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Project Title: A New Approach in Design of Antimalarials Selectively Toxic to the Parasite

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Introduction:

This project began with our early observation of the *in vitro* growth-inhibition by lipophilic chelators (i.e. of iron and copper for the most part), our study of their possible mechanism of action and the role of these and other cations in malaria metabolism. Subsequently as a result of this approach we recognized the vital role of calcium to parasite metabolism and the unrecognized potential of a large number of drugs presently in clinical use for a variety of human diseases and the potential these offer in malaria chemotherapy. While the calcium study was an offshoot of the iron study, the studies are quite distinct and consequently they will be presented in two separate sections of the final progress report.

Final Progress Report

1. Lipophilic chelators and iron metabolism:

In the last few years considerable progress has been made in understanding the metabolism of the malaria parasite (1-4). Points of departure between the metabolism of the parasite and host have been described by us (5-7), several of which have the potential of yielding rational approaches to chemotherapy of malaria. Therefore, our efforts to develop new compounds which exploit these unique characteristics of parasite metabolism offer a novel approach which promises new chemotherapeutic agents where cross resistance to antimalarials currently in use would not be an immediate problem, since conventional anti-malarials appear to involve other mechanisms.

The growing malaria parasite degrades glucose almost quantitatively to lactate over 16 hours (8-9). There is no evidence of a classical Pasteur Effect in either the primate malaria parasite, *Plasmodium knowlesi* (5), or the human parasite, *P. falciparum*[1]. In addition, there is no evidence of the existence of

[1] L.W. Scheibel and A. Adler, unpublished results.

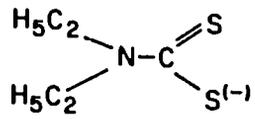
a tricarboxylic acid cycle in P. knowlesi (5,6); and optimum growth in P. falciparum occurs in vitro at 3% O_2, with good growth resulting at 0.5% O_2, but not below (7). This would suggest that in the malaria parasite oxygen does not mediate to any significant degree an ATP generating electron transport system[2], but instead participates in biosynthetic reactions through metalloprotein oxidases (10-12). These would presumably involve oxygen transferases and mixed function oxidases. This is a significant departure from the metabolism of most normal mammalian host cells.

The rationale of our approach in choosing compounds selective toxic to the intracellular malaria parasite involves lipophilic chelators known to inhibit these metalloprotein oxidase enzymes in cell-free systems (13-14). An example of this can be seen in the homolactate fermenter Schistosoma mansoni where small amounts of O_2 are required for tanning eggshells by oxidation of phenolic compounds, not energy generation (1). We, however, felt that only those compounds which exhibit: (1) lipid/H_2O partition coefficients favoring penetration and (2) high binding constants enabling them to compete effectively with naturally occurring intracellular chelators for metal ions, would be logical candidates. The alkylthiocarbamates (e.g., diethyldithiocarbamate) and the 8-hydroxyquinolines are chelators of this type (Fig. 1).

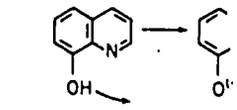
Scheibel et al. first demonstrated that growth of the human malaria parasite is sensitive to inhibition by low doses of these agents (8,9,15-17) and this antiparasitic activity depends directly on chelation. In addition, the alkylthiocarbamates (Fig. 1) and 8-hydroxyquinoline (oxine) (Fig. 1), at pharmacologic doses, inhibited the glycolysis of infected erythrocytes with little effect on the same processes in normal red cells. Several of these

[2]Similar to what is observed in tumor cells [see Boxer & Devlin, Science 134:1495-1501, 1961; Papaconstantinou & Colowick, J. Biol. Chem. 236:278-284; 285-288, 1961].

Figure 1.

