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In Vitro and In Vivo Studies of the Effects of Halogenated Histidine Analogs on *Plasmodium falciparum*†

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The effects of four halogenated analogs of histidine on in vitro growth of *Plasmodium falciparum* malaria parasites were monitored by measurement of the incorporation of ^{3}H-labeled amino acids into parasite proteins and by light and electron microscopy. The uptake of [^{3}H]isoleucine was reduced to 50% of the control value by addition of 70 μM 2-fluoro-L-histidine (2-F-HIS) or 420 μM 2-iodo-L-histidine (2-I-HIS). [^{3}H]histidine uptake into acid-insoluble material was affected equally by these two compounds, 50% inhibition resulting at 200 μM concentration. Morphological analysis of parasite development proved a sensitive assay, since development of mature trophozoites was inhibited 50% by 25 μM 2-F-HIS or 100 μM 2-I-HIS. Electron microscopy studies suggested different mechanisms of action of 2-F-HIS and 2-I-HIS on *P. falciparum*. 2-F-HIS produced a decrease in knob number at the erythrocyte surface and accumulation of electron-dense material under the parasite membrane. 2-I-HIS had no obvious effect on knobs or electron-dense material but affected parasite morphology. Surprisingly, 2-chloro-L-histidine and 2-bromo-L-histidine did not inhibit *P. falciparum* in vitro, even though their halogen atom substituents are intermediate in size between F and I atoms. 2-F-HIS and 2-I-HIS were tested in vivo against *P. falciparum* in owl monkeys (*Aotus* sp.) but were ineffective at doses that were nontoxic.

More than half of the population of the world live in areas where malaria is endemic. *Plasmodium falciparum*, the agent of the most pathogenic form of the disease, is responsible for 80% of cases, and its incidence has remained unchanged over the past decade (16). Possibly the most important single reason for the failure of control measures is the spread of parasites resistant to available antimalarial drugs. Therefore, there is an urgent need for new compounds with antimalarial activity, especially for those unrelated to previously used drugs.

Asexual *P. falciparum* parasites express a number of unusual proteins including at least three histidine-rich proteins (HRPs), containing 7.5 to 33% histidine (17). It has been suggested that analogs of this amino acid would selectively disrupt the synthesis or function of these proteins and thereby inhibit parasite growth (7). The functions of these proteins are unknown, although HRP1 has been localized by immunoelectron microscopy to electron-dense material below surface membrane knobs on erythrocytes containing mature asexual parasites (2, 5, 10, 11, 13, 15). Knobs are required for cytoadherence of *P. falciparum*-infected erythrocytes to capillary endothelial cells, and this acquired functional property of infected cells allows them to avoid destruction in the spleen. Thus, HRP1 appears to play a structural and/or functional role in knobs and is therefore advantageous for parasite survival in vivo (6).

In an earlier in vitro study, we screened 24 analogs of histidine for antimalarial activity against *P. falciparum* and found 2-fluoro-L-histidine (2-F-HIS) and 2-iodo-D,L-histidine to be the most effective at the two concentrations tested (7). 2-F-HIS has also been shown to inhibit knob formation, the number of knobs decreasing as the drug concentration was increased (1). Coincident with the decrease in knob number was an increase in the electron-dense material associated with the parasitophorous vacuole, suggesting that 2-F-HIS inhibited the transport of the electron-dense knob material from the parasite to its normal position below the membrane of infected erythrocytes (1).

In the work described in this communication, we extended the study of the in vitro effects of 2-F-HIS and 2-iodo-L-histidine (2-I-HIS) by monitoring the incorporation of ^{3}H-labeled amino acids into parasite proteins and by light and electron microscopy. The effects of two other halogenated histidine analogs, 2-chloro-L-histidine (2-Cl-HIS) and 2-bromo-L-histidine (2-Br-HIS), were also tested. We also examined the antimalarial action of two of these histidine analogs against *P. falciparum* in experimentally infected owl monkeys (*Aotus* sp.).

MATERIALS AND METHODS

Histidine analogs. 2-F-HIS (molecular weight, 173), 2-Cl-HIS (molecular weight, 190), 2-Br-HIS (molecular weight, 234), and 2-I-HIS (molecular weight, 284) were synthesized in the Laboratory of Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md. All compounds were pure when assayed in two thin-layer chromatography systems (V. M. Labroo, K. L. Kirk, E. Chang, and L. A. Cohen, manuscript in preparation).

