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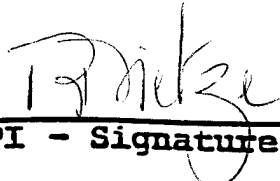
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**STUDIES FOR THE PREVENTION AND TREATMENT OF MALARIA, LEISHMANIA  
AND OTHER EMERGING INFECTIOUS DISEASES IN BRAZIL**

ANNUAL REPORT  
(Sept 1995 - Sept 1996)

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## 1. INTRODUCTION

Surveillance programs have allowed the Development of Disease assessment teams in conjunction with the Brazilian Army in Rio de Janeiro and the development of a field site in Peixoto de Azevedo. Major focuses of this program include identification of pathogens as important causes of human and emerging infectious diseases (EID), evaluation of parasite resistance to drug therapy, development of a potential malaria vaccine study site in the Amazon, diagnostic strategies for leishmaniasis, a trial of WR6026 in the treatment of visceral leishmaniasis, and Dengue surveillance. The USAMRU-B site has been established as a reference lab/repository to isolate and characterize *Leishmania* and malaria strains on the basis of drug resistance using molecular and biochemical techniques. Efforts to identify and isolate pathogens concentrating on leptospirosis and hantavirus among fever of unknown origin patients at Peixoto clinics and hospital also continue.

2. WR6026 LEISHMANIA DRUG STUDY: Clinical Trial Of Oral WR6026.2HCl In Patients With Brazilian Visceral Leishmaniasis due to *L. chagasi* : Initial Dose Range Determination For Efficacy, Safety And Tolerance.

### 2.1 BACKGROUND

Visceral leishmaniasis (VL) results from infection with protozoan parasites of the genus *Leishmania*. In Brazil, where the disease has an extensive area of transmission, the causative parasite is *L. chagasi*

Promastigote forms of the parasite, inoculated into the skin via the bite of the female sandfly, (genus *Lutzomya* in the New World) are rapidly transformed into mammalian forms (amastigotes) that are mainly seen within the phagolysosomes of mononuclear phagocytes. Infection of liver, spleen and bone marrow macrophages with parasites of *L. chagasi* results in syndromes ranging from asymptomatic infection to full-blown VL with fever, weight loss, hepatosplenomegaly, and pancytopenia (Badaró et al., 1986; Lainson & Shaw, 1987). If untreated, symptomatic disease is characteristically fatal due to concurrent infections such as diarrhea and pneumonia. More recently, a related syndrome with fever, hepatosplenomegaly, and abdominal symptoms due to visceral infection with *Leishmania tropica* has been described in soldiers returning from Operation Desert Storm (Magill et al., 1993)

Approximately 90% of the VL reported in the Americas are from Brazil (Anonymous, 1984). During the decade of 1982-91, 14,736 new cases were reported to the Brazilian Ministry of Health (Anonymous, 1992). In the last three years, there have been approximately 3,000-4,000 cases of the disease reported each year to the Ministry of Health. The disease is endemic in all coastal states from Pará to Paraná as well as in the inland states of Minas Gerais, Mato Grosso do Sul, Goiás and Roraima. Endemic visceral leishmaniasis in Brazil, as in Kenya and the Mediterranean countries, is predominantly a pediatric disease with the majority of cases occurring in patients less than 18 years of age.

