

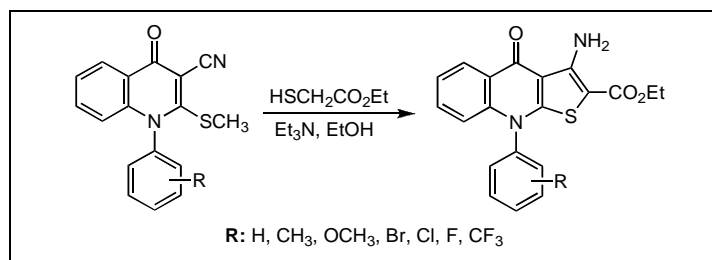
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A series of thieno[2,3-*b*]quinolone derivatives were synthesized and investigated for their abilities to inhibit β -hematin formation, hemoglobin hydrolysis and *in vivo* for their efficacy in rodent *Plasmodium berghei*. Compound **3b** was the most promising as inhibitor of hemoglobin hydrolysis, and its effects as inhibitor of β -hematin formation was promising. When the aromatic ring was substituted in 2 (Me), in 3 (CF₃) or in 2,4 (Cl) the inhibition of hemoglobin proteolysis was maximal (88%), the rest of compounds maintained a low inhibition. The most active compound to emerge *in vitro* and in murine studies, was **3b** suggesting an antimalarial activity *via* multiple mechanisms.

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INTRODUCTION

Malaria is one of the most important infectious disease problems of humans, particularly in tropical regions of the world. As per WHO reports, annually there are more than 1 million deaths and 500 million new cases, resulting in at least 1-2 million deaths [1,2]. In addition, since resistance to currently used antimalarials is spreading rapidly, there is a great need for new effective drugs. Thus, there is a compelling and urgent need for new antimalarials with mechanisms of action different from those of existing ones in order to replace those that are becoming obsolete and to identify new drug targets [3]. Chloroquine has recently been shown to inhibit hemozoin formation within the parasite food vacuole [4]. This process is also thought to be the molecular target of other quinoline antimalarials [5]. Hemozoin was originally considered to be formed by polymerization of heme [6], but it has now been demonstrated to be a crystalline cyclic dimer of ferriprotoporphyrin IX [7]. Thus, hemozoin synthesis, a process unique to the malaria parasite, offers a logical and valuable potential target for new antimalarial drug development. New drugs that attack the same vital target of chloroquine but that are not subject to the same resistance mechanism would be highly desirable.

Fluoroquinolones, such as ciprofloxacin, gatifloxacin, moxifloxacin and trovafloxacin have been reported for their antimalarial activities [8]. We have recently described the preparation and antimalarial activities of several tricyclic quinolone analogs [9]. In continuation of our studies directed toward synthesis of quinolones annelated with various five and six member heterocycles, we report here the synthesis of thienoquinolones and their abilities to inhibit β -hematin formation and hemoglobin hydrolysis *in vitro* and *in vivo* for their efficacy in rodent *Plasmodium berghei*.

RESULTS AND DISCUSSION

We have prepared (2E) ketene S,N-acetals **1a-k** by reaction of *o*-chlorobenzoylacetone with phenyl isothiocyanate respective, methyl iodide and potassium hydroxide in 1,4-dioxane [10]. The configurations of the compounds **1a-k** were determined by ¹H NMR and that of **1a** confirmed by X-ray crystallography [11] (Figure 1). The (2E) ketene S,N-acetals **1a-k** were mixed with potassium carbonate and irradiated without solvent in a domestic microwave oven to afford compounds **2a-k** [12]. The target compounds were easily recovered by adding water to the final substrate, removing the solid products by filtration, and recrystallization from ethanol-water 1:1

to give pure samples. The identities of compounds **2a-k** were established by comparison of their physical and spectroscopic properties with those reported in the literature [10,12]. Products **3a-k** were obtained when **2a-k**, were reacted with ethyl 2-mercaptoacetate, triethylamine in dry ethanol under an inert atmosphere of nitrogen (Scheme I).