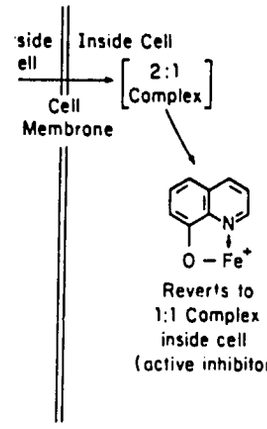


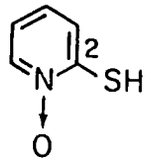
diethyldithiocarbamate



8 hydroxy quinoline
(Oxine)

But this is your original

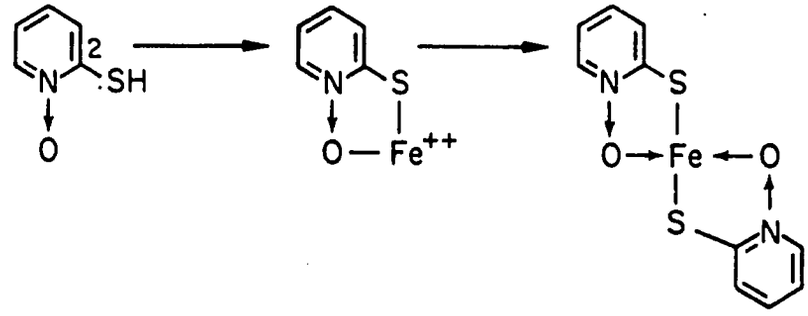


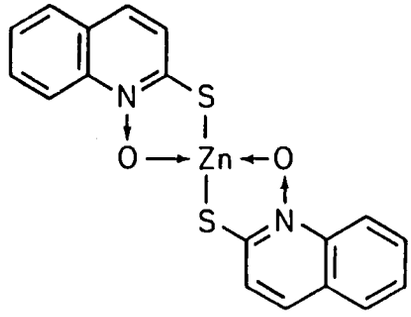


2—mercaptopyridine—N—oxide

(pyridine-2-thiol-N-oxide)

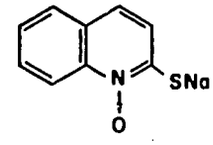
2 mercapto pyridine-N-oxide





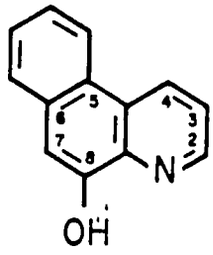
bis—2—mercaptoquinoline—N—oxide, zinc salt

(quinoline-2-thiol-N-oxide:zinc)

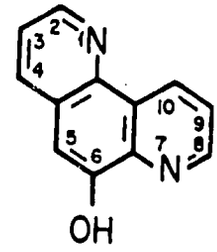


Na 2—mercaptoquinoline—N—oxide

(quinoline-2-thiol-N-oxide)



5,6 benzo-oxine



6 Hydroxy-m-phenanthroline
(6 Hydroxy-1, 7-phenanthroline)

agents are well tolerated by higher animals and are more plasmodiacidal than quinine *in vitro*, even against chloroquine resistant Plasmodia. Recently, other investigators reported the antiparasitic activity of a number of compounds capable of chelating metal ions (see 17 for references); unfortunately, the only instance where there is conclusive chemical evidence that this antimicrobial or antiprotozoal activity is directly related to chelation is with the oxine-type of chelators (9,18). Shapiro et al. (19) and Raventos-Suarez (20) postulated that selected chelators may act by blocking iron or copper utilization by the parasite thereby depriving the organism of an obligate nutritional requirement. Apparently, the intra-erythrocytic *P. falciparum* takes up iron from transferrin and, according to Pollack, it is this step that explains the sensitivity to desferrioxamine (21,22). Indeed, it has even been suggested that the clinically useful quinoline antimalarials (chloroquine, primaquine, etc.) also block iron uptake in the parasitized cell (23).

Many years ago it was proposed that the antimicrobial action of agents such as the dithiocarbamates and of 8-hydroxyquinoline was by formation of chelate complexes or precipitates with various heavy metal ions indispensable to microbial growth. Growth was thought to be prevented by inhibiting the production of essential enzyme systems containing these elements. Since then, this hypothesized mode of action of these agents has been questioned (24,25). Evidence against the precipitation theory is offered in the case of copper:8-hydroxyquinoline which has been reported to be even more active as a fungicide than oxine.

