PN-ABN-504
81700

U.S.-ISRAEL CDR PROGRAMME
PROJECT ID # C7-171

FINAL REPORT (Reported period: Aug. 1987- July 1991)

MODULATION OF MEMBRANE TRANSPORT: A BIOCHEMICAL APPROACH TO THE CHEMOTHERAPY OF MALARIA

Principal investigators: Z.I. Cabantchik (Hebrew University, Jerusalem, Israel)
Y. Yuthavongh (Mahidol University, Bangkok, Thailand)

SUMMARY OF RESULTS

The major goal of this research proposal was the development of novel approaches to malaria chemotherapy. The second major goal was to delineate the biochemical basis for the specific cytotoxicity of novel drugs to malaria parasites. The approaches were based on rational design of agents tailored to selectively affect malaria parasitized cells by either one or by a combination of the following mechanisms: a. selective permeation into infected cells via permeation routes induced/modified by the intracellular parasite; b. selective blockage of entry routes of substances into infected cells and c. selective molecular "hit" at parasite's targets. We feel that we have accomplished the major goals of the projects, and although we have diversified the lines of candidate antimalarial agents (AMG) originally proposed, we have purposely done that in order to further strengthen the major aims of the project. By taking advantage of the enormous potential offered by new lines of agents we have merely changed some of the chemical tools, but not the basic concepts or methodological approaches set in the original proposal. On the contrary, the new tools expedited accomplishments of the original goals, as follows:

1. We delineated the permeation pathways operating in infected cells vis a vis normal cells. This comprised development of new methodologies or adaptation of existing ones for measuring transport in a temporal sensitive and reliable fashion, at various stages of intracellular development of parasites. The immediate applicative bonus of these studies was the development of the technique for separating of all developmental stages of erythrocytic parasites in a single sorbitol-Percoll step gradient (25), based on the identification of the progressive nature of permeation which parasites induce/modify in the host cell membrane. The biophysical information gained was comprehensively reviewed (1,2& 14), with particular emphasis on how it could be used for rational drug design (1). The picture which emerged from these studies indicated the operation of 3 distinctly

new permeation pathways: one with pore-like properties which admits hydrophiles with limiting radius, another with features of diffusion through a lipid-like domain and a third unspecific one.

2. We developed a blocker for the pore-like system, phlorizin (a bioflavonoid glycoside) which also acts as an AM. Because this naturally occurring agent is also toxic to mammalians (causes glycosuria by blocking Na-glucose absorption in the gut and reabsorption in the kidney), we sought to improve its therapeutic efficiency and index by synthesis of structural congeners. The synthetic effort demanded adaptation of schemes for chemical synthesis, purification and structural analysis and extensive testing in various biological systems and establishment of structure-activity schemes. This provided agents with 10 fold higher in vitro AM efficiency and apparent lack of toxicity to mammalian cells as well as new tools for identification of the putative parasite induced targets and routes of entry of substances into infected cells (47). (Unlike the reviewers, we feel that the have widely -but not necessarily exhaustively-exploited the "promising phlorizin", as witnessed in our published work and in the progress report).

3. We studied the mode of action of phlorizin and analogues as AMG. We found these agents to block only the pore-like pathways, but blockage required first their gaining access into infected cells, via the same pore they eventually block from within the cells (35,47).

4. We sought to study the biochemical properties of the pores. We initially attempted a crude identification of the pore components by chemical and affinity labelling, using disulfonic stilbenes/protection experiments (52). These studies gave an indication for the presence of putative targets which might be associated with the blockage of the function. In parallel we estimated the upper limit of "specific" phlorizin binding sites with ^{3}H-labelled forms and prepared a photoreactive and affinity labelling congener for tagging the putative pore sites (35). We prepared also an affinity purified anti-phlorizin antibody for identification of tagged sites on membranes. However, because of the minute number (300) phlorizin-sensitive (high affinity binding) sites per cell, and their endofacial location in the host membrane and the order of magnitude higher non-specific binding sites, it became essentially impossible to unequivocally identify the relevant sites with the tools available.

We therefore opted for further exploiting the differences in permeation between infected and uninfected cells in order to come up with improved therapeutic schemes. This was, after all, the ultimate goal in the project and judging by the results, we can fully justify the decision made.

5. We undertook a systematic, rational and targeted approach of synthesis and *in vitro* evaluation of agents which had the potential (i.e. flexibility) for molecular tailoring and for biochemical analysis (which bioflavonoids had not, at least in our hands). At the 3rd year of the preceding grant, we commenced a joint

project with Dr. Shanzer on iron chelators as AMG, based on earlier success of others with desferioxamine (DFO) *in vitro* and *in vivo*. (Dr. Shanzer's agents were developed previously and independently by his group at the Weizmann Institute). The then accepted rationale for the AM action of DFO) (a hydrophilic iron(III) iron chelator) proved quickly to be incorrect-since DFO demonstrably acted within the infected cell and not on serum sources, apparently by scavenging iron from some unidentified sources (17,18,71). At that time, and hitherto, little was/is known about how and from where does the parasite acquire the critical iron needed for its growth and replication (3,67,68). The sensational view that it does acquire it from serum transferrin by endocytosis at the level of the host membrane, was probably incorrect. What is known is that DFO, which is used successfully in various iron-storage diseases (72), affects parasites with a slow-action profile and stage dependency, demanding continuous and prolonged drug exposures due to fast clearance from circulation, with obvious consequences (72).

