Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6585–6589, August 1991
Medical Sciences

Reversed siderophores act as antimalarial agents

(malaria/*Plasmodium falciparum*/chemotherapy/iron)

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Communicated by William Trager, April 22, 1991

ABSTRACT **We describe here a family of biomimetic iron carriers that display high binding efficiency for ferric ions and favorable permeation properties across erythrocytic membranes. These carriers inhibit *in vitro* growth of *Plasmodium falciparum* by scavenging intracellular iron. The chemical features were realized by reproducing the iron-binding cavities of natural iron carriers (siderophores) and by systematic substitutions of their hydrophilic envelopes for more hydrophobic ones. In contrast to natural carriers, which participate in receptor-mediated iron uptake in cells and act as growth promoters, our synthetic carriers were designed to penetrate cellular membranes by diffusion, scavenge intracellular iron, and thereby act as growth inhibitors. Based on these properties we designate the compounds reversed siderophores and refer to the specific analogs of the natural ferrichrome as synthetic ferrichromes. The antimalarial activity of the synthetic ferrichromes correlated with their lipophilicity, and this antimalarial activity was averted when the chelators were applied as iron(III) complexes. The sites of synthetic ferrichrome action reside in the intraerythrocytic parasite and not in serum or on normal erythrocyte components. The agents were effective against all stages of parasite growth and against a variety of multidrug-resistant strains of *P. falciparum*. The most potent agent of this synthetic ferrichrome series, SF1-ileu, was not toxic to mammalian cells in culture and was 15-fold more potent and 20-fold faster acting than desferrioxamine. Taken *in toto*, these agents constitute a series of promising candidates for future use in malaria chemotherapy.**

Other types of antimalarial agents are urgently needed to combat the global expansion of malaria. This expansion and the resurgence of drug resistance in various regions of the world have prompted the development of innovative chemotherapeutic strategies (see refs. 1–4 for reviews). Because iron is an essential element for virtually all living species (5), the development of compounds that can exert differential sequestration of essential iron(III) could prove useful for arresting parasite growth, including strains of *Plasmodium falciparum* of demonstrable drug-resistance nature. Previous attempts to use natural and synthetic iron binders as *in vitro* and *in vivo* growth inhibitors of intraerythrocytic parasites scored some success (6–14). However, each class of compounds thus far examined has had some drawbacks. The hydroxamate-based natural siderophores, which are highly specific chelators, are only moderately efficient antimalarials because of their poor permeability into infected erythrocytes, their putative site of action (14). Synthetic dithiocarbamates and hydroxyquinolines, on the other hand, proved efficacious, apparently because of a combination of high permeation features and metal complexation; however, their propensity for forming cytotoxic metal complexes might curtail their use *in vivo* (6–10). Synthetic catecholates, which acted as specific iron scavengers and showed satisfactory membrane penetration, appeared also of limited use because they demonstrably deplete serum iron pools (11).

This study focuses on iron(III) carriers that were designed to overcome the above limitations and to display (*i*) high binding specificity, (*ii*) requisite permeation properties for gaining rapid access to sensitive intracellular iron sources, and (*iii*) compartmental selectivity, so as to affect cell iron pools while sparing those in serum.

The chemical design is basically biomimetic, using as a guiding model the natural ferric ion carriers known as siderophores. Siderophores are microbial ferric ion binders of hydrophilic character that bind iron in the medium and transport it into the cell via a receptor-mediated mechanism (15–18). We chemically reproduced the iron-binding properties of the siderophores but replaced their hydrophilic envelopes with hydrophobic ones to facilitate penetration into infected erythrocytes. Because the functions of such binders are opposite, or reversed, to those of natural siderophores, we coined the term "reversed siderophores" (RSs) for them.

The RS design was based on a tripodal topology that mimics the natural ferrichrome model (Fig. 1). These synthetic ferrichromes (SFs) are assembled in a modular fashion with amino acid residues of variable hydrophobicity. Such an approach facilitates systematic chemical modifications for obtaining optimal antimalarial performance. Moreover, by tagging the molecules with fluorescent or radioactive markers, the drug can also be traced in the various cellular compartments (S.D.L. and Z.I.C., unpublished work).