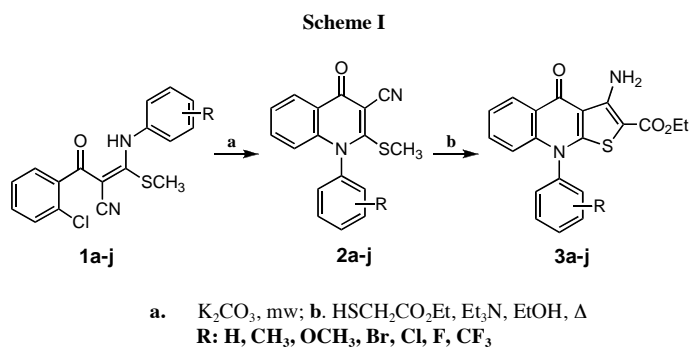
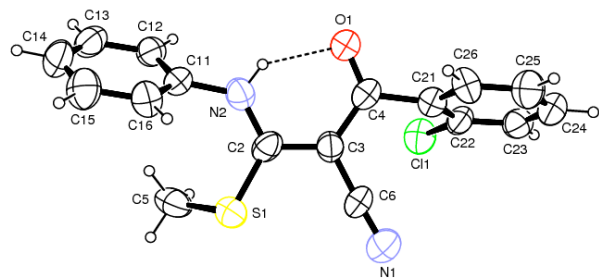


Figure 1. Molecular structure of compound **1a**. The intramolecular hydrogen bond is indicated by a dashed line. The displacement parameters are drawn at 50% probability.



Eleven analogs of *N*-phenylthieno[2,3-*b*]quinolone derivatives were tested *in vitro* for their activity against cultured of β -hematin formation and hemoglobin hydrolysis and *in vivo* for their efficacy in rodent *Plasmodium berghei* (Table 1, 2). The *in vitro* assay was used to assess the abilities of the *N*-phenylthieno[2,3-*b*]quinolone derivatives to inhibit β -hematin formation. In that assay, hemin was allowed to form β -hematin under acidic conditions. Among the eleven compounds tested, ten showed no measurable activity; only compound **3b** inhibited β -hematin formation to (94%) compared to chloroquine (87%), and inhibited hemoglobine proteolysis by (75%).

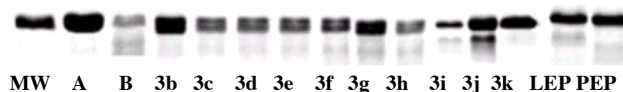
Consequently compounds **3b-k** were tested for inhibition of globin proteolysis, in an *in vitro* assay which uses rich extract of trophozoite to digest the native hemoglobin of mice. Electrophoretic analyses indicated that compounds **3b, j, k** were effective as inhibitors of hemoglobin degradation 74.42, 88.61, 84.53% respectively (band at 14.4 KDa) (Figure 2).

Table 1

Inhibition of β -hematin synthesis (IBHS) and globin proteolysis (IPG) by quinolone derivatives. The results are expressed by the mean \pm standard error of the mean. $\dagger p > 0.05$ compared to chloroquine (CQ). $* p > 0.05$ compared to leupeptin (LEP). $** p < 0.05$ compared to LEP and pepstatin (PEP).

No	R	% IBHS	% IPG
3a	H	< 5	26.84 \pm 1.15
3b	2'-Me	94.2 \pm 0.64 \dagger	74.42 \pm 1.46
3c	4'-Me	< 5	26.52 \pm 1.16
3d	3'-OMe	< 5	28.55 \pm 1.03
3e	4'-OMe	< 5	30.43 \pm 1.16
3f	3',4'-OMe	< 5	30.12 \pm 1.67
3g	4'-Br	< 5	55.89 \pm 1.55
3h	4'-Cl	< 5	11.81 \pm 1.51
3i	4'-F	< 5	22.25 \pm 2.96
3j	2',4'-Cl	< 5	88.61 \pm 1.26 $**$
3k	3'-CF ₃	< 5	84.53 \pm 1.1 $*$
LEP		-----	89.06 \pm 0.69
PEP		-----	92.94 \pm 0.66
CQ		86.6 \pm 2.75	-----

Figure 2. Effects on globin proteolysis by quinolone derivatives.