Early work by Albert (26) and later work by Scheibel (17,27) attribute a different mechanism to both the thiocarbamate and oxine chelators. Albert (28) maintains that toxic complexes are formed with metals of variable valency which are present in the medium, a mechanism he feels applies to many of these agents used in medicine. Oxine exerts its lethal effect by combining with these

metallic ions without which there is no antibacterial activity (28). The chelates formed from the reaction of oxine with these metals, e.g., iron or copper, are themselves as toxic as, or even more strongly antimicrobial than, oxine (Fig. 1) (29). This was found to be the case in P. falciparum cultures by Scheibel (15,27) with the 2:1 chelator:metal complex of quinoline-2-thiol-N-oxide:zinc. It presumably penetrates the cell as a lipophilic 2:1 complex with the metal and then reverts to a 1:1 oxine:metal complex, a true toxic agent which initiates oxidative chain reactions inactivating labile enzymes and killing the cell (Fig. 1) (30,31). The mode of action of the dialkylthiocarbamates is closely comparable with that of oxine and pyridine-2-thiol-N-oxide (32) except that the latter has a lower stability constant and lower partition of coefficient than oxine but is more active biologically being more able to liberate 1:1 complexes within the cell (30). Scheibel extended this proposed mechanism to suggest that the free chelator (liberated when the 2:1 complex reverts intracellularly to a 1:1 complex) may also exert profound biological effects (27). In fact, there are significant differences between the results found in the plasmodial system by Scheibel (27) and that by others working on bacteria and fungi, suggesting "toxic complexes" may not play the inhibitory role in malaria in vitro cultures that they have been reported to do in bacteria and fungi. The addition of copper or iron salts does not change the lethal effects of chelators against malaria; the increase in the concentration of chelators does not result in "concentration quenching" of growth curves; an increase in the lipophilicity of the chelating agent does not result, in some instances, in a proportional increase in plasmodiacidal activity (6-hydroxy-m-phenanthroline or oxine are less lipid soluble but more potent than 5,6-benzo-oxine, and pyridine-2-thiol-N-oxide is less lipophilic but more potent than quinoline-2-thiol-N-oxide) (9,15,16); (Fig. 1) and EDTA or cobalt added to

growing cultures did not antagonize the activity of chelators as had been found in other microbial systems (33). However, in agreement with Albert's "toxic complex theory" the rate of uptake by P. falciparum of 8-hydroxyquinoline: radiolabeled ^{59}Fe complexes within 6 hr under conditions similar to our original growth inhibition studies (9), occurs at doses corresponding to, or less than, those producing the lethal effects and metabolic changes (34). In addition, the presence of 8-hydroxyquinoline facilitates entry of the radiolabeled cations and uninfected erythrocytes take up less cation, especially in the absence of chelator. Consequently, while this suggests the chelator plays an important role in the increased activity on parasitized red cells, it remains to be seen how much activity is due to the chelator itself (and how much is due to the participation of iron), and what is their discrete site of action. Future studies utilizing radiolabeled oxine are necessary to answer this, particularly since recent evidence has been presented indicating that iron deficiency may not be protective in clinical malaria (35), so iron may not be as important to the parasite as once thought. [This would be at variance with Shapiro's (19) and Raventos-Saurez's (20) theory that the chelators act by depriving the parasite of obligate nutritional requirements.] In addition, Peto and Thompson have published data to indicate P. falciparum does not directly utilize serum iron and the chelator, desferrioxamine, does not inhibit the parasite by interfering with the supply of iron form the incubation medium (36). In fact other ions may play as important a role as iron. X-ray fluorometry studies show that while iron level remains constant throughout the parasite cell cycle that of zinc increases parallel with parasite maturation to reach a 2-3 fold higher level than that of uninfected red cells. Most of this gain is associated with the parasite (37). It is also known that there is an obligate calcium requirement for invasion and replication in P. falciparum and some clinically effective antimalarials function in part by inhibiting this vital cellular mechanism (38) (to be

discussed in part 2 of report). In any event, iron and iron chelators appear to play an important role in malaria metabolism and applications for chemotherapy, especially since Pollack et al. (39) demonstrated that desferrioxamine (an iron specific chelating agent) does suppress *in vivo* parasitemia in *P. falciparum*-infected *Aotus* monkeys. *In vitro* this effect is overcome by addition of iron to the culture, suggesting it is this cation (iron) which is involved.

