# Army Malaria Institute – its Evolution and Achievements Third Decade (2nd Half) : 1990-1995

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## Abstract

The second half of the third decade (1990-1995) after the establishment of the Army Malaria Research Unit was characterised by substantial progress in meeting the challenges posed by drug-resistant malaria. In view of the rapid emergence of drug resistance, laboratory/field studies were carried out to develop novel and improved methods to assess and monitor changes in patterns of parasite susceptibility to antimalarial drugs. The high malaria attack rates observed during training exercises in Papua New Guinea (PNG) in 1988/1989 underscored the urgent need to provide better protection for soldiers deployed to malarious areas. This led to doxycycline substituting pyrimethamine/dapsone (Maloprim®) for prevention of falciparum malaria, but relapsing vivax malaria remained a problem. Laboratory investigations with tafenoquine indicated that this long-acting drug could eventually replace the cumbersome primaquine eradication course for prevention of vivax malaria. Furthermore, alternative antibiotics and halofantrine might be able to be used for prevention and treatment of falciparum malaria. Other studies showed that, apart from acting synergistically with dapsone, proguanil and PS-15 (experimental antifolate) potentiated the activity of atovaquone (new antimalarial) against drug-resistant parasites. Further investigations with Mannich base compounds revealed some of them to be more potent than amodiaquine or pyronaridine. Personal protection measures against insect bites were expanded by testing the effectiveness of various formulations of insect repellents when applied to skin, uniforms and bednets. After completing the survey of *Anopheles farauti* in northern Australia during the previous quinquennium, its spatial distribution was analysed in relation to detailed topography and historical climate records. Further anopheline surveys were performed in the Torres Strait and four PNG provinces to enhance future malaria control activities in these areas.

## Background

The malaria situation continued to deteriorate in many tropical areas of the world during the mid- to late- 1980s.[1] In fact, most countries had made little progress in controlling malaria during the two decades following the establishment of the small Army malaria research laboratory in 1965.[2,3] The ability of developing countries to control malaria was hampered by the slow pace of development, rudimentary health infrastructures and various administrative, social and technical problems. Parasites were also becoming increasingly resistant to antimalarial drugs. This was facilitated by the widespread movement of people looking for work, food or a peaceful environment, and the proliferation of mining, agricultural or deforestation projects. Although chloroquine resistance was considered to affect only *Plasmodium falciparum*, the Army Malaria Research Unit (AMRU) documented in 1989 that *P. vivax* could also develop resistance to this drug.[4]

Significant progress was made at AMRU during the first half of the third decade (1985-1990) in using *in vitro* cultivation of *P. falciparum* for identifying the presence and degree of parasite drug resistance and for screening the activity of potentially useful antimalarial drugs.[5] In addition, the introduction of high performance liquid chromatography (HPLC) technology for measuring drug concentrations in body fluids and of the bioassay for estimating serum/plasma drug activity greatly increased the ability of AMRU to undertake investigations concerning the *in vivo* effectiveness of various drugs and drug combinations.[5] In fact, the use of these newly-established procedures suggested that daily proguanil (Paludrine®, 200 mg), in combination with a low dose of dapsone (diamino-diphenyl sulfone, 10 mg), might be more effective against drug-resistant malaria than a standard weekly regimen of pyrimethamine (12.5 mg)/dapsone (100 mg) (Maloprim®) prophylaxis. This was subsequently

confirmed in a field trial conducted in a malarious area of Papua New Guinea (PNG) during 1987/1988.[5]

During and after a 3-4 week field deployment of 163 Australian Defence Force (ADF) personnel to PNG in 1988/1989, about 6% of them developed falciparum malaria and about 20% developed vivax malaria.[6] Not only was weekly pyrimethamine/ dapsone/ chloroquine no longer providing adequate protection against falciparum malaria, but the 14-day (42-dose) primaquine eradication course was unable to prevent acute attacks of vivax malaria in an unacceptably high number of soldiers following their return to Australia. Alternative prophylactic regimens were obviously needed. Although favourable results had been obtained with proguanil plus low-dose dapsone, this drug combination was not registered for clinical use. Two other drugs – doxycycline and mefloquine – were registered, the latter only in 1988. Consequently, 224 Australian soldiers volunteered to take one of these two drugs while in PNG. Preliminary results showed that both drugs suppressed the blood stages of *P. falciparum* and *P. vivax*, and that both were potential candidates to replace pyrimethamine/dapsone/chloroquine for malaria prophylaxis.[6]

Studies were also carried out with anopheline mosquitoes and non-human primates to determine their susceptibility to different strains of *P. falciparum* and *P. vivax*.[5] Routine malaria transmission would provide suitable models for assessing the activity of experimental antimalarial agents against different stages of the human malaria life cycle.

The survey of anopheline mosquitoes in northern Australia, spanning a period of 6 years, was completed in 1990.[5] This was the most extensive characterisation of malaria vectors in a region that remains receptive to the re-introduction of malaria. During earlier field investigations with *Culicinomyces*, another mosquito pathogen – *Amblyospora* – had been discovered parasitising *Culex annulirostris*.[5] The complete life cycle of this microsporidian parasite in mosquitoes and its copepod intermediate host was able to be established and the feasibility of using it as a biological control agent was explored. However, the complexity of the parasite-host system, coupled with technical problems relating to larger-scale production, precluded the use of *Amblyospora* as a biological control agent for mosquitoes, at least at that time. Studies were also initiated to evaluate alternatives to the standard ADF mosquito repellent because it was not particularly well accepted by soldiers in the field and, besides, did the repellent require such a high deet concentration (95%)? The investigations showed the potential value of alternative repellent approaches to reduce contact with mosquitoes and chigger mites, including the impregnation of uniforms and bednets with permethrin, a synthetic pyrethroid compound.[5]

### Staff and facilities

Professor Karl Rieckmann continued to direct activities at AMRU and was ably supported by Deputy Director and Commanding Officer, Lieutenant Colonel Anthony Sweeney. During this time, the Unit was privileged to receive the advice and support of the Army Malaria Research Advisory Board (AMRAB), chaired by the Director General of Army Health Services (DGAHS).

*Figure 1. Army Malaria Research Advisory Board (1995)*

*Standing (L to R): LTCOL A. Gill, LTCOL A.W. Sweeney, COL M. Heugh, X, Prof J. Egerton.*

*Sitting (L to R): Prof W.J. O' Sullivan, BRIG B.G. Stevens, Prof K.H. Rieckmann, BRIG P.T.R. Buckley (DGAHS), Prof T.C. Sorrell.*

Pharmacological investigations were somewhat curtailed because of the unexpected re-assignment of Major Robert Veenendaal to Canberra in 1990. Although Major Michael Edstein started a 3-year exchange posting at the Armed Forces Research Institute of Medical Services (AFRIMS), Bangkok in the same year, he was able to make important pharmacological contributions to drug studies of mutual interest to AMRU and AFRIMS. In 1991, Captain Anthony Yeo (Medical Officer) started a 3-year assignment during which he examined the activity of various drugs and drug combinations on *P. falciparum*. With the further development of close ties with the US Army, Lieutenant Colonel G. Dennis Shanks was posted by the Walter Reed Army Institute of Research (WRAIR) to AMRU in 1992 for 3 years. During that time he was involved with malaria field studies and provided clinical support and advice to the ADF regarding malaria. Also in 1992, Major Steve Frances replaced Major Edstein at AFRIMS with a 3-year exchange posting to identify insect repellents providing improved protection against mosquitoes

and chigger mites. Ms Barbara Kotecka and Major Robert Cooper continued their studies investigating the *in vitro* activity of antimalarial drugs and the distribution/speciation of anopheline mosquitoes, respectively.

During this quinquennium, various overseas investigators spent several weeks to years at AMRU and contributed significantly to malaria research activities. Sponsored by the World Health Organization, Australian International Development Assistance Bureau, or Rotary Against Malaria, they included Mr George Taleo (Vanuatu), Dr Li Xiuhong and Dr Tian Liping (China), Prof Bui Dai, and Dr Nhuyen Thi Nhu Mai (Vietnam). With the wholehearted support of AMRAB, the scope and impact of research activities were advanced substantially through collaboration and interaction with other national and international institutions (Table 1).

