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Final Report

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New Strategies for the Chemotherapy of Malaria through the Use of Biomimetic Iron Carriers

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- Synthesis and antimalarial activities of iron chelators linked Artisiminin

Reprints of papers baed on the project

Scientific Summary

It is a pleasure for us to present the final report concerning the research project which encompassed three lines of interdisciplinary research activities The research, which was carried out in Israel and in Thailand included the following topics

*Development of the synthetic methodology for the preparation of iron scavenging molecules that possess two major structural elements (i) iron binding groups, and (ii) reporter groups or synergetically acting elements

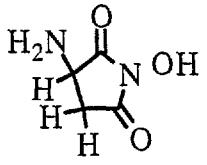
The synthetic methodology involved (i) the preparation of tetra, tris-, or bis-carboxyphenolates, (ii) their coupling with one equivalent of reporter group, drug or synergetically acting element, and (iii) subsequent coupling with three, two or one equivalents of iron binding groups (WI, Rehovot, Israel)

*Development of novel formulations for *in vivo* examination of combination therapies involving iron scavengers Particular emphasis has been placed on the generation of slow release formulations, which are expected to pertain an adequate serum level of active drug (HU, Jerusalem, Israel)

*Preparation of novel conjugates between artemisinine and iron binding molecules for *in vitro* studies as potential antimalarials The iron binding elements have been prepared and supplied by the Rehovot group (Bangkok, Thailand)

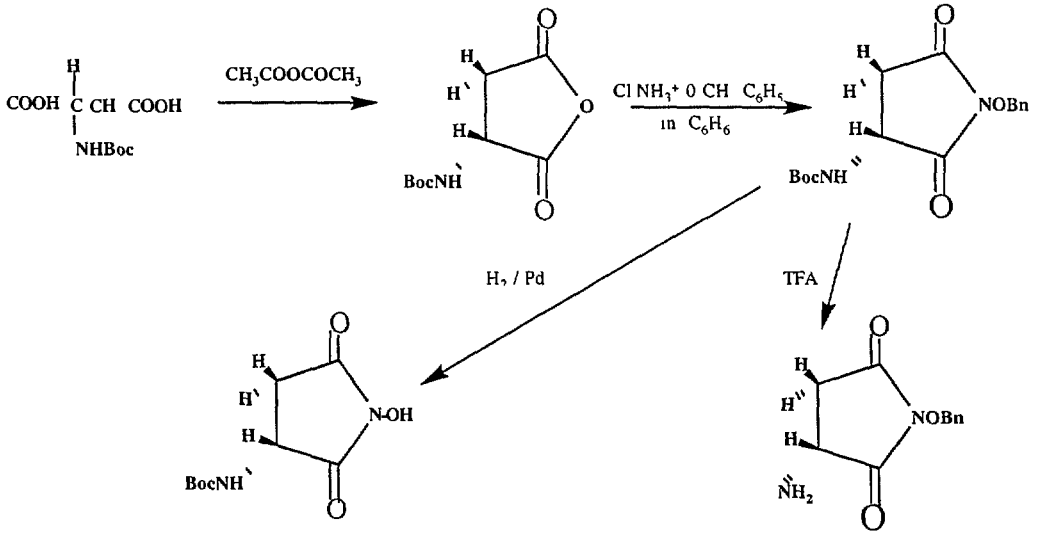
Final Report of The Chemical Aspects (Rehovot Group)

The synthesis of a new iron binding ligand (**1**) a derivative of N-hydroxy succinimide was pursued



1

the synthetic route is described in the Scheme



Three of these molecules are combined to form the tridentate molecule **2** which is expected to be a new siderophore that binds iron

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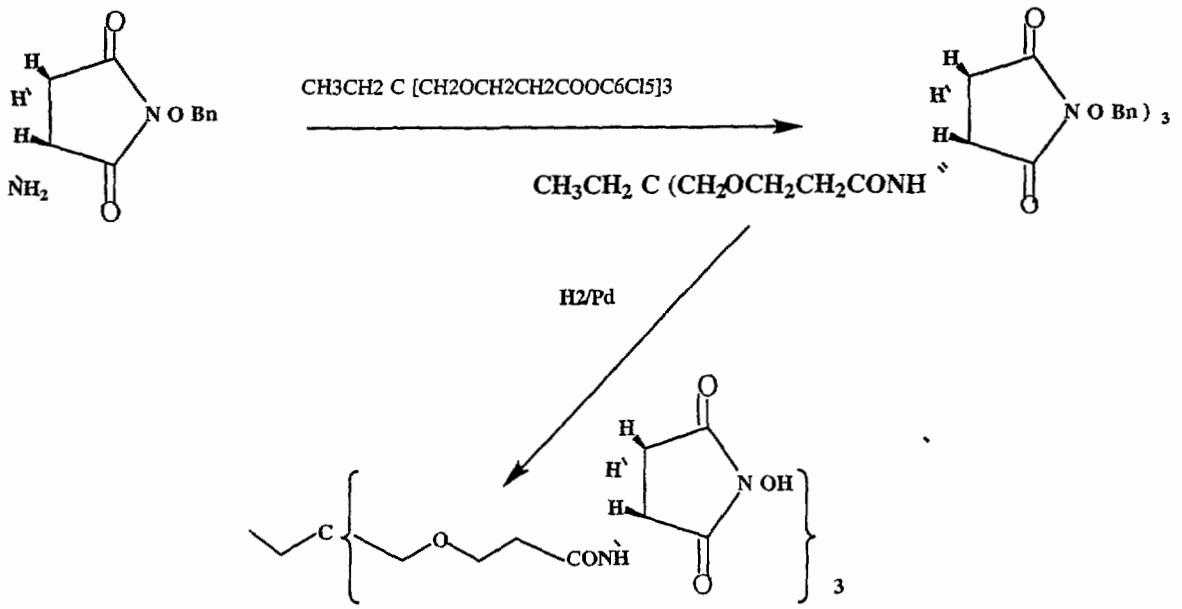

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Titration experiments of the tripodal imide 2 with iron established binding by the appearance of a new absorption around 470 nm, which was attributed to the Fe(III)-N-hydroxy-succinimide complex pH-titration of the iron complex established optimal binding around pH 5, while the corresponding tris-hydroxamate exhibited optimal binding around pH 8 8 The novel tripodal imide (2) is thus expected to be a more potent iron scavenger than the corresponding trishydroxamate at cell compartments of lower pH

5

AID-CDR Final Report (Jerusalem and Rehovot)

The original aim of the proposal was based on empirical observation that the hydroxamate-based iron chelator DFO, arrested parasite growth *in vitro*, in animal models and in the clinic The project aimed at introducing novel iron chelators (ICHs) with improved AM efficacy by using molecular probe design and modular chemical synthesis Those were combined in order to obtain drugs with defined features such as a **membrane accessibility** (for faster speed of action), b **selective action** on infected RBCs, by differential accessibility to *P falciparum* infected cells and c **irreversible action on iron metabolism** in parasite infected cells The two major groups of ICHs used were based either on N-derivatized forms of the natural DFO or the synthetic ferrichrome-like siderophores (SFs) (called here **reversed siderophores, RSFs**) A third group of chelators was based on the bidentate acyl-hydrazone conjugates (AHs) of various cyclic aldehydes The novel ICHs were initially characterized for their iron binding capacity and octanol water partition coefficient and screened in *in vitro* cultures of *P falciparum* for a AM potency, speed and stage dependence of action and c cytotoxicity and iron extraction capacity in animal cells They were evaluated in terms of quantitative structure-activity relationship (SAR) schemes The most specific and potent AM agents found in *in vitro* cultures of parasites were also preliminary assessed for AM activity in animal models of malaria

Summary of work and goals accomplished

i chemical design of two novel series of reversed siderophores (RSFs) and of N-derivatized DFOs and their assessment in terms of quantitative SAR on *in vitro* inhibition of *Plasmodium falciparum* growth,

ii assessment of RSFs and DFOs on parasite nucleic acid synthesis as a function of concentration, time of exposure to drug and developmental stage of parasites,

iii assessment of RSFs and DFOs capacity to irreversibly arrest parasite growth *in vitro* as a function of parasite developmental stages,

iv delineation of drug effects on parasite development in terms of permeability properties and of routes of access of drugs into parasite *vs* host compartments,

v comparative evaluation of cytostatic and cytotoxic effects of RSF and DFOs on parasitized cells and mammalian cells in culture,

vi integration of the collected information into a working model of RSF mode of action as AMs and its implication for drug design and therapeutic improvement,

vii assessment of the working model on parasites grown *in vitro* and *in vivo* with combinations of SFs that display different action profiles (analysis of drug synergism),

viii preliminary assessment of new means of drug delivery in animal models of malaria

Most of the above studies are covered in papers which appeared in peer reviewed journals and in invited reviews The studies indicated that

a fast, effective and selective AM activity (*in vitro)* can be achieved with the most lipophilic members of both, the RSF and DFO families (*e g* RSFm2phe and its propyl-ethyl ester and MA-DFO, respectively) and with various AHs (*e g* SIH*)* They outperformed DFO's AM action in *in vitro* cultures, but acted synergistically with DFO, leading to long lasting inhibition of parasite proliferation

b all ICHs used were well tolerated by mammalian cells in the inhibitory antimalarial range, some even showing no cytostatic effect on mammalian cells

c the ICHs used were active against strains which display different degrees of multi-drug resistance

d the mode of action of the ICHs were rationalized in a working model in which drug permeation and parasite stage susceptibility to iron deprivation played a central role The model was put to test initially on *in vitro* malaria cultures It led to the development of three concepts regarding drug design and application Those were incorporated in the new proposal i coverage of the entire parasite life cycle in terms of antimalarial action by combining the action of ICHs with different activity (*i e* permeation) profiles, ii improvement in the antimalarial potential of ICHs by increasing their cell retention or iii their exposure to cells by application chemical devices for slow-drug release Preliminary information showed the feasibility of three concepts *in vitro* and in animal models of malaria

Publications

Golenser, J , Tsafack, A , Amitai, Y , Shanzer, A and Cabantchik, Z I (1995) Antimalarial action of hydroxamate-based iron chelators and potentiation od desferrioxamnine action by reversed siderophores *Antimicrcrob Agents. Chemother* 39 61-65

Tsafack, A , Golenser,J , Libman, J , Shanzer, A and Cabantchik, Z I (1995), Mode of action of iron chelators as antimalarial III Overadditive effect in the combined action of hydroxamate-based agents on in vitro growth of Plasmodium falciparum *Mol. Pharmacol* 47 403-409

Tsafack, A , Loyevsky, M , Ponka, P and Cabantchik, Z I (1996) Mode of action of iron chelators as antimalarials IV Potentiation of desferral action by benzoyl and isonicotinoyl-hydrazone derivatives *J Lab Clin Med* 127 574-582

Glickstein,H, Breuer, W , Loyevsky, M ,Konijn, A , Libman, J , Shanzer, A and Cabantchik, Z I (1996) Differential cytotoxicity of iron chelators on malaria infected cells versus mammalian cells *Blood* 87 4871-4878

DETAILED REPORT

IRON METABOLISM AND PARASITE DEVELOPMENT

In the infected cell, the erythrocyte environment serves the parasite as a route of communication with the outer world, as a source of nutrients and as a disposal ground for metabolites The bi-directional properties of the host-parasite relationship are most profoundly manifested in the advanced stages of growth (*i e* trophozoites), when metabolic activities are at their peak These activities include iron mobilization and integration into essential functions and removal or neutralization of by-products of metabolism, including oxidative radicals Like with most organisms, it can be assumed that parasites need not only to mobilize iron but also to coordinately regulate iron levels bv balancing metal import and storage, and thus reduce te risk of oxidative damage

Iron is apparently supplied to parasites from the internal sources of the host, since parasites can propagate *in vitro* in media nominally free of iron or transferrin (91) The supply of iron and/or its integration into vital parasite functions would seem to be confined to a discrete stage of the parasite RBC life cycle This is based on our observations that infected cells treated with permeant chelators resume growth after drug removal only when treated at the advanced but not at the early stages of parasite growth (14,71) The proposed operation of an iron mobilization/integration phase at the trophozoite stage coincides also with the phase of phagocytosis of the host The putative source of iron is hemoglobin which is endocytosed and proteolytically processed (40) Iron was proposed to be chemically released from heme either in the parasite's food vacuole or in the host cytosol (4,32, 89a) However, provisions have still to be made for how such a mechanism operates in a regulated fashion in terms of meeting parasite demands while avoiding excess supply

This projects aims at designing and applying ICHs as antimalarials and as probes for assessing the unique cytotoxicity displayed by these agents The **rationale** rests on two basic and unique properties associated with iron metabolism in parasitised cells and in reticulo-endothelial (RE) cells of the host **One** is the nature of parasite mobilization of vital iron from within the infected cells, which is restricted in terms of both the metal source and the stage of parasite development, the trophozoite These features which are described in our working model (Fig 6), indicate that parasites can be deprived of iron and led to an irreversible or deadly path, if substantial uptake of the ICH occurs outside the active phase of iron mobilization and/or can be intracellularly retained during the alleged active phase The **second** rests on the restricted capacity of monocyte/macrophages to clear parasitised cells due to release of toxic agents from digested cells (83a,96a) Such toxicity seemingly reduces the host defense capacity, but can be overcome, in part, by ICH treatment, as animal (38a) and clinical studies (104a) to imply The challenge is therefore to design specific chelators which express defined permeation and cell distribution properties in parasitised and mammalain cells so as to attain, in a step-wise, systematic fashion, improved antimalarial performance and selectivity These approaches constitute a rational drug design whose products we assess first in *in vitro* cultures and subsequently in animal models, with selected agents

ANTIMALARIAL EFFECTS AND SELECTED PROPERTIES OF IRON CHELATORS

Iron chelators I *Chemical properties*

We focused most of our previous work on hydroxamate type SFs which form octahedral binding cavities for hexadental complexation of a single iron(III) **3-CONOH-+Fe^{3+}=Fe(CONO-)$_3$+3H^+** These SFs have several advantages a they form high affinity complexes with iron(III) but hardly affect transferrin iron, b binding of a single iron(III) does not modify their electroneutral character and c the chelated iron is markedly reduced in chemical reactivity, so that the SF-iron complexes are not toxic to cells The two major structures used were based on natural SFs and ferrioxamine B (log K=30 6 and 29 1 respectively) In type A SFs (Fig 1A), we have chemically mimicked the basic iron binding cavity of Ferrichrome (in terms

of ion selectivity and affinity) The cavity connects to a symmetric **anchor** via a tripodal bridge with amino acids which bear different side chains (R) $CH_3CH_2C(CH_2O(CH_2)_mCONHCHRCONOHCH_3)_3$

The **first line** of SFs (type A) included m1 and m2 congeners (m=# of CH_2 in the tripodal bridge) with amino acid residues of variable hydrophobicity By modulation of R we controlled the hydrophilic/lipophilic balance (HLB) of the SFs This in turn conferred upon the SFs the requisite properties for improved and in specific cases, selective penetration across RBC membranes and accessibility to cellular iron (III) pools (14) We refer to the compounds with the name of "reversed siderophores" **(RSF)**, because their hydrophobic character is opposed to those of natural SF's (which are hydrophilic and extra-cellular chelating) (14,97) Modification of the chain length in m allowed us also to control the size of the chelator, another determinant of membrane permeation Some of the physicochemical properties of RSFs and their AM activity are summarized in Table I and in Figs 3-5 and reviewed in ref 14,15

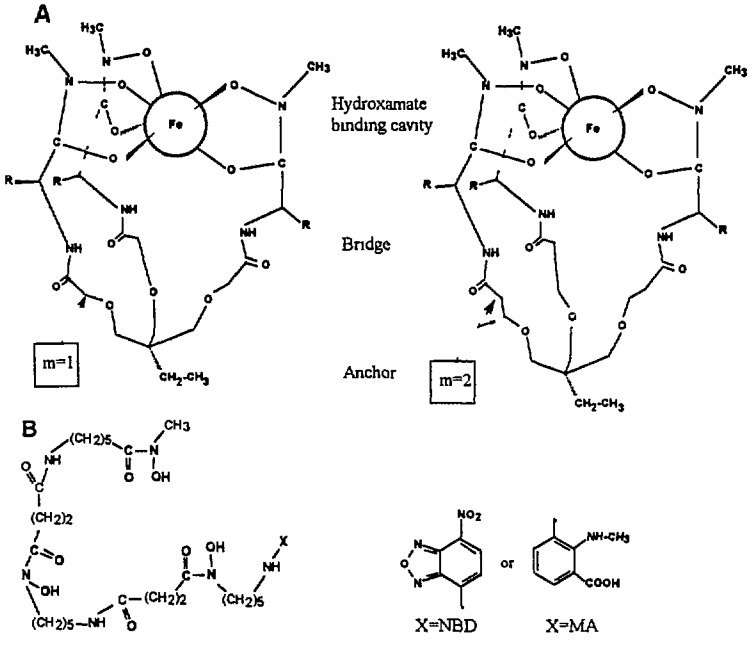
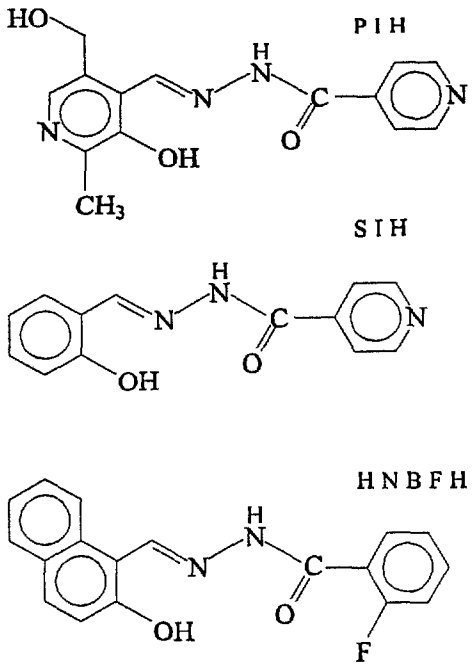


Fig 1 Stucture of reversed siderophores (RSFs) and desferrioxamine derivatives (DFOs) RSFs are built as hydroxamate binding cavity (for hexadentate iron coordination in octahedral geometry as found in DFO) which is connected via tripodal bridges to anchor linked on a modifiable base m=1 and m=2 represent families of RSFs identified by the number of methylene groups in the bridge while R denoted the amino acid side groups The fluorescent DFO derivatives are N t substituted NBD or MA (methylanthranilic)

The **second line** of chelators (type B) was made of N-terminal derivatives of DFO, which bind iron(III) by looping around the ion as in a coil The added groups were usually hydrophobic and fluorescent such as nitrobenz-oxa-furazan (NBD)-, anthranilic (A) or methylanthranilic (MA) (66) **(Fig 1B)** The groups conferred to DFO higher partition coefficients, but did not interfer with their iron binding properties The unique feature of those derivatives is their metal sensing properties, which are manifested as fluorescence quenching upon stoichiometric binding of the metal

The **third line** of agents was composed of lipophilic RSFs which carry ester groups as in **Et-C-[$CH_2OCH_2CH_2CONOHCH_2CH_2COOEt]_3$** As esters, these compound were envisioned to swiftly penetrate into parasites and undergo enzymatic hydrolysis to yield the charged acids and the respective alcohols **Et-C-[$CH_2OCH_2CH_2CONOHCH_2CH_2COO^-]_3$**

Fig 2 Acyl hydrazone conjugates



The **fourth line** of chelators was based on acyl-hydrazone (AH) conjugates (Fig 2, PIH, SIH and HNBPH) of various cyclic aldehydes of variable hydrophobic character (provided by Dr P Ponka, McGill University, Canada) (6) In addition to their relatively small dimension, high iron binding affinity (logK=29) and 2 1 stoichiometry of iron binding (bidentate), those agents were found to swiftly enter into cells and markedly reduce the labile (or chelatable) iron pool (17) Moreover, due to their fast iron binding kinetics, they could also serve as iron donors to suitable high affinity acceptors (such as DFO derivatives) (111)

Iron Chelators II *Antimalarial activity*

In what follows we shall summarize only some of the collected (mostly published) data to illustrate major points and integrate them into working models of RSF action

1 **The AM efficacy of drugs** administered continuously to growing cultures of *P falciparum* was best **correlated with the partition coefficient (PC)** of the drug (an indicator of permeation properties across membranes) (illustrated in **Table 1 & Fig 3 for RSFs)** This was reflected both in the IC_{50} on nucleic acid synthesis (NAS) (24-48 h starting at the ring stage) or the **speed of drug action**, measured as inhibition of NAS during the first 8 h of exposure of rings at IC_{50}-levels of drug The empirical SAR served as guide for the development of type-A RSFs **The optimal RSF is about 60 fold more potent and 14 fold faster acting than DFO The AM efficacy of RSFs and DFO were similar in strains of *P falciparum* spanning a wide range of drug resistance (97)**

