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Dehydroepiandrosterone effect on *Plasmodium falciparum* and its interaction with antimalarial drugs

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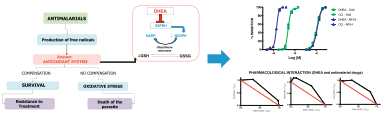
HIGHLIGHTS

- DHEA showed antiplasmodial activity, but the effect has low potency *in vitro*.
- DHEA inhibits glucose 6-phosphate dehydrogenase from healthy erythrocyte.
- DHEA decrease *P. falciparum* and erythrocyte GSH.
- The combination of DHEA with antimalarials drugs showed indifferent interactions.
- It is possible that DHEA analogs could act with higher potency and selectivity.

GRAPHICAL ABSTRACT



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ABSTRACT

Dehydroepiandrosterone (DHEA) inhibits glucose 6-phosphate dehydrogenase (G6PDH) of different species and may potentially decrease intracellular glutathione. Therefore, it can have and enhance anti-parasitic action against *Plasmodium* spp. We evaluated the antiplasmodial activity and the interaction of DHEA with several antimalarial drugs. The inhibitory effect of DHEA on erythrocytic and G6PDH activity and changes in the content of total and reduced gluthatione *Plasmodium falciparum* content were also evaluated. DHEA showed antiplasmodial activity *in vitro*, but the potency was low (IC_{50} 118.5 μM). DHEA inhibits G6PDH from healthy erythrocyte and decreases GSH content in both erythrocytes and *P. falciparum*. DHEA did not synergize or antagonize the antiplasmodial effect of several antimalarial drugs. The most important actions of DHEA were the inhibition of G6PDH activity, and the decrease in both *P. falciparum* and erythrocyte GSH. Since most of the GSH in *Plasmodium* spp. infected erythrocytes comes from the parasite itself, it is possible that DHEA analogs could act with higher selectivity, better potency, and might interact synergistically with antimalarials drugs.

1. Introduction

Malaria, a disease caused by the apicomplexan parasite *Plasmodium* spp., still affects many people in the world and constitute a public health problem by its prevalence, economic burden and annual mortality (WHO, 2010). Nowadays, the development of new antimalarial drugs is an urgent need due to a high frequency of treatment failure, absence of drugs that inhibit the development of liver stages and block the transmission, and development of resistance to antimalarial drugs by *Plasmodium* parasites. (malERA, 2011; Nwaka and Hudson, 2006). Moreover, other treatment strategies, such as the potentiation of conventional antimalarial drugs, have been proposed as alternative resources. Studies to explore this possibility are currently relevant.

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Plasmodium falciparum is highly dependent on the redox state for its survival; therefore, the antioxidant system in this parasite has been proposed as a therapeutic target (Krauth-Siegel et al., 2005). The main component of the antioxidant system in *P. falciparum* is reduced glutathione (GSH) (Muller, 2004). The maintenance of adequate levels of GSH relays on the availability of precursors and proper enzyme function for the synthesis de novo, and depends on the elimination of oxidized forms (generated after participating as electron donors in detoxification processes) through efflux to the extracellular space and enzymatic reduction (Ayi et al., 1998; Patzewitz et al., 2012). The latter involves NADPH dependent steps such as glutathione reductase activity (Muller, 2004). Since the pentose phosphate pathway is the source of NADPH for parasites, it has been proposed as suitable for therapeutic targeting (Muller, 2004). NADPH is produced in this pathway by glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase. Moreover, this pathway is also involved in the production of carbohydrate intermediates used in nucleotide synthesis and other biosynthetic pathways; thus, providing key elements for parasite survival (Muller, 2004).

While the exact mechanism of action of many antimalarial drugs is still unclear, one of the explanations proposed for their antiplasmodial action involves death through oxidative stress by direct or indirect production of free radicals (Becker et al., 2004). During this process, reduced thiols become oxidized to counter the stress condition (Becker et al., 2004). Therefore, compounds able to inhibit the synthesis of thiols may potentiate the action of drugs that produce reactive oxygen species (ROSs) as part of their mode of action (Raj et al., 2009). Moreover, they might be effective as antimalarial agents.