6. We adopted the approach outlined in our previous work-that of drug targeting to parasitized cells by exploiting the ostensible differences in permselectivity between uninfected and infected cells. We opted for systematically changing the lipophilic/hydrophilic balance of fully synthetic siderophores designed on the basis of a biomimetic approach or by modifying natural siderophores, all this in order to fit the permeation window induced by intracellular parasites. This is how the concept of reversed siderophores (RSF's) was generated-that is siderophores (SF's) with modulatable lipophilic character for faster permeation and hence faster removal of iron from cells. Comprehensive SAR studies of novel agents as AMs were established in *in vitro* models and preliminary studies were also done in an *in vivo* model. Fluorescent labelled probes were synthesized based on concepts drawn from the biophysical aspects of the project and in conjunction with synthetic schemes tailored to the special needs of the project. Since submission of the previous continuation grant proposal we published 2 full papers (9&10) and submitted 5 MS's which cover: The mode of action of RSF's as AMG I. permeation properties and cytotoxicity; II. identification of pharmacological targets (55&58), The AM action of desferal involves a direct access route to erythrocytic (*P. falciparum*) parasites (56), Monitoring of iron(III) removal from biological sources using a novel fluorescent SF (57) and *In vivo* AM action of a lipophilic iron(III) chelator: suppression of *P. vinckei* infection by RSF (59). All these were covered in MS's and in 3 separate *addenda* appendixed to the previous grant.

Significance Malaria is considered a major threat to the life of millions of human beings in various sections of the world. On the background of widening spread of drug resistance, the demand for new chemotherapeutic strategies has increased even further. Concomitant to those demands are the needs for basic and applied studies which could provide the requisite scientific background for the development of novel strategies. The present study was constructed with those ideas in mind based on fundamental studies carried by two laboratories, one specializing on molecular design and the other on studying basic transport phenomena in health and disease (malaria,etc.).

Participants (Jerusalem group):

Z.I. Cabantchik (50%), PI.

J. Silfen (100%), Research Associate

H. Glickstein (100%), Research Assistant

D. Baruch (50%), doctoral fellow

S. Lytton (100%), doctoral fellow

O. Eidelman (50%) postdoctoral fellow

The major goal of our study was to define in biophysical and biochemical terms the permeation pathways (pores and others) induced in red cell membranes by malaria (*Plasmodium falciparum*) parasites and exploit them as chemotherapeutic targets or vehicles for chemotherapeutic agents. To that extent we have accomplished the major goals of our study and laid the grounds for a comprehensive program aimed at exploiting the information gained to tailor agents for selective arrest of malaria infected cells.

The stage to the project was set by our initial biophysical studies which indicated that red blood cells undergo stage dependent alterations in their permselectivity properties and that a natural bioflavonoid agent, phlorizin, demonstrably arrested parasite growth apparently by blocking entry pathways of essential solutes (14, 25 and recently reviewed in ref. 1 & 2). *On the basis of phlorizin structure we synthesized a variety of analogs with and without covalent binding capacity with the aim of 1. obtaining more efficient blockers of the new pathways-based on SAR studies; 2. define in pharmacological terms the various pathways induced by the parasites in terms of selectivity and susceptibility to bioflavonoids; 3. define the targets in biophysical and biochemical terms by using covalent binding analogs of bioflavonoids and anti-bioflavonoid antibodies-for identification of the putative targets in Western blots of membranes isolated from modified cells; 4. define the sidedness of phlorizin effects on the host cell membrane (outer or inner membrane surface) using permeant and impermeant versions of phlorizin in intact infected cells and in cells encapsulated with phlorizins which wre*

subsequently invaded with parasites. In the last stages of the project we joined forces with chemists from the Weizmann Institute with the aim of assessing a novel series of synthetic siderophores (SF's) as antimalarial agents. *We have basically diversified the tools used, but not the basic concepts and rationale on which the project was based.* The unique feature of those SF's is their modular design and synthesis which allowed systematic substitutions of amino acid side groups as a means to modify the probe's size and lipophilicity (9), the two major determinants of selective admission into infected cells (1,2). An additional series of SF's were based on the hydrophobic N-terminal derivatizion of desferrioxamine (DFO) with the purpose of increasing the agent's hydrophobicity (i.e. permeation properties). This approach yielded also fluorescent analogs of the SF which fully retained their iron(III) chelating capacity (10) and imparted to the molecules unique spectroscopic properties. With both series of agents we obtained potent antimalarial derivatives which hardly affected mammalian cells in culture, and for the case DFO, we obtained derivatives which penetrated selectively into malaria infected cells. We have demonstrated that those agents site of action is intracellular, that they spare serum transferrin, but could scavenge iron from ferritin as well from infected cells (10,60). The derivatization of DFO was shown to markedly enhance the SF's ability to penetrate into infected cells, although the iron(III)-conjugated siderophore was somewhat less mobile through the host membrane than the free SF.

Our attempts to chemically identify the membrane components involved in host cell mebrane permeabilization have been only partially successfull and still demand further efforts. Affinity and photoaffinity labels for the putative pores based on phlorizin derivatives were not specific enough to allow unequivocal assignement of the pore function to particular polypeptides, because of the few number of such polypeptides (300 per cell) relative to the large number of non-spefic binding sites. The sidedness of binding of these labels, posed serious difficulties in terms of purification by afinity chromatography using phlorizin-NCS labelled membranes and anti-phlorizin antibodies.

We therefore opted for concentrating most of our efforts towards the chemical design of novel antimalarial agents based on the knowledge gained about the properties of the novel permeation properties. Judging by the results, we believe that the choice made was fully justified. The new approaches have opened new routes of research on malaria chemotherapy of both basic and applicative nature.

Biophysical and biochemical properties of the new permeation pathways

The development of novel methodologies for studying transport phenomena in normal and infected cells was essential for the delineation of permeation pathways in cells (schematically depicted in **Fig. A1**). They provided the following information:

1. In addition to the battery of tranporters constitutively present in red cell membranes (**Fig. A2**), three

distinctly diferent permeation pathways were found to be induced by *P. falciparum* parasites (Strains FCR knobby or knobless and ItG knobby in human red blood cells). These pathways are identified by their selectivity and susceptibility to bioflavonoids (2,47): Pathway (a) admits hydrophobic molecules (polyols and amino acids) and behaves as a pore in terms of energy of activation (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. Some agents might permeate through more than one pathway, although to different degrees (reviewed in 1,2 and 14).

