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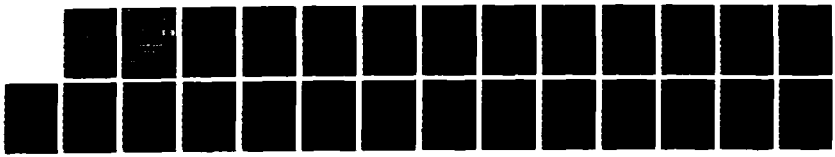

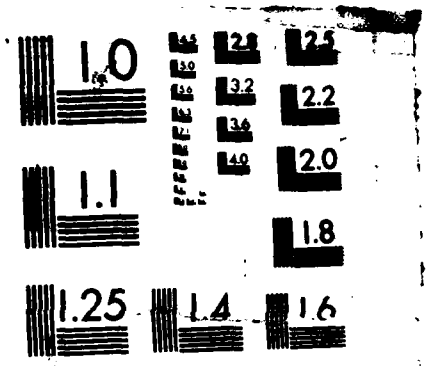

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TESTING OF EXPERIMENTAL COMPOUNDS FOR EFFICACY AGAINST LEISHMANIA

Annual Report

William L. Hanson, Ph.D., Virginia B. Waits, B.S.,
and Willie L. Chapman, Jr., D.V.M.

February 1987

(For the period 1 January 1986 - 31 December 1986)

Supported By
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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The efficacy of six selected compounds was compared against L. donovani in hamsters. The results indicated that the antileishmanial activity of WR06026 > sinefungin > amphotericin B > glucantime > 9-deazainosine > pentamidine.

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FOREWORD

In conducting the research described in this report the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources. National Research Council. (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION
(Statement of the Problem and Background)

The leishmaniases, the group of diseases caused by protozoan parasites of the family Trypanosomatidae, genus Leishmania, are widely distributed throughout the world and are found on every inhabited continent except Australia (Kinnamon et al., 1). These diseases occur in such important countries as Russia, China, India, Pakistan, Egypt, Sudan, Israel, Syria, Iran, Brazil, Venezuela, Panama, Mexico, Argentina, and many others. These parasites are transmitted by several species of phlebotomine flies and in most areas the leishmaniases are zoonoses with canines, rodents, or other mammals serving as reservoir hosts.

These parasites are a significant health hazard to humans in these areas. Visceral leishmaniasis, the most severe type, is endemic in many areas where epidemics occur (TDR Publ, 7th Program Rpt., 2) with mortality reported to reach as high as 98 percent in untreated cases (Biagi, 3; Steck, 4). While it is difficult to obtain an accurate estimate of the number of human beings infected with the leishmaniases throughout the world, (TDR Publ, 7th Program Rpt., 2) one estimate indicates that at least 12 million persons have one of the different forms of the disease caused by infection with these parasites (Mahmoud and Warren, 5) and outbreaks involving additional thousands of persons occur periodically (Peters, 6; TDR Publ, 7th Program Rpt., 2).

Infection with these parasites represents a significant health hazard to military personnel operating in many areas of the world. For example in World War II where troops were operating in an endemic area of the Persian Gulf 630 cases were reported in a three-month period (Most, 7). Subsequently during troop movements in another endemic area, 50 percent of certain Israeli forces experienced infections (Naggan et al., 8). In addition, although relatively few troops were involved, 10 to 45 cases per year have been reported among U.S. troops in the Canal Zone (Walton et al., 9) and a subsequent report indicated an overall infection rate of 1.6% in one U.S. Army batallion that was deployed to Fort Sherman in the Canal Zone for jungle warfare training (Takafuji et al., 10). Although mortality may occur, the primary problem is the considerable loss in duty time in infected individuals. For example it has been estimated that each individual having visceral leishmaniasis lost at least one year duty time (Most, 7) and in one instance in which 20 cases of cutaneous leishmaniasis occurred in troops in

the Canal Zone, two man-years of duty time were lost (Walton et al., 9).

Efforts to control the leishmaniases have met with only limited success. The first line drugs currently available to treat the leishmaniasis are the pentavalent antimony compounds which are toxic (James and Gilles, 11) with side effects which include vomiting, nausea, lethargy, and electrocardiographic changes. Another liability associated with available drugs is that they must be administered via the parenteral route and often repeated injections are required. In addition, these compounds are often not curative and evidence for antimony resistance among the Leishmania is increasing. A strain of L. donovani from Kenya has been shown to be considerably more insensitive to antimony than a strain which has been in the laboratory for many years (Hanson et al., 12) and antimony resistant strains of L. donovani and L. braziliensis panamensis have been developed in this laboratory (Waits, et al., unpublished).