**Table 1. List of institutions and individuals collaborating with the Army Malaria Research Unit.**

Australian National University, John Curtin School of Medicine, Department of Medicinal Chemistry, Canberra, Australia. (Dr. G. Barlin)

Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. (Dr T. Brewer, Dr. G. D. Shanks)

Australian International Development Assistance Bureau, Canberra, Australia. (Vietnam-Australia Malaria Control Project)

Center for Disease Control, Atlanta, USA. (Dr. W. Collins)

Gorgas Memorial Laboratory, Panama City, Panama. (Dr. O. Nicanor)

Jacobus Pharmaceutical Company, USA (Dr. D. Jacobus)

ICT Diagnostics, Sydney, Australia. (Dr. M. Garcia)

Ministry of Health and Medical Services, Honiara, Solomon Islands. (Dr. N. Kere)

National Health and Medical Research Council, Canberra, Australia. (Dr. G. Davis)

Ok Tedi Mining Limited, Tabubil, PNG. (Dr. P. Spicer, Dr. J. Schuurkamp)

Papua New Guinea Institute of Medical Research, Maprik, PNG. (Dr. B. Genton, Mr. K. Lorry)

Rotary Against Malaria, Sydney, Australia. (Dr. B. Hanley)

Solomon Islands Malaria Training and Research Institute, Honiara, Solomon Islands. (Dr. B. Bakote'e)

University of Sydney, Department of Biochemistry, Sydney, Australia. (Dr. R. Christopherson)

US Army Malaria Research Institute, Nairobi, Kenya. (Dr. G.D. Shanks)

US Naval Medical Research Unit No. 2, Jakarta, Indonesia. (Dr. K. Baird)

Walter and Eliza Hall Institute, Melbourne, Australia. (Dr. J. Reeder)

Walter Reed Army Institute of Medical Research, Experimental Therapeutics Division, Washington DC, USA. (Dr. W.K Milhous)

Wellcome Research Laboratories, Bangkok, Thailand. (Dr. N. White)

World Health Organization, Geneva, Switzerland

World Health Organization, Western Pacific Region, Manila, Philippines. (Dr. Matsushima, Dr. D. Parkinson, Dr. K. Palmer)

### Malaria situation

Malaria continued to be a problem for Australians travelling overseas because of parasite resistance to an increasing number of antimalarial drugs.[7] The high incidence of malaria in Australian soldiers during 1988 and 1989 declined dramatically following the replacement of weekly pyrimethamine/dapsone by daily doxycycline (100 mg) at the start of the 1990-1995 quinquennium. During the deployment of almost 2,000 Australian soldiers to Cambodia, Somalia and Rwanda, only 8 soldiers developed malaria while on daily doxycycline prophylaxis.[8] This was probably related to inadequate compliance rather than parasite resistance to doxycycline. The antibiotic was usually well tolerated by soldiers taking it with food and protecting themselves from sunlight. Between 1-2% of soldiers were placed on weekly mefloquine (250 mg) prophylaxis because of gastrointestinal intolerance, sun-sensitisation, or contraindications precluding the use of doxycycline.

Although mefloquine was recommended widely as first-line malaria prophylaxis in drug-resistant areas, AMRU preferred the use of doxycycline. In addition to killing asexual blood stages, the tetracyclines acted against the pre-erythrocytic liver stages of *P. falciparum*,[9] a feature not shared by mefloquine. A further reason to use doxycycline was mefloquine's exceedingly long pharmacological half-life which would favour the development of parasite resistance to the drug if it were used extensively for malaria prophylaxis. In fact, it was already no longer able to suppress or cure falciparum malaria in some parts of Cambodia and Thailand. For example, during the deployment of various military contingents to the United Nations Transitional Authority in Cambodia (UNTAC), mefloquine was ineffective in protecting soldiers from countries other than Australia against falciparum malaria. On the other hand, only 2 of 500 Australians on doxycycline prophylaxis developed malaria during their deployment to Cambodia during 1992. Because far more malaria was observed in other contingents, prophylaxis with doxycycline, introduced in Cambodia by the Australian contingent, was later adopted as the standard malaria prophylaxis by UNTAC. The value of doxycycline was further proven by the low malaria attack rates observed by ADF personnel during deployments to Rwanda and Somalia.[8]

### Activities

The emergence of chloroquine resistance in *P. falciparum* along the Thai-Cambodian border in the late 1950s led to world-wide efforts to develop alternative drugs for the prophylaxis and treatment of falciparum malaria.[10] In the meantime *P. vivax*, the other main species infecting humans, remained susceptible to chloroquine everywhere. However, the landmark discovery by AMRU in 1989 that *P. vivax* had developed resistance to chloroquine in PNG[4] was a reminder that parasites and vectors in Australia's neighbourhood could be appreciably different from those encountered elsewhere in the world. Furthermore, the large number of ADF soldiers who developed falciparum and vivax malaria in 1988/1989[6] indicated the need for more effective alternative drug regimens and improved measures to control and avoid contact with anopheline mosquitoes.

With this in mind, more and more of AMRU's activities during this quinquennium were focussed on addressing practical problems confronting the ADF. Research activities were usually performed under one of the following broad categories:

1. Drug resistance and diagnosis;
2. Drug development and evaluation; and
3. Vector control, biology and geographical distribution.

### Drug resistance and diagnosis

#### Field assessment of drug resistance

Assessment of parasite resistance to drugs in Australian soldiers while deployed overseas or after return to Australia was proving difficult to carry out for a variety of operational reasons. However, in view of the rapid changes in patterns of parasite susceptibility to drugs, it was important to obtain information regarding the degree and extent of drug resistance from malarious areas of possible future deployment.[11,12] Not only could this be helpful in the formulation of more appropriate prophylactic regimens, but medical personnel in the field would be alerted to the possibility of treatment failures, thereby guiding them in the selection of alternative drug regimens. To meet these objectives, studies were initiated in collaboration with other institutions to determine the parasite susceptibility of parasites from different geographical areas using both established and novel procedures. The findings would of course also assist local health authorities in controlling malaria more effectively.

- Assessment of drug resistance needed for optimum prophylaxis and treatment

#### Drug resistance in PNG

Assessment of the drug resistance pattern in PNG was commenced in collaboration with the PNG Institute of Medical Research (PNGIMR) facility at Maprik, Sepik Province. Using the WHO *in vitro*

microtest,[13] about three-fourths of the isolates were found to be resistant to chloroquine, with one-fourth of them also being resistant to amodiaquine. Another significant finding was the greatly reduced susceptibility of about one-third of the isolates to pyrimethamine, indicating that a substantial number of non-immune patients were no longer being cured by the current standard treatment used in PNG, viz. 3 days of quinine and a single dose of sulfadoxine/pyrimethamine. On the other hand, the 10-fold greater parasite susceptibility to cycloguanil, the active metabolite of proguanil, supported earlier findings[5] that proguanil, used in combination with dapsone, should be evaluated for the treatment and prophylaxis of malaria. Subsequent examination of these specimens at the Walter and Eliza Hall Institute (WEHI) found no point mutations in dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) genes in any of the 13 patients who were found to be sensitive to pyrimethamine and proguanil by the *in vitro* test.[14] The findings confirmed the value of the *in vitro* field test whenever local circumstances render it difficult or impossible to use highly sophisticated (and expensive) genotyping technology to determine the parasite susceptibility to antifolate drugs.