Table 1 Summary of chemical properties and antimalarial effects of RSFs and DFO

RSF	m	Partition coefficient[1]		Iron(III) binding[2]	Rel speed of action[3]	IC_{50}-48 h M	Relative potency[4]
m2	2	1 7	3 2	0 52	7	3±1	21
m2(H)*	2	0 11	0 2	0 35	-	92±18	0 7
m2-PEE	2	13 5	26	0 61	20	3 0±0 3	21
m2-PA	2	-	-	0 46	-	>100	<0 6
D-ala	2	0 53	1	1 00	1	70±13	0 9
D-ileu	2	14	26	0 23	3	9±3	6 9
L-ala	2	0 53	1	1 00	1	62±4	1
L-ileu	1	15 3	29	0 80	21	3±1	21
L-ileu	2	14	26	0 23	6	3±2	21
L-leu	1	29	55	0 18	5	5±2	12
L-leu	2	12 5	24	0 11	3	4±2	15
L-phe	2	40	75	1 00	43	0 6±0 2	103
L-phe-PEE	2	100	189	0 72	67	0 3±0 1	206
L-pro	2	0 7	1 4	0 13	0 1	>100	<0 6
L-val	1	1 7	3 2	0 80	6	4±1	15
L-val	2	3 6	6 8	1 05	7	6±2	10
L-trp	2	-	-	-	9	3±1	21
L-leu-NBD	2	-	-	-	10	5±2	12
DFO	-	<0 1	-	0 93	5	17±1	3 6

* Methyl groups of the binding cavity were substituted with H See Fig 1 for structural formula and nomenclature

1 Octanol/saline partition coefficient (left-absolute value, right relative to L-ala_{m1})

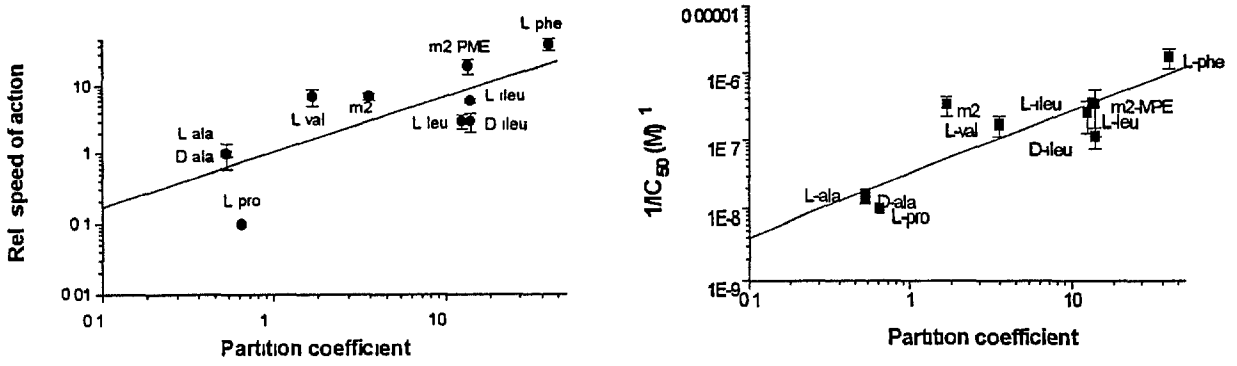
2 Relative to L-ala_{m1}

3 Relative speed of action % inhibition of nucleic acid synthesis after 8 h exposure of rings to the indicated IC_{50} concentration normal

divided) by the actual 48h- IC_{50} value

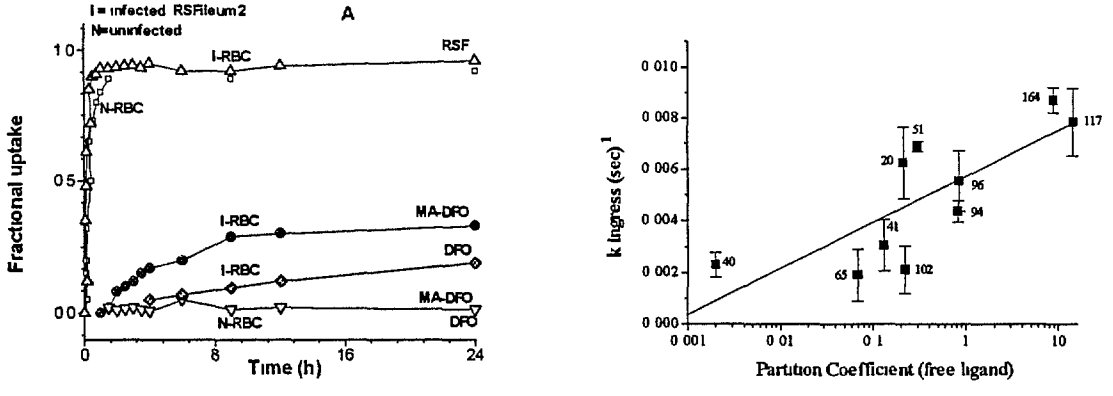
4 The reciprocal of IC_{50} normalized (*i e* divided) by the reciprocal of the L-ala$_{m1}$ ' IC_{50}

Fig 3 Correlation between AM activity and RSF partition coefficient (Data were taken from Table I)



ii The ability of chelators to permeate across membranes was studied in three experimental systems a as ^{55}Fe-RSF or ^{59}Fe-RSF uptake into normal or infected RBCs (Fig 4A) (71), b as RSF ability to de-quench the fluorescence of calcein-Fe complexes encapsulated in red cell ghosts (17) and c the same as b but in cultured mammalian cells loaded with calcein-AM (10-12,17) (Fig 4B) The **permeation of the RSFs clearly correlated with their PC** in all the experimental systems, despite the fact that each method measured a different aspect of RSF a permeation of the iron complex across the RBC cell membrane, b permeation of free RSF across the RBC membrane and c permeation of free RSF across the K562 cell membrane (10-12,17) Similar properties were also found for DFO derivatives (35)

Fig 4 A Uptake of SFs into normal and infected cells over time B (right) ICH ingress and binding of the labile iron of K562 cells as a function of their partition coefficient



On-line follow-up of drug effects on nucleic acid synthesis throughout all stages of parasite growth clearly indicated that the onset of inhibition (also referred to as drug speed of action) depended on drug lipophilicity and also on the particular developmental stage of the parasite (14)

iii The ability of chelators to chelate and extract intracellular iron from infected RBCs varied with the stage of parasite growth The chemical measurements, which were done after 4h incubation of cells with either of two ICHs, show that iron extracted from cells into medium by either ICH varied with the parasite stage of development (trophozoites> ring>schizont>uninfected)

chelator	uninfected	rings	trophozoites	schizonts
DFO	0	0	2 0±0 5	0 7±0 2
RSF m2pa	0	0	0	0
RSF m2(H)	0	1 2±0 4	2 1±0 5	1 0±0 3
RSF m2	0 8±0 3	2 2±0 7	3 1±1 2	3 0±1 0
RSF-ileu$_{m2}$	<0 1	ND	4 6±0 9	ND
RSF m2pee	1 7±0 9	3 7±1 8	8 5±2 6	3 0±0 7
RSF-phem2	1 6 ± 0 9	3 1±1 5	8 5±2 6	3 0±0 7
SIH	1 2 ± 0 5	2 3±0 7	6 5±1 2	1 9±0 4

Values are given as nmol/10^{10} cells of iron extracted from cells

Although the two chelators,an RSF and an AH-conjugate, differed in their PC and the stoichiometry and mode of iron complexation, they showed remarkably similar membrane permeation rates and intracellular iron binding properties If anything, SIH would seem to be more efficient than RSFm2phe in all those respects Nonetheless, the RSFm2phe was about 30 fold more potent than SIH in inhibiting parasite growth (IC_{50} 0 6μ vs 20 M) Clearly, these (and other) results indicate that **the AM potency of some RSFs is dictated not only by the speed and amount of iron it can extract from infected cells, but presumably, also by its accessibility to specific cell targets (enzymes ?)**

iv Access paths and site of action of ICHs host or parasite ?

Although normal cells can be easily accessed by RSFs but not by DFOs, infected cells are seemingly accessible to DFOs and also to fluorescent DFOs, but only to a fraction of their volume (65,66) Since this fraction was identified in trophozoites with the parasite compartment (by confocal fluorescence microscopy) (74), it could be implied that parasites can be reached by externally added DFOs while bypassing the host cytosol Although such routes of access to parasites might coincide with the proposed (and still controversial) membrane duct (29,83,100), other alternatives (*e g* vesicle trafficking) are also feasible

In order to assess whether DFOs access the host cytosol prior to the parasite, normal RBCs were loaded with mM concentrations of DFOs by encapsulation, and the resulting red cell containers were examined for their capacity to support parasite invasion and growth (the drugs were largely retained in biologically active forms) While none of the encapsulated DFOs interfered with parasite development in drug-laden, containers, they were demonstrably inhibitory to other cultures after being released from RCC by lysis (IC_{50} of 20-30 μM for DFO and 3-5 μM for MA-DFO) These and other observations indicated that either DFOs apparently does not access the parasite from the RBC host, but through some fenestrations (14) as shown also for phlorizin (66), or that it might be neutralized by iron released from parasite to host

vi Stage dependence of inhibition and irreversibility of effects by RSFs and DFOs in *in vitro* cultures of *P falciparum* and mammalian cells

The assumed target of iron deprivation is nucleic acid synthesis, via inactivation of iron-dependent ribonucleotide reductase (RNRase) However, unlike RNRase inhibitor hydroxyurea, prolonged treatment with DFO and RSFs affected parasite growth irreversibly, *i e* parasites failed to resume growth after drug removal (Fig 5)

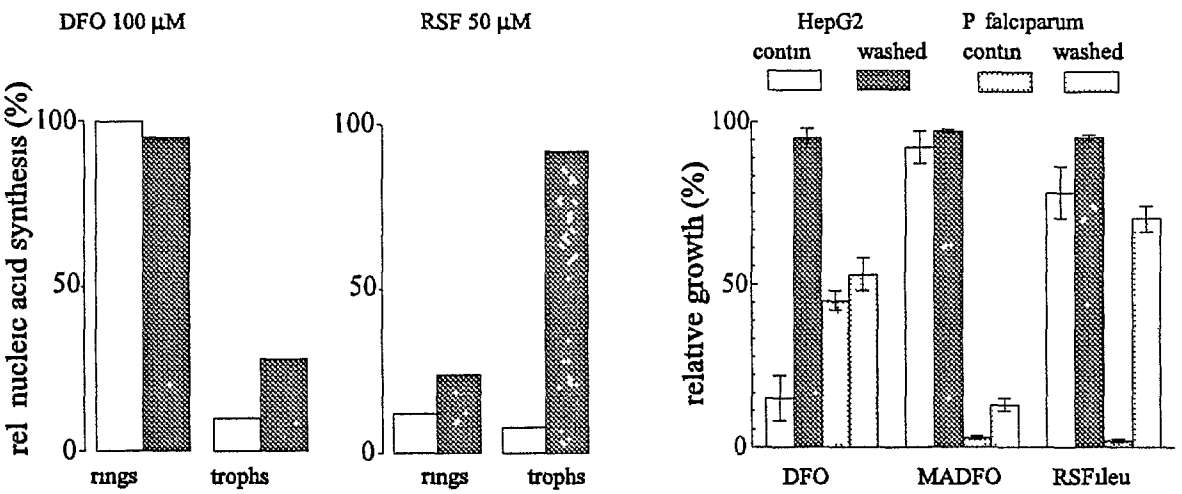


Fig 5 Effect of DFOs and RSFm2ileu on growth of *P falciparum* and human hepatoma cells (HepG2)
Drugs were given either at the indicated concentrations *(left)* or at 50 M each *(right)* (**cont** refers to continuous exposure of drug for 24 h followed by 6 h measurement of nucl acid synthesis in the presence of drug and **washed,** refers to cultures exposed for 24 h to drug followed by washing and measurement of nucl acid synthesis for 6 h)

Moreover, replenishment of iron in forms which are utilizable by mammalian cells, also failed to restore viability (73) This

contrasted with the marked reversibility found in mammalian cells (35) In general, the effects of DFOs and RSFs varied with parasite-stage of growth as

a RSF's inhibited both rings and trophs when the latter were continuously exposed to the agents, while DFO inhibited primarily trophs and schizonts,

b RSFs inhibited progression of rings to trophozoites in an irreversible fashion, but failed to do so on trophozoite onwards while DFO blocked growth of trophozoites, mostly irreversibly

In contrast to DFO which completely suppresses mammalian cell proliferation, MA-DFO, which is about 10 fold more potent than DFO as AM, hardly affected mammalian cells up to tens of μM concentrations

In terms of the mode of action of SFs as growth suppressors, the results clearly indicate a major difference in parasites and mammalian cell susceptibility This point will be addressed in the following sections, in which the various properties of DFO and RSFs were integrated into a working model which rationalized their mode of action as Ams

A WORKING MODEL FOR THE MODE OF ACTION OF IRON CHELATORS (14,15,74)

The model depicts the various stages of parasite growth within one cycle It rests on the concept that parasites mobilize and metabolically integrate iron only at a specific stage of their life cycle (the active iron phase) (Fig 6)

The efficacy of ICHs in arresting parasite growth resides on their potential to cause **irreversible damage** to parasites either a by intracellular iron deprivation when acting outside the active phase or b by acting on a sustained basis within the active iron phase and possibly also by generating toxic iron complexes The permeation properties of the ICHs determine to a large extent at which parasite growth stage they act permeant RSFs and SIH affect all stages, but only at the ring stage do they act irreversibly, since at that stage the iron mobilizing mechanisms are apparently inoperative (rings fail to resume growth because of their inability to reactivate iron dependent functions) Trophs and schizonts, however, do have such mechanisms operative and show, therefore, major recovery from RSF treatment The hydrophilic DFO which is impermeant to rings but slowly permeant to trophs, accumulates apparently in an intraparasitic compartment, and it is retained there for relatively long period of times, possibly contributing to inhibition also by complexing with iron and forming potentially toxic complexes

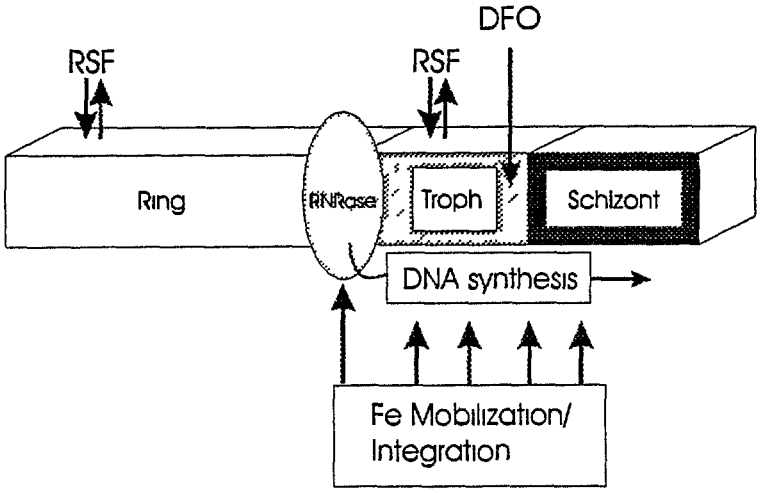


Fig 6 Model of iron chelator mode of action in malaria parasites The metabolic cycle of malaria parasite (shown in the box) is affected by all-stage permeant RSFs and by trophozoite-schizont-permeant DFO The permeation rates are indicated by the width of the arrows on the top portion of the diagram (DFO shows poor ingress into cells and virtually no egress) The lower part depicts the active stage of iron mobilization (from hemoglobin degraded in the food vacuole ?) and integration into DNA synthesizing enzymes, which, like RNRase (ribonucleotide reductasee)(18,90), are part of the replication apparatus

PHARMACOLOGICAL IMPLICATIONS OF THE MODEL DRUG SYNERGISM

The working model highlights also the possible shortcomings of therapy based on single ICH Drugs like RSFs or DFO cause sustained inhibitions only at particular stages of parasite development While RSF has a fast action profile on all parasite stages, it acts reversibly at the mature stages Due to its relatively slow permeation, DFO requires prolonged exposures at high doses to attain sufficiently high concentrations in mature parasites This is apparently also the case *in vivo* We reasoned that the inherent limitations in the usage of individual drugs such as RSFs or DFO, can be largely overcome by simultaneous application of both types of drugs This could be achieved by either one of the following 1 by drug complementation, that is by using a combination of drugs which act at different stages of parasite growth and 2 by drug synergism, that is by a drug which not only complements but also potentiates the effect of the other These modes were experimentally verified with various fast acting RSFs (phe, m2-pee, leum2 and m2) which complemented DFO effects by acting irreversibly on rings However, they also potentiated the persistent effects of the slow acting DFO by allowing its intracellular accumulation in trophs while attenuating their growth (15,110)

An analogous mode of synergy in drug action was also observed in rings using SIH (111), which is not only highly permeant, but which can also scavenge iron from infected cells and transfer it to an extracellular acceptor, like DFO DFO, which is virtually inaccessible to rings, served here as a factor which potentiated SIH inhibition of rings by serving as an iron sink for SIH-extracted metal The two modes of action of chelator combinations are depicted in the following scheme for both stages of parasite development (Fig 7)

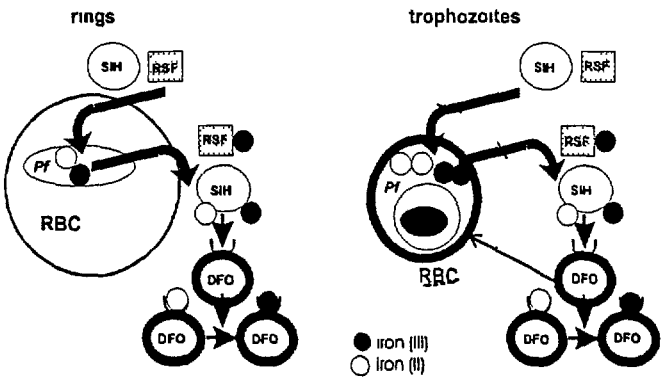


Fig. 7 Pathways of entry of RSF, SIH and DFO into different developmental stages of intraerythrocytic parasites and extraction of intracellular iron The arrows indicate the permeation of the respective chelator into the cell (as free chelator) and out from the cell (as a complex with iron) In rings, the impermeant DFO serves only as a sink whereas in trophs it serves as a sink and acts as a permeating-inhibitor SIH can transfer the extracted iron to extracellular DFO

We focused primarily on the restricted cytotoxicity displayed by each class of chelators and whether it could be overcome by simultaneous application of complementary acting agents A predicted feature of the model is that the combined effect of agents which act at potentially similar parasite targets but access them at different rates is likely to display overadditive action, with fast acting RSFs potentiating the persistent effects of the slow acting DFO The RSFs derivatives (m2phe, m2leu and m2) and SIH were applied here either separately or together with DFO, at either equal or at different molar ratios and their antimalarial effects were followed kinetically These were assessed in terms of inhibof NAS and parasitemia during drug treatment or after drug removal

The results indicated that the combined application of both agents demonstrably improved the antimalarial efficacy of individual drugs beyond additivity and stage restricted cytotoxicity, as manifested in the IC_{50} values (Table II)

Combined drug actions were assessed according to Poch and theoretical values for "additive" and "independent " modes of action of drug combination were compared with experimental ones by statistical t-tests We have found that combinations of fast acting RSFs or SIH potentiated the effects of DFO, both in terms of profiles of concentrations and time-dependence of effects (38,109,111) We depict here only results obtained with various concentrations of SIH± DFO (10 M), administered to rings for 24h (Fig 8)

	Growth inhibition IC_{50} (M)	
	(-) DFO	(+) DFO
RSFm2phe	0 6±0 2	0 3±0 1 (s)
RSFm2ileu	4 4±1 0	1 1±0 1 (s)
RSFm2leu	10±1	7 4±1 0 (s)
RSFm2	2 9±0 5	1 0±0 2 (s)
SIH	23±3	5 0±1 2 (s)
DFO	-	17 ±1

Table II.Individual and combined effects of RSFs, SIH and DFO on in vitro cultures of *P falciparum* Data were obtained as in Table I (+)DFO depicts in the presence of 10 M DFO , (s) denotes synergistic mode of actionaccording to Pochs' analysitical schemes

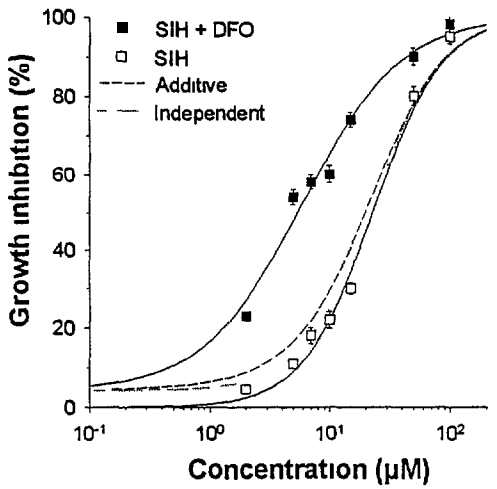


Fig 8 Inhibition of nucleic acid synthesis by SIH alone and in combination with DFO Parasite nucleic acid synthesis was measured for 20h after exposing early rings for 24h with SIH±DFO (10 µM) The lines are best fits to experimental points (mean±SE, n=3) obtained by NLSQ analysis to a sigmoidal function The theoretical curves are best fits through points obtained for "additive" and "independent" modes of action (following Poch's analysis)(78) The combined effects of RSF with DFO were significantly different (higher) from the theoretical values for "independent" (P<0 01) and "additive" (P<0 05) effects (Student t-tests) Similar profiles were obtained for the RSFs and similar conclusions could be drawn for the time dependence of the effects The combined drug action was clearly higher than additive

