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INHIBITORY EFFECTS OF HISTIDINE ANALOGUES ON GROWTH AND PROTEIN SYNTHESIS BY *PLASMODIUM FALCIPARUM IN VITRO*

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Abstract—The human malaria parasite *Plasmodium falciparum* synthesizes several proteins that are unusually rich in histidine. We therefore screened histidine analogues for their capacity to inhibit *in vitro* parasite growth. Analogues were added to cultures of ring-stage parasites, and parasite morphological development was assessed by light microscopy after a 22-hr culture. Inhibition of morphological development was identified as the appearance of condensed or pycnotic parasites rather than mature trophozoites. Inhibition of parasite protein synthesis was assessed by radioactivity counting of [^{3}H] isoleucine incorporated into acid-insoluble products and by sodium dodecyl sulfate polyacrylamide gel electrophoresis and fluorography of [^{3}H]histidine-labeled malarial proteins. 2-F-L-Histidine and 2-I-D, L-histidine exerted the most pronounced inhibitory effects, the fluoro-analogue being the more effective of the two. At a 0.125 mM concentration, both compounds inhibited parasite growth and 2-F-L-histidine also inhibited protein synthesis. At a 1.0 mM concentration, 2-azido-L-histidine, α-methyl-L-histidine and WR 177589A also inhibited *P. falciparum* growth and protein synthesis. Twenty other histidine analogues, including 5-F-L-histidine and 5-I-L-histidine, showed little or no effect under these conditions. The inhibitory histidine analogues may be of interest for antimalarial chemotherapy if they should prove to have greater effect on *P. falciparum* protein synthesis than on host protein synthesis.

The fast-acting antimalarial compounds (quinine, chloroquine, amodiaquine, and 4-quinolinemethanols) currently used to kill asexual blood stages represent three structurally related groups of compounds: cinchona alkaloids, 4-aminoquinolines and quinolinemethanols, all of which have a quinoline nucleus in common. Several other compounds (e.g. proguanil, pyrimethamine, sulfonamides, and dapsone) that are structurally distinct can be used in various combinations to cure blood infections rapidly [1, 2]. These compounds presumably have different mechanistic bases for antimalarial activity compared to the quinoline-containing compounds. However, this spectrum of antimalarials is too restricted, given the increasing problem of drug resistance. The most lethal human malaria, *Plasmodium falciparum*, exhibits widespread and expanding resistance to the most commonly used antimalarial, chloroquine. Furthermore, many chloroquine-resistant strains are also resistant to other 4-aminoquinolines, as well as to pyrimethamine and/or proguanil, or the combination of pyrimethamine plus sulfadoxine [3]. New classes of antimalarial compounds which inhibit *P. falciparum* by different mechanisms are urgently required.

In this study we set out to determine whether simple analogues of histidine can inhibit *in vitro* growth of *P. falciparum*, based on the following rationale. Asexual *P. falciparum* parasites synthesize two proteins which appear to have an exceptionally high histidine content [4, 5]. The larger of these histidine-rich proteins (HisRP‖), with an M_r of 80,000–120,000 in different strains of *P. falciparum* [5, 6], cross-reacts antigenically [7] with a HisRP from the avian malaria parasite *P. lophurae* which contains 72% histidine [8]. The histidine content of this *P. falciparum* protein is likely to be much higher than that of any mammalian proteins studied to date, including those known to have a relatively high histidine content—such as a serum glycoprotein which contains 9% histidine by weight [9]. The larger HisRP of *P. falciparum* is synthesized only by parasites which produce knobs on the surface of infected erythrocytes [4–6] and is associated in subcellular fractions with a sub-membrane electron-dense structure at the knobs [5]. Laboratory-derived knobless isolates of *P. falciparum* do not synthesize this HisRP [4–6]. Knobs mediate cytoadherence of infected erythrocytes to capillary endothelium, thereby sequestering parasitized cells and preventing their

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‖ Abbreviations include: HIS, histidine; HisRP, histidine-rich protein; Me, methyl; F, fluoro; Cl, chloro; Br, bromo; I, iodo; SH, thiol; N_3, azido; CF_3, trifluoromethyl; diF, difluoro; diCl, dichloro; diBr, dibromo; diI, diiodo; TAA, 1,2,4-triazole-3-alanine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; M_r, apparent molecular weight on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

destruction during passage through the spleen [10, 11]. Knobless isolates are much less virulent *in vivo* [12]. It is therefore likely that the larger HisRP has a function important for parasite survival as a structural element of knobs. Thus, histidine analogues might selectively inhibit the synthesis or function of the *P. falciparum* HisRP under conditions that cause little perturbation of host proteins due to their much lower histidine content.