Parasite lines. Uncloned *P. falciparum*, Malayan Camp

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(MC) (3), was grown in *Aotus trivirgatus*. In vitro culture and labeling with tritiated amino acids have been described previously (7). Metabolic labeling was carried out with 10 μCi of L-[4,5-^{3}H]isoleucine, L-[4,5-^{3}H]leucine, or L-[2,5-^{3}H]histidine (Amersham Corp., Arlington Heights, Ill.) per well. Cultures were incubated at 37°C in an atmosphere of 3% oxygen, 6% carbon dioxide, and 91% nitrogen for 20 to 22 h. Each parasite-drug combination was tested in triplicate wells, and each well was sampled twice for measurement of hot trichloracetic acid-insoluble radioactivity (7).

For electron-microscopic analyses, *P. falciparum* MC that had been adapted to continuous culture in vitro was grown in human erythrocytes in RPMI 1640 medium as above but supplemented with 10% (vol/vol) human serum rather than horse serum.

Electron microscopy. Human erythrocytes containing ring-stage *P. falciparum* were incubated alone or with 2-F-HIS or 2-I-HIS at concentrations of 0.25 or 1.0 mM for 16 or 28 h and fixed in 2% glutaraldehyde–50 mM phosphate buffer (pH 7.4)–4% (wt/vol) sucrose for 1 h at 4°C. The cells were postfixed with 1% OsO_4 after several washes with 20 mM phosphate buffer (pH 7.4) containing 15 mM NaCl and dehydrated in a graded series of ethyl alcohol solutions. Samples were embedded in Epon 812 and sectioned with a diamond knife on a Porter-Blum Mt-2 ultramicrotome. After being stained with uranyl acetate and lead citrate, the sections were examined with a 100CX electron microscope (JEOL U.S.A. Inc., Peabody, Mass.).

In vivo drug tests. In vivo trials of 2-F-HIS and 2-I-HIS were performed with adult male and female Panamanian owl monkeys (*A. lemurinus* subsp. *lemurinus*), each weighing between 730 and 943 g, and maintained as previously described (12). Each animal was inoculated with 5×10^6 asexual blood stage parasites of the Uganda Palo Alto strain of *P. falciparum* (12). Six monkeys were infected for each trial.

Blood films were prepared once or twice daily and stained with Giemsa stain. Blood was collected by lancing the marginal ear vein, and the number of parasites per cubic millimeter was determined (4).

(i) Trial A. Two infected monkeys were each given 12.5 or 25 mg of 2-F-HIS per kg intravenously (i.v.) in sterile saline twice daily for 5 days. Drug administration began on day 2 postinoculation (p.i.), when the animals had low parasitemias (1 to 1,000/mm^3).

(ii) Trial B. Two infected monkeys were each injected i.v. with one of the following drug doses: 2-F-HIS, 50 or 100 mg/kg, and 2-I-HIS, 200 or 400 mg/kg. On the afternoon of day 7 p.i. and the morning and afternoon of day 8 p.i., all four animals previously treated with 2-I-HIS were given 100 mg of 2-I-HIS per kg orally in sterile water through a urethral catheter. The drug was followed with 2 volumes of water given by the same method. Control animals were injected with a similar volume of sterile saline.

Necropsies were performed on animals that died under treatment or that were sacrificed.