The potentiating action of RSFs on DFO's long lasting effects on parasite growth, conformed with the postulated mechanistic model of ICH action and iron handling by parasites (14,15)

DIFFERENTIAL EFFECTS OF ICHS ON PARASITES AND MAMMALIAN CELLS

In the above mentioned model, we highlighted the molecular properties underlying the mode of action of the chelators in terms of iron mobilization by parasites and drug accessibility (*i e* permeation) and intracellular retention A most extreme example of a differential effect of an ICH on parasites vis a vis mammalian cells, is given by DFO and its methylanthranilic (MA) derivative MA-DFO Both the parent compound and its derivative have comparable affinities for iron(III), although MA-DFO was considerably less hydrophilic than DFO and therefore more permeant to cells (65) As anticipated by our model, outperformed DFO in arresting parasite growth both, in terms of dosage, speed of action and spectrum of activity on parasite stages (Fig 5) All these properties are commensurate with the lipophilic character conferred to DFO by the MA group However, the opposite appeared to be the case with mammalian cells in culture, both in terms of growth inhibitory capacity and iron sequestration, as visualized by cellular ferritin levels (35) The demonstrably permeant MA-DFO, failed to affect mammalian cells when given at equivalent concentrations to those of DFO Taken together, MA-DFO displayed a markedly higher therapeutic index and higher efficacy as antimalarial agent than DFO We tentatively attribute the "poor" performance of MA-DFO on mammalian cells to its limited capacity for chelating the cytosolic form of the metal, iron(II) (35) This might explain the apparent lack of effects on mammalian cell proliferation In parasites, iron(III) is most likely the prevailing chelatable form of iron This differential mechanism of action was deduced based on a novel technology we developed for assessing then iron status in living cells with fluorescent probes (10-11a,28*, 65)

ASSESSMENT OF ICHs IN ANIMAL MODELS

Animal models We have assessed the AM effects of ICHs in two animal models of malaria

a *Plasmodium vinckei petteri* parasites (strain 279, originally provided by Dr I Landau, Paris) because of their highly synchronized cell cycle in mice (Swiss) and the possibility of timing the administration of drug at equivalent stages in the cycle for a given population of animals and

b Cerebral malaria (CM) model which has been described for *Plasmodium berghei K173* infections in mice (C57BL) This model is characterized by sequestration of mononuclear cells that migrate between the endothelial and post-capillary venules and cause widespread petechial hemorrhages (6a,44) The parasitemia (P) and body temperature (BT) were monitored for development of CM, inasmuch as a strong correlation was shown to exist between a drop in BT (< 30°C) during the last 24 h of life (death usually occurs in the 2nd week post-infection) and the presence of lesions in the brain (analyzed *post mortem*) (6a) The model differes from the *Plasmodium yoeli 17XL*, which, in CM, resembles more closely the human *P falsiparum*

The **ICHs** used for these studies were selected on the basis of their performance as growth inhibitors in *in vitro* studies of *P falciparum* DFO, MA-DFO, SIH or RSFm2phe They were used either as individual drugs or in combination with DFO

Mode of drug administration The major problem encountered with the RSFs is the choice of an appropriate vehicle for animal administration, due to poor solubility in aqueous solutions and precipitation in mixed solvents All the RSFs and DFO could be administered at doses of 2 5 or 5 mg/mouse(20-25g body weight) as DMSO solutions, either i p or s c For RSFm2ileu, we also succeeded in dissolving sufficient material either in 25% DMSO-saline or in ethanol and preparing a dispersion in miglyol 840 (propylene glycol diester of dicaprylate/dicaprate from coconut oil (German pharmacopoeia, DAB 9) (5% final ethanol conc) This rather viscous material could be administered i p or s c However, we were forced to discontinue the use of this material because of local reaction DFO was dissolved in saline and could be administered either i p , or with the aid of an infusion pump (s c)

In order to overcome the solubility problems encountered with most RSFs, we opted for entrapping sufficient amounts of ICHs inside polymerized fatty acids, which decompose in body fluids and provide a means for slow release of the drugs (1-3,25-27) A 1cm patch of polymer can entrap sufficient amounts of drug equivalent to a full course treatment (9 injections), and be released during a 5-7 day course Preliminary experiments were conducted with DFO and SIH and the polymer poly(dimer erucic acid-sebacic acid)22 78, which has been approved by the US Food and Drug Administration for human clinical trials (101) It was first melted on a hot plate at 70°C, and immediately mixed with DFO or SIH powder and pressed between two metal plates to obtain the appropriate thickness (~2 mm) A 35% DFO polymer (wt wt) was found suitable according to *in vitro* slow release determinations the polymer tablet was placed in 20 ml PBS (0 1 M, pH 7 4), incubated at 37°C and the release was monitored daily DFO was determined spectrophotometrically after complexation with $FeCl_3$ Degradation of the polymer was estimated by weighing after drying The polymer released the DFO and SIH within 1 week as a first order process (28a) **The rate of release could be controlled by the chemical composition of the polymer and the ratio of drug to polymer**

Treatment Swiss mice were infected by i p injection of blood ($3x10^6$parasitized erythrocytes) from a donor animal (parasites were maintained by weekly transfer of infected blood to naive animals) Treatment with drug was initiated at patency (parasites are usually detected within 2-3 days after infection) by injection i p (or as indicated) with, for example, 2 5 or 5 mg DFO (or any other ICH), 3x/day during 3 consecutive days Alternatively, the polymer (cut to obtain the desired size/drug dose) was implanted s c or i p in the lower abdominal region and the incision closed P was monitored daily by Giemsa stained blood smears The C57Bl mice were infected with 10^3 parasitized erythrocytes and 6 dayslater (when P 0 5%), the patch containing 140mg DFO or control patches were implanted s c

Results (selected studies) The examples given are representative and are meant to provide examples of data obtained

i Early studies with DFO and RSFm2 ileu (given to mice post-patent, thrice daily for 3 days s c in miglyol) showed arrest of parasitemia within 24 h of treatment and significant prolongation of life of infected animals (followed up to 10 days) DFO effects were similar but slower to develop This approach was discontinued because of incompatibility of the carrier with most other RSFs and some reactions observed at the site of injection

ii Recent studies were aimed at assessing the new technology of slow-drug release with chemical devices and its applicability to the drugs of interest (28a) Data presented are primarily of DFO, because this drug was also used on CM models of malaria (see below) The efficacy of DFO given i p in saline solution (3x2 5mg or 3x5mg injections/day for 3 days, days 2-4 post-patent on P vinckei petteri infected mice) (Fig 10) was compared with that of a single patch (70 or 140 mg) implanted s c on day 2 (Fig 11) Data show that with either treatment, development of parasitemia was significantly delayed relative to control (no drug) and death was largely prevented Similar data were obtained with i p implanted patches of DFO (not shown)

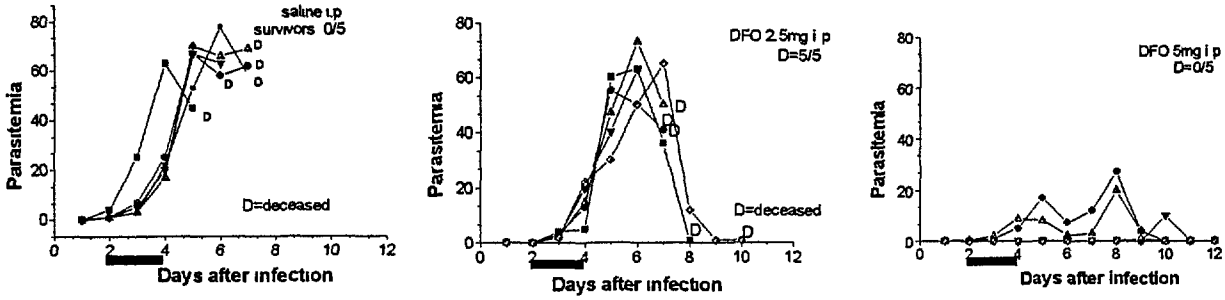


Fig 10 Effect of DFO on in vivo development of P vinckei petteri in mice Days of treatment (3 daily i p injections for 3 consecutive days) indicated as dark bars

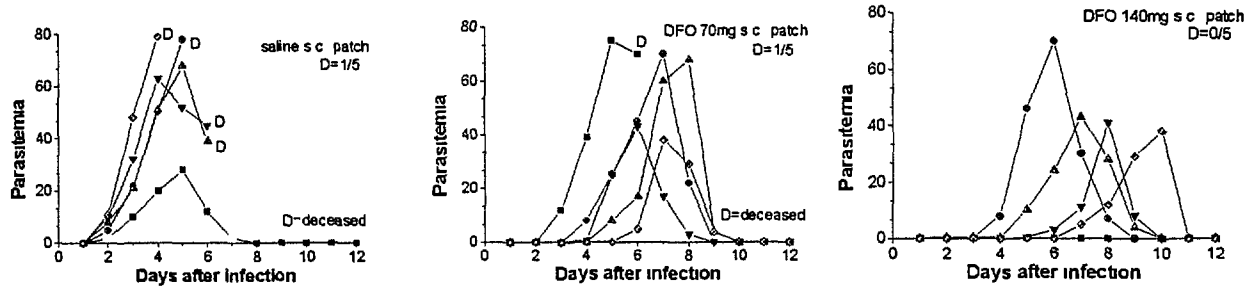


Fig 11 Effect of DFO on *in vivo* development of *P vinckei petteri* in mice Treatment (a s c patch was implanted on day 2)

iii. Similar studies showed that SIH outperformed DFO in terms of speed of action (28a*) although, caution had to be exercised with the amount of drug administered or speed of release from the polymer, so as not to attain toxic levels

iv DFO and SIH were also used in various combinations and shown also *in vivo* to have potentiating effects (28a)

v DFO patches were also used to preliminary assess its effects on rodent model of cerebral malaria CM (Table III) (30), which was made available to us by Dr W M S Elin (Netherland) Although we are aware of differences that exist between the tested model and that of *P falciparum* CM, we have used it because preliminary studies indicated that DFO given to the infected mice, either by i p injections or by infusion pumps, was totally inefficient in relieving them from symptoms and from progression of the disease leading to death (Table III)

	day 8			day 9			day 10			day 11		
	P	T	S	P	T	S	P	T	S	P	T	S
control	34	31 7	11/11	36	27	8/11	ND	27 7	2/11	-	-	0/11
DFO	7	35 4	5/5	5	31 7	4/5	ND	36 6	3/5	0	36 9	3/5
DFO-Zn	8	37 1	5/5	10	35 2	5/5	17 9	34 2	5/5	4 5	36 0	5/5

Table III Effect of DFO (±Zn) patches on the progression of infection and CM symptoms in *P berghei* infected mice Experiments with DFO administered i p or with infusion pumps gave similar results to those of control
The following symbols denote P= % parasitemia, T= body temperature in °C and S=mice survival, ND, not determined Days refer to time after infection, with polymeric patch implanted on day 6

Based on these series of studies, and two others not shown (38a), we obtained that the slow release of DFO had significant effects on parasite survival and on development of symptoms similar to those of CM Using a new DFO formulation (Zn-DFO) (19) administered via an infusion pump (1 mg/day/mouse), P was also markedly reduced, CM was prevented and mice survival was fully attained (5/5 infected animals)

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Final Report by Y Yuthavong, June 1998

1 Effect of Combination of Iron Chelators with Artemisinin

We have proposed that artemisinin acts by binding with its target and exerting oxidative damage within its vicinity through combined action with redox metal ions, which could be Fe(III)/Fe(II) The reactive intermediates may include both reactive oxygen species, and radical derivatives of the drug Generation of these intermediates would, like generation of oxygen intermediates, require participation of iron or other redox metals These reactions are equivalent to the Fenton reaction which gives rise to the hydroxyl radical This hypothesis therefore deserves to be tested further, and may lead to other agents with strong anti malarial effects Our approach was to test the validity of the concept by studying the anti malarial effect of artemisinin and iron chelators, and to synthesized and study the anti malarial effect of iron-chelator-linked artemisinin derivatives We reason that artemisinin and iron chelators should be antagonistic in combination On the other hand, if moderately strong iron chelators can be covalently linked to artemisinin, the linked compounds might be better anti malaria agents than the parent artemisinin, since they could deliver required iron at the site of action

In support of our hypothesis, we observed strong antagonism in anti malarial effect between arteether and chelators desferrioxamine, pyridoxal benzoyl hydrazone, or 1,2-dimethyl-3-hydroxypyrid-4-one Other iron chelators were tested for combination effect with artemisinin As have been observed before with other iron chelators and arteether, desferrioxamine, pyridoxal benzoyl hydrazone (PBH) and 1,2-dimethyl-3-hydroxypyrid-4-one (DMP), kojic acid, pyrogallol, and salicylhydroxamic acid each showed a marked antagonistic effect with artemisinin Benzohydroxamic acid showed an antagonistic effect only at low concentrations of the chelator Methyl caffeate showed an additive effect in combining with artemisinin These results taken

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together show that the action of artemisinin and derivatives are indeed augmented by the iron chelators

We then showed that the reversed siderophores, provided to us by Prof A Shanzer, are moderately to strongly antagonistic with artemisinin in the activity against *Plasmodium falciparum* K1 in culture RSF-Leu (IC_{50} = 3 7 μM) shows only a moderate antagonism with artemisinin (IC_{50} = 8 4 nM) at low artemisinin concentrations (less than 3 nM) and an additive effect at higher artemisinin concentrations RSF-Phe (IC_{50} = 1 8 μM) shows a strong antagonism with artemisinin at all concentration ranges, with Berenbaum fractional inhibition value of 1 31 RSF-COOEt (IC_{50} = 9 μM) shows strongest antagonism, with the fractional inhibition value of 1 80 These results confirm further our previous conclusion that iron is important in the action of artemisinin

2 Synthesis and Anti malarial Activities of Iron-Chelator-Linked Artemisinin[1]

Various derivatives of artemisinin covalently linked to iron chelators were synthesized and their anti malarial activities were evaluated Our first generation of linked compounds were derivatives of dihydro artemisinin, linked through the 17-O to an alkyl chain, further linked to a chelator Although results show no indication that the presence of an iron chelator in the vicinity of artemisinin potentiates its action, the linked compounds prepared still retain comparable activities to that of artemisinin One problem with this group of linked compounds is the instability of the ether linkage It is known that this linkage is unstable and easily hydrolyzed We therefore developed another group of linked compounds which would be more stable in aqueous conditions

Such linked compounds can be synthesized through conversion of artemisinin **[1]** to artemisitene **[2]**, an intermediate which can be used to link with iron chelators through a C-C bond We established a procedure which gave artemisitene with 71% yield in one pot whereas a prior published procedure gave about 58% yield through four steps of reactions

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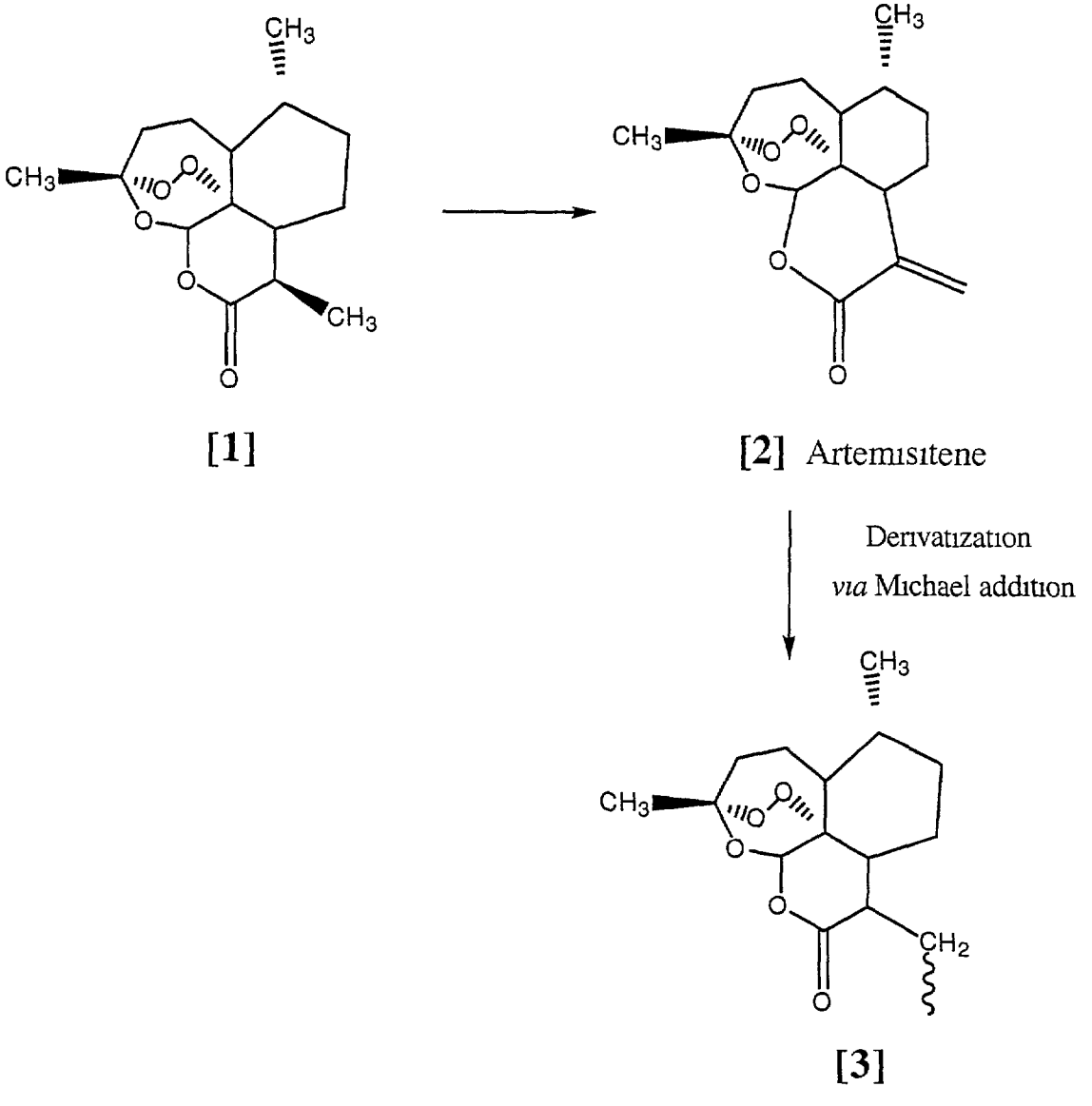
The reversed siderophores (RSFs) from Israel were linked to exocyclic double bond at C-9 position of artemisitene In preliminary exploration, we used for a model Benzyl amine, with NH_2 as the functional group to attack the double bond to acts as a model In the same manner, imidazol was also linked to the double bond of artemisitene



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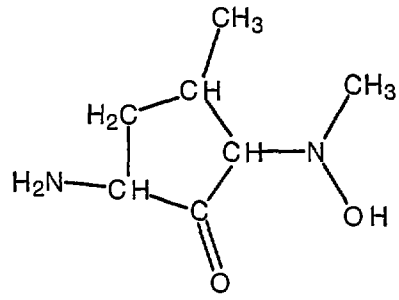
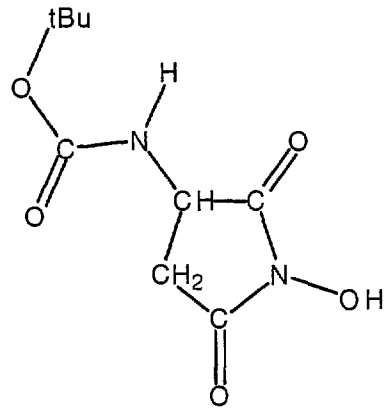


Compound A



Compound B

We then tried to link compounds A and B, delivered to us from the Israeli group, to the exocyclic double bond at C-9 position of artemisitene using the same conditions as the previous models However, we found that the compounds could not be linked under the same conditions So we attempted to find an optimal condition by varying various parameters(time, temperature, solvent system, chemical reagents to generate anion for attack, etc) Eventually, we found the condition (a large excess of lithium di-isopropyl amine as anion generator, $ZnCl_2$ as stabilizer of the keto function, -78°C, 6 h), to produce the desired products However, the desired products could not be purified from crude reaction mixtures, either on silica gel or alumina gel The products also proved to be unstable Alternative plans for linkage are being tried out

Although we have not yet achieved our primary objective, we obtained a number of stably linked compounds which together proved to give novel insight

into the mechanism of action of artemisinin, and the prediction of anti malarial activity of derivatives