Peto and Thompson (36) suggest chelators, such as desferrioxamine, may inhibit growth of *P. falciparum* by blocking the synthesis or action of ribonucleotide reductase, an iron containing enzyme necessary for DNA synthesis. Desferrioxamine is a potent inhibitor of DNA synthesis *in vitro* by human B and T lymphocytes, and this was associated with diminution in ribonucleotide reductase activity (40). In addition, a series of iron chelating agents (the catecholamide siderophores), parabactin, vibriobactin, GABA and Compound II are reported to have similar activity in L1210 leukemia cells (41-43) and their activity reflects their lipophilicity, as seen with 8-hydroxyquinoline chelators in bacteria, fungus, and Plasmodia. These compounds are reported to be relatively non-toxic to higher animals (44,45). This may suggest a common mechanism for a number of these agents, many of which are well tolerated by higher animals, enabling one to mount a rational approach to the problem of antimalarial chemotherapy.

In a preliminary study, these catecholamide siderophores were assessed by us for *in vitro* antimalarial activity to determine if antitumor potential might be reflected in antimalarial activity. Activity was assessed, as described in previous studies (16). The catecholamide siderophores were generously supplied by Dr. Raymond Bergeron, University of Florida, Gainesville, FL. As can be seen from Table 1, the most potent chelator appears to be vibriobactin and the least potent is reduced compound II. Antimalarial potency *in vitro* appears to

correlate with lipophilicity, in general, and it is the same order of magnitude (micromolar) as that required to inhibit L1210 leukemia cells.

Table I

Concentration (in uM) required to reduce *in vitro* growth of *P. falciparum* 50% (ED_{50}) after exposure for 2 days and 3 days

	Day 2	Day 3
Vibrobactin	4.5	1.8
Parabactin	2.6	2.3
Compound II	4.5	3.7
GABA	5.1	4.3
Reduced Compound II	28.5	25.0

It has been suggested these chelators can exist for sometime in a free state in the media during which time they diffuse into cells at a rate associated with their favorable partition coefficients (43). Vibriobactin is more lipid soluble than parabactin as indicated by an octanol-water partition constant of 65 vs 35 (41). Parabactin appears equal to GABA and in general the antileukemic and antiherpetic activity of the spermidine catecholamide (ferric) iron chelators is prevented by exogenous iron (43) similar to what is seen in the malaria system with the hydroxamic acid chelators, desferrioxamine[3], and the poor penetration seen with either rhodotorulic acid or mycobactin P complexed with iron (34). Parabactin initiates inhibition earlier than compound II also reflecting greater lipophilicity (42). This would suggest *in vitro* antimalarial activity of drugs in Table I largely reflects their lipophilicity, which in general is similar to the oxines in our earlier malaria studies (9,15,16,34).

[3]Desferrioxamine not saturated with iron is taken up by red cells infected by *P. falciparum* (Fritsch & Jung. Z. Parasitenkd 72:709-713, 1986.

There are so many redox and biosynthetic enzymes in which a metal serves as the prosthetic group that it is impossible to predict with absolute certainty which enzyme is being inhibited by these chelators. It appears, however, (at least from what is known about their mechanism in tumor cells, that the activity of these agents is associated with the inhibition of the rate limiting iron dependent enzyme ribonucleotide reductase, an obligate step in DNA synthesis. This iron containing enzyme is activated by oxygen and catalyzes the conversion of ribonucleotides to deoxyribonucleotides. This is not, however, an isolated event. A number of iron chelators such as desferrioxamine and related hydroxamates, alpha-picclinic acid, certain thiosemicarbazones as well as other types of inhibitors, such as deoxynucleosides have been shown to inhibit tumor growth by interfering with ribonucleotide reductase activity (for complete list of refs see 42). The mode of action of the anticancer agent, hydroxyurea, is believed to inhibit cell growth through inhibition of this enzyme (42,43). What makes the future consideration of these siderophore compounds so compelling is the combination of lipophilicity and very high affinity for iron plus low host toxicity. They could extract iron from critical binding sites within the cell not accessible to other agents.

