Pan American Health Organization

ADVISORY COMMITTEE ON MEDICAL RESEARCH

Sixth Meeting

Washington, D.C., 12-16 June 1967

Item 5.3 of the Agenda

RESEARCH IN THE UNITED STATES OF AMERICA ON DRUG-REFRACTORY MALARIA

Ref: RES 6/17
8 June 1967

PAN AMERICAN HEALTH ORGANIZATION
Pan American Sanitary Bureau, Regional Office of the
WORLD HEALTH ORGANIZATION

Washington, D.C.

RESEARCH IN THE UNITED STATES OF AMERICA ON DRUG-REFRACTORY MALARIA*

The first strain of Plasmodium falciparum demonstrated conclusively to be resistant to "standard" doses of chloroquine originated in Sabana de Torres, Puerto Wilches, Colombia. These 1961 studies of Young and his coworkers,** in neurosyphilitic patients at the Public Health facility in Columbia, South Carolina, indicated the strain also was resistant to mepacrine. Confirmatory data soon was obtained by Alving at the University of Chicago--Army Medical Research Project, where studies in malaria in volunteers had been under way since 1944.

For several years prior to these disturbing observations the University of Miami had been examining a variety of derivatives of known antimalarial drugs as a part of the Cancer Chemotherapy Program. The antimalarial activity of these derivatives was not known and in late 1961 the Army Medical Research and Development Command established a contract with Drs. R. Jones and Leo Rane to permit such studies after a preliminary examination in a subhuman test model. This was the initial step taken in the development of the current Army research effort on malaria.

In December of 1961 an individual in Southern Thailand developed chloroquine-refractory malaria. This strain was studied extensively in the two clinical units mentioned

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**References to the literature and to tables and figures will appear in the final text of this report.

earlier and in the Public Health Service unit at Atlanta, Georgia. In rapid succession came strains from other areas and by the end of 1962 it was clear that drug-refractory strains of P. falciparum existed not only in Colombia but in Thailand, Malaysia, Cambodia, and Vietnam. These careful studies in volunteers in nonmalarious areas indicated not only refractoriness to the 8-aminoquinolines but to many or all of the other synthetic drugs. Similar findings, based on clinical response of patients, had been reported from Brazil.

The historical impact of malaria on military operations and the possibility of an increase of our commitments in Southeast Asia resulted in the establishment and funding early in 1963 of a specific U.S. Army research program seeking a solution to this threat. The requirement for new antimilarials was clearly apparent but before such a program could be put into operation four important capabilities had to be developed.

The first was an expansion of the clinical facilities, since the existing two units did not have the capacity to support a major program. Additional units were established, one at the University of Missouri under the direction of Dr. John Arnold and a second at the University of Maryland, now headed by Dr. David Clyde. At the University of Chicago unit Dr. Robin Powell succeeded Dr. Alf Alving as director. These facilities, with the primary mission of characterizing new

strains of malaria and performing clinical evaluation of new agents, infection being initiated by blood or by sporozoite inoculation, are each also engaged in a variety of basic studies. These four units cannot meet the world's requirements for such studies, and the recent announcement that the Australian unit at Cairns is to be reactivated is of much importance. The physician who will be in direct charge of this unit has been with Dr. Powell for the past year.

The second major concern in 1963 was the requirement to reawaken an interest in malaria in the scientific community. To focus attention on the problems, an International Panel Workshop on Cultivation of Plasmodia and Immunology of Malaria was held at the Walter Reed Army Institute of Research in August of 1963. From these efforts developed the program in basic biology which as now constituted contains, both in-house and by contract, some thirty projects including cultivation of mammalian and avian parasites, immunology, fine structure of the parasites, mosquito taxonomy and ecology, colonization of vectors, cyclic transmission, metabolic studies, development of drug-resistant strains, and the transmission of human malaria parasites to subhuman hosts. Many of these findings have been noted in a recent publication and only those directly relevant to the structure of the present drug research program will be noted later in this discussion.