2. *Based on our studies with phlorizin (which effectively blocks the major pathway of permeation and acts as an antimalarial in "in vitro" cultures), we synthesized a series of structural congeners with the aim of obtaining improved blockers, better antimalarials and less toxic agents (i.e. with no effects on epithelial Na-glucose cotransport systems). A varitey of agents were synthesized and a few proven to meet the above demands (Figure A3-A5). Among the most potent of the agents were also naturally occurying bioflavonoid glycoside products such as cosmetin, from parsley. The sites of actions of those agents was shown to be on the inner membrane surface of the infected cells. Thus, in order to block the pathways, the agents need to gain access to the host cell cytosol (35,47).*

3. We have established the experimental conditions for chemical modification of pathway (a) by phlorizin-isothiocyanate or phlorizin-diazonium Cl, two agents which we designed and synthesized. These agents block the pathways at μM concentrations, producing an irreversible effect on both the pathways and on parasite growth. We have produced and affinity purified anti-phlorizin antibodies on affinity columns using antithyroglobulin-phlorizin as the antigen (raised in rabbits). We have obtained sufficient amounts of the affinity purified antibodies for immunodetection of phlorizin tagged on membranes from metabolically labeled parasitized cells. Western blots of membranes isolated from phlorizin-NCS labelled trophozoites (+/- phlorizin) detected phlorizin protected bands. However at this point we can not say whether those bands are directly phlorizin protected, or the protection reflects blockade of entry of the labelling agent (53,53).
Using radiolabelled (^{3}H)phlorizin-NCS at 20Ci/mmol, we did not manage to obtain specific phlorizin-protectable labelling which could be localized on a particular band. We have concluded, that we can not meet the requisite sensitivity necessary for identifying at most 300 sites per cell. The same applies to antiidiotypic antibodies to phlorizin which we raised.

4. We have developed a sensitive assay for detecting surface antigens in intact infected cells (cell fluorescence ELISA)(45). We have used this assay to identify putative reactive sera from malaria endemic areas, from malaria patients (children and adults). Reactivity was found only if infected cells were sterol-treated-a

method used for inducing surface exposure of cryptic antigens. We have screened the positive sera from malaria patients and monoclonal antibodies against host cell membranes as potential blockers of the induced pathways, thus far, with no clear success (54). Some sera which proved positive by various functional tests, gave a too complex pattern of immunoprecipitation bands (metabolically labelled cells) to discern between relevant and irrelevant bands.

As mentioned above, we felt at this point that it will be more rewarding to exploit the knowledge gained from the biophysical studies for designing more versatile tools which should allow not only specific targeting of tailor made drugs but also understand their mode of action at the cellular level. We focused on siderophores because we had the unique opportunity to design molecules and synthesize them the project's major objectives.

Novel Antimalarial agents

The rationale for using siderophores (SF's) as antimalarial agents was essentially empirical, as it was based on previous experience with desferal applied both in vitro and in vivo. Scavenging of iron from erythrocyte infected cells by RSF or by any SF could a priori be assumed to be neither specific in terms of selective accessibility to SF or selective damage inflicted by SF. The basis for susceptibility of parasites to SF's must reside on the mode parasites acquire iron for producing active iron-proteins and on the mode SF's scavenge that iron.

We have focused on hydroxamate type siderophores for selective targeting into infected cells and for significant improvement of AM therapeutic potential and index. This encompassed:

1. **selective targeting of SF's.** Previous studies showed that natural and synthetic iron binders have antimalarial activity in cultures of human erythrocytes infected with *P. falciparum* and also in rodent and simian malaria due to induction of iron deprivation (4-10,15-19). Each class of compounds tested so far had limitations because of either low permeability (38), depletion of serum iron pools (8) or generation of cytotoxic metal complexes (6,7). The initial purpose of our research was to provide compounds that would overcome these limitations. We synthesized hydroxamate based iron (III) binders that mimick the natural siderophore ferrichrome, in terms of the binding site's ion selectivity and affinity, but have a symmetric anchor with amino acids which bear different side chains (**Figure B**). By introducing amino acid residues of variable hydrophobicity into the arms of these artificial siderophores we modified the overall lipophilicity of the compound and confer properties for improved and in specific cases selective penetration acrosss erythrocyte membranes and access to cellular iron (III) pools *(9, 10 and 55-60)*. On the basis of membrane permeation properties we coined the compounds with the name of "reversed siderophores" (**R-SF**), because their properties (hydrophobicity) are opposed or reversed to those

of natural siderophore (hydrophilic and extracellular chelating) (9,75). We found that R-SF's are potent antimalarial agents against **all the erythrocytic stages of *in vitro P. falciparum* growth**, in both chloroquine sensitive and resistant strains. The inhibitory properties derive from the agent 's high selectivity for scavenging intracellular Iron (III) and not serum iron or other metal ions in body fluids. The most versatile member of this family, ileu-containing RSF1-ileu, displayed 10 fold higher IC_{50} and 20 fold faster activity than the classical desferrioxamine (DFO) and was not toxic to mammalian cells in culture for 24-48 hours (9). The inhibitory potency of a first series of RSF's was shown to correlate with the product of the partition coefficient of the drug and the iron binding affinity (58). This quantitative SAR serves as the basis for present and future drug design and assessment of mode of permeation and action. The second series of chelators were N-terminal derivatives of DFO which displayed higher hydrophobic character than the parent compound and traceable properties) fluorescent moieties such as NBD-nitrobenzyl diazole or MA- methylantharanilic acid) (10,56). The introduction of these groups conferred to DFO higher partition coefficients, higher permeation rates into parasitized cells, faster antimalarial action and lower IC_{50} values.