Parasite susceptibility to various drugs using the *in vitro* test[13] was also determined at Ok Tedi Mining Limited (OTML) located in a highland area of the Western Province of PNG. There was no malaria transmission in the township, but many employees, recruited from different areas of PNG, became ill with malaria after their 3-week annual vacation to their home provinces. In collaboration with the Medical Department of the company, AMRU was able to document the widespread prevalence throughout PNG of *P. falciparum* resistance to chloroquine, amodiaquine and pyrimethamine. Furthermore, *P. vivax* resistance to chloroquine was common, especially so in the island provinces. However, this species remained sensitive to amodiaquine, pyrimethamine and cycloguanil.[15]

- Widespread parasite resistance to standard antimalarial drugs in PNG demonstrated by the *in vitro* test

#### Field *in vitro* test using disposable environmental bag

In many malarious countries, specially-prepared gas mixtures needed to support the growth of parasites *in vitro* are not readily available, or are unaffordable, or the field incubators are too small to accommodate standard environmental chambers. These problems were partly circumvented in the WHO microtest kit by the use of candle jars in which pure paraffin candles are able to generate a suitably $O_2$-depleted and $CO_2$-enriched environment.[13,16] As the "candle-jar" was more bulky and expensive than other items in the kit, investigations were carried out to determine whether disposable plastic environmental bags, used by many hospital and pathology laboratories to culture other micro-organisms, might facilitate assessment of the drug susceptibility of malaria parasites. After examining various bags, the Becton Dickinson Bio Bag type C was found to be very satisfactory, with parasite maturation being comparable to that obtained in the specially-prepared gas mixture (5% $O_2$, 5%$CO_2$ and 90% $N_2$) and superior to that obtained in the candle-jar.[17]

- Disposable environmental bag used successfully for the *in vitro* test

#### Bioassay for field use

As was pointed out previously,[5] the bioassay developed at AMRU could be employed in various ways to complement drug analysis of serum/plasma specimens by HPLC, and it was also useful for assessing drug resistance under field conditions.

After earlier development of a bioassay for cycloguanil,[5] further investigations at AMRU produced bioassays for mefloquine,[18] artemisinin,[19] and chloroquine.[20] During more detailed studies with chloroquine, it became clear that the bioassay measured the total antimalarial activity of the parent drug and its active metabolites, desethylchloroquine and bis-desethylchloroquine, whereas HPLC analysis measures parent drug and metabolite concentrations.[20]

In collaboration with the Department of Health, Vanuatu and the Oxford Group, the bioassay was used in 1993 to determine whether observed treatment failures were due to true drug resistance or whether they were related to difficulties in administering chloroquine to children under field conditions[21] (young children dislike the bitter taste of chloroquine and, in understaffed clinics or hospitals located in endemic areas, it is often difficult to supervise adequate ingestion of the entire treatment course). The findings showed that most children who had a recurrence of parasitaemia after treatment also had much lower-than-average chloroquine plasma concentrations. This demonstrated the value of the bioassay, used in conjunction with the *in vitro* microtest, to exclude inadequate ingestion/absorption before ascribing treatment failures to drug resistance. A distinct advantage of the bioassay was that only small amounts of blood (usually finger-tip specimens) were required for assessment. Furthermore, this assay could be employed in many malarious areas where HPLC equipment, expensive reagents and specially-trained staff are often not available.

As part of the medical support to the Australian contingent of UNTAC, the bioassay was also used to determine chloroquine concentrations in plasma specimens obtained from 26 Australian soldiers on chloroquine plus doxycycline for malaria prophylaxis.[22] In addition, plasma chloroquine and doxycycline concentrations were monitored by HPLC analysis. Although the bioassay relies on the measurement of total antimalarial plasma activity, chloroquine equivalent concentrations could still be measured by the bioassay (with similar results to HPLC analysis) despite the presence of doxycycline in the plasma samples. The reason for this was that chloroquine is a rapidly-acting drug which inhibits schizont maturation during the first 30 hours of incubation (endpoint of the *in vitro* test), whereas therapeutic concentrations of doxycycline have a delayed effect upon parasite growth and maturation which only becomes apparent after 3 to 4 days of incubation.[23] This is in accord with doxycycline's mode of action against the *P. falciparum* apicoplast, with a loss of apicoplast function occurring late in the life-cycle with a delayed inhibition of protein synthesis. These results suggested that the bioassay could be used successfully in the field to estimate the concentration of rapidly-acting drugs when administered in combination with slower-acting ones.

- Bioassay used to determine drug resistance in Vanuatu and Cambodia

#### Non-microscopic malaria diagnosis

Definitive diagnosis of malaria can only be established by microscopic examination of blood films. However, in highly synchronous infections of *P. falciparum*, parasites are sometimes missed if blood is not examined during the first half of the 48-hour asexual blood cycle before they disappear from the peripheral blood circulation into the brain and other organs.[24] But more commonly, diagnostic problems are due to technical difficulties experienced in identifying parasites during routine examination of blood films, particularly at low parasite densities. Although this can be partly remedied by special training in malaria microscopy, the availability of a non-microscopic test would be a distinct advantage. In 1994, ICT Diagnostics developed the ICT Malaria Pf test card which used an immuno-chromatographic approach to capture Pf Histidine Rich Protein-2 antigen by antibodies specific for this antigen.

The first field evaluation of this test card was carried out by AMRU in collaboration with ICT Diagnostics, Sydney, and the Solomon Islands Malaria Training and Research Institute (SIMTRI), Honiara. After testing finger-tip specimens of blood from 251 symptomatic patients reporting to the outpatients clinic of the Honiara Central Hospital, the accuracy of this test proved to be good, with a sensitivity of 100% and a specificity of 96.2% for parasite densities exceeding 80/μL blood.[25] These findings suggested that this 6-minute card test might prove to be a simple and practical means of making a rapid diagnosis of falciparum malaria at all levels of health care. As a result, the card test was introduced for use in the ADF under circumstances in which a microscopic diagnosis would be difficult or unreliable, such as under field conditions where pathology support is limited.

- Diagnosis of falciparum malaria achieved without a microscope

### Drug development and evaluation

The proven effectiveness of doxycycline in preventing malaria in Australian soldiers in PNG led to further field and laboratory investigations with this and other antibiotics. As doxycycline does not affect the tissue stages of *P. vivax*, further studies were carried out with primaquine and a potentially new alternative to primaquine – tafenoquine, in efforts to decrease the inordinately high number of soldiers returning to Australia with relapsing vivax malaria. Studies were also carried out with halofantrine, a new drug which had been developed primarily as a possible replacement for mefloquine in drug-resistant areas.

Meanwhile, the long-standing interest of AMRU in proguanil, used in combination with other drugs, continued unabated. Does proguanil metabolism affect its effectiveness in combination with dapsone? What about its combination with the new antimalarial - atovaquone? Might another antifolate – PS-15 (phenoxypropoxybiguanide) - be more potent than proguanil?

Investigations were also initiated with the artemisinins which appeared ready to replace quinine as the main drug for treating chloroquine- or amodiaquine-resistant falciparum malaria. In addition, further *in vitro* and *ex vivo* studies were carried out with Mannich base compounds to identify candidates with a greater antimalarial activity than amodiaquine or pyronaridine.

In carrying out these investigations, new *in vitro*, *ex vivo* and analytical methods and procedures were developed, and used alongside older ones, to assess drug activity against parasites with varying degrees of drug susceptibility. They were also used during pharmacokinetic studies to optimise drug regimens for malaria prophylaxis and treatment.

#### Investigations with antibiotics other than doxycycline

As already pointed out, doxycycline became the cornerstone for malaria prophylaxis during this period. Nevertheless, studies with other antibiotics were initiated because doxycyline cannot always be used, e.g. during pregnancy or early childhood, and because parasites may eventually become resistant to it. When multidrug-resistant parasites were incubated *in vitro* with various antibiotics for 96 hours (rather than 30 or 48 hours), inhibitory concentrations were similar to plasma drug concentrations observed after *in vivo* administration of chloramphenicol,[26] ciprofloxacin,[27] or azithromycin.[23] For example, the mean minimum inhibitory concentration (MIC) of azithromycin was reduced from 6.2 μg at 48 hours to 0.08 μg at 96 hours after the start of incubation. Since peak and trough serum concentrations after 500 mg azithromycin were about 0.4 and 0.04 μg, respectively, and serum protein binding was low, courses of azithromycin treatment lasting 3-4 days might be sufficient to cure malaria infections. However, because azithromycin (and other antibiotics) clear parasites slowly, it would probably have to be taken in combination with a rapidly-acting, non-curative blood schizontocide, such as artesunate, to quickly abort an acute attack of malaria. In addition to its potential value for treating malaria infections, this drug might also prove to be useful for preventing drug-resistant malaria.

