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# Mode of Action of Iron(III) Chelators as Antimalarials. III. Overadditive Effects in the Combined Action of Hydroxamate-Based Agents on *In Vitro* Growth of *Plasmodium falciparum*

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**SUMMARY**

Hydroxamate-based iron(III) chelators exhibit potent antimalarial effects on the asexual stages of *Plasmodium falciparum* grown *in vitro*. Antimalarial activity varies with the parasite growth stage and the drug permeation properties. The hydrophilic drug desferrioxamine (DFO) is ineffective on early stages (ring forms) of the parasite due to its poor permeability but irreversibly blocks the growth of advanced stages of parasites. On the other hand, hydrophobic reversed siderophores (RSFs) are more membrane permeable and affect all parasite developmental stages; they affect ring forms irreversibly and trophozoite/schizont forms reversibly and at relatively faster rates, compared with DFO. These observations have provided the basis for postulating a possible overadditive action of the two, distinctly acting, iron chelator types for enhanced antimalarial activity. This was assessed in this study by using novel fast-acting chelators such as RSF derivatives (RSFleum2 and RSFm2) in combination with the relatively slow-acting DFO. Parasite growth was assessed in terms of nucleic acid synthesis and parasitemia. The results indicate that, at any molar ratios of the two types of drugs, the combined inhibitory effect was faster and more potent than the sum of individual effects. The combined drug action showed neither additive nor independent but overadditive properties, as well as sustained inhibition even after drug removal. The potentiating action of RSFs on the long-lasting effects of DFO on parasite growth conformed with the postulated mechanistic model of iron chelator action and iron handling by parasites. Iron chelator combinations might be of therapeutic value.

The life-threatening malaria caused by the human parasite species *Plasmodium falciparum* has steadily spread throughout the tropical world (1). The expansion of the disease and the growing problem of acquired drug resistance has prompted the demand for novel therapies. Iron chelation therapy was considered a suitable treatment for various infectious diseases, including malaria. Recent experimental data obtained *in vitro* (2, 3), in rodent (4) and primate models (5), and in clinical studies (6–9) showed that treatment with the natural siderophore DFO suppressed parasite growth. The drug-affected target was surmised to be ribonucleotide reductase (9), a key enzyme in the DNA synthetic apparatus.

The biochemical basis for the selective cytotoxicity of iron(III) chelators in malaria has remained elusive (10). Malaria parasites, like all living organisms, require iron for development and their growth is suppressed by iron(III) chelators such as the hydroxamate-based DFO (2–9, 11–13), ferrichromes (13–16), and others (17). For chelators such as DFO, their action on malaria parasites (11, 18) or mammalian cells (19) depends on drug access to intracellular compartments and its capacity to scavenge iron (20). The iron pool affected by drugs like DFO was localized within the parasite (11, 18) and is presumably associated with hemoglobin breakdown (21). Nonetheless, the mode of action of iron(III) chelators on cell development differs in parasites, compared with mammalian cells (10). Whereas mammalian cells fully recover after iron chelation treatment, parasites do so to only a very limited extent. Failure to regenerate metabolic activities was observed in parasites after extracellular replenishment of iron either in free ionic form [iron(III) or iron(II)] or as transferrin [iron(III)] (13). This phenomenon arises both from the intrisically restricted capacity that parasites have for mobilizing and integrating bioavailable iron and from failure to regenerate biological activities after treatment with particular iron chelators (10, 13).

The cytotoxic action of iron chelators is parasite stage dependent (12, 15, 16) and is largely dictated by three factors (10), i.e., iron(III)-binding capacity, chelator ingress into in-

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**ABBREVIATIONS:** DFO, desferrioxamine; RSF, reversed siderophore; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

fected cells, and chelator egress from parasites after treatment. The hydrophilic DFO, which displays poor permeation across membranes (11), is relatively slow in eliciting biological effects on intracellular parasites (11, 12). However, DFO effects, which are manifested primarily in the trophozoite/schizont stage, persist even after drug removal (10, 13). On the other hand, recently synthesized hydroxamate siderophores (RSFs) display good permeation properties and relatively fast and potent antimalarial action on all stages of parasite growth (13, 16). However, persistent effects of RSFs on parasites were observed primarily in rings (13).

The mode of action of iron(III) chelators such as DFO and RSFs was rationalized in a working model (10, 13), which provided an explanation for the cytotoxic actions of both types of agents and the stage specificity of the reversible and irreversible effects. The model highlighted the advantages but also the limitations of using either type of chelator, i.e., the lack of DFO effects on rings and the reversibility of RSF effects on trophozoites. In this work we assessed the model experimentally, as a basis for designing iron chelation schemes for the management of the disease. We focused primarily on the restricted cytotoxicity displayed by each class of chelators and whether it could be overcome by simultaneous application of both agents. A predicted feature of the model (10, 13) is that the combination of agents that act at potentially similar parasite targets but access them at different rates is likely to produce overadditive action, with fast-acting RSFs potentiating the persistent effects of the slow-acting DFO. The RSF derivatives (RSFleum2 and RSFm2) (Fig. 1) were applied here either separately or together with DFO, at either equal or different molar ratios, and their antimalarial effects were determined kinetically. These were assessed in terms of inhibition of both nucleic acid synthesis and parasitemia, either during drug treatment or after drug removal. The results, as predicted from the model, indicated that the combined application of both agents demonstrably improved the antimalarial efficacy of individual drugs beyond additivity and stage-restricted cytotoxicity.