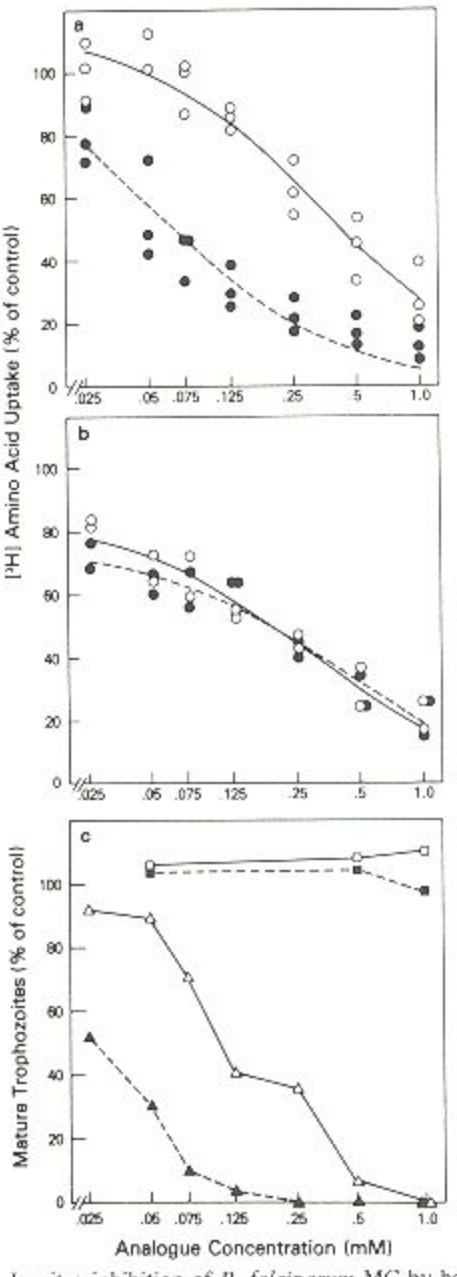

FIG. 1. In vitro inhibition of *P. falciparum* MC by halogenated histidine analogs. (a and b) Uptake of [^{3}H]isoleucine or [^{3}H]leucine (panel a) or [^{3}H]histidine (panel b) into hot trichloroacetic acid-insoluble material, expressed as a percentage of the untreated control. Each point represents the mean of triplicate culture wells. The lines were fitted by the MLAB modeling system. ---, 2-F-HIS; ——, 2-I-HIS. (c) Representative samples of the number of mature trophozoites which developed from ring stage parasites after in vitro culture with histidine analogs, expressed as a percentage of the untreated control. Differential parasite counts were performed by light microscopy of Giemsa-stained slides as described earlier (11). Symbols: ▲, 2-F-HIS; ■, 2-Cl-HIS; □, 2-Br-HIS; △, 2-I-HIS.

RESULTS AND DISCUSSION

In vitro cultures. Methods designed to assess the total growth of the parasites, namely, incorporation of [^{3}H]leucine or [^{3}H]isoleucine into malarial proteins and microscopic analysis, clearly showed 2-F-HIS to be more active than 2-I-HIS. The 50% inhibitory concentrations, as measured by incorporation of [^{3}H]leucine into acid-insoluble material, were 0.07 and 0.42 mM, respectively (Fig. 1a). Similar results were obtained with [^{3}H]isoleucine. Higher levels of 2-I-HIS than 2-F-HIS were also required to affect the morphological development of mature trophozoites (Fig. 1c). In contrast to their differences in inhibiting parasite growth, these two compounds exerted similar inhibitory effects on the uptake of [^{3}H]histidine (Fig. 1b), giving 50% inhibitory concentrations of 200 μM. We have no explanation at present for this discrepancy in the labeling studies, but it is