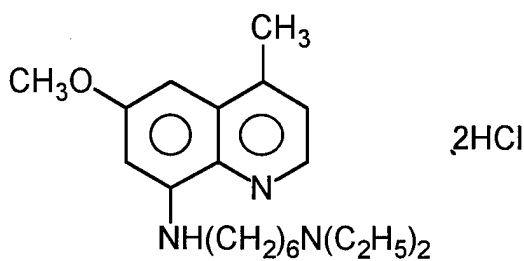
The first effective antileishmanial chemotherapeutic agents, the pentavalent antimonials ( $Sb^V$ ), were introduced in the 1920's-1940's and are still first-line agents for all forms of leishmaniasis (Herwaldt & Berman, 1992). The formulations available since World War II include sodium stibogluconate (Pentostam<sup>®</sup>) and meglumine antimonate (Glucantime<sup>®</sup>). Though it is possible to achieve cure rates as high as 90% in VL using a single course (Berman J, 1988) of  $Sb^V$  there are several disadvantages to its use. The standard regimen recommended by the World Health Organization and the US Centers for Disease Control and Prevention is 20 mg  $Sb^V$ /kg/day parenterally (IM or IV), for a minimum of 28 days (Herwaldt & Berman, 1992). This therapy is of long duration and results in moderate to intolerable dose-related toxicity including: myalgia/arthralgia, hepatocellular damage, cardiac repolarization abnormalities, pancreatitis, renal dysfunction and gastro-intestinal side effects. Furthermore there is a significant relapse rate ranging from 15% to 36% in some areas in Kenya (Anabwani et al. 1983). In Brazil the efficacy of 28 days of Glucantime is > 95%. Both antimonial formulations lack standardization, and *in vitro* efficacy varies among different lots (Jackson et al., 1990) and neither drug is approved for use in treatment of leishmaniasis in the United States. Treatment failures and relapses require extended antimony treatment or the use of second-line drugs such as amphotericin B or pentamidine.

Conventional Amphotericin B desoxycholate is an extremely effective agent against *Leishmania* sp. both *in vitro* (Berman & Wyler, 1980) and clinically (Prata, 1963; Sampaio et al., 1971). The antileishmanial activity of this antifungal agent is due to its preferential interaction with a 24-substituted sterol (ergosterol or episterol), the major membrane sterol shared by both leishmania and fungi (Berman et al. 1986; Hart et. al., 1989). However, amphotericin B also interacts with the major mammalian sterol, cholesterol, and despite its clinical efficacy, amphotericin B has not been widely utilized in the treatment of leishmaniasis because of its toxicity. Recently, a new formulation of amphotericin B (liposomal

amphotericin B) has been used in the treatment of kala-azar with promising results (Davidson et al., 1991; Dietze et al., 1993).

For Leishmania the mechanism of action of pentamidine is not well defined but appears to be related to inhibition of RNA polymerase, ribosomal function, and protein and phospholipid synthesis. It binds selectively to the parasite kinetoplast DNA, resulting in swelling and loss of function of the kinetoplast. (Brack et al. 1972; Williamson 1979). Adverse effects of pentamidine are protean and include fatigue, anorexia, nausea, abdominal pain, prolonged hypoglycemia, tachycardia and other cardiac arrhythmias, reversible renal failure (in 25% of pts.), hypotension, and pancreatitis (sometimes resulting in permanent pancreatic endocrine dysfunction).

WR6026·2HCl is an 8-aminoquinoline originally synthesized at the Walter Reed Army Institute of Research in the malaria research program during World War II. It was originally tested in clinical trials against vivax malaria, but it showed limited efficacy and was not developed as an antimalarial. Subsequently the drug was considered as an oral alternative in the treatment of VL. If WR6026 can be shown to be safe and effective treatment for VL (even if efficacy is less than 100%), it will provide a major therapeutic advance since it would be the only orally administered drug for this disease. As such, WR6026 would become the primary treatment of choice for visceral leishmaniasis.



WR6026·2HCl

WR6026 (6-methoxy-8-(6-diethylaminohexylamino) lepidine dihydrochloride) is an 8-aminoquinoline (primaquine) analog. WR6026 has been shown highly active against Leishmania *in vitro* and in animal models. In hamsters inoculated intraperitoneally with *L. donovani*, for example, the drug produced a 700-fold greater suppression of infection (at a dose of 0.025 mg/kg orally BID for 4 days) than intramuscular administration of the reference antimonial compound, Na<sup>+</sup> stibogluconate (Kinnamon et al. 1978). In the human macrophage-

*L. tropica* system, WR6026 was approximately seven times more active than Na<sup>+</sup> stibogluconate (Berman and Lee, 1983).