3. Mechanism of RSF action We have shown by pretreatment of serum and red cells with chelators that R-SF site of action is confined to the parasite itself but is definitely neither in the serum nor in the uninfected cell (9,10). Antimalarial activity of R-SF is correlated with physiochemical properties, iron binding and lipophilicity, the latter property determined by the hydrophobic/hydrophilic balance of the amino acid side chain (55). By measuring uptake of pre-formed ^{59}Fe-carrier complexes into normal and infected red cells we assessed permeability of compounds for erythrocyte membranes and demonstrate that fast cytotoxic action of RSF-1 isoleu in all stages of erythrocytic growth relates to its rapid uptake into cytosol (55,56). *The usage of complexes as measure for permeation of free drugs is justified to the extent that the partition coefficients of the iron complexes and free drugs differ very little (by about 20-30%).* The permeation rates for the leu and ileu congeners of RSF-iron(III) complexes were extremely fast: $t_{1/2} < 2$ min.). For fluorescent derivatives of DFO we measured permetion of both the free drug (by fluorescence) and the complex (by radioactivity). Methyl-anthranilic-DFO (MA-DFO) (partition coeff. 3.5 as free drug and 2.7 as iron complex) and NBD-DFO (part. coeff. 5 for free drug and 1.7 for complex) permeated specifically into infected cells. Unlike MA-DFO which behaved similarly to RSF's, NBD-DFO permeated about 5 fold faster in the free form. All these agents were efficient antimalarial agents, both in terms of IC_{50} values (lower μM range), speed of action (2-6 hrs exposure for onset of growth inhibitin), irreversibility of effects (inhibition is not abolished after removal of drug) and effects on all developmental stages of parasite growth. The classical DFO, on the other hand affects only the advanced stages of parasite growth, demands prolonged exposures and shows IC_{50} values in 30-50 μM range. None of the RSF's showed any any antimalarial effect when used in the iron(III)-complexed form-implying that

iron(III) extraction from parasites in involved in the mode of action of these drugs. Using NBD-DFO, we demonstrated >5 fold higher extraction of iron(III) from infected cells than form uninfected cells.

Based on these studies we postulated that the relevant parameters which confer RSF's with AM activity are:

1) efficient and selective binding capacity of intracellular iron(III);
2) the chelator's hydrophobicity which confers requisite permeation properties and
3) molecular dimensions which guide accessibility to critical iron pools.

In summary, in this section of the project we :

1) introduced several novel synthetic ferrichrome analogues;
2) evaluated DFO derivatives which carry hydrophobic fluorescent tags and which display similar properties to RSF's.
3) showed RSF-mediated depletion of intracellular iron pool of parasitized cells;
4) obtained agents which were 10 fold more potent and 5 fold faster acting than DFO on development of *P. falciparum* in culture;
5) obtained RSF's and DFO's active against *P. falciparum* strains of multidrug resistance properties;
6) showed that RSF's acted against all stages of parasite growth;
7) obtained AM agents which were non-toxic to mammalian cells in culture.

Publications (full papers/reviews)

Kutner,S., Ginsburg,H. and Cabantchik,Z.I.(1986) Molecular specificity of the permeation pathway induced in human red blood cells by malaria parasite Biochim. Biophys. Acta 861, 194-196.

Cabantchik,Z.I.,Keren-Zur,Y.,Zangwill,M. and Ginsburg,H.(1986) The modulatory effect of membrane microviscosity on structural and functional properties of the red cell membrane. Membr. Biochem. 6, 197-216.

Breuer,W.V.,Silfen,J.,Ginsburg,H. and Cabantchik,Z.I.(1987) Covalent modification of permeation pathways induced in human red blood cells by malaria parasites. J. Cell Physiol. 133, 55-63.

Kutner,S.,Ginsburg, H. and Cabantchik,Z.I. (1987) On the mode of action of phlorizin as antimalarial agent Biochem. Pharmacol. 36, 1 23-129.

Silfen, J. , Yanai, P. and Cabantchik, Z.I. (1988) Bioflavonoid effects on permeation pathways induced in erythrocytes by malaria parasites. Biochem. Pharmacol. 37, 4269-4276.

Cabantchik, Z.I. (1989) Altered membrane transport of malaria-infecte erythrocytes: a possible pharmacological target. Blood 74/75:1464-1471.

Cabantchik, Z.I., Silfen, J. Krugliak, M. Firestone, J.F., Nissani, E. and Ginsburg, H. (1988) Effects of lysosomotopic detergents on the human malarial parasite *Plasmodium falciparum* in *in vitro* cultures Biochem. Pharmacol. 38:1271-1277.

Cabantchik, Z.I., Glickstein, H, and Silfen J. (1991). Discrete sites of permeation pathways induced in *Plasmodium falciparum* infected humans erythrocytes.in Cellular and Molecular Biology of Normal and abnormal Erythroid membranes (C.M. Cohen and J. Palek, eds.), Wiley-Liss, New York, pp. 267-282.

Baruch, D. and Cabantchik, Z.I. (1989) Passive modulation of antigenic expressions on the surface of malarial infected red blood cells. Mol. Biochem. Parasitol. 36:127-138.

*Baruch,D.,Glickstein,H. and Cabantchik,Z.I. (1991) *P. falciparum*: modulation of surface antigenic expression as revealed by fluorescence-ELISA. Exp. Parasitol. 73:440-450.

Cabantchik, Z.I.(1990). Properties of permeation pathways induced in the human red cell membrane by malaria parasites. Blood Cells 16:421-432.

Eidelman, O., Yanai,P., Lang, H.C., Englert, H.C., Greger, R. and Cabantchik, Z.I. (1991) Macromolecular conjugates of transport inhibitors: new tools for probinfg the topography of anion transport proteins. Am. J. Physiol. 260:C1094-C1103.

*Shanzer, A., Libman, J., Glickstein, H., Lytton, S. and Cabantchik, Z.I. (1991). Reversed siderophores act as antimalarial agents. Proc. Natl. Acad. Sci. 88:6585-6589.

*Lytton, S., Cabantchik, Z.I., Libman, J. and Shanzer, A (1991). NBD-DFO, a fluorescent iron chelator with improved antimalarial activity. Mol. Pharmacol. 40,584-590.