Therefore, complete resolution of the mechanism of these chelators in malaria awaits the measurement of uptake and the metabolic fate of the radiolabled chelator itself. In addition, the sensitivity of ribonucleotide reductase to these agents must be measured in a cell-free system. With few mechanistic approaches to explore in the rational development of new antimalarial drugs, it has been said by Desjardins and Trenholm in their recent review of Antimalaria Chemotherapy: "the observation of such a potentially novel mechanism of antimalarial activity (metal chelators) warrants further investigation" (46).

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2. Calcium-calmodulin antagonists

Thommen-Scott, (1) and Nickell, Scheibel and Cole, (2) independently reported on the antimalarial activity of cyclosporin A (CsA). The later report resulted form an attempt by Bueding to use this immunosuppressive agent to ameliorate the formation of granulomas around *Schistosoma mansoni* eggs in the livers of infected mice (3). The unexpected *in vivo* antischistosomal effects of CsA are a consequence of hemoglobinase inhibition in the parasite (4,5).

Another antimalarial, Quinghaosu, (a natural product used by the Chinese since antiquity) is structurally distinct from cyclosporin but also produces worm reductions approaching 80% in *S. mansoni* and even greater in *S. japonicum* infections *in vivo* (6). In addition, it appears to also inhibit the hemoglobinase of this helminth (Bueding, personal communication). Paradoxically, however, Scheibel reported that neither CsA nor Quinghaosu inhibit the hemoglobinases of the human malaria parasite (7) as assayed at the 3 pH optima using either the method of Bueding et al. (4), or that of Gyang et al. (8), (also see ref. 2). It would, therefore, appear that the mechanism of antischistosomal activity is different than the antiplasmodial activity of these drugs or that other mechanisms are also involved in antischistosomal activity, or that agents such as these inhibit another vital cellular process yet to be elucidated in the malaria parasite. It would also appear antiparasitic activity of CsA is not the result of immunosuppression but is instead a direct effect of the drug on the parasite (2,7).

Calcium (Ca^{++}) has been shown to be absolutely indispensable for the normal growth of *P. falciparum* cultures, particularly during the maturation phase, 20-26 hours after invasion, and also either in the process of reinvasion or final maturation of a fully infective merozoite (9). Malarial parasites actively accumulate Ca^{++} within themselves to levels 30 times those found in

uninfected erythrocytes. The rate of influx is 7 times that which occurs in normal red cells (10,11,12). This enhancement of calcium uptake was more pronounced with increasing parasitemeia and maturity of parasites (13), and is accomplished in cells almost impermeable to this ion and against the active extrusion gradient by Ca^{++}ATPase (14). The regulation of calcium is also important to another protozoan parasite, the African trypanosome. For example, calcium influx triggers the surface coat release in antigenic variation and synergizes the action of some trypanocidal drugs (15). Calmodulin, a low molecular weight intracellular calcium binding protein present in eukaryotic cells, has been identified in three species of African trypanosomes, Trypanosoma brucei rhodesiense, T. congolense and T. vivax (16) and trypanosome calmodulin is physically and functionally distinct from that of host tissues (17). Calmodulin regulates a number general cellular responses such as contraction, transport, motility, proliferation and metabolic control, intermediary and cyclic nucleotide metabolism in particular (18). The phenothiazines, known calmodulin antagonists (19), markedly inhibit the African trypanosomes (17,20), and the mechanism of drug action is reported to be mediated through specific interaction of the phenothiazines with the pellicular, but not flagellar microtubules (20). Microtubular polymerization is regulated by calmodulin (18). Consequently, it would appear calcium uptake may play some role in the growth and metabolism of the malarial parasite and an understanding of this process may have chemotherapeutic implications. In fact, experimental evidence was published over 40 years ago to suggest quinine inhibits the growth and reproduction of the malaria parasite by reducing the amount of calcium available in the red cell (21). In addition, the malaria parasite has been shown by us to be very sensitive to inhibition by chelators which are capable of interacting with extracellular cations (22).