The bioavailability and pharmacokinetics of intravenous and oral doses of 5 mg/kg of WR6026(base) were studied in beagle dogs (Hawkins et al. 1989). Plasma and urine concentrations of the parent drug and its desethyl metabolite were measured by high pressure liquid chromatography (HPLC). After intravenous administration, the plasma concentration of WR6026 declined in a bi-exponential manner to below the limit of detection (<10 ng/ml) at 16 hours after dosing. The results obtained in this study characterize WR6026 as a high-extraction drug (mean extraction ratio  $0.955 \pm 0.068$  SD) with a relatively high systemic clearance ( $43.5 \text{ ml/min/kg} \pm 8.0$  SD mean clearance), a high volume of distribution (mean  $7.69 \text{ L/kg} \pm 2.4$  SD), and a short terminal half-life (mean of 2.0 hours  $\pm 0.3$  SD). Orally administered WR6026 is of low absolute bioavailability with respect to the intravenous dose (mean 4%), possibly as a result of significant "first pass" metabolism in the liver. There is also evidence to suggest that WR6026 is subject to extensive extra-hepatic, extra-renal elimination and that it is markedly distributed into the extravascular space and bound in tissues.

The urinary excretion in humans of the parent drug and two metabolites was quantified over 6 days after an oral dose of 60 mg. (Theoharides et al. 1987). Twelve hour serial urine collections from eight subjects were obtained for 96 hours after dosing. WR6026 and two unidentified peaks with identical chromatographic mobilities as the 4-hydroxymethyl and desethyl metabolite were present. The didesethyl or 6-desmethyl metabolites were not detected. The elimination half-life was calculated from excretion over time and found to be  $30.0 \pm 5.3 \pm 1\text{SD}$  and  $15.0 \pm 4.9 \pm 1\text{SD}$  hours for 4-hydroxymethyl-WR6026 and WR6026, respectively. The excretion of WR6026, 4-hydroxymethyl-WR6026 and desethyl-WR6026 accounted for 14.1% of the dose (range 6.2%-30%).

The major toxicity of WR6026 results from the oxidant action of the drug on hemoglobin, NADPH, glutathione and sulfhydryl groups. The resulting methemoglobinemia causes a reduced oxygen carrying capacity and a shift of the oxyhemoglobin dissociation curve which causes a somewhat reduced oxygen release to tissues at any given oxygen partial pressure. Glucose-6-phosphate dehydrogenase can reduce the oxidized hemoglobin, glutathione, and sulfhydryl-containing enzymes through an NADPH-dependent mechanism. Red cells deficient in glucose-6-phosphate dehydrogenase (G6PD) are unable to resist this oxidant stress, and, in patients with G6PD deficiency, hemolysis may result (Marr, 1985). Methemoglobin (MHb) results when the heme iron of hemoglobin is in the oxidized (Fe<sup>+3</sup>)

state. It normally accounts for  $0.78 \pm 0.37\%$  of total hemoglobin, or 0.06 g of methemoglobin (MHb)/100 ml of blood in a normal adults. Methemoglobinemia can occur after ingestion of several different drugs or chemicals or result from congenital methemoglobin reductase deficiency. Methemoglobin levels of up to 10-30% are generally asymptomatic but may be associated with symptoms of decreased oxygen transport at levels above 30% of total hemoglobin.

WR6026, similar to primaquine and other quinoline drugs can lead to methemoglobinemia when given to animals and humans. MHgb levels have been seen in volunteers and volunteer patients given in phase I and phase II studies (See below). Headache was the only reported symptom. As noted above, a methemoglobin level of 10% to 25% of total hemoglobin is asymptomatic; 30% to 50% is associated with dyspnea on exertion and headache; 50% to 75% of total hemoglobin is associated with collapse, coma and death can occur at levels above 75% (Davidson and Henry, 1969).