In this work we describe a series of RSs with demonstrable antimalarial activities. These activities are shown to correlate with the lipophilicity and iron-binding capacity of the agents. Antimalarial effect was largely determined by the ability to selectively scavenge intracellular iron(III) from intracellular compartments. The isoleucine RS derivative SF1-ileu was found 15-fold more potent and 20-fold faster acting than the classical desferrioxamine B (Dfo), and it was effective against all stages of parasite development. Although mammalian cells in culture were hardly affected by treatment with these agents, intracellular growth of *P. falciparum* strains spanning a wide spectrum of drug resistances was markedly arrested by SF1-ileu and other related congeners.

MATERIALS AND METHODS

Synthesis of RSs. Synthesis of the RSs relied on a three-stage strategy. The first stage involved preparation of the C_3-symmetric anchor as its active trisphenolate ester from 1,1′,1″-trishydroxymethylpropane by (*i*) treatment with acrylonitrile, (*ii*) hydrolysis, and (*iii*) condensation with pentachlo-

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Abbreviations: RS, reversed siderophore; SF, synthetic ferrichrome; Dfo, desferrioxamine B; DMSO, dimethyl sulfoxide.

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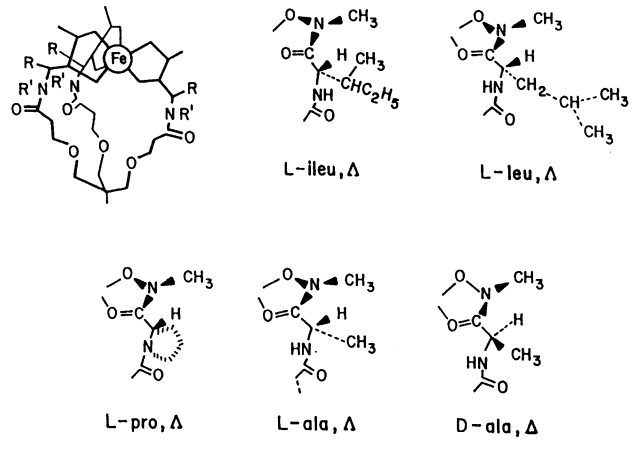

FIG. 1. Chemical structure of a prototypic RS (upper left) with a tripodal design and the iron-chelating cavity. Structures of the hydroxamate- and amino acid-containing moieties are depicted for the different amino acid derivatives used.

rophenol. The second stage involved preparation of the amino acid bridges with the hydroxamate-bearing residues. This stage included (*i*) converting the protected benzyloxycarbonyl-amino acids to the corresponding carboxyphenolates, (*ii*) treating the phenolates with methylhydroxylamine, and (*iii*) removing the protecting group by hydrogenation. The third and final stage involved coupling of the trisphenolate ester with the amino acid residues. The final products were purified by chromatography on silicagel and fully characterized by their analytical and spectroscopic properties. Experimental details of the synthesis will be published elsewhere (A.S. and J.L., unpublished work).

Physicochemical Properties of RSs. The ion-binding stoichiometry of the RSs to Fe^{3+} was determined spectrophotometrically at 430 nm by titration with $FeCl_3$ in aqueous MeOH [80% (vol/vol) MeOH/20% (vol/vol) 0.1 M aqueous NaOAc]. All ligands were found to form 1:1 complexes. The absolute configuration of complexes in aqueous MeOH was determined by the absolute signs of their CD-Cotton effects at ≈470 and 370 nm. Positive Cotton effects at the longer wavelength and negative ones at the shorter one indicate Λ cis configuration; the opposite signs stand for Δ cis configuration. All ligands with amino acid constituents of L configuration formed complexes with Λ cis configuration, whereas the ligand with D-alanine formed a Fe^{3+} complex with Δ cis configuration.

The Relative Binding Efficiencies of the RSs. The binding efficiencies of the RSs [0.75 mM and 0.15 mM Fe^{3+} in aqueous MeOH (MeOH/0.1 M NaOAc)] were determined by competition with EDTA (0.15 mM). After equilibration overnight, the fraction of Fe^{3+} complex present was determined at 430 nm.