4 Binding of Artemisinin Derivatives to Ferroprotoporphyrin IX Correlates with Their Anti malarial Activity (Ref 2)

The anti malarial activity of a number of artemisinin derivatives, both newly synthesized and currently used as drugs, against *Plasmodium falciparum* in culture shows a correlation with their affinity of binding with ferroprotoporphyrin IX, as measured from the spectral change of the latter The new C-16-functionalized artemisinin derivatives were obtained through a novel one-pot synthesis of artemisitene **[2]** from naturally abundant artemisinin **[1]**, followed by Michael addition with nucleophiles The correlation points to the biological significance of the interaction of these derivatives with ferroprotoporphyrin IX and may provide a basis for primary screening of peroxidic anti malarial agents of similar structures The correlation also raises a number of questions to be pursued further, such as how the drug activation occurs after binding, what is the protein target, if any, and how the parasite is killed It can also be asked whether the interaction with heme derivatives also occurs in the erythrocyte, and whether this leads to drug inactivation before it reaches the parasite, hence partly explaining the ineffectiveness of the drug with parasite infecting thalassemic erythrocytes (see below)

5 Ineffectiveness of Artemisinin Derivatives against Plasmodium falciparum Infecting α-Thalassemic Erythrocytes (Ref 3)

Dihydro artemisinin and other artemisinin derivatives are relatively ineffective against *Plasmodium falciparum* infecting α-thalassemic erythrocytes, namely hemoglobin (Hb) H or HbH/Hb Constant Spring erythrocytes, as compared with those infecting genetically normal erythrocytes The variant erythrocytes accumulate radiolabelled dihydro artemisinin to a much higher extent than the normal ones, and the accumulated drug was retained after extensive washing, in contrast to the drug in normal erythrocytes which was mostly removed At initial drug concentration of 1 μM, most (82-88 %) of the

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drug was found in the cytosol fraction of both variant and normal erythrocytes Binding of the drug to hemoglobins accounted for 40-70% of the total uptake Hb H, accounting for 10 9 ± 2 7 % and 12 4 ± 6 2 % of total protein in HbH and HbH/Hb Constant Spring erythrocytes, bound with 28 7 ± 6 7 % and 21 8 ± 8 3 % of the drug respectively Binding experiments showed that Hb H had 5-7 times the drug-binding capacity of Hb A For Hb H, the maximum binding capacity (B_{max}) = 1 67 ± 0 17 mol/mol Hb, and the dissociation constant (K_d) = 66 ± 17 μM, and for Hb A, B_{max} = 0 74 ± 0 18 mol/mol Hb and K_d = 224 ± 15 μM It is concluded that preferential binding of dihydro artemisinin to Hb H over Hb A accounts partly for the higher accumulation capacity of the α-thalassemic erythrocytes, which leads to its anti malarial ineffectiveness In addition to binding to Hb H, other factors such as inactivation of the drug by heme derivatives present in the thalassemic erythrocytes may also contribute to ineffectiveness of the drugs

6 Publications

1 Kamchonwongpaisan, S ,Paitayatat, S , Thebtaranonth, Y , Wilairat, P and Y Yuthavong (1995) Mechanism-Based Development of New Antimalarials Synthesis and Derivatives of Artemisinin Attached to Iron Chelators *J Med Chem* **38**, 2311-2316

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Mechanism-Based Development of New Antimalarials: Synthesis of Derivatives of Artemisinin Attached to Iron Chelators

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Various derivatives of artemisinin covalently linked to iron chelators were synthesized, and their antimalarial activities were evaluated Although results show no indication that the presence of an iron chelator in the vicinity of artemisinin potentiates its action, the linked compounds prepared still retain comparable activities to that of artemisinin

Malaria continues to be a major endemic infectious disease in many developing countries, requiring both global and national efforts in research toward its control and eradication The disease is caused by *Plasmodium* spp , especially *Plasmodium falciparum*, the pathogen responsible for the life-threatening form of the disease The problems have been further complicated by emergence of resistance to many drugs Therefore there is a great need for new antimalarials, preferably with radically different structures and modes of action, in order to deal with development of resistance to the drugs in current use Artemisinin offers exciting new possibilities in drug development due to its unique structure, mode of action, rapid action, and efficacy against chloroquine-resistant parasite [1-3]

Artemisinin or qinghaosu had been isolated from *Artemisia annua* Linn , an ancient Chinese herbal used for treatment of fever and malaria Its structure had been identified to be an endoperoxide-containing sesquiterpene lactone The presence of endoperoxide linkage is essential for its action,[1 4] and many reports showed that the drug probably acts through generation and exertion of oxidative stress on the parasite [5-7] Furthermore, the presence of free iron is likely to be important in the drug action, as simultaneous *in vitro* application of artemisinin or its derivatives, arteether and artesunate, and one of a number of iron chelators, which by themselves also have antimalarial property, leads to antagonism [8 9] Antemisinin or its derivatives may thereore act, after reaching the site of action in the parasite, by exerting oxidative damage through combined action with Fe(III)/Fe(II) which generates reactive intermediates The reactive intermediates may include reactive oxygen species and radical derivatives of the drug If this is so, then it should be expected that the reactive intermediates will combine with parasite components at or very close to the site of their formation It should furthermore be expected that if iron can be made available within the vicinity of artemisinin or its derivatives, the drug should become much more potent Such strategy may be achieved through application of new drug agents with the structure of artemisinin covalently linked to a suitable iron chelator Such a chelator should not bind iron too strongly since it must be able to deliver free iron to the site of drug action or allow reversible redox reactions of the iron and yet must have the capacity to carry iron to that site In order to test this hypothesis, approaches to the synthesis of such linked compounds should be developed In this report we show a general scheme for such synthesis, with a number of examples of linked compounds, some of which are shown to have *in vitro* antimalarial activities comparable to artemisinin

Chemistry

Artemisinin **1** was reduced with sodium borohydride into dihydroartemisinin **2** [4] Treatment of the benzene solution of **2** with bromopropanol at room temperature in the presence of boron trifluoride etherate catalyst[4] afforded a single β-isomer of the corresponding bromide **3** Similar treatment of **2** with propanediol, on the other hand, gave both α- and β- isomers of artemisinin–propanol derivatives, **4** and **5**, in respectable yields (11% for α- and 30% for β-isomers), which can be crystallized from hexane at −20 °C (Scheme 1) Elucidation of the stereochemistry of the above products was achieved by their NMR data whereupon the small coupling constant (J = 3 5 Hz) between the vicinal protons, H_9–H_{10}, was observed in the case of the β-isomer The *trans* relationship of these two protons in the α-isomer was evident from a large coupling constant of 9 Hz

Artemisinin–chelator derivatives were prepared by reactions depicted in Scheme 2 The iron chelator was dissolved in DMSO and subjected to sodium hydride (1 5 equiv) treatment to generate the corresponding anion for further reaction with artemisinin–bromide derivative **3** The method provided a series of artemisinin hybrids whose structures were proved by high-resolution NMR Linking of artemisinin to methyl caffeate regiospecifically afforded compound **6** as evidenced by the chemical shift of aromatic proton at C-2′ on the caffeate nucleus which remained unchanged upon linking Alkoxylation of artemisinin to kojic acid provided compound **7** which resulted from the reaction of hydroxyl function at C-2′, causing an up field shift of the adjacent C-3′ proton by 0 4 ppm as compared to that of kojic acid itself Reaction of artemisinin with pyrogallol yielded three products, **8**, **9**, and **10** Signals of the equivalent (compound **8**) and unequivalent (compound **9**) aromatic protons at C-4′ and C-6′ on the pyrogallol nucleus indicated the linkage of artemisinin molecule to the hydroxyl group at C-2′ and C-1′, respectively The NMR spectrum of the dialkylated product **10** not only showed the unequivalent protons at C-4′ and C-6′ but also the presence of two sets of signals of artemisinin

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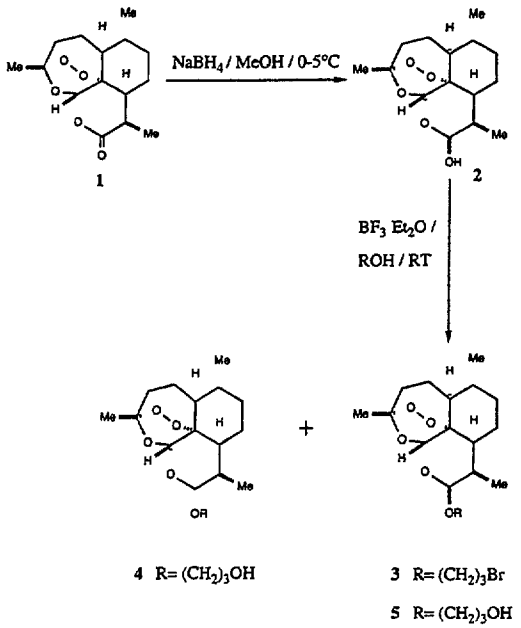
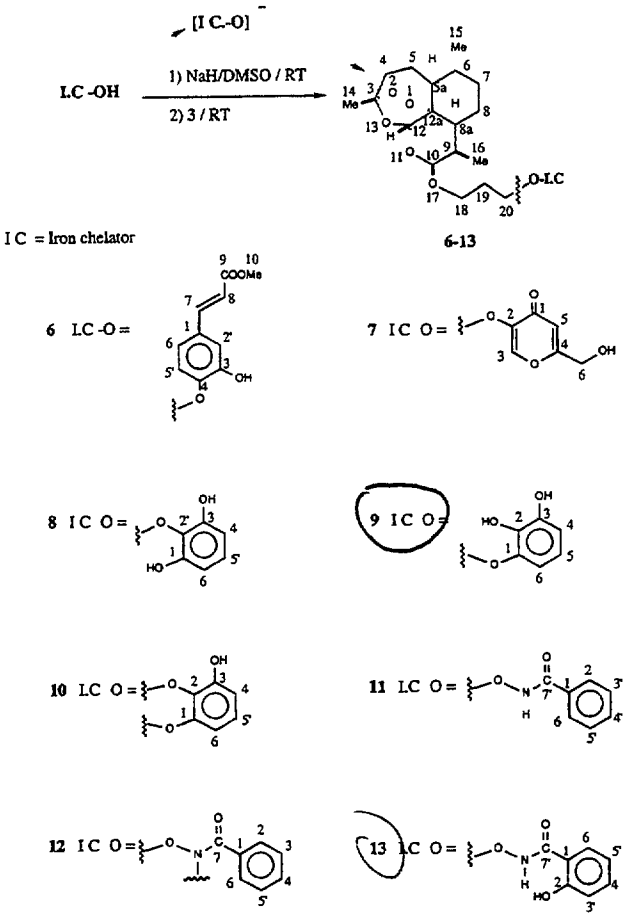
Scheme 1

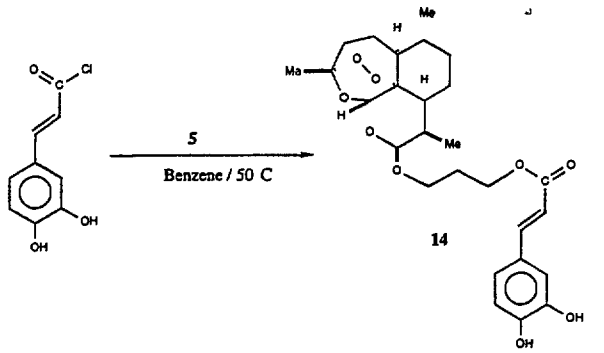


Scheme 2

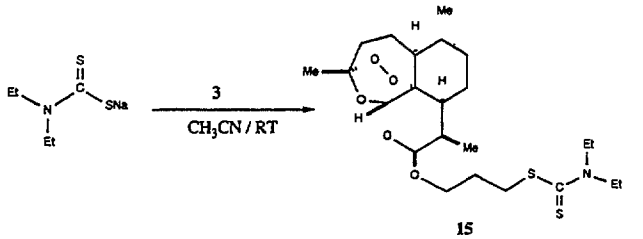


moieties, indicating the coupling of two artemisinin molecules to C-1′ and C-2′ hydroxyl groups Reaction of artemisinin with benzohydroxamic acid gave the mono- and dialkylated products **11** and **12**, respectively Salicylhydroxamic acid also linked to artemisinin *via* its hydroxamic OH giving rise to compound **13** as the sole product Further experiment was performed to provide a better understanding of the site of covalent bonding between artemisinin and the hydroxamate

Scheme 3



Scheme 4



Results from a model reaction between salicylhydroxamic acid and benzyl bromide indicated that the reactivity toward alkylation at each heteroatom of this chelator was in the order of hydroxamic OH > hydroxamic NH > phenolic OH

It was worth noting that although reactions described above could be regarded as straightforward, manipulations were sometimes difficult due to the fact that dihydroartemisinin nucleus was unstable under the strong basic conditions employed in the study Also, some of these products, bearing both oxidizing and reducing moieties, were rather unstable and underwent spontaneous decomposition

An alternative method to construct artemisinin–caffeic acid hybrid was shown in Scheme 3 Caffeic acid was treated with thionyl chloride to afford the acid chloride which upon reaction with artemisinin–hydroxy derivative **5** provided the hybrid **14**, but in very low yield and the product very unstable Scheme 4 also shows the construction method of compound **15**, a derivative of artemisinin coupled with diethyl dithiocarbamate, an iron chelator which is also a potent inhibitor of superoxide dismutase, an antioxidant enzyme present both in the erythrocyte and the parasite [10]

Results and Discussions

Based on drug combination studies, we proposed[8 9] that artemisinin acts by binding with its target and exerting oxidative damage within its vicinity through combined action with redox metal ions, probably Fe(III)/Fe(II) Compounds with the structure of artemisinin cross-linked to suitable chelators can therefore have greater antimalarial activity, provided a number of important conditions can be fulfilled The main criteria for effectiveness of such new compounds against the malarial parasite should be the ability to penetrate infected cells and bind to the same target as artemisinin, the ability to carry the iron to that target, and the ability to allow the iron to participate in reversible redox reactions A number of iron chelators were chosen based on the presence of suitable groups which can be

Table 1 Antimalarial Activity of Iron Chelators, Diethyldithiocarbamate (DDC) and Their Derivatives against *P falciparum in Vitro*

agents	IC_{50} (M)
caffeic acid	1.22×10^{-4}
methyl caffeate	4.65×10^{-5}
kojic acid	1.58×10^{-3}
pyrogallol	5.66×10^{-6}
benzohydroxamic acid	1.76×10^{-5}
salicylhydroxamic acid	1.07×10^{-4}

Table 2 Antimalarial Activity of Artemisinin and Its Novel Derivatives against *P falciparum in Vitro*

compounds	IC_{50} (nM)	compounds	IC_{50} (nM)
artemisinin (1)	5.82 ± 1.4	compound 9	12.7
dihydroartemisinin (2)	1.1	compound 10	6.64
compound 3	3.68 ± 0.55	compound 11	23.64
compound 6	4.78 ± 3.0	compound 12	4.25
compound 7	9.55 ± 5.0	compound 13	52.45
compound 8	7.67	compound 15	7.67

Table 3 Effect of Iron (20 μM $FeCl_3$) on the Antimalarial Activity of Artemisinin and Its Novel Derivatives against *P falciparum in Vitro*

	IC_{50} (nM)		
compounds	control (*a*)	with 20 μM $FeCl_3$ (*b*)	ratio of *b*/*a*
artemisinin 1	6.04	5.41	0.9
compound 6	2.82	3.38	1.2
compound 7	14.79	16.80	1.1
compound 8	10.61	9.21	0.9
compound 11	19.36	32.91	1.7
compound 13	45.53	48.71	1.1

used to link covalently with artemisinin. The iron chelators and the novel artemisinin derivatives were investigated for antimalarial activity against a multidrug-resistant *P falciparum* K1 strain *in vitro*. The results are summarized in Tables 1 and 2. All the chosen iron chelators possessed intrinsic antimalarial activity. Derivatization of artemisinin into compound **3**, which is an ether derivative of dihydroartemisinin, exerted the antimalarial activity in the same range as its parent compound. Modifications of compound **3** into compounds **6**–**13** by covalent linking with iron chelators did not increase the antimalarial potency of these derivatives over that of artemisinin but did not diminish the potency remarkably either. Although compounds **9** and **14** structurally possess iron binding capability, they were impaired by their intrinsic instability and therefore might not be expected to show any superior antimalarial potency. Compound **15**, with a linked molecule of diethyl dithiocarbamate, which is both an iron chelator and an inhibitor of the antioxidant enzyme superoxide dismutase,[10] also has comparable activity with artemisinin.

In order to check whether the antimalarial acitivity might depend on previously chelated iron in the medium, $FeCl_3$ was added to antimalarial testing system. Table 3 shows that the presence of 20 μM $FeCl_3$ did not enhance the killing effect of these synthetic derivatives. The antimalarial activities of these derivatives therefore did not depend on the presence of exogenous iron.

In the testing of our hypothesis, it is important that the derivatives can chelate iron under the conditions of our experiment. We have ascertained, from the change in visible spectrum, that the salicylhydroxamate derivative **13** could indeed chelate with Fe(III) although not as effectively as the parent salicylhydroxamic acid.

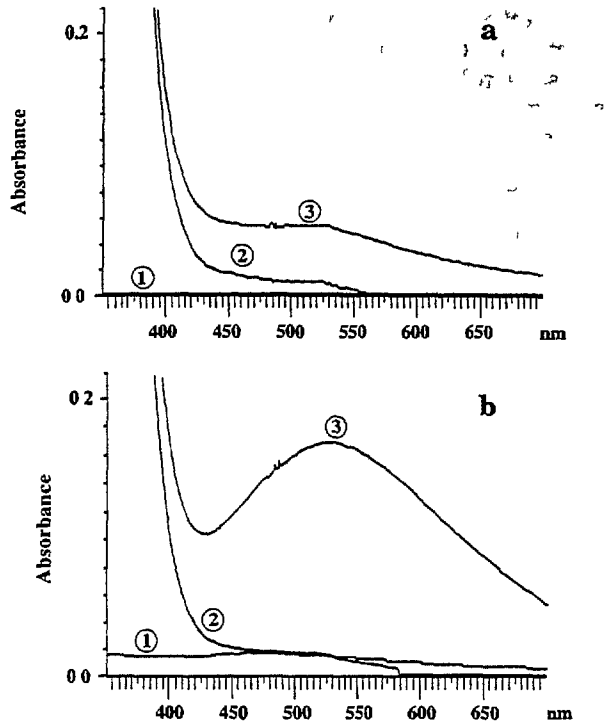


Figure 1 (A) Spectra of (1) 0.19 mM salicylhydroxamate derivative dissolved in 5 mM sulfuric acid, (2) 2.5 mM ferric nitrate dissolved in 5 mM sulfuric acid and (3) 0.19 mM salicylhydroxamate derivative plus 2.5 mM ferric nitrate dissolved in 5 mM sulfuric acid. (B) Spectra of (1) 0.075 mM salicylhydroxamic acid dissolved in 5 mM sulfuric acid, (2) 2.5 mM ferric nitrate dissolved in 5 mM sulfuric acid and (3) 0.075 mM salicylhydroxamic acid plus 2.5 mM ferric nitrate dissolved in 5 mM sulfuric acid.

Figure 1A shows the appearance of a peak at 515 nm when Fe(III) is present together with the derivative **13**. A similar, but stronger, peak also appears at 525 nm with the chelated parent salicylhydroxamate.

Although the results from our study have not given any indication that the presence of an iron chelator in the vicinity of artemisinin can potentiate its action, the fact that the linked compounds still retain antimalarial activities nevertheless encourages us to explore other derivatives which will meet the criteria anticipated for synergistic interaction.