Several histidine analogues have already been shown to affect growth of prokaryotic and eukaryotic cells. For example, α-methyl-L-histidine (α-Me-L-HIS) causes a temporary inhibition of bacterial growth by inhibiting the charging of $tRNA_{his}$ by histidine [13]. This analogue is not incorporated into bacterial proteins. 1,2,4-Triazole-3-alanine (TAA) inhibits bacterial growth by repression of the synthesis of the histidine-biosynthetic enzymes and is also incorporated into protein replacing histidine [14]. 2-Fluoro-L-histidine (2-F-L-HIS) interferes with protein synthesis in bacteria, viruses and eukaryotic cells [15], possibly due to its incorporation into protein in place of histidine. This modification may result in perturbation of protein structure and enzymic functions because of the highly electronegative fluorine atom on the imidazole ring group [16]. The results of screening these and other histidine analogues for inhibitory effects on *in vitro* growth of *P. falciparum* are described below.

MATERIALS AND METHODS

Histidine analogues. The nomenclature for numbering positions of substitution on the imidazole ring is shown in Fig. 1. L-HIS, D-HIS, 1-Me-L-HIS, 3-Me-L-HIS, α-Me-L-HIS and TAA were purchased from the Sigma Chemical Co., St. Louis, MO. *N*-α-Acetyl-L-HIS and 3-benzyl-L-HIS were from the Cyclo Chemical Co., Miami, FL. The following compounds were obtained from the inventory of the Division of Experimental Therapeutics, Walter Reed Army Institute of Medical Research, Washington, DC: WR177589A (III), N-α-*p*-toluenesulfonyl-L-HIS, 2-SH-L-HIS, 3-cyclohexyl-L-HIS, 3-phenyl-L-HIS, 3-isopropyl-L-HIS and 2,5-diI-L-HIS. Some compounds were synthesized in the Laboratory of Chemistry, NIADDK, NIH, Bethesda, MD: 5-F-L-HIS, 5-Cl-L-HIS, 5-Br-L-His, 5-I-L-HIS, 2-F-L-HIS, 2-I-D,L-HIS, 2-N_3-L-HIS, 2-CF_3-L-HIS, 2,5-diF-L-HIS, 2,5-diCl-L-HIS, and 2,5-diBr-L-HIS. Stock solutions of each compound (10 mM) were made the day before use in "Basic Medium" (see below). WR177589A and *N*α-*p*-toluenesulfonyl-L-HIS were not completely soluble at 10 mM but were soluble at 1 mM.

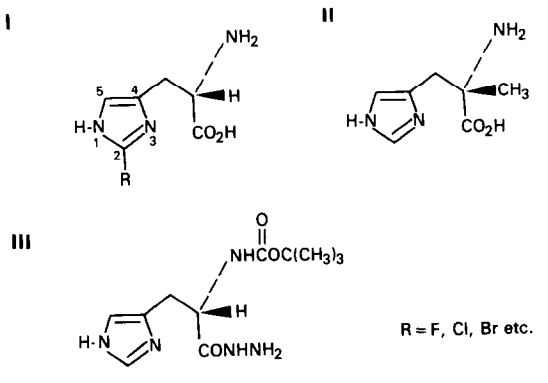

Fig. 1. Structures of histidine analogues.

Malaria parasites. An isolate (not cloned) of knob-positive *P. falciparum*, Malayan Camp strain, was obtained from non-splenectomized *Aotus trivirgatus* monkeys. Infected blood containing ring-stages was cryopreserved [17].

In vitro *culture with histidine analogues.* Thawed infected blood was washed twice in "Basic Medium": RPMI 1640 containing 0.025 mM histidine (25% of the normal concentration) and supplemented with 30 mM HEPES, pH 7.2, 10 mg/ml hypoxanthine, 2 mg/ml D-(−)-glucose and 50 mg/ml gentamicin (Schering Corp., Kenilworth, NJ). A preliminary experiment showed that levels of L-histidine from 0 to 0.5 mM had no effect on parasite morphological development or uptake of [^{3}H]isoleucine. The percentage of parasitized erythrocytes was determined by Giemsa staining and light microscopy and the cells were resuspended at 3.0×10^7 parasitized erythrocytes/ml in "Complete Medium": "Basic Medium" plus 15% (v/v) horse serum (M. A. Bioproducts, Walkersville, MD) and 3 ml/100 ml of 7.5% (w/v) $NaHCO_3$. Aliquots (100 μl) of cell suspension were distributed into the wells of flat-bottomed 96-well culture trays (Costar, Cambridge, MA). Each well also received 100 μl of "Complete RPMI" containing a histidine analogue such that, in a final volume of 210 μl, the analogue concentration was 0.125 or 1.0 mM. Control wells received "Complete RPMI" alone. For metabolic labeling with [^{3}H]histidine or [^{3}H]isoleucine, 10 μl of L-[2,5-^{3}H]histidine (60 Ci/mmole, 1 mCi/ml) or L-[4,5-^{3}H]isoleucine (85 Ci/mmole, 1 mCi/ml) (Amersham, Arlington Heights, IL) was added per well. The trays were placed in a Perspex incubation box which was flushed with a gas mixture of 3% O_2, 6% CO_2 and 91% N_2, and sealed. Incubation was done at 37° for 22 hr.