Extractions. Extractions were performed by overnight equilibration of 0.3 mM chloroform solutions of RS with aqueous solutions of 0.3 mM $FeCl_3$/0.3 mM citric acid/40 mM Tris, pH 6.9. The amount of iron taken up into the organic phase was determined by absorption of the iron complex at 430 nm. No iron uptake into chloroform solutions devoid of SF was seen by ion chromatography.

Partition Coefficients. These coefficients were obtained by overnight equilibration of the free RSs between equal volumes of 1-octanol and saline. Concentrations of ligands in each phase were determined by adding excess iron(III) and measuring the ferric complexes at 430 nm.

Parasite Cultures. *P. falciparum* strains used in this study were as follows: ItG2G1 (Brazil, provided by L. H. Miller, National Institutes of Health, Bethesda, MD), D6 (West African, provided by A. J. M. Oduola, Walter Reed Army Institute of Research, Washington), FCR-3 (Gambian, provided by J. B. Jensen, Michigan State University), and W2 (Indochina, provided by A. J. M. Oduola, Walter Reed Army Institute of Research, Washington) were grown in culture flasks of human erythrocytes by a modified version of Trager and Jensen's method (19), as described elsewhere (20).

Bioassay of Iron Carrier Antimalarial Activity. The antimalarial activity of agents was assayed by adding them from concentrated stock solutions [in dimethyl sulfoxide (DMSO)] to microcultures (24 wells, Costar) containing infected erythrocytes (2.5% hematocrit and 2% parasitemia). The cultures were usually synchronized (4- to 7-hr windows) by incubation in 300 mM alanine/10 mM Tris hydrochloride in conjunction with gelatin flotation (19) and used either at the trophozoite (1–2% parasitemia) or at the ring stage (4–6% parasitemia) of the erythrocytic cycle. After the indicated time of incubation with drug and either before or after washing three times with 100 vol of growth medium, the cells were supplemented with 6 μCi (1 Ci = 37 GBq) of either [^{3}H]hypoxanthine or [^{3}H]isoleucine (Amersham) per well, and parasite growth was assessed after 24–48 hr by harvesting the labeled cells onto glass-fiber filters (Tamar, Jerusalem) (21) and counting the radioactivity.

Effect of Preincubation of Erythrocytes or Plasma with Drug on Parasite Growth. Erythrocyte suspensions (5% hematocrit) in RPMI 1640 medium, pH 7.4 (no plasma), were treated for 24–48 hr with either chelator at 100 μg/ml or DMSO alone (<1% final concentration) in culture conditions. Chelator was removed by washing the cells four times with RPMI medium followed by 1-hr incubation at 37°C. Parasite growth was assayed after addition of gelatin-enriched schizonts (>90%) as described above. To deplete serum components from bound iron(III), human plasma (2 ml) from O^+ donors was adjusted to either pH 5 with 2-(*N*-morpholino)ethanesulfonic acid (to enhance iron removal) or pH 7.4 (with Tris base) and treated for 18 hr with chelators at 100 μg/ml or with DMSO. The plasma was subsequently dialyzed for 24 hr against 800

Table 1. Chemical and antimalarial properties of RSs (SF series)

Compound	Relative binding*	Extraction efficiency*	Partition coefficient*	Hydrophobicity†	IC_{50}, μg/ml* (μM)
Dfo	100				30 (46)
SF1-pro	14	38	0.65	1.5	>100
SF1-L-ala	108	88	0.53	1.0	40 (62)
SF1-D-ala	108	88	0.53	1.0	45 (70)
SF1-Leu	10	35	12.5	3.5	17 (22)
SF1-ileu	25	52	14.0	5.0	2 (3)

*For determination of tabulated values, see *Materials and Methods*. IC_{50} values were obtained by exposing cell cultures (trophozoites of *P. falciparum*, strain ItG2G1) to compounds for 48 hr and determining the incorporation of [^{3}H]hypoxanthine into nucleic acids during the last 24 hr of growth, as described.

†Hydrophobicity values are for amino acid chains (Tanford/Segret's scale) as given by D. Eisenberg (23).

ml of 0.1% bovine serum albumin/10 mM glucose/phosphate-buffered saline, pH 7.4, followed by an additional 24-hr dialysis against 400 ml of the same buffer and supplemented to the medium for parasite growth assays.