Several facts support these ideas. For example, *in vivo* studies in mice infected with *Plasmodium berghei* and *Plasmodium vinckey* showed an increase in the antimalarial effect of sub-therapeutic doses of CQ and AQ with acetaminophen, indomethacin and disulfiram (Deharo et al., 2003). Although none of the three drugs is a direct inhibitor of GSH metabolism, they decrease this thiol indirectly. Thus, these drugs could reverse CQ and AQ resistance at least in some strains of *P. falciparum*. Moreover, the resistance to the 4-aminoquinolines antimalarials, chloroquine (CQ) and amodiaquine (AQ), was associated with increased glutathione in *P. falciparum*. DL-buthionine-(S,R) sulfoximine (BSO), a glutathione synthesis inhibitor, and methylene blue (MB), an inhibitor of GSH reduction, had antiplasmodial effect and were more potent against CQ-sensitive strains (*Pf*3D7) as compared with resistant strains (*Pf*Dd2). These results correlated with higher levels of GSH in resistant strains (Meierjohann et al., 2002).

DHEA is a pre-hormone synthesized from cholesterol in the adrenal cortex cells (Dillon, 2005; Oberbeck and Kobbe, 2010). This substance has an inhibitory effect on G6PDH (Gordon et al., 1995), which leads to NAPDH decrease, and lower reduction of glutathione. The hormone has shown *in vitro* antiplasmodial activity against *P. falciparum*, while its analogs have shown activity *in vivo* against *P. berghei* (Ayi et al., 2002; Freilich et al., 2000). The combination of CQ with the sulfated form of DHEA (DHEAS) increased the sensitivity of CQ resistant *P. berghei* and increased the survival of infected mice (Safeukui et al., 2004).

This evidence supports that inhibition of either synthesis or reduction of glutathione could enhance antimalarial drug activity. Thus, DHEA analogs might have a role as a therapeutic alternative for *P. falciparum* infection in combination with antimalarial drugs.

2. Materials and methods

2.1. Antiplasmodial activity of DHEA

To assess the *in vitro* antiplasmodial activity of DHEA, two *P. falciparum* strains were employed. One sensitive (NF54, from East Africa) and other resistant to chloroquine, (Dd2, from Indochina) (Basco, 2007). Both strains were cultured *in vitro* according to the method described by Trager and Jensen (Trager and Jensen, 1976). Antiplasmodial activity was assessed in continuous cultures by using the fluorometric SYBR Green I assay (Smilkstein et al., 2004). Minor modifications included an optimized starting parasitemia 1% and hematocrit of 2% and a wash step with PBS 1X after incubation with the treatment to avoid interference from serum with the fluorescence measurement.

2.2. Determination of the most sensitive stage of P. falciparum to DHEA

The NF54 strain of *P. falciparum* was used to determine the most sensitive stage to DHEA, this strain has a replication cycle lasting between 42 and 48 h. Cultures were synchronized ($\geqslant$90% of the total ring forms at baseline) with sorbitol (Lambros and Vanderberg, 1979), then reincubated during 42 h (to ensure their viability) and finally the assay was started with predominance of late rings. The antiplasmodial effect of DHEA was assessed in four treatment periods: 0–12 h, late rings (>12 h of maturation); 12–24 h, late rings (>20 h of maturation) and mature trophozoites; 24–36 h, mature trophozoites and schizonts (<10 h of maturation); 36–48 h, schizonts (>10 h of maturation) and young rings (<12 h of maturation). The culture medium was removed after each incubation period and the PBS-resuspended pellet was kept frozen until performance of fluorescence analysis. Because the most sensitive stage of *P. falciparum* to CQ is already known, this drug was tested here as a validation control for these experiments (ter Kuile et al., 1993). Parasite cultures treated with DHEA and CQ during 48 h were also included as internal controls.

2.3. Effect of DHEA on G6PDH and glutathione

G6PDH activity was measured through the rate of appearance of NADPH during 2 min (absorbance increase at 340 nm). The reaction mix contained the enzyme (erythrocyte or parasite), 0.1 M Tris–HCl, 0.01 M $MgCl_2$, 0.2 mM NADP, 0.6 mM G6P and distilled water. To determine whether DHEA had an effect on erythrocyte G6PDH, healthy red blood cells were suspended in a stabilizing solution (2 mM dithriotheitol, 2 mM n-caproic amino acid, and 2 mM EDTA in deionized water), and enzyme activity was measured under different concentrations of DHEA.