Standard Molecular Weight (MW) is expressed in kilodaltons (14.4 kDa). A: Under graded globin (control hemoglobin without trophozoites). B: Control hemoglobin with trophozoites of *P. berghei*. **3b-k**: quinolone derivatives. LEP: hemoglobin with trophozoites and leupeptin. PEP: hemoglobin with trophozoites and pepstatin.

Compound **3b** was tested in mice infected with *P. berghei* ANKA, a chloroquine-susceptible strain of murine malaria. Mice were given the compound (chloroquine or **3b** in 20 mg kg⁻¹, i.p. once daily) for 4 consecutive days (days 0-4 post infection). At day fourth post infection, the parasitemia was determined; the survival days were monitored and compared with control mice receiving saline (untreated mice). Control mice died between days 10 post-infection, compound **3b** increased the survival time for 24 days, while chloroquine prolonged the survival time of the infected mice to 30 days. Compound **3b** was able to reduce and delay the progression of malaria 6.2% but did not eradicate the infection (Table 2). Compounds **3b, j, k** were not tested as specific proteases inhibitors *in vitro*. The mechanism of action of these compounds on hemoglobin degradation could be related to the inhibition of some aspartic, cysteine or metalloproteases due to the presence of globin band. The activity of compound **3b** on hemoglobin degradation and β -hematin formation could relate the

mechanism of the action of **3b** with the blockade of hemoglobinolytic proteases and inhibition of β -hematin formation.

Table 2

Effect of quinolone derivatives (20mg/kg) on parasitemias at fourth day post-infection (%P) and survival days (SD) of *P. berghei* infected-mice. The results are expressed by the mean \pm standard error of the mean. * $p < 0.05$ and ** $p < 0.01$ compared to untreated mice (saline). n=5

Treatment	%P	SD
Saline	21.8 \pm 2.31	11.66 \pm 1.66
3b	6.2 \pm 3.88 *	23.42 \pm 2.65 **
3j	11.2 \pm 3.21 *	11.6 \pm 2.69
3k	16.6 \pm 3.75	11 \pm 1.67
CQ	1.3 \pm 0.3	30

In conclusion, we have described the syntheses and evaluation of a series of *N*-phenylthieno[2,3-*b*]quinolone derivatives, where the intermediates **2a-k** were prepared by using a microwave device. The solvent-free reaction using microwave proceeds with significant decreases in reaction times, and comparable high chemical yield [12]. From this evaluation, we could say that ring system thieno[2,3-*b*]quinolones 9-substituted seems to be a new class of potential antimalarial compounds. As shown for compounds **3d-f**, the presence of strong electron-releasing (OMe) groups results in complete loss of activity at the maximum concentration used for both experiments.

The halogen substituted compounds **3g-k** also have good activities and selectivities against the blockage of hemoglobinolytic proteases *in vitro*. The introduction of a methyl group in position 2 of the aryl **3b** had an increasing effect on antimalarial activity, which would be imposed by two different mechanism of action. The active species may still be working as protease inhibitors in their antimalarial role, but inhibiting a parasitic enzyme. A different regimen of drug treatment might be more effective in order to produce best results *in vivo* assays. The relatively short and easy syntheses of these molecules make them potential candidates for further development.

EXPERIMENTAL

Melting points were determined on a Thomas micro hot stage apparatus and are uncorrected. Infrared spectra were determined as KBr pellets on a Shimadzu model 470 spectrophotometer. The ^1H NMR spectra were recorded using a Jeol Eclipse 270 MHz spectrometer. Chemical shifts are expressed relative to residual chloroform. Elemental analyses were performed by Central Service of Research, Universidad de Málaga, Málaga, Spain; results were within $\pm 0.4\%$ of predicted values for all compounds. Chemical reagents were obtained from Aldrich Chemical Co. USA. All solvents were distilled and dried with the usual desiccant. *N*-phenylquinolone 2,3-substituted **2a-k** were obtained by following the method previously reported [12].