Neutralization of Drug-Induced Inhibition by Addition of Iron(III). Preformed ferric iron–carrier complexes were prepared by adding increased amounts of $FeCl_3$ (in methanol) to a DMSO solution containing the indicated chelator concentration. Mixtures were incubated for 1 hr at room temperature and added to microculture wells at the trophozoite stage at final concentrations of 30 μg/ml (for Dfo) and 5 μg/ml (for RSs).

RESULTS AND DISCUSSION

Design of SFs. Our design of SFs as antimalarial agents relied on hydroxamate groups as iron(III)-binding sites because hydroxamate-based chelators have been shown to scavenge iron(III) poorly from transferrin (13). To achieve high binding efficiency and selectivity for iron(III), we adopted the tripodal topology, which is ideal for generating octahedral binding cavities and is common among siderophores, such as enterobactin, ferrichrome, etc. (22). We, however, replaced the nonsymmetric structure of ferrichrome by a C_3-symmetric one, substituted the chiral hexapeptide anchor for a tricarboxylate residue, and compensated the resulting chirality loss by extending the anchor with chiral amino acids (Fig. 1). Using amino acids as variable extensions constitutes one modular element that permitted systematic modification of the lipophilicity of the molecule.

Following this approach, a series of C_3-symmetric iron(III) chelators were prepared based on the following reaction scheme:

$$\begin{array}{ll} \text{Et-C-(CH}_2\text{OH)}_3 & \text{Cbz-NHCHRCOOH} \\ \downarrow\ 1.\ \text{CH}_2=\text{CHCN} & \downarrow\ 1.\ \text{C}_6\text{Cl}_5\text{OH/DCC} \\ \quad 2.\ \text{H}^+/\text{H}_2\text{O} & \quad 2.\ \text{CH}_3\text{NOHH} \\ \quad 3.\ \text{C}_6\text{Cl}_5\text{OH/DCC} & \quad 3.\ \text{H}_2/\text{Pd-C} \end{array}$$

$$\text{EtC(CH}_2\text{OCH}_2\text{COOC}_6\text{Cl}_5)_3 + \text{H}_2\text{NCHRCONOHCH}_3$$

$$\downarrow$$

$$\text{EtC(CH}_2\text{OCH}_2\text{CH}_2\text{CONHCHRCONOHCH}_3)_3,$$

where R = methyl (alanine), isobutyl (leucine) or secondary butyl (isoleucine); DCC = dicyclohexyl carbodiimide; Et = ethyl; Cbz = benzyloxycarbonyl; and Pd-C = palladium on carbon.

Physicochemical Properties. The physicochemical properties of the free molecules and of their iron(III) complexes are summarized in Table 1. Lipophilicity of the various agents, as measured by their partition between octanol and saline, correlated highly with hydrophobicity of the amino acid side chain.

All the SFs synthesized displayed high binding affinity for iron and formed 1:1 stoichiometric complexes with the iron, similar to what has been found for natural siderophores. Absolute configuration of the complexes was predominantly left-handed, Δ cis, when natural L-amino acids were used, and right handed, Δ cis, when D-amino acids were used. The function of these agents as potential iron(III) scavengers and carriers is clearly demonstrated by their capacity to extract and transfer ferric ions from aqueous to organic phases, which resemble the hydrophobic domains of biological membranes. A 26-fold preference of SF extraction of iron(III) as compared with copper(II) from an aqueous into an organic phase was thus observed when SF-ileu was used as extractant (S.D.L. and Z.I.C., unpublished work).

Antimalarial Activity. The antimalarial activity of the different chelators was initially evaluated on cultures of trophozoites that were exposed for 24 hr to different concentrations of chelators. After that period, [^{3}H]hypoxanthine or [^{3}H]isoleucine was added, and 24 hr later the parasitemia and incorporation of label into macromolecular material were determined. Typical dose–response curves for Dfo and SF1-ileu (Fig. 2) were used for determining IC_{50} values (Fig. 2 *Inset*). A compilation of these values for various structural congeners of SF1-ileu is presented in Table 1.

