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High Accumulation and In Vivo Recycling of the New Antimalarial Albitiazolium Lead to Rapid Parasite Death

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1 **High accumulation and *in vivo* recycling of the new antimalarial albitiazolium**
2 **rapidly condemn the parasite to death**

3
4 **Sharon Wein^{a#}, Nicolas Taudon^{b*}, Marjorie Maynadier^a, Christophe Tran Van Ba^a,**
5 **Delphine Margout^b, Yann Bordat^a, Laurent Fraisse^c, Kai Wengelnik^a, Rachel Cerdan^a,**
6 **Françoise Bressolle-Gomeni^{b†} and Henri J. Vial^a**

7
8 ^aDynamique des Interactions Membranaires Normales et Pathologiques, CNRS UMR 5235,
9 Université de Montpellier, Montpellier, France.

10 ^b Pharmacocinétique Clinique, Faculté de Pharmacie, Université de Montpellier, Montpellier,
11 France.

12 ^c Sanofi Research & Development, Marcy L'Etoile, France

13
14 **Running Title: Albitiazolium pharmacodynamics in murine malaria**

15
16 # Address correspondence to Sharon Wein, sharon.wein-gratraud@umontpellier.fr

17
18 * Present address: Nicolas Taudon, Unité de Toxicologie Analytique, Institut de Recherche
19 Biomédicale des Armées, Brétigny sur Orge, France

20
21 † Present address : Françoise Bressolle-Gomeni, R&D Department, Pharmacometrica, Longcol,
22 La Fouillade, France

ABSTRACT

Albitiazolium is the lead compound of bis-thiazolium choline analogues and exerts powerful *in vitro* and *in vivo* antimalarial activities. Here we provide new insight into the fate of albitiazolium *in vivo* in mice and how it exerts its pharmacological activity. We show that the drug exhibits a rapid and potent activity and has very favorable pharmacokinetic and pharmacodynamic properties. Pharmacokinetic studies in *Plasmodium vinckei*-infected mice indicated that albitiazolium rapidly and specifically accumulates to a great extent (cellular accumulation ratio > 150) in infected erythrocytes. Unexpectedly, plasma concentrations and the area under concentration-time curves increased by 15% and 69% when mice were infected at 0.9% and 8.9% parasitemia, respectively. Albitiazolium that had accumulated in infected erythrocytes and in the spleen was released into the plasma, where it was then available for another round of pharmacological activity. This recycling of the accumulated drug, after the rupture of the infected erythrocytes, likely extends its pharmacological effect.

We also established a new viability assay in the *P. vinckei*-infected mouse model to discriminate between fast- and slow-acting antimalarials. We found that albitiazolium impaired parasite viability in less than 6 and 3 hours at the ring and late stages, respectively, while parasite morphology was affected more belatedly. This highlights that viability and morphology are two parameters that can be differentially affected by a drug treatment, an element that should be taken into account when screening new antimalarial drugs.

INTRODUCTION

47
48
49 Malaria remains one of the major human infectious diseases, with more than 40% of the
50 world's population at risk, and is responsible for 150-300 million annual clinical cases with
51 around 430,000 deaths, mostly children under five years old and in sub-Saharan Africa (1). In the
52 absence of a marketed vaccine and facing the recent emergence of resistance to the artemisinin
53 derivatives, the need for new antimalarial drugs has never been greater. These new drugs must be
54 structurally unrelated to existing antimalarials and should have a new mechanism of action (1, 2).

55 In this context, our laboratory has developed a new class of antimalarial drugs that target
56 membrane biogenesis in *Plasmodium falciparum*, the most lethal malaria-causing parasite, during
57 its intraerythrocytic development. During its blood stage, *Plasmodium* synthesizes considerable
58 amounts of membranes for its growth and proliferation. These membranes differ in lipid
59 composition from those of the host. Cholesterol is almost completely absent while phospholipids
60 with phosphatidylcholine (PC) and phosphatidylethanolamine constitute the bulk of malarial
61 lipids (3-5). We designed and developed compounds which mimic the structure of choline in
62 order to target choline metabolism and thus prevent PC biosynthesis that is essential for
63 *Plasmodium* during the asexual blood stage. Over the past years, compounds were optimized for
64 *in vitro* antimalarial activity (6-8) leading to bis-thiazolium salts which inhibit *P. falciparum*
65 asexual blood stages at concentrations in the low nanomolar range (9, 10). These compounds are
66 also able to cure *in vivo* malaria infections, at very low doses, in rodent (< 1 mg/kg by
67 intraperitoneal (i.p.) route) and primate models (10). The lead compound albitiazolium (formerly
68 named T3 and SAR97276) (Fig. 1) (10) was successfully evaluated during ADME (absorption,
69 distribution, metabolism, and elimination) preclinical studies and human phase I and phase II
70 clinical trials. Due to the absence of an oral formulation to treat uncomplicated malaria,

71 albitiazolium was developed by Sanofi for the treatment of severe malaria by the intramuscular
72 route. After successful phase II trials in adult patients, trials in a pediatric population revealed
73 higher drug clearance as compared to adults without significantly changing the highest observed
74 concentration (C_{max}). The high C_{max} value did not allow increasing the dose without risking
75 potential toxic effects. This constraint stopped the clinical development of albitiazolium.
76 Nevertheless, understanding the mode of action of this drug is of utmost interest for future
77 developments.

78 The bis-thiazolium salts exert their antimalarial activity, through inhibition of *de novo* PC
79 biosynthesis, by blocking the parasite choline carrier (10-12). An additional interaction with
80 heme inside the food vacuole contributes to the antimalarial activity (13). The impressive potency
81 and specificity of these dual effectors are likely due to their ability to accumulate several
82 hundred-fold in a non-reversible way in *P. falciparum*-infected erythrocytes. Drug accumulation
83 in *in vitro* cultures is so significant that it depletes the incubation medium. Accumulation is
84 restricted to infected erythrocytes and the major part of the accumulated drug is found in the
85 intraerythrocytic parasite (10, 13, 14).

86 We observed that albitiazolium rapidly and irreversibly affects *P. falciparum* viability
87 (contact of less than 2 h at 80 nM) and condemns the parasite to death before the end of the cycle
88 (15). However, morphological changes and arrest of the cell cycle progression are apparent only
89 at the late trophozoite stage (11, 15). Here, we conducted *in vivo* studies in *P. vinckei*-infected
90 mice to quantify the level of accumulation in infected erythrocytes and to analyze whether this is
91 accompanied by depletion of albitiazolium from plasma. The results demonstrated that massive
92 accumulation also occurs *in vivo* in *P. vinckei*-infected mice and showed unexpected
93 pharmacokinetic (PK) and pharmacodynamic (PD) properties of this choline analogue revealing
94 that plasma concentrations increased with the degree of parasitemia. This unique property is

95 likely due to the release of the drug from the infected erythrocytes into the plasma, leading to the
96 recycling of pharmacologically active albitiazolium, and a subsequent extension of its
97 pharmacological activity.

98 Dissociation between the pharmacological effect and the effect on parasitemia was measured
99 using a new *in vivo* viability assay. This led us to conclude that albitiazolium exerts its
100 pharmacological activity through a Trojan horse effect leading to the rapid reduction of parasite
101 viability as a consequence of a high cytotoxic drug activity.