- Short azithromycin course might cure falciparum malaria

#### Doxycycline and primaquine combination for causal prophylaxis

Doxycycline acts against blood stages of both *P. falciparum* and *P. vivax* but primaquine is needed to eliminate residual hypnozoite liver stages of *P. vivax*. Co-administration of daily doxycycline (100 mg) and primaquine (22.5 mg) for 14 days was required to prevent both falciparum and vivax malaria after leaving malarious areas. For various reasons, compliance with this lengthy "eradication course" was not optimal. But even with good drug compliance, an increasing number of individuals were developing vivax malaria after returning to Australia, presumably because the hypnozoite liver stages were becoming increasingly tolerant to primaquine.

Earlier observations in ADF personnel deployed to PNG in 1988/1989 had indicated that doxycycline, in combination with a low dose of primaquine, might be effective in killing the pre-erythrocytic liver stages of both *P. falciparum* and *P. vivax*.[5] If such causal prophylactic activity could be confirmed, this would be operationally very significant because doxycycline and primaquine would have to be taken for only a few days, instead of 14 days, after leaving a malarious area. Although the tetracyclines, such as doxycycline, are able to kill pre-erythrocytic liver stages of *P. falciparum*, they have little or no activity against the hypnozoite liver stages of *P vivax*.[9] Based on earlier observations with rhesus monkeys infected with *P. cynomolgi*,[28] it was hoped that a low dose of 7.5 mg primaquine, administered together with 100 mg doxycycline, might be able to exert sufficient synergistic drug activity against the pre-erythrocytic liver stages of *P. vivax* to enable prophylaxis to be discontinued within a few days after return to Australia.

The deployment of a 53-man detachment of 3rd Combat Engineer Regiment to PNG in 1993 for a 42-day road and bridge building exercise provided a good opportunity to verify the effectiveness of such a drug combination in a heavily malarious area of the country.[29] Malaria was not observed when men took a daily dose of 100 mg doxycycline and 7.5 mg primaquine while in PNG and for 3 days after return to Australia, despite intense exposure to malaria. However, 2 of the soldiers taking this drug combination developed falciparum malaria within 3 weeks after return and 7 of them developed vivax malaria between 3 and 16 weeks after leaving PNG. This occurred despite adequate doxycycline plasma concentrations. Clearly, discontinuation of this prophylactic regimen 3 days after return to Australia could not be relied upon to suppress all pre-erythrocytic parasites in some individuals who had been exposed to heavily-infected mosquitoes.[29] Consequently, 14-day courses of doxycycline and primaquine would still need to be taken after leaving an endemic area until less cumbersome post-exposure courses became available.

- Short courses of doxycyline and primaquine are unable to replace cumbersome 14-day courses for malaria eradication

#### Tafenoquine as a possible replacement for primaquine

Tafenoquine (also known as WR238605 and Etaquine) was a new 8-aminoquinoline drug being developed as an alternative to primaquine by the Walter Reed Army Institute of Research (WRAIR), Washington, DC, USA. Preliminary results at AMRU showed that tafenoquine had a relatively low level of intrinsic blood stage activity and acted slowly *in vitro* against *P. falciparum* and *P. vivax*. Slow clearance of parasitaemia was also observed after administration of a 3-day course of tafenoquine to six *Aotus* monkeys infected with the chloroquine- and pyrimethamine-resistant AMRU 1 strain of *P. vivax*.[30] Because of the paucity of *Aotus* monkeys at

AMRU, collaborative studies were undertaken with Gorgas Memorial Laboratory in Panama where a larger group of *Aotus* monkeys could be inoculated with the AMRU 1 isolate of *P. vivax*.[31] The studies confirmed that tafenoquine was a slow-acting blood schizontocide which persisted in the body for much longer than primaquine. Although 3mg/kg was not curative, the addition of 30 mg/kg chloroquine (well-below curative dosage) usually cured monkeys of their infections. However, identical dosages of primaquine combined with chloroquine failed to cure any monkeys. The findings indicated that these drug combinations were more effective than either drug used alone and that tafenoquine was far more effective than primaquine in achieving clearance of parasitaemia and curing blood-induced vivax infections.

Since the main value of tafenoquine would be to replace primaquine as a tissue schizontocide (active against liver stages of *P. vivax*), studies were initiated to determine whether the AMRU 1 strain could be transmitted via mosquitoes (*Anopheles farauti*) to either *Aotus* or *Saimiri* monkeys. Although early results were encouraging,[5] too few monkeys were available at AMRU to carry out definitive tissue schizontocidal studies. After consultation with Dr William Collins at the Center for Disease Control in Atlanta, the parasite isolate was forwarded to him, resulting in the routine transmission of this first chloroquine-resistant isolate of *P. vivax* between mosquitoes and monkeys in his large simian colony.

- Tafenoquine has a much longer half-life and greater blood schizontocidal activity against *P. vivax* than primaquine

#### Halofantrine for prevention of falciparum malaria

Halofantrine, a phenanethrenemethanol drug initially developed by WRAIR, was used successfully for treating drug-resistant falciparum malaria starting in the late 1980s. As the pharmacokinetics of halofantrine had mostly been studied in healthy volunteers, a collaborative study was carried out, in collaboration with SIMTRI, to determine the disposition of halofantrine and its principal metabolite, N-desbutyl-halofantrine after treatment of 6 adult Melanesian patients infected with falciparum malaria. All patients responded well to treatment with 500 mg halofantrine given 3 times at 6-hourly intervals, indicating that the observed pharmacokinetic parameters, such as maximum plasma concentrations and elimination half-lives of halofantrine and N-desbutylhalofantrine, were adequate to achieve good therapeutic outcomes.[32]

By the early 1990s, however, halofantrine was failing to cure falciparum malaria in Thailand.[33] Although taking it with food increased the absorption of the drug, about half of the patients were not cured after treatment.[34] But as with many other drugs, halofantrine was still effective in areas outside of southeast Asia. In French troops deployed to Central Africa for 4 months, a short course of halofantrine at the end of deployment prevented soldiers from developing malaria after return to France.[35]

The role that post-exposure treatment with halofantrine might play in preventing malaria was further investigated in PNG. In collaboration with Ok Tedi Mining Ltd located in a non-malarious highland area of the Western Province, 345 copper miners received a 3- or 6- day course of halofantrine after returning to the mine from 2-4 weeks home leave in various rural areas of the country. None of the men receiving either regimen of halofantrine developed falciparum malaria, whereas 36% of accompanying family members developed malaria within 1 month after return.[36] Although halofantrine cannot be used for long-term prophylaxis and has been largely superseded due to cardiovascular adverse events, the PNG findings suggested that individuals exposed to malaria for a short period (preferably shorter than the malaria incubation period) might not develop symptomatic malaria if an effective treatment course of halofantrine is administered immediately after leaving an endemic area.

- Falciparum malaria is suppressed by halofantrine administered after short visits to malarious areas

#### Proguanil conversion to cycloguanil affects synergy with dapsone

The marked synergism between proguanil and dapsone, observed during earlier studies,[5] led to further *in vitro*, *ex vivo* and *in vivo* investigations using both drugs. Proguanil, a biguanide prodrug, is metabolised by hepatic enzymes to the active triazine metabolite, cycloguanil. Synergism occurs between cycloguanil and dapsone with the triazine inhibiting the malarial parasite enzymes DHFR and the sulfone inhibiting DHPS, resulting in inhibition of folate metabolism. In addition to confirming that cycloguanil, not proguanil, was the main determinant of antimalarial activity,[37] studies at AMRU showed that cycloguanil's activity could not be potentiated by combining it with another DHFR inhibitor – pyrimethamine.[38] Furthermore, the pharmacokinetics of dapsone were not altered when administered in combination with proguanil in healthy volunteers.[39]

Additional investigations were also carried out to determine whether inter-individual variability in the oxidative activation of proguanil to cycloguanil could influence the effectiveness of malaria prophylaxis

or treatment. Was the poor protection to proguanil/dapsone prophylaxis in Thai soldiers solely due to a high level of parasite resistance to antifolate drugs or was poor metabolic conversion from proguanil to cycloguanil a contributing factor? The latter could have been partly responsible because 23% of 207 Thai soldiers were classified as poor metabolisers (PMs) of proguanil, resulting in low plasma concentrations of cycloguanil.[40] By contrast, all 25 Australian soldiers receiving daily 200 mg proguanil and 8 mg dapsone during a short-term deployment to Thailand were extensive metabolisers (EMs) of proguanil, and this may have contributed to their protection against malaria.