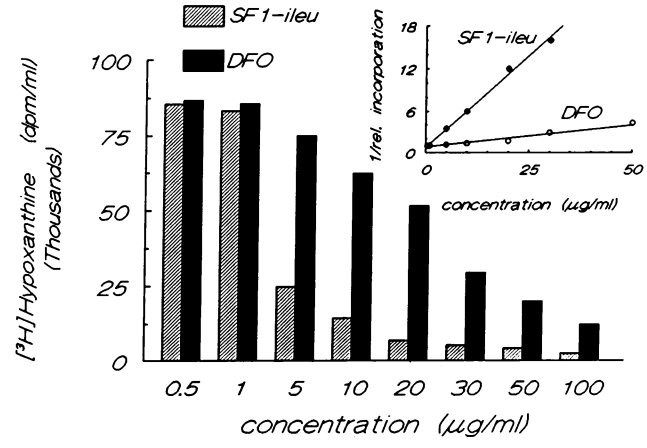

FIG. 2. Dose–response curves of Dfo (DFO) and SF1-ileu on parasite growth measured over 48-hr drug exposure. (*Inset*) Dixon plot of data used for computation of IC_{50} values (Table 1).

The efficacy of the agents is clearly primarily correlated with their respective octanol/saline partition coefficient or the hydrophobicity of the amino acid side chain and only secondarily with their iron-binding efficiencies, which are relatively high for all agents. The most potent congener of this series, the isoleucine-containing SF1-ileu, was ≈6- to 13-fold more efficient than Dfo when administered for 24- to 48-hr exposure periods to various strains of parasites that display a wide spectrum of drug-resistance to antimalarial agents (Table 2).

The antimalarial activity of the various chelators was assessed over a 24-hr period of exposure on infected cells (*P. falciparum* trophozoite stage), as described. W2 and FCR-3 represent chloroquine-resistant strains of *P. falciparum*, whereas D6 and ItG2G1 strains represent chloroquine-sensitive strains.

Mechanism of Inhibition. Plasma and uninfected erythrocytes were separately treated with relatively high concentrations of chelators (100 μg/ml) to evaluate whether the site of action of the lipophilic chelators was associated with components of the plasma and/or the uninfected erythrocyte. The 24-hr exposure of either plasma or cells to 40-fold higher concentration of the SF1-ileu IC_{50} followed by washing or dialysis of the free drug was found to have no significant effect on the ability of plasma and cells to support parasite growth (data not shown). This finding demonstrates that the inhibition by the lipophilic iron chelators was at the level of the infected cell. Because in an independent study we observed that lipophilic chelators, such as SF1-ileu, can easily

Table 2. Antimalarial activity of siderophores in various strains of *P. falciparum*

Parasite strain	Dfo IC_{50}, μg/ml (μM)	SF1-ileu IC_{50}, μg/ml (μM)
D6	40 ± 9 (61)	6 ± 2 (7.7)
ItG2G1	35 ± 3 (53)	5 ± 2 (6.4)
W2	31 ± 6 (47)	4 ± 1 (5.2)
FCR_3	32 ± 7 (49)	3 ± 1 (3.8)

IC_{50} values (μM in parentheses) are for 48-hr-exposure of a trophozoite culture to different drug concentrations; the last 24 hr were in the presence of [^{3}H]hypoxanthine, as described.

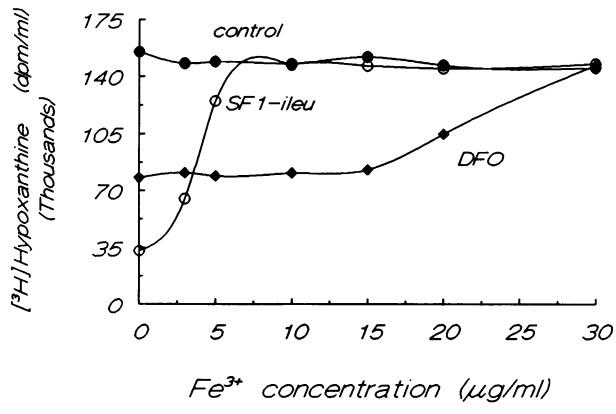

FIG. 3. Effect of extracellular iron on chelator-induced inhibition of parasite growth (trophozoites). Concentration of drug used was the IC_{50} value depicted in Table 1. DFO, Dfo.