The only Phase 2 study of the efficacy of WR6026 was performed in Kenya and data published in 1994 (Sherwood, et al, CID 1994). In this study, sixteen patients with VL underwent treatment with WR6026 at doses ranging from 0.75-1.0mg/kg/d for fourteen days (8 patients) or 1mg/kg/d for four weeks (8 patients). The results included one cure in the 2-week group and four cures in the four-week group. Other patients in both groups experienced a one or two-logarithmic decrease in the number of parasites seen by histologic examination. All eight patients treated for four weeks experienced improvement in clinical variables such as weight, liver and spleen size, hemoglobin concentration, leukocyte count, and eosinophil percentage. The adverse effects of the drug were mild to moderate and were considered transient. Adverse effects included headaches in four patients, and mild abdominal complaints in two patients. Elevations in methemoglobin levels were mild and transient and the authors concluded that these results supported the use of WR6026 at higher doses and longer courses of treatment

Because further study of WR6026 is required before its routine use, we designed an open-label Phase 1/Phase 2 trial to evaluate efficacy, safety, and tolerance of WR6026 in the treatment of VL caused by *L. chagasi*. This study will examine the above parameters for escalating doses of the study drug over a four-week course of treatment.

## 2.2 METHODS

In our study, a progressive dose regimen will be used, starting at 1mg/kg/d and increasing to a maximum of 3mg/kg/d for 28 days. The number of patients enrolled will be a maximum of 60 in cohorts of six each, with three cohorts currently enrolled. Subject ages will range from 6 to 50 years old (10 to 50 years old in the first cohort enrolled). Both male and non-pregnant females will be enrolled in open-label consecutive fashion until cohorts are filled. All patients must have a documented VL infection by evidence of amastigotes in splenic aspiration tissue. Major exclusion criteria include known HIV infection, G-6PD deficiency, and prior anti-leishmania therapy.

Each patient will be evaluated for clinical response and evidence of toxicity. Clinical parameters include fever curve, liver and spleen enlargement, and complete blood counts. In addition, splenic aspirations will be obtained at baseline and day 42 (two weeks after completion of therapy). Parasitological cure is difficult to document for VL, since elimination of amastigotes may occur after treatment is stopped and relapses can occur after apparent elimination of parasites at the completion of therapy. Therefore, definitive cure will be based on the absence of clinical signs and symptoms of VL at one year post-therapy.

## 2.3 RESULTS AND CONCLUSIONS

Currently, we have completed a four-week course of therapy in two cohorts and filled the third cohort. The pretreatment patient demographics are displayed in Table 1. The first cohort was treated at 1mg/kg/d and was closed after four patients were enrolled due to a poor response seen in three patients as evidenced by continued positive splenic aspirations at 42 days. Of these patients, one had a 50% decrease in the number of parasites seen on aspiration, but the other two had no decrease in number of parasites. The fourth patient was removed from the study after 7 days of treatment due to lack of response as evidenced by progression of the disease. The patient remained febrile during treatment, splenic size increased, and there was a 50% reduction in total leukocytes from 2,000 to 1,000. Toxicity was ruled out as a cause of the leukopenia after a bone marrow biopsy revealed no maturation arrest in any cell line.

The maximum methemoglobin level was 9.9% in one patient in this cohort. The other two patients had levels of 1.8% and 2.0%. The only other side effect related to the drug other

than methemoglobin level in this cohort was T-wave inversion seen in leads V1, V2, and V3 in one patient during weeks three and four of treatment. However, these abnormalities disappeared by the time of two-week post-treatment follow up.