A further collaborative study with AFRIMS showed that Thai patients had a limited ability to convert proguanil to cycloguanil, even at a very high dose of proguanil.[41] Additional *in vitro* and *ex vivo* studies with cycloguanil-resistant isolates of *P. falciparum* suggested that low plasma cycloguanil concentrations, coupled with high levels of drug resistance, limited the value of proguanil plus dapsone in Thailand.[42] However, this drug combination might still be useful in other ethnic groups who were better able to biotransform proguanil to cycloguanil, especially in areas with a greater parasite susceptibility to cycloguanil.

This was demonstrated by collecting blood samples from Australian soldiers on proguanil/dapsone prophylaxis and incubating their sera with parasites previously obtained from Thai patients who had not been cured by proguanil/atovaquone or proguanil/dapsone. Parasite growth was inhibited at plasma dilutions of 4, 8 and 16, indicating that Australian soldiers on proguanil/dapsone prophylaxis, most of whom are EMs of proguanil, would probably be protected if exposed to parasites with a drug susceptibility similar to these isolates from Thailand.[42]

- Proguanil/dapsone synergistic activity is affected by the ability to biotransform proguanil to cycloguanil

#### Proguanil plus Atovaquone – a new antimalarial drug combination

Atovaquone, a novel hydroxynaphthoquinone which inhibits pyrimidine biosynthesis in parasite mitochondria, was developed primarily for treating *Pneumocystis carinii* and *Toxoplasma gondii* in HIV-infected patients. It was also shown to abort clinical attacks of malaria, but about 30% of patients had a recrudescence of parasitaemia.[43] When proguanil was combined with atovaquone, cure rates above 90% were obtained.

Unlike dapsone, atovaquone was potentiated *in vitro* by proguanil to a much greater degree than by its cycloguanil metabolite.[44,45] Studies at AMRU also showed that the MIC of atovaquone was considerably higher in cultures containing 50% serum than in those containing 10% serum. Presumably this was due to the substantial binding of atovaquone to plasma proteins, with less "free" drug being available to inhibit parasite growth (similar findings were observed with pyrimethamine, well-known for its high protein binding to plasma of about 94%). By contrast, proguanil and cycloguanil had similar MIC values at high and low serum concentrations. These findings were a reminder that chemical analysis of "total" drug concentrations does not necessarily equate to the "free" drug available for killing parasites.

As part of a collaborative effort with AFRIMS and Wellcome Research Laboratories in the United Kingdom, AMRU determined the pharmacokinetics of proguanil in Thai patients receiving either proguanil alone or in combination with atovaquone. Proguanil and cycloguanil plasma concentrations were similar in the two groups, with the group that received the drug combination showing slightly higher values for proguanil and its metabolite.[46] In a further study involving Thai malaria patients treated with proguanil plus atovaquone, *ex vivo* antimalarial plasma activity (measured by bioassay) was similar in PM and EM patients, indicating that an individual's ability to metabolise proguanil did not appear to affect the *in vitro* antimalarial activity of this drug combination. This suggested that the phenotypic status of individuals was of little importance in determining the outcome of treatment with proguanil plus atovaquone and that, unlike proguanil plus dapsone, the synergistic activity of this combination was determined primarily by proguanil rather than by that of its metabolite.[46]

In collaboration with the Department of Biochemistry at the University of Sydney, studies were also conducted to investigate the effects of pyrimidine antagonists, such as atovaquone, on the third and fourth steps of the pathway for the de-novo biosynthesis of pyrimidine nucleotides.[47,48] The results indicated the need for further studies to understand the regulation of nucleotide metabolism and the mechanism of action of pyrimidine antagonists in preventing DNA synthesis in the parasite.

- Proguanil/atovaquone synergistic activity is not affected by the ability to biotransform proguanil to cycloguanil

#### Other potential atovaquone drug combinations

In view of the above findings, a further study was started to determine whether the addition of

dapsone might enhance the synergistic activity between proguanil and atovaquone by potentiating the activity of proguanil's metabolite – cycloguanil. Preliminary results seemed to indicate that this might be the case, raising the possibility that two synergistic actions – that of proguanil/atovaquone and cycloguanil/dapsone - in a triple combination of proguanil, atovaquone and dapsone might enable the dosage of the component drugs to be reduced, thereby possibly reducing potential adverse drug reactions.[49] Additional *in vitro* and *in vivo* studies involving *Saimiri* monkeys showed that folinic acid did not reverse antifolate activity against *P. falciparum*, suggesting that it could be used, if necessary, to prevent bone marrow depression.[50,51]

So far, these various studies with atovaquone had demonstrated synergism between this drug and inhibitors of the folate pathway. How might doxycycline, already the main drug used for malaria prophylaxis, interact with atovaquone? As both drugs depress the activity of dihydroorotate dehydrogenase - suppressing *de novo* pyrimidine synthesis in the parasite - they might potentiate each other's antimalarial activity. In fact, when serum samples from 52 Australian soldiers on doxycycline prophylaxis were incubated *in vitro* with atovaquone, this proved to be the case, indicating that these drugs were rational partners for malaria prophylaxis or treatment.[52,53]

- Atovaquone activity is increased by combining this new drug with proguanil/dapsone or doxycycline

**A new generation antifolate: PS-15 and its metabolite (WR99210)**

WR99210, a potent diamino triazine developed by WRAIR during the early 1970s, was shown to be remarkably effective against multidrug-resistant strains of *P. falciparum*.[54] Unfortunately, severe gastrointestinal reactions and poor bioavailability during subsequent clinical trials precluded the further development of this antifolate drug. Nevertheless, interest in WR99210 was maintained, particularly because of its lack of cross resistance with other antifolates, such as pyrimethamine and cycloguanil.

Recently, PS-15 (also known as WR250417) had been developed as a prodrug for WR99210.[55] The concept for its design was based on the conversion of proguanil to its active metabolite, cycloguanil. It was hoped that PS-15 would circumvent the intolerance and poor bioavailability seen with WR99210, but still maintain potent antimalarial activity.

In collaboration with the Jacobus Pharmaceutical Company, Princeton, USA, AMRU administered PS-15 and WR99210 to non-infected *Saimiri sciureus* monkeys and subsequently used HPLC analysis to determine serum drug concentrations[56] and the bioassay to determine the serum *ex vivo* activity against *P. falciparum*. Previous studies had shown that these monkeys provided excellent preliminary information about the degree and duration of serum antimalarial activity of promising experimental drugs because of the ability of their serum to support the growth of *P. falciparum in vitro*.[57] After drug administration, substantial and sustained serum antimalarial activity was observed in monkeys receiving PS-15, but not in those receiving WR99210 (probably due to poor bioavailability).[58] Although PS-15 had some intrinsic antimalarial activity, the results indicated that WR99210 was primarily responsible for the antimalarial activity observed after PS-15 administration.

As a result of these observations, and the lack of any observable gastrointestinal toxicity with PS-15, *in vitro* studies were carried out which compared the activity of various antifolate compounds against six multidrug-resistant isolates or clones of *P. falciparum*.[59] They showed that WR99210 had a complete lack of cross-resistance to other antifolates and that, if humans were able to tolerate, absorb and metabolise PS-15 equally as well as *Saimiri* monkeys, it should be a prime candidate for further development as an antimalarial drug. Furthermore, marked synergistic activity was observed *in vitro* between the metabolite WR99210 and dapsone or sulfamethoxazole, but not atovaquone.[45] When PS-15 was co- administered with sulfamethoxazole to *Saimiri* monkeys, serum antimalarial activity (after biotransformation from PS-15 to WR99210) was more pronounced than in monkeys receiving PS-15 alone. But at 24 and 48 hours there was little difference in activity, probably because most of the sulfonamide had been eliminated from the blood circulation.[60] Unlike the *in vitro* findings with WR99210, the activity of PS-15 was not potentiated by dapsone, but it was potentiated by atovaquone.[45] This suggested that the synergistic interactions of proguanil and PS-15 may be similar: namely antimalarial activity of parent drugs potentiated by atovaquone, and that of their triazine metabolites potentiated by dapsone or sulfamethoxazole.