The effect of DHEA on GSH was measured in healthy erythrocytes and *P. falciparum*. Healthy red blood cells and a synchronized culture with predominance of mature forms of *P. falciparum* (NF54 strain) were treated with 750 μM of DHEA during 2, 4 and 6 h of incubation. The culture was followed after each period of incubation through direct microscopic visualization to rule out parasitemia decline or changes in morphology. Healthy erythrocytes were lysed with a stabilizing solution, while erythrocytes from the *P. falciparum* culture were lysed with 5% sorbitol in water. After centrifugation at 4800*g* the parasite pellet was reconstituted in 50 mM Tris/HCl buffer with protease inhibitor cocktail (Roche®) and the parasites were lysed by ten cycles of freeze/thawing in liquid nitrogen and sonication. Finally, the hemozoin-free supernatant was taken for quantification of GSH and total glutathione (GS–T) by a high performance liquid chromatography (HPLC) method previously described (Cereser et al., 2001). Additionally, the effect of DHEA on Glutathione levels from healthy erythrocytes was measured over 48 h.

The total protein concentration was measured by the BCA micromethod (Pierce®) and was used to normalize G6PDH activity and glutathione concentration.

We also combined DHEA with GSH (1 mM and 10 mM) to evaluate the interdependence of the antiplasmodial effect of DHEA on

the GSH decrease in continuous cultures of *P. falciparum* (NF54 and Dd2 strains).

2.4. In vitro pharmacological interaction

The antiplasmodial effect of several antimalarials in combination with DHEA was assessed in continuous cultures of *P. falciparum* (NF54 and Dd2 strains). Antimalarials were tested alone and mixed with a fixed concentration of DHEA (11.72 μM). In the same way, DHEA alone and combined with a fixed concentration of CQ, (2.34 nM for strain NF54 or 62.5 nM for Dd2 strain), mefloquine (MQ) (12.5 nM) or dihydroartemisinin (DHA) (1.25 nM), were evaluated. The fixed concentrations employed for each combination produced less than 10% of the maximum antiplasmodial effect.

Stock solutions of DHEA, MQ, and DHA were prepared in DMSO and sterile water for CQ. The incubation of the cultures with drugs was 48 h at 37 °C with 5% CO_2, 5% O_2 y 90% N_2.

2.5. Statistical analysis

Nonlinear regression analysis for antiplasmodial activity was used to estimate the IC_{50} values. To determine the most sensitive stage of *P. falciparum* to DHEA, the observed IC_{50} for each incubation point was compared with the 0–48 h treatment curve by Dunnett's multiple comparisons test. The concentrations of GSH, GSSG and GS–T of erythrocytes or parasites treated and untreated with DHEA were compared using Student's *t*-test.

Data from interaction experiments were analyzed by calculating the fractional inhibitory concentration (FIC) (FIC index = IC_{50} drug A in combination/IC_{50} drug A alone) + IC_{50} drug B in combination/IC_{50} drug B alone, for each fixed concentration), (Odds, 2003). A FIC index $\leqslant$0.5 was defined as synergism, >4 as antagonism and >0.5–4 as no interaction or indifferent interaction. Isobologram constructions were also used to analyze the data.

Results correspond to means ± standard deviations for at least three independent experiments. All experiments were done in triplicate. Statistical analyses were performed using Prism 5.0 (GraphPad).

3. Results

3.1. DHEA is active against P. falciparum, but has no synergistic interaction with several antimalarial drugs

DHEA had antiplasmodial effect and the IC_{50} was similar in the CQ sensitive (NF54) and resistant (Dd2) *P. falciparum* strains, (125.80 ± 7.5 μM and 118.50 ± 8.5 μM, respectively) (Fig. 1a and b). The IC_{50} values found here for CQ, MQ and DHA, were similar to those reported historically for these strains (Fig. 1a and b). On the other hand, the combination of DHEA with antimalarials showed neither synergistic nor antagonistic interactions in two strains of *P. falciparum* (FIC between 0.5 and 4 in all cases) (Table 1, Fig. 2a–c).