102

103

MATERIALS AND METHODS

104

105 **Chemicals.** Albitiazolium (albitiazolium bromide, 3,3'-dodecane-1,12-diylbis[5-(2-
106 hydroxyethyl)-4-methyl-1,3-thiazol-3-ium] dibromide) and [thiazole-2,2'-¹⁴C]albitiazolium were
107 provided by Sanofi. In the entire document, doses of albitiazolium in mg/kg are expressed in the
108 form of bis-charged compound.

109 **Animals and parasites.** Specific-pathogen-free female albino Swiss mice, weighing 22 to 26
110 g were obtained from Charles River Laboratories (Saint Aubin-les-Elbeuf, France). They were
111 kept at constant temperature (20-24°C) and 40-70% relative humidity with a standard 12 h
112 light/dark cycle. Animals were housed in stainless steel cages with suspended wire-mesh floors
113 (maximum of 9 mice per cage). The mice were fed with a standard rodent controlled pelleted diet
114 (SAFE, Epinay Sur Orge, France) and allowed free access to tap water. All animals underwent a
115 period of one week of observation and acclimatization before treatment. Mice were randomly
116 distributed in three different groups. They were infected with *P. vinckei petteri* (279 BY strain)
117 (16, 17) (I. Landau, Paris, France) maintained by weekly passage by i.p. administration.

118 This research is conform to the national and European regulations (EU directive no. 86/609
119 modified by the directive 2010/63 regarding the Protection of Animals used for Experimental and
120 Other Scientific Purposes) and the laws of the member states of the partners. The animal studies
121 were performed at the “Centre d’Elevage et de Conditionnement Experimental des Modèles
122 Animaux”, Montpellier, under permission n°. B-34-172-23 (University of Montpellier) after
123 approval by the animal experimenting commission.

124
125 **Counting of parasites.** Parasitemia was routinely monitored on thin blood smears fixed in
126 methanol and stained with Diff-Quick™ (pH 7.2) (Dade Behring, France) or by flow cytometry
127 on blood samples using Yoyo-1 iodide (491/509) (Invitrogen, France)(18). For microscopy
128 determination, at least 1000 cells were counted.

129
130 **Pharmacokinetics/pharmacodynamics study of albitiazolium.**

131 Two groups of 94 mice were inoculated intravenously with 10^7 (first group) or 10^8 (second
132 group) *P. vinckei*-infected erythrocytes (resuspended in 0.2 ml of sterile isotonic saline solution).
133 A third group of 90 mice remained uninfected. Thirty additional untreated animals were used to
134 provide pre-dose blood and tissue samples to prepare calibration curves and quality control
135 samples.

136 Mice were fasted overnight (12 h) before drug administration and then weighed. Drug
137 treatment was initiated when the parasitemia was between 0.5 to 1% (first group) and between 8
138 to 12% (second group). In each infected group, four mice (control) did not received treatment to
139 evaluate the development of parasitemia. Albitiazolium was prepared in 0.9% sterile isotonic
140 saline on the day of administration and was administered i.p. at the dose of 3.7 mg/kg. The
141 administered volume was 3.12 ml/kg (i.e., ~ 100 µl per mouse).

142 Parasitemia counting was performed on samples at each blood sampling time.

143 Blood and tissues (heart, liver and brain) were obtained at the following times: 5, 15 and 30
144 min and 1, 2, 4, 6, 11, 24 and 35 h after treatment. At each time point, 9 mice were sacrificed and
145 three pools of 3 mice prepared. Blood samples were collected in heparinized polypropylene
146 tubes. Thereafter, tubes were gently agitated to prevent coagulation and then centrifuged ($2,000 \times$
147 g) at 4°C for 15 min. The plasma was separated and immediately stored at -80°C . The cell pellet
148 was washed twice with an equal volume of 0.9% sodium chloride to limit plasma contamination
149 before storage at -80°C . Tissues were washed twice for 30 s in 0.9% sodium chloride to limit
150 blood contamination, dried on gauze, placed in a polypropylene tube and then frozen at -80°C .

151
152 **Quantification of albitiazolium in erythrocytes and plasma.** Albitiazolium was quantified
153 in red blood cells (RBC), infected red blood cells (IRBC) and plasma using a liquid
154 chromatography/electrospray ionization mass spectrometry (LC-ESI-MS) method (19). Briefly,
155 the method involved solid phase extraction using Oasis[®] HLB columns after protein precipitation.
156 LC separation was performed on a Zorbax eclipse XD8 C8 column ($5 \mu\text{m}$) with a mobile phase
157 of acetonitrile containing $130 \mu\text{l/l}$ trimethylamine and 2 mM ammonium formate buffer (pH 3).
158 MS data were acquired in single ion monitoring mode at m/z 227.3 for albitiazolium and m/z 326
159 for the internal standard. Precision was below 14% and accuracy was 91.4-104%. The lower
160 limits of quantitation were 3.65 nM in plasma, and 7.26 nM in RBC.

161
162 **Quantification of albitiazolium in tissues.** Albitiazolium concentrations were quantified in
163 tissues using the rapid resolution LC-ESI-MS method (20). Before sample pre-treatment using
164 Oasis[®] WCX cartridges, tissues were powdered under liquid nitrogen. LC separation was
165 performed on a Zorbax Eclipse XDB C8 ($1.8 \mu\text{m}$) column using a mobile phase consisting of

166 acetonitrile-trimethylamine-formate buffer at a flow rate of 1 ml/min. Assay precision was <13%
167 and accuracy was 90–107%. The lower limit of quantitation was 7.26 nmol/kg in brain and 72.6
168 nmol/kg in liver and heart.

169 Animal samples were processed with a standard curve, and quality control samples were
170 included in each analytical sequence to verify the stability of study samples during storage and
171 the accuracy and the precision of analysis.

172

173 ***In vivo* cellular accumulation ratio.** At each time of sampling, in both infected groups,
174 albitiazolium concentration was calculated in 100% infected RBC (C_{IRBC}) as follows:

$$175 \quad C_{IRBC} = [C_{RBC+IRBC} - (100 - P) \times C_{unpar}] / P$$

176 where $C_{RBC+IRBC}$ is the measured albitiazolium concentration in the erythrocyte fraction
177 (infected + uninfected); C_{unpar} is the concentration in uninfected RBC, assuming that it
178 corresponds to the concentration found in treated healthy mice; and P is the parasitemia.

179 The cellular accumulation ratio (CAR) was defined as the ratio of the concentration of
180 albitiazolium in 100% infected cells to that of albitiazolium in the same volume of plasma. The
181 CAR was also calculated in the uninfected group.

182

183 **Pharmacokinetic analysis.** PK analysis was carried out based on the average concentration
184 values at each time point using the Pk-fit software (21, 22).

185 From plasma, RBC or IRBC data, PK parameters were determined using a compartmental
186 approach. The following parameters were calculated: the total area under concentration-time
187 curves (AUC_{0-inf}), elimination half-life ($t_{1/2\ elim}$), total clearance (CL/F) and steady-state volume of
188 distribution (Vd/F). CL and Vd were uncorrected for bioavailability (F). The highest observed

189 concentration was designated C_{\max} . The time of the C_{\max} concentration relative to the time of
190 dosing was designated T_{\max} . C_{\max} and T_{\max} were observed values.

191 From tissue data, AUC and $t_{1/2 \text{ elim}}$ were calculated using a non-compartmental approach.

192
193 **Comparative pharmacokinetics of [^{14}C]albitiazolium administered in saline solution or**
194 **in loaded-IRBC.**

195 Radioactive experiments were performed in a room dedicated to radioactivity manipulation
196 at the UMR5235 (under permission n° T340403 delivered by Autorité de Sûreté Nucléaire) after
197 approval by the animal experimenting commission.