Each concentration of compound to be used with a particular radioactive amino acid was tested in triplicate wells distributed in at least two 96-well trays. Parasite development was assessed by Giemsa staining and light microscopy using wells in a different tray without added radioisotope. The test trays were harvested when a majority of parasites in control wells had matured to late trophozoites. The cells in each well were gently resuspended by pipetting, and two aliquots of 20 μl from each well were applied as streaks to strips of glass fiber paper (934-AH, Whatman Inc., Clinton, NJ) for assay of incorporation of radioactivity into hot-acid-insoluble material. The remaining cells from two wells of each triplicate were pooled and used for light microscopy analysis of parasite morphology. The contents of the third well were mixed with 120 μl of 2× SDS-electrophoresis sample buffer and stored at −70° for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and fluorography. Malarial proteins labeled by [^{3}H]histidine or [^{3}H]isoleucine uptake were analyzed by SDS-PAGE using the Laemmli buffer system [18] and 5–10% polyacrylamide gradi-

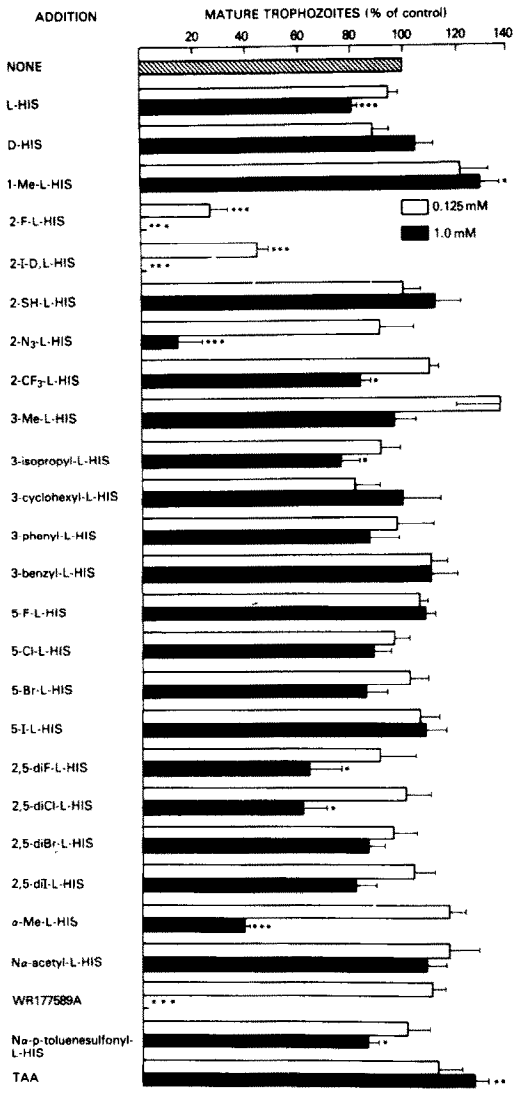

Fig. 2. Number of mature trophozoites of *P. falciparum* which developed from ring-stage parasites after *in vitro* culture with histidine analogues. Values are expressed as a percentage of the number of trophozoites which developed in cultures without analogues. Infected erythrocytes were obtained from cryopreservatives of *P. falciparum*-infected *Aotus* monkeys and cultured at 1.5×10^7 parasitized cells/ml in culture medium containing 0.025 mM L-histidine. Histidine analogues were added at the initiation of culture at final levels of 0.125 or 1.0 mM and remained throughout the 22-hr period required for development of trophozoites. Parasite morphology was assessed by smearing duplicate incubations onto glass slides, staining with Giemsa, and differential counting of the number of trophozoites per 600 parasites by light microscopy. The values represent mean ± S.E.M. of four to six analyses. Statistical significance (Student's *t*-test) is expressed as follows: (*) $2P < 0.05$, (**) $2P < 0.01$, and (***) $2P < 0.001$. Abbreviations: HIS, histidine; and TAA, 1,2,4-triazole-3-alanine.

ent gels. The electrophoresis sample buffer contained 5% β-mercaptoethanol and 5% SDS. The gels were fixed in acetic acid/methanol/H_2O [19], and radioactive proteins were detected by fluorography [20] using Enhance from New England Nuclear, Boston, MA.

Measurement of protein radiolabeling. Strips of glass fiber paper streaked with radioactive samples were air-dried and washed in a series of trichloroacetic acid (TCA) solutions [20% (w/v) TCA ×1, 10% (w/v) TCA ×2, 5% (w/v) TCA ×2]. The first three washes and the fifth wash were for ≥ 8 hr at 23°. The fourth wash was for 1 hr at 90°. The strips were air dried, and radioactivity in each streak was