3.2. Mature trophozoites and schizonts are more sensitive to DHEA

The mature trophozoite and schizont stages of *P. falciparum* were the most sensitive to treatment with DHEA. IC_{50}s were obtained for each treatment interval and compared against a control IC_{50} (same treatment during 48 h) (Table 2). The lowest IC_{50} was observed during the treatment periods from 24 to 36 h, and was not different when compared with the control IC_{50} (Table 2 and Fig. 3). For other treatment intervals, IC_{50} values were significantly higher than the control (Table 2 and Fig. 3).

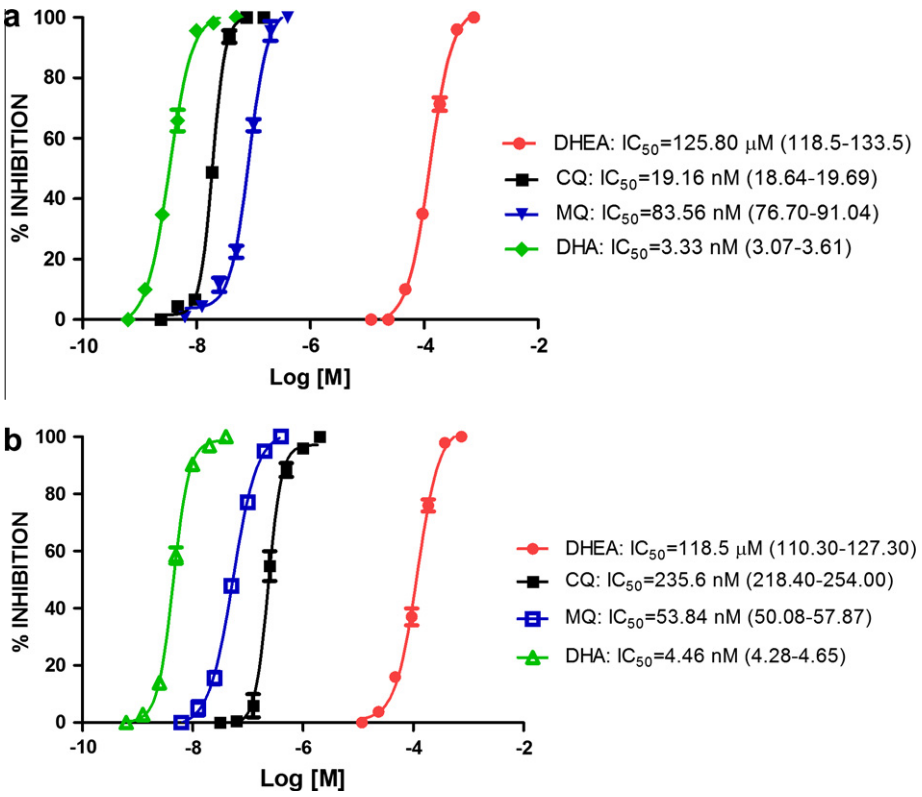

Fig. 1. Antiplasmodial activity. Effect of DHEA and antimalarials in continuous cultures of CQ-sensitive of *P. falciparum* (NF54) (a) and CQ-resistant (Dd2) (b) treated during 48 h. Values are expressed as the mean (± SD) of three independent experiments.

Table 1
Interaction between DHEA and antimalarial drugs.[a]

Strain	Combination	FIC	Lower limit–Upper limit
NF54 *P. falciparum*	CQ + DHEA	2.00	1.75–2.30
	MQ + DHEA	2.00	1.46–2.73
	DHA + DHEA	1.80	1.38–2.43
Dd2 *P. falciparum*	CQ + DHEA	1.46	1.07–1.98
	MQ + DHEA	1.67	1.12–2.48
	DHA + DHEA	1.80	1.64–1.99

[a] Antiplasmodial activity for combinations of a fixed concentration of DHEA plus increasing concentrations of antimalarial drugs, and increasing concentrations of the latter plus a fixed concentration of DHEA. Values shown are averages of three independent assays.