198 Naïve mice received intravenously (i.v.) [^{14}C]albitiazolium in the form of either 100 μl of
199 loaded erythrocytes (RBC + IRBC)(50% hematocrit) or 100 μl of saline solution, both either
200 containing 0.15 or 0.09 mg/kg. Mice were weighed just before the injection. Distribution of
201 albitiazolium between RBC and IRBC was evaluated. Albitiazolium is almost entirely found in
202 the IRBC which contain more than 90% of the drug in both suspensions (data not shown). This
203 means that almost all the drug released in the plasma is provided by IRBC. For this reason and
204 for clarity, we decided to use the term of “loaded-IRBC” in the text.

205 Fifteen mice were inoculated intravenously with 10^8 *P. vinckei*-IRBC (resuspended in 0.2 ml
206 of 0.9% sterile isotonic saline). When parasitemia of mice was between 20-50%, blood was
207 collected in heparinized polypropylene tubes. RPMI 1640 (Invitrogen) was added to obtain a
208 suspension at 5% hematocrit. This suspension was pre-incubated during 5 min at 37°C before
209 adding 500 μM (suspension at 29% parasitemia) or 100 μM [^{14}C]albitiazolium (suspension at
210 50% parasitemia) with a specific activity of 23.8 mCi/mmole to each tube. Suspensions were
211 incubated for 90 min at 37°C then centrifuged ($805 \times g$) at 4°C for 10 min and cells were washed

212 twice with 0.9% NaCl. Cells were resuspended in 0.9% NaCl at 50% hematocrit.
213 [¹⁴C]Albitiazolium contained in IRBC incubated at 37°C was quantified by liquid scintillation
214 counting as described below for total blood. After quantification, we determined that injection in
215 mice of 100 µl of these suspensions corresponds to a dose of 0.15 mg/kg or 0.09 mg/kg
216 albitiazolium for IRBC incubated in the presence of 500 µM or 100 µM albitiazolium,
217 respectively.

218 Blood, spleen and liver were collected at 1, 5, 15 and 30 min and 1, 2, 3, 5, 8, 11, 23, 33 and
219 46.33 h after the i.v. injection. At each time point, parasitemia and hematocrit were determined
220 for each mouse (3 mice/time point). Blood samples were collected and aliquots of blood were
221 taken to dose radioactivity in total blood, IRBC and plasma. After collection, tissues were washed
222 twice in 0.9% NaCl, dried on gauze and frozen at -80°C.

223
224 **Quantification of [¹⁴C]albitiazolium.** Albitiazolium in total blood was measured directly in
225 a scintillation vial. 100µl of total blood were lysed by the addition of 200 µl distilled water and
226 then solubilized and decolorized by the addition of 500 µl of a decolorization cocktail containing
227 5 parts of tissue solubilizer (SOLVABLE[®], Perkin Elmer), 2 parts of 30% H₂O₂, and 2 parts of
228 glacial acetic acid.

229 For IRBC, 100 µl of total blood were overlaid onto 400 µl of dibutylphtalate and were
230 centrifuged at 10,000 × g for 1 min at 4°C, sedimenting the cells below the oil. The supernatant
231 was removed and the walls of the microtube washed once with 200 µl of 0.9% NaCl. After
232 centrifugation (1 min at 10,000 × g), the washing buffer and the dibutylphtalate were discarded.
233 Pellets of cells were lysed and treated as total blood samples (see above).

234 Albitiazolium concentration was also determined in the plasma. After centrifugation of a
235 blood aliquot at 4°C for 10 min (1,800 × g) the plasma was transferred to scintillation vials.

236 Samples of total blood, IRBC and plasma were then counted by liquid scintillation counting
237 after addition of 2.5 ml of scintillation cocktail (Ultimagold®, Perkin Elmer).

238 Spleens were powdered under liquid nitrogen in scintillation vial. Then, cells were
239 completely lysed with 300 µl of H₂O, frozen/thawed and solubilized and decolorized by the
240 addition of 500 µl of the decolorization cocktail. Radioactivity was determined by liquid
241 scintillation counting after addition of 3 ml of scintillation cocktail.

242 Livers were crushed directly in 20 ml scintillation vials. Then, residues were completely
243 lysed with 1 ml of H₂O, frozen/thawed twice then solubilized and decolorized by the addition of
244 2 ml of the decolorization cocktail. Radioactivity was determined by liquid scintillation counting
245 after addition of 10 ml of scintillation liquid.

246 The quenching factor was determined for the different sample types and the values corrected
247 accordingly. PK analysis was carried out as described above.

248

249 **Pharmacological activity of albitiazolium released in mouse plasma.** Plasma samples
250 containing released albitiazolium were collected from mice that had received 0.14, 0.15 or 0.18
251 mg/kg albitiazolium injected as loaded-IRBC. IRBC had been loaded with 100µM non-
252 radioactive albitiazolium as described above but albitiazolium had been quantified by LC-MS.
253 The three batches of loaded-IRBC (batches 1, 2 and 3) contained 7.1, 6.9 and 8.6 nmoles per 200
254 µl of suspension, respectively. Naïve mice received intravenously 200 µl of these suspensions
255 (five mice per batch) corresponding to doses of 0.11, 0.10 and 0.13 mg/kg albitiazolium for
256 batches 1, 2 and 3, respectively. Blood was collected 15 min after administration and the plasma
257 fraction, containing released albitiazolium, was obtained by centrifugation of blood sample at
258 4°C for 5 min (1,800 × g). The three batches of plasma containing released albitiazolium were
259 dosed by LC-MS and contained 404, 315 and 395 nM albitiazolium for batch 1, 2 and 3,

260 respectively. Then, an independent batch of *P. vinckei*-IRBC collected from one mouse with a
261 parasitemia of 26% was incubated with the three batches of plasma containing released
262 albitiazolium or with plasma from naïve mice (control) at 10% hematocrit for 1 h at 37°C.
263 Thereafter, 200 µl of these suspensions (IRBC + plasma) were injected in naïve mice (3 per
264 batch). The development of parasitemia was followed by Giemsa stained smears in the recipient
265 mice and is a function of the number of infectious (viable) injected parasites after incubation in
266 the plasma.

267
268 **Effect of albitiazolium administration on parasitemia and parasite viability.** The number
269 of parasites visible in a blood smear after albitiazolium treatment does not necessarily correspond
270 to the number of viable parasites. To distinguish between both, we determined the number of
271 viable parasites using inoculation of treated parasites from donor mice in recipient (uninfected)
272 mice and monitored their parasitemia. Initially, we investigated the effect of a single dose of
273 albitiazolium at ring or mature stages. Donor mice were infected by 2×10^7 *P. vinckei*-infected
274 erythrocytes by i.v. route in the caudal vein on day 0 (at about 11:00 am). On day 1 (at 5:00 pm),
275 parasites were predominantly at the ring stage at 2.6% of parasitemia. On day 2 (at 5:00 am),
276 parasites were at mature stages with a parasitemia of 5.8%. At each of these stages, the donor
277 mice were treated (or not for controls) by a single dose of 7.4 mg/kg albitiazolium by i.p. route.

278 Subsequently, we compared the effect of repeated doses on mature parasites. Donor mice
279 were infected as above on day 0. On day 1, when parasites were at 2% mature stages (T_0), one
280 group of mice was not treated, the second group received a single dose i.p. of 7.4 mg/kg
281 albitiazolium at T_0 and the third group received two injections at 12 h interval. The dose at T_0
282 was 7.4 mg/kg and the dose at $T_{12\text{ h}}$ was 3.7 mg/kg to avoid a possible toxic effect of
283 albitiazolium.