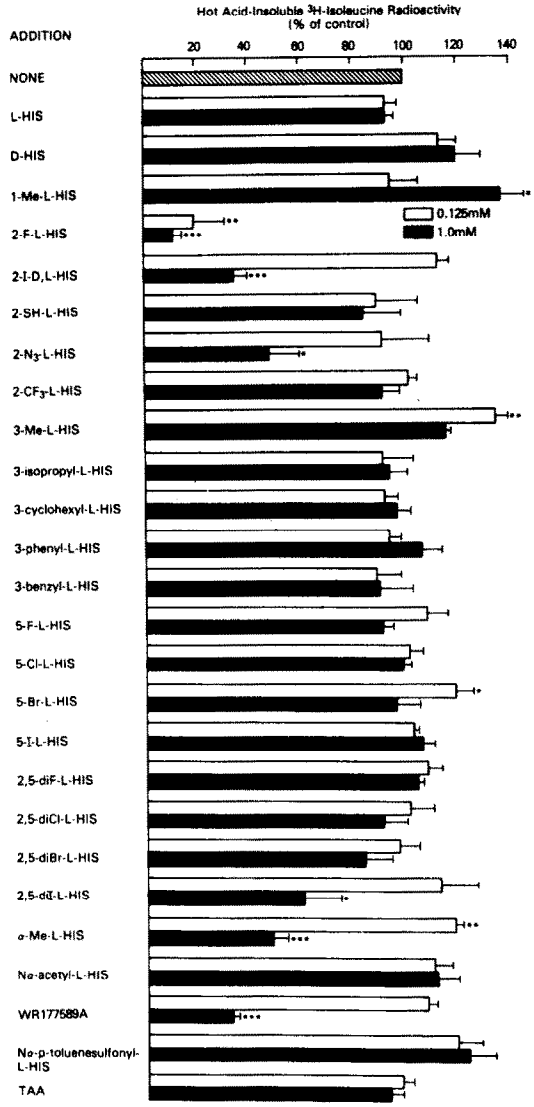

Fig. 3. Amount of hot-acid-insoluble [^{3}H]radioactivity incorporated into asexual *P. falciparum* after a 22-hr culture with [^{3}H]isoleucine and various histidine analogues. Values are expressed as a percentage of the amount of radioactivity incorporated by cultures without analogues. Data are from the experiments described in Fig. 2. Duplicate aliquots from resuspended cultures were applied onto glass fiber filter paper and washed with trichloroacetic acid solutions. Radioactivity on the washed paper was assayed, and mean uptake was determined and expressed as a percentage of the control value for that experiment. Four to six such percentage values were then used to calculate the mean ± S.E.M. shown in this figure. Statistical significance (Student's *t*-test) is expressed as follows: (*)$2P < 0.05$, (**)$2P < 0.01$, and (***)$2P < 0.001$. Abbreviations: HIS, histidine; and TAA, 1,2,4-triazole-3-alanine.

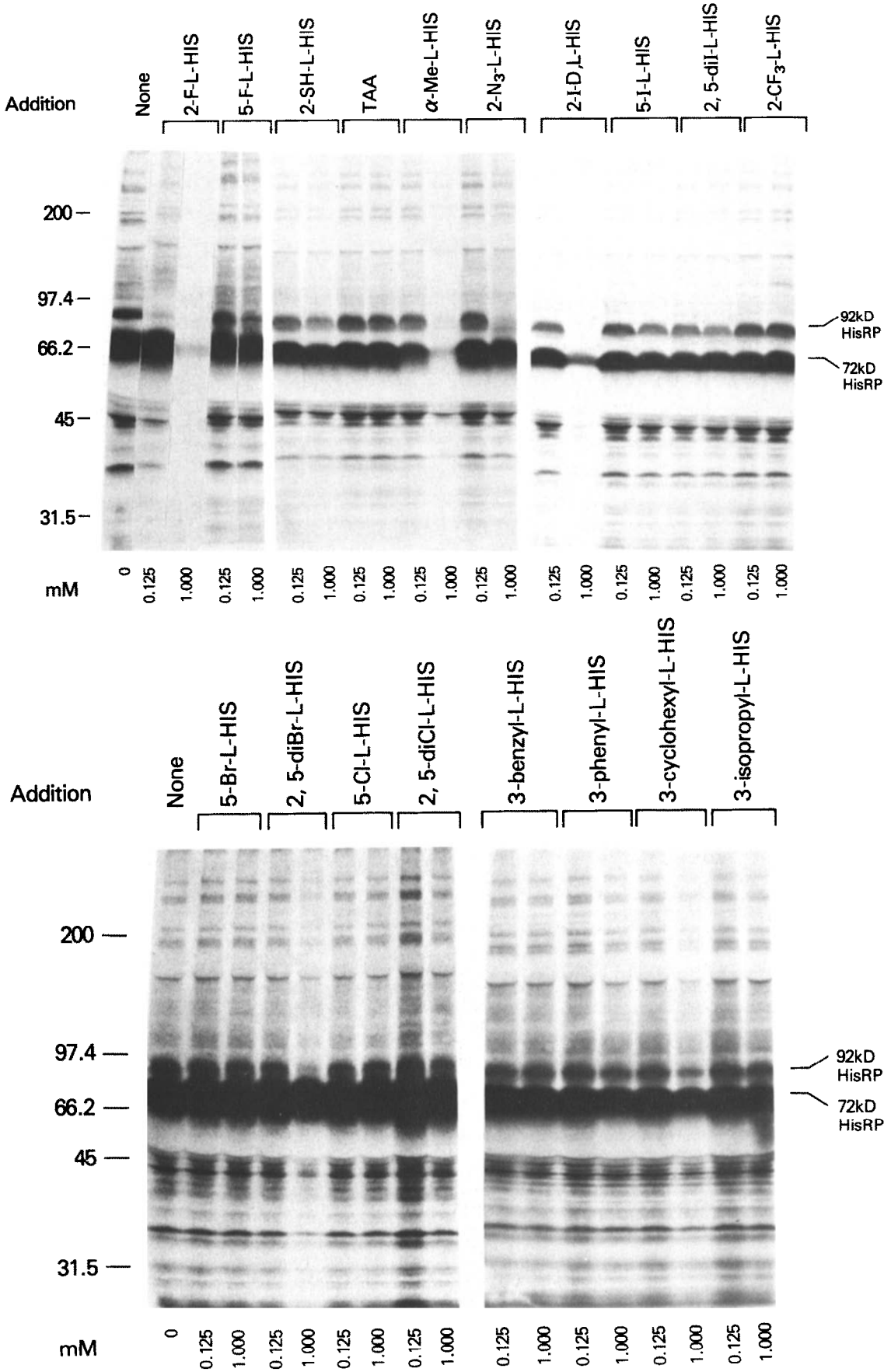
Addition
None
2-F-L-HIS
5-F-L-HIS
2-SH-L-HIS
TAA
α-Me-L-HIS
2-N3-L-HIS
2-I-D,L-HIS
5-I-L-HIS
2, 5-diI-L-HIS
2-CF3-L-HIS
200
97.4
66.2
45
31.5
92kD HisRP
72kD HisRP
mM
0
0.125
1.000
5-Br-L-HIS
2, 5-diBr-L-HIS
5-Cl-L-HIS
2, 5-diCl-L-HIS
3-benzyl-L-HIS
3-phenyl-L-HIS
3-cyclohexyl-L-HIS
3-isopropyl-L-HIS