Conclusions

Several novel artemisinin derivatives have been synthesized based on the requirement of iron in the mechanism of action of artemisinin. The general route for establishing a linkage of iron chelators to the C-10 lactol of artemisinin nucleus in this study is accomplished by a displacement reaction. Although the novel hybrids obtained did not show enhanced antimalarial activities, they were nontheless comparable to that of artemisinin. Hence they might be useful drugs if they exhibit less toxicity than artemisinin or other known derivatives. Furthermore, the study reveals the use of other iron chelators which might interact synergistically with artemisinin. Some of the linked derivatives reported here do not have high chelating ability (e.g., compounds **6**, **7**, **8**, **10**, **11**, **12** and **13**, and **15**). However, we have shown that compound **13** has a finite chelating ability, although not as high as the parent chelator salicylhydroxamate. Some of the compounds synthesized in this study (e.g., compounds **9** and **14**) turn out

reaction (between peroxide and iron chelator moieties) Consequently, more suitable chelators for optimum synergistic interaction with artemisinin still have to be found

Experimental Section

Chemistry NMR spectra were recorded either on a Varian EM 360L or on a Bruker AM 400 spectrometer The IR spectra were determined on a JASCO model A 302 or a Perkin Elmer 2000 NIR FT Raman spectrometer Elemental analyses were carried out on a Perkin Elmer elemental analyzer 2400 CHN and the results are within 0 4% of the theoretical values Mass spectra were recorded on a JMS-DX 300 JEOL mass spectrometer Melting points were determined on an electrothermal melting point apparatus (Electrothermal 9100) Merck's Kiesegel 60 PF254 were used for preparative layer chromatography

Conversion of Dihydroartemisinin to the Bromide or Hydroxy Derivatives (3–5) Dihydroartemisinin 2[4] (500 mg, 1 76 mmol) in dry benzene (5 mL) was treated with bromopropanol (2 mL, 20 mmol) under nitrogen in the presence of freshly distilled boron trifluoride etherate ($BF_3 Et_2O$ 1 mL of 5% (v/v) $BF_3 Et_2O$ in dry benzene) at room temperature for 2 h Purification of the bromo derivative 3 (90% yield) was performed by crystallization from hexane at −20 °C

The reaction of dihydroartemisinin with propanediol gave the corresponding alcohols 4 and 5 which after purification by TLC (silica gel using 50% ethylacetate in hexane as eluent) followed by crystallization from hexane at −20 °C provided pure samples in 11 4 and 30 11% respectively

Compound 3 white crystals, mp 85–86 5 °C (from hexane) IR ν_{max} (Nujol) 1100, 880, and 825 cm^{-1}, MS *m/e* (relative intensity) 388 ($M^+ - H_2O$, 0 4), ^1H NMR ($CDCl_3$) δ 0 90 (d 3H J = 7 36 Hz H 16), 0 90 (m 1H H-7α), 0 95 (d, 3H J = 6 25 Hz, H 15), 1 25 (m 1H, H-5a), 1 33 (m, 1H H 6), 1 44 (s, 3H, H-14) 1 48 (m, 1H H 8a) 1 50 (m 1H H 5β) 1 64 (m, 1H H 7β), 1 75 (m, 2H H-8) 1 87 (m 1H, H 5α) 2 03 (ddd, 1H, J = 14 32, 4 67, 2 92 Hz H-4β) 2 10 (m 2H, H-19), 2 37 (ddd, 1H, J = 14 32, 13 9, 3 94 Hz, H-4α), 2 63 (ddq, 1H, J = 2 8, 3 4, 7 36 Hz, H 9), 3 49 (t, 2H, J = 6 41 Hz, H-20), 3 49 (dt, 1H, J = 10 06, 5 73 Hz, H 18b) 4 00 (dt 1H, J = 10 06, 5 73 Hz H-18a), 4 80 (d 1H, J = 3 4 Hz, H 10), 5 42 (s 1H H-12), ^{13}C NMR (δ in $CDCl_3$) 12 92, 20 31, 24 47, 24 62 26 13, 30 53, 30 84 32 50, 34 58, 36 38, 37 40, 44 34, 52 54 65 66 81 00, 87 88 102 06, 104 06 $[\alpha]_D$ +122 1° (*c* 0 35, $CHCl_3$) Anal ($C_{18}H_{29}O_5Br$) C, H

Compound 4 white crystals, mp 106–109 °C (from hexane) IR ν_{max} (Nujol) 3500 (sharp), 1100, 880 and 830 cm^{-1}, MS *m/e* (relative intensity) 310 ($M^+ - 32$, 1), ^1H NMR ($CDCl_3$) δ 0 8–2 8 (m, 23H), 3 35 (s 1H OH disappeared after D_2O), 3 45–4 3 (m 4H 2 × H-18 2 × H-20) 4 50 (d, 1H, J = 9 Hz, H-10), 5 40 (s, 1H H 12), $[\alpha]_D$ +73 3° (*c* 0 03, $CHCl_3$) Anal ($C_{18}H_{30}O_6$) C, H

Compound 5 white crystals mp 72–73 °C (from hexane) IR ν_{max} (neat) 3500 (sharp), 1100 880 and 830 cm^{-1} MS *m/e* (relative intensity) 310 ($M^+ - 32$, 1) ^1H NMR ($CDCl_3$) δ 0 85–2 8 (m 23H), 3 13 (s 1H OH disappeared with D_2O) 3 3–4 3 (m, 4H 2 × H-18 2 × H 20), 4 75 (d, 1H, J = 3 5 Hz H-10), 5 38 (s, 1H, H 12), $[\alpha]_D$ +141 1° (*c* 0 48, $CHCl_3$) Anal ($C_{18}H_{30}O_6$) H, C calcd, 63 14 found, 64 21

Coupling of Artemisinin Derivative 3 with Iron Chelators 6–13 Typical Procedure A mixture of 50% sodium hydride in oil (48 mg, 1 mmol) and methyl caffeate (1 5 mmol) in dimethyl sulfoxide (5 mL) was stirred under nitrogen at room temperature for 1 h The reaction mixture was cooled down to 4 °C and a solution of compound 3 (405 mg 1 mmol) in dimethyl sulfoxide (5 mL) was introduced and the solution was left stirring at room temperature for 2 h The reaction mixture was cooled down to 4 °C and acidified with dilute aqueous hydrochloric acid The organic material was extracted into ethyl acetate and the combined ethyl acetate extract was washed with water, dried ($MgSO_4$) filtered, and finally evaporated to dryness The crude product was purified

Compounds 6 white crystals, mp 141–144 °C (from chloroform), IR ν_{max} ($CHCl_3$) 3550, 1700, 1640 1620 1590, 1515, 1100, 990, 880, and 830 cm^{-1}, MS *m/e* (relative intensity) 472 ($M^+ - 46$, 4) ^1H NMR ($CDCl_3$) δ 0 85 (m, 1H H-7α), 0 85 (d 3H, J = 6 27 Hz, H 15), 0 89 (d, 3H, J = 7 36 Hz, H 16), 1 02 (m, 1H, H 6), 1 19 (ddd 1H, J = 11 47, 11 21, 6 54 Hz, H-5a), 1 42 (s 3H, H 14), 1 43 (m, 1H, H 8a), 1 45 (m, 1H, H 5β), 1 50 (m, 1H, H 7β), 1 70 (m, 2H, H 8) 1 83 (dddd, 1H, J = 13 6, 3 08, 3 64, 6 84 Hz, H 5α), 2 02 (ddd, 1H, J = 14 48, 4 69, 3 05 Hz H-4β) 2 11 (m, 2H, H-19), 2 35 (ddd, 1H J = 14 48 13 63, 3 95 Hz H-4α) 2 64 (ddq, 1H, J = 7 36 4 40, 3 36 Hz, H-9), 3 55 (dt, 1H, J = 9 97, 5 54 Hz, H-18b) 3 79 (s, 3H H-10) 4 07 (ddd, 1H, J = 9 97, 7 08, 5 82 Hz H-18a), 4 19 (t, 2H, J = 6 41 Hz, H 20), 4 81 (d, 1H, J = 3 36 Hz, H 10), 5 33 (s 1H, H 12) 5 82 (s, 1H, OH), 6 29 (d 1H, J = 15 93 Hz, H 8) 6 83 (d, 1H, J = 8 34 Hz, H-5), 7 00 (dd, 1H, J = 8 34, 2 06 Hz, H 6′), 7 15 (d, 1H, J = 2 06 Hz, H-2) 7 59 (d, 1H, J = 15 93 Hz, H-7′), ^{13}C NMR ($CDCl_3$) δ 12 94, 20 31, 24 54 24 59, 26 09, 29 03, 30 82, 34 50, 36 37, 37 35, 44 28, 51 53, 52 44, 64 04, 65 82, 80 92, 87 82, 101 91, 104 15 111 34 113 36, 115 88, 121 61, 128 11, 144 59, 146 05, 147 73, 167 63 $[\alpha]_D$ +84 2° (*c* 0 30 $CHCl_3$) Anal ($C_{28}H_{38}O_9$) C, H

Compound 7 semisolid IR ν_{max} (Nujol) 3370 (broad) 1650 1620 1600, 1110, 880 and 835 cm^{-1}, *m/e* (relative intensity) 420 ($M^+ - 46$ 0 2) ^1H NMR ($CDCl_3$) δ 0 81 (d, 3H, J = 7 35 Hz H 16) 0 82 (m, 1H, H 7α) 0 87 (d 3H, J = 6 00 Hz H 15) 1 15 (m 1H, H-5a) 1 21 (m, 1H H 6), 1 35 (s 3H H-14) 1 38 (m 1H H 8a) 1 41 (m, 1H H 5β), 1 53 (dq, 1H J = 13 16 3 10 Hz H 7β) 1 68 (m, 2H H 8) 1 80 (m, 1H, H-5α) 1 96 (ddd 1H, J = 14 05 4 42 3 18 Hz H-4β) 2 00 (quin 2H J = 6 29 Hz, H 19), 2 28 (ddd, 1H, J = 14 05, 13 91 3 9 Hz, H-4α) 2 54 (ddq, 1H J = 7 35, 4 10 3 5 Hz H-9), 3 47 (dt 1H J = 10 19, 6 06 Hz H-18b), 3 88 (dt, 2H, J = 6 41 1 76 Hz H-20), 3 92 (dt 1H J = 10 19 6 06 Hz, H 18a) 4 41 (s, 2H H-6′) 4 71 (d, 1H, J = 3 5 Hz H-10) 5 30 (s 1H H 12) 6 46 (s 1H, H-5′), 7 55 (s, 1H H-3), ^{13}C NMR ($CDCl_3$) δ 12 87, 20 19 24 35 24 49 25 95, 29 12 30 72, 34 41 36 22, 37 28, 44 18, 52 34 60 29, 64 41, 66 61, 80 91, 87 70, 101 85, 104 01, 111 41 139 37 147 49, 168 04 174 87 $[\alpha]_D$ +96 4° (*c* 0 29 $CHCl_3$) Anal ($C_{24}H_{34}O_9$) C, H

Compound 8 pale yellow needles, mp 140–141 °C (from a mixture of ethyl acetate–hexane), IR ν_{max} (Nujol) 3530, 3450, 1600, 1500, 1480, 1110, 875, and 820 cm^{-1}, MS *m/e* (relative intensity) 404 ($M^+ - 46$ 5), 266 (7), 249 (3), 221 (60), 184 (18), 180 (24) 163 (100), 149 (22), 137 (12) 126 (52) 93 (12), 81 (10), 71 (9), 55 (16), 46 (17), 43 (42), ^1H NMR ($CDCl_3$) δ 0 90 (m 1H H 7α), 0 93 (d, 3H J = 7 39 Hz H 16), 0 94 (d, 3H, J = 6 12 Hz, H-15), 1 25 (m, 1H, H-5a), 1 30 (m, 1H, H 6), 1 45 (s, 3H, H-14), 1 48 (m 1H, H 8a), 1 50 (m, 1H, H-5β) 1 60 (dq, 1H, J = 13 19, 3 24 Hz H-7β), 1 75 (m, 2H, H-8), 1 88 (m, 1H H 5α) 2 04 (m 2H, H 19), 2 06 (m 1H, H-4β), 2 38 (ddd, 1H J = 14 34, 13 70, 3 98 Hz, H-4α), 2 68 (ddq, 1H, J = 2 92, 3 49, 7 39 Hz H 9) 3 76 (ddd, 1H, J = 10 89 6 57 4 32 Hz, H 18b), 4 06 (m, 1H, H-18a), 4 10 (m, 2H, H 20), 4 87 (d, 1H, J = 3 49 Hz, H-10), 5 44 (s, 1H, H 12), 5 72 (s, 1H, OH, disappeared with D_2O) 6 49 (d 1H, J = 8 19 Hz H-4), 6 49 (d 1H, J = 8 19 Hz H-6′), 6 85 (t, 1H, J = 8 19 Hz H-5), ^{13}C NMR ($CDCl_3$) δ 12 83, 20 27, 24 43, 24 66 26 07, 29 90 30 89, 34 59 36 42, 37 43 44 36, 52 52 65 23, 71 35, 81 06 88 00, 101 95 104 23 108 10, 124 65 133 69, 149 31, $[\alpha]_D$ +101 5° (*c* 0 62, $CHCl_3$) Anal ($C_{24}H_{34}O_8$) C H

Compound 9 pale yellow oil, IR ν_{max} ($CHCl_3$) 3560 (sharp), 3460 (broad) 1620 1510, 1470, 1100, 880, and 830 cm^{-1}, MS *m/e* (relative intensity) 404 ($M^+ - 46$, 2), 361 (1), 267 (5), 266 (4), 234 (1) 221 (67), 184 (21) 163 (100), 162 (59), 149 (17), 137 (14), 126 (41) 107 (90), 93 (15), 81 (12), 71 (9) 55 (17), 46 (20) 43 (45) Due to rapid decomposition in solution no respectable NMR spectrum of 9 was obtained

Compound 10 colorless oil MS *m/e* (relative intensity) 502 ($M^+ - 272$ 1) ^1H NMR ($CDCl_3$) δ 0 88 (d 3H, J = 6 02 Hz H-15) 0 88 (m 2H H-7α), 0 89 (d 3H J = 7 30 Hz, H 16) 0 92 (d 3H J = 7 30 Hz H 16), 0 93 (d, 3H, J = 6 02 Hz, H 15), 1 10 (m 1H H-6) 1 22 (m 2H H-5a) 1.29 (m 1H H 6) 1 42 (s, 3H, H-14), 1 44 (m, 2H, H 8a), 1 44 (s, 3H, H-14), 1 45 (m,

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2H, H 5β), 1 51 (m, 2H H-7β), 1 59 (dq, 1H, J = 13 14 3 19 Hz, H-7β), 1 73 (m, 4H, H-8), 1 83 (m, 1H, H 5α), 1 87 (m 1H, H 5α), 2 02 (m, 2H, H-4β), 2 04 (m 2H H 19) 2 09 (m, 2H, H-19), 2 36 (m, 2H, H-4α), 2 64 (m, 2H, H 9), 3 53 (dt, 1H J = 9 90, 5 63 Hz, H 18b), 3 66 (m, 1H H 18b) 4 04 (m 2H, H-18a), 4 08 (m, 2H, H 20), 4 17 (m, 2H, H 20), 4 81 (d, 1H, J = 3 38 Hz, H-10), 4 84 (d, 1H, J = 3 44 Hz H-10) 5 33 (s 1H, H 12) 5 42 (s, 1H H 12) 6 04 (s, 1H, OH, disappeared with D_2O) 6 44 (dd 1H, J = 8 30, 1 15 Hz H 6′) 6 58 (dd 1H, J = 8 30, 1 15 Hz, H-4′), 6 88 (t, 1H J = 8 30 Hz H 5′) ^{13}C NMR ($CDCl_3$) δ 12 92, 12 97, 20 26, 20 28, 24 43 24 50 24 62, 24 65, 26 12 26 14, 29 41 30 22 30 86, 34 55 34 60 36 40 37 28, 37 35 44 34 44 39, 52 46, 52 51, 64 33 65 25, 65 40 70 81 81 00 81 05, 87 74, 87 89 101 90 101 93, 104 00 104 09, 104 77 108 13, 123 77, 134 76 149 87, 151 84 No satisfactory elemental analysis was obtained

Compound 11 pale yellow semisolid, IR ν_{max} (Nujol) 3550 (broad), 3250 (broad) 1740 1605 1580 1520, 1490 870, and 825 cm^{-1}, MS *m/e* (relative intensity), 415 (M^+ − 46, 1), 60 MHz ^{1}H NMR ($CDCl_3$) δ 0 7−2 9 (m 23H) 3 32−4 33 (m 4H 2 × H 18, 2 × H 20), 4 77 (d, 1H, J = 3 5 Hz, H-10), 5 43 (s, 1H H 12), 7 5 (m, 3H, H 3 , H-4 , H 5) 7 75 (m, 2H, H 2′, H 6) 9 47 (s, 1H, NH, disappeared with D_2O) $[\alpha]_D$ +103 4° (*c* 0 21 $CHCl_3$) Anal ($C_{25}H_{35}O_7N$) C H N calcd 3 03 found, 3 46

Compound 12 pale yellow semisolid IR ν_{max} (Nujol) 1740 1660, 1600 1580, 1500, 1460 1095 870 and 820 cm^{-1} MS *m/e* (relative intensity) 514 (M^+ − 271, 2), ^{1}H NMR ($CDCl_3$) δ 0 81 (d 3H J = 7 35 Hz, H-16) 0 88 (d 3H, J = 7 32 Hz H 16) 0 90 (m, 2H H 7α), 0 94 (d 3H J = 6 21 Hz H 15) 0 95 (d 3H J = 5 98 Hz H 15), 1 22 (m 1H H 5a) 1 24 (m, 1H H 5a) 1 27 (m 1H H 6) 1 33 (m 1H H 6) 1 43 (s 6H H-14) 1 45 (m 2H H 8α) 1 48 (m 2H H 5β) 1 60 (m 2H H 7β) 1 65 (m, 2H, H 8) 1 70 (m 2H H 8) 1 72 (m 2H H 19) 1 87 (m, 2H H 5α) 2 01 (m 2H H 19) 2 05 (m, 2H H 4β) 2 36 (ddd 2H J = 14 25 13 77 3 94 Hz H 4α) 2 58 (m 1H H 9), 2 62 (m 1H H 9) 3 26 (dt 1H, J = 10 01 6 36 Hz H 18b), 3 47 (dt 1H J = 10 12 6 24 Hz, H 18b) 3 71 (dt 1H J = 10 01 6 05 Hz H 18a), 3 79 (m 4H, H 20), 3 94 (dt 1H, J = 10 12 5 97 Hz, H 18a), 4 64 (d, 2H J = 3 37 Hz H 10), 4 78 (d, 1H J = 3 40 Hz H 10), 5 29 (s, 1H, H 12), 5 38 (s, 1H, H-12), 7 39 (m, 2H H 3 H 5) 7 42 (m 1H H 4) 7 64 (m 2H, H 2 , H 6), ^{13}C NMR ($CDCl_3$) δ 12 92 12 97, 20 30, 24 42, 24 66 26 14 27 63, 28 44, 30 74, 30 85, 34 59, 34 64 36 40 36 43 37 35, 37 46 44 33 44 42 52 53 52 58, 64 68, 65 61 71 42 80 95 81 05, 87 81 87 88, 101 85, 102 07 104 03, 104 06 127 93 127 93, 128 11 128 11, 130 48 134 43 169 88, $[\alpha]_D$ +128 3° (*c* 0 15 $CHCl_3$) Anal ($C_{43}H_{63}O_{12}N$) C H N

Compound 13 pale yellow semisolid IR ν_{max} ($CHCl_3$) 3425 (sharp), 3250 (broad) 1650, 1605, 1590, 1495 1450 1305 1240, 1095 870 and 820 cm^{-1} MS *m/e* (relative intensity) 431 (M^+ − 46, 1), ^{1}H NMR ($CDCl_3$) δ 0 90 (d 3H J = 7 32 Hz H 16), 0 90 (m 1H H-7α), 0 93 (d, 3H J = 6 19 Hz, H 15) 1 25 (m 1H H 5a), 1 32 (m, 1H H 6) 1 43 (s, 3H, H-14), 1 50 (m 1H H-8a) 1 51 (m, 1H H-5β) 1 61 (dq 1H J = 13 20 3 11 Hz H 7β) 1 75 (m, 2H H-8), 1 88 (m, 1H H 5α), 1 99 (m, 2H H-19) 2 05 (m 1H H-4β) 2 37 (dd 1H J = 14 03 13 97 3 89 Hz, H-4α) 2 63 (ddq 1H J = 3 41 4 12 7 32 Hz H-9), 3 55 (dt 1H J = 10 05 5 86 Hz H 18b) 3 99 (dt 1H J = 10 05, 6 20 Hz H 18a), 4 14 (m 2H H 20) 4 80 (d 1H, J = 3 41 Hz, H-10), 5 45 (s 1H H 12) 6 83 (m 1H H 5) 7 00 (m 1H, H 3) 7 40 (m, 1H H 6) 7 41 (m, 1H H 4) 9 36 (s 1H, NH disappeared with D_2O) 11 58 (s 1H OH, disappeared with D_2O) ^{13}C NMR ($CDCl_3$) δ 12 96, 20 27, 24 51, 24 66 26 14 28 54, 30 90, 34 59, 36 44, 37 46, 44 40 52 55, 65 09 74 25, 81 11, 87 97, 102 21 104 23 112 33 118 68, 118 85 125 37 134 66, 161 23, 169 10 $[\alpha]_D$ +96 3° (*c* 0 58, $CHCl_3$) Anal ($C_{25}H_{35}O_8N$) C, H, N

Preparation of Artemisinin−Caffeate Derivative 14 Compound **5** (100 mg, 0 3 mmol) in dry benzene (5 mL) was added to caffeyl chloride at 10 °C, and the mixture was left stirring at 50 °C for 7 h The crude product was purified by TLC (silica gel, using 50% ethyl acetate in hexane as eluent) to obtain compound **14** (15 5 mg, 0 031 mmol, 10 05% yield)

Compound 14 yellow oil, IR ν_{max} ($CHCl_3$) 3550 (sharp), 3300 (broad), 1700, 1640, 1600, 1510, 1450, 1110, 980 875 and 820 cm^{-1}, ^{1}H NMR ($CDCl_3$) δ 0 3−2 8 (m, 25H), 3 2−4 6 (m 4H 2 × H-18, 2 × H 20) 4 82 (d, 1H, J = 3 5 Hz, H-10) 5 50 (s 1H H 12) 6 30 (d 1H, J = 16 Hz, H 8′), 7 08−7 22 (m, 3H, H 2′,5 ,6′), 7 63 (d, 1H, J = 16 Hz, H 7′) Due to rapid decomposition no mass ion or elemental analysis of this compound was obtained