## Experimental Procedures

**Materials.** Iron chelators of the RSF family were prepared and analyzed by previously described methods (14). RSFm2 is a member of the RSF tripodal family that, unlike the other congeners, lacks amino acid substitution, whereas RSFleum2 carries a two-carbon spacer and a leucine residue on each arm (Fig. 1). Iron(III) binding affinities and octanol/water partition coefficients were determined by methods described elsewhere (15, 16). DFO was obtained from Ciba Geigy (Basel, Switzerland). All other chemicals were from Sigma Chemical Co. (St. Louis, MO) and were the best available grade. [$^3$H]Hypoxanthine was from the Radiochemical Centre (Amersham, UK).

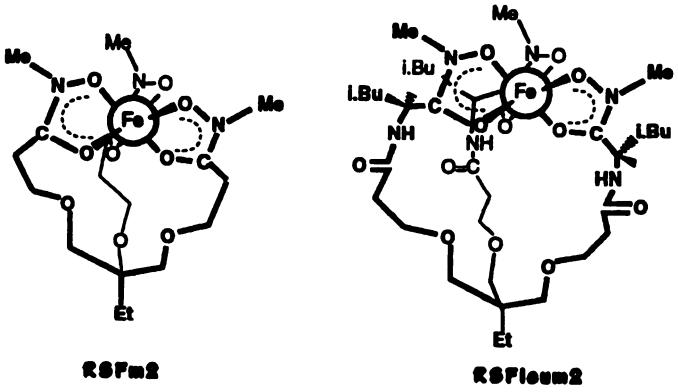


**Fig. 1.** Structures of RSFm2 (*left*) and RSFleum2 (*right*). *Me*, methyl; *Et*, ethyl; *i.Bu*, isobutyl. The two RSF congeners had the following properties: partition coefficients (octanol/saline): RSFm2, 1.7; RSFleum2, 12.5; binding efficiency with iron(III), relative to DFO (100): RSFm2, 47; RSFleum2, 10.

**Parasite and cell cultures.** *P. falciparum* (strain FCR-3) used for all experiments was maintained in culture flasks (Nunclon) in human $A^+$ or $O^+$ red blood cells, in RPMI 1640 medium supplemented with 25 mM HEPES, 10 mM glucose, 20 mM $NaHCO_3$, and 10% (v/v) heat-inactivated, $A^+$, human plasma pooled from different blood donors. The cultivation method used was a modified version of the method of Trager and Jensen (22), as described elsewhere (15). Parasitemia and growth stage distribution were determined on methanol-fixed and Giemsa-stained smears. Chinese hamster ovary cells, human erythroleukemia K-562 cells, and human hepatoma Hep G2 cells were grown under standard culture conditions (in Dulbecco's modified Eagle's medium at 37° in a 5% $CO_2$ atmosphere) in 24-well plates (Nunc, Roskilde, Denmark).

**Bioassay of the individual and combined chelator activities.** The antimalarial activity of RSFs alone or in combination with DFO was assayed as described previously (15). The compounds were added from concentrated stock solutions in dimethylsulfoxide to microcultures (24 wells; Costar, Cambridge, MA) containing infected red blood cells (2.5% hematocrit and 2–5% parasitemia). The cultures were synchronized to the ring stage by incubation in 300 mM alanine, 10 mM Tris·HCl, pH 7.4. Twenty-four hours after incubation with the indicated drug, either the cells were supplemented directly with 6 μCi/ml [$^3$H]hypoxanthine or the drugs were washed off three times with a large volume of RPMI 1640 medium and replaced with fresh growth medium before addition of radiolabel. All systems were run in sextuplicate. Parasite growth was assessed after an additional 24 hr by harvesting the freeze-thawed lysate of labeled cells onto glass fiber filters (Tamar Inc., Jerusalem, Israel). Incorporation of label into nucleic acids was measured with a Beckman scintillation counter. The effects of drugs on mammalian cells in culture were assessed as described previously (15).

The $IC_{50}$ values were determined by nonlinear least-squares fits to sigmoidal functions as described by Desjardins *et al.* (23). Combined drug actions were assessed according to the method of Poch (24), and theoretical values for "additive" and "independent" modes of action of drug combinations were compared with experimental values by statistical *t* tests. Those were performed according to the Pharmacologic Calculation System statistical package, based on Ref. 25. The Microsoft Windows-based computer software MicroCal Origin (MicroCal Software) was used for curve simulation and for graphic display.