Fig. 4.

Table 1. Effects of histidine analogues on light microscopy appearance of *P. falciparum*

Analogue	Concn (mM)	Morphology of Giemsa stained parasites* (% of total parasites)		
		Condensed	Intermediate	Mature trophozoite
None		30	22	48
L-Histidine	0.125	24	23	52
	1.0	40	18	42
2-F-L-Histidine	0.125	81	10	9
	1.0	93	7	0
2-I-D,L-Histidine	0.125	62	18	20
	1.0	87	13	0
2-N_3-L-Histidine	0.125	26	28	46
	1.0	67	26	7
2,5-diF-L-Histidine	0.125	32	23	45
	1.0	35	38	26
2,5-diCl-L-Histidine	0.125	32	20	48
	1.0	44	27	29
2,5-diBr-L-Histidine	0.125	37	16	47
	1.0	44	16	40
α-Me-L-Histidine	0.125	25	20	55
	1.0	54	28	16
5-F-L-Histidine	0.125	29	18	53
	1.0	24	21	55
5-I-L-Histidine	0.125	33	16	51
	1.0	23	25	52
WR177589A	0.125	32	21	47
	1.0	94	6	0

* Infected blood of 20–50% parasitemia was cultured with histidine analogues from the immature ring stage until maturation of approximately 70% of the parasites to trophozoites (22 hr). Duplicate slides were taken from duplicate cultures for each analogue, and parasite morphology was assessed by Giemsa staining and light microscopy. Parasites (600) were counted on each slide, and the differential parasite counts were converted to the percentages of total parasites counted. The four percentage values were used to calculate mean values shown in this table for a typical experiment. Trophozoites were scored as large mature forms ("Mature trophozoite") or as smaller forms with detectable parasite cytoplasm ("Intermediate"). The remaining parasites were very small, heavily stained, and without obvious cytoplasm ("Condensed"). Other analogues tested but not shown here gave results almost identical to the control.

assayed by liquid scintillation spectrometry. A preliminary experiment established that cultures of uninfected erythrocytes incorporated less than 1% as much radioactivity from [^{3}H]histidine or [^{3}H]isoleucine compared with infected cells. We also established that there was a direct linear relationship between the number of parasitized cells applied to the paper and the radioactivity recovered.

RESULTS

Cultured parasites were stained with Giemsa and examined by light microscopy to determine the effects of histidine analogues on parasite morphological development. The majority of analogues had no effect at 0.125 mM on the proportion of parasites developing into mature trophozoites (Fig.