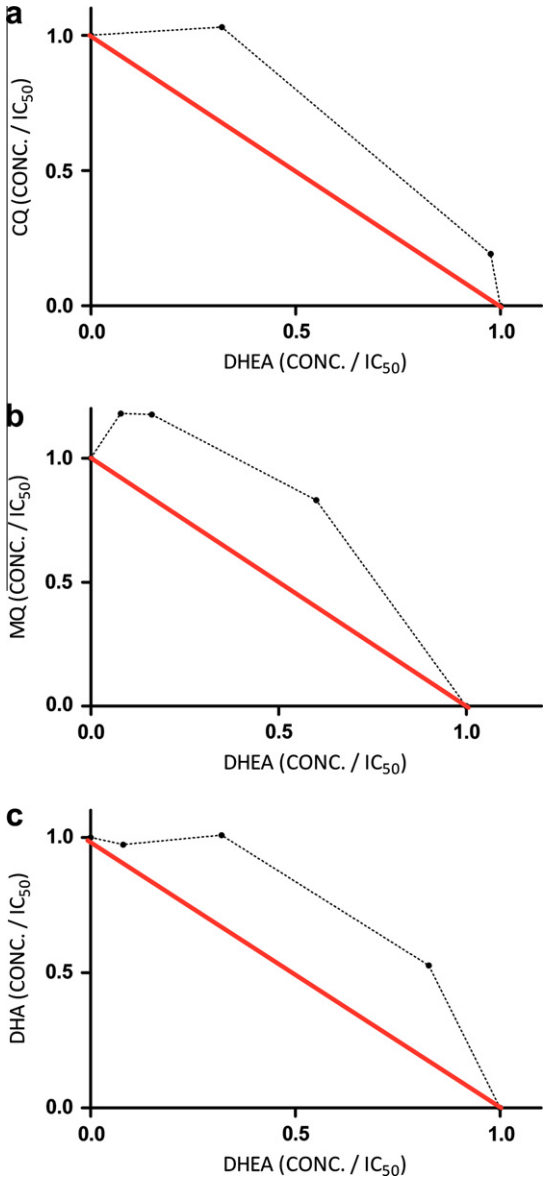

Fig. 2. Isobolograms of the interaction of DHEA with antimalarials. FIC average calculated from two separate assays of drug interactions *in vitro* in NF54 strain of *P. falciparum*. DHEA plus chloroquine (a), DHEA plus mefloquine (b), and DHEA plus dihydroartemisinin (c). Values shown are averages of three independent assays.

We also found that DHEA required more than 8 h to reach its maximum inhibitory effect. When treatment periods were performed for 8 h, the maximal inhibitory effect for DHEA was not observed, and the highest concentration used only reached about 60% inhibition (data not shown).

Table 2
IC_{50} for sensitive stage test of *P. falciparum* to DHEA and CQ.

Time[b]	CQ		DHEA	
	IC_{50} (nM) ± SD	P^*	IC_{50} (μM) ± SD	P^*
0–12 h	15.91 ± 0.91	>0.05	604.4 ± 29.55	<0.0005
12–24 h	16.76 ± 0.69	>0.05	687.4 ± 47.95	<0.0005
24–36 h	109.2 ± 20.56	<0.0005	193.6 ± 40.15	>0.05
36–48 h	>150	–	>750	–
0–48 h (CONTROL)	15.20 ± 1.18		161.4 ± 14.15	

[b] Treatment interval with DHEA or CQ. $^*P$ values for Dunnett's multiple comparisons test. Values shown are averages of three independent assays.

For CQ, the IC_{50}s for treatment intervals from 0 to 12 h and from 12 to 24 h were not statistically different when compared with the control IC_{50} (Table 2). Thus, the most sensitive stages of this antimalarial were late rings (>12 h of maturation) and mature trophozoites (Fig. 3).

3.3. DHEA inhibits erythrocytic G6PDH, but decreases glutathione levels in *P. falciparum* exclusively

DHEA inhibited G6PDH enzyme activity in healthy red blood cells. The IC_{50} was 135.6 ± 28.7 μM. It was not possible to assess the effect of DHEA on *P. falciparum* G6PDH.

A treatment of isolated *P. falciparum* parasites with DHEA 750 μM for 6 h decreased GSH and GS–T significantly ($P < 0.05$, treatment vs. control) (Table 3). Shorter treatments did not affect GSH and GS–T content ($P > 0.05$) (Table 3). DHEA also caused a reduction of GSSG (approximately 40%) at 6 h of treatment, but such change did not reach statistical significance (Table 3).