*Ex vivo* studies were also performed to compare the *in vitro* antimalarial activity of serum samples obtained from *Saimiri* monkeys treated with PS-15 and proguanil triple drug combinations. The results showed that the synergistic activity of PS-15/atovaquone/dapsone was greater than for proguanil/atovaquone/dapsone.[49] Significantly, folinic acid did

not reverse the antimalarial activity of these drug combinations.[48,49] In view of these findings, it was felt that further studies were needed to assess the value of this antifolate class of biguanide precursors for the prevention and treatment of multidrug-resistant falciparum malaria.

- PS-15 (and metabolite) is far more potent than proguanil (and metabolite), either alone or in combination with atovaquone and/or dapsone

#### Artemisinin compounds – pharmacokinetic information needed

Artemisinin (qinhaosu) is derived from *Artemisia annua*, (qinghao), a medicinal plant used extensively in China as a febrifuge for hundreds of years. As artemisinin and its derivatives, such as artesunate and artemether, act more rapidly than quinine (and other drugs) in aborting acute attacks of malaria, increasing consideration was being given to using them to treat acute infections of drug-resistant malaria. However, recrudescence rates of 20-50% were observed when mainly empirical regimens of these drugs were given for less than 5 days. Clearly, more information was needed on the pharmacokinetic properties of the artemisinins to support clinical studies aimed at improving treatment regimens.

Chemical assays were proving to be rather insensitive and unreliable because these drugs were thermally labile and lacked an ultra-violet or fluorescent chromophore. In a collaborative study with WRAIR, plasma samples were analysed at AMRU using an HPLC method incorporating reductive electrochemical detection. Although artemisinin, artesunate and dihydroartemisinin (the principal metabolite of artesunate) could be detected at concentrations as low as 10 ng/mL, this method was too cumbersome for routine drug analysis, with the need for rigorous deoxygenation of samples and the mobile phase.

The bioassay appeared to offer a more accurate (detection limit down to about 1 ng/mL) and a considerably cheaper alternative for use in pharmacokinetic studies, although it obviously could not differentiate between serum concentrations of parent compounds and their metabolites.[19] By measuring total antimalarial serum activity, preliminary pharmacokinetic information was obtained about the degree and duration of artemisinin and its derivatives after their administration to *Saimiri* monkeys. The results indicated that the artemisinins were more potent but were eliminated more rapidly than standard antimalarial drugs. Significantly, antimalarial activity was reduced when serum samples were incubated at higher erythrocyte concentrations, suggesting that the artemisinins, similar to chloroquine, were selectively concentrated by erythrocytes. In view of these findings, the bioassay might play a significant role during clinical evaluation of alternative artemisinin therapeutic regimens.

- Bioassay provides a sensitive method for use during pharmacokinetic evaluation of various artemisinin compounds

*Figure 2. Dr Barbara Kotecka preparing cultures to assess the in vitro activity of drugs against Plasmodium falciparum*

#### Mannich bases with greater activity than amodiaquine or pyronaridine

Early studies with amodiaquine[61] and pyronaridine[62] had shown these two Mannich base drugs to have greater antimalarial activity than chloroquine or pyrimethamine.

Since parasites were becoming increasingly tolerant to both Mannich bases, the search for more potent compounds in this class[5] was continued as part of an ongoing collaborative effort with Dr Gordon Barlin at the Australian National University, Canberra.[63-70] Apart from *in vitro* screening by standard morphological and radioisotopic methods, a new visual technique was developed and used successfully to screen 44 compounds.[71]

Ten of the most active compounds were administered to *Saimiri* monkeys as a single dose and their antimalarial serum activity over the next 7 days was compared with that of amodiaquine and pyronaridine.[72] Four of the compounds had a greater and more prolonged *ex vivo* activity than pyronaridine against the standard K1 isolate. They were also similarly active against four other drug-resistant strains of *P. falciparum*, one of which was resistant to mefloquine and halofantrine, and the other resistant to atovaquone. The marked degree and duration of activity after a single dose of these drugs indicated the need for further studies in

infected *Aotus* monkeys to determine the potential value of this class of compounds for the prophylaxis and treatment of multidrug-resistant malaria.

These findings also provided further evidence of the value of the *ex vivo* monkey model for the development of antimalarial drugs. Furthermore, by using non-infected monkeys for obtaining preliminary information about potential antimalarial drugs, *in vivo* evaluation could be restricted to those drugs showing the greatest promise for further development. Since evaluation of drugs in infected *Aotus* monkeys has significant ethical and economic implications, any experimental approaches minimising the use of such monkeys deserved strong support.

- Visual test developed for the *in vitro* screening of antimalarial drugs
- Some Mannich bases are substantially more active *ex vivo* than amodiaquine and pyronaridine

## Vector control, biology and distribution

### Insect repellents

Deet – applied to skin. The topical application of mosquito repellents was (and continues to be) an important means of protecting oneself against malaria. As the current ADF repellent (95% deet) was not popular with soldiers in the field,[5] laboratory and field tests were conducted in Thailand during 1992-1993 to evaluate protection provided by novel repellents and other formulations of deet.

Early on, it was established that deet (diethylmethylbenzamide) and dimethylphthalate provided shorter protection against *Anopheles dirus*, an important malaria vector in Thailand, than against *Aedes albopictus*.[73] With this background information, the efficacy of deet was compared with two experimental chemicals developed by the US Army - AI3-37220 and CIC-4. Using 25% ethanol solutions of each repellent, *An. dirus* was more susceptible to CIC-4 than either deet or AI3-37220 in the laboratory. However, a field study in Chantaburi province, southeastern Thailand, showed that AI3-37220 provided significantly better protection (>95% for 4 hours) than the other two repellents (<95% after 2 hours).[74]

In a further study, different deet formulations were evaluated in Sisaket province, northeastern Thailand. An applicator stick containing 33% deet provided >87.1% protection against *Culex* sp. for 5 hours, and 50% deet provided >93.3% protection against *Anopheles* spp. for 8 hours. By comparison, the US Extended Duration Repellent Formulation containing 33% deet provided complete protection for 6 hours against *An. dirus* and *Cx. vishnui*. Although similar levels of protection were observed with 50 and 75% deet in ethanol, 25% solutions were less effective.[75]

The repellent trials in Chantaburi and Sisaket provinces also presented the opportunity to examine *Anopheles* mosquitoes from untreated participants for the presence of *Plasmodium* circumsporozoite (CS) proteins using an ELISA technique. *P. vivax* CS protein was detected in 3.4% (2/54) of *An. karwari* and 4.8% (2/42) of *An. barbirostris*, but *P. falciparum* CS protein was detected in only 0.3% (1/276) of *An. dirus*. This suggested that *An. barbirostris* may play a role in transmission of *P. vivax* in Chantaburi Province, Thailand.[76]

- Deet and other repellents provide good protection against malaria vectors in Thailand

#### *Permethrin – impregnated in uniforms and bednets.*

Following earlier work with permethrin-impregnated uniforms and bednets,[5] further studies revealed that the knockdown and mortality of *An. farauti* and *Ae. aegypti* were similar following 15-180 seconds exposure to permethrin treated bednets and polyester/cotton fabrics.[77] Despite the presence of permethrin (0.007-0.068 mg/cm$^2$) in bednet material, 18-50% of *An. farauti* s.s. adults successfully blood fed through the nets and, although all were knocked down after 60 minutes, up to 78% recovered within 24 hours. The results indicated that this important vector of malaria could obtain a bloodmeal through treated bednet material and they emphasised the importance of avoiding contact with bednets while sleeping.