Fig. 4. Fluorography of [^{3}H]histidine-labeled proteins of asexual *P. falciparum* parasites separated by SDS-PAGE after culture with various histidine analogues. (A) 2-F-L-HIS, 5-F-L-HIS, 2-SH-L-HIS, TAA, α-Me-L-HIS, 2-N_3-L-HIS, 2-I-D,L-HIS, 5-I-L-HIS, 2,5-diI-L-HIS, and 2-CF_3-L-HIS. (B) 5-Br-L-HIS, 2,5-diBr-L-HIS, 5-Cl-L-HIS, 2,5-diCl-L-HIS, 3-benzyl-L-HIS, 3-phenyl-L-HIS, 3-cyclohexyl-L-HIS, and 3-isopropyl-L-HIS. *P. falciparum* was cultured for 22 hr with a 0.125 or 1.0 mM concentration of each analogue and a sample of resuspended culture mixed with an equal volume of 2 × SDS-sample buffer. SDS-PAGE was performed on 5–10% gradient gels with molecular weight standards in adjacent lanes (positions of migration indicated on the left). The M_r 92,000 histidine-rich protein (HisRP) and the M_r 72,000 HisRP of this *P. falciparum* isolate (indicated on the right) could be identified after fluorography by their mobility and by comparison with the protein patterns for knob-negative parasites from the same isolate [5]. Abbreviations: HIS, histidine; and TAA, 1,2,4-triazole-3-alanine.

2). However, 2-F-L-HIS and 2-I-D,L-HIS were markedly inhibitory at this concentration, decreasing the number of mature trophozoites by approximately 70 and 60%, respectively, compared to control cultures. A higher concentration (1.0 mM) of several other analogues also inhibited trophozoite development: L-HIS, 2-N_3-L-HIS, 2-CF_3-L-HIS, 3-isopropyl-L-HIS, 2,5-diF-L-HIS, 2,5-diCl-L-HIS, α-Me-L-HIS, WR177589A and N-α-*p*-toluenesulfonyl-L-HIS (Fig. 2). The most inhibitory analogues at 1.0 mM (2-F-L-HIS, 2-I-D,L-HIS, 2-N_3-L-HIS, α-Me-L-HIS and WR177589A) prevented the development of between 65 and 100% of parasites into mature trophozoites. In contrast to the 2-substituted compounds, the 5-substituted fluoro- and iodo-histidines had no inhibitory effects. Two analogues increased the proportion of parasites which developed into mature trophozoites (1-Me-L-His and TAA) (Fig. 2).

Compounds which decreased the number of mature trophozoites produced a corresponding increase in the number of immature trophozoites ("intermediate" forms) or pycnotic, condensed parasites (Table 1). Differential analysis of parasite morphology showed that, at 0.125 mM, 2-F-L-HIS was more inhibitory than 2-I-D,L-HIS (81% vs 62% of parasites were pycnotic respectively). Nearly 100% of parasites were pycnotic with 2-F-L-HIS, 2-I-D,L-HIS, 2-N_3-L-HIS and WR177589A at 1.0 mM.

2-F-L-HIS was the only analogue which inhibited incorporation of [^{3}H]isoleucine into acid-insoluble radioactivity at 0.125 mM (80% inhibition) (Fig. 3). 3-Me-L-HIS, 5-Br-L-HIS and α-Me-L-HIS stimulated [^{3}H]isoleucine incorporation at this level. At 1.0 mM, 2-F-L-HIS was the most inhibitory compound, reducing parasite radiolabeling by 90%. 2-I-D,L-HIS, 2-N_3-L-HIS, 2,5,-diI-L-HIS, α-Me-L-HIS and WR177589A also inhibited [^{3}H]isoleucine incorporation at 1.0 mM (45–70% inhibition). 1-Me-L-HIS stimulated incorporation at 1.0 mM. 5-F-L-HIS and 5-I-L-HIS had no effect on [^{3}H]isoleucine labeling at 1.0 mM, in contrast to the inhibitory effects of the 2-substituted compounds (Fig. 3).

The effects of histidine analogues on protein synthesis were also examined by SDS-PAGE and fluorography of proteins labeled by [^{3}H]histidine. Cultured cells were solubilized in SDS-sample buffer and equal volumes electrophoresed without correction for the number of parasites which developed into trophozoites (Fig. 4). The positions of migration of the M_r 92,000 and M_r 72,000 HisRP are indicated. At 0.125 mM, 2-F-L-His reduced [^{3}H]histidine incorporation into the M_r 92,000 HisRP, with little or no effect on labeling of the other malarial proteins (Fig. 4A). The higher concentration of 2-F-L-HIS (1.0 mM) markedly reduced the radiolabeling of all malarial proteins by [^{3}H]histidine. α-Me-L-HIS, WR177589A (not shown) and 2-I-D,L-HIS also reduced [^{3}H]histidine labelling markedly at 1.0 mM. The pattern of [^{3}H]histidine-labeled proteins for 1.0 mM 2-N_3-L-HIS was similar to that of 0.125 mM 2-F-L-HIS, with greater inhibition of [^{3}H]histidine uptake into the M_r 92,000 HisRP than with the other malarial proteins. None of the other histidine analogues at 0.125 mM or 1.0 mM consistently produced a major change in the patterns of [^{3}H]-histidine-labeled proteins apparent on visual inspection of gel fluorographs.