General Procedure for the Synthesis of Ethyl 3-Amino-9-Phenylthieno[2,3-*b*]4-Quinolone-2-Carboxylates (3a-k**).** A

mixture of the appropriate quinolones (1.3 mmol), ethyl mercaptoacetate (1.3 mmol), and ethylamine (3 mmol) in dry ethanol 10 ml was refluxed for 5 h. The solvent was evaporated to dryness under reduced pressure, water was added 10 ml and the solid thus obtained was collected by filtration. Further purification was accomplished by recrystallization from ethanol-water (4/1).

Ethyl 3-amino-4-oxo-9-phenyl-4,9-dihydrothieno[2,3-*b*]quinoline-2-carboxylate (3a**).** Yield 68%, mp 215-217 $^\circ$; ir: 3479 (NH $_2$), 1732 (C=O), 1691 (C=O) cm $^{-1}$; ^1H nmr: δ 1.27(t, 3H, CH $_3$, J=7.2 Hz), 4.35(c, 2H, CH $_2$, J=7.2 Hz), 6.79(d, 1H, 8-H, J=8.5 Hz), 7.28-7.63(m, 7H, phenyl protons), 8.46(dd, 1H, 5-H, J=8.7, 1.5 Hz); ^{13}C nmr: 14.7, 60.1, 89.5, 113.5, 115.8, 123.6, 124.6, 126.9, 128.7, 131.1, 132.7, 132.9, 137.3, 141.6, 153.3, 158.6, 165.2, 175.2. *Anal.* Calcd. for C $_{20}$ H $_{16}$ N $_2$ O $_3$ S: C, 65.92; H, 4.43; N, 7.69. Found: C, 66.03; H, 4.48; N, 7.49.

Ethyl 3-amino-4-oxo-9-(2-methylphenyl)-4,9-dihydrothieno[2,3-*b*]quinoline-2-carboxylate (3b**).** Yield 55%, mp 178-180 $^\circ$; ir 3475 (NH $_2$), 1725 (C=O), 1695 (C=O) cm $^{-1}$; ^1H nmr: δ 1.27(t, 3H, CH $_3$, J=7.2 Hz), 1.99(s, 3H, CH $_3$), 4.23(c, 2H, CH $_2$, J=7.2 Hz), 6.72(d, 1H, 8-H, J=8.2 Hz), 7.28-7.56(m, 6H, phenyl protons), 8.5(dd, 1H, 5-H, J=8.7, 1.48 Hz); ^{13}C nmr: 14.7, 17.1, 60.1, 89.5, 113.5, 115.8, 123.6, 124.6, 126.9, 128.7, 131.1, 132.7, 132.8, 132.9, 136.8, 137.3, 141.6, 153.3, 158.6, 165.2, 175.2. *Anal.* Calcd. for C $_{21}$ H $_{18}$ N $_2$ O $_3$ S: C, 66.65; H, 4.79; N, 7.40. Found: C, 66.51; H, 4.83; N, 7.36.

Ethyl 3-amino-4-oxo-9-(4-methylphenyl)-4,9-dihydrothieno[2,3-*b*]quinoline-2-carboxylate (3c**).** Yield 60%, mp 221-223 $^\circ$; ir 3480 (NH $_2$), 1735 (C=O), 1700 (C=O) cm $^{-1}$; ^1H nmr: δ 1.26(t, 3H, CH $_3$, J=7.2 Hz), 2.51(s, 3H, CH $_3$), 4.22(c, 2H, CH $_2$, J=7.2 Hz), 6.85(d, 1H, 8-H, J=7.9 Hz), 7.28(d, 2H, 2'- and 6'-H, J:8.2 Hz), 7.35(t, 1H, 6-H, J:8.0 Hz), 7.46(d, 2H, 3' and 5'-H, J:8.2 Hz), 7.51(m, 1H, 7-H), 8.46(d, 1H, 5-H, J:8.1 Hz); ^{13}C nmr: 14.7, 21.5, 59.9, 89.7, 113.4, 116.4, 123.4, 124.6, 126.6, 128.1, 131.7, 132.5, 136.1, 141.3, 142.3, 153.5, 159.6, 164.6, 175.2. *Anal.* Calcd. for C $_{21}$ H $_{18}$ N $_2$ O $_3$ S: C, 66.65; H, 4.79; N, 7.40. Found: C, 66.73; H, 4.76; N, 7.53.