penetrate into erythrocytes (S.D.L., unpublished work), the possibility that the antimalarial action of the drugs was associated with chelation of intraerythrocytic or parasitic iron is strongly implied. To assess this point, we examined whether the RSs cause growth inhibition by virtue of iron deprivation (extraction at the level of the infected cell) as compared with formation of toxic iron complexes. The effect of chelator on trophozoite cultures was therefore examined with increased amounts of ferric salt. The results (Fig. 3) clearly indicate that in analogy with the mode of Dfo action (14, 28) but not that of the synthetic hydroxyquinolines and

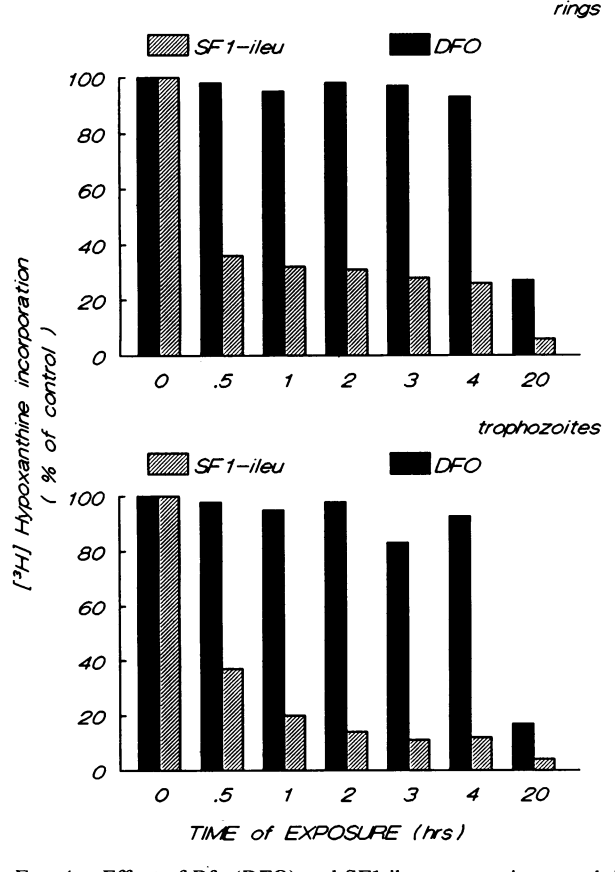

FIG. 4. Effect of Dfo (DFO) and SF1-ileu on parasite growth in different developmental stages. Effect of exposure time of infected cells to drug. After indicated time, cells were washed from extracellular drug by repeated washings with growth medium and were subsequently tested for [³H]hypoxanthine incorporation for 16 hr in growth medium.

dithiocarbamates (6–8), stoichiometric addition of iron(III) salt to SF fully averted their antimalarial activity. This preventive effect afforded by iron was not caused by formation of impermeant ferric siderophore complexes because the latter were shown to be demonstrably permeant both to uninfected and infected cells (unpublished work). The complexes were evidently nontoxic to parasites, thus ruling out chelated iron-mediated lipid peroxidation as the *modus operandi* of the drugs. Such mechanism of antimalarial activity has been proposed for other iron chelators (24, 25). The most likely explanation for the antimalarial effect of SFs is related to siderophore-mediated sequestration of iron from essential sources—i.e., enzymes (5, 9) or degraded hemoglobin (26).

Growth Stage of Parasites and Time Dependence of Inhibition. The time dependence of the inhibitory effect of SF1-ileu (10 μg/ml) *vis-à-vis* that of Dfo (80 μg/ml) was assessed on both synchronized cultures of rings and cultures of trophozoites. Cells were exposed to the indicated concentrations of agents in culture conditions; at the indicated times (0–4 hr) samples were withdrawn, and the cells were washed and assessed for [³H]hypoxhantine incorporation after the last sample completed 4-hr drug incubation period. Fig. 4 shows that major inhibitory effects of SF1-ileu were already evident after 0.5-hr incubation with drug at either ring or trophozoite stage. Almost maximal inhibition was attained after 2-hr drug incubation, which was ≈70% for rings and 90% for trophozoites. In both stages, more prolonged drug incubations (>20 hr) led to >95% inhibition of parasite growth. On the other hand, with 8-fold higher concentrations of Dfo, inhibitory effects were dismal after 6-hr drug exposure, and only after 20-hr exposure was substantial inhibition attained.