## Results

**Properties of iron chelators.** The antimalarial effects of RSFs and DFO are manifested at different developmental stages of intracellular parasites (11, 16). These properties are dictated primarily by the membrane permeation profiles of the various agents or, to a first approximation, by their partition coefficients (16). The RSFs RSFleum2 and RSFm2 used in this study displayed iron(III) binding affinities and partition coefficients indicated in the legend to Fig. 1.

According to the structure-activity relationship scheme presented elsewhere (16), the inhibitory potency of RSFleum2 in *in vitro* cultures of *P. falciparum* should follow the empirical relationship between $IC_{50}$ and the product of the partition coefficient and the iron(III) binding affinity. RSFleum2 represents an RSF congener with greater hydrophobicity and therefore faster permeation properties than DFO, yielding the expected higher potency. RSFm2 is a RSF congener with no amino acid substituent and relatively shorter side chains. How-

ever, the relatively smaller molecular radius was anticipated to impart improved permeation properties across membranes and thus compensate for the moderate value of its partition coefficient.

### Stage Dependence and Inhibitory Efficacy of DFO Action in the Presence of RSFs

The individual and combined effects of RSFm2 or RSFleum2 and DFO were assessed on synchronized ring cultures after exposure to drug for either 24 or 48 hr. The individual drug concentrations were set at levels that produced very minor inhibition of growth, as determined by parasitemia values and differential counting of stages on Giemsa-stained smears. For the particular drug treatments used, all cultures progressed normally up to the trophozoite stage (Fig. 2A), and most cultures completed a cycle of growth including reinvasion, except in the combined presence of both drugs (Fig. 2B). In the combined presence of both drugs, parasites were arrested at the trophozoite stage and showed signs of deterioration. Clearly, the combined effects of RSFm2 and DFO exceeded those of individual drugs or their additive action.

The trophozoite was identified as the most drug-sensitive stage. A qualitatively similar picture was found when drugs were applied at the trophozoite stage and the cultures were inspected 24 hr later (data not shown).

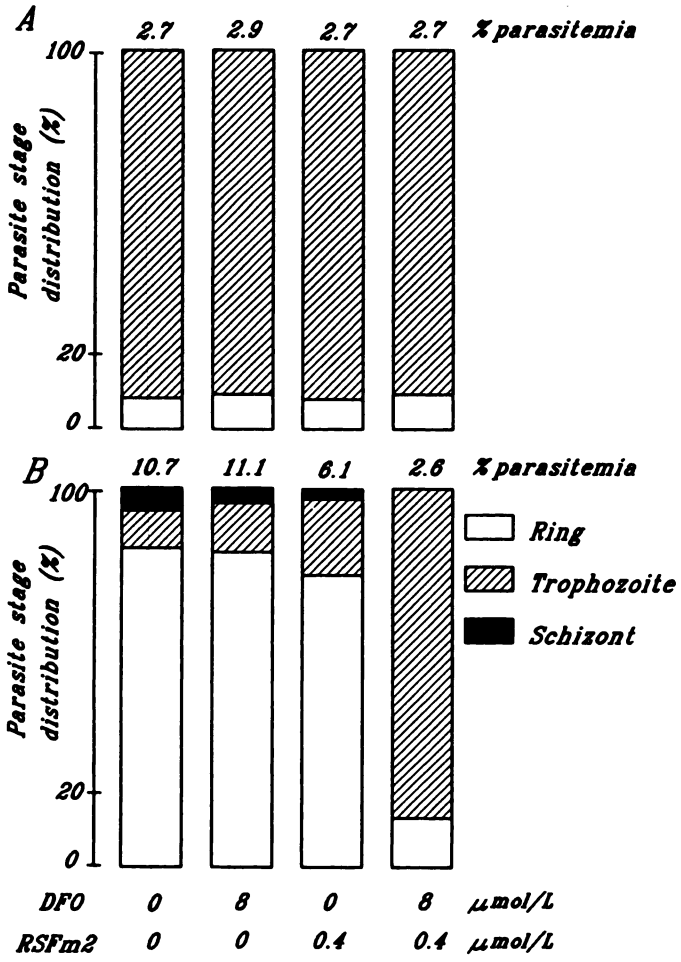


**Fig. 2.** Effects of DFO and RSFm2 on the growth of ring forms. Cultures of early rings (1% parasitemia) were exposed for either 24 hr (A) or 48 hr (B) to 8 μM DFO and 0.4 μM RSFm2, either alone or combined. Microscopic examination and differential counting were done after methanol fixation of blood smears and staining with Giemsa stain. When both chelators were jointly applied, many more abnormal trophozoites appeared than when agents were individually applied.