The current prospects for new drugs for the treatment of visceral leishmaniasis are quite limited (WHO Publication, 13). Drugs with proved efficacy in laboratory animals and which are currently undergoing pre-clinical or clinical studies are WR06026 and allopurinol riboside. Drugs or delivery systems in various stages of development and showing some promise are sinefungin, formycin B, miconazole, and liposomes. Formycin B has been observed recently in this laboratory to be extremely toxic in dogs and only marginally active and thus additional study of this compound is probably not warranted. In our experience sinefungin is also toxic and this toxicity probably will preclude further consideration of this compound for future practical use. Considerable work remains to be done before any of the others will be useful on a practical basis. Furthermore, the possibility that Leishmania already exists which are resistant to WR06026 (an 8-aminoquinoline) must be considered since these infections occur in areas of the world where 8-aminoquinolines have been used against malaria in humans.

Because of the potential importance of leishmaniasis to the health and performance of military personnel in many parts of the world and the need for improved and more satisfactory chemical compounds for consistent successful treatment of this disease, this project was initiated to test experimental compounds for efficacy against Leishmania donovani and L. braziliensis panamensis infections in the golden hamster as the primary test systems and in non-human primates as a secondary test system. This is the second annual progress report for this project and this report covers the period 1 January 1986 through December 31, 1986. It describes the test procedures used and summarizes the results obtained. The test results obtained have been sent to appropriate officials at The Walter Reed Army Institute of Research as they became available during the contract year.

MATERIALS AND METHODS
(Approach to the Problem)

I. Studies Involving Leishmania donovani

1. Primary Visceral Test System

A Khartoum strain of L. donovani (WR 378) was used in this part of the studies and the golden hamster (Mesocricetus auratus), 40-60 gm, served as the host animal. Suspensions of amastigotes for infection of experimental hamsters were prepared by grinding heavily infected hamster spleens in sterile saline in a Ten Broeck tissue grinder and diluting the suspensions so that 0.2 ml contained approximately 10×10^6 amastigotes. Each experimental hamster was infected via the intracardiac injection of 0.2 ml of the amastigote suspension.

The testing procedure used was that described by Stauber and his associates (14, 15, 16) as modified by Hanson et al. (17). On day 3 following infection, hamsters were divided randomly into experimental groups consisting of a minimum of 6 animals per group, initial group weights were obtained, and administration of test compounds was initiated. Each compound was tested at 2 or 3 drug dosage levels dependent on the priority rating of the compound. Generally the test compounds with high priority ratings were studied initially via the intramuscular route (I.M.) at 208, 52, and 13 MKD, (milligrams/kilogram/day) while those compounds received with a routine or low priority rating were studied at 104 and 13 MKD only. Other drug dosage levels determined by the quantity of compound available or previous toxicity data were used also.

The vehicle for the test compounds was 0.5% hydroxethylcellulose-0.1% Tween 80 (HEC-Tween). Each test group contained 6 hamsters and received one of the desired drug dosage levels. A control group of 6 to 8 hamsters received the 0.5% HEC-Tween vehicle only and the reference compound, glucantime, was given at 2 or 3 drug dosage levels (104, 13, and 3.25 MKD, or 104 and 13 MKD, dosage levels based on antimony content). All test compounds were administered routinely twice daily via the intramuscular route on days 3 through 6. Final group weights were obtained on all experimental hamsters on day 7 and all animals were killed, livers removed, weighed and liver impressions made for enumeration of amastigotes. Subsequently, the total number of parasites per liver was determined as described by Stauber (15).

In addition to recording body weight changes as a general indicator of toxicity of the test compounds, experimental hamsters were observed for such clinical signs of toxicity as nervous disorders, roughened hair coat, and sluggish activity. Deaths also were recorded. Weight loss of 15% or greater and/or death of the animals was considered indicative of significant drug toxicity.

After determining the ratio of numbers of amastigotes/host cell nucleus, the weight of organ, and initial and final weights of the hamster, the raw data was evaluated with a IBM PC XT microcomputer using a program which calculates percent weight change, total numbers of parasites, mean numbers of parasites/liver, and percent parasite suppression.