On the other hand, levels of GSH, GSSG and GS–T were not affected in healthy red blood cells after 2, 4 and 6 h of treatment with DHEA ($P > 0.05$, vs. control treatment for each time) (Table 3). However, after 48 h of treatment with DHEA higher than or equal to 46.87 μM, the GSH and GS–T levels were decreased (approximately 50% and 20%, respectively) (Table 3).

When we combined DHEA and GSH in *P. falciparum* infected erythrocytes, there was no change in the IC_{50} for the hormone. Thus, GSH failed to counteract the antiplasmodial effect of DHEA (Fig. 4a and b).

4. Discussion

This work was aimed at study the antiplasmodial effect of DHEA because this physiological form has been available as an alternative therapy for the treatment of some allergies and immunological disorders (Dillon, 2005; Hazeldine et al., 2010; Hinson et al., 2003). Other DHEA analogs are not commercially available and were not considered here.

DHEA was active and effective *in vitro* against *P. falciparum*, but its potency was very low ($IC_{50} \approx 120$ μM). Although the effect of DHEA on *P. falciparum* has not been published, the sulfated form of DHEA (DHEAS) was previously reported as active against *P. falciparum* (T996/86 strain, IC_{50} of 19 μM) (Ayi et al., 2002). Other analog has also been reported to be more potent against *P. falciparum* (Ayi et al., 2002; Freilich et al., 2000). In antiplasmodial activity assays, the 16α-Bromo epiandrosterone (16αBr-EA), a derivative of DHEA, showed an IC_{50} of 3 μM on strain Dd2 and 7.5 μM on a CQ-sensitive strain (Ayi et al., 2002; Freilich et al., 2000).

In addition, it has been reported that DHEA has *in vitro* effect against *Trypanosoma* spp., and DHEA analogs have a more potent

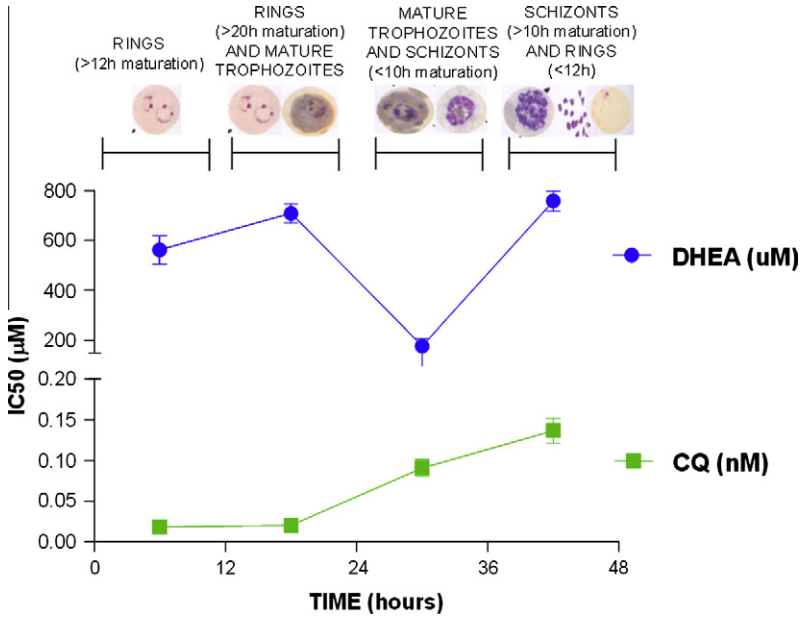

Fig. 3. Sensitive stage of *P. falciparum* to DHEA and CQ. IC_{50} obtained for each treatment interval of 12 h with CQ or DHEA, in synchronous cultures of strain NF54. The lower IC_{50} for each treatment curve corresponds to the most sensitive stage of *P. falciparum*. Values are expressed as the mean (± SD) of three independent experiments.