284 For both experiments, blood of control and treated mice was collected at different times after
285 the treatment (two mice per point), the drug was washed off and the cells were immediately
286 injected in two recipient mice (100 μ l reconstituted blood/mouse). The parasitemia in these
287 recipient mice is a function of the number of injected infectious (viable) parasites. This number
288 was calculated on the basis of the parasite multiplication ratio (PMR), which we had observed to
289 be linear up to 30% parasitemia. The PMR over 24 h is the number of parasites on day (x)
290 divided by the number of parasites on day (x-1). As expected, the PMR was similar between
291 recipient and donor mice.

292 The parasitemia in the recipient mice just after inoculation of treated parasites was back-
293 calculated by the formula: Parasitemia of mice treated on day 1 = Parasitemia on day (x)/PMR^{(x-}
294 ¹⁾. Parasitemia of mice treated on day 2 = Parasitemia on day (x)/PMR^(x-2). The total number of
295 viable parasite in donor mice was then calculated (total blood volume of mouse \sim 7 ml/100 g of
296 body weight, and 9×10^9 RBC/ ml of total blood volume).

297
298 ***In vitro P. falciparum* culture.** *P. falciparum* 3D7 strain (MRA-102 from MR4) was
299 cultured in human RBC (Etablissement Français du Sang, Montpellier, France) at 5% hematocrit
300 in complete medium composed of RPMI 1640 medium supplemented with 25 mM HEPES buffer
301 (pH 7.4) and 10% AB⁺ human serum (23). Cultures were incubated at 37°C in a culture gas
302 chamber under a gaseous mixture of 5% CO₂, 5% O₂ and 90% N₂. For *in vitro* parasite viability
303 assays, asynchronous cultures at 2% hematocrit and 0.5% parasitemia were maintained under
304 shaking to reduce the number of RBCs invaded by more than one parasite.

305
306 ***In vitro* parasite viability assay.** Drug effects on parasite viability were determined as
307 described in Sanz *et al.* (24). IRBC were treated (or not) with albitiazolium at concentrations

308 corresponding to 3, 10 or 100 times its IC₅₀ (concentration required to inhibit the parasite
309 viability by 50%) (albitiazolium IC₅₀ = 3 nM). Culture medium with or without drug was
310 changed every 24 h during the entire treatment period. At times 0, 2, 4, 6, 24, 48, 72 and 96 h,
311 thin blood smears were realized to calculate the number of visible parasites, and samples of
312 untreated or treated parasites were taken to determine the number of viable parasites. Samples
313 were washed twice to eliminate the drug and then added to 96 well plates and 3-fold serial
314 dilutions were performed. Parasites were cultured during 21 days to reach detectable parasitemia
315 in wells started from only one viable parasite. Culture medium was changed twice a week. The
316 presence of parasites was detected by [³H]hypoxanthine incorporation during 24 h. The most
317 diluted well with significant [³H]hypoxanthine incorporation corresponds to the well containing
318 initially only one parasite after serial dilution. The numbers of viable parasites were back-
319 calculated by using the formula X^{n-1} where n is the number of wells able to render growth and X
320 the dilution factor (24).

321

322

RESULTS

323

324 **Pharmacokinetics of albitiazolium in healthy and *P. vinckei*-infected mice**

325 The goal of this study was to investigate the PK and PD properties of albitiazolium in a
326 murine malaria model following the administration of a single dose of the choline analogue to the
327 mouse. The capacity for choline analogues to clear high parasitemia in mice (10) incited us to
328 perform these experiments at both moderate and high parasitemia using a dose able to cure both
329 levels of infection. Control groups (n=4) and two groups of *P. vinckei*-infected mice with $0.9 \pm$
330 0.29% and $8.9 \pm 1.92\%$ parasitemia (n = 9) thus received a single i.p. administration of 3.7 mg/kg

331 albitiazolium. The erythrocyte fraction collected from mice was composed of either RBC (control
332 mice) or both RBC and IRBC (infected mice).

333

334 **Albitiazolium plasma concentrations are increased in infected mice.**

335 The plasma, RBC and IRBC concentration-time curves were modelled according to a two-
336 compartment model (Fig. 2A and B). The PK parameters are presented in Table 1. Following
337 albitiazolium administration, similar C_{\max} values of $\sim 8 \mu\text{M}$ were found in the plasma of the three
338 groups of mice. Plasma concentrations and AUC, however, increased with the parasitemia of *P.*
339 *vinckei*-infected mice. This increase was more important at high parasitemia (Fig. 2A, Table 1).
340 In uninfected mice, albitiazolium concentration was below the limit of quantification after 12 h,
341 but was measurable for up to 24 h and 36 h in mice with 0.9% and 8.9% parasitemia, respectively
342 (Fig. 2A). In addition, the terminal elimination half-lives in the infected mice were significantly
343 longer. The AUC ratios (infected / uninfected) of plasma indicated an increase of 15% and 69%
344 of AUC for 0.9% and 8.9%, respectively, compared to uninfected mice. In plasma, the total
345 clearance decreased with the increase of parasitemia.

346

347 **Albitiazolium accumulates in infected erythrocytes.**

348 Uninfected erythrocytes do not accumulate albitiazolium. Following albitiazolium
349 administration in uninfected mice, C_{\max} value in RBC was $0.35 \mu\text{M}$ after 15 min (Table 1) while
350 at the same time plasma concentration was $5 \mu\text{M}$. Thus the cellular accumulation ratio (CAR),
351 *i.e* the ratio of the intracellular concentration in RBC to the plasma concentration, was
352 below 1 (Fig. S1).

353 The AUC of the erythrocyte fraction increased from $0.57 \mu\text{mol}\times\text{h/l}$ in healthy mice to 1.9
354 and $11.7 \mu\text{mol}\times\text{h/l}$ in 0.9 and 8.9% infected mice, respectively. Both AUC_{IRBC} were largely

355 increased compared to AUC_{RBC} in healthy mice. The AUC_{IRBC} (deduced from $AUC_{RBC+IRBC}$ but
356 corrected from contribution of uninfected erythrocyte) were 187-190 $\mu\text{mol}\times\text{h/l}$ *i.e.* remarkably
357 similar in both 0.9 and 8.9% infected mice (Table 1). The ratios of albitiazolium content of IRBC
358 to the one of RBC (AUC_{IRBC}/AUC_{RBC}) were 335 and 331 for 0.9% and 8.9% of parasitemia,
359 respectively, *i.e.* also very similar (Fig. 2B and Table 1).

360 Remarkably, in both infected groups, the CAR in the IRBC was very high indicating an
361 important accumulation of albitiazolium in IRBC (Fig. S1). The CAR increased until 4 h after
362 drug administration with maximal values of 527 ± 126 at low parasitemia and 145 ± 14 at high
363 parasitemia (Fig. S1). Considering that the concentrations of albitiazolium in IRBC in lowly and
364 highly infected mice were comparable (Fig. 2B), the difference in the CAR values was mainly
365 attributed to the albitiazolium concentration in the plasma of the two groups of infected mice
366 (Fig. 2A).

