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US-Israel CDR Program Project # C7-171

Modulation of Membrane Transport: A. Biochemical Approach to the Chemotherapy of Malaria

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In this report we shall deal primarily with the collaborative aspects of our project, which comprise the definition of iron transporting mechanisms in malaria parasitized cells and the development of specific iron delators as potential antimalarial agents.

In the second part we shall report about progress made in the other aspects of the subject, namely, the characterization of the novel transport pathways which appear in the red cell membrane after parasitization and new attempts at chemical identification of the putative funtional components of those pathways.

Part I (A. Jerusalem)

Iron is essential for the maintenance of living systems, and parasites are no exception (1). One of the first microscopic changes a parasite induces after penetrating a red blood cell is at the level of the host cell membrane. Proteins of parasitic origin have been shown to be associated with the red cell membrane and it has been suggested that one of them might be a receptor for transferrin (mammalian iron carrier) (2). This observation complements the findings that intracelluair parasites which demand acquistion of nutrients from extracelluair sources (3) display increased permeability properties for a variety of substrates, a property which increases with parasite maturation (4). Based on the need of parasites for iron, attempts have been made to cause iron starvation by additon of desferal (5), a "natural" iron sequestering agent. This approach proved promising, as

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the agent inhibited in vitro growth of Plasmodium (6) and surpressed malaria propagation in mice (7) and monkeys (8). However, desferal from being an ideal drug, in as much as experience with extensive use of desferal for the treatment of iron overload has established, that it has to be administered by infusion, because of its fast metabolic degradation, and that it can cause severe side effects , including death.

In this project we focused on the design, synthesis and in vitro testing of artificial iron binders and carriers that can penetrate infected erythrocytes and remove entrapped iron (III). These compounds were examined as iron sequestering agents and growth inhibitors of Plasmodium falciparum in in vitro cultures of erythrocytes. The project was conducted in collaboration with Dr. A. Shanzer, from the Dept. of Organic Chemistry, Weizman Institute of Science.

This project thereby suggests a new approach towards chemotherapy of malaria which, is anticipated to become complementary to conventional drug treatment. The latter is limited by the capability of malaria parasites to develoop drug resistance. However, drug treatment based on iron starvation can hardly be counteracted by the development of drug resistance. Moreoever, iron sequestering agents might well be used synergisticaaly with conventional drugs as they both rely on different antimalarial mechanisms. Vaccines appear to be promising solutions for the future once the many technological obstacles have been overcome. Iron sequestering agents thus appear to be intermitant soltuions that are based on rather simple and readily accessible compounds of low molecular weight which are unlikely to cause immunological responses. Yet the relationship between iron sequestering capability and growth inhibition and the mechanisms by which iron scavengers act as growth inhibitors will have to be further established.

The design and synthesis of the novel iron binders suggested in this project has relied on earlier experience (10-14) in the preparation of artificial iron carriers, while considering the specific requirements when applied to cells and microoorganisms.

In order to cause iron starvation of Plasmodium falciparum, the iron sequesterign agent should fulfill the following requirements:

(i) It should not remove iron from transferrin and other mammalian iron transport proteins and interact minimally with serum proteins;

(ii) It should permeate selectively the infected erythrocyte membrane as a free ligand;

(iii) It should bind Fe^{3+} once in the RBC and

(iv) leave the infected erythrocyte membrane as a ferric complex;

(v) It should release the iron to the extracellualr medium and then resume its action in a "shuttle mechanism".

Although it might appear very demanding to satisfy this set of seemingly contradictory requirements, prel ary assays proved promising. Our first iron carrier (type B) were found to be powerful growth inhibitors of Plasmodium falciparum in erythrocytes and to surpass the efficiency of desferal by 30-fold: compound 1367 showed 60% growth inhibiton at 1 μg/ml while desferal reached 50% inhibition only at 30 μg/ml.

TABLE I

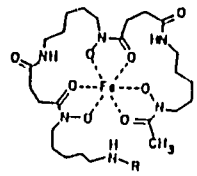
Inhibition of *in vitro* *p. falciparum* growth by ion chelating agents (IC_{50} values are given in μg/ml).

Compound Code	IC_{50} (± 15% SD)
Desferal	30.0
4112	7.5
1392	70.0
1367	50.0
E110	1.0
RL271	2.0
E109	18.0
RL266	70.0
V1	20.0
MP14	30.0

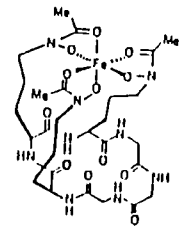
All experiments were performed in triplicates with standard deviations of ±15%. Cultures were exposed for 24 hrs at the indicated concentrations of scavengers and then incubated for 24 hrs with tritiated isoleucine under culture conditions. Crontols were exposed to equivalent amounts of DMSO.