*Lytton, S., Mester, B., Glickstein,H., Dayan, I., Libman, J., Shanzer, A. and Cabantchik, Z.I. (1992). The mode of action of reversed siderophores as antimalarial agents. I. Permeation properties and cytotoxic effects. submitted to Blood.

I. Literature cited

1. Cabantchik, Z.I. (1989). Blood 74/75 1464-1471.
2. Cabantchik, Z.I.(1990). Blood cells 16:421-432.
3. Sherman, I.W. (1988) Parasitology 96:8576-891.
4. Raventos-Suarez,C., Pollack,S. and Nagel,R.L. (1982) Am.J.Trop. Med. Hyg. 31, 919-922.
5. Fritsch, G., Treumer, J., Spira, D.T. and Jung, A. (1985), Exp. Parasitol. 60, 171-174.
6. Scheibel, L.W. and Stanton, G.G. (1986) Mol. Pharmacol. 30, 364-369.
7. Scheibel, L.W. and Rodriguez, S.(1989) In "Malaria an the red cell", G. Brewer ed., Alan R.Liss. Inc. vol. 2pp. 119-149.
8. Heppner, D.G., Hallaway, P.E., Kontoghiorghes, G.J. and Eaton, J.W. (1988), Blood 72, 358-361.

9. Shanzer, A., Libman, J., Glickstein, H., Lytton, S. and Cabantchik, Z.I. (1991). Proc. Natl. Acad. Sci. 88:8561-8569.

10. Lytton, S., Cabantchik,Z.I., Libman,J. and Shanzer,A. (1991). Mol. Pharmacol.(in press.

11. Hommel, M. and Semoff, S. 1988. Biology of the Cell 64: 183-203.

12. Howard, R.J. 1984. Philos. Trans. of Royal Soc. London B 307:141-158.

13. Howard, R.J. 1986. *In* "Parasitology - Quo Vadis ?" (M. Howells, Ed.) pp. 17-29. Australian Academy of Sciences, Canberra.

14. Cabantchik, Z.I., Silfen, J. and Glickstein, H. (1989) in : Cellular and Molecular Biology of Normal and abnormal Erythroid membranes (C. Cohen and J. Palek,eds.), Wiley-Liss, New York, pp.267 282.

15. Pollack, S., Rossan, R.N., Davidson, D.E. and Escajadillo, A. (1987). Proc. Soc. Exp. Biol. Med. 184,162.

16. Pollack, S. (1983) British J. Haematol, 53, 181-183.

17. Hershko, C. and Peto, T.E. (1988). J. Exp. Med. 168, 375-387.

18. Peto, T.E.A. and Thompson, J.L. (1986). British J. Haematology 63, 273-280

19. Yinnon,A.M., Teanacho E.N., Grady, R.W., Spira, D.T. and Hershko, C. (1989) Blood 74:2166-2171.

20. Shanzer, A. and Libman, J. (1983) J. Chem. Soc. Chem. Commun. 846 .

21. Shanzer, A., Libman, J., Lifson, S., Felder (1986), C.E. J. Am. Chem. Soc. 108,7609.

22. Lifson, S., Felder, C.E., Shanzer, A. and Libman (1987), J. Prog. Macroc. Chem. 3, 241.

23. Tor, Y., Libman, J., Shanzer, A. and Lifson (1987), S. J. Am. Chem. Soc. 109, 6511.

24. Tor, Y., Libman, J. and Shanzer (1987), A. J. Am. Chem. Soc. 109, 6518 .

25. Kutner, S., Breuer, W.V., Ginsburg, H., Aley, S.B. and Cabantchik, Z.I., J. Cell. Physiology, 125, 521 (1985).

26. Schwarzenbach, G. and Schwarzenbach (1963) K. Helv. Chim. Acta 46, 1391.

27. Raymond, K.N., Muller, G. and Matzanke, B.F. Top. Current Chem. 123, 49 (1984).

29. Jensen, J.B. Am. J. Top. Hyg. (1978), 27, 1274-1276 .

30. Lambros, C., Vanderberg, J.P. (1979) J. Parasitol, 65, 418-420 .

31. Desjardins, R.E., Canfield, C.J., Haynes, J.D. and Chulay, J.D. (1979) Antimicrob. Agents Chemother. 16, 710-718 .

32. Letenere, E.D. and Giggons, W.A. (1985). Biochem. Biophys Res, Commun. 129:262-267.

33. Chwyn, B. and Neilands, J.B. (1987). Anal. Biochem. 160:47-56.

34. Clyde, D.F. (1986). Epidem. Rev. 9:250-253.

35. Kutner, S., Ginsburg, H. and Cabantchik, Z.I.(1987) Biochem. Pharmacol. 36, 1 23-129.

36. Peters, W. (1985) Parasitology 90:705-715.

37. Schlegel, R.L. and Reichsteiner, M. (1986). in :Microinjection and organelle transplantation techniques (J.E. Celis et al., eds.), Academic press , New York, pp. 66.

38. Fritsch, G. and A. Jung. Z. Parasitenkd 72:709-713 (1986).

39. Blake, D.R., P. Winyard, J. Lunec, A. Williams, P.A. Good, S.J. Crewes, J.M.C. Gutteridge, D. Rowley, B. Halliwell, A. Cornish and R.C. Hider (1985). Quaterly J. Med. 56:345-355

40. Whitehead, S. and T.E.A. Peto. . Blood 76:1250-1255 (1990).

41. Barany, G.and Merifield, R.B. (1984) in The Peptides, Analysis, Synthesis, Biology (E. Gross and E. Meinhofer, eds.), Academic Press, N.Y. vol. 2, 1-284.

42. Eidelman, O., Yanai,P., Lang, H.C., Englert, H.C., Greger, R. and Cabantchik, Z.I. (1991) Am. J. Physiol. 260:C1094-C1103.