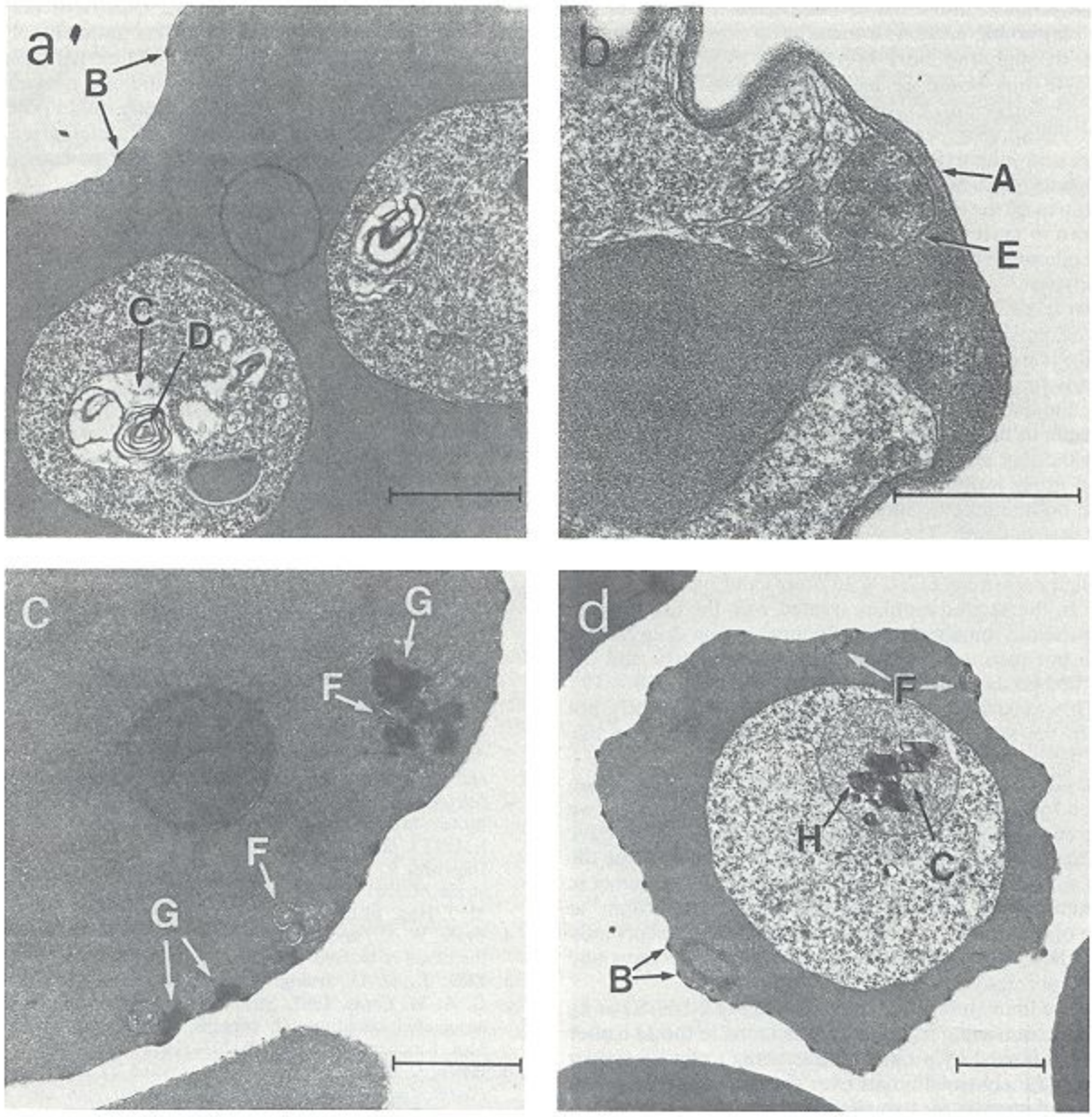

FIG. 2. Electron microscopy of *P. falciparum* MC treated with histidine analogs in vitro. (a) 2-I-HIS (0.25 mM) 16-h culture showing abnormal myelin whorls in the food vacuole and knobs at the erythrocyte surface. Magnification ×19,000; bar, 1 μm. (b) 2-F-HIS (0.5 mM) 20-h culture showing accumulation of electron-dense material below the parasitophorous vacuole membrane and lack of knobs. Magnification ×47,000; bar, 0.5 μm. (c) 2-I-HIS (0.25 mM) 28-h culture showing abnormal electron-dense material, Maurer's clefts, and knobs. Magnification ×19,000; bar, 1 μm. (d) Control untreated culture at 28 h. Magnification ×12,000; bar, 1 μm. Key to all panels: A, erythrocyte membrane; B, knob; C, parasite food vacuole; D, myelin whorl; E, parasitophorous vacuole membrane; F, Maurer's cleft; G, abnormal electron-dense material; H, parasite pigment.

possible that both 2-F-HIS and 2-I-HIS have multiple and different effects. 2-Cl-HIS and 2-Br-HIS had no inhibitory effects on parasite maturation (Fig. 1c) or on [^{3}H]leucine uptake (data not shown) at levels up to 1.0 mM. Since fluorine and iodine are at the extremes of size in the halogen series, these results also suggest that 2-F-HIS and 2-I-HIS exert their antimalarial effect by different mechanisms.