DISCUSSION

Five structural analogues of L-histidine have been identified which have significant antimalarial effects *in vitro* against a knob-positive isolate of asexual *P. falciparum*. Nineteen other histidine analogues had no significant inhibitory effects, two of which caused some stimulation of parasite growth. All of the inhibitory compounds were simple chemical modifications of L-histidine: halogen atom substitution on the imidazole 2-position (2-F-L-HIS and 2-I-D,L-HIS), azido group substitution at the same position (2-N_3-L-HIS), and methyl substitution on the α-C atom of the amino acid (α-Me-L-HIS). The most structurally complex inhibitory compound identified, WR177958A, was substituted on both the amino and carboxyl groups of the amino acid (see III).

Antimalarial effects were measured quantitatively by light microscopy analysis of the development *in vitro* of immature ring stages into mature trophozoites and by determination of [^{3}H]isoleucine incorporation into hot acid-insoluble material. Uninfected erythrocytes incorporate only very low levels of [^{3}H]isoleucine into hot-acid-insoluble material, and this amino acid, which is essential for growth of Plasmodia, must be supplied in the culture medium [21, 22]. Incorporation of radioactivity from [^{3}H] isoleucine or other labeled amino acids into hot-acid-insoluble material from infected erythrocytes is therefore a measure of malarial protein synthesis.

For the five most inhibitory compounds, the results obtained by the two quantitative methods were complementary. 2-F-L-HIS at 0.125 mM markedly arrested morphological development of trophozoites, increased the number of pycnotic parasites after 24 hr *in vitro*, and markedly inhibited [^{3}H]isoleucine uptake. 2-I-D,L-HIS, 2-N_3-L-HIS, α-Me-L-HIS and WR177958A had no significant effects at 0.125 mM but inhibited morphological development and [^{3}H] isoleucine uptake at 1 mM.

The position of substitution on the imidazole ring was a critical determinant of antimalarial activity. Some 2-substituted analogues (2-F-L-HIS, 2-I-D,L-HIS) were strongly inhibitory while the 5-substituted analogues with the same functional groups (5-F-L-HIS, 5-I-L-HIS) were without significant effects even at 1.0 mM. These observations conform to a pattern of biological activity that has emerged for the 2- and 5-F-substituted histidines in diverse biological systems. 2-F-L-HIS has been shown to inhibit protein synthesis in organ explants [23] and cell cultures [15] and to inhibit hormonally-induced stimulation of enzyme activities [23]. Furthermore, 2-F-L-HIS inhibits the cytopathogenicity of DNA and RNA viruses in primary and continuous cell cultures [15]. This histidine analogue also blocks transformation of normal mouse cells by murine sarcoma virus and suppresses release of murine leukemia virus by a continuously infected mouse cell line [15]. In the same experiments, 5-F-L-HIS (called 4-F-L-HIS according to a different nomenclature in earlier reports) was uniformly without effect. We also observed that replacement of the H-atom at position

5 on the imidazole ring by fluorine (i.e. 2,5-diF-L-HIS) rendered the markedly inhibitory 2-F-L-HIS structure noninhibitory in the antimalarial assays. 2,5-dI-L-HIS was also noninhibitory, in contrast to 2-I-D,L-HIS. The 2-substituents on 2-F-L-HIS and 2-N_3-L-HIS are relatively small, producing little structural perturbation of histidine but leading to marked differences in electron distribution (the *pKa* of the imidazole ring is reduced by 5 pH units in 2-F-L-HIS compared with L-HIS [24]). The 2-substituent on 2-I-D,L-HIS is significantly larger and yet this compound is still inhibitory. (It should be noted that this iodo compound was a mixture of optical isomers, making the actual concentration of the L-isomer half that of the other analogues tested.) We are currently synthesizing 2-Cl-L-HIS and 2-Br-L-HIS in order to determine if the remaining 2-halo-histidines also show inhibitory effects.

The chemical nature of the substituent at the 2-position of the imidazole ring was also a determinant of antimalarial activity; 2-SH-L-HIS had no antimalarial effect, in contrast to 2-F-L-HIS, 2-I-D,L-HIS and 2-N_3-L-HIS.

Several other histidine analogues with simple substitutions on the imidazole ring were also not inhibitory (e.g. 1-Me-L-HIS, 3-Me-L-HIS), but in these examples the lack of activity may reflect both the position and chemical nature of the imidazole substituent.