Ethyl 3-amino-4-oxo-9-(3-methoxyphenyl)-4,9-dihydrothieno[2,3-*b*]quinoline-2-carboxylate (3d**).** Yield 38%, mp 234-236 $^\circ$; ir 3485 (NH $_2$), 1735 (C=O), 1695 (C=O) cm $^{-1}$; ^1H nmr: δ 1.27(t, 3H, CH $_3$, J=7.4 Hz), 3.85(s, 3H, OCH $_3$), 4.23(c, 2H, CH $_2$, J=7.4 Hz), 6.89(d, 1H, 8-H, J=8.3 Hz), 6.93(s, 1H, 2'-H), 7.00(d, 1H, 4'-H, J=7.2 Hz), 7.16(dd, 1H, 6'-H, J=8.1, 2.0 Hz), 7.35(t, 1H, 6-H, J= 7.2 Hz), 7.48(t, 1H, 7-H, J=7.2 Hz), 7.57(t, 1H, 5'-H, J=7.2 Hz), 8.46(d, 1H, 5-H, J=8.1 Hz); ^{13}C nmr: 14.7, 55.8, 59.9, 90.3, 113.2, 113.7, 116.4, 120.3, 123.5, 124.7, 126.6, 126.7, 131.9, 132.6, 139.6, 142.1, 153.2, 159.8, 161.66, 164.6, 175.2. *Anal.* Calcd. for C $_{21}$ H $_{18}$ N $_2$ O $_4$ S: C, 63.94; H, 4.60; N, 7.10. Found: C, 63.85; H, 4.57; N, 7.29.

Ethyl 3-amino-4-oxo-9-(4-methoxyphenyl)-4,9-dihydrothieno[2,3-*b*]quinoline-2-carboxylate (3e**).** Yield 53%, mp 238 $^\circ$; ir 3480 (NH $_2$), 1725 (C=O), 1685 (C=O) cm $^{-1}$; ^1H nmr: δ 1.26(t, 3H, CH $_3$, J=7.2 Hz), 3.92(s, 3H, OCH $_3$), 4.22(c, 2H, CH $_2$, J=7.2 Hz), 6.86(d, 1H, 8-H, J=8.2 Hz), 7.14(d, 2H, 2'- and 6'-H, J=8.8 Hz), 7.33(d, 1H, 4'- and 5'-H, J=8.8 Hz), 7.35(t, 1H, 6-H, J=7.2 Hz), 7.50(t, 1H, 7-H, J=7.2, 1.5 Hz), 8.46(dd, 1H, 5-H, J=8.1, 2.4 Hz); ^{13}C nmr: 14.7, 55.8, 59.9, 89.3, 113.4, 116.9, 116.4, 123.4, 124.6, 126.6, 129.6, 131.2, 132.5, 142.5, 153.5, 159.5, 161.0, 164.6, 175.2. *Anal.* Calcd. for C $_{21}$ H $_{18}$ N $_2$ O $_4$ S: C, 63.94; H, 4.60; N, 7.10. Found: C, 63.92; H, 4.72; N, 7.17.