The third area which had to be developed prior to the initiation of a full-scale program was a test system capable of looking at large numbers of chemicals for antimalarial activity at a relatively small cost per chemical tested both in terms of money to do the test and the amount of chemical consumed. Such a system came as a direct outgrowth of the previously mentioned contract with the University of Miami. The theoretical superiority of a mammalian parasite test system over the earlier used avian models was obvious. Plasmodium berghei, discovered by Vincke in 1948, was chosen because it appeared to be the only mammalian parasite which could be adapted to a high volume test. This prediction has been borne out and the test, in its present form, will be described later.

The fourth basic area which had to be developed was an administrative structure to provide for the coordination and control of a large-size drug development program. This included the development of an appropriate legal mechanism whereby commercial and private concerns could participate in the program on a basis satisfactory to them and to the Department of the Army. The result of this was the so-called "no-dollar" contract which in its simplest form allows the submission of chemical compounds for antimalarial screening. The submitter retains patent rights while the Army receives the right to future procurement on a royalty-free basis. Any

structures so submitted are considered to be "commercially discreet" and their identity is protected for a reasonable period of time. There has been a steady rise in the number of participants and approximately half of the compounds thus far screened have come through such a mechanism. Also involved was the development of specialized programs and techniques to permit multiple participants to obtain information on material submitted for testing, to provide the administrative group with the mechanism for proper distribution of agents submitted for testing, and with a series of computer programs to permit the chemical categorization, sorting, comparison and retrieval of data. Happily, a similar although much smaller requirement had arisen in 1960 in another Army Medical drug development effort and many of the basic computer techniques were in advanced stages of development.

Concurrently with the development of the foregoing obvious requirements, detailed plans were made for the total drug development program. It was necessary to make an estimate about the size of the program required and about the "proper" mix of screening, synthesis, and biology. W. Mansfield Clark terminated his introduction to the history of the World War II antimalarial program in the United States with this succinct comment: "Who would dare to predict the source of the still-to-be desired agent?" Further it was obvious that to put his question in the light of current events, "agent" should be

replaced by the plural form "agents" since it appeared probable that any antimalarial might be limited to use in only certain geographical areas and be limited in the length of its useful life. There was a need for therapeutic and for prophylactic drugs. Hence a requirement clearly existed to look at any family of compounds exhibiting activity, and to seek for new leads as well.

The United States World War II program involved the synthesis of approximately 4,000 compounds. Specific data concerning the numbers of compounds examined for antimalarial activity by industry in other searches for antimalarials were not available but it was clear that many thousands of compounds had been examined for each successful drug, save for the program which had developed proguanil and paludrine based on reasoning from the sulfonamides. According to a 1945 report, with the demonstration of activity in 3349, the guanidine precursor of these compounds, "hundreds of new substances were then synthesized."

The level of research effort in other chemotherapeutic programs provided some yardsticks. Approximately 1,300 compounds synthesized in a search for antihistamine activity have been recorded in the open medical literature in addition to an estimated 4,000 to 5,000 compounds which have not been disclosed by the pharmaceutical houses. In 1960, the Pharmaceutical Manufacturers Association presented to the Kefauver

Committee a survey of the research efforts of 140 pharmaceutical companies for the year 1958 which indicated that 2,865 compounds were tested for each marketable product.

As initial guides in the synthesis program, there were, in addition to the quinolines and pyrimidines, 12 other broad chemical families with antimalarial activity, derived from the World War II program and from subsequent efforts. It was decided that these plus additional hoped-for new leads would support the rational synthesis of 1,000 target compounds per year.

With regard to the screening program, preliminary studies indicated that tests could be devised with a cost low enough to permit examination of all compounds submitted, thereby increasing the chance of developing new leads. Obviously all intermediate and target compounds from the synthesis program would be so examined.

With these parameters set, the remainder of the program could be envisioned readily. It was submitted and funded early in 1965 with an estimated annual expenditure of 10-12 million dollars. This program has the following main components:

Primary Screening. At this time three screens are in use and each operates at a cost of less than $20 per compound tested. The capability exists to handle several hundred compounds per week in each. The identity of the compound undergoing test is unknown to the investigator and multiple control samples are included in each run.