Relying on this study, two stucturally different familites of iron sequestering agents, types A and B, were synthesized, based on two natural ferric ion carriers: namely desferal and ferrichrome(5). They are, however, modified so as to overcome the inherent disadvantages of the natural compounds, such as low membrane permeability, metabolic susceptibility and toxicity.

Desferal



Ferrichrome

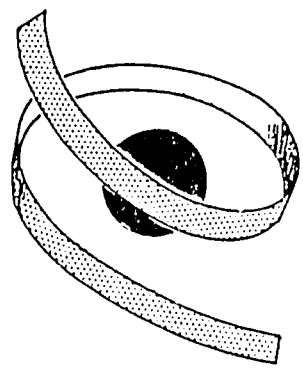


Both these carriers make use of hydroxamate groups as ion binding sites and thereby form neutral complexes when binding iron, which is advantageous in order to achieve membrane permeability (15).

$$3\ \text{-CO-NOH-} + Fe^{3+} \rightleftharpoons (\text{CO-NO-})_3\text{Fe} + 3H^+$$

Structurally however, desferal and ferrichrome are inherently different. While desferal forms the octahedral binding cavity by coiling around the guest ion in a loop, ferrichrome creates the binding cavity with its three side arms. As to their ion binding efficiency(16) ferrichrome is slightly superior to desferal (log K = 30.6 and 29.1, respectively), presumably due to entropy factors. This demonstrates the sensitivity of ion binding to structural variations.

Desferal-like carriers, type A, are synthesized by assembling in an alternating fashion amino acids and N-hydroxy-amino acids to a string containing three hydroxamate and three amide groups.

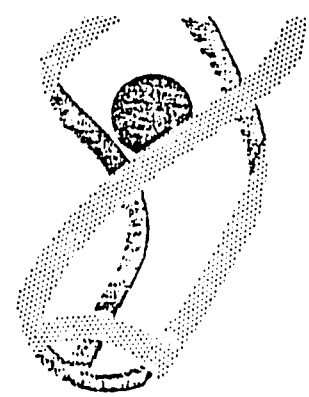


$R'O(COCH_2CH_2NOHCOCHR(CH_2)_mNH)_3H$

Type A

$m = 0,1,2,3,4$; $R,R' =$ alkyl, $CONEt_2$, CONHR

The principle design of the ferrichrome-like iron binders, type B, relies on C_3-symmetric molecules that are assembled from a (i) tricarboxylate as stem, (ii) extended by amino acids and (iii) terminated by hydroxamate groups.



$R''C(CH_2O(CH_2)_nCO(NHCHR(CH_2)_mCO)_qNOHR')_3$

$N-((CH_2)_n-CO-(NHCHR(CH_2)_mCO)_qNOHR')_3$

Type B

$n = 1,2$; $m = 0,1,2$; $q = 0,1$, $R,R',R'' =$ alkyl

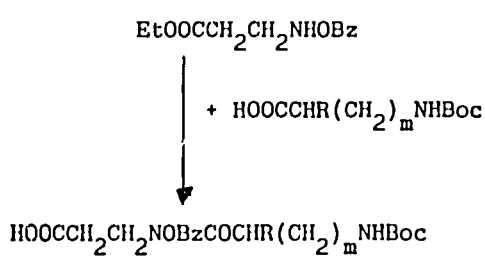
The use of a whole range of R,R',R'' or amino acids in this design allows for regularly changing the cavity size and, thereby, iron binding efficiency and selectivity. It enables bulkiness and lipophilicity to be modified (and, thereby, also membrane permeability), by replacing large aise chains by smaller ones, and hydrophilic by lipophilic ones. Moreover, the possibility of using in place of amino acids of the natural L-configuration amino acids of the unnatural D-configuration may drastically increase the metabolic stability of these compounds and simultaneously reduce their toxicity. Both type A and type B binders thus have the advantage of being designed in a "modular" fashion that makes systematic modification of specific parameters possible.

Synthesis

Two inherently different families of iron sequestering agents are synthesized and examined. Type A, that rely on the structural principles of desferal, and type B, that rely on those of ferrichrome.