43 Trager, W. and Jensen, J.B. (1978) Science 193, 673-675.

44. Haynes J.D. et al. (1978) Nature 263:767-769.

45. Baruch, D., Glickstein, H. and Cabantchik, Z.I. (1991). Exp. Parasitol. .In press.

46. Yayon, A., Cabantchik, Z.I. and Ginsburg, H. (1984) . EMBO J. 3, 2695-2200.

47. Silfen, J., Yanai, P. and Cabantchik, Z.I.(1988). Biochem. Pharmacol. 37:4269-4276.

48. Neilands, J.B.(1981) Ann. Rev. Biochem. 50:715-731.

49. Ismail, A., Beddel, G.W. and Lupan, D.M. (1985) . Biochem. Biophys. Res,Commun. 130:1160-1165.

50. Letenere,E.D. and Gibbons,W.A.(1985) Biochem. Biophys. Res. Commun.129:262-267.

51. Chwyn, B. and Neilands,, J.B. (1987). Anal. Biochem. 160:47-65.

52. Breuer, W.V., Silfen, J. , Ginsburg, H. and Cabantchik, Z.I. (1987). J. Cell Physiol. 133, 55-63.

53. Baruch, D., Berzins, H., Howard, R.J. and Cabantchik, Z.I. (1991) (in prep.).

54. Yudilevich,D. and Cabantchik,Z.I. (eds) (1991). New methodologies for the study of transport across the cell membrane. Plenum Press. London.

55. Lytton, S.J., Mester, B., Glickstein, H., Dayan, I., Libman, J., Shanzer, A. and Cabantchik, Z.I.(1991). (in preparation).

56. Loyevsky, M., Lytton, S.J., Mester, B., Libman, J., Shanzer, A. and Cabantchik, Z.I..(1991).(in preparation).

57. Lytton, S.J., Mester, B., Libman, J., Shanzer, A. and Cabantchik, Z.I.(1991).(in preparation).

58. Lytton, S.J., Libman, J., Shanzer, A. and Cabantchik, Z.I. (1992).(Submitted to Blood). (in preparation).

59. Lytton, S.J., Mester, B., Landau, I., Libman, J., Shanzer, A. and Cabantchik, Z.I.. (1992).

60. Lytton, S.J., Loyevsky, M., Libman, J., Shanzer, A. and Cabantchik, Z.I. (1991). (in preparation).

61. Elledge, S.J. *et al.* (1992). TIBS 17:119-122.

62. Reichart, P. (1988). Ann. Rev. Biochem. 57:349-374.

63. Stubbe, J. (1990). J. Biol. Chem. 256:5329-5332.

64. Inselburg, J. and Banyal, H. (1984). Mol. Biochem. Parasitol. 10:79-87.

65. Chulay, J. *et al.* *(1983)*.. Exp. Prasitol. 55:138-146.

66. Lederman, H.M. et al. (1984). Blood 64:748-753.

67. Whitehead, S and Peto, TEA (1989). Blood 76:1250-

68. Hershko, C and Peto, TEA (1988): J Exp Med 168:375-.

69. Traore, O, Carnevalle, P, Kaptue-Noche, L, M'NBede,J, Desfontaine, M, Elion, J, Labie, D, R Nagel. (1991) Am J Hematol 37:206-208.

70. Pollack, S and Schnelle, V: Brit J Haematol (1988) 68:125-.

71. Carter, T, Bayne, MT, Gordeuk, VR, Brittenham, GM and Aikawa, M. (1991). Am J Trop Med Hyg 45:593-.

72. Hershko, C and Wheatherall, DJ:Iron chelating therapy. CRC Critical reviews in Clinical Laboratory Sciences, vol. 26, CRC Press, Boca Raton, Florida, 1988, p 303

73. Porter, J (1989). Eur J Haematol 43:271-.

74. Pouvelle, B, Spiegel, R, Hsiao, L, Howard, RJ, Morris, RL, Thomas, AP and Taraschi, TF (1991). Nature 353:73-.

75. Winkelmann,G, van der Helm, D., Neilands, JB eds.: Iron Transport in Microbes, Plants and Animals. VCH Verlagsgesellschaft mbH, Weinheim, Germany, 1987.

C7-171
Final

US-Israel CDR Program Project #C7-171

(ID no. 936-5544-G-00-6026-00)

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

Final Report from the Thai component (PI: Y. Yuthavong, Mahidol University, Bangkok, Thailand), Aug. 87 - Jul. 90.

A. Transport Folate Cofactors in Malaria-Infected Red Blood Cells

(Y. Yuthavong, S. Suttikulpitag)

Our studies over a number of years have shown that the malarial parasite *Plasmodium falciparum* can not only synthesize folate cofactors de novo, but also salvage exogenous folates for its own use. Two possible routes for salvage exist. The first is uptake of folate, which is probably degraded to pterin aldehyde and PABG (p-aminobenzoyl-glutamate) which can re-enter the synthetic pathway. The second is uptake of 5-methyltetrahydrofolate, the major form of folate cofactors in plasma, or its polyglutamate derivatives in red cells. Studies on transport of folate and 5-methylatetrahydrofolate can therefore throw light on these salvage pathways.

P. falciparum infected red cells in culture took up folate from extracellular medium at the higher rate than normal red blood cells. The rate of uptake was maximal at the schizont stage and the uptake of whole cells in culture was increased with percent parasitemia. To ascertain that the increase in folate uptake was due to the intracellular parasite, uninfected red cells from the culture were separated from infected red cells and folate uptake subsequently determined for both types of cells. It was found that uninfected cells showed no alteration in folate uptake from control normal cells, while increase in transport occurred only in infected cells. Therefore, the increase in folate uptake was a direct result of the malarial infection and not an indirect result from factors released into the medium during infection.

The folate taken up by the parasitized red cells was not significantly metabolized after 60 minutes. Therefore, the kinetics of folate uptake could be studied without significant interference from metabolism. From the kinetic study, it was found that folate uptake was a non-saturable process and was not inhibitable by any folate analogues.

MAR - 1 1993
CC: CDIE + BOST 10

This indicated that the uptake was through a non-carrier mediated system. Furthermore, the folate uptake was not an active transport process because it could not be inhibited by the metabolic inhibitors such as NaF, KCN, and CCCP.