Additional information on the antileishmanial activity (estimation of potency) of each active compound was obtained by comparing the percent suppression of numbers of amastigotes it exhibits with the percent suppression observed with Glucantime, the reference compound. This comparative measure (referred to as the Glucantime Index or "G") was determined by the following formula:

$$\underset{(G)}{\text{Glucantime Index}} = \frac{SD_{50} \text{ for Glucantime}}{SD_{50} \text{ for new test compound}}$$

Drug dosage levels (MKD) required for a given degree of effect, such as 50% parasite suppression (SD_{50}) was estimated graphically from computer plots.

2. WR06026 Metabolite Studies in Hamsters

The activity of a total of 8 metabolites of WR06026 was compared with that of WR06026 against L. donovani in hamsters in 2 separate experiments.

The procedures used in this aspect of the work were the same as those used for the primary visceral test system with the following exceptions. In the first experiment BL18736 and BL18765 along with WR06026 were adminstered via the intramuscular route in some groups and oral route in others twice each day on days 3-6 following infection. Dosage levels used were 13, 3.25 and 0.81 MKD. In the second experiment BK40735, BK50713, BK56573, BK56733, BL24361 and BK99014 along with WR06026 were adminstered via the intracardiac route as a single injection on day 3 following injection. Dosage levels used were 1.6, 0.4, 0.1, and 0.025 MKD.

3. Comparison of the Efficacy of Selected Compounds against L. donovani.

A comparison of the efficacy of sinefungin, 9-deazainosine, pentamidine, amphotericin B, WR06026, and the reference compound, glucantime, was done using procedures outlined for the routine primary

visceral test system (Sect. I, 1) with the following modifications.

Compounds in this experiment were administered via the route determined to be the most efficacious during previous studies (i.e. glucantime, sinefungin, and pentamidine via the intramuscular route, 9-deazainosine and WR06026 per os, and amphotericin B via the intracardiac route). All of this group of compounds were administered twice each day on days 3-6 after infection except amphotericin B which was given once daily during this interval. The dosage levels used were determined from data obtained from prior studies of these compounds in the primary visceral test system.

II. Studies involving Leishmania braziliensis panamensis

1. Primary Cutaneous Test System

Leishmania braziliensis panamensis (strain WR 539) was used in these studies. Male golden hamsters, 40-60 grams, served as experimental hosts.

Promastigotes for establishing experimental infections in hamsters were grown in Schneider's Drosophila Medium (Hendricks et al., 18) and quantitated using procedures described previously (Hanson and Roberson, 19). In preparation for infection and weekly during the experiment, the hair was clipped on the dorsal tail head and a commercial dipilatory agent applied to the area to remove the remaining hair. Each hamster was inoculated via the intradermal route with 1.5×10^7 promastigotes of L. braziliensis panamensis near the base of the tail using a 0.25 ml glass syringe equipped with a 30 gauge x 1/2" needle. Each experimental group consisted of six hamsters. Initial body weights were obtained and administration of therapy, generally via the intramuscular route, was initiated on day 19 post infection, and continued through day 22 post infection. Glucantime was included at two dosage levels (208 and 52 mg Sb/kg/day) as the reference compound and a group of six hamsters received vehicle only (HEC-Tween). Test compounds were administered generally at 104 and 52 MKD.

Lesion areas of each experimental hamster was determined with the aid of a template made at WRAIR and calibrated according to the formula $r_1 r_2 \pi$ where r_1 is the major radius of the lesions and r_2 is the minor radius of the lesion (Wilson et al., 20). The mean lesion area of each experimental group was obtained and the percent suppression of lesion size calculated by comparing the mean lesion area of each treated group with that of the group receiving vehicle only with the aid of a computer program and an IBM PC XT microcomputer. Comparison of the suppressive activity of test compounds with that of the reference compound, glucantime, was made from computer plots and a Glucantime Index for active compounds was calculated as described in the preceding section (Sect. I, 1). Toxicity of test compounds was determined as indicated in the primary visceral test system.