Since the role played by calcium in the malaria parasite is not understood, studies were therefore undertaken by us to determine the effects exerted by agents inhibitory to calcium-mediated regulatory processes such as calmodulin antagonists or calcium antagonists which inhibit extracellular calcium influx (see enclosed PNAS manuscript). We found by radioimmunoassay that free parasites contain calmodulin. Schizont-infected erythrocytes had calmodulin levels of 23.3±2.7 ng/10^6 cells compared to normals (11.2±1.5 ng/10^6 cells). Calmodulin levels were proportional to parasite maturity. Immuno-electronmicroscopy identified calmodulin diffusely within the cytoplasm of mature parasites, and at the apical end of merozoites, within the ductule of rhoptries, which may explain the calcium requirement for invasion. This process is quite sensitive to drug inhibition (23). Cyclosporin A (CsA), a drug which we demonstrated possessed *in vitro* and *in vivo* antimalarial activity (2,7,24) was reported to bind and inhibit calmodulin like the clinically useful antimalarials (i.e. quinine, mepacrine, chloroquine)[1]. We also found by electron microscopic autoradiography CsA concentrated in the food vacuole as does chloroquine and mefloquine and distributed within the cytoplasm of mature parasites. The binding of dansylated CsA to schizont-infected erythrocytes was higher than to normal erythrocytes as analyzed by flow cytometry. Kinetic analysis revealed that binding was saturable for normal and infected erythrocytes and possibly free parasites. Competition for binding existed between dansylated CsA and native CsA as well as the calmodulin inhibitor, W-7, and the classic antimalarial chloroquine. The *in*

[1]CsA was reported to be used successfully in treating human cerebral malaria patients in Viet Nam by Drs. C.R. Jerusalem & W. Eling (Nijmegen, The Netherlands). The report was given at the meeting of "Antiparasitic Effects of Cyclosporin A and Analogues" SANDOZ Ltd. Basel, Switzerland, 15 Feb. 1985.

vitro growth of *P. falciparum* was sensitive to calmodulin antagonists and in large part inhibition of the parasite was proportional to known anti calmodulin potency, suggesting there is a possible role for calmodulin in parasite cell functions and chemotherapy. Antagonism existed between combinations of these drugs in multi-drug resistant strains of *P. falciparm* suggesting possible competition for the same binding site and implicating calmodulin reactions in drug resistance. This would agree with our earlier observations that unlike many other cells, parasite cytochrome P450 does not explain drug resistance (25) in Plasmodia. In addition, the malaria parasite was also susceptible to calcium channel blockers. It would appear therefore that sequential processes (i.e. calcium flux into and out of the cell, calmodulin itself, secondary calcium-dependent enzymes) in the parasite are all viable targets of chemotherapeutic interdiction and an understanding of the relationship of these processes would aid in the proper choice of drugs and synergistic drug pairs to treat drug resistant *P. falciparum*. In addition anticalmodulin activity appears to partially explain the actions of the antimalarial drugs, quinine, mepacrine, and chloroquine as well as the well known immunomodulating effect of chloroquine in collagen vascular diseases (26).

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The following is a list of the publications and the presentations at national or international meetings as a result of my AID support. Please note that the first 11 publications and 6 presentations were a result of my work in the AID supported lab of Dr. Wm. Trager (Rockefeller University, NY) and forms the basis of the work done on this AID/SCI: 2H-01 project. There are 18 publications (16 full length) and 15 presentations resulting from AID funding on this grant.

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2. The Antimalarial Effect of Antabuse on Plasmodium falciparum. 63rd Annual Meeting, Federation of American Societies for Experimental Biology, Dallas, Texas 1-10 April 1979 (Abstracted - see Bibliography).