The synthesis of type A carriers was approached by a series of reactions that involve essentially two parts:

i) preparation of the artificial amino acid monomers possessing the ion binding hydroxamate groups in their backbone,



m = 0,1,2,3,4 R = iBu, $CONEt_2$, CONHEt, etc.

ii) coupling of the amino acid monomers to trimers with the use of the Merrifield method, which is commonly employed in peptide synthesis and proved adequate in prelimary experiments also for the oliogomerization of such artificial amino acids.

$$\mathrm{HOOCCH_2CH_2NOBzCOCHR(CH_2)_mNHBoc}$$

$$\Big\downarrow \text{ Merrifield Polymer method}$$

$$\mathrm{R'O\text{-}(COCH_2CH_2NOBzCOOCHR(CH_2)_mNH)_3Boc}$$

The synthesis of type B carriers has been approached by two sequences:

i) preparation of the hydroxamate bearing chains by condensation of amino acids with hydroxyl amine and hydroxyl amino derivatives:

$$\mathrm{H_2NCHR(CH_2)_mCOOH} \longrightarrow \mathrm{H_2NCHR(CH_2)_mCONOHR'}$$

ii) attachment of the chains to a C_3-symmetric tris-carboxylate to provide the ferrichrome-type binders.

$$\mathrm{R''(CH_2O(CH_2)_nCONHCHR(CH_2)_mCONOHR')_3}$$

$$\Big\downarrow \mathrm{R''C(CH_2O(CH_{2n}COOH)_3}$$

$$\mathrm{H_2NCHR(CH_2)_mCONOHR'}$$

$$\Big\downarrow \mathrm{N((CH_2)_nCOOH)_3}$$

$$\mathrm{N((CH_2)_nCONHR(CH_2)_mCONOHR')_3}$$

Once at hand, the structural features, iron-binding properties and lipophilicities will be examined in an attempt to later deduce a structure-activity relationship of the different sequestering agents. Structural features were deduced by Dr. Shentzer using a combination of IR- and NMR spectrometry in conjunction with CD; iron binding was tested by titration in conjunction with UV and CD, and lipophilicity by partition experiments.

The various agents were tested for in vitro suppression of malaria parasite growth at the various stages of intraethrocytic development with the aim of evaluating:

a. IC_{50} values under condition of continuous exposure to drug.
b. IC_{50} values under exposure to drug for defined periods of time.
c. Stage of parasite growth which is most sensitive to drug.

The parasite targets most sensitive to the drugs examined in biological terms (nucleic acid synthesis, protein synthesis, protein degradation, permeability properties and energy supply) were the trophozoites.

Once the first round of these experiments will be completed, the efficiency of the compounds as iron scavengers will be compared with their efficiency as growth inhibitors in order to establish the relationship between these two phenomena.

Other Procedures Used:

EFFECT OF INHIBITORS ON MALARIA PARASITES

Cultures: Cultures of P. falciparum (FCR_3 uncloned strain from Gambia, obtained from Dr. J.B. Jensen and ITG-2F6 cloned strain from Brazil, obtained from Dr. L.H. Miller) were grown in cuture flasks containing growth medium (RPMI-1640 from Gibco, supplemented with 25mM HEPES, 23 mM na-bicarbonate, 10 mM glucose and 10% heat-inactivated AB^+ or A^+ washed human erythrocyte at 2-2.5% hematocrit. The

growth medium was replaced daily and the cultures were gassed with a micture of 90% N_2, 5% CO_2, and 5% O_2. Cells were normally harvested or subcultured when reaching 15-20% parasitemia (determined microscopically by thin blood smears stained with Giemsa). Trophozoite and early schizont stages were routinely isolated by the gelatin floatation method as described elsewhere (17).

Effect on parasite growth: Cultures of parasites are synchronized according to the method of Lambros and Vanderberg (18) except that sorbitol is substituted by the relatively less toxic alanine. After reaching the trophozoite stage (10-20% parasitemia), the cells are washed aseptically with growth medium, resuspended to a 2% hematocirt (1-2% parasitemia) and distributed into wells of a 24 well microtiter plate (0.6 ml/well). Inhibitors are added to the indicated concentrations, the plate transferred to a candle jar and incubated at 37°C for the indicated periods of time (0-4 hrs). After this period, the supernate is removed, the cells are washed with fresh growth medium and returned to culture condition (without inhibitor) for an additonal 18 hr period. Samples are withdrawn for microscopic estimation of newly formed rings and [^{3}H]ILEU or [^{3}H]HYPOX are added to final activity of 4 µCi/ml. Following an additonal 24 hrs incubation period, cells are transferred in triplicate samples to 96 well plate and harvested in a Cell Harvester (Dynatech, Inc.). The filters are washed with distilled water, then dried for 2 hrs at 60°C and transferred into tolunene-based scintilation fluid for counting of radioactivity.