367

368 **Tissue distribution of albitiazolium.**

369 Albitiazolium was widely distributed throughout the major organs with high concentrations
370 found in liver and heart tissue, but not in brain, in both healthy and infected mice (Fig. 2C, D and
371 E). Maximum tissue concentrations were obtained between 30 min and 1 h after administration
372 for the three organs. For the liver, a decrease in AUC was observed in the infected mice
373 compared to healthy mice and was dependent on parasitemia (Fig. 2C and Table 2).
374 AUC_{liver}/AUC_{plasma} ratios were 38 in healthy mice, 29 in 0.9% infected mice and 10 in 8.9%
375 infected mice. In heart tissue, similar concentrations were observed in all three groups of mice
376 and concentrations remained relatively stable over the 35 h study period and the elimination half-
377 life was very high (> 100 h) (Fig. 2D). For the brain, AUC in infected mice was higher than in
378 healthy mice (Fig. 2E and Table 2). However, AUC_{brain}/AUC_{plasma} ratios were 0.39, 0.46 and 0.52

379 in healthy, 0.9% and 8.9% infected mice, respectively, demonstrating that albitiazolium uptake in
380 the brain is very low.

381

382 **Effect of albitiazolium administration on parasitemia.**

383 Simultaneously, we determined the parasitemia in all infected mice treated or not with
384 albitiazolium. At 0.9% initial parasitemia, parasitemia rose to $13.8 \pm 2.2\%$ at 35 h in control mice
385 while albitiazolium treatment decreased the parasitemia to $0.42 \pm 0.08\%$ (Fig. 2F). At 8.9% initial
386 parasitemia, the treatment induced a decrease in parasitemia from 6 h on; at 35 h, parasitemia was
387 $2.4 \pm 0.3\%$, as compared to $47.3 \pm 4.6\%$ in the untreated mice (Fig. 2F).

388 Taken together, this first set of experiments showed that high accumulation of albitiazolium
389 in IRBC also occurred *in vivo* and most interestingly, that the PK profiles in plasma were
390 different in healthy and infected mice, the plasmatic AUCs being substantially increased with
391 high parasitemia.

392

393 **Albitiazolium contained in treated *P. vinckei*-IRBC is released into the plasma.**

394 The increased albitiazolium concentration in plasma of infected mice could be due to a
395 release of the accumulated drug from IRBC and/or alternatively to a lower drug uptake in the
396 liver. The first case would constitute the recycling of albitiazolium that had accumulated in
397 IRBC. We investigated this hypothesis by comparing plasma concentrations when equal doses of
398 albitiazolium were injected intravenously either as a saline solution or in the form of loaded-
399 IRBC. In the latter case, albitiazolium recovered in the plasma must have been released from
400 loaded-IRBC.

401 In two concomitant but independent experiments, [¹⁴C]-albitiazolium was administrated
402 intravenously to healthy mice at 0.15 and 0.09 mg/kg, either directly in the blood circulation as
403 saline solution or after being accumulated within IRBC collected from a set of infected mice.
404 Albitiazolium was then quantified in the different RBC compartments, plasma and spleen at
405 different time points after injection.

406 When [¹⁴C]-albitiazolium was injected directly into the blood circulation a classical plasma
407 concentration-time curve was observed (Fig. 3) with a profile similar to the one determined by
408 LC-MS in the above PK/PD experiment (Fig. 2A). When data were normalized with respect to
409 the injected dose, similar plasma C_{max} values were found. Trace levels of albitiazolium were
410 found in RBC (Fig. 3B) while concentrations were relatively high in liver (Table 3) and
411 corresponded to those detected in the PK/PD study (Table 2) after normalization (not shown).
412 Concentrations in the spleen were very low, not exceeding 0.2 μmol/kg (Fig. 3C and Table 3).

413 When [¹⁴C]albitiazolium loaded-IRBC were injected, substantial amounts of drug were
414 detected in the plasma and the kinetic profile suggested a continuous and progressive release of
415 the radioactive drug from IRBC. The plasma level of albitiazolium remained very high over time
416 as compared to the injection in saline solution (Fig. 3A). With [¹⁴C]albitiazolium loaded-IRBC
417 (Fig. 3A) an AUC increase of 3 and 1.6 times was observed after administration of 0.15 and 0.09
418 mg/kg, respectively as compared to injection of saline solution (Table 3). We observed a
419 concomitant decrease in albitiazolium levels in the total erythrocyte compartment (RBC + IRBC)
420 (Fig. 3B). The spleen whose function is to remove infected or damaged cells from the circulation,
421 showed a rapid and important increase in albitiazolium concentration in less than 3 h. The C_{max}
422 (2.1-2.5 pmol/mg) was 10 times higher than after administration of similar doses in saline
423 solution (Fig. 3C and Table 3). This strong increase in drug concentration in the spleen likely
424 reflects the capture and/or destruction of the loaded-IRBC. Compared to the injection in saline

425 solution, the spleen AUC was 20 and 193 times higher for doses of 0.15 and 0.09 mg/kg,
426 respectively (Table 3). There was no significant difference in C_{max} and AUC between saline
427 solution and loaded-IRBC administration was observed in the liver (Table 3).

428

429 **Released albitiazolium is pharmacologically active.**

430 The detection of substantial quantities of drug in plasma after administration of IRBC loaded
431 with [^{14}C]albitiazolium indicated that plasma can be supplied with the albitiazolium released
432 from loaded-IRBC (Fig. 3A). But is this recycled albitiazolium still pharmacologically active?
433 Degradation or metabolism of albitiazolium has never been observed in IRBC and albitiazolium
434 is only weakly bound by human plasma proteins (33% at 20 ng/ml) (unpublished observations),
435 suggesting that the drug remains active. Also, a part of the accumulated albitiazolium is strongly
436 bound to hemozoin (13), which could prevent drug activity.

437 We thus investigated the pharmacological activity of the released albitiazolium by measuring
438 its capacity to block the proliferation of *P. vinckei*-IRBC injected in naïve mice. Three groups of
439 mice received loaded-IRBC at 0.14, 0.15 or 0.18 mg/kg albitiazolium. Their plasma was
440 collected and the released albitiazolium content quantified. These plasma samples were mixed
441 with fresh *P. vinckei*-IRBC suspensions (10% hematocrit) resulting in final albitiazolium
442 concentration of 315, 404 and 395 nM. Albitiazolium-free plasma served as control. After 1 h of
443 incubation at 37°C, the treated IRBC were injected in naïve mice and the development of
444 parasitemia was followed. As compared to the control, parasitemia was strongly reduced (by $48 \pm$
445 3%, mean \pm sem for the three batches after 1 day) indicating that the albitiazolium released from
446 IRBC still affected parasite viability and was at least partially pharmacologically active (Fig. 4).

447

448

449 ***In vivo* albitiazolium treatment induces a rapid loss of *P. vinckei*-parasite viability.**

450 In *P. falciparum in vitro* cultures, the accumulation of albitiazolium in the intraerythrocytic
451 parasite has been shown to be a rapid process requiring less than 2 h to reach the steady state
452 (14), and being associated with a rapid irreversible toxic effect. However, morphological
453 alterations and the destruction of the parasite were observed only much later (15). It is difficult to
454 extrapolate *in vitro* PD data to *in vivo* models because both PK and PD properties of the
455 compound must be considered. However, morphological alterations of parasites seem also to be
456 delayed *in vivo*, since we observed that after albitiazolium administration to *P. vinckei*-infected
457 mice (PK/PD study), the parasitemia decreased only 6 h after the treatment (Fig. 2F). In order to
458 determine whether albitiazolium acts as rapidly *in vivo*, we designed and validated a test that
459 measures the viability of circulating parasites in mice treated with albitiazolium. IRBC taken
460 from infected and treated mice at different times after treatment were washed and then transferred
461 to healthy mice. The viability of the transferred parasites was assessed by their ability to
462 proliferate and was deduced from the parasite multiplication rate (PMR) over a 24 h period (*P.*
463 *vinckei* life cycle time). The PMR method has been validated in independent experiments
464 conducted with untreated infected mice where development of parasitemia between 1 and 30%
465 was linear and the PMR appears constant. Analysis of the parasitemia profile in the recipient
466 mice allowed tracing back the number of initially injected infectious parasites. Simultaneously,
467 the parasitemia was determined by counting unaltered parasites on Giemsa-stained thin blood
468 smears.

