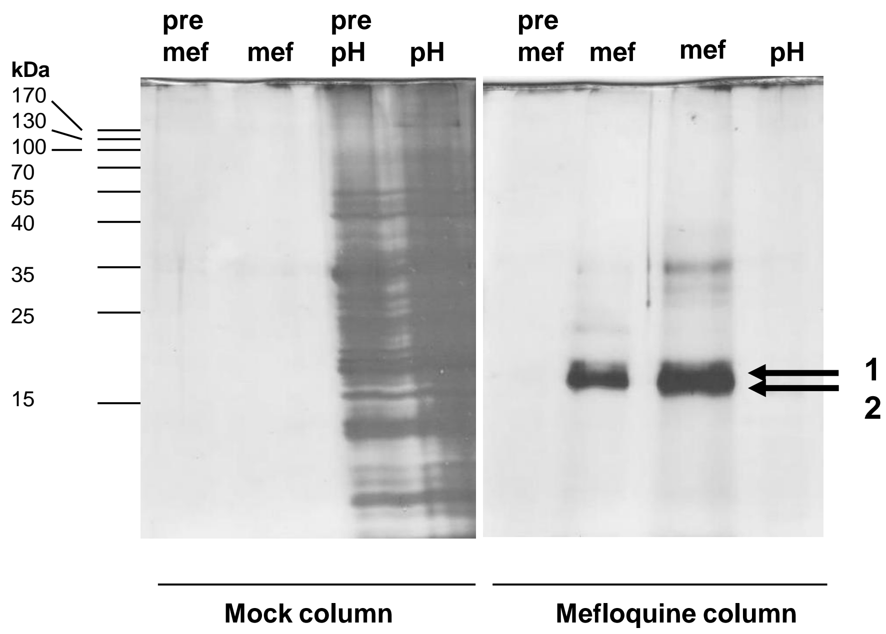
488 **Table 1. Analysis of in-gel digestion LC-MS/MS of two bands (1 and 2), the two**
489 **mefloquine-binding proteins eluted by affinity chromatography using mefloquine-**
490 **epoxy-sepharose.** Represented are the accession numbers (AC) of the identified proteins in
491 the database http://www.genedb.org/Homepage/Emultilocularis; their description; the PMSS
492 values that are the sum of z-scores of all peptides of a protein, thus representing a
493 semiquantitative measure of the abundance of the respective proteins; the protein score; the
494 percentage of coverage (cov %) of each protein; the number peptide scan matches (PSMs);
495 the number of peptides.
496
497