Table 3
GS–T, GSH and GSSG level in free parasites (NF54 strain) and healthy red blood cells treated with DHEA.[c]

		GHS (nmol/mg proteína) ± SD				GSSG (nmol/mg proteína) ± SD			
		2 h	4 h	6 h	48 h	2 h	4 h	6 h	48 h
Control	Free parasites	0.097 ± 0.003	0.081 ± 0.010	0.114 ± 0.011	–	1.042 ± 0.296	1.103 ± 0.134	1.147 ± 0.168	–
	Healthy RBC	0.690 ± 0.042	0.810 ± 0.028	0.845 ± 0.007	0.829 ± 0.051	0.350 ± 0.155	0.410 ± 0.099	0.335 ± 0.007	0.402 ± 0.126
Treatment	Free parasites	0.133 ± 0.003	0.098 ± 0.005	0.044 ± 0.005[d]	–	1.078 ± 0.079	0.922 ± 0.106	0.603 ± 0.174	–
	Healthy RBC	0.650 ± 0.028	0.825 ± 0.021	0.820 ± 0.014	0.330 ± 0.018	0.330 ± 0.184	0.460 ± 0.028	0.330 ± 0.127	0.269 ± 0.009

[c] Results are the average of three independent assays.
[d] $P < 0.05$ (treatment vs. control).

antitrypanosomal effect (Cordeiro and Thiemann, 2010; Cordeiro et al., 2009). All these findings suggest that chemical modification can be used to generate analogs with higher potency.

There is no previous information about which blood stage of *P. falciparum* is the most sensitive to DHEA or its analogs. We found that the most sensitive blood stages to DHEA were the mature trophozoites and schizonts. CQ, used as a validation control for the assay, had a maximal effect in late rings and mature trophozoites which is consistent with previous reports (ter Kuile et al., 1993). Moreover, DHEA needed more than 12 h to exert its antiplasmodial effect. This could indicate slow passage through membranes, reduced accumulation at its site of action, or gradual changes in metabolites essential for parasite survival.

DHEA inhibited erythrocytic G6PDH within the same antiplasmodial concentration range. Probably, low NADPH levels increase susceptibility to oxidative stress in the infected erythrocytes, and then affect the viability of *P. falciparum*. It is also possible that redundant mechanisms maintaining the balance of antioxidant system in *P. falciparum* are able to counteract G6PDH inhibition. Perhaps, DHEA affects other parasitic targets, but the combined actions lead to the antiplasmodial effect.

Although there are no reports in the literature about the effect of DHEA on *P. falciparum* G6PDH, DHEAS treatment in *P. berghei* infected mice inhibited parasite G6PDH activity (Safeukui et al., 2004). Also, IC_{50}s for DHEA, EA, 16α Br-DHEA and 16α Br-EA on G6PDH from *Trypanosoma cruzi* were 25 µM, 5.6 µM, 0.216 and 0.086 µM, respectively (Cordeiro and Thiemann, 2010). Therefore, the inhibitory action of DHEA on G6PDH is probably the mechanism responsible (at least in part) for the antiparasitic effect, but the selectivity is poor since it also inhibited human erythrocytic G6PDH (Gupta et al., 2011; Marks and Banks, 1960). DHEA did not inhibit the analog enzyme in plants, yeast and *Leishmania* spp. (Gupta et al., 2011). Again, structural modifications of DHEA could lead to better selectivity and may be a feasible way to further study DHEA analogs.

We found a decrease in GSH level of *P. falciparum* after 6 h of DHEA treatment. This could be explained by parasite G6PDH inhibition (which could not be confirmed in this study). Alternatively, inhibition of erythrocyte G6PDH could cause an increase in *P. falciparum* GSH/GSSG output in response to increased oxidative stress in the host cell. Despite the effect of DHEA on GSH *P. falciparum*, but because high concentrations of exogenous GSH were not able to reverse the antiplasmodial effect, we propose that such mechanism does not explain this effect. We confirmed that exogenous GSH accessed erythrocytes (data not shown) and a previous report showed that exogenous GSH is able to penetrate the parasite (Raj et al., 2009).

DHEA decreases both erythrocytic forms of glutathione (GSH and GSSG) only after 48 h of DHEA treatment, whereas shorter treatments (2, 4 and 6 h) did not affect GSH/GSSG from this cell. Perhaps the erythrocyte is able to correct the inhibitory effect of DHEA on G6PDH during the first hours of treatment, but it cannot compensate a persistent inhibitory effect on G6PDH when the treatment with DHEA is extended (data not shown).