The dose-response curves for RSFm2, RSFleum2, DFO, and the equimolar combinations of RSFs with DFO are depicted in Fig. 3. Parasite development was assessed in terms of nucleic acid synthesis, which was monitored during the last 24 hr of exposure as [$^3$H]hypoxanthine incorporation into macromolecules. At nearly all drug levels used, the inhibitory effect of either RSFleum2 or RSFm2 was demonstrably greater than the effect of the equivalent treatment with DFO (Fig. 3A). When used in combination with DFO doses that produced no detectable inhibition, the action of either RSF was apparently potentiated or, conversely, DFO action became apparent. This feature of overadditivity was particularly evident when the effects of two concentrations of RSFs and DFO were depicted in terms of percentage inhibition, relative to control (Fig. 3C and 3D).

To gain insight into the nature of the combined action of the two type of drugs, RSFs and DFO were used in various combinations to inhibit parasite growth. Data in Fig. 4 depict the degree of inhibition attained with either compound alone ($I_a$ and $I_b$) at the indicated concentrations, as well as their combined effects ($I_{ab}$). The values given above the bars (Fig. 4) depict the theoretical $Y = (I_a + I_b)/I_{ab}$, where $Y = 1$ denotes additive effects and $Y < 1$ indicates overadditive effects. For all drug combinations tested the results conform with overadditivity. A more rigorous analysis is given below.

### Quantitative Analysis of the Combined Effects of Iron Chelators

The analytical treatment used for assessing the combined effect of DFO and the RSFs was based on the method described by Poch (24). The experimental points given in Fig. 5 correspond to inhibition of parasite nucleic acid synthesis by RSFleum2 alone or in combination with a constant concentration of DFO (10 μM) (Fig. 5a). Fig. 5b depicts the same for RSFm2. The lines through the experimental points were obtained by best fits based on Lorentzian distributions. The experimental data were used to construct theoretical curves based on the "dose approach" (additive action) and the "effect approach" (independent action). Statistical comparison between observed (experimental) and calculated (theoretical) values for both additive and independent action demonstrated that the combined effects of either RSFleum2 and DFO or RSFm2 and DFO on *P. falciparum* growth were significantly different from either additive or independent responses (Fig. 5, legend). The mode of action of DFO combined with either RSF was clearly overadditive.

### Mode of Action of DFO and RSFs

**Precomplexation of chelators with metals.** To establish that the observed inhibitions resulted from drug-mediated sequestration of iron rather than formation of toxic chelator-metal complexes or interaction between different chelators, DFO and RSFs chelators were precomplexed with various metals and assessed for inhibitory effects at 30 μM concentrations. The metals included iron(III) as $FeCl_3$ and iron(II) as $Fe(NH_4)_2(SO_4)_2$, $ZnCl_2$, $MgCl_2$, and $CaCl_2$. After a 2-hr preincubation of chelator and metal, to ensure maximum interaction, the complexes were added to *P. falciparum* cultures. The fact that full complexation of iron(II) and iron(III) indeed occurred was corroborated spectrophotometrically by monitoring the

