

Research brief

Plasmodium berghei: Antiparasitic effects of orally administered Hypoestoxide in mice [☆]

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Abstract

Hypoestoxide (HE) is a diterpene isolated from *Hypoestes rosea* (Acanthaceae), a plant indigenous to Nigeria. Previous studies demonstrated that HE exhibited potent anti-inflammatory and anti-cancer activities in well established animal models but weak *in vitro* activities in both the anti-inflammation and anti-cancer *in vitro* screening systems. We now report a similar observation in the *in vitro* and *in vivo* screening systems for antimalarial activity. The results indicate that while HE exhibits a relatively weak *in vitro* activity ($IC_{50} = 10 \mu\text{M}$ versus $0.11 \mu\text{M}$ for chloroquine) against different strains of cultured *P. falciparum* parasites, the dose of HE required to reduce parasitemia by 90% in *Plasmodium berghei*-infected mice, is much lower than standard antimalaria drugs ($SD_{90} = 250 \mu\text{g}/\text{kg}$ versus $5 \text{ mg}/\text{kg}$ for chloroquine). Furthermore, lower doses of HE were much more effective than higher doses in inhibiting parasite development. The implications of these findings are discussed.

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Index Descriptors and Abbreviations: HE, Hypoestoxide; *P. berghei*, *Plasmodium berghei*; *P. falciparum*, *Plasmodium falciparum*; *P. berghei*; Hypoestoxide; Mice; Malaria; *P. falciparum*

1. Introduction

Malaria remains one of the most important infectious diseases of mankind, killing 1–3 million people and causing morbidity in more than 500 million people annually (WHO, 2005). Infection with *Plasmodium falciparum*, the most virulent human malaria parasite is responsible for all of the mortalities and morbidities (Guinovart et al., 2006). The increasing resistance of malaria parasites to available drugs increases the burden of disease and the need to develop new and effective antimalarial agents (Guinovart et al., 2006; Barat and Bloland, 1997).

2. Materials and methods

HE, prepared as previously reported (Adesomoju et al., 1983), is a novel pharmaceutical agent under development for a variety of indications (Ojo-Amaize et al., 2001, 2002). To assess the effect of HE on the development of different strains of cultured *P. falciparum* parasites, cultures were incubated with different concentrations of HE (from 100× stocks in DMSO) for 48 h, beginning at the ring stage. Concentrations yielding 50% inhibition were extrapolated from plots of mean percent control activity over inhibitor concentration (Olson et al., 1999). To evaluate the antimalarial effect of HE *in vivo*, we utilized a model for treatment of murine malaria (Olson et al., 1999). Swiss Webster mice, 6–8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA) and maintained

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according to institutional regulations in facilities approved by the American Association for Accreditation of Laboratory Animal Care. The mice were infected with 1×10^5 *Plasmodium berghei*-infected erythrocytes (purchased from ATCC, Manassas, VA), and treatment with varying doses of HE administered orally once daily for 3 days, was initiated 3 days after infection.

3. Results and discussion

The results indicate that HE exhibits relatively weak inhibitory activities against all strains of *P. falciparum* parasites tested (IC_{50} for ItG2 strain = $10.0 \mu\text{M}$; chloroquine-sensitive NF54 strain = $9.5 \mu\text{M}$; chloroquine- and pyrimethamine-resistant strain = $10.6 \mu\text{M}$). This weak activity *in vitro* is most probably due to the fact that certain metabolites of HE once formed intracellularly may be more active than the parent natural product, HE which needs to be administered *in vivo* in order to be converted to the putative more potent metabolite(s). Unlike the *in vitro* results, the *in vivo* results demonstrate a remarkable effect of HE on *P. berghei* parasite development (Table 1). The maximum inhibitory effect of HE was obtained at a nominal dose of $250 \mu\text{g}/\text{kg}$. Thus, a disconnect exists between *in vitro* and *in vivo* activities of HE which might reflect a *falciparum/berghei* disconnect. However, this remains to be determined in human clinical studies with *P. falciparum*.