Preparation of Artemisinin−Diethyl Dithiocarbamate Derivative 15 Sodium diethyl dithiocarbamate (148 mg, 0 87 mmol) in acetonitrile (3 mL) was stirred with artemisinin bromide derivative **3** (350 mg, 0 87 mmol) in acetonitrile (3 mL) at room temperature for 1 h The white precipitate of sodium bromide that formed in the reaction was filtered out The filtrate was evaporated and diluted with ethyl acetate washed with water followed by saturated aqueous sodium chloride solution dried over $MgSO_4$ filtered, and evaporated to dryness The crude product was purified by TLC (silica) using 30% ethyl acetate in hexane as eluent to obtain pure **15** as yellow oil (332 mg, 0 7 mmol 80 68% yield)

Compound 15 yellow viscous oil, IR ν_{max} (neat) 1500, 1260, 1200, 1100, 880, 820, and 780 cm^{-1}, MS *m/e* (relative intensity) 473 (M^+ 1), 440 (3), 428 (0 2), 324 (6), 267 (3), 266 (2), 249 (2) 237 (6) 220 (17), 207 (14) 190 (76), 162 (100), 149 (66), 135 (15) 116 (92), 100 (15), 88 (51), 72 (30), 60 (36), 55 (23) 44 (74) 43 (59), ^{1}H NMR ($CDCl_3$) δ 0 7−3 0 (m, 29H), 3 2−4 4 (m 8H 2 × H 18 2 × H-20 2 × H 24 2 × H 26), 4 77 (d, 1H J = 3 5 Hz H 10) 5 43 (s 1H H 12) Anal ($C_{23}H_{39}O_5NS_2$) C, H N calcd 2 96 found 3 69

Biology

Antimalarial Activity of the Novel Artemisinin Derivatives and Iron Chelators The antimalarial activity of iron chelators and artemisinin novel derivatives against *P falciparum* infected red cells was measured by using a modification of the [^{3}H]hypoxanthine incorporation method reported by Desjardins [11] Briefly, the drug was dissolved in dimethyl sulfoxide (DMSO) and diluted with the culture medium to the required concentration The final concentration of DMSO was less than 0 1% usually 0 001% which had no effect on the parasite growth A mixture of 25 μL of the medium containing the drug and 200 μL of 1 5% cell suspension with 1−2% parasitemia at immature schizont stage was cultured in triplicates for 24 h after which 25 μL of 0 5 μCi [^{3}H] hypoxanthine was added After an additional 18 h in culture the cells were harvested onto glass-fiber filters The radioactivity was measured by a Beckman liquid scintillation counter mode LS 1801 The IC_{50} the concentrations required for 50% reduction of the radioactivity as compared to control without the drug of the drug against these infected cells were obtained from dose−response curves

In testing for effect of iron on the antimalarial activity of the novel artemisinin derivatives a sterilized solution of freshly prepared 2 mM $FeCl_3$ in double distilled water was mixed with the parasitized cell suspension The cell mixture was immediately used for testing the antimalarial activity of the prepared compounds as mentioned above The final concentration of $FeCl_3$ in the test system was 20 μM

Acknowledgment This work received financial support from the US AID/CDR (Grant TA-U01-C09-060) and the NIH/ICIDR (Grant 1-U01-AI35827-01) S.P received a scholarship from China Medical Board

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Differential Cytotoxicity of Iron Chelators on Malaria-Infected Cells Versus Mammalian Cells

By Hava Glickstein William Breuer Mark Loyevsky, Abraham M Konijn Jacqueline Libman Abraham Shanzer and Z. Ioav Cabantchik

Iron chelators of the hydroxamate class arrest in vitro proliferation of malaria parasites and of mammalian cells. The factors determining the biological activity of the chelators have classically been attributed to the chelators' capacity for binding iron and to their ability to traverse membranes as free chelators and as chelator-iron complexes. We show in this work that the nature of the chelatable pool of cell iron also contributes to the susceptibility of cells to iron chelators. A class of N-terminal (N_t) derivatives of desferrioxamine (DFO), (N_t-DFO) is shown here to differentially affect growth and replication of intraerythrocytic parasites (*Plasmodium falciparum*) Methyl-anthranilic DFO (MADFO), the relatively less hydrophilic member of the N_t-DFOs series reduced parasite proliferation (48 hour test) with an IC_{50} of 4 ± 1 μmol/L and mammalian cell (K562 and HepG2) proliferation with an $IC_{50} > 100$ μmol/L. On the other hand the more hydrophilic N_t free DFO displayed IC_{50} values of 21 ± 5 μmol/L for parasites and 7 ± 1 μmol/L for mammalian cells. The selective antiparasitic activity of MA-DFO as reflected in the speed of action and IC_{50} values on cell proliferation is attributed primarily to membrane permeation and iron(III) binding properties of the drug In contrast, the relatively low antiproliferative activity of the more permeant MA-DFO on mammalian cells resulted from MA-DFO's reduced capacity for scavenging intracellular iron This is apparent from MA-DFO reduced effects on (1) the chelatable iron(II) pool that is associated with the cell cytosol (2) the cell chelator-extractable iron, and (3) cell ferritin levels. The potent antimalarial efficacy and biological selectivity of MA-DFO relative to the parent DFO, is of importance for improved design of chemotherapeutic agents.
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DESFERRIOXAMINE (DFO) the hydroxamate-based chelator of iron, is an agent with a proven therapeutic record in iron overload diseases[1,2] and with demonstrable antimalarial activity in vitro,[3] in animal models,[4,5] and in human clinical trials.[6-9] The basis for its relatively high therapeutic index resides, in part, in the fact that animal cells can tolerate doses of chelator treatments that are demonstrably effective in reducing iron-load and can even cause transient cell metal deprivation.[1] Indeed, in vitro studies indicated that such DFO treatments lead to a transient arrest in cell proliferation primarily via blockage of obligatory deoxyribonucleotide synthesis [10] The cytostatic blockage has been attributed to the scavenging of intracellular iron,[1] which is presumed to be composed of low molecular weight iron(II)- or iron(III)-complexes[11,12] (ie, chelatable iron pool) On chelator removal iron is restored and nucleic acid synthesis is swiftly resumed. However, in the process of iron(II) oxidation/complexation by DFO there is also a risk of oxidative radical formation[13] that can also cause DNA breaks and induce apoptosis [14] These adverse properties of DFO, might contribute to some of the toxic effects of the drug as previously reported.[1]

In malaria infected cells, the major biological effect of DFO is cytotoxic rather than cytostatic.[15,16] Thus, unlike mammalian cells that largely recover on DFO removal, parasites can neither replenish iron stores and resume metabolic activities, nor, apparently, can they discard and/or neutralize the chelator-metal complexes [16-18] These properties are associated with the restricted mode of iron acquisition by parasites from parasite-mediated degradation of host hemoglobin[19] and with DFO's differential permeation to parasitized red blood cells (RBCs) [20] Ring stages, being essentially impermeant to DFO, are relatively insensitive to the drug, whereas trophozoite-schizonts, being markedly more permeable, are highly susceptible to it. These properties dictate the chemotherapeutic regimens, which comprise prolonged treatments[1] and expose the host to potentially deleterious drug effects

With the aim of understanding the selective nature of DFO effects on malaria parasites as compared to mammalian cells we assessed several N-terminal derivatized DFOs (X-N_t-DFOs) in terms of their effects on cellular iron stores and antiproliferative action. Preliminary studies indicated that N_t-derivatization of DFO did not impair its iron(III) binding properties [20,21] The Nt-lipophilic substitution of DFO conferred to the chelator improved permeation capacity into infected cells and markedly higher antimalarial potency However the improved antimalarial efficacy of N_t-DFOs was also accompanied by a major reduction in the adverse action of DFOs on mammalian cells Therefore, we have assessed whether the differential action of N_t-DFOs might be associated with an apparently lower capacity of N_t derivatized-DFOs particularly methyl anthranilic (MA)-DFO for affecting the cytosolic labile (or chelatable) iron pools of mammalian cells Such differential performance of N_t-DFO derivatives on parasites vis a vis mammalian cells may provide new guides for improving the antimalarial drug perfor-

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mance of iron chelators Moreover this class of agents can also be of use as biochemical or pharmacological tools for assessing the mechanisms of metal acquisition and handling by cells

MATERIALS AND METHODS

Materials

DFO and DFO-E were kindly provided by Dr H. Schnebli (Ciba-Geigy Basel Switzerland) and were used for X, N-terminal derivatization with either anthranalic (A-) or MA groups to yield A-DFO and MA-DFO respectively [20] Nitrilo-DFO (N-DFO) was kindly provided by Dr G Gasparini, Ciba-Geigy Basel) [^{3}H]hypoxanthine, [^{3}H]isoleu and [^{3}H]-thymidine were from the Radiochemical Centre (Amersham, UK) Unless specified otherwise, all other chemicals were from Sigma Chemical Co (St Louis MO) or best available grade

Parasite Cultures

Plasmodium falciparum, strain FCR-3 used for all experiments was maintained in Nunclon culture flasks (Nunc, Roskilde, Denmark) in human A$^+$ or O$^+$ RBCs in RPMI 1640 medium (Biological Industries Beth Haemek, Israel) supplemented with 25 mmol/L HEPES 10 mmol/L glucose and 20 mmol/L $NaHCO_3$ and 10% (vol/vol) heat inactivated A$^+$ human plasma pooled from different blood donors The cultivation method used was a modified version of Trager and Jensen's[22] as described elsewhere.[15] Parasitemia and growth stage distribution were determined on methanol fixed and Giemsa stained smears Parasite growth synchronization and assessment of antimalarial activity of iron chelators were done as described previously [15] The compounds were added from concentrated stock solutions in dimethyl sulfoxide (DMSO) to microcultures (24 wells Costar Cambridge MA) containing infected RBCs (2.5% hematocrit and 2 to 5% parasitemia) The cultures were synchronized to the ring stage by incubation in 300 mmol/L alanine and 10 mmol/L Tris-HCl pH 7 4[15] or by Percoll gradient centrifugation.[23] After 18 to 24 hours exposure to the indicated drug the cells were either supplemented directly with 6 μCi/mL of [^{3}H]hypoxanthine or washed 3 times with large volume of RPMI 1640 medium and replenished with fresh growth medium before addition of the radiolabel incubation for an additional 18 to 24 hours and lysis by freezing The freeze thawed lysate of labeled cells was passed through glass fiber filters (Tamar Inc, Jerusalem, Israel) Incorporation of label into nucleic acid was measured in a Beckman Scintillation Counter (Beckman Corp Fullerton, CA) All systems were run in hexatuplicates The 50% inhibitory concentrations (IC_{50}) were determined by nonlinear least square fit to sigmoidal functions as described elsewhere.[24] Statistical *t*-tests were performed according to the statistical package PCS (Pharmacologic Calculation System, MicroComputer Specialists, New York) based on the method of Tallarida and Murray [24a] The software program Microcal Origin (MicroCal Software Inc, Northampton, MA) was used for curve simulation and for graphic display

Mammalian Cell Cultures

Mammalian cells were maintained in Nunclon culture flasks (Nunc) and grown at 37°C in a CO_2-incubator (5% CO_2) in 24-well plates (Nunc) as follows human K562 erythroleukemia cells as suspensions in α-Modified eagle s Medium (αMEM) human hepatoma cells (HepG2) as monolayers in Earle s MEM (EMEM) and chinese hamster ovary cells (CHO) as monolayers in Dulbecco s MEM (DMEM) Cells in growing phase were exposed to iron chelators for 24 to 48 hours and assayed in duplicates for macromolecular synthesis (5-hour incorporation) of either [^{3}H]-hypoxanthine or [^{3}H]-thymidine (5 μCi/mL) into nucleic acids, respectively K562 cells were processed with a Matrix cell harvester (Packard Instruments Meriden CT) Radiolabeled HepG2 and CHO cells were washed with isotonic buffer fixed on the plates with ice-cold 5% trichloroacetic acid (TCA) and TCA precipitable materials were solubilized with 0 1N KOH and counted for radioactivity

Total cell protein was estimated on 96 well plates either with the BCA method (Pierce Chemical Co, Rockford, IL) or the sulforhodamanine method as described elsewhere [-5] The microplates were read in an enzyme linked immunosorbent assay (ELISA) Microplate EL 309 autoreader (Bio-Tek Instruments Inc Winooski, VT) at the appropriate wavelengths.

Ferritin Assay

Cells (K562 or HepG2) grown in 12-well plates and treated with chelators for 24 hours, were washed with isotonic buffer and subsequently extracted with 300 μL Tris-HCl 10 mmol/L, NaCl 150 mmol/L, Triton X-100 (Sigma) (0.5%) NaN_3 (0.2%), pH 7 4 containing a cocktail of antiproteases as specified elsewhere [26] The extract was centrifuged at 2,000*g* × 10 minutes at 5°C and kept at 5°C. Aliquots were used for determinations of protein (BCA method, Pierce Chemical Co) and of ferritin by fluorogenic ELISA, using rabbit antihuman spleen ferritin antisera, and purified antiferritin antibodies conjugated to β-galactosidase.[27] Ferritin was also checked on immunoblots of samples run on Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10 to 15% acrylamide) gels[28] using rabbit antihuman placental ferritin antibodies [27] goat antirabbit γ globulin conjugated to horse radish peroxidase (Jackson Immunoresearch, West Grove, PA) in conjunction with enhanced chemiluminescence (ECL) detection (Radiochemical Center Amersham, UK) [26] No attempts were made to discriminate between ferritin H and L subunits

Iron Extraction Capacity of DFO Derivatives

K562 cells were preloaded for 1 hour with ^{55}Fe-diferric Transferrin (6 25 × 10^6 dpm/nmole Fe 0 05 μg/mL transferrin) extensively washed with complete growth medium and buffered saline solutions and incubated for 24 hours with the respective chelators in 24 well plates. The suspensions were separated into cell supernates (S1) and pellets by centrifugation. The pellets were extensively washed with full growth medium and buffered saline solutions, treated with 0.2% Triton X-100 (in 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7 4) supplemented with an antiprotease mixture.[26] An aliquot of the detergent-dispersed cells was taken for protein determination (BCA method, Pierce Chemical Co) and the remainder was centrifuged at 15 000*g* × 5 minutes. Radioactivity was counted in both cell supernate (S1) and soluble (ie, supernate) fraction of detergent extract (supernate TS2) after decoloration with H_2O_2 (5%)

Chelation of Intracellular Iron Assessment by a Fluorescent Method

Human erythroleukemia K562 cells were treated overnight with chelators in full growth medium, washed extensively and loaded with 0 125 μmol/L calcein-acetoxymethyl ester (calcein-AM) for 5 minutes at 37°C in HEPES-buffered, bicarbonate-free α-MEM medium containing 20 mmol/L HEPES pH 7.3 (α-MEM-HEP) containing 1 mg/mL bovine serum albumin, washed from excess calcein-AM and resuspended in α MEM-HEP medium.[29,30] Just before measurements 1 mL of calcein loaded cell suspension (~5 × 10^6 cells) was centrifuged in a microcentrifuge and the cells were resuspended in 2 mL of prewarmed 150 mmol/L NaCl 10 mmol/L

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HEPES-Tris pH 7 3 (HBS buffer) The cell suspension was transferred to a stirred, thermostated (37°C) cuvette and fluorescence measurements were initiated. Fluorescence measurements (Exc 488 nm, Em. 517 nm) were done in a PTI Spectrometry Station (Oscar configuration) (PhotoMed, Wedel Germany), equipped with a temperature controlled cuvette holder and magnetic stirrer The magnitude of the baseline fluorescent signal remained relatively stable throughout the measurements the estimated leakage of the probe was lower than 10% per hour at 37°C A fluorescence-quenching anticalcein antibody (10 μL/cuvette) was added to eliminate all extracellular fluorescence, which amounted to ~2% of the total fluorescence The baseline signal under this condition remained stable for ≥20 minutes, indicating minimal leakage of the probe during the experimental period. At the indicated time salicylaldehyde isonicotinoyl hydrazone (SIH) was added (100 μmol/L final concentration) to obtain the maximal level of chelatable iron, as shown elsewhere [30,31]

RESULTS

The repertoire of DFOs used in this study comprises the hydrophilic DFO and its nitrilo derivative N-DFO and the less hydrophilic methylanthranilic derivative MA-DFO and the cyclic DFO-E (Fig 1) Although these agents differ primarily in the nature of the substituent group at the terminal amino group they display comparable binding affinities for iron(III) (pKa 29-31) (measured as in ref 20)

Effect of DFOs on Macromolecule Synthesis in Parasitized Cells

P falciparum infected cells were exposed to various concentrations of DFOs starting at mid-rings After 24 hours

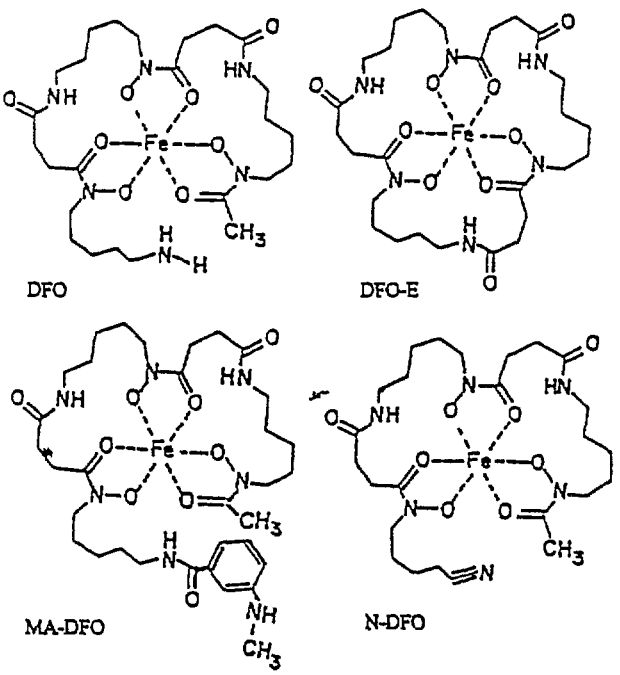

Fig 1 Structure of DFO derivatives N-derivatized DFOs used in the present study DFO N-DFO cyclic DFO (DFO-E) and N-methylanthranilic DFO (MA)

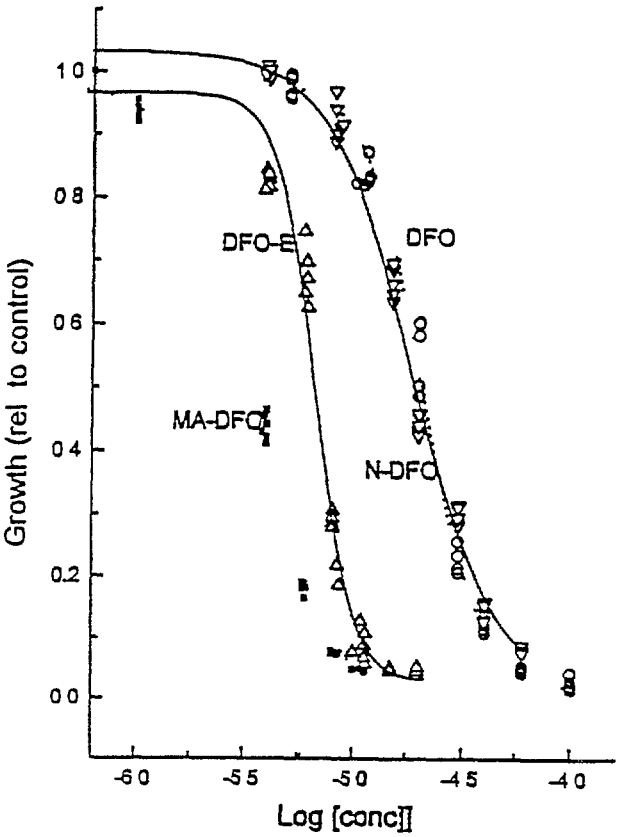

Fig 2. Dose-response curves of DFOs' effects on nucleic acid synthesis of *P falciparum* infected erythrocytes: Cultures of early rings were exposed to the indicated concentrations of DFOs and supplemented 20 hours later with [^{3}H]-hypoxanthine The incorporation of ^{3}H label into nucleic acid was assessed 20 hours later and is given as a percent relative to control. The 50% inhibitory concentration IC_{50} (in μmol/L) was obtained from nonlinear square analysis of sigmoidal curves. MA DFO 4 ± 1 DFO-E 7 ± 2, DFO 21 ± 4 and N-DFO 17 ± 3

treatment, they were assessed for nucleic acid synthesis in terms of [^{3}H]hypoxanthine incorporation into nucleic acids The dose-response curves for the various DFOs are given in Fig 2 Two groups of agents can be identified in terms of their growth inhibitory profiles The first is composed of the N-derivatized and relatively less hydrophilic MA-DFO and DFO-E, which gave IC_{50} values of 4 ± 1 and 7 ± 2 μmol/L, respectively The second is composed of the more hydrophilic DFO and N-DFO which gave IC_{50} values of 21 ± 5 μmol/L and 17 ± 4 μmol/L, respectively A fifth DFO derivative, anthranilic DFO (A-DFO) (not shown), performed comparably to MA-DFO (IC_{50} = 6 ± 2 μmol/L) None of the structural congeners of DFO had any detectable effect on parasite cultures when administered as iron(III) complexes (not shown) Having previously shown that iron(III)-DFO complexes and even more so iron(III)-MA-DFO complexes were demonstrably permeant to malaria infected cells [20] it can be implied that DFO effects on malaria are primarily caused by iron deprivation.