Effect of RSs on Mammalian Cells. That the RSs might affect mammalian cells in culture was assessed in conditions identical to those used for parasites in cultures. In the concentration range and exposure time that are demonstrably effective, neither SF1-ileu nor Dfo affected proliferation of mouse NIH 3T3 or human HT29 cells, as measured by

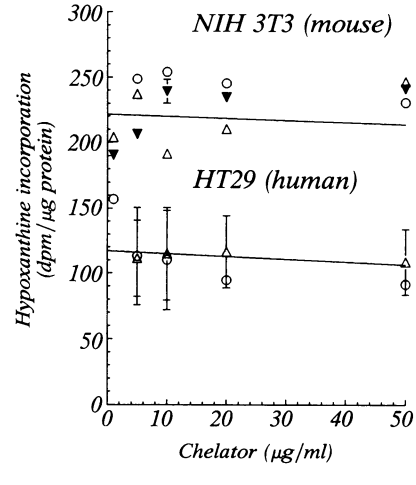

FIG. 5. Effect of chelators on proliferation of mammalian cells in culture. Nucleic acid synthesis was measured in cultures of mouse NIH 3T3 fibroblasts and human HT29 colonic carcinoma cells with either SF1-ileu (○) in DMSO, Dfo (▲) in DMSO, or DMSO alone (△; control; <1% final) at the same concentrations. Cultures at confluence were exposed to chelators for 24 hr—the first 3 hr without radiolabel and 21 hr with [³H]hypoxanthine at 6 μCi/ml. Extracellular hypoxanthine was removed by several washes with phosphate-buffered saline, and the cells were treated with 5% trichloroacetic acid for 15 min at 5°C and solubilized with hot (60°C) 2.5% SDS. Samples were taken for counting of radioactivity and measurement of protein (BCA, Pierce). Data are given as dpm/μg of protein and SD (duplicate samples). Analysis of variance (ANOVAR) shows no significant difference between groups at <1% level Snedecor's *F* test.

nucleic acid synthesis (Fig. 5) or protein synthesis ([^{3}H]isoleucine incorporation, data not shown).

CONCLUSIONS

The modular design of another family of iron chelators of defined chemical properties provided a series of antimalarial agents for which efficacy is determined, to a large extent, by the hydrophobic character of the amino acid side chains (Table 1). In agreement with these findings is the observation that SF1-ileu, the most potent member of the SF1 series, penetrates rapidly (in the uncomplexed form) into infected erythrocytes (unpublished work). The inhibitory effects of SF1-ileu on DNA and protein synthesis were already manifested within <1/2 hr of exposure of either rings or trophozoites to IC_{50} concentrations of drug (Fig. 4). Other SF1s as well as Dfo required >10 hr to elicit substantial inhibitory effects; the trophozoite stage was apparently most susceptible to the antimalarial of these drugs. Site of action of the RSs is definitely not either in the serum or on uninfected cells. The most likely site of action is the infected cell, although the precise compartment cannot be specified. SF-ileu displays specificity at four levels: (*i*) it acts on infected cells while sparing serum iron (i.e., transferrin), (*ii*) it does not affect irreversibly the ability of uninfected cells to support parasite growth, (*iii*) it acts swiftly on intracellular parasites, and most important, (*iv*) it does not affect the growth of mammalian cells in culture. With the knowledge that infected cells have marked but defined changes in host cell membrane permeation properties (2, 19, 27), it should be possible, in principle, to use the present modular assembly of agents so as to construct RSs with the requisite hydrophobicity and/or molecular dimensions for selective penetration into infected erythrocytes.

The authors kindly acknowledge Mrs. R. Lazar for her skillful assistance in chemical synthesis and Mrs. J. Silfen for her technical help in the initial stages of this work. This work was supported, in part, by National Institutes of Health Grant AI-20342 and AID-CDRC7-171 (to Z.I.C.).

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