Electron microscopy of parasites grown in human erythrocytes and treated with 2-I-HIS confirmed the results observed under light microscopy and supported the theory that the mechanisms of action of 2-I-HIS and 2-F-HIS are different, since they exerted different effects on cell structure at the ultrastructural level. At 1.0 mM 2-I-HIS, almost all of the parasites seen were shrunken and pyknotic. After 16 or 28 h at 0.25 mM, some parasites appeared to be normal. In others, the presence of abnormal myelin whorls (8) in the food vacuoles showed that the parasites had been affected by the drug (Fig. 2a). Knob formation occurred even in the abnormal cells, and there was no accumulation of electron-dense material below the membrane of the parasitophorous vacuole as has been described for parasites treated with 2-F-HIS (Fig. 2b) (1); however, unusual areas of electron-dense material, sometimes associated with Maurer's clefts (Fig. 2c), were seen at both 0.25 and 1.0 mM 2-I-HIS in cells containing dead parasites and in cells with no visible parasites but in which the presence of knobs indicated infection.

These may reflect abnormal or obstructed transport of the several plasmodial proteins known to be exported from the parasite through the host erythrocyte cytoplasm to the erythrocyte membrane or into the external medium (reviewed in reference 6). Erythrocytes showing no evidence of parasite infection appeared normal. The ultrastructure of parasites and infected erythrocytes from control untreated cultures was identical to that described previously (Fig. 2d) (1). Cultures of the same parasites taken from owl monkeys and grown in vitro for 20 to 22 h in the presence of histidine analogs showed the same effects as cultures in human erythrocytes.

In vivo trials. (i) Trial A. Panamanian owl monkeys with low parasitemias (1 to 1,000/mm^3) of *P. falciparum* Uganda Palo Alto were given 12.5 or 25 mg of 2-F-HIS per kg i.v. twice daily for 5 days beginning on day 2 p.i. The higher dose cleared the parasites in one animal and suppressed the parasitemia to below 10/mm^3 in the other, but both animals died on the day after termination of treatment. Necropsies revealed gross lesions in the kidneys and bleeding gastric ulcers in both monkeys, and drug toxicity was considered to be the cause of death. The lower drug dose appeared to have no effect in one animal, and the monkey died on day 12 p.i. with a high parasitemia (710×10^3/mm^3) and typical malarial lesions. In the second monkey treated with the lower dose, the parasitemia remained below 10/mm^3 during drug administration but rose when treatment was terminated, and the animal died on day 14 p.i. with a parasitemia of $1,300 \times 10^3$/mm^3. This experiment showed that 2-F-HIS is clearly not suited to repeated i.v. administration at levels effective against the parasite.

(ii) Trial B. In view of the high level of suppression produced by a total of 50 mg of 2-F-HIS per kg per day, we considered that a single high-dose regimen might be effective against acute infections. However, a single dose of 50 or 100 mg/kg i.v. had no effect on the course of the parasitemia in three animals with parasitemias of 100 to 230×10^3/mm^3 at the time of treatment. In a fourth monkey with an aberrantly low infection, the parasitemia was reduced for 3 days and then rose normally.

The i.v. administration of 200 or 400 mg of 2-I-HIS per kg produced a transient decrease in parasitemia in the 24 h after injection, followed by a rise in parasitemia to levels similar to those in the control animals over the next day. In view of the initial decrease in parasitemia, the animals were retreated. The oral route was chosen because of its relevance to repeated administration. Since there was no difference between the animals treated with 200 or 400 mg/kg, each monkey was treated with 100 mg/kg once on day 7 p.i. and twice on day 8 p.i. This regimen of repeated oral administration had no detectable effect on the course of the parasitemia. All of the monkeys died. Necropsies revealed typical malarial lesions.

The studies described in this communication and our earlier experiments on histidine analogs as potential antimalarial agents (7) were stimulated by the discovery of the HRPs (17), especially HRP1 (or the knob-associated HRP) (10), and the hypothesis that synthesis of these proteins would be inhibited to a greater extent than synthesis of other parasite or host proteins which have normal, very low histidine contents. Numerous *P. falciparum* proteins other than the HRPs have been shown to have high contents of particular amino acids in comparison with normal proteins (e.g., the asparagine-rich protein containing 40% asparagine [14] and extended sequences of tandem repeats [reviewed in reference 9]). Although these studies with halogenated histidine analogs indicate that the HRPs may not be suitable targets for amino acid analogs used as antimalarial agents, the concept that malarial proteins of unusual amino acid composition may be preferentially affected by amino acid analogs may still be valid. This approach would probably be most successful if we could identify a *P. falciparum* protein of unusual amino acid composition that had an essential function in parasite survival.

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