469 In a first experiment, two groups of *P. vinckei*-infected mice received 7.4 mg/kg
470 albitiazolium ($7 \times ED_{50}$) when the synchronous growing parasites were either in ring or 12 h
471 later, in mature stages. Analysis of thin blood smears revealed a significant decrease in
472 parasitemia only 12 h after the drug administration, for both groups. Then, the parasitemia

473 decreased slowly to ~15% of the control, 12 h later (Fig. 5A). To the contrary, the viability test
474 revealed a very rapid and drastic effect for both treatments. When treated with albitiazolium at
475 the ring stage, the number of viable parasites decreased by 22.4% after 3 h and by 99.93% after 6
476 h and did not decrease further, thereafter (Fig. 5B). When treated at the mature stages, viability
477 was affected even faster, decreasing by 43.6% after only 10 min of treatment and by 99.98% after
478 3 h, again without further decrease thereafter (Fig. 5C).

479 It can thus be concluded that albitiazolium exerts its toxic effect on 99.9% of parasites very
480 rapidly, in less than 6 hours at the ring stage and in less than 3 h at the mature stages. The fact
481 that beyond this time, there was no further decline of parasite viability indicates that at least one
482 additional albitiazolium administration would be required to kill the 2.4 to 5×10^5 of parasites
483 that escaped the first dose.

484 We therefore monitored the effect of a second dose of albitiazolium given 12 h after the first
485 administration. The first dose showed the same delayed effect on morphology and very rapid
486 irreversible toxicity (Fig. 5D and E). The second dose again eliminated 99.9% of the remaining
487 very low number of viable parasites in less than 2 h (Fig. 5E), reducing the total number of viable
488 parasites to less than 650 per mouse.

489
490 **A highly sensitive assay confirms that albitiazolium treatment also rapidly affects**
491 **parasite viability *in vitro*.**

492 Considering the difference between the very rapid effect on parasite viability and the delayed
493 appearance of morphological alterations in treated mice, we analysed this phenomenon *in vitro*
494 with *P. falciparum* parasites. The number of viable parasites after treatment was determined
495 using an assay recently described by Sanz *et al.* (24). This method is based on serial parasite

496 dilutions and shows higher sensitivity compared to the direct incorporation of [³H]hypoxanthine
497 in parasite cultures.

498 Similarly to the effects observed with *P. vinckei* in mice, *P. falciparum* morphology was
499 affected late. After one developmental cycle (48 h), parasitemia decreased by 80% for all
500 albitiazolium concentrations. After 96 h, the decrease in parasitemia was 97%, 99% and 99.98%
501 for albitiazolium concentrations of 3×, 10× and 100× its IC₅₀, respectively (Fig. 6A). On the other
502 hand, the viability assay revealed that parasites were rapidly affected (Fig. 6B and C). After only
503 6 h, the number of viable parasites already decreased by 50%, 79% and 72% for 3×, 10× and
504 100× IC₅₀, respectively. After 48 h, parasite viability was reduced by 67%, 98.8% and ≥ 99.99%
505 for 3×, 10× and 100× IC₅₀, respectively, while the number of untreated parasites tripled (Fig. 6B
506 and C). Finally, after 96 h *P. falciparum* viability was reduced by 92%, 99.98% and 100% for 3×,
507 10× and 100× IC₅₀, respectively, reflecting continuous drug action over time (Fig. 6B).

508

509

DISCUSSION

510

511 In *P. falciparum* *in vitro* cultures, the massive accumulation of albitiazolium in infected
512 erythrocytes results in a concomitant depletion of the drug from the incubation medium. In
513 infected patients the consequence could be an extremely short *in vivo* half-life of the drug or low
514 plasma concentrations. We thus investigate the fate of albitiazolium *in vivo* in the *P. vinckei*-
515 infected mouse model.

516 As observed *in vitro* in *P. falciparum*, albitiazolium rapidly and massively accumulated in *P.*
517 *vinckei*-infected erythrocytes *in vivo*, while no accumulation was observed in uninfected
518 erythrocytes (Fig. 2 and S1). This massive accumulation, however, did not lead to albitiazolium
519 depletion in plasma. Unexpectedly, albitiazolium plasma concentrations and their AUC

520 significantly increased with the level of parasitemia and the total drug clearance decreased (Table
521 1, Fig. 2). These results suggest that the plasma level of albitiazolium in infected humans would
522 be similar or even higher (depending on the parasitemia) as compared to healthy subjects. Data
523 obtained during phase I and II clinical trials showed that there was no albitiazolium depletion
524 from the plasma in *P. falciparum* infected patients. PK profiles were similar between patients and
525 healthy subjects except for a longer half-life in plasma of patients (Sanofi, unpublished data).
526 This extended elimination half-life was also observed in mice (Table 1). These results indicate
527 that the *P. vinckei* mouse model is a reliable model for exploring the PK/PD profiles of new
528 antimalarials even if we must keep in mind the shorter life cycle duration of *P. vinckei*.

529 There was a rapid and high uptake of albitiazolium in liver and heart (Fig. 2C and D). It is
530 noteworthy that albitiazolium concentrations in heart tissue remained relatively stable during the
531 35 h of the study period. These high concentrations likely reflect a rapid uptake of the drug when
532 plasma concentrations are high, followed by a slow release of the drug into the plasma (Fig. 2C,
533 2D and 7). Given that, at 35 h, plasma concentrations were low or even below the limit of
534 quantification, the elimination rate of albitiazolium from the body was thus likely controlled by
535 the slow release of the drug from the heart tissue. In contrast, there was a low uptake of
536 albitiazolium in the brain even at high plasma concentrations (Fig. 2E, Table 1 and Fig. 7). The
537 slight increase in brain AUC with parasitemia may be due to the presence/sequestration of IRBC
538 in the blood capillaries.

539 Administering IRBC loaded with [¹⁴C]albitiazolium to healthy mice showed that
540 albitiazolium plasma levels were up to 3 times higher than those observed after administration of
541 an equal dose of albitiazolium in saline solution (Fig. 3A, Table 3). The drug release
542 phenomenon was demonstrated by incubating the loaded-IRBC in vitro (3 h in RPMI 1640 /10%
543 FCS). Under these conditions, albitiazolium concentration decreased by 20 μM in the cellular

544 fraction and concomitantly increased by 15 μM in the supernatant (Fig. S2). This clearly
545 indicated that albitiazolium can be directly released from IRBC into the bloodstream and behave
546 as a drug reservoir.

547 In the spleen, the AUC increased to a much higher extent after administration of
548 albitiazolium loaded-IRBC, compared to drug injection in saline solution (Fig. 3 and Table 3).
549 This likely reflects the capture and/or destruction of the loaded-IRBC in the spleen. The amount
550 of albitiazolium in the spleen (calculated using the weight of the spleen and the albitiazolium
551 spleen exposure) of $0.027 \mu\text{mol}\times\text{h}$ was similar to the total amount of the drug in the intra- and
552 extracellular space ($0.029 \mu\text{mol}\times\text{h}$, calculated using the initial distribution volume and the
553 albitiazolium plasma exposure). The spleen was thus a substantial source of albitiazolium and
554 also constituted a reservoir that slowly released the drug into the plasma. Therefore, the observed
555 albitiazolium concentrations in the plasma seem to be the result mainly of spleen activity and, to
556 a lesser extent, of the direct release from IRBC (Fig. 7).