Increasing doses of HE resulted in decreased efficacy (Table 1). This phenomenon cannot be explained by toxicity since the maximally tolerated oral dose in mice was established in earlier studies to be greater than $750 \text{ mg}/\text{kg}$ (Ojo-Amaize et al., 2002). Even at this very high dose, no indications of drug toxicity were observed, such as weight loss, hair loss, elevation of liver and cardiac enzymes, etc. However, inhibition of pro-inflammatory cyto-

kine production with high doses of HE may be responsible for its decreased efficacy at increasing doses. In support of this notion is the fact that HE has been shown to inhibit the production of pro-inflammatory cytokines, IL-1 β , IL-6, TNF- α (Ojo-Amaize et al., 2001) and IL-12 (Ojo-Amaize, unpublished results). The suppression of these cytokines has been correlated with exacerbation of the early phase of blood-stage malaria infection in mice and human (Bastos et al., 2002; Walther et al., 2006). Paradoxically, the anti-inflammatory property of HE at higher doses can be useful in protecting against malaria-induced fever which is associated with high levels of TNF- α . Thus, HE clearly induces a biphasic response which may just be a case of hormesis, which is the response of a biological entity to an effector, with beneficial results at low doses and detrimental results at high doses depending on the biological context in which it occurs (Murado and Vazquez, 2007).

The antimalarial activity of HE was compared with other antimalarial drugs at their respective $SD_{90\text{s}}$. The results demonstrate that the activity of HE was comparable to the activities of these drugs and the dose of HE required to inhibit parasite development by 90% was much lower than standard antimalarial drugs (Table 2). The dried leaf powder of *Hypoestes rosea*, the parent plant of HE was also active in this mouse system and supports the herbal use of the plant material for the management of malaria by Nigerian natives. A similar study (partly sponsored by Paraquest, Inc.) on the anti-plasmodial activity and toxicity of HE was conducted independently in mice, at the National Institute for Pharmaceutical Research and Development in Abuja, Nigeria. The results were similar to those reported here. HE was shown to possess over 10 times the potency of chloroquine in that study (Okogun, unpublished results).

Table 1
Therapeutic effects of varying doses of orally administered Hypoestoxide in *P. berghei*-infected mice

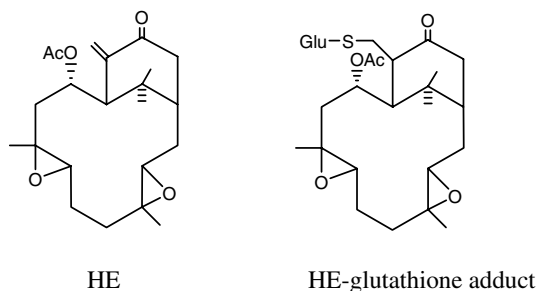
Drug dose ($\mu\text{g}/\text{kg}$)	% Reduction of parasitemia	MST \pm SE (days)	% ILS	Survivors # in group
<i>Vehicle control</i>				
2% Tween 80/PBS	0	8.6 ± 1.5	0	0/10
<i>HE</i>				
31.25	2	9.7 ± 1.8	12.8	0/10
62.5	5	10.4 ± 1.3	20.9	0/10
125	67	24.8 ± 3.6	188.4	1/10
250	83	28.6 ± 4.0	232.6	3/10
500	45	18.8 ± 3.1	118.6	0/10
1000	42	16.8 ± 4.3	95.3	0/10
<i>Positive control</i>				
Chloroquine (5 mg/kg)	87	30.4 ± 5.9	253.5	2/10

Ten female mice per group were infected i.p. with 1×10^5 *P. berghei berghei* (EI strain) parasitized RBCs and 3 days later when parasitemia was about 0.4%, begun on therapy once daily with either HE or chloroquine, which was continued for 3 days. Parasitemias were evaluated daily on Giemsa-stained smears and the day of death of each mouse that died was recorded. The mean survival time (MST) was calculated from the period between parasite infection and the day of death. The % increase in life span (ILS) was calculated using MST for each drug-treated mouse. % ILS = [(MST of drug-treated mouse - MST of Vehicle Control) / MST of vehicle control] \times 100. The mice that survived were clear of parasites and kept for 6 months before they were sacrificed. Percent reduction of parasitemia is based on day 6 parasitemia levels after infection and following three treatments with drug or vehicle.