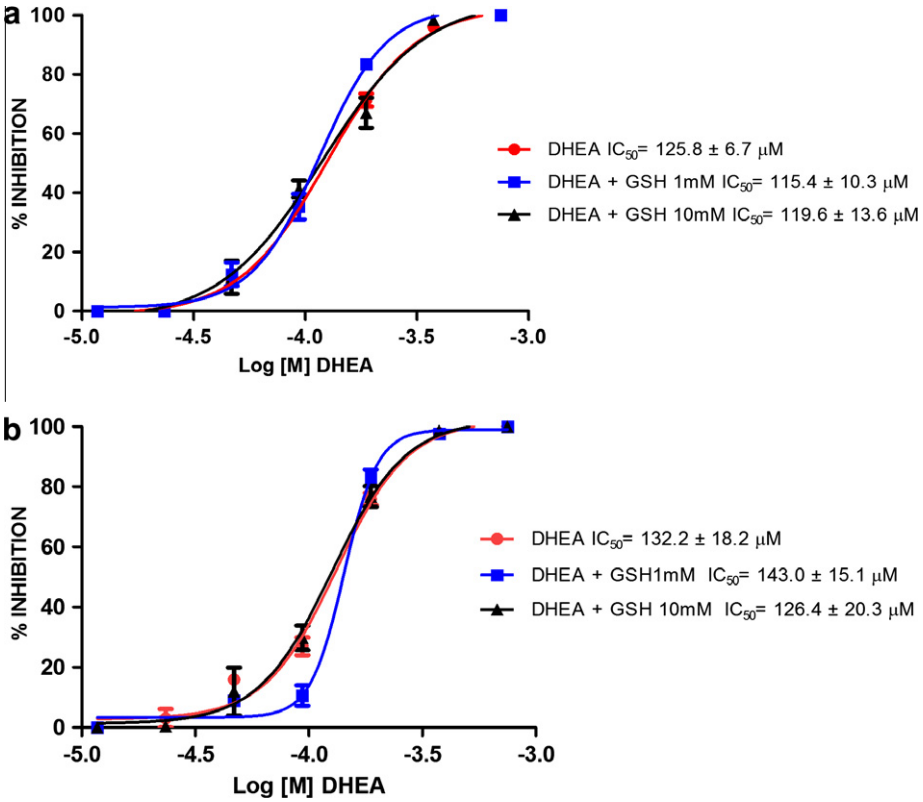

Fig. 4. Effect of GSH on antiplasmodial activity of DHEA. Dose–response of DHEA alone or in combination with constant concentrations of reduced glutathione (GSH) in strains NF54 (a), and Dd2 (b). The results of the graph are the average of three independent assays.

Although DHEA has a direct effect of DHEA on *P. falciparum*, there is evidence of a potential inmunomodulatory effect. This effect de DHEA on immune system was reported in studies with *T. cruzi*. Receptors for the hormone have been described in different immune cells, mainly in macrophages (Brazao et al., 2010; Caetano et al., 2009). *T. cruzi* infected macrophages treated with DHEA showed an increase in the concentration of TNF α, IL-12 and nitric oxide which in consequence activate NK cells and lymphocytes which ultimately secrete pro- and anti-inflammatory cytokines that promote the control of infection (Kuehn et al., 2011; Santos et al., 2010). Recent reports also suggested that this hormone increases resistance to experimental infections (Brazao et al., 2010; Caetano et al., 2009). The effect of DHEA on the immune system in *P. falciparum* infections has not been explored.

In this study, we found no synergistic or antagonistic effects between DHEA and antimalarial drugs. While the effect of DHEA on the immune system may favor a synergistic interaction with the antimalarial drug, this that cannot be detected with *Plasmodium* cultures. Therefore, the results of *in vitro* interactions do not necessarily predict the outcome in the host. An *in vivo* study using a combination of DHEAS with CQ seems to illustrate such a case (Safeukui et al., 2004). Nevertheless, only DHEA analogs with very interesting pharmacodynamic properties on *P. falciparum* must be selected from screenings *in vitro* for further evaluation *in vivo*.

5. Conclusion

We conclude that DHEA has *in vitro* antiplasmodial effect, but its potency is very low. Probably, this effect cannot be explained by a unique mechanism on the parasite. Instead, it could be related to the deleterious effects resulting from inhibition of G6PDH activity in parasites and host cells. Although most those findings are discouraging, they constitute a starting point for further studies aiming to develop DHEA analogs with better potency, higher selectivity, and able to interact synergistically with other antimalarial drugs.

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