3. Carbohydrate and Energy Metabolism, Malaria Chemotherapy Using In Vitro Cultures. 11th International Congress of Chemotherapy and the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston, Massachusetts. 1-5 Oct. 1979 (Abstracted - see Bibliography). (Invited Presentation)

4. Effect of 8-Hydroxyquinoline and other Chelators on the Growth of Plasmodium falciparum. 75th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Marriot Hotel, Tucson, Arizona 14-16 Nov. 1979.

5. Studies on the Energy Metabolism of In Vitro Cultured Plasmodium falciparum. 80th Annual Meeting, American Society for Microbiology, 11-16 May 1980, Miami Beach, Florida. (Invited Presentation)

6. Antimalarial Activity of Selected Quinolines and Quinoline-N-Oxides. 65th Annual Meeting, Federation of American Societies for Experimental Biology, Atlanta, Georgia, 12-17 April 1981 (Abstracted - see Bibliography).

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Chairmanship: Presiding Biochemistry and Physiology, Section A, 11 August 1981.

8. New Approaches and Achievements in Malaria Chemotherapy. Joint All Staff Conferences and Isthmian Medical Society Conferences. Gorgas Hospital, Ancon, Republic of Parama, August 1982. (Invited Presentation)

9. Overview of the design and methods in the development of antimalarial drugs, including Plasmodium in vitro cultivation. Conference of Panamanian Association of Microbiology, University of Panama, School of Medicine, August 1982. (Invited Presentation)

10. Chelating Agents in the Chemotherapy of Malaria. John Jacob Abel Symposium on Drug Development: Molecular Parasitology. The Johns Hopkins School of Medicine, Baltimore, Maryland. June 13-15 1983. (Invited Presentation)

11. Protease Inhibitors and Antimalarial Effects. Malaria Workshop 6th International Conference on Red Cell Metabolism and Function, Ann Harbor, Michigan, October 2-5, 1983. (Invited Presentation)

12. *In Vitro* Inhibition of the Human Malaria Parasite by Selected Lipophilic Chelators. 6th International Conference on Red Cell Metabolism and Function, Ann Harbor, Michigan, 2-5 Oct. 1983. (Invited Presentation)

13. Session on Parasitic Diseases and Blood Transfusion. Infection, Immunity and Blood Transfusion, XVI Annual Scientific Symposium, Washington, D.C., 9-11 May 1984. (Invited Presentation)

Chairmanship: Of Session and Member of Symposium Planning Committee.

14. New Approach in Design of Selectively Toxic Antimalarials in Symposium on New Strategies in Anti-parasitic Chemotherapy. IUPHAR 9th International Congress of Pharmacology, London, England, 29 July - 3 August 1984. (Invited Presentation)

15. Biochemistry of Malaria Parasites. Herbsttagung der Gesellschaft fur Biologische Chemie, Giessen, West Germany 17-20 September 1984. (Invited Presentation.)

16. The Metabolism of the Erythrocytic Stage of the Malaria Parasite. The Albert Einstein and Tufts School of Medicine and Mass. Inst. Technol. Joint Workshop on Magnetic Resonance in the Study of Intracellular Parasitism. Marine Biological Lab., Woods Hole, Massachusetts, 15-18 October 1984. (Invited Presentation)

17. Anti Malarial Effects of Cyclosporin and Analogues, and Activity Against Parasite Hemoglobinase. Workshop on The Antiparasitic Effects of Cyclosporine (Cyclosporin A) and Analogues, Sandoz Ltd., Basel, Switzerland, 14-15 February 1985. (Invited Presentation.)

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19. High Resolution Autoradiography and Ultra Structural Effects of Cyclosporin A on Plasmodium falciparum Grown in vitro. 61st Annual Meeting of the American Society of Parasitologists. Denver, CO 7-11 December 1986 (Abstract).

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21. Comparative Biochemistry, A Major Factor in Helminth Control. U.C.L.A. Symposium: Molecular Paradigms Eradicating Helminthic Parasites. Steamboat Springs, CO 24 January - 1 February, 1987.