All compounds received are examined in the P. berghei-mouse system. The infection is produced by blood inoculation and, when a variety of conditions are properly controlled, deaths routinely begin on the sixth day. The data on control animals derived from some three years of on-line tests are shown in Fig . The test is performed by administering various concentrations of the drug subcutaneously in oil on the third day after blood inoculation. Any death on day 3, 4, or 5 is ascribed to drug toxicity. Prolongation of survival is taken as evidence of drug effect. Arbitrarily a compound is coded as "active" if the mice survive for 14 days or about 10 standard deviations longer than the mean death time. Blood and tissues from animals surviving 60 days are subinoculated and if no infection develops the arbitrary term of "cure" is used. Full details of survival time data are recorded so that other measures of significance can be used if desired. At the present time a compound goes on test within six to eight weeks after it has been received. Telephone reports are made on all compounds having greater than 14-day survival time with detailed machine printouts following thereafter. Thus far, the major technical error has been due to mislabeling of samples somewhere in the system so that all compounds of interest are retested using, if necessary, new procurement or resynthesized materials.

The second screen is based on Terzian's 1947 observation that drugs which interfered with the development of the sexual forms in the mosquito frequently are active against tissue schizonts in man. The test system shown in Fig is performed by the Insect Control & Research Inc. This system uses P. gallinaceum in Aëdes mosquitoes and involves the administration of the candidate drug in a sucrose solution, through an eye dropper suspended on the top of a screened cage housing mosquitoes. These mosquitoes must take the sucrose solution in order to obtain nutrition and water. They then are starved for two days in order to induce them to bite. Following a blood meal on a malarious bird or mammal they are returned to the sucrose drug solution for a period of 14 days. At this time they are dissected and the numbers of sporozoites and oocysts within the stomach are counted. For selected compounds an identical test is run in Anophelines by Terzian and by Powell using the human malarial strains of interest. It, therefore, is possible, for the first time, to bring into the laboratory a test system which examines a candidate compound at the 25 mg level for its effectiveness against human malarias. The basic mosquito-gallinaceum system is currently operating at the rate of 300 chemicals per week.

The third prime test system also requires 25 mg of drug per test. P. berghei infected erythrocytes are incubated in vitro for a short time and the ability of the test compound to modify various metabolic activities is determined. These

include the synthesis of nucleic acids, of proteins, of lipids, the rate of consumption of carbohydrates, and the rate of production of free amino acids. This system will probably not produce interpretable data until most of these parameters are run simultaneously. With these automated techniques an eventual throughput of 2,000 structures per week is anticipated.

Secondary Test Systems. As a standby the primary rodent screen is duplicated at another laboratory. Compounds of interest also are administered orally and subcutaneously with a determination of the influence on parasitemia. Strains of P. berghei made resistant to chloroquine, to triazines, to sulfones, and to pyrimethamine are also available. Tests are run in the chick using P. gallinaceum to permit a comparison with World War II findings. Finally, tests with P. knowlesi and P. cynomolgi in primates are utilized.

It seems safe to predict that this pattern of secondary (and even the primary) testing will undergo change rapidly due to developments coming from the basic biology program. Cyclic mosquito-mouse passage of P. berghei has been established in Most's laboratory and the activity of various selected compounds is under examination. There are obvious, but probably not insurmountable, problems in adapting this system to large-scale testing, but if the present promising findings continue this effort will be made.

Most, if not all, of the present strains of drug-refractory P. berghei require continuous drug pressure to maintain this resistance and are transmitted only by blood inoculation. It is not known whether such laboratory strains can be used to provide meaningful predictions; and indeed there is some evidence to indicate, in the case of the 4-aminoquinolines, that the mechanisms of resistance in the human and in the animal strains are not identical. The "naturally" chloroquine-refractory strain of P. berghei yoeli reported recently by London workers may be much more useful and cyclic transmission of this refractory strain has been established.