RESULTS

I. Studies Involving Leishmania donovani

1. Primary Visceral Test System

A total of 298 compounds were studied at various drug dosage levels for suppressive activity against L. donovani in hamsters in the primary visceral test system. Twenty-nine of these compounds were noted to be active at one or more dosage levels. Glucantime Indexes were calculated for 28 of these. Based on the parameters of the test, the Glucantime Indexes ranged from at least approximately 0.1 to 88. Included among these was sinefungin (G index, ranged as high as 30.2) and WR06026 (G ranged as high as 87.8). The activity of several others was at least as great as the reference compound, glucantime (BL20649, $\geq$ 1.17; BL09533, G $\geq$ 1.37; BK99121, $\geq$ 1.38; AJ15304, G $\geq$ 2.58; BH32724, G $\geq$ 2.82). Some of these active compounds were toxic at some of the drug dosage levels used.

2. WR06026 Metabolite Studies in Hamsters

The antileishmanial efficacy of metabolites of WR06026 were compared with WR06026 against L. donovani. Four of the metabolites (BL18756, BK40735, BK50713, BK99014) were active at dosage levels of 3.25 mg/kg/day or greater. WR06026 was greater than 90% suppressive at a dosage level as low as 0.4 mg/kg/day and when studied at this dosage level and lower, only 2 of the metabolites were significantly active (BK50713, 45.2%, and BK99014, 65.9%). Thus none of the metabolites studied were noted to be as active against L. donovani in hamsters as the parent compound, WR06026. Neither WR06026 nor the metabolites were toxic at dosage levels used in these studies.

3. Comparison of the Efficacy of Selected Compounds against L. donovani in Hamsters.

When compared in a single experiment, the antileishmanial efficacies based on SD_{50}'s of amphotericin B, glucantime, 9-deazainosine, pentamidine, sinefungin, and WR06026 were observed to be WR06026 > sinefungin > amphotericin B> glucantime > 9-deazainosine > pentamidine. When studied at dosage levels resulting in parasite suppression approaching 90-100%, sinefungin was toxic as indicated by roughened hair coat of the treated hamsters, and amphotericin B and pentamidine were toxic as indicated by

weight loss and/or mortality in treated hamsters.

II. Studies Involving L. braziliensis panamensis

1. Primary cutaneous test system

A total of 88 compounds were studied for suppressive activity against L. braziliensis panamensis. Eighty-three of these were studied at 104 and 52 MKD x 4 days while five (sinefungin, 9-deazainosine, pentamidine, amphotericin B and WR06026) were studied at three dosage levels determined by toxicity information. Five of the 88 compounds were active (greater than 50% lesion suppression). Among these five were sinefungin (Glucantime Index ranged from at least 12.7 to 30.2) and WR06026 (Glucantime Index was at least 15.7). Two of the active compounds (AR80315 and ZP43609) were less active than Glucantime (G Index = .379 and .857 respectively) while the remaining active compound, AJ15304, was approximately equal to Glucantime (G Index = 2.58).

Twenty-four of the 88 compounds studied were found to be toxic as indicated by death of hamsters and/or greater than 15% loss of weight.

DATA PROCESSING

During the period covered by this report, work was completed on all historical data for the four test systems dating back as far as 1975. This completes the master data base on leishmanial drug screening and on test compounds supplied by WRAIR for the Trypanosoma cruzi screen for the 12 year period. All results can be sorted by bottle number, experiment number, percent parasite suppression and/or Glucantime or Lampit Indexes enabling quick retrieval of information by WRAIR officials, as deemed necessary, in planning future drug screening pursuits.

New data from current experiments are now being sent via Hayes Smartcom Modem directly to WRAIR's VAX computer where the file is edited, data is automatically added to the master data base, and reports are generated.

DISCUSSION

The potential threat of the leishmaniases to the health of military and other personnel operating in many areas of the world (Kinnamon et al., 1; Mahmoud and Warren, 5; Takafuji et al., 10; Chance, 21) is significant and current means of therapy for these important diseases are not satisfactory for a number of reasons (James and Gilles, 11; Kern, 22). Thus continued efforts to obtain better chemotherapeutic agents for their treatment is warranted. The studies reported herein were conducted to identify new and better compounds with significant potential for consideration for future use in the treatment of human beings infected with protozoan parasites of the genus Leishmania. Several significant developments have been forthcoming from these efforts.