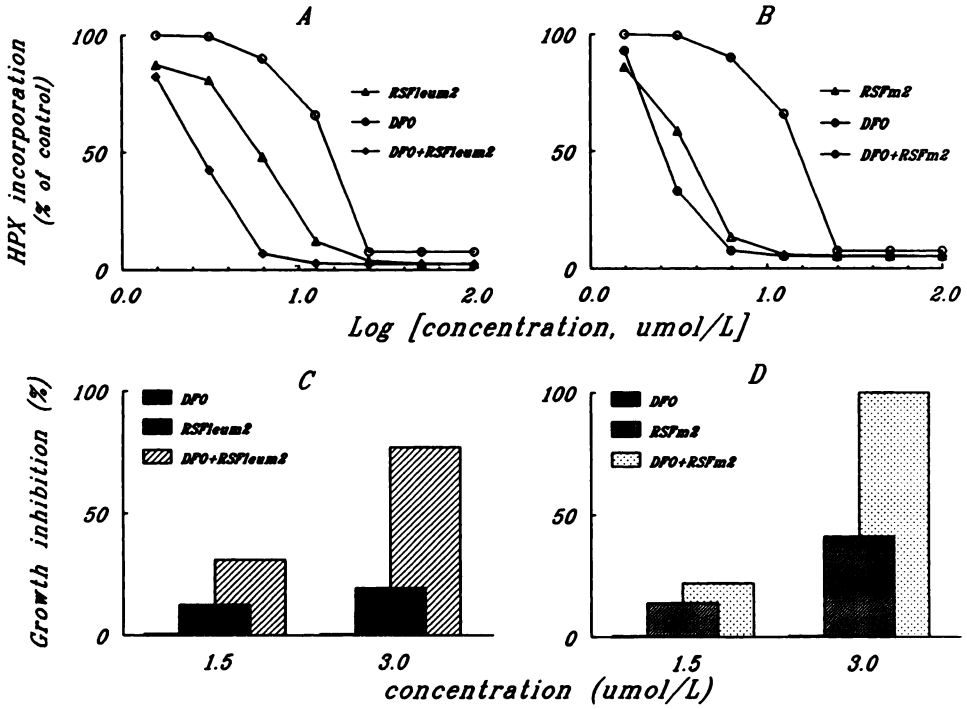


**Fig. 3.** A and B, Dose-response curves for RSFleum2, RSFm2, and DFO used individually or in equimolar combinations of DFO and RSFs. Parasites were synchronized by successive treatments with 300 mM alanine and 10 mM Tris·HCl, pH 7.4. Ring cultures were treated with drug for 48 hr, with the last 24 hr being used also for assessing nucleic acid synthesis in terms of [$^3$H]hypoxanthine (*HPX*) incorporation. The experimental errors for all systems were <10% of the average of sextuplicate samples. The respective $IC_{50}$ values for growth inhibition, determined according to the method of Desjardins *et al.* (23), were 4 ± 2 μM for RSFleum2, 3 ± 1 μM for RSFm2, 17 ± 1 μM for DFO, 3 ± 0.3 μM for RSFleum2 and DFO, and 2.2 ± 0.2 μM for RSFm2 and DFO. C and D, Apparent overadditive activities of RSFs and DFO.

iron-hydroxamate absorption characteristics (15). Iron(II) normally undergoes hydroxamate-catalyzed oxidation to iron(III) before stable complexes are formed (26). We determined that all of the magnesium, calcium, and zinc salts had virtually no effect on the antimalarial activity of either DFO alone, RSFleum2 or RSFm2 alone, or the respective combinations of DFO and RSFs. These agents caused 76–97% reduction in nucleic acid synthesis, relative to control. However, comparable studies with iron(II) or iron(IIII) salts showed complete abrogation (±16%) of the inhibitory activity of the chelators.

**Kinetics of inhibition.** In the design of experiments with combinations of the two types of agents, we selected RSF concentrations that were in the range of the $IC_{50}$ (micromolar) values. The time-dependent synthesis of nucleic acids was monitored for 24 hr in the presence of either individual agents or pairs of agents applied to ring forms (Fig. 6, *insets*). The results clearly indicate that RSFleum2 (Fig. 6, *upper*, *inset*) and RSFm2 (Fig. 6, *lower*, *inset*) decreased nucleic acid synthesis at equivalent concentrations of DFO that were virtually ineffective on parasites during the first 24–30 hr of growth. However, both RSF congeners produced a markedly greater effect when combined with otherwise ineffective doses of DFO than when used alone.

The time dependence of RSF inhibition was also analyzed in terms of windows of exposure to drugs. The data are given as percentage of nucleic acid synthesis, relative to control. They show marked inhibitory effects of RSFs at the earliest times of exposure. As expected from its hydrophobicity, RSFleum2 reached the plateau level of inhibition at shorter exposure times, compared with RSFm2. The performance of RSFs, or conversely the performance of DFO, improved in the presence of the other drug by an apparently synergistic mechanism, inasmuch as DFO alone failed to inhibit parasites. The onset of the individual action of DFO became clearly apparent only after 16-hr exposure to drug, similarly to previously published observations (11). This indicates that the transition from the individual effects of RSFs to the overadditive effects of RSFs and DFO is limited by the DFO penetration rate.

**Irreversible action of chelators.** To assess the reversibility of the inhibition observed with particular combinations of chelators, cells were exposed for 24 hr to individual agents or to their combinations and were assessed for [$^3$H]hypoxanthine incorporation after the agents were washed from the cells. Table 1 shows that RSFleum2 was partially reversible when it was applied alone. However, in the presence of DFO, the combined effect was sustained even after removal of the chelators. Thus, the persistent inhibition remaining after treatment with RSF and DFO was apparently also overadditive.

**Effects on animal cells in culture.** Individual RSFs and DFO tested at 10 μM final concentrations or combinations of RSFs and DFO each at 10 μM concentrations were assesed in terms of their capacity to inhibit mamalian cell growth in culture (Chinese hamster ovary, K-562, and Hep G2 cell lines). Cells exposed for 24 hr to the individual drugs or their 1:1 combinations were not significantly affected in terms of their nucleic acid synthesis capacity (data not shown). As shown earlier for similar chelators, the selective action of RSFs and of DFO on malaria parasites, compared with mammalian cells, clearly underscored the great susceptibility of parasites to iron deprivation.