Drug-refractory P. falciparum strains are being maintained in the splenectomized chimpanzee and our unit in Bangkok has established drug-refractory P. falciparum infections in the gibbon (Hylobates lar) using either blood or sporozoite inoculation. This was followed by the demonstration by Young et al. that the Central American night monkey (Aotus trivirgatus) is susceptible to infection with P. vivax, an observation confirmed by Geiman. The latter investigator recently has succeeded in establishing both drug-sensitive and drug-refractory strains of P. falciparum in Aotus. Both splenectomized and intact animals are susceptible. High levels of parasitemia associated with morphologically mature gametocytes are obtained, thus providing the possibility of a direct test of drug activity against the parasite of primary concern. Of almost equal interest is that this system provides an excellent source of cells parasitized

with P. falciparum to be used for in vitro studies. Geiman has continued such an approach and now has a system which will permit maturation of erythrocytic parasites in a serum-free medium of defined chemical composition, excluding, of course, the presence of malaria parasites and of the mammalian erythrocytes. A continuous tissue culture line of P. fallax has been developed by Huff. Drug effects can be examined in these in vitro systems by changes in metabolism, alterations in numbers of parasites, and by morphologic alterations. There are a variety of other interesting findings coming from the basic biology program but the foregoing have been selected for the present discussion because of their direct bearing.

Chemistry. The chemical synthesis program is designed to exploit leads deriving from any source. The synthesis of compounds is sponsored only after activity in allied compounds has been confirmed. The average cost per compound is about $2,500 and is approximately the same whether the work is performed in an academic environment, by a nonprofit research organization or a company organized for profit. There are some 80 contracts now in existence in this endeavor. The categories of compounds under study are shown in Fig . For this portion of the program we have in the past and continue to rely on the advice provided by the Chemistry Advisory Committee currently chaired by Dr. Kenneth E. Hamlin, Jr.

The second phase of the chemistry program involves the production of selected chemicals in 3-kilogram amounts to permit preclinical and clinical study. Coupled with this is the capability to perform independent assays for purity and stability as well as the development of appropriate methods of formulation.

Pharmacology. There are two principal objectives--the first is to guide the synthesis effort in obtaining active chemical compounds without adverse side effects, and the second to obtain the initial safety and toxicological information on new compounds. Promising chemical compounds are examined for undesirable side effects using various intact animal test systems and standardized isolated organ systems. In addition, information on metabolic pathways, systemic distribution, gastrointestinal absorption, rate and route of excretion essential in the design of candidate chemical compounds is sought, using radiolabeling and other conventional pharmacologic approaches. The isolation and determination of active metabolic products of candidate drugs in higher animals is an essential component. Any drug considered for eventual testing in man is extensively and critically studied in appropriate mammalian systems, including the higher primates. These include determination of dose requirements, duration of effects, and associated pharmacologic responses. Combinations of candidate drugs are also evaluated.

Thus far in describing the pharmacology program the approaches are to a degree identical to those used to examine any new candidate drug for any purpose. Because of the known effects of certain antimalarials the program also includes intensive examination of the influence of new compounds on the folic acid metabolism of the host and of the parasite. The interest in quinoline and phenanthrene methanols has demanded the development of extensive test systems to examine the photosensitization capacity of these compounds. Currently this includes in vitro tests for inhibition of yeast growth when the colonies are exposed to drug and light, the response produced in normal and in hairless albino mice, a preliminary skin patch test in man, and, finally, drug administration to black and white swine. It is too early to make a prediction as to whether these potent antimalarial compounds can be tailored in such a manner as to retain activity against the parasite and yet be devoid of their very long-term photosensitizing effect.

In this portion of the program we rely heavily on the recommendations of our Pharmacology Advisory Committee, currently chaired by Dr. Walter Modell.

Clinical Evaluation. Eventually each candidate antimalarial must be examined in man. The well-recognized hazardous nature of the clinical illness of drug-resistant falciparum malaria makes exceedingly competent clinical supervision

mandatory. The clinical facilities have been described earlier and the approach is based on World War II experience.

The clinical studies fall into three categories. The first are evaluations of the candidate drugs for their pharmacologic effects in man so that, if necessary, an effort can be made to eliminate undesirable side effects by molecular modification. These initial clinical studies are designed to reflect information back into the chemical synthesis and pharmacology programs.

The second category consists of the crucial testing of candidate drugs in a limited number of volunteers infected with drug-refractory falciparum malaria. A fully responsive strain of P. falciparum from Uganda is available for study if desired as is the Chesson strain of P. vivax. Infection with these several strains is induced either by blood or sporozoite inoculation depending on the anticipated action of the test compound.

The third category is a long-term evaluation of an apparently acceptable drug prior to its general use. Drugs that pass the first preliminary clinical phases for both toxicity and effectiveness require at least two years of intensive clinical and field evaluation before they become available for general use.