The mechanism of inhibition of asexual *P. falciparum* by these histidine analogues has not yet been elucidated. 2-F-L-HIS, the most inhibitory antimalarial identified here, has been studied in detail for its effects on protein synthesis in other systems. The results of experiments with cell-free protein-synthesizing systems suggested that 2-F-L-HIS does not inhibit protein synthesis *per se*, but competes with HIS for attachment to the $tRNA_{his}$ and is then incorporated into protein instead of HIS. For example, 2-F-L-HIS blocked uptake of [^{3}H]histidine into protein in a cell-free protein-synthesizing system from mouse L cells directed either by endogenous mRNA or viral-mRNA. There was no effect on incorporation of [^{3}H]leucine into protein, even with a ratio of 2-F-L-HIS/L-HIS of 7500/1 [25]. In an *Escherichia coli* extract with poly (U)-directed incorporation of [^{3}H]phenylalanine into acid-insoluble material, there was no effect of 2-F-L-HIS. In contrast, this analogue had a marked inhibitory effect on poly (A, C)-directed incorporation of [^{3}H]histidine into acid-insoluble material [25]. The incorporation of 2-F-L-HIS into newly synthesized protein has been demonstrated directly using [^{3}H]-2-F-L-HIS and cultures of mouse L cells [25] or pineal glands [26]. Incubation of organ explants or cell cultures for longer periods with 2-F-L-HIS does inhibit protein synthesis in general [15, 23] and the activity of various enzymes [23]. These effects may result from the replacement of L-HIS by 2-F-L-HIS at sites in proteins that are essential for enzymatic or structural activity [27]. As chemically-altered, nonfunctional proteins containing 2-F-L-HIS accumulate, secondary effects on protein synthesis and cell growth would be observed. With *P. falciparum* parasites which synthesize proteins of unusually high histidine content [4–7], there may even be additional inhibitory effects in the presence of 2-F-L-HIS, other than those produced by incorporation of the analogue into malarial proteins.

α-Me-L-HIS was markedly inhibitory to *P. falciparum* at the higher level tested (1.0 mM). This analogue causes a transitory growth inhibition of *E. coli* and *Staphylococcus typhimurium* and has been shown to act on the bacterial HIS-$tRNA_{his}$-synthetase to compete reversibly with L-histidine for charging of tRNA. Unlike 2-F-L-HIS, α-Me-L-HIS was not incorporated into bacterial protein [13].

The inhibitory histidine analogue WR177958A has rather bulky substituents on the amino acid carboxyl and amino groups (III). Its mode of inhibition of *P. falciparum* is therefore likely to be different from the imidazole-substituted and α-carbon-substituted analogues. This compound appears to be less inhibitory than 2-F-L-HIS at the same concentration.

The failure of TAA to inhibit *P. falciparum*, even at 1.0 mM, is significant in view of previous studies with this histidine analogue in bacterial systems. Bacterial growth is inhibited by TAA, initially rather slowly, but then there appears an increasing bacteriostatic effect [14]. In fact, TAA has several modes of action against bacteria. The first enzyme in the histidine biosynthetic pathway (Compound III synthetase) is inhibited *in vitro* by TAA [14]. This compound is also incorporated into newly-synthesized protein, replacing histidine [14]. Furthermore, TAA acts as a false co-repressor of the histidine operon of *S. typhimurium*, thereby repressing the synthesis of the histidine biosynthetic enzymes [28, 29].

A semiquantitative analysis of the effects of histidine analogues on the synthesis of the two major *P. falciparum* HisRPs was made by fluorography of SDS-polyacrylamide gels after [^{3}H]histidine labeling. The M_r 92,000 HisRP could be identified on SDS-polyacrylamide gels by its characteristic mobility [4–6] and by comparison of the patterns of [^{3}H]histidine-labeled proteins of knob-negative parasites of the same *P. falciparum* strain [5]. 2-F-L-HIS, α-Me-L-HIS, and 2-I-D,L-HIS (at 1.0 mM) clearly inhibited the labeling of all malarial proteins through inhibition of parasite growth. 2-F-L-HIS at 0.125 mM and 2-N_3-L-HIS at 1.0 mM appeared to inhibit selectively the uptake of [^{3}H]histidine into the M_r 92,000 HisRP, since there was a greater reduction in radioactivity incorporated into this protein than in the other malarial proteins. As yet, we do not know if a reduction in [^{3}H]histidine uptake into this knob-related HisRP [4–6] reflects decreased synthesis, replacement of [^{3}H]-L-HIS by 2-F-L-HIS, or both processes.

Of the five most inhibitory analogues for *P. falciparum in vitro*, only 2-F-L-HIS has been studied for toxic effects on vertebrates *in vivo*. In mice the LD_{50} of 2-F-L-HIS is 250 mg/kg at 5 days [30]. When this analogue was administered to mice at 125 mg/kg, there were several acute toxic effects including leukopenia, reduced hematopoiesis and reduced lymphocyte levels in spleen, lymph nodes and thymus. These effects appeared to be completely reversible after 6–10 days [30]. The other histidine analogues with antimalarial activity *in vitro*, α-Me-L-HIS, 2-I-D,L-HIS, WR177589A and 2-N_3-L-HIS,

are also likely to have toxic effects on the vertebrate host of the asexual malaria parasite. However, it may be possible to use sufficiently low concentrations of histidine analogues that do not significantly affect the host, but which affect *P. falciparum*, since synthesis of proteins of unusually high histidine content appears to be a specific property of the malaria parasite.

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