Control samples of uninfected cells and of infected cells exposed to DMSO alone at the same final concentrations in the different systems, are subjected to the same treatments as those described above. When necessary, cells are exposed to inhibitors for a continuous 22 hour period.

The IC_{50} values of inhibiton of growth are calcumlted from the profiles of parasite growth obtained in the presence of various concentrations of inhibiotr using the equation:

$$Y_i = Y_{iw} + (Y_{io} - Y_{iw} / [1 + (i/IC_{50})^f]$$

where Y_i is the amount of [^{3}H]ILEU incorporated into protein in the system subjected to a given concentration of inhibitor i, Y_{io} is at

i=0, Y_{iw} is at i concentration where the inhibitory effect is maximal and f is a scaling factor (19). The IC_{50}±SD and the correlation coefficient values are obtained by non-linear regression analysis of the data according to the above equation, based on the Lavenberg-Marquardt algorithm. The analysis is done on a Wang PCSII system.

Effect on protein synthesis: The effect of agents on [^{3}H]ILEU incorporation into parasite proteins is assessed essentially as described above for the parasite growth, except that [^{3}H]ILEU and either the test agent in DMSO or DMSO alone, are added to the cultures and are present during the indicated time of incubation (0-4 hrs). Triplicate samples from each system are processed for determination of the amount of [^{3}H]ILEU incorporated into proteins as described above. Other methods were described in previous reports.

The suggested approach to malaria chemotherapy relies on the dependence of parasites on iron and the enhanced permeabilization of infected erythrocytes. It is based on the possiblility of inhibiting growth of Plasmodium falciparum once inside the red blood cell by iron starvation. In contrast to conventional drug treatment, this approach has low probability for evolving the development of drug resistance. Moreover, the iron-sequestering agents suggested are rather simple, low molecular weight compounds that are unlikely to induce immunological response.

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US-Israel CDR Program Project #C7-171

Modulation of Membrane Transport: A Biochemical Approach to the Chemotheray of Malaria

Progress Report from the Thai component (PI: Y. Yuthavong , Mahidol University, Bangkok Thailand), Aug. 87-Jul. 88

A. Folate Transport of Malaria-Infected Red Blood Cells and Its Modulation

Our study on folate transport into red blood cells infected with *P. falciparum* by using [^{3}H] Folic acid as a tracer showed that these cells transport much higher amounts of folate than normal red blood cells, probably through a novel transport pathway. The rate of transport was increased with maturation of the parasite and was highest at schizont stage. In addition, the rate of folate trasnport was also linear with percent parasitemia. Characteristics of folate transport by *P. falciparum* infected red blood cells were studied extensively. Initial rates of folate transport at various temperatures; 37, 25 and 0°C were measured and found to be highest at 37°C, with almost no transportation at 0°C. This implies that cellular energy may play a role in folate transport, and will be studied further. The K_m of folate transport was 11.33 ± 1.8 uM and V_{max} was 2.8 ± 0.35 pmol/10^9 cells/min.

B. Mefloquine and Chloroquine Transport

While chloroquine resistance is widespread in *P. falciparum* infection, mefloquine is an effective drug even for chloroquine-resistant parasites. Yet the structure of mefloquine is similar to chloroquine, and their modes of action remain a mystery. Understand-

ing the transport mechanisms of both drugs would pave the way toward understanding their chemotherapeutic mechanisms. Mefloquine is known to be more hydrophobic, and to bind substantially with membranes. Yet the site of action of chloroquine, and possibly also mefloquine, is probably the parasite food vacuole where both drugs are accumulated. We have new information which points to the likelihood that the transport of these drugs occur be through secondary active transport, whereby the proton gradient between the food vacuole and the cytoplasm is used to drive uptake of both drugs. Chloroquine transport with proton antiport is electroneutral, but mefloquine transport is electrogenic, hence explaining the higher uptake of the latter drug and providing a possible explanation for its higher effectiveness. Our proposal accommodates all the data which can also be explained by the "lysosomotropic" theory of chloroquine transport, but can also explain the higher vacuolar uptake of mefloquine, and provides new clues to the search for new antimalarials of similar structure.