**Table 1.** Pretreatment demographics of patients.

Characteristic	Cohort 1 (n = 4)	Cohort 2 (n = 6)	Cohort 3 (n=6)
Male/female ratio	4/0	5/1	5/1
Age (y)	19±2.58(16-22)	32.8±12.9(11-49)	23.8±13.8(9-39)
Duration of disease (months)	4.7±2.9(1-8)	3.5±1.2(1-4)	6.2±4.3(2-12)
Amastigotes (splenic aspiration)	3±1.1(2+-4+)	3.5±0.8(2+-4+)	3±0.7(2+-4+)
<b>Physical examination</b>			
Axillary temperature ( <sup>o</sup> C)	37.9±1.2(37-39.7)	38.6±1.3(37.2-40.5)	38.7±1.46(36.4-40.3)
Spleen size (cm)	8.2±3.3(5-12)	9.2±3.2(5-13)	9.4±3.2(6-14)
<b>Hematologic and serum chemistry values</b>			
Hemoglobin g/dl (normal 12-17)	9.2±0.93(7.8-9.8)	7.7±1.5(6.5-10.1)	8.86±1.05(7.3-10.2)
WBC 1.000/mm <sup>3</sup> (normal 5-10)	2.9±0.76(2-3.7)	2.3±0.89(1.1-3.3)	2.4±0.88(1.4-3.6)
Platelet 1.000mm <sup>3</sup> (normal 200-400)	157±58(99-210)	95±39(32-138)	99±35(55-127)
Albumin g/dl (normal 3.5-5.5)	2.8±0.52(2.3-3.3)	2.9±0.81(1.9-4.1)	3.3±0.56(2.9-3.7)
BUN mg/100mL (normal 10-20)	13.2±1.7(11-15)	13.8±3.6(10-19)	13.2±2.9(9-16)
Creatinine mg/100mL (normal 06-1.2)	0.7±0.2(0.5-1)	0.7±0.14(0.5-0.9)	0.8±0.36(0.4-1.3)
SGOT U/L (normal 4-32)	34±10.5(21-43)	34.6±22.6(16-74)	43.4±21.3(8-65)

Data represent the mean±SD(range)

The second cohort involved six patients taking 1.5mg/kg/d. One patient achieved possible cure with a negative bone marrow aspiration (performed in place of splenic aspiration since the spleen was not palpable below the costal margin without inspiration). This patient has completed 6-month follow-up without clinical symptoms of disease and will complete 12 months of follow-up next December. Two patients had a 50% reduction in the number of parasites seen on splenic aspiration. One patient had 75% reduction in parasites and two final patients evidence no reduction in the number of parasites seen on 42-day follow-up splenic aspiration.

The maximum methemoglobin levels were 3.8, 6.9, 1.6, 6.1, 8.7, and 2.7% respectively in patients 2A-2F in this cohort. Patient 2F evidenced T-wave inversion during the first and second week of treatment, however this finding was not present by the third week.

Because of poor response seen in cohort 2, the dosage for cohort three was incremented by 0.5mg to 2.0mg/kg/d. This cohort has six patients enrolled, with one patient

having completed the four-week course of treatment. Follow-up splenic aspiration for this patient will be performed this week.

The results of the first two cohorts were disappointing in their lack of efficacy at doses that had previously been reported to include up to 50% cure rates. Cohort number 3 will be important to determine whether higher doses will increase the cure rate without increasing toxicity. Up to now, clinically important toxicity has not been observed. We will use the results of cohort 3 to determine the direction of future therapeutic investigation of WR6026.

### 3. CURRENT PROGRAMS AND RESULTS.

A 2-year prospective study of Amazon settlers (April 96) and gold miners (late 1995) was started at a now established study site in Peixoto where disease surveillance is ongoing. Hepatitis E has been identified as a significant EID pathogen constituting 25% of active hepatitis cases and holding a 6% seroprevalence in the Southern Amazon. Leptospirosis has been identified as a significant EID pathogen in urban Rio de Janeiro as well as in the Southern Amazon where an 11% IgM antibody seroprevalence has been detected. Seroprevalence of IgG antibodies for hantavirus is currently 2% in the Southern Amazon.