From studies in these clinical centers and in the field in Southeast Asia has come the Army's current method of

treatment of P. falciparum. This employs quinine for a 14-day period with the concurrent administration of 50 or 75 mg of pyrimethamine for the first three days. This produces a cure rate of over 90 per cent in P. falciparum cases treated in Vietnam. (Chloroquine, in a "standard" dose, used under similar circumstances produces a cure rate of about 50 per cent and does not control clinical disease in 10-15 per cent of the patients.) Diaminodiphenylsulfone, in daily doses of 25 mg, has been shown to be a useful and safe suppressive drug when combined with weekly chloroquine-primaquine administration. The long-acting sulfonamides combined with pyrimethamine have a demonstrated curative effect. 2,4,diamino-5-(3',4',5' trimethoxy-benzyl)pyrimidine (Trimethoprim) alone, or in combination with pyrimethamine, is effective for the control of clinical disease. More recently has come additional preliminary evidence that the weekly administration of primaquine will markedly reduce the capacity of a drug-refractory P. falciparum gametocyte carrier to infect mosquitoes. Properly used, this would appear to offer a method of significantly reducing the possibility of introducing such strains into controlled areas.

In concluding this presentation, recognition must be extended to the numerous investigators in government, in universities, and in industry whose participation makes the current program. With the exception of the work attributed

to other government agencies and the report on *P. b. yoeli*, all investigations noted in this summary are directly supported by the U. S. Army Medical Research and Development Command through some 160 contracts. About 20 per cent of the scientific work and all of the administration is in-house, principally in the Walter Reed Army Institute of Research including those components located in Saigon, Bangkok, and Kuala Lumpur. Dr. David P. Jacobus plays a major role in the screening, chemistry, and data handling components. Dr. Elvio Sadun has the responsibility for much of the supporting biology, and the late Dr. Donald B. McMullen contributed in a very material way to the overall scientific administration. Numerous investigators throughout the world have provided the various human and animal malaria strains, and the paramount importance of the contribution of the prisoner volunteers is obvious. The program is supported actively by the Armed Forces Epidemiological Board through the Commission on Malaria.

For readily apparent reasons this program to the present has been preoccupied with Southeast Asia. The previous speakers have summarized the importance of drug-refractory malaria in this hemisphere. This opportunity to describe our current program is much appreciated, since it is our belief that there is much to be gained by the active participation of appropriate agencies in your several countries at both the basic and the applied level of investigation.

Summary of Group Means - University of Miami

Control Groups

	No. of Groups	Mean	Standard Deviation
Jul - Dec 63	55	6.57	.36
Jan - Jun 64	61	6.86	.43
Jul - Dec 64	149	6.83	.58
Jan - Jun 65	62	7.00	.49
Jul - Dec 65	50	6.64	.38
Jan - Jun 66	58	6.36	.32
Jul - Sep 66	13	6.32	.20
Total	448	6.73	.51
All Males	211	6.73	.48
All Females	236	6.73	.53

Units are survival times in days

Fig. 1

SUMMARY OF SYNTHESIS AREAS

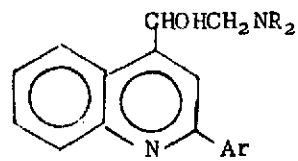
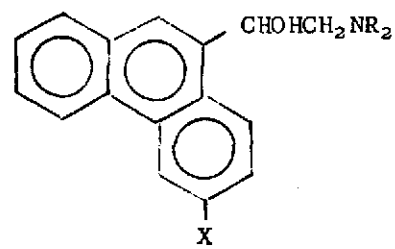
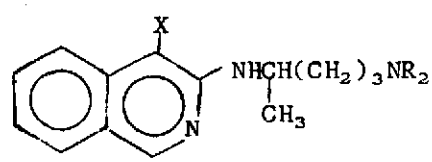
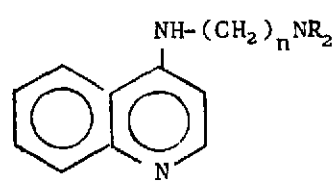
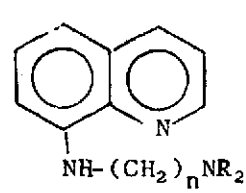
TYPE	STRUCTURE	% OF EFFORT
Quinoline Methanols and Analogs		15
Phenanthrene Methanols		3
Isoquinolines		2
4- and 8-Amino Quinolines	 	6

Fig. 2. a.