Graphical abstract

Figure

Figure 1

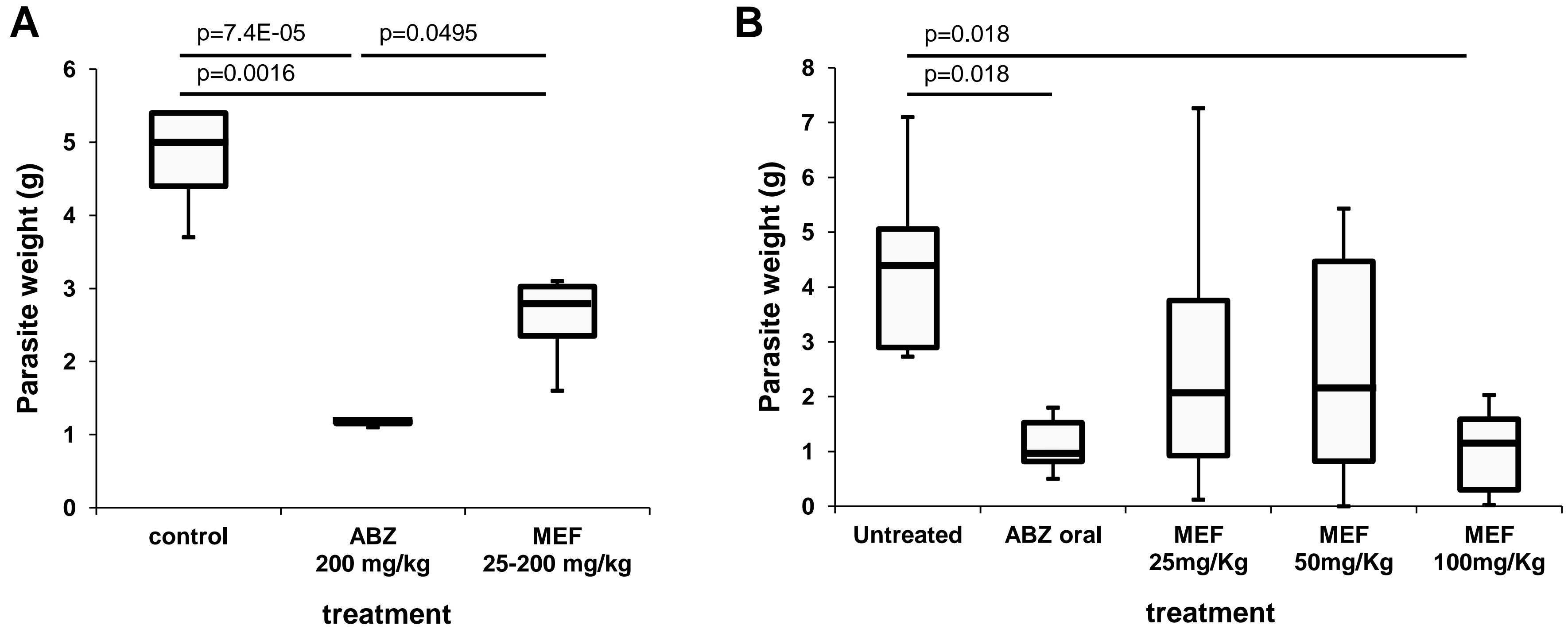


Figure 2

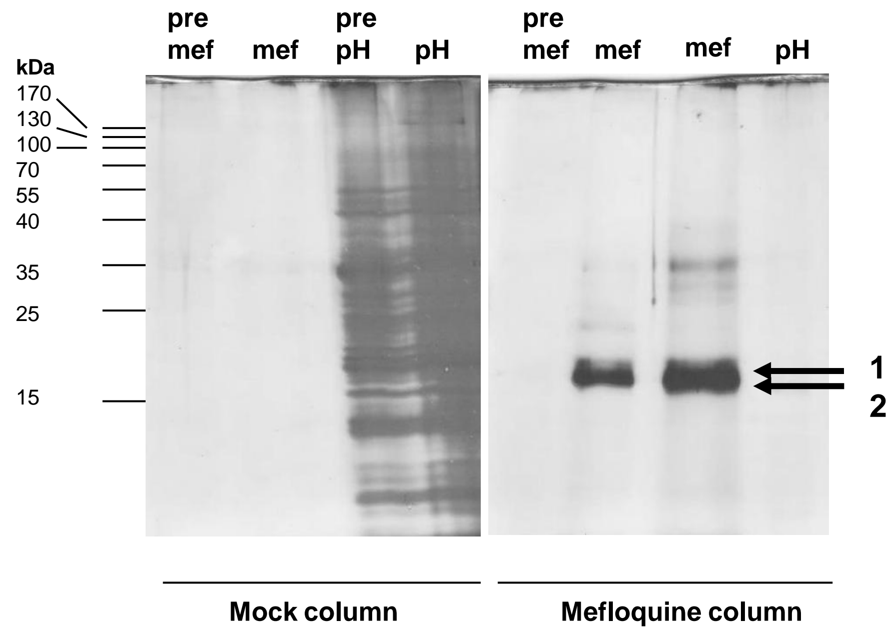
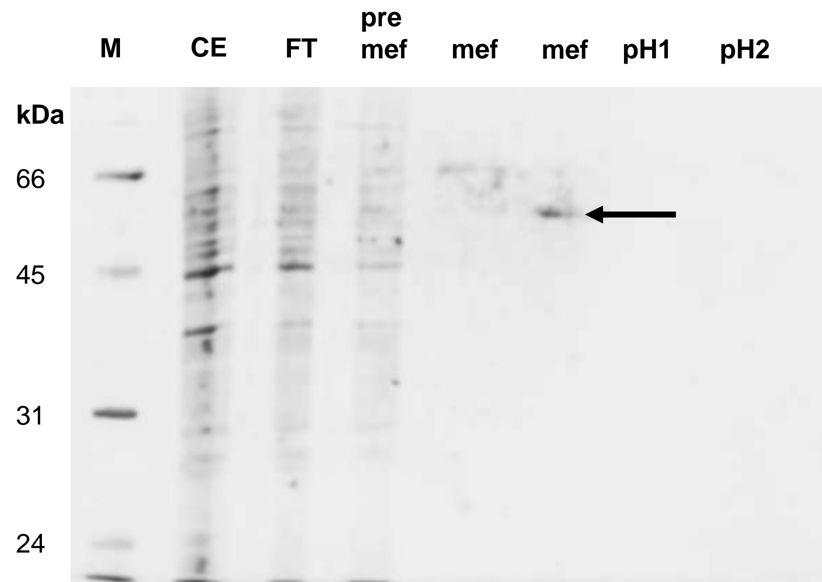


Figure 3



Supplementary Figure 1