Malaria among Amazon settlers accounts for 20-30% of febrile illnesses and monthly incidence ranges from 3-15% for *P. falciparum* and 15-50% for *P. vivax*. A cohort of 350-500 people have been followed with 50% *P. falciparum* and 50% *P. vivax* prevalence. Daily malaria surveillance at 2 Ministry of Health health clinics and 1 private health clinic in Peixoto de Azevedo involves a total of 1500 - 2000 cases per month in peak season (November - April). Surveillance has allowed the identification of high risk occupational exposure (50%) attack rates in Brazilian Entomology field technicians. This group may serve for future studies of drug prophylaxis. In strains of *P. falciparum* in Brazil, 13/13 strains tested were chloroquine resistant. Two point mutations correlating with chloroquine resistance in the Pfmdrl gene at Cys-1034 and Asp-1042 positions have been discovered.

Surveillance of Brazilian Army troops deployed to Angola was initiated in February 1996. In these troops, *P. falciparum* malaria has been identified as the chief threat with 20% attack rates in Angola and an 11% asymptomatic parasitemia rate on return. A Northern Amazon surveillance

site planned in collaboration with Instituto Evandro Chagas in Belém has not been successfully established.

With the emergence of increasing drug resistance, new effective therapies must be developed and tested. A 3-day regimen of Atovaquone and Proguanil has been tested in uncomplicated *P. falciparum* cases (n=160) and has been found to be highly (>95%) effective and comparable to a 3 day quinine + 7 day tetracycline regimen.

*Leishmania* research continues to progress with efforts to develop rapid assays for diagnosis and to better understand the pathogenesis of disease. The antigenic profile of *Leishmania braziliensis* isolated from cutaneous lesions was compared with that in mucosal lesions. Cross-reactive antigens may play a role in the severe tissue destruction in the absence of amastigotes observed in mucocutaneous cases. An "all or none" animal model of *Leishmania* tropism has been established and validated which can be used for selection of tropism mutants following chemical mutagenesis and of revertants following transfection. *L. tropism* mutants with altered temperature sensitivity have been developed and suggest that temperature resistance is important for visceralization. Concordantly, an in vitro system for studying parasite temperature sensitivity has been developed and correlates well with dermatropic or viscerotropic manifestations in humans. Urine *L. chagasi* antigens present possibilities for a non-invasive rapid diagnostic assay to replace the present invasive spleen/bone marrow aspirates to diagnose visceral disease and further research is necessary for development.

Drug resistant strains of *Leishmania* and malaria species have been studied and we described a novel paromomycin resistance mechanism in *Leishmania tropica* by an increase in the amount of rRNA which can account for broad spectrum resistance to other ribosome inhibitor drugs. Another method of resistance determined by transfection studies is via the *LmpgpA* gene coding for pentavalent antimonial glutamine. This gene serves as a marker for antimony resistance in leishmaniasis and can be used for screening. Plans include the development and validity testing of a PCR assay using *LmpgpA* as a rapid diagnostic assay for antimony resistance in *Leishmania*. A *Leishmania* clone with a 180 kb amplification containing P-glycoprotein has been developed and is resistant to both Glucantime and Pentostam providing a mechanism for further study of resistance.

A new-onset dengue type 2 outbreak was discovered in Vitória in 1995-96 and Dengue was found to account for about 50% of Dengue like illness during May-July outbreaks.

As insects are the vectors transmitting all discussed diseases, entomology research is crucial. Twelve monthly surveys at two locations resulted in the collection of 14 species of *Anopheles*, 7 of which are known or suspected vectors. Behavior data is being collected. Free-mating colonies of *A. albitalis* and *A. aquasalis* were developed at the Rio de Janeiro main laboratory and will allow clear behavioral data and study opportunities as these varieties of *Anopheles* are known to be malaria vectors. Twenty bi-weekly surveys at two locations have resulted in 35 *Lutzomyia* species four of which are suspected vectors of ACL and behavior data is ongoing. DEET and A13-37220 skin repellents against potential sandfly vectors of ACL in Peixoto have been field tested.

#### 4. DISCUSSION

Continued programs are necessary for surveillance of resistance to reference drugs in clinical use in malaria and *Leishmania* strains around the world. All necessary pieces are now present to enhance understanding parasite factors involved in the pathogenesis of visceral and mucosal leishmaniasis in order to improve patient care and permit the safe development and testing of new therapies and attenuated vaccines.

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