## Discussion

The antimalarial potency of hydroxamate-based iron chelators that form hexadentate complexes, such as DFO and RSFs, resides in their capacity to cause parasite iron deprivation. The parasite stage that is apparently most susceptible to chelation treatment is that of trophozoites, although membrane-permeant chelators demonstrably affect rings as well (16). This is consistent with the idea that the major metabolic pool of iron is presumably mobilized by parasites at the trophozoite-schizont stage (11, 13). Data shown in the present study for various RSFs and DFO are consistent with that notion. They also

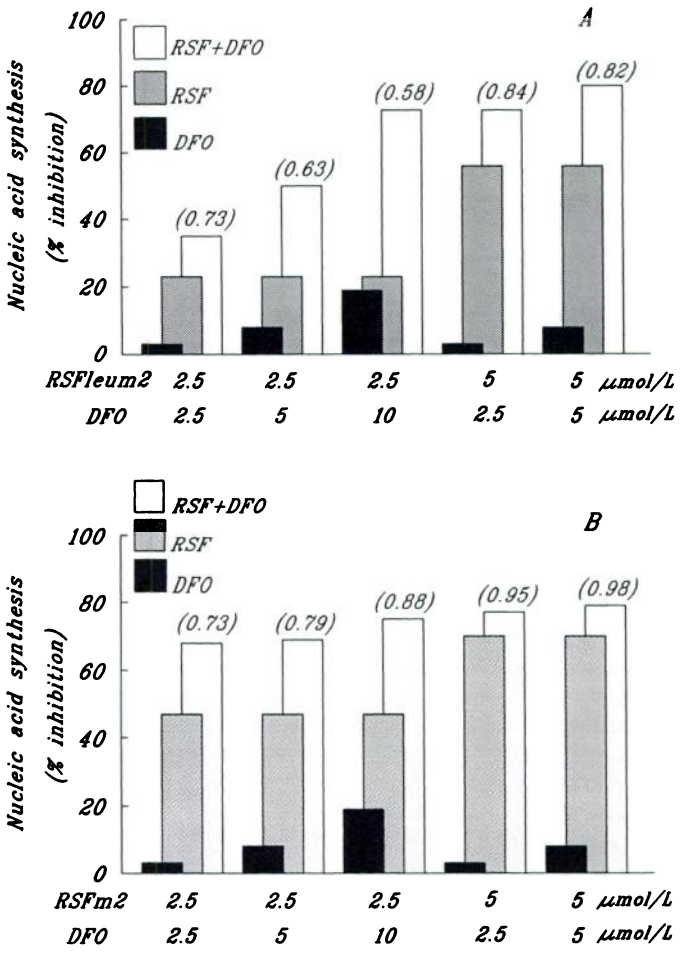


**Fig. 4.** Inhibition of [$^3$H]hypoxanthine incorporation by RSFs and DFO used at various combinations. Experiments were carried out as described in Fig. 3. Data are given as percentage inhibition of nucleic acid synthesis by the individual chelators (RSFleum2, RSFm2, and DFO) and by the indicated combinations of chelators. *Numbers above the bars*, indices $Y = (I_a + I_b)/I_{ab}$, where $I_a$ and $I_b$ denote the individual inhibitory effects of RSFs and DFO and $I_{ab}$ indicates the combined effects of the indicated RSF and DFO. The experimental values for each drug or combination of drugs are given as the mean of sextuplicates (three experiments), with the standard error being <10% of the mean.