Ethyl 3-amino-4-oxo-9-(3,4-dimethoxyphenyl)-4,9-dihydrothieno[2,3-*b*]quinoline-2-carboxylate (3f**).** Yield 47%, mp 250-

252^o; ir 3475 (NH₂), 1715 (C=O), 1695 (C=O) cm⁻¹; ¹H nmr: δ 1.29(t, 3H, CH₃, J=7.2 Hz), 3.89(s, 3H, OCH₃), 4.02(s, 3H, OCH₃), 4.24(c, 2H, CH₂, J=7.2 Hz), 6.87(s, 1H, 2'-H), 6.91(d, 1H, 8-H, J=8.2 Hz), 7.01(d, 1H, 5'-H, J=7.9 Hz), 7.10(dd, 1H, 6'-H, J=7.9, 2.3 Hz), 7.37(t, 1H, 6-H, J=7.2 Hz), 7.52(t, 1H, 7-H, J=7.2 Hz), 8.48(dd, 1H, 5-H, J=8.7, 1.4 Hz); ¹³C nmr: 14.7, 55.8, 56.3, 56.4, 59.9, 89.5, 112.2, 113.2, 120.3, 116.4, 123.5, 124.7, 126.6, 131.2, 132.6, 138.4, 142.7, 150.7, 150.9, 153.2, 158.9, 164.9, 175.3. *Anal.* Calcd. for C₂₂H₂₀N₂O₃S: C, 62.25; H, 4.75; N, 6.60. Found: C, 62.41; H, 4.77; N, 6.76.

Ethyl 3-amino-4-oxo-(4-bromophenyl)-4,9-dihydrothieno[2,3-b]quinoline-2-carboxylate (3g). Yield 39%, mp 278-280^o; ir 3480 (NH₂), 1725 (C=O), 1685 (C=O) cm⁻¹; ¹H nmr: δ 1.27(t, 3H, CH₃, J=6.9 Hz), 4.24(c, 2H, CH₂, J=6.9 Hz), 6.82(d, 1H, 8-H, J=8.3 Hz), 7.34(d, 2H, 2'- and 6'-H, J=8.2 Hz), 7.36(t, 1H, 6-H, J=7.2 Hz), 7.50(t, 1H, 7-H, J=7.2 Hz), 7.82(d, 2H, 4'- and 5'-H, J=8.2 Hz), 8.45(dd, 1H, 5-H, J=8.7, 1.5 Hz); ¹³C nmr: 14.7, 59.9, 89.6, 113.6, 116.0, 123.8, 124.6, 126.8, 128.4, 130.3, 132.8, 134.6, 137.5, 141.9, 154.0, 158.4, 164.5, 175.1. *Anal.* Calcd. for C₂₀H₁₅BrN₂O₃S: C, 54.19; H, 3.41; N, 6.32. Found: C, 53.97; H, 3.67; N, 6.46.

Ethyl 3-amino-4-oxo-9-(4-chlorophenyl)-4,9-dihydrothieno[2,3-b]quinoline-2-carboxylate (3h). Yield 43%, mp 268-270^o; ir 3475 (NH₂), 1725 (C=O), 1700 (C=O) cm⁻¹; ¹H nmr: δ 1.27(t, 3H, CH₃, J=7.1 Hz), 4.21(c, 2H, CH₂, J=7.1 Hz), 6.82(d, 1H, 8-H, J=8.2 Hz), 7.35(t, 1H, 6-H, J=7.3 Hz), 7.40(d, 2H, 2'- and 6'-H, J=8.4 Hz), 7.51(t, 1H, 7-H, J=7.2 Hz), 7.66(d, 2H, 4'- and 5'-H, J=8.4 Hz), 8.44(dd, 1H, 5-H, J=8.7, 1.5 Hz); ¹³C nmr: 14.7, 59.9, 89.1, 113.6, 116.0, 123.7, 124.6, 126.8, 130.0, 131.6, 132.8, 137.0, 139.7, 142.0, 153.6, 158.6, 164.5, 175.1. *Anal.* Calcd. for C₂₀H₁₅ClN₂O₃S: C, 60.22; H, 3.79; N, 7.02. Found: C, 60.17; H, 3.76; N, 7.17.