Our determination of activation energy of folate uptake from temperature dependence of transport indicated that the folate uptake by *P. falciparum* infected red cells could be trhough newly formed pores. The pores were compared with the pores induced by chemical treatment, i.e., diamide linkage of membrane proteins. Diamide treated normal cells showed a slight increase in folate uptake, less than the uptake of untreated parasitized red cells. It can be concluded that the folate pores are not of the same nature as diamide-induced pores.

Studies of transport of 5-methyltetrahydrofolate and methotrexate also showed significant transport enhancement for *P. falciparum*-infected red cells.

The enhanced folate uptake could not be increased further when the parasites were grown in folate depleted medium for 96 h and up to 10 days. This was interpreted as inability of the parasite to induce the pores in medium foalte depletion. In addition, most of the transported folate accumulated in the cytoplasm of red blood cells, while only small amount of folate were taken up into parasite cytoplasm. In contrast, radiolabeled 5-methyltetrahydrofolate introduced into red cells through a reversible lysis procedure was effectively incorporated into the parasite and the methyl carbon transferred to methionine. this showed that the transport of this cofactor is metabolically significant.

B. <u>Mefloquine and Chloroquine Transport</u> (Y. Yuthavong, G. Vanderkooi, P. Prapunwattana)

While chloroquine resistance is widespread in *P. falciparum* infection, mefloquine is an effective drug even for chloroquine-resistant parasites, although resistance to this drug is also increasing rapidly. The structure of mefloquine is similar to chloroquine, and their modes of action remain a mystery. Understanding 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 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 were in the past explained by the "lysosomotropic" theory of chloroquine transport, but our data can also explain the higher vacuolar uptake of mefloquine, and provides new clues to the search for new antimalarials of similar structure.

C. Iron uptake and metabolism of malaria-infected red cells and malaria parasites (D. Wititsuwannakul, S. Srichairatanakool)

Different opinions concerning the iron uptake of malaria parasite were proposed by various investigators. One group reported that malaria parasite took up iron from plasma transferrin probably by receptor-mediated endocytosis. Another group showed contradicting results, that parasitized cells did not utilize transferrin-bound iron. The bona fide iron source for parasite growth and development was investigated and reported in this study. The results obtained suggested the possibility that malaria parasite might be dependent on intraerythrocytic endogenous iron source which was very likely to be ferritin iron. One approach explored was the possible existence and utilization of specific parasite-derived low MW iron chelator (Plasmodium siderophore). This was investigated both in the cytosol of infected red cells as well as the parasite cytosol. The siderophore that might be participating in the iron uptake process of malaria was assessed and characterized as follows.

Iron uptake study of *P. berghei*-infected red cells was investigated both *in vitro* and *in vivo*. The results showed no enahnced iron uptake by the infected cells in both systems. Various sugars and other common metabolic intermediates that could chelate iron showed no effect

on the iron uptake. Possible existence and the presence of a siderophore in the lysate of infected cells was detected and evidenced in this investigation. Iron mobilization studies showed that deproteinized infected lysate could chelate the iron from CAS-Fe (III) complex as well as from transferrin. More significantly, ferritin iron was strongly chelated and mobilized by the infected lysates (both *P. berghei* and *P. falciparum*). The results strongly suggested that ferritin iron might possibly be serving as endogenous iron source for the parasite and that the infected lysates contained parasite-derived iron chelator for iron acquisition from ferritin. Organic solvent extraction of the deproteinized infected lysate showed that the siderophore was extractable with benzyl alcohol but not with ethyl acetate. The results indicated the presence of hydroxamate type siderophore in the infected lysate. Methanol/water (1:1) was found to be most suitable solvent for resolving and characterizing the plasmodial siderophores by paper chromatography and autoradiography. The R_f value of 0.44 for the infected lysate iron chelator was found to be different from that of DFO (R_f = 0.94) while the uninfected lysate remained at the origin, indicating the absence of siderophore. Iron competitive mobilization study showed DFO to be stronger chelator than that of the parasite iron chelator.

To substantiate these findings, the presence of ferritin in the infected red cell cytosol was investigated. Ferritin was assayed and shown to be present in the red cell cytosol (3-40 ag/cell). The ferritin molecule contained up to 4,500 atoms of iron which could be sequestered by the parasite. Infected lysate could strongly mobilize iron from the labeled ferritin forming iron chelator complex. The mobilization was almost complete and comparable to DFO. On the contrary, the uninfected control lysate was unable to mobilize the ferritin iron. These results supported the possibility that ferritin could serve as iron source for the intraerythrocytic parasite. It could also help explain the failure to observe iron uptake by parasitized cells from exogenous source in the plasma and in culture media. These results projected significant implication so far as intraerythrocytic parasite iron uptake and acquisition process was concerned.

D. Red blood cell Ca^{2+} transport and Ca^{2+}-ATPase in malaria infection

(D. Wititsuwannakul, K. Promsongk)

Malaria infected red cells showed an increase of intracellular Ca^{2+} content due to decreased Ca^{2+} efflux. This investigation aimed to see the effect of malaria infection on the host cell membrane Ca^{2+}-ATPase. Changes in the kinetic properties, both K_m and V_{max}, were observed on the infected membrane enzyme. Maximum Ca^{2+}-ATPase activity decreased almost 40% on the infected membrane. Iron-porphyrin containing compounds (hemin, hemozoin, ferriprotoporphyrin IX) were found to exert specific inhibitory effect on the Ca^{2+}-ATPase. The enzyme was very sensitive to inhibition by these compounds while (Na^++K^+) ATPase was found to be insensitive. The inhibition of enzyme activity might be at the calmodulin binding site on the enzyme, or alternatively the compounds might be acting as calmodulin antagonists. The inhibitory effect of these compounds on the enzyme was related to Ca^{2+} chelating effect. They caused change on both K_m and V_{max} values of the enzyme. The inhibitors seemed to have a strong binding affinity to the membrane. Moreover, the presence of specific Ca^{2+}-ATPase inhibitor in the infected lysate was also found. The characteristics of infected lysate Ca^{2+}-ATPase inhibitor were quite similar to those heme compounds. This included the maximum absorption peak at 400 nm which is the characteristic of iron-porphyrin compounds. It was shown to be the factor in the infected lysate responsible for Ca^{2+}-ATPase inhibition.