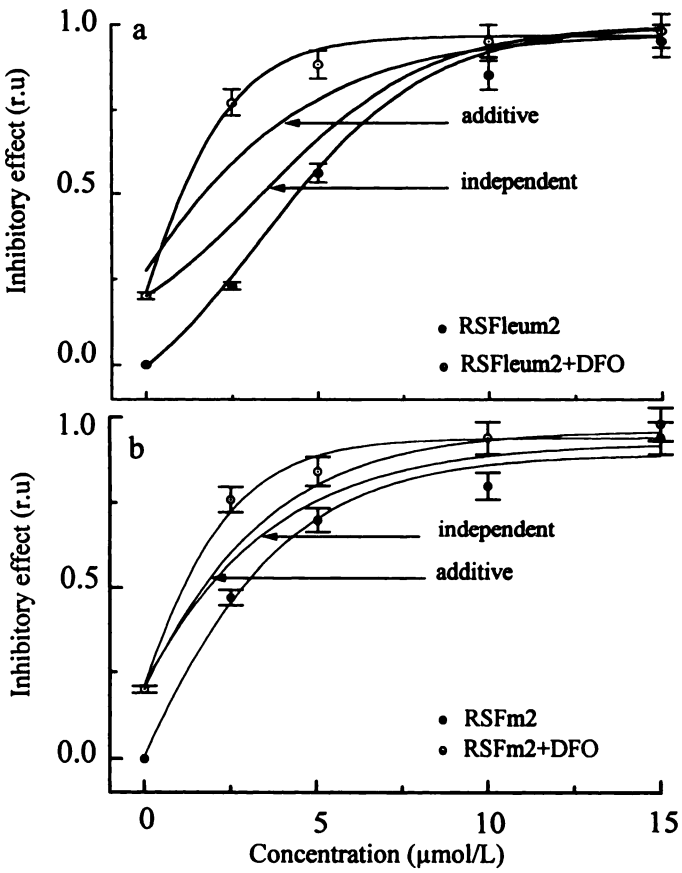


**Fig. 5.** Dose-response curves for iron chelators. Single drug and combined drug effects are shown. A fixed concentration of 10 μM DFO was used with the indicated concentrations of either RSFleum2 (a) or RSFm2 (b). The effect is expressed as inhibition of nucleic acid synthesis units relative to control, which contained no drug (*r.u*). Experimental data points are depicted as average ± standard error values (three experiments) for RSFs alone (●) and for their combination with DFO (⊙). *Lines*, best fits obtained by nonlinear regression analysis with the function described by Desjardins *et al.* (23). The theoretical additive and independent curves are best fits (23) to calculated points (24). Statistical analysis was based on Student *t* tests; the experimental data for the combined effects of either RSF with DFO were significantly different from the theoretical values for independent ($p < 0.01$) and additive ($p < 0.05$) action.

indicate that precomplexation with metals such as iron(III) or iron(II), but not Zn(II), abolished the antimalarial effects of DFO, RSFm2, and RSFleum2 and also of combinations of RSFs and DFO. Thus, these agents do not apparently form toxic metal complexes, as do other chelators (27–29), but act as intracellular iron scavengers, as shown for other structural congeners (16). As shown here and elsewhere (15), the effects of the chelators on mammalian cells in culture were minimal, compared with those obtained with cultures of human malaria parasites.

However, RSFs differ from DFO in their antimalarial action profile, despite their similar iron(III) binding affinities and metal binding specificities (16). Due to their favorable hydrophobic/hydrophilic balance, RSFs affect parasites at considerably faster rates and at all stages of parasite growth (Fig. 4). Their effects are mostly irreversible in rings but not in trophozoites. DFO, on the other hand, affects primarily the advanced stages of parasite growth, in a rather irreversible manner (13). The different antiparasitic properties of both types of chelators were recently explained by a model in which mobilization/integration of iron(III) into essential activities was assumed to be restricted to the trophozoite stage (10, 13). The efficacy of the chelators in arresting parasite growth was attributed to two major factors, (a) the capacity of the chelator to access the parasite at the various stages of parasite growth and (b) the capacity of the parasite to overcome the iron deficit after removal of chelator. Included in factor b is also an aspect of factor a, namely the speed at which and the extent to which the chelator exits the cell after its removal from the medium. As indicated elsewhere (10), cell retention of chelators such as DFO might play an essential role in limiting the capacity of the parasite to recover from treatment, even at those stages in which iron is actively mobilized by the parasite.

In this study we tested one of the predictions of the working model, in both basic and applied aspects. We used as a basis the finding that DFO effects on mature stages of parasite growth were slow to develop but were mostly persistent, whereas those of RSFs were quick to develop but were transient, except in rings. Thus, improvement of drug performance should