Ethyl 3-amino-4-oxo-9-(4-fluorophenyl)-4,9-dihydrothieno[2,3-b]quinoline-2-carboxylate (3i). Yield 37%, mp 210-212^o; ir 3475 (NH₂), 1715 (C=O), 1695 (C=O) cm⁻¹; ¹H nmr: δ 1.27(t, 3H, CH₃, J=6.9 Hz), 4.23(c, 2H, CH₂, J=6.9 Hz), 6.81(d, 1H, 8-H, J=8.4 Hz), 7.34-7.45(m, 5H, phenyl protons), 7.51(t, 1H, 7-H, J=7.8 Hz), 8.45(dd, 1H, 5-H, J=7.8, 1.5 Hz); ¹³C nmr: 14.7, 59.9, 89.3, 113.6, 116.0, 118.4, 123.8, 124.6, 126.8, 130.7, 132.7, 134.5, 142.2, 153.5, 158.9, 163.6, 164.5, 175.1. *Anal.* Calcd. for C₂₀H₁₅FN₂O₃S: C, 62.82; H, 3.95; N, 7.33. Found: C, 63.01; H, 3.83; N, 7.43.

Ethyl 3-amino-4-oxo-9-(2,4-dichlorophenyl)-4,9-dihydrothieno[2,3-b]quinoline-2-carboxylate (3j). Yield 34%, mp 196-198^o; ir 3485 (NH₂), 1725 (C=O), 1695 (C=O) cm⁻¹; ¹H nmr: δ 1.28(t, 3H, CH₃, J=7.1 Hz), 4.23(c, 2H, CH₂, J=7.1 Hz), 6.69(d, 1H, 8-H, J=8.4 Hz), 7.39(m, 1H, 6-H), 7.46-7.59(m, 3H, phenyl protons), 7.75(d, 1H, 3'-H, J=2.2 Hz), 8.48(dd, 1H, 5-H, J=8.7, 1.5 Hz). ¹³C nmr: 14.7, 60.3, 89.9, 113.8, 115.3, 123.9, 124.6, 127.0, 129.9, 131.6, 132.1, 133.0, 134.3, 134.8, 137.9, 141.2, 153.2, 157.9, 164.4, 175.2. *Anal.* Calcd. for C₂₀H₁₄Cl₂N₂O₃S: C, 55.44; H, 3.26; N, 6.47. Found: C, 55.48; H, 3.29; N, 6.51.

Ethyl 3-amino-4-oxo-9-(3-trifluoromethylphenyl)-4,9-dihydrothieno[2,3-b]quinoline-2-carboxylate (3k). Yield 48%, mp 274-276^o; ir 3475 (NH₂), 1725 (C=O), 1685 (C=O) cm⁻¹; ¹H nmr: δ 1.28(t, 3H, CH₃, J=7.4 Hz), 4.23(c, 2H, CH₂, J=7.4 Hz), 6.31(d, 1H, 8-H, J=8.4 Hz), 7.01-7.39(m, 6H, phenyl protons), 7.95(dd, 1H, 5-H, J=7.7, 1.5 Hz); ¹³C nmr: 14.7, 59.9, 89.8, 115.2, 115.3, 116.8, 117.0, 120.8, 122.8, 125.6, 125.7, 131.2, 132.5, 132.7, 135.6, 135.7, 142.2, 153.8, 160.3, 164.3, 176.4. *Anal.* Calcd. for C₂₁H₁₅F₃N₂O₃S: C, 58.33; H, 3.50; N, 6.48. Found: C, 58.47; H, 3.53; N, 6.23.

Biological assays

Inhibition of β-hematin formation. The β-hematin formation assay was performed according to [13], briefly, a solution of hemin chloride (50 μL, 4mM), dissolved in DMSO (5.2 mg/mL), was distributed in 96-well micro plates. Different concentrations (50-5 mM) of the compounds dissolved in DMSO, were added in triplicate in test wells (50 μL). Controls contained either water (50 μL) or DMSO (50 μL). β-hematin formation was initiated by the addition of acetate buffer (100 μL 0.2 M, pH 4.4). Plates were incubated at 37 °C for 48 h to allow for completion of the reaction and centrifuged (4000 RPM x 15 minutes, IEC-CENTRA, MP4R). After discarding the supernatant, the pellet was washed twice with DMSO (200 μL) and finally, dissolved in NaOH (200 μL, 0.2 N). The solubilized aggregates were further diluted 1:2 with NaOH (0.1 N) and absorbance recorded at 405 nm (Micro plate Reader, BIORAD-550). The results were expressed as a percentage of inhibition of β-hematin formation.