C. <u>*In vivo* study of iron transport into malaria parasite and possible existence of parasite siderophores</u>

Two approaches were used in the *in vivo* iron uptake study. When *P. berghei* infected mice were given ^{59}Fe for different time periods (6-24 hrs), the infected cell as well as the parasite showed enhanced Fe uptake with time. The results showed exogenous iron was translocated into malaria parasite from plasma. The other approach was to use mice with ^{59}Fe-prelabelled red cell by labelling phenylhydrazine-induced reticulocyte with ^{59}Fe. After the plasma was clear of ^{59}Fe the mice were infected with *P. berghei* and followed the Fe uptake into

parasite for 2-4 days. The results showed very little parasite Fe uptake and the iron was located mainly in particulated fraction. These results suggested that heme-bound iron is probably not utilized by the parasite due to its potential toxicity and hence the iron must be obtained from the plasma instead.

Free *P. berghei* prepared from infected mouse erythrocyte was incubated with ^{59}Fe at varied time and varied amount of ^{59}Fe. Effect of lysate derived from the infected cell was shown to have profound influence on parasite iron uptake. The heated lysate still showed enhanced effect on iron uptake of the free parasite. The results suggested possible existence of parasite siderophore in the infected red cell cytoplasm for iron transport into the parasite. Identification of the parasite siderophore is being carried out for detailed studies. Several possible compounds with high affinity for iron and capable of chelating Fe^{3+} from transferrin were investigated (nucleotides, sugars, amino acids, and common metabolic intermediates including 2,3 diphosphoglycerate). None of these potential candidates showed significant enhanced iron uptake. The deproteinized infected lysate showed the presence of iron chelator extractable by benzyl alcohol in a manner similar to desferrioxamine (DFO) extraction. The iron chelator was not detectable in uninfected control lysate even at several folds higher concentration. Comparative studies with DFO suggested possible hydroxamate compound present in infected cell lysate which might be of parasite origin. The same results were also observed with parasite cytosol extract. Extractions with ethyl acetate for detection of catecholate siderophore were all negative, indicating the absence of catecholate iron chelator. Characterization of

the parasite hydroxamate siderophore by high-voltage paper electrophoresis in conjunction with other biological assays are in progress. These studies might open up an alternative explanation for mechanism of iron acquisition by malaria parasite.

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Part II: Definiton of permeation pathways induced in red cell membranes by intraeythroytic parasites (For details see enclosed manuscripts)

Jerusalem

As part of our continuing effort to elucidate the permeation pathways induced in red cell membranes by malaria parasites and exploit them as chemotherapeutical targets, we expanded our original studies with bioflavonold glycosides particularly phlorizin and its isothiocyano derivative.

1. We have found that three distinct pathways are induced by parasites. These pathways are identified by their susceptibility to the above bioflavonoids.:

Pathway (a) admits hydrophobic molecules (polyols, amino acids) and behaves as a pore (Ea, exclusion limits, anion conductive channel) and is fully blockable by bioflavonoid glycosides.

Pathway (b) admits hydrophobic molecules (amino acids) and behaves as a quasi-hydrophobic phase and lacks susceptibility to bioflavonoid-glycosides or any covalent modification of infected cells.

Pathway (c) is usually associated with changes in the lipid constituents of the membrane and behaves as a classical leak pathway.

Those studies have recently been summarized in an Editonal Review on Blood (herewith submitted).

2. We have established the experimental conditions for chemical modification of pathway (a) by phlorizin-isothiocyanate or phlorizin-diazonium chloride, two agents which we designed and synthesized. These agents block the pathways at μM concentrations, producing an irreversible effect on both the pathways and parasite growth.

We have recently managed to affinity purify an anti-phlorizin on an affinity column using antithyroglobulin-phlorizin as the antigen (raised in rabbits).

It is our plan to gather sufficient amounts of the affinity purified antibodies for immunodetection of phlorizin tagged on membranes from metabolically labeled parasitized cells.

3. We are continuously screening sera from malaria patients and monoclonal antibodies as potential blockers of the induced pathways. We have recently isolated a fraction from the serum of malaria patients which can be specifically absorbed on CHS-treated-malaria infected cells (glutoraldehyde fixed on solid substrata) and eluted with acidic glycine. We are presenlty testing whether this fraction reacts specifically with the phlorizin labeled proteins of the membrane. For that purpose we have invested efforts in synthesizing [^{3}H] labeled phlorizin-isothiocyanate of high specific acitivity (> 20 Ci/mmole), as this is required for identification of the few bounded putative sites per infected red cell.

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