557 Our results presented here clearly demonstrate that released albitiazolium has antimalarial
558 activity (Fig. 4). Whether the totality or a fraction of the drug is still active is currently unknown.
559 Previous studies had shown that a part of accumulated bis-thiazolium salts is bound to hemozoin
560 (13, 25), forming a complex that could prevent the pharmacological activity of the bound
561 compound. This study revealed that the albitiazolium accumulated *in vivo*, by infected
562 erythrocytes, can be released into the plasma when the parasites die. This allows drug recycling,
563 thereby extending its pharmacological activity.

564 Treating *Plasmodium* with bis-thiazolium salts results in delayed morphological alterations
565 and in the destruction of the parasites *in vitro* (14) and *in vivo* (Fig. 2F). We therefore wanted to
566 define the timing of the irreversible damage done to the parasites through the measurement of
567 their viability early after treatment. Every marker of cell death processes such as necrosis,

568 apoptosis, mitotic catastrophe or arrest of metabolism possesses its own limit (26). The test
569 developed here quantifies the viability of parasites collected from *in vivo* treated mice by
570 measuring their ability to proliferate in healthy mice, thereby changing from morphological to
571 biological definitions of the pharmacological activity.

572 Our results showed that the pharmacological activity of albitiazolium is indeed very rapid.
573 The number of viable parasites was reduced by 99.9% in less than 6 h when treatment started at
574 the ring stage and less than 3 h for mature stages (Fig. 5A to C). A second drug administration
575 had a similar efficiency, killing 99.9% of the 0.1% parasites that had escaped the first drug pulse
576 (Fig. 5D and E).

577 Knowing the number of viable parasites after the treatment allowed us to determine the
578 parasite reduction ratio (PRR), which is the ratio of the total parasite burden of the mice at the
579 time of treatment to the count one cycle later (24 h for *P. vinckei*). This represents the fractional
580 reduction per asexual life cycle, or the killing rate (27). The PRR were found to be 1138 and
581 3380 after treatment at the ring and mature stages, respectively. After two doses at 12 h intervals,
582 the PRR was 2.8×10^6 . These high PRR are similar to those obtained for artemisinin derivatives
583 (27) indicating that albitiazolium might be suitable for short course treatments.

584 Very similar results were obtained *in vitro* with *P. falciparum* cultures. As controls we tested
585 atovaquone and dihydroartemisinin and obtained similar results to those published by Sanz *et al*
586 (24) (data not shown). Parasite viability was rapidly affected by albitiazolium treatment, and long
587 before parasite morphology was affected. The PRR was 1.4×10^4 at $100\times IC_{50}$. The viability of
588 *P. falciparum* was less rapidly affected than that of *P. vinckei*. This discrepancy could be due to
589 the different lifecycle duration (48 h for *P. falciparum* and 24 h for *P. vinckei*) and/or to the
590 difference between *in vitro* and *in vivo* experiments, *i.e.* the action of the spleen in the removal of
591 infected erythrocytes.

592 The rapid onset of the pharmacological action is likely due to the fast and irreversible
593 accumulation of albitiazolium within the intracellular parasite that is then condemned to die, even
594 though morphological effects occur only later. Thus, the parasite accumulates the drug and
595 creates a 'Trojan Horse' to its own detriment. This also means that the real pharmacological effect
596 is dissociated from the effects on parasite morphology and on the parasitemia.

597 We hypothesize that this Trojan horse effect explains why the dose leading to the total cure
598 (0.37 mg/kg) is very close to the efficient dose to decrease the parasitemia by 50% ($ED_{50} = 0.22$
599 mg/kg) after four days of treatment by i.p. route at initial parasitemia of 0.5-1% (S. Wein and H.
600 Vial, unpublished data). At higher initial parasitemia (5-10%), both the ED_{50} and complete cure
601 were 0.37 mg/kg. We explain this lack of difference by the fact that the parasites still counted
602 under the microscope are already condemned to death, thereby distorting the determination of the
603 ED_{50} value.

604 The new *in vivo* viability test that we present here discriminates fast- and slow-acting
605 antimalarials by determining the time needed to impair parasite viability. Indeed, this is a crucial
606 parameter in the development of new antimalarials and should ideally be as fast as possible. In
607 this test, PK parameters are taken into account and the effect on the viability is the result of both
608 the mode of action and the elimination half-life unlike under *in vitro* conditions. Moreover, this
609 test can be helpful to determine the minimal time of exposure to lethal drug concentrations for
610 clinical development.

611 In conclusion, we determined the fate of albitiazolium *in vivo* and the kinetics of its
612 pharmacological activity. Comparison with our clinical data showed that the *P. vinckei* infected-
613 mouse model is a reliable model to understand drug fate *in vivo* before starting phase II clinical
614 trials. Comprehensive PK/PD experiments and the new *in vivo* assay of viability could be a

615 valuable support during the development program of new antimalarials and could be used as a
616 guide for the future design of clinical studies.

617

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623

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630

631

632 **AUTHORSHIP CONTRIBUTIONS**

633 S.W. planned and designed the study, acquired and interpreted the data, and wrote the
634 manuscript; M.M., C.T.V.B, Y.B., N.T. and D.M. acquired, analyzed and interpreted the
635 data; R.C and K.W. interpreted the data and wrote the manuscript, F.B. performed PK
636 experiments and modelled the PK; L.F. designed the study; H.V. planned and designed
637 the study, and wrote the manuscript.

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FIGURE LEGENDS

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725
726 **FIGURE 1. Structure of the bis-thiazolium salt albitiazolium.** Albitiazolium has potent
727 activity against the *in vitro* growth of *P. falciparum* with an IC₅₀ of 2.3 nM (10)

728
729 **FIGURE 2. Pharmacokinetic study of albitiazolium in healthy and *P. vinckei*-infected mice.**
730 Panel A to E show albitiazolium concentration-versus-time curves after a single 3.74 mg/kg i.p.
731 administration. Concentrations in plasma (A), RBC or IRBC (B), liver (C), heart (D) and brain
732 (E) are given as mean ± SD (n=3) in healthy mice (□) or *P. vinckei*-infected mice at 0.9% (▲)
733 and 8.9% parasitemia (●). All dosages have been performed until 35 h after albitiazolium
734 administration. Data not shown on the graph(s) were below the analytical limit of quantitation
735 (3.63 nM in plasma, 7.26 nM in RBC, 7.26 nmol/kg in brain and 72.6 nmol/kg in liver and heart)
736 (F) Follow up of parasitemia in mice treated (closed symbol) or not (open symbol) with 3.74
737 mg/kg albitiazolium. Treatment was started when infected mice had an initial parasitemia of 0.9
738 (▲,△) or 8.9% (●,○). Values are given as mean ± SEM (n≥3).

739
740 **FIGURE 3. Comparative fate of albitiazolium after administration in healthy mice of**
741 **[¹⁴C]albitiazolium in saline solution or loaded in *P. vinckei*-IRBC.** Naïve mice received by
742 intravenous route 0.15 mg/kg of [¹⁴C]albitiazolium either in 0.9% NaCl (▲) or loaded in IRBC
743 (□). At different time points after the injection, mice were sacrificed and plasma (A), RBC or
744 RBC + IRBC (B), and spleen (C) were collected and albitiazolium concentration was determined.
745 Data presented are from one representative experiment and are expressed as mean ± SEM (n=3).