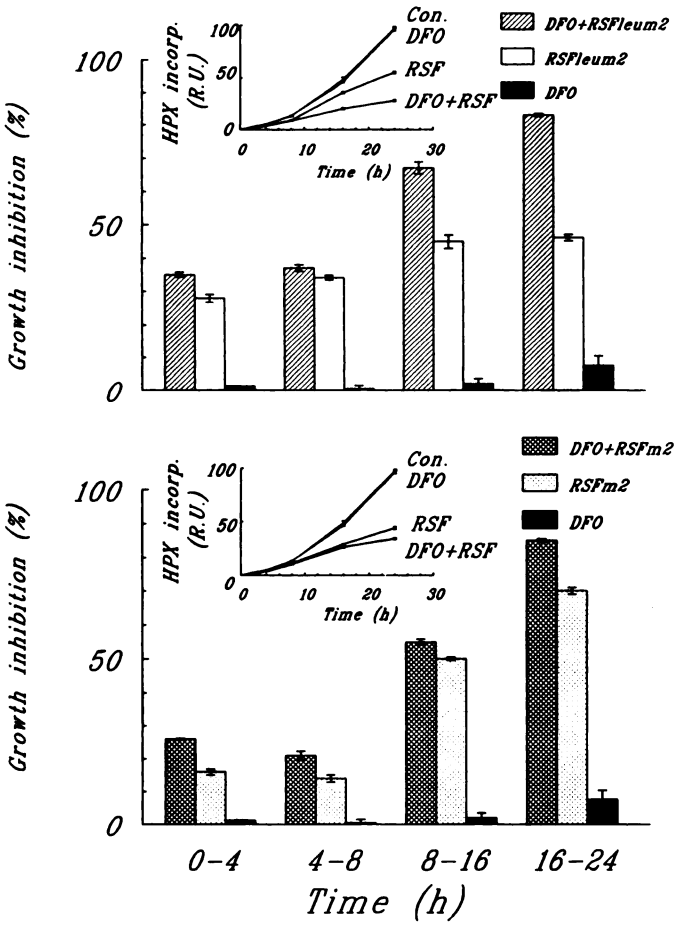


**Fig. 6.** Time dependence of the combined action of DFO and RSFs on nucleic acid synthesis by *P. falciparum*. Ring cultures were exposed to 5 μM doses of individual drugs or combination of drugs, concomitant with [$^3$H]hypoxanthine. *Insets*, data depict the time-dependent [$^3$H]hypoxanthine incorporation (*HPX incorp.*) into nucleic acids, given relative to the control (*Con.*) value at 24 hr (*R.U.*). *Main panels*, data depict the percentage inhibition of nucleic acid synthesis for given drug treatments, which include the indicated drug levels and the particular time windows used for assessing the rate of growth.

TABLE 1

***P. falciparum* growth with continuous exposure and after exposure to RSFs and DFO**

Ring forms of the parasite were exposed to the indicated chelators for 24 hr, and either nucleic acid synthesis was assessed after drug removal by washing from the cell suspension, i.e., after exposure (mean ± standard error, three experiments) or drugs were maintained during measurement of nucleic acid synthesis, i.e., continuous exposure. For each assessment, appropriate controls (no drugs) were used.

| Chelators | Inhibition | | | |
|---|---|---|---|---|
| | 12.5 μM | | 25 μM | |
| | After exposure | Continuous exposure | After exposure | Continuous exposure |
| | % | | | |
| RSFleum2 | 46 ± 1 | 86 | 54 ± 2 | 93 |
| RSFm2 | 64 ± 2 | 91 | 72 ± 1 | 93 |
| DFO | 7 ± 1 | 46 | 75 ± 1 | 85 |
| DFO + RSFleum2 | 68 ± 1 | 97 | 80 ± 1 | 98 |
| DFO + RSFm2 | 75 ± 1 | 93 | 90 ± 1 | 94 |

be achieved by using combinations of RSFs and DFO. Those combinations should not only widen the spectrum of action of chelators to all developmental stages of parasite growth but also increase the irreversible mode of action of the agents. The basic strategy resided in the use of particular combinations of RSFs and DFO, so that all stages of parasite growth could be rapidly affected by RSFs, rings irreversibly and trophozoites reversibly. The anticipation was that RSF-induced arrest of trophozoite growth should also allow the slowly permeating DFO to accumulate to sufficient levels and elicit effects that were sustained even after drug withdrawal. Thus, RSFs would not only elicit faster inhibition across the entire lifespan of the parasite but also increase the antimalarial potential of DFO. This mechanistic interpretation conforms both with the suggested model of iron(III) chelator action on parasites and with the phenomenon of overadditivy (24). The chelators chosen for this study, RSFm2, RSFleum2, and DFO, had similar metal binding specificities, stoichiometries, and apparent binding affinities for iron(III) ($K_a$ values of $10^{-31}$ M for DFO and $10^{-27}$ to $10^{-29}$ M for RSFs) but differed markedly in their partition coefficients and therefore in their expected permeation properties, speed of action on parasites, and stage susceptibility. As shown in Fig. 6, RSFleum2 was first to elicit inhibition in rings and markedly potentiated DFO effects after 8–16 hr of exposure, commensurate with DFO permeation into early trophozoites (11). As expected, the relatively less hydrophobic RSFm2 was more slowly acting on rings and relatively weaker in potentiating DFO effects in trophozoites. Similar patterns were obtained in the dose-response curves for continuous exposure of infected cells to drugs (Figs. 2–6) and for sustained inhibitions remaining after drug removal (Table 1). The conclusions described above were also supported by more rigorous analysis of the experimental data (Fig. 5). The combined RSF and DFO action was significantly higher than the additive or independent mode of action of drugs (24). This, however, was apparently less pronounced for RSFm2 and DFO than for RSFleum2 and DFO. A possible reason for that quantitative difference in the behavior of RSFs might reside in their different speed and stage of action on parasites, relative to those of DFO. The actions of the less hydrophobic RSF congeners resemble the action spectrum of DFO, and their combined action with DFO would approach additivity. Although potentiation of the action of DFO by RSFs might be indicative of synergistic actions of the two drugs, in the absence of more detailed mechanistic studies such a contention could be premature (30, 31).

In summary, selective intervention with iron(III) chelators and particularly with their combinations, as exemplified in this work and others (32–34), might be of value for the treatment of malaria. The levels of chelators used in this work and in a parallel study with other classes of RSFs (34) were considerably subtoxic to mammalian cells in culture, whether used individually or in combinations and even after 48-hr exposure of cells. If drugs are to be applied *in vivo*, pharmacological consideration should also be given to other essential factors, such as drug pharmacokinetics and chemical stability in body fluids.

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