Effect of DFO Derivatives on Mammalian Cells

Nucleic acid and protein synthesis The two human cell lines K562 and HepG2 (exponential growth phase) were

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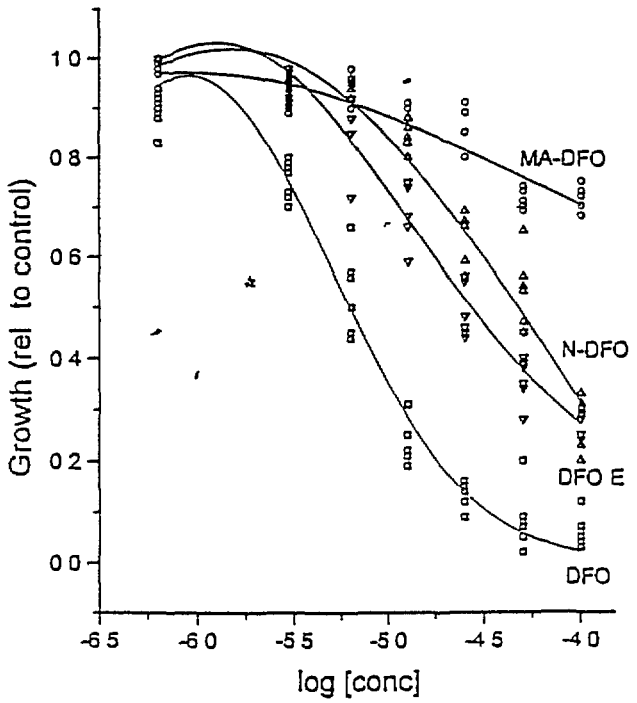

Fig 3 Dose-response curves of DFOs effects on nucleic acid synthesis of mammalian cell cultures: K562 cells were exposed to the indicated concentrations of drug for 48 hours in culture conditions and subsequently supplemented with [^{3}H] hypoxanthine incubated for 5 hours and assessed for incorporation of radiolabel into nucleic acids Data of a representative experiment are given as nucleic acid synthesis relative to control (no chelator) Data pooled from 6 independent experiments and analyzed as in Fig 2 gave the following IC_{50} values (in μmol/L) MA-DFO > 100 μmol/L, DFO E 7 ± 2 DFO 9 ± 2, and N DFO 17 ± 3

exposed for 43 hours to the various DFOs and pulsed for 5 additional hours with the [^{3}H]-labeled precursors hypoxanthine or thymidine Figure 3 depicts the dose-response curves of the various agents on nucleic acid synthesis and Table 1 summarizes the calculated IC_{50} values for chelator effects on DNA protein, and nucleic acid synthesis Although quantitatively different, the rank order of inhibitory potencies was essentially similar for the three parameters DFO was considerably more potent than all its derivatives on either mammalian cell line whereas MA-DFO was the least potent of the four The relative potencies of the two other DFO derivatives were intermediate to those of DFO and MA-DFO We have found the IC_{50} values for drug effects on [^{3}H]-thymidine incorporation into DNA particularly variable for HepG2 cells Factors contributing to that phenomenon were found to be associated with the state of the culture such as culture passage number phase of cell growth and the time of exposure to drug Most of the variability could be minimized by extending the drug exposure to 48 hours As shown above for antimalarial activity, none of the structural congeners of DFO had any detectable effect on mammalian cells proliferation when administered as iron(III) complexes (not shown)

Because the various DFOs differ in the lipophilic nature of the N-terminal group and therefore in their putative permeation properties, we assessed whether any correlation existed between these drug properties and the onset of growth inhibition, ie, the drugs' speed of action (Fig 4) For that purpose we followed the kinetics of nucleic acid synthesis in the presence of drugs' in both K562 and HepG2 cells Cells exposed to a single concentration of 100 μmol/L DFO derivative were time monitored for [^{3}H]-hypoxanthine incorporation into nucleic acid (Fig 4) The onset of inhibition for all four DFO derivatives was between 4 to 6 hours suggesting that permeation into cells per se did not apparently limit the rate of chelator action This was found to be the case for the least potent of the drugs tested, MA-DFO This fluorescent derivative[30] accumulated in the cells within 4 to 6 hours as visualized by fluorescence microscopy image analysis (not shown)

Cytosolic-chelatable iron (i) and ferritin levels (ii) The effect of DFOs on mammalian cells was also assessed in terms of cytosolic chelatable iron and cell ferritin levels Both cell parameters provide a measure for the iron status in the cytosol The first gives a direct measure of the chelatable metal[29,30] and the second indicates how changes in cytosolic iron affected ferritin levels

(1) The cytosolic chelatable iron pool that might be affected by the various DFOs was assessed with the recently developed fluorescence-quenching method based on the metal sensor calcein [29,30] K562 cells treated with DFOs were loaded with the fluorescent calcein The initial level of fluorescence attained represents the amount of free calcein loaded into cells The increase in fluorescence resulting from addition of the highly permeant chelator SIH provides an in situ measure for the chelatable iron pool in the cell A typical experiment is depicted in Fig 5 Control cells showed a 0.25 μmol/L level of intracellular chelatable iron as shown previously [30] After pretreatment with chelators, the basal level of cell fluorescence increased relative to control as relatively less chelatable iron is present in cells Consequently the amount of fluorescence restored by SIH decreased. For all concentrations used (20 to 100 μmol/L) DFO was considerably more potent than MA-DFO The cell chelatable iron was reduced from 0 25 μmol/L to 0 013 to

Table 1 IC_{50} Constants of Chelator Effects on HepG2 and K562 Cells

	HepG2 Cells			K562 Cells	
Chelator	T	H	P	T	H
DFO	7 ± 1	13 ± 3	10 ± 2	21 ± 5	13 ± 3
MA DFO	>100	>100	>100	>100	>100
N DFO	45 ± 4	39 ± 3	35 ± 6	29 ± 4	33 ± 5
DFO-E	26 ± 2	19 ± 4	23 ± 5	22 ± 5	27 ± 5

Cells were exposed to various concentrations of chelators (3 to 100 μmol/L) for 48 hours and analyzed for DNA synthesis by [^{3}H]thymidine (T) incorporation nucleic acid synthesis by [^{3}H]hypoxanthine (H) incorporation and total protein (P) by the sulforhodamine method as described in the text. Data were analyzed as described in Fig 2 the IC_{50} values (50% inhibitory concentrations) (± SEM n = 7) given in μmol/L

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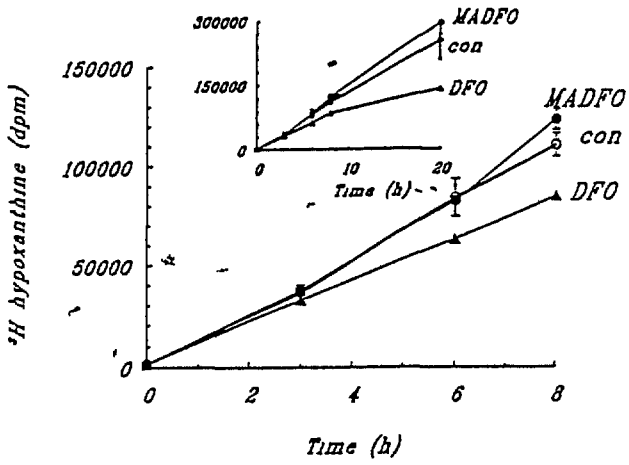

Fig 4 Time-dependence of DFOs action on nucleic acid synthesis in mammalian cells K562 cells were exposed to a single concentration of drug supplemented with [^{3}H]-hypoxanthine and processed at the indicated times Incorporation of radiolabel into nucleic acids is given as a function of time of incubation.

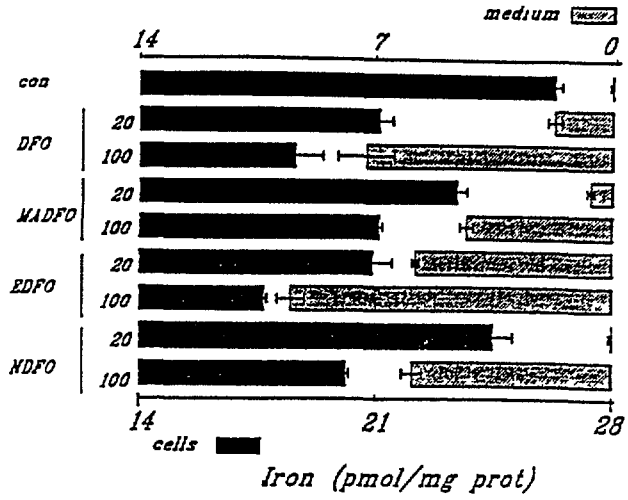

Fig 6. Intracellular iron scavenging by DFOs as assessed with ^{55}Iron K562 cells were loaded for 1 hour with ^{55}Iron-human transferrin washed extensively with growth medium and then incubated for 24 hours in growth medium alone (control) or in medium supplemented with DFOs at either 20 or 100 μmol/L. Radioactivity was measured in the medium and in cells following extensive washing Data are given in terms of pmol-iron (using the ^{55}Iron-transferrin specific activity) per mg cell protein In the medium (top scale) and in cells (bottom scale)

0 04 μmol/L (85% to 95% reduction) after treatment with 20 μmol/L DFO and to undetectable levels (→100% reduction) by treatment with 100 μmol/L DFO However by equivalent treatments with MA-DFO the chelatable iron pool of cells was considerably less affected 42% and 74% reduction with 20 and 100 μmol/L, respectively This clearly indicates that the relatively more permeant MA-DFO, was less efficient in intracellular chelation of iron than the parent, more hydrophilic DFO Measurements of chelator extractable iron were also assessed in cells loaded with ^{55}Fe-transferrin and analyzed for radioactivity in cells and medium (Fig 6) Despite the relatively higher partition coefficient and permeation capacity of MA-DFO versus DFO,[20] the latter was considerably more efficient than MA-DFO in extracting iron from cells This was particularly reflected in the parameters obtained following a 24 hour treatment with 20 μmol/L chelator the iron appearing in the medium was 1 7 ± 2 and 0 5 pmol/mg protein after DFO and MA-DFO respectively The iron retained in the cells after 24 hour treatment was 26 ± 1 21 ± 1 and 23 ± 1 pmol/mg protein, in control DFO and MA-DFO treated cells, respectively

(2) A similar pattern of chelator activities was observed with K562 and HepG2 cells analyzed for ferritin levels after a 24 hour incubation in the presence of chelators (Table 2)

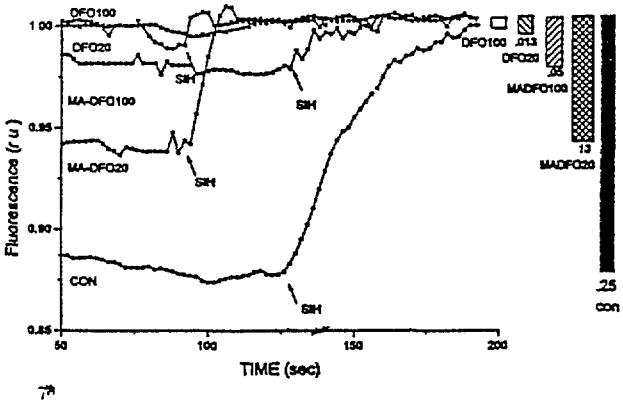

Fig 5 Intracellular iron scavenging by DFO and MA-DFO as assessed by fluorescence K562 cells were incubated for 24 hours in growth medium alone (control) or in medium supplemented with either DFO or MA-DFO (20 or 100 μmol/L) After washing they were incubated in buffered saline medium with 0.25 μmol/L calcein-AM washed twice and suspended in 2 mL of the HBS-Hepes-buffered saline solution placed in a thermostated cuvette (37°C) cuvette under constant stirring Anticalcein antibodies were added to eliminate any extracellular fluorescence[29,30] and SIH (100 μmol/L, final) to recover full fluorescence by iron removal from cells. Traces of fluorescence are given in terms of normalized levels 1 00 indicating maximal fluorescence attained after addition of SIH Bars on the right indicate the μmol/L values of SIH-chelated cell iron

Table 2. Ferritin Levels in HepG2 and K562 Cells Treated With Chelators

	HepG2 Cells	K562 Cells
Treatment	% (Relative to Control)	% (Relative to Control)
DFO 100	25 ± 5 (17)	10 ± 2 (5)
DFO 20	33 ± 4 (33)	23 ± 4 (25)
MA-DFO 100	73 ± 7 (58)	40 ± 5 (25)
MA-DFO 20	91 ± 4 (89)	85 ± 6 (95)
CHX 5	70 ± 7 (75)	65 ± 8 (25)
FAC 10	>500 (>300)	>190 (250)

Cells were treated for 24 hours and analyzed for cytosolic ferritin by ELISA and by immunoblotting (data in parenthesis were based on densitometry tracings based on Fig 7) Data are given as percent of control HepG2 100% = 42 ng ferritin/mg protein and K562 100% = 200 ng/mg protein)

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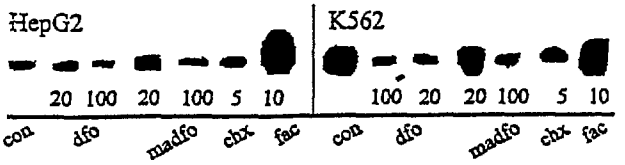

Fig 7 DFO effects on ferritin levels in mammalian K562 cells: Cells grown for 24 hours in growth medium alone (control) or in medium supplemented with either DFO (20 or 100 μmol/L) MA DFO (20 or 100 μmol/L) FAC (10 μmol/L), or cycloheximide (10 μmol/L) were washed with buffered saline and their cytosol extracted and analyzed for ferritin levels by SDS-PAGE immunoblotting Each slot on the gel was loaded with an aliquot derived from a culture well which contained the same number of cells at the beginning of the treatment. The blots were scanned in a Umax scanner (Umax Data Systems Inc Industrial Park, Hsiuchn, Taiwan) and densitographed with the aid of the ImagePro program (Media Cybernetics Silver Spring MD) Data of ferritin levels normalized per milligram cell protein (BCA method) are given in Table 2 (details in Materials and Methods)

For the ELISA method, we used rabbit antihuman spleen ferritin antibodies and rabbit antihuman placental ferritin antibodies For SDS-PAGE (Western) immunoblotting, we used rabbit antihuman spleen ferritin antibodies (Fig 7A and B respectively) The picture emerging from both assays was essentially equivalent. Supplementation of iron(III) to the growth medium in the form of ferric ammonium citrate (FAC) induced a major increase in cytosolic ferritin levels DFO markedly reduced cytosolic ferritin even at 20 μmol/L concentrations whereas the less hydrophilic MA-DFO was considerably less effective The other derivatives showed a pattern of effects similar to that obtained for iron extraction These experiments indicate that in mammalian cells the iron extraction capacity of the various DFOs is dictated not only by the partition coefficients, but also by their ability to form stable complexes with the chelatable iron form that prevails in cells

DISCUSSION

Malaria parasites are among the most susceptible organisms to the action of iron chelators[9,18,31,32] The mechanisms by which those chelators curb the proliferative capacity of parasites have recently been reviewed for hydroxamate-based chelators [16-18] They include primarily properties associated with the chelators, such as iron binding affinity and membrane partition coefficients of the free chelator and its iron complexes, which provide a measure of their membrane permeation properties [32] These properties can be assumed to be similar in parasite infected RBCs and mammalian cells as previously shown [32] However the mode of action of the chelators on the different cell systems is associated with additional properties These include (1) the mechanisms of iron mobilization by the different cells, (2) the dependence of iron mobilization on the developmental stage of the cell or organism and (3) the nature of the chelatable form of bio-available iron that prevails in parasites as compared to mammalian cells [16] Parasites apparently differ from mammalian cells in all 3 properties, thus making them unique targets for iron chelating agents The reversed siderophores used previously,[15,33] and the DFOs used in this study, were found to irreversibly affect *P falciparum* growth in vitro, whereas all mammalian cells tested (K562, human hepatoma HepG2, human colon carcinoma HT-29, and CHO cells), fully recovered from 24 to 48 hour treatment with up to 100 μmol/L chelators (not shown) We attributed these findings to the differential capacity of mammalian cells for restoring intracellular bio-available iron on drug removal[16,17,34,35] and for removing the accumulated drug either in the free form or as drug-iron complexes Because none of the drugs used had any effect on either parasite or mammalian cell proliferation when applied as iron complexes, we assume that their antiproliferative mode of action is most likely associated with their capacity to deprive cells of metabolic iron However, at present, we cannot reject the possibility of in situ formation of toxic iron-chelator complexes, particularly in the unique parasite environment.[17,34,35]

In this work we showed that differential effects of iron chelators on parasites versus mammalian cells can be more profound than previously described with RSFs [16,17] The effects were found with a series of N_t-derivatives of the DFO family (Figs 2 and 3 and Table 1) First, their biological efficacy as suppressers of cell growth correlated with the putative permeation properties of the DFOs in parasites but not in mammalian cells Second, the relatively low susceptibility of mammalian cells to MA-DFO (Fig 3 and Table 2) the most potent antimalarial N_t-DFO tested was apparently associated with the chemical nature of the iron present in the chelatable pool of the mammalian cell (Figs 5 and 6) In particular MA-DFO, which has similar iron(III) binding affinity and even higher partition coefficient and membrane permeation capacity than DFO,[20] was less effective than DFO in chelating and extracting intracellular iron from mammalian cells (Figs 5 and 6) and reducing the ferritin stores (Fig 7)

The relatively lower cytotostatic potency of MA-DFO in mammalian cells, which correlates with its relatively lower effects on cell iron stores, could be attributed to several factors Limited drug access into cells was highly unlikely, because MA-DFO was found to be demonstrably permeant to mammalian cells [20] The possible association of N_t-DFO properties with blockage of the terminal $-NH_2$ group or modification of the pK_a of *ca* 10^{36} in DFO to values >5 in MA-DFO or A-DFO[37] should be considered. Such a modification might confer on DFO different accessibility properties, which, in turn, might affect its accumulation in particular cell compartments or its interactions with the chelatable form of intracellular iron. The biological effects of the two other N-blocked or N-modified DFO derivatives provide some support for the previously discussed ideas, but do not necessarily prove them. However, alternative explanations should also be considered, such as specific intracellular modifications of the drugs or different interactions with the form of chelatable iron prevailing in cells, ie, iron(II) Although the affinities of DFOs for iron(II) are relatively small ($<10^5$) compared to those for iron(III) (ca. 10^{30}), in ambient air, even iron(II) might swiftly and avidly bind to DFO following hydroxamate-catalyzed oxidation to iron(III) [38] Thus, the ca-

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pacity of DFOs for forming stable complexes with the iron(II) prevailing in cells,[12,30,39] might also depend on their capacity to induce oxidation to the iron(III) form. This property might vary not only with the flexibility of the chelator backbone and the physicochemical nature of the N_t group, but also with additional factors present in cells Our in vitro studies of the interaction of DFO and MA-DFO with iron(II) bound to the calcein, support only in part, such a notion They indicate that MADFO's capacity for chelating cytosolic iron from mammalian cells, most of which is apparently in the iron(II) form, is significantly lower than that of DFO [40] Although the reasons for the differential behavior of MA-DFO towards mammalian cells as compared to malaria infected cells remain to be fully elucidated, the implications of the results of the present work are highly relevant for future antimalarial drug design

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Antimalarial Action of Hydroxamate-Based Iron Chelators and Potentiation of Desferrioxamine Action by Reversed Siderophores

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Hydroxamate-based chelators of iron are potent inhibitors of in vitro growth of *Plasmodium falciparum* Two types of such chelators, the natural desferrioxamine and the synthetic reversed siderophore RSF_{ileum-}, are prototypes of antimalarial agents whose action spectra differ in the speed of action, stage dependence, and degree of reversibility of effects This work explores the possibility of improving the antimalarial efficacy of these agents by using them in various combinations on in vitro cultures of *P falciparum* Growth assessment was based both on total nucleic acid synthesis and on parasitemia The results indicate that the synthetic reversed siderophore more than complements the antimalarial action of desferrioxamine when applied during either ring, trophozoite, or mixed stages The combined drug effects were significantly higher than the additive effect of the individual drugs Qualitatively similar results were obtained for both reversible effects and irreversible (i e , sustained) effects Following an 8 h window of exposure the combined drug treatment caused parasite growth arrest and prevented its recovery, even 3 days after the treatment The fact that such a combination of iron chelators displays a wider action spectrum than either drug alone has implications for the design of chemotherapy regimens