Collaborative studies with the US Department of Agriculture, Agricultural Research Service, Gainesville, Florida, showed that permethrin persisted in uniforms and provided good protection after 5 gentle cold water washes. These findings corroborated recommended guidelines of re-treating after 3-4 washes. As a result, AMRU recommended the use of permethrin treated uniforms, and this protective measure was instituted by the ADF in the early 1990s.[78]

In 1992 a joint trial was conducted with AFRIMS in northeastern Thailand to compare the incidence of malaria in Thai soldiers whose uniforms, covering both arms and legs, had received a high-pressure spray of permethrin with those whose uniforms had not been sprayed.[79] Bioassays of treated clothing, using laboratory reared *An. dirus*, showed permethrin remained in treated clothing for up to 90 days. Both permethrin-treated and untreated fabric provided >84% protection from biting *An. dirus* for the duration of the study. However, the impregnated

uniforms did not provide protection against malaria in this highly malarious area of Thailand. This was probably related to soldiers wearing casual attire (shorts and T-shirts) after work hours, exposing them to vector mosquitoes when they were at their most active.

During this trial, the prevalence and incidence of antibodies to *Orientia tsutsugamushi*, the aetiologic agent for scrub typhus, was also tested in these soldiers.[80] The point prevalence of antibodies varied from 0 to 4.1%, which was low compared to other regions in Thailand. An increased incidence of antibodies was observed during the wetter months of the year, indicating an increased risk for local and foreign military personnel.

- Permethrin treatment of uniforms and bednets is effective against mosquitoes. Limitations in the use of impregnated uniforms against malaria in Thailand

**"Chiggers"**

Previous work at AMRU with chiggers, insect vectors of scrub typhus (*Orientia tsutsugamushi*),[5] was continued during Major Frances' assignment at AFRIMS in Thailand from 1992 onwards. Following the establishment of a laboratory colony of *Leptotrombidium deliense* naturally infected with *O. tsutsugamushi* at AFRIMS,[81] the development and persistence of antibodies to *O. tsutsugamushi* in laboratory rats and mice could be observed.[82] In addition, it allowed investigation of the vertical[83] and horizontal[84] transmission of this pathogen. The observed novel transmission of *O. tsutsugamushi* to co-feeding mites could possibly explain the presence of rickettsiae in trombiculid genera not considered to be the main vectors of this organism.[85] Subsequent field studies in Nonthaburi Province (near Bangkok) showed that, contrary to findings with laboratory mice, rats were good sentinels, with chiggers attached to 44% of 202 rats and 2 rodents developing infections with *O. tsutsugamushi*. Out of a total of 314 engorged *L. deliense* obtained from sentinel rats, 2 (0.6%) were naturally infected with *O. tsutsugamushi*. This study also showed that the risk of exposure to *O. tsutsugamushi* is greater during the wet season, and that only a relatively small number of chigger attachments are needed to infect potential hosts.[86] A further field study in Phitsanulok Province, central Thailand, in 1993, showed that, contrary to expectations, *Blankaartia acuscutellaris* was not a vector of scrub typhus.[87]

Insect repellent studies against two vectors of scrub typhus – *L. deliense* and *L. fletcheri* - showed that low concentrations of permethrin, dimethylphthalate, deet, benzyl benzoate, di-n-propyl 2-5 pyridine-dicarboxylate, AI3-37220, 2-hydroxymethyl-cyclohexyl acetic acid lactone and a high concentration of dibutylphthalate (DBP) were toxic for uninfected larvae of both species. The median knockdown times for all chemicals were longer for *L. deliense* infected with *O. tsutsugamushi* than uninfected larvae. The study indicated that low concentrations of test chemicals, except DBP, should be effective against two important vectors of scrub typhus in southeast Asia.[88]

- Ecology of scrub typhus vectors and evaluation of repellents in Thailand

**Distribution of Anopheline mosquitoes in northern Australia**

The work on the anophelines in northern Australia was completed in 1991 with a survey of the Torres Strait, an area of scattered islands lying between Cape York and Western Province of PNG. While the mainland of Australia is malaria free, outbreaks of the disease regularly occur throughout these islands because people bring malaria parasites with them when moving between the coastal villages of Western Province and the Australian islands (Boigu and Saibai are only 7 km off the coast of Western Province). During the survey of the Torres Strait conducted in April-May 1991, *Anopheles farauti* 1 and *Anopheles hilli* were found on the larger islands of Boigu, Saibai, Badu, Moa, Horn, and Prince of Wales. Both species have been known to transmit malaria in Australia. Their larvae tolerate brackish water, an adaptation which would support their dispersal through these island groups.

Retrospective spatial analysis of potential malaria vectors, surveyed in northern Australia between 1985-1990,[5] showed that the three sibling species of *Anopheles farauti* were distributed around the coast and 50-100 km inland north of 20° S latitude and east of 129° E longitude. In some areas the three species were found together but the overall patterns of occurrences for each species were different.[89-91] These apparent dissimilarities in realised distribution suggested that there might be differences in ecological factors influencing the range of the individual species. This was investigated with the use of computer based Geographical Information Systems (GIS) data sets and the ecological niche modelling (ENM) software GARP (Genetic Algorithm for Rule-set Prediction). The prediction of species range by GARP uses point localities where species are known to occur together with environmental data for the geographic region of interest. Inputs for model construction included species occurrence data from field surveys together with high resolution environmental information for northern Australia

based on historical climate records. Two data mining software packages, CART (classification and regression tree analysis) and KnowledgeSeeker, were selected to search for significant environmental factors associated with species presence. The overall objective was to identify the key environmental factors responsible for defining the geographical ranges of the different vector species, as such factors are of epidemiological significance for malaria control.

The results revealed consistent agreement in the variables ranked by both data mining methods. This permitted the selection of reduced sets of environmental data to develop GARP models for the three target species with equivalent predictive accuracy to those which used all of the environmental information. Atmospheric moisture was shown to be a key predictor for all three species of *Anopheles farauti* in Australia.[91]

However, the GARP results were less satisfactory for describing the realised distribution of *Anopheles farauti* s.s. The distribution of this important vector of malaria in the Southwest Pacific Region includes New Guinea, the Solomon Islands and Vanuatu, as well as northern Australia. Systematic surveys in Australia's Northern Territory and Queensland have indicated that its distribution is predominantly within 5 km from the coast.[92] This species utilises brackish water sites for larval development, but larvae have also been found in freshwater environments,[93] suggesting that its coastal distribution is determined by environmental factors other than simple proximity to the sea. But the GARP models were unable to identify combinations of environmental parameters that successfully delimited the coastal distribution of this species.

Further analyses were undertaken with a novel exploratory method which permits visualisation and analysis of environmental gradients across distribution boundaries. The use of this range boundary tool to investigate environmental variable changes across the known range boundary limits of *An. farauti* s.s. in northern Australia identified the importance of elevation. The inclusion of this topographical variable resulted in models which included all of the record sites of this species in northern Australia and successfully reconstructed its narrow coastal distribution.[94]

- Environmental factors are shown to influence the distribution of *Anopheles farauti*.

**Distribution and Speciation of Anopheles mosquitoes in Papua New Guinea.**

Following on from Operation Anopheles in northern Australia, similar mosquito surveys were conducted in PNG. This highly malarious country has close ties with Australia and, historically, was administered by it until gaining independence in 1975. During training exercises in PNG, Australian soldiers are at a high risk of acquiring malaria when exposed to malaria vectors.[6] Although the primary vectors were considered to be members of the *An. punctulatus* group - *An. farauti* 1, *An. punctulatus* and *An. koliensis*, very little was known about their distribution or what other species might be present throughout PNG.

Faunal surveys were conducted in Western Province (1992), the Sepik region (1993), Gulf Province (1994), and Madang Province (1995). As this vast region is sparsely populated and has a limited road network, the work could only be accomplished with the support provided by crew and helicopters from Army Aviation's 162 Reconnaissance Squadron.[5] Excellent support was also provided by Preventive Medicine personnel from the PNG Defence Force.

These surveys relied heavily on the use of dry ice ($CO_2$) baited light traps to attract and collect adult mosquitoes. Up to 10 traps were set up each evening and retrieved the next morning, thus enabling a wide area to be surveyed with much less effort than the laborious human landing catches. As dry ice was unavailable, it had to be made from $CO_2$ gas cylinders. Because of the inaccessibility of field sites, this survey would not have been possible without Caribou aircraft from 25 Squadron pre-positioning these cylinders at various sites. Towards the end of the survey, another potential mosquito attractant – octenol - was being trialled for collecting culicine mosquitoes for arbovirus surveillance. As it appeared to have distinct advantages over $CO_2$ for use in remote locations, a trial was conducted during 1995 in the Madang area comparing different trapping methods for various species of anopheline mosquitoes. While octenol alone attracted more mosquitoes than light alone, it lured fewer mosquitoes into its traps than those with $CO_2$ alone or $CO_2$ plus octenol.[95] Continued reliance on dry ice for attracting anopheline mosquitoes appeared to be necessary.