746
747

748 **FIGURE 4. Evolution of parasitemia in mice infected with *P. vinckei*-IRBC pre-treated with**
749 **plasma containing released albitiazolium.** Mice received 0.14 (A), 0.15 (B) or 0.18 (C) mg/kg
750 of albitiazolium injected as loaded-IRBC. After 15 min, plasma were collected and dosed for
751 released albitiazolium concentration. Then, a new batch of 10^8 *P. vinckei*-IRBC was incubated
752 for 1 h at 37°C in the presence of naïve plasma or of collected plasma containing 315 (A), 404
753 (B) or 395 (C) nM released albitiazolium. These parasite preparations were then injected
754 intravenously into healthy mice and the development of the parasitemia was followed by thin
755 blood smears. Parasitemia was expressed as percent of control and data are mean \pm SEM (n=3).

756
757 **FIGURE 5. Comparative effect of albitiazolium on parasitemia and on parasite viability in**
758 **mice.** (A) Follow up of donor mice parasitemia in absence of treatment (■) or after
759 administration of 7.4 mg/kg albitiazolium at ring (▲) and mature (▼) stages. (B and C) Follow
760 up of the number of parasites in the donor mouse treated at ring (B) or mature (C) stages. The
761 number of visible parasites per mouse was determined by counting thin blood smears (open
762 symbols and dotted lines). The number of viable parasites (closed symbols and solid lines) was
763 experimentally determined by measuring their capacity to proliferate in a second set of mice
764 (recipient mice) (untreated (■,□), treated at the ring stage (▲,△), treated at mature stages
765 (▼,▽). (D) Follow up of parasitemia of untreated (■) or treated donor mice with 7.4 mg/kg
766 albitiazolium (●) or 7.4 then 3.7 mg/kg albitiazolium (◆) at mature stages. Arrows indicate
767 albitiazolium administrations. (E) Number of visible (open symbols and dotted lines) and viable
768 (closed symbols and solid lines) parasites per mouse after no (■,□), one (●,○) or two (◆,◇)
769 administrations of albitiazolium. For all experiments, two mice per time point were used.

770

771 **FIGURE 6. Effect of albitiazolium on *P. falciparum* parasite viability.** The study was carried
772 out in the context of the Sanz *et al.* paper (24) (A) Follow up of parasitemia of untreated (●) or
773 treated cultures with an albitiazolium concentration corresponding to 3× (■), 10× (▲) or 100×
774 (▼) its IC₅₀. (B) Effect of albitiazolium on parasite viability expressed as percent of initial
775 inoculum. (C) Follow up of the number of parasites per 100 μl of culture. The number of visible
776 parasites per 100 μl (open symbols and dotted lines) was determined by counting thin blood
777 smears of treated or untreated cultures. The number of viable parasites (closed symbols and solid
778 lines) was calculated using serial dilutions of treated or untreated cultures. Results are expressed
779 as mean ± SEM (n=3) of one representative experiment.

780
781 **FIGURE 7. Schematic representation of distribution and fate of albitiazolium in non-**
782 **infected and *P. vinckei*-infected mice.** The exposition of mice to albitiazolium is represented by
783 their AUC. AUC of plasma, RBC and IRBC are in μmol × h/l. AUC of organs are in μmol ×
784 h/kg. (*) AUC of spleen was obtained after administration of 0.09 mg/kg [¹⁴C]albitiazolium and
785 recalculated for a dose of 3.74 mg/kg. The flow of albitiazolium in the body, based on the PK
786 profiles, is represented by red arrows (high for solid arrows and low for dashed arrows).

787 **TABLES**

788

789 **TABLE 1. Pharmacokinetic parameters in blood after intraperitoneal administration of 3.74 mg/kg (8.2 μ mol/kg) albitiazolium.**

790 Pharmacokinetic 791 parameters	Uninfected		Parasitemia 0.9%			Parasitemia 8.9%		
	mice							
	RBC	plasma	RBC+IRBC	IRBC	plasma	RBC+IRBC	IRBC	plasma
793 C_{max} , μ M	0.35	8.05	0.86	64.24	8.73	3.48	39.82	8.22
794 T_{max} , h	0.25	0.083	0.083	0.083	0.083	0.50	0.50	0.083
795 AUC_{0-inf} , μ mol \times h/l	0.57	2.49	1.9	190.74	2.86	11.73	188.54	4.20
796 AUC ratio ^a	-	-	3.3	335	1.15	20.6	331	1.69
797 $t_{1/2\ elim}$, h	7.9	4.52	8.01	8.72	9.05	8.8	37.7	8.8
798 CL/F, l/h/kg	-	3.30	-	-	2.84	-	-	1.95
799 Vd/F, l/kg	-	21.5	-	-	37.1	-	-	24.7

800 C_{max} , the highest observed concentration; T_{max} , the time of the C_{max} concentration relative to the time of dosing; AUC_{0-inf} , total801 area under concentration-time curves; $t_{1/2\ elim}$, elimination half-life; CL/F, total clearance; and Vd/F, steady-state volume of802 distribution. ^aInfected / uninfected

803

804 **TABLE 2. Pharmacokinetic parameters in tissues.**

805	Pharmacokinetic		Uninfected	Parasitemia 0.9%	Parasitemia 8.9%
806	parameters		mice		
807	AUC _{0-inf} ,	Liver	93.5	82.5	42.0
808	μmol×h/kg	Brain	0.98	1.32	2.19
809		Heart	220 ^b	262 ^b	204 ^b
810	t _{1/2 elim} , h	Liver	9.0	14.3	11.5
811		Brain	4.91	10.80	8.00

812 AUC_{0-inf}, total area under concentration-time curves; t_{1/2 elim}, elimination half-life. ^bAUC_{0-35 h}

813

814 **TABLE 3. Kinetic parameters after administration of albitiazolium directly in the blood circulation in saline solution or after**
 815 **being accumulated within IRBC**

		Saline solution			Loaded-IRBC		
Dose injected		C _{max}	T _{max}	AUC	C _{max}	T _{max}	AUC
		(μ M)	(h)	(μ mol \times h/l)	(μ M)	(h)	(μ mol \times h/l)
0.15 mg/kg*	(I)RBC	0.059	0.017	0.51	3.91	0.083	28.21
	Plasma	1.27	0.017	0.64	0.33	0.083	1.92
0.09 mg/kg*	(I)RBC	0.018	0.5	blq	1.326	0.25	5.00
	Plasma	0.117	0.083	0.40	0.221	0.083	0.64
		(μ mol/kg)	(h)	(μ mol \times h/kg)	(μ mol/kg)	(h)	(μ mol \times h/kg)
0.15 mg/kg*	Spleen	0.2	0.017	13	2.1	1	269
	Liver	nd	nd	nd	nd	nd	nd
0.09 mg/kg*	Spleen	0.04	0.083	1	2.5	2	193
	Liver	2.2	0.5	17	1.5	2	21

828 C_{max}, the highest observed concentration; T_{max}, the time of the C_{max} concentration relative to the time of dosing; AUC: area under concentration-time
 829 curves; (I)RBC: RBC + IRBC; nd: not determined; blq: below the limit of quantification. * 0.15 mg/kg corresponds to 0.33 μ mol/kg and 0.09 mg/kg to
 830 0.2 μ mol/kg