Parasite, experimental host and strain maintenance. Male Balb-C mice, weighing 18-22 g were maintained on a commercial pellet diet and housed under conditions approved by Ethics Committee. *Plasmodium berghei* (ANKA strain chloroquine sensible), a rodent malaria parasite, was used for infection. Mice were infected by i.p. passage of 1 x 10⁶ infected erythrocytes diluted in phosphate buffered saline solution (PBS, 10 mM, pH 7.4, 0.1 mL). Parasitemia was monitored by microscopic examination of Giemsa stained smears [14].

Parasite extracts. Blood of infected animals, at a high level of parasitaemia (30-70%), was collected by cardiac puncture with a heparinized syringe and the blood pool was centrifuged (500g x 10 minutes, 4 °C). Plasma and buffy coat were removed and the red blood cells (RBC) pellet was washed twice with chilled PBS-Glucose (5.4 %). The washed RBC pellet was centrifuged on a discontinuous percoll gradient (80-70% percoll in PBS-Glucose, 20000g x 30 min x 4 °C) [15]. The upper band (mature forms) was removed by aspiration, collected in eppendorf tubes and washed twice with chilled PBS-Glucose and the infected erythrocytes were lysed with the nonionic detergent saponin (0.1% in PBS x 10 min). Cold PBS (1 mL) was added and the samples were centrifuged (13000g x 5 minutes, 4 °C) to remove erythrocyte cytoplasm content (including erythrocyte hemoglobin). The free parasites were mixed PBS-Glucose (5.4 %), and subjected to three freeze-thaw cycles (-70°C + 37°C). The final homogenate was used in the hemoglobin hydrolysis inhibition assay [16].

Mice native hemoglobin. Native hemoglobin from non-infected mice was obtained by treating one volume of pellet erythrocytes with two volumes of water. The resulting solution was used as the substrate in the inhibition of the hemoglobin hydrolysis assay.

Inhibition of hemoglobin hydrolysis. The proteolytic effect of the parasite extract on the native mice hemoglobin was assayed using 96-wells tissue culture plate (Greiner Bio-One). The assay mixture contained: mice native hemoglobin (10 μL), parasite extract (50 μL), GSH (10 μL, 10 μM), and acetate buffer (0.2 M, pH 5.4) to a final volume of 200 μL. The compounds, chloroquine, leupeptin and pepstatin (2.5 mM) were incorporated in the incubation mixture dissolved in DMSO. The incubations were carried out at 37 °C for 18 hours and the reactions were stopped by addition of reduced sample buffer. The degree of digestion was evaluated electrophoretically by

SDS-PAGE by visual comparison of the globin bands (14.4 kDa). A DMSO control was electrophoresed at the same time. Once we get the bands, the densitometer registers the band density values and it reports as intensity/mm² ± SD, so we proceed to check the densities of them in order to have a % of inhibition of hemoglobin hydrolysis.

Four-day suppressive test. NIH mice (18-22 g) were infected *ip* with 1 x 10⁷ *Plasmodium berghei* parasites. Two hours after infection, treatment began with the best compounds tested in the globin hydrolysis assay. These were dissolved in DMSO (0.1 M), diluted with Saline-Tween 20 solution (2 %). Each compound (20 mg/kg) was administered once by *ip* for 4 days. At day four, the parasitaemia was counted by examination of Giemsa stained smears. The chloroquine (25 mg/Kg) was used as a positive control. The survival time after infection was recorded. The results were expressed as percentage of parasitaemia at 4th day post-infection and survival time after infection comparing to control-mice [17].

Data analysis. Data were statistically analyzed using t-tests for specific group comparisons, assuming 95 % of confidence according GraphPad Prism 3.02

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