In studying possible mechanism of hemin inhibition of Ca^{2+}-ATPase, the results suggested that hemin might inhibit the enzyme by interfering with the calmodulin function. When the hemin inhibited Ca^{2+}-ATPase was assayed in the presence of varying amount of added calmodulin, the enzyme activity was restored to the full maximum level. On the other hand, addition of hemin to the calmodulin activated enzyme led to inhibition of the enzyme activity down to the basal level. Both calmodulin and hemin could antagonize or reverse the effect of each other.

To look into the interaction of malaria parasite with erythrocyte membrane Ca^{2+}-ATPase, hemozoin and its component FP were tested with Ca^{2+}-ATPase in a similar manner as the hemin study. Quantitative assays

were made for measuring the iron porphyrin content in the testing specimens. With the assay of the two membrane bound enzymes, Ca^{2+}-ATPase and (Na^+ + K^+) ATPase, malarial pigment caused Ca^{2+}-ATPase to be strongly and completely inhibited while (Na^+ + K^+) ATPase was insensitive to the inhibition. The results in this study suggested that hemozoin and FP had specific effect on Ca^{2+}-ATPase activity. Comparison between the two enzymes helped supporting the specificity observation. The degree of inhibition by hemozoin and FP was not significantly different. However, FP showed a slightly higher degree of inhibition than hemozoin on Ca^{2+}-ATPase.

Partially purified calmodulin could be shown to reverse the hemozoin or FP inhibited enzyme activity. The results suggested that the inhibitors might interfere with calmodulin interaction with the enzyme. The inhibitors seemed to exert the effect at calmodulin binding site on the enzyme in a competitive manner.

These observations helped provide the evidence ot suggest that the porphyrin structure in hemozoin, FP, and hemin played an important role in the inhibition of membrane-bound enzyme, especially Ca^{2+}-ATPase. However, it was not exactly known whether the porphyrin part in those compounds affected directly at the enzyme or at other site on the membrane. It might be possible that the porphyrin compound bind with the membrane and affect the enzyme indirectly, especially phospholipids which regulate the membrane bound-enzyme.

Comparison of the level of calmodulin between normal and infected lysates was investigated. The results showed that the Ca^{2+}ATPase activation by normal lysate was higher than the infected lysate. The activation of the Ca^{2+}-ATPase by infected lysate was lower by 35% as compared to normal lysate control. This bioassay indicated that calmodulin in the lysate after infection was lower than the uninfected control by about 35-40%. This results agreed with the previous observation that membranes from infected cells showed a 30-40% reduction in Ca^{2+}-ATPase activity. This indicated that the lysate of infected cell might be defective to a certain degree as compared to the normal lysate on control of Ca^{2+}-ATPase activities.

Calmodulin in the heated lysates was eliminated by trypsin digestion and tested on the enzyme. The results showed that Ca^{2+}-ATPase was significantly inhibited by the infected lysate while no inhibition was observed for the normal lysate. The results suggested the presence of Ca^{2+}-ATPase inhibitor in the infected lysate. This inhibitor in the infected lysate was specific for Ca^{2+}-ATPase and the enzyme was quite sensitive to the inhibition. No effect on the (Na^+ + K^+) ATPase activity was observed. After dialysis the inhibition of Ca^{2+}-ATPase by the infected lysate totally disappeared.

The study showed that the inhibitor was a small molecule present only in the infected lysate. It was assumed that the dialysable Ca^{2+}-ATPase inhibitor might be hemozoin or some form of hemoglobin degraded product. The porphyrin containing compound in the infected lysate would be expected to exert the inhibitory effect observed on Ca^{2+}-ATPase. This assumption was confirmed by comparing the absorption spectrum of various iron-porphyrin compounds Ca^{2+}-ATPase inhibitors. The results of these absorption spectrum showed similar patterns with maximum absorption at 400 nm. This absorption peak was the characteristics of iron-porphyrin compound.

The infected lysate which exerted Ca^{2+}-ATPase inhibitory effect also showed an absorption peak at 400 nm similar to hemozoin. No absorption peak at 400 nm was observed for the normal control lysate. It could be assumed that the presence of 400 nm absorption peak in the infected lysate might be the factor responsible for Ca^{2+}-ATPase inhibition. The absorption peak at 400 nm disappeared after dialysis and showed the same pattern as the normal lysate. The disappearance of 400 nm absorption in the infected lysate suggested that the inhibitor might be an iron-porphyrin containing compound. The true nature of the infected lysate Ca^{2+}-ATPase inhibitor remained to be further studied in a more refined experiment.

Better understanding on the interaction between the infected lysate and Ca^{2+}-ATPase would provide us wuth a clearer picture on alteration of Ca^{2+} metabolism upon parasite infection of the red cell. The results obtained in this study provided a partial contribution to the better understanding of Ca^{2+} metabolism in malaria parasite infected cells.

Publications

1. Vanderkooi, G., Prapunwattana, P. and Yuthavong, Y. (1988) Evidence for Electrogenic Accumulation of Mefloquine by Malarial Parasites. Biochem. Pharmacol. 37, 19, 3623-3631.
2. Suttikulpitag, S. (1989) Transport of Folate Cofactors by *Plasmodium falciparum* Infected Red Blood Cells. M.Sc. Thesis, Mahidol University.
3. Srichairatanakool, S. (1989) Iron Uptake and Metabolism in Malaria Parasite. M.Sc. Thesis, Mahidol University.
4. Promsongk, K. (1990) Studies of Erythrocyte Membrane Ca^{2+}-ATPase in Malaria Infection. M.Sc. Thesis, Mahidol University.