Collections of larvae and adults were processed in the same way as described previously.[5] Initially, DNA probes were only available to identify the sibling species of *An. farauti*: *An. farauti* 1, *An. farauti* 2, and *An. farauti* 3.[96] However, by 1994, DNA probes had been developed for all the known members of the *An. punctulatus* group and were made available to support this work.[97]

*Anopheles farauti* 1 was found to be a dominant coastal species throughout the survey area. Preferred larval habitats were coastal swamps and

lagoons where the flow of streams is blocked by sand bars. Such breeding sites are ubiquitous along the coastline of PNG, their large size allowing huge population densities which were reflected in adult trap collections exceeding 7000 mosquitoes per night. *An. farauti* 1 is usually the primary vector of malaria in PNG, but it will also feed on domestic animals (pigs and dogs). In these surveys it was often found well outside its flight range of humans and domestic animals, indicating that this species also feeds upon native birds and animals.

*Anopheles farauti* 2 occurred throughout the surveyed region and it was the dominant species in Western Province. Significantly, this species had previously not been recorded to be present in PNG.[98] Although it was most abundant in inland lowland river valleys and flood plains, it was also found breeding along the coast and up to 1500 m in highland areas. This species was also found in human-made sites, such as wheel ruts and earthen drains. Although it was observed biting humans, nothing is known of its possible role in malaria transmission.

*Anopheles farauti* 3, *An. meraukensis* and *An. novaguinensis* were recorded from only a few localities in Western Province. In this region the climate tends to be different from the rest of PNG - it is monsoonal with distinct wet and dry seasons similar to northern Australia where these species are common. Larval habitats included swamps and the edges of rivers and streams, though it was also found in smaller ground pools. This is the first record of these species in PNG and nothing is known about their behaviour. However, due to their limited distribution and low numbers, they are unlikely to play a role in malaria transmission.

*Anopheles farauti* 4 larvae were commonly found in small ground pools to large swamps of inland river valleys and flood plains north of the central highlands. Nothing is known about the malaria transmission potential of this species.

*An. punctulatus* and *An. koliensis* are considered to be primary vectors of malaria in PNG. Both species were abundant throughout the survey region in inland, lowland river valleys and flood plains. *An. punctulatus* was also commonly collected in the highlands >1000 m. Larvae of both species were found breeding in natural pools of water, but the highly invasive *An. punctulatus* was also breeding in small pools created by human or animal activity, such as foot or hoof prints, pig wallows, shallow drains around village dwellings, and wheel ruts on roads.

*Anopheles karwari*, an Asian immigrant first discovered in the Sepik region in 1957,[99] was found in several localities in the Sepik region. It is a dangerous vector of malaria in Asia but nothing is known of its role in malaria transmission in PNG.

Anopheles longirostris and An. bancrofti were also found throughout the survey area. The former species was identified from adult mosquitoes collected mainly in the inland regions of the Sepik and Madang provinces. Larvae of the latter species were most frequently collected from swamps and lagoons of inland flood plains of the Sepik and Fly rivers. Their role in malaria transmission has not been determined.

- New information has been obtained about Anopheles vectors of malaria in four provinces of Papua New Guinea

## Conclusions

The second half of the third decade (1990-1995) experienced a marked expansion of activities. Novel and well-established procedures were used to assess drug resistance of malaria parasites and to develop and evaluate antimalarial drugs in laboratory and field settings. The value of doxycycline for malaria prophylaxis was confirmed and, in addition, other antibiotics were investigated as possible alternative drugs. Despite efforts to curtail the cumbersome and lengthy primaquine eradication course, the prevention of vivax malaria remained difficult. Results of initial studies with a long-acting analogue of primaquine – tafenoquine – appeared to indicate that this new drug might be more effective than primaquine. Further studies with proguanil combinations indicated that another new drug – atovaquone – might be a suitable partner for prophylaxis of drug-resistant falciparum malaria. Investigations with PS-15 showed this experimental antifolate compound to be considerably more potent than proguanil. In the search for better tools to combat drug-resistant malaria, progress was also made during laboratory-based studies with other drugs, including artemisinin and Mannich base compounds. Personal protection measures against mosquitoes and other disease vectors were broadened to delineate the efficacy and persistence of different formulations of standard and novel repellents applied to the skin and impregnated into uniforms and bednets. Retrospective spatial analysis of potential malaria vectors, surveyed in northern Australia between 1985-1990, showed atmospheric humidity to be a key predictor of the widespread distribution of the three sibling species of *An. farauti*. After completing the survey of the Torres Strait islands, from which malaria is intermittently imported into Australia, surveys of four provinces in PNG revealed the presence of numerous species

of anopheline mosquitoes, some of which had not been identified previously in PNG. New knowledge gained about larval breeding sites and geographic distribution of these potential malaria vectors should not only be of benefit for future field deployments, but also assist local health authorities in their malaria control activities.

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## Highlights

1990 Major M.D. Edstein commences 3-year exchange posting to Armed Forces Research Institute of Medical Sciences, Thailand.

Doxycycline replaces pyrimethamine/dapsone as standard malaria prophylaxis.

1991 Tafenoquine (new primaquine analogue) acts slowly against blood stages of *P. falciparum* and *P. vivax*.

Proguanil biotransformation in Australian and Thai soldiers.

Mosquito survey in Torres Strait.

1992 Major S. P. Frances commences 3- year posting to Armed Forces Research Institute of Medical Sciences, Thailand.

Lieutenant Colonel G. D. Shanks (US Army) commences 3-year posting to AMRU.

Evaluation of halofantrine at OK Tedi Mining Ltd.

Tafenoquine active against liver stages of chloroquine-resistant *P. vivax* (AMRU1) strain in *Aotus* monkeys.

*In vitro* assessment of non-tetracycline antibiotics, e.g. azithromycin, and artemisinin derivatives

Mannich base compounds screened by *in vitro* visual test.

Absorption of PS-15 (new antifolate prodrug) by *Saimiri* monkeys much better than its active metabolite WR 99210.

Persistence of permethrin in uniforms during field trial in Thailand.

Mosquito survey in Western Province, Papua New Guinea (PNG)

1993 Doxycycline prophylaxis proven effective and well-tolerated in 2000 Australian soldiers, with only 1-2% switching over to mefloquine prophylaxis.

Shorter courses of doxycycline plus primaquine unable to replace 14-day post-exposure course.

*In vitro* results suggest that 3-4 day azithromycin course probably sufficient to cure falciparum infections, but must be taken with rapidly acting drug.

Atovaquone (new drug) potentiated by proguanil, but not its metabolite cycloguanil (reverse of proguanil/dapsone synergy)

Marked degree and duration of activity of some Mannich compounds against highly resistant isolates of *P. falciparum*.

Permethrin-impregnated uniforms provide good protection after 5 cold water washes

Mosquito survey in Sepik region, PNG

1994 Tafenoquine more effective than primaquine against *P. vivax* (AMRU 1 isolate).

Proguanil/atovaquone synergistic activity not affected by patients' ability to transform proguanil to cycloguanil.

PS-15 combined with atovaquone and dapsone far more effective than proguanil triple drug combination.

Studies with Mannich bases confirm the value of using uninfected monkeys to obtain preliminary information about the degree and duration of activity of antimalarial drugs.

Evaluation of different deet formulations and new insect repellents.

Investigations with insect vectors of scrub typhus completed.

Retrospective spatial analysis of potential malaria vectors in Australia.

Mosquito survey in Gulf region, PNG.

1995 First field evaluation of non-microscopic test card for falciparum malaria.

Mannich bases more active than chemically-related drugs against drug-resistant malaria.

Pharmacokinetic information on artemisinin derivatives obtained more reliably by bioassay than chemical analysis.

Proguanil biotransformation not increased by administering high proguanil dose to poor metabolisers of the drug.

Mosquito survey in Madang Province, PNG

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