The antimalarial action of various classes of agents is determined by a variety of factors which are associated with properties of the drug itself the biological system in question and the interplay between the two factors Malaria parasites have a complex life cycle which encompasses continuous progression through developmental changes of a distinct morphological and chemical nature For *Plasmodium falciparum* the most dangerous of the human malaria parasites the changes are also manifested in membrane properties such as permeability and in the appearance of susceptible biochemical targets for drug action (4) The efficacy of a potential antimalarial drug is therefore dictated by its speed of entry into parasitized cells and by the speed selectivity extent and irreversible nature of the chemical damage to putative parasite targets Vis-a-vis all these properties are the parasite s ability to counteract those effects by mechanisms which contribute to drug resistance and the limitations posed by the selectivity of the drug toxicity to the parasite versus that to host (5) For in vivo applications there are other considerations which also need to be taken into account such as the pharmacokinetics and drug tolerance of the organisms

Iron chelators are a class of agents which have proven useful as antimalarial agents in vitro (2, 9, 15 18–23), in rodent models (6), in primate hosts (17), and in humans (reviewed in reference 8) For the hydroxamate type chelators, the biological action has been attributed to chelation of internal iron pools (9, 13, 19) and, in turn, to interference with the supply of iron to different parasite components (27), possibly to ribonucleotide reductase (1, 10, 27) For other types of chelators which bind iron(III) but do not yield hexadentate complexes other modes of action have been suggested including formation of toxic chelator metal complexes (9, 20 21) and induction of oxidant stress (7) In general the speed of antimalarial action of any of the above type of agents the stage specificity of the effects and the growth arrest efficacy are largely a result of their membrane permeation properties and iron(III)-binding capacity (5 12 14) Thus the in vitro antimalarial action of the relatively hydrophilic desferrioxamine B (DFO) is manifested after 8 to 10 h of continuous exposure of *P falciparum* trophozoites to the drug (the 50% inhibitory concentration in a 24-h culture was 30 μM [14]) Less hydrophilic derivatives of DFO such as N terminal-modified DFOs (12) or a series of synthetic reversed siderophores (RSFs) (5 13, 14) showed up to 10-fold higher potency, as reflected in the speed of action and the 50% inhibitory concentration as well as a wider spectrum of activities at all developmental stages (12) However whereas the effects of DFO on trophozoites were relatively slow to develop (12 19, 27) but were largely irreversible in nature (12), those of RSFs were irreversible only on the ring stage but reversible on trophozoites (14)

In a working model for the antimalarial action of siderophores (5, 14) we proposed that the differential efficacies of DFOs and RSFs on the various stages of the life cycle of *P falciparum* (13 14, 27) reflect not only the drugs' permeation properties at the different stages of parasite development but also their retention within parasites after drug removal. Thus despite the fact that either DFO or RSF cause parasite growth arrest as a result of intracellular iron scavenging, for the drugs to be of therapeutic value, two drawbacks would need to be overcome the reversibility of RSF effects on trophozoites and the lack of DFO effects on rings The aim of this work was to assess whether the combined action of DFO and RSF would increase the antimalarial spectrum of activity of both agents

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above their individual effects or even their expected additive action Expanding a parasite s susceptibility to a drug over most of the life span of the parasite should improve the ther apeutic efficacy in terms of speed of action and irreversible mode of inhibition Various aspects of the combined use of drugs (3, 15 16) and their application to antimicrobial (15) and antimalarial (2, 26) therapy have been previously considered The effects of iron chelators in combinations with chloroquine, pyrimethamine and quinine were neither synergistic nor higher than the additive effects (2) Moreover, depending on the range of concentrations used, some iron chelators showed antagonistic rather than synergistic antimalarial action (25)

MATERIALS AND METHODS

Parasites. *P falciparum* (strain FCR 3) was cultured according to the method of Trager and Jensen (24) and its growth was synchronized by sorbitol treatment as described by Lambros and Vanderberg (11) every second day The experiments were initiated within 14 days after collection of blood from normal healthy donors

Iron chelators RSF_{lewis} was obtained as described elsewhere (22) and DFO (DFO B) was purchased from Ciba Geigy (Basel Switzerland)

Drug treatment. The number of parasitized erythrocytes containing either rings or advanced forms (late trophozoites and schizonts) were adjusted to constitute a 5% erythrocyte suspension (5% hematocrit in RPMI 1640 containing 10% plasma) with a 1% level of parasitemia in medium containing the test drug The cells were dispensed into a 96-well microplate and incubated at 37°C by the candle jar method (24) The supernatant was replaced daily with fresh test medium unless otherwise stated The concentrations of the drugs are expressed in micrograms per milliliter.

Parasite development. Parasite development was assessed in terms of nucleic acid synthesis [^{3}H]hypoxanthine (Dupont New England Nuclear) was added to the culture (18.5 kBq per well) for various periods of time the cells were collected by filtration on glass microfiber filters and the radioactivity was counted (Minaxi Tri Carb 4000 Packard) The incorporation of label in uninfected erythrocytes exceeded no more than 3% the value obtained with untreated parasitized erythrocytes Parasitemia was estimated by using Giemsa stained blood smears

Experimental design Various concentrations of DFO and RSF either alone or in combinations were compared for effects on the in vitro development of parasites The parasites were exposed to the drugs for 4 8 24 or 48 h The experiments were performed in triplicate cultures and repeated at least three times. For each individual experiment the standard deviation exceeded no more than 10% of the mean value The variability between results obtained in different experiments was relatively higher than that in individual experiments We attributed the higher degree of variability to variations inherent in the biological system which include plasma and erythrocytes from different donors and experimental factors such as the degree of synchronization of the cultures and the exact stage in the life cycle of the plasmodium at which the drug was administered However despite variations all individual experiments led to the same qualitative conclusions Consequently we opted for depicting a graphically representative set of results for each type of experiment

The degree of additive effects of combinations of drugs A and B (DA) was initially calculated for each combination of drugs as DA = $I(AB)/[I(A)+I(B)]$ where $I(A)$ and $I(B)$ are the percent inhibition caused by the respective single drugs and $I(AB)$ is the inhibition caused when both drugs A and B were present. A value of 1 represents additivity and values of >1 indicate levels higher than the additive (3) The DA values are given in the graphs within the appropriate bars. Statistical comparison between treatments (Student s *t* test) is given in the respective legends. A more rigorous analysis of additivity and independent drug effects was carried out according to the method of Poch (16)

RESULTS

The effects of 48-h exposure of asynchronously grown parasites to DFO and RSF are depicted in Fig 1A. A fraction of cells was washed to remove the drug and incubated for an additional 24 h (Fig 1B) Nucleic acid synthesis was measured starting at either 24 (Fig 1A) or 48 (Fig 1B) h by following [^{3}H]hypoxanthine incorporation for an additional 24-h incubation period The individual drug effects on parasite growth were usually below 20% ± 2% inhibition (mean ± standard error of the mean), and in some cases there was even an apparent enhancement of growth relative to that of the control However, for each drug combination, the combined action

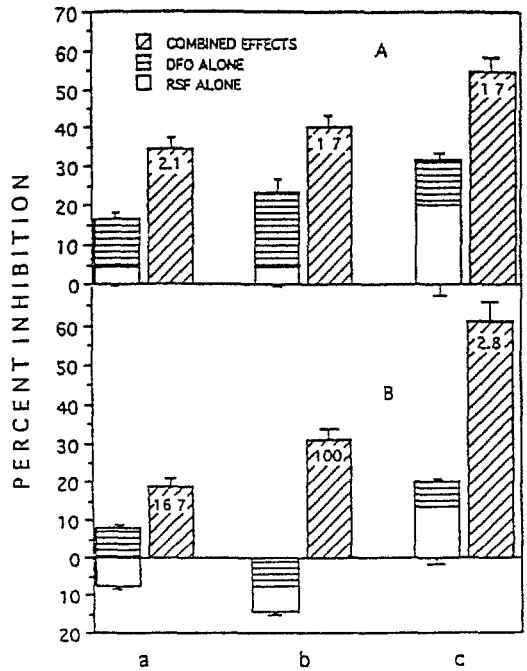

FIG 1 Effects of DFO RSF and their combinations on *P falciparum* cultures (asynchronous) Cultures were exposed for either 48 (A) or 72 (B) h to 16 μg of DFO per ml or 1.5 μg of RSF per ml or their combination (column a) 32 μg of DFO per ml or 1.5 μg of RSF per ml or their combination (column b) or 16 μg of DFO per ml or 3 0 μg of RSF per ml or their combination (column c) The results are expressed as the mean percent inhibition of [^{3}H]hypoxanthine incorporation during the last 24 h of treatment relative to that of controls (untreated cells) Error bars indicate standard errors of the means Statistical comparisons between the combined effects of drugs and the sum of effects of the corresponding individual drugs were significantly different ($P < 0.01$) The numbers inside the columns (DA) indicate the degree of additivity (see Materials and Methods)

of both drugs was significantly higher than the sum of the individual drug effects ($P < 0.01$) For example whereas the sum of the individual inhibitory effects of 16 μg of DFO and 3 μg of RSF was only 22% ± 2% (Fig 1B column c) the combined action was 61% ± 5% This is also reflected in the degree of additivity (DA ≫1) as depicted in the respective bars (Fig 1) A qualitatively similar picture was obtained whether the drugs were removed after a given incubation period

A similar experiment was performed on a synchronic culture of trophozoites exposed to the drugs for a continuous 48-h period in which the last 24 h included assessment of nucleic acid synthesis (Fig 2A) or for a 24-h drug exposure followed by an additional 24-h period of assessment of nucleic acid synthesis in the absence of drugs (Fig 2B) In the range of concentrations used removal of the drugs after 24 h of exposure virtually reversed the effect of RSF and slightly reduced the effects of DFO However, in all systems tested, the combined action of both drugs was markedly greater than that of the additive effect. These experiments, when complemented with determinations of parasitemia, revealed that the removal of the drugs enabled the recovery of parasites treated with RSF (1 5 or 3 0 μg/ml) or DFO (32 μg/ml) but not with 64 μg of DFO per ml or any combinations of RSF with DFO Thus removal of RSF or DFO (32 μg/ml) increased the level of parasitemia from 5 to 6% to 8 to 9% whereas those cultures which were previously treated with the combinations failed to recover and remained at a low level of parasitemia of 3 to 4%

Experiments in which parasitized erythrocytes were treated

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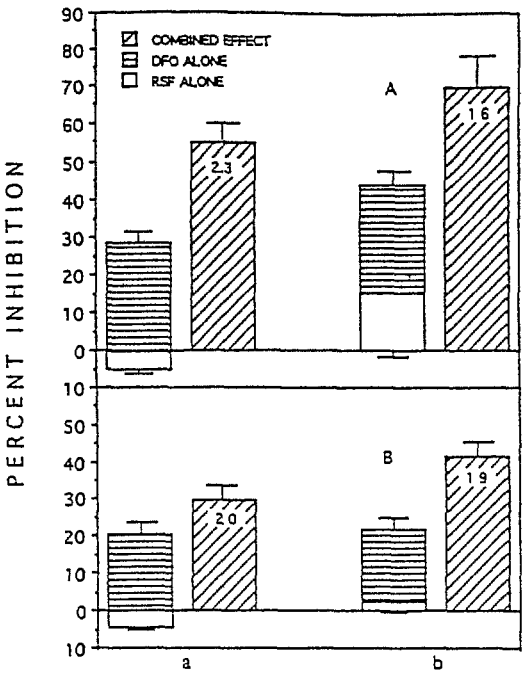

FIG 2 The effects of DFO RSF and their combinations on *P falciparum* cultures (trophozoite stage) Cultures were exposed to the indicated compounds for 24 h subsequently washed, and reincubated in culture medium with (A) or without (B) compounds for an 24 additional h Columns a, 32 μg of DFO per ml or 1.5 μg of RSF per ml or their combination b 32 μg of DFO per ml 3 0 μg of RSF per ml or their combination The results are expressed as the mean percent inhibition of [^{3}H]hypoxanthine incorporation during the last 24 h in culture relative to that of the control (untreated cells) Error bars indicate standard errors of the means Statistical comparisons between the combined effects of drugs and the sum of effects of the corresponding individual drugs were significantly different ($P < 0.01$ [A and B column b] and $P < 0.05$ [B column a]) The numbers inside the columns (DA) indicate the degree of additivity (see Materials and Methods)

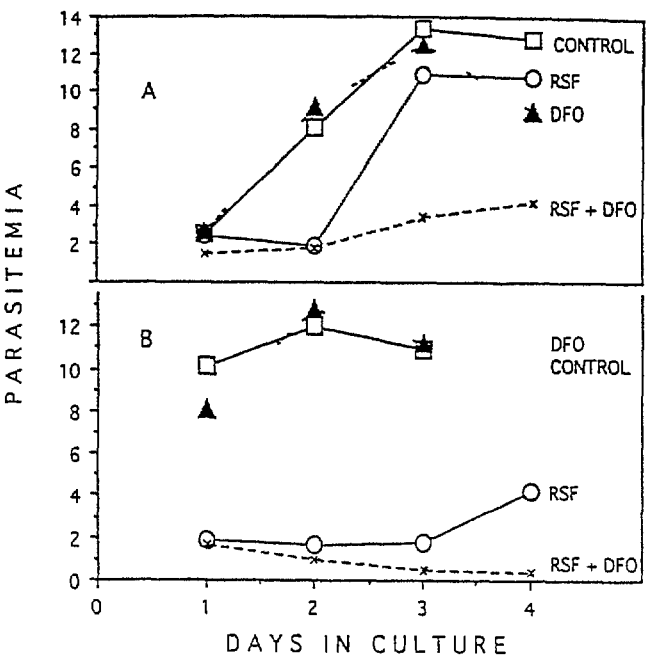

FIG 3 Parasitemia of *P falciparum* treated with either DFO (16 μg/ml) RSF (12 μg/ml) or both. Cultures of rings (A) or trophozoites (B) were treated with drugs for 8 h washed and reincubated in drug free medium Data for the Control or DFO were not given for day 4 (panel B) since the respective cultures could not survive for an additional day in culture at those levels of parasitemia

for relatively long periods (>24 h) revealed the same pattern of results as those shown previously irrespective of the initial parasite stage In order to examine the stage specific effects of individual drugs or their combinations, the drug exposure time had to be relatively shorter A 4-h treatment of either ring forms or trophozoites was found to be inadequate (too short) for obtaining reliable estimates of parasite growth Therefore most experiments were conducted by exposing parasitized erythrocytes to drugs for 8 h Ring or trophozoite forms grown in the same conditions, were exposed to the drugs on two successive days Upon completion of drug exposure the cultures were washed and resuspended in medium free of drugs The residual effects were estimated in terms of parasitemia (Fig 3) RSF caused pronounced delay in parasite growth up to the second day after treatment of ring forms but was followed by a marked recovery whereas DFO alone was totally ineffective However only with the two drugs combined did the antimalarial effect persist throughout the experiment (Fig 3A) At the level of RSF and DFO used, the effects on trophozoites were more pronounced for RSF although signs of recovery were apparent at day 3 posttreatment (Fig 3B) However, despite the fact that an 8-h treatment of trophozoites with DFO alone had no apparent effect on the propagation of parasites, the combined action of RSF and DFO demonstrably persisted over the entire period of the culture

A more rigorous analysis of the combined action of RSF and DFO was carried out according to the method of Poch (16) Parasitized erythrocytes at the ring stage were treated with various concentrations of RSF in the presence or absence of a given concentration of DFO The activities of the drugs were measured in terms of their capacities to inhibit nucleic acid synthesis, which were determined during the last 6 h of treatment (total exposure time of 30 h) The solid lines depict the fit curves through the experimental points (Fig 4) The dotted lines depict the theoretical curves for additive or independent effects The inhibition obtained with 8 μg of DFO per ml was 6 1% ± 0 6% The results indicate that simultaneous treatment with both compounds leads to an effect significantly greater than additive effect Comparison of the RSF and DFO effects with either theoretical line for additive or independent effect showed statistically significant differences at P of <0 05

DISCUSSION

Acquired resistance to standard antimalarial drugs has created an urgent need for new drugs which are effective against multidrug-resistant strains The application of drugs with different chemical characters and modes of action has been suggested as a strategy for overcoming resistance It has been used in the past against bacteria (16) and malaria (26), including the application of iron-chelating agents (2 25)

Various iron chelators inhibit malaria growth in vitro and in vivo and some are clinically effective Inhibition of parasite growth was usually detected while in vitro cultures were being treated with the drug but recovery of growth was observed after discontinuation of the treatment (8 27) Similar results were obtained with DFO as a therapeutic agent in humans, since most of the patients suffered from recrudescences 7 to 10 days after termination of the therapy (8)

DFO is a hydrophilic agent which permeates very slowly into parasitized cells, so that its antimalarial activity develops relatively slowly even under continuous exposure Thus, the relatively short time window of action of such an agent limits its

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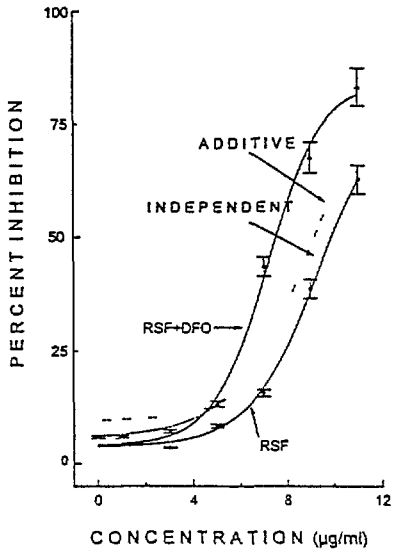

FIG 4 Dose response curves for RSF in presence or absence of DFO (8 µg/ml) and analysis of additivity and independence of drug action Cultures of *P falciparum* were treated at the ring stage for 30 consecutive h with the indicated concentrations of drugs Parasite development was assessed in terms of [^{3}H]hypoxanthine incorporation into nucleic acids during the last 6 h of culture Data are expressed as the mean percent inhibition relative to that of the control (without drugs) Error bars indicate standard errors of the means The inhibition obtained with 8 µg of DFO per ml was 6 1% ± 0 6% (mean ± standard error of the mean) Solid lines, sigmoidal fits to the experimental points obtained at the different concentrations of RSF and RSF plus DFO respectively Dotted lines sigmoidal fits to theoretical points for the combined action of the drugs according to the method of Poch (16) The points for the additive effects were obtained by shifting the experimental points obtained with RSF alone to a new value corresponding to the effect generated by DFO alone but in terms of the equieffective RSF concentrations the points for the independent effect were generated in terms of the following formula (16) I(AB) = I(A) + I(B) − [I(A) I(B)] where I(AB) denotes the percent inhibition when both A and B were present and I(A) and I(B) denote the respective percent inhibitions caused by the individual drugs The effects of combinations of RSF and DFO were significantly higher than the effect of the additive or of the drugs individually ($P < 0.05$)

applicability as an antimalarial agent At present, the nature of the selective parasite cytotoxicity of DFO and other hydroxamate iron chelators remains unresolved, although the possibility that it involves sequestration of intracellular iron en route to integration into macromolecules has been proposed (5 14) The proposed model also raised the possibility that parasites acquire iron from the host only during a restricted period of the parasite's life cycle so that chelators acting outside that period could irreversibly affect parasites

A possible means of improving the therapeutic potential of iron chelators as antimalarial agents is by increasing their accessibility to parasites and thus leading to an increase in their speed of action (12) Another means is by augmenting the retention of the drug by the parasites to ensure a more-persistent cytotoxic effect (5 27) These seemingly contradictory properties of potential drugs which on the one hand act rapidly and on the other hand cause a long lasting inhibition could be accomplished either by application of permeant prodrugs which produce intracellular impermeant drugs or by application of a combination of drugs with different action profiles, as shown in the present study For the case of malaria parasites, the combined action of such drugs was anticipated to outperform the sum of the individual effects (15, 16, 22, 26) Where the mechanism of drug action is known, a scheme of synergistic action can be drawn (16) Demonstration of such an action was assessed in this work for a pair of iron chelators of the hydroxamate type, whose action on malaria parasites is associated with their capacity to permeate into parasites and scavenge essential iron (5, 13, 14) The distinctly different permeation features of the two chelators confer them with markedly different accessibilities to parasite compartments and determine their speed of action and stage specificity (19, 20) Since they are assumed to affect similar parasite targets, by scavenging iron(III) (5), they should theoretically inhibit in a classically additive manner (16) However, the faster permeating drugs although showing faster inhibitory action on parasites, also allow parasites to recover from drug treatment upon drug removal (14) Therefore, the combined action of slow and fast permeating drugs was expected to inhibit beyond the theoretical additive level and cause more-persistent inhibitory effects on parasites exposed for a given period to drugs (5) Data shown in this work are in accord with the above hypothesis They also show that the combined in vitro antimalarial action of RSF and DFO was neither additive nor independent but demonstrably greater than additive action The potentiation of one drug effect by another drug, both in the continuous and discontinuous modes of drug treatment, might be indicative of a synergistic mechanism In view of results obtained with a mouse model of *Plasmodium vinckei* infection in which RSF outperformed DFO in terms of speed of action but not in terms of cure after discontinuation of treatment (5), we propose that a drug combination of this type should lead to improved antimalarial performance of DFO in in vivo animal studies and also in humans

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ADDENDUM IN PROOF

Recent studies indicated greater than additive effects for four different types of reversed siderophores used in combination with DFO (A. Tsafack et al , unpublished data)

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