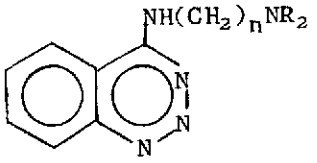
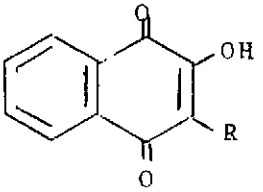
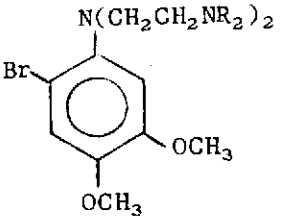
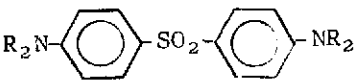
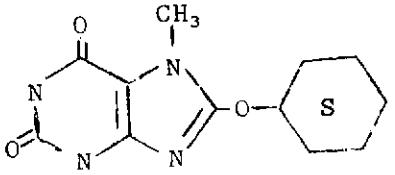
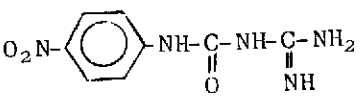
TYPE	STRUCTURE	% OF EFFORT
Azaquinolines		2
Quinones		7
RC-12 Analogs		3
Special Element Compounds	Sn, P, Si	4
Sulfones		5
Terephthalanilides and Caffeine Analogs		4
Urea and Biguanide Analogs		2

Fig. 2. b.

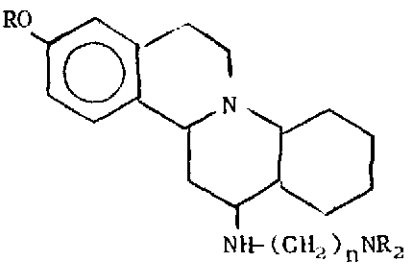
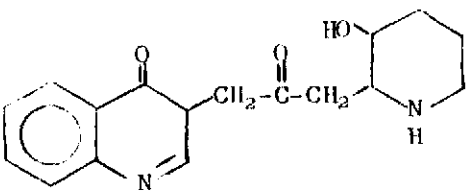
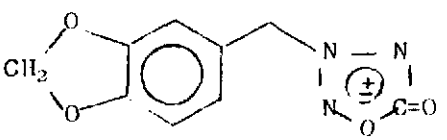
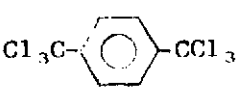
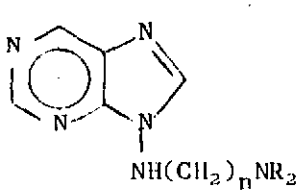
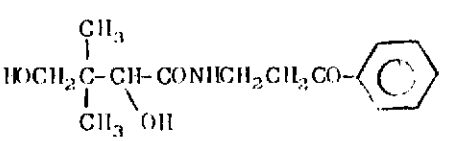
TYPE	STRUCTURE	% OF EFFORT
Bridged N Compounds		1
Febrifugine Analogs		4
5-Membered Ring Heterocycles		10
Polyhalogen Compounds		2
Purines, Pyrimidines Pyrimethamine Analogs		14
Pantothenic Acid Analogs		2
Miscellaneous Triazines Pyridazines Pentadieneoic Acid Analogs Diphenyl Amines Benzothiazoles Azepines Folic Acid Analogs		15

Fig. 2. c.

SUMMARY OF CONTROL DATA - INSECT CONTROL RESEARCH

Control Group	Mosquitoes Per Group	No. Of Groups	Mosquito Mortality At Dissection Day	Oocysts	Sporozoites
10% Sucrose Controls	Approx. 30	34	Mean 3 Range 0 - 14	0 = 0 1 - 10 = 5 10+ = 277	All Positive
0.01% Proguanil	Approx. 30	34	Mean 4 Range 0 - 9	0 = All 1 - 10 = 0 10+ = 0	All Negative

Fig. 3