Table 2
Comparison of anti-malarial activity of Hypoestoxide with standard anti-malarial drugs in *P. berghei*-infected mice

Drug	SD ₉₀	% Parasitemia	% Reduction of parasitemia
Untreated control (olive oil)	Vehicle	20.56 ± 3.20	0
Hypoestoxide	250 µg/kg	1.28 ± 0.19	93.8
Artemisinin	150 mg/kg	1.80 ± 0.14	91.2
Chloroquine	5 mg/kg	1.25 ± 0.16	93.9
Mefloquine	5 mg/kg	1.20 ± 0.22	94.2
<i>H. roseao</i> leaf powder	10 mg/kg	4.96 ± 1.57	75.9

Ten female mice per group were infected i.p. with 1×10^5 *P. berghei berghei* (EI strain) parasitized RBCs and 3 days later when parasitemia was about 0.4%, begun on therapy once daily for 3 days with indicated doses of drug. Parasitemias were evaluated daily. Percent parasitemia is shown for day 6 after infection and following three treatments with drug or vehicle. Percent parasitemia results are expressed as \pm standard error of the mean (\pm SE). SD₉₀ is defined as the dose that suppresses parasitemia by 90% or more.



Although the mechanism by which HE inhibits malaria parasite development is presently unknown, it is worthwhile to speculate on a structural feature of HE, the α/β -unsaturated ketone moiety as shown above. It is possible that this moiety may interfere with mitochondrial electron transport in the parasite as has been shown with β methoxyacrylates (Alzeer et al., 2000).

Glutathione (GSH) is an endogenous sulfur-containing tripeptide and is a major constituent of cells. GSH serves several independent functions such as in red blood cells in which GSH levels are normally found in high concentrations and serves to reduce methemoglobin back to hemoglobin. The intraerythrocytic malaria parasite uses GSH to detoxify heme, which is produced when the parasite digests hemoglobin in the host cell cytosol. If this heme is not detoxified in the parasite, it accumulates in the membranes and results in permeabilization of the membranes to ions. Indeed, the antimalarial activity of chloroquine has been found to be due to its ability to inhibit the degradation of heme by glutathione (Ginsberg et al., 1998).

Because GSH is used in detoxification reactions to conjugate with chemicals containing certain functional groups such as found in halogenated and nitro compounds as well as allylic compounds and epoxides (Devlin, 1992), it is expected that HE, which contains one allylic and two epoxide functions, would therefore be a very good substrate for cellular glutathione conjugation as evidenced by one of its metabolites, HE-glutathione adduct (structure shown above). This metabolite was detected in rat plasma by LC/MS/MS during preliminary pharmacokinetics studies

of HE conducted in the rat. This unique chemical structure may explain the potent *in vivo* antimalarial activity of HE. In an effort to address this possibility, we synthesized the HE-glutathione adduct and tested its antimalarial activity *in vivo*. When administered orally to *P. berghei*-infected mice, the HE-glutathione adduct was not significantly more active than HE. However, this may be due to a lack of bioavailability of the glutathione adduct when administered exogenously compared to the metabolite formed intracellularly. Nevertheless, HE may be active by lowering GSH levels in the parasite via direct scavenging of GSH and/or by inhibiting the enzymes involved in GSH synthesis and recycling. These possibilities will be explored in future studies.

A more likely mechanism of action of HE however involves the possible interference with parasite actin assembly. The movement of the malaria parasite into a host erythrocyte during invasion is thought to involve polymerization of actin (Tardieux et al., 1998) since pharmacological agents such as cytochalasin B can inhibit host cell invasion by the parasite. In earlier studies directed toward the antitumor properties of HE (Ojo-Amaize et al., 2002) we demonstrated that HE inhibits endothelial cell migration and that this is due to the finding that HE interferes, either directly or indirectly, with actin assembly and cytokinesis. In addition, HE has been shown to inhibit the motility of sporozoites (Dr. Ana Rodriguez, unpublished results). It is possible that the actual target of HE could be an actin-associated protein or that HE is acting upstream from actin assembly so that the dramatic changes in the actin cytoskeleton represent an indirect effect of the drug. Studies to investigate these possible mechanisms are ongoing.

In conclusion, we report here for the first time our preliminary data on the antimalarial activity of HE and speculate on its possible modes of action.

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