First, the results from the primary visceral test system have identified several new compounds with antileishmanial activity equal to or greater than the currently used antimonial, glucantime. The activity of one of these was at least 8 times greater than the reference compound, glucantime. These results also confirmed the relatively high antileishmanial activity of sinefungin which has been reported by others (Neal et al., 23), and confirmed the high antileishmanial efficacy of WR06026 previously reported (Kinnamon et al., 1) by this laboratory. Although the new compounds were tested "blind" (i.e. without knowledge of chemical structure), it is possible that some of these may warrant further study to determine their future potential.

Since previous recent in vivo and/or in vitro work in this laboratory and others (Neal et al., 23) has verified the promising antileishmanial efficacy of the 8-aminoquinolin, WR06026, and the antifungal agent, sinefungin, studies were carried out to compare under standardized conditions, the efficacy of these with that of several compounds known for many years to have antileishmanial activity. Compounds of the latter type which were chosen for comparison were the polyene macrolide antibiotic, amphotericin B, the diamidine, Pentamidine, and the pentavalent antimonial compound, glucantime. The studies indicated that WR06026 was the most active of this group. The next most active compound was sinefungin followed by amphotericin B, glucantime, 9-deazainosine, and pentamidine in decreasing order of activity.

Although the antileishmanial activity of several of these was promising, several are toxic at dosage levels effecting parasite

suppression approaching 80-90%. Hamsters receiving sinefungin at these dosage levels had roughened hair coats, and those receiving amphotericin B had signficant weight loss and/or mortality. This in our opinion, limits the potential of these two compounds for significant use in human beings unless they are encapsulated into liposomes (Berman et al., 24) or other carriers which decreases toxicity to the host and possibly enhance antileishmanial activity. The other compounds of this group were not toxic at these dosage levels.

In studies reported in the previous paragraphs as well as in previous publications (Kinnamon et al., 1) WR06026 was noted to be highly active against L. donovani in hamsters and appears to have a reasonable therapeutic index in these experimental hosts. Evidence is accumulating that metabolites of this compound may be the active antileishmanial agent(s). This evidence includes the considerably greater activity of this compound in hamsters (Kinnamon et al., 1) than in mice (Neal et al., 23), and there is some suggestion that this compound is more active in the livers of treated hamsters than in the spleen or bone marrow (Hanson, unpublished results).

Efforts have been underway at WRAIR (by Dr. A. D. Theoharides) to identify and isolate metabolites of WR06026 and when possible have these synthesized and forwarded to our laboratory for testing for antileishmanial efficacy in vivo. During the previous project period one of these (BK90014) was noted to have suppressive activity similar to that of the parent compound while another (BL05884) was much less active when administered orally. These studies were extended during the period covered by this report utilizing BK90014 and several recently isolated metabolites administered via the intracardiac route. In addition to confirming the activity of BK90014, the results of these studies showed that three additional metabolites of WR06026 were active. The activity of these (BL18756, BK40735, BK50713) was less than that of WR06026.

These findings are significant in that they suggest that the metabolic products of WR06026 are important in the high antileishmanial efficacy observed in hamsters with WR06026. Since several are active, the question arises regarding the relative importance as well as possible synergistic or additive activity of the various metabolites. If adequate quantities of these compounds can be obtained, it would be of considerable interest to study their efficacy in other hosts such as the squirrel monkey (Madindou et al., 25) and to study the efficacy of combinations of these metabolites in hamsters and non-human primates. These studies hopefully would help point the direction for clinical trials with this group of compounds and should indicate the most efficacious and least toxic treatment regimen.

The cutaneous leishmaniases are especially difficult to treat and the list of available useful compounds is short. This difficulty in therapy is reflected in the results obtained in our testing of compounds for activity against L.b. panamensis during the period covered by this report.

Although a total of 88 compounds were tested for activity against cutaneous lesions caused by Leishmania braziliensis panamensis, limited success was achieved in this area. This reflects the considerable difficulty generally experienced in obtaining successful chemotherapy of some species of Leishmania causing cutaneous leishmaniasis. Generally, compounds which are active against visceral leishmaniasis are not active against cutaneous species. During this period, an exception was noted, namely that sinefungin is active against both visceral and cutaneous species. Unfortunately, this compound is toxic which severely limits its future potential as a candidate drug for consideration for human use.

CONCLUSIONS

1. The primary visceral and primary cutaneous test systems are useful and valid in identifying new compounds with antileishmanial activity against Leishmania donovani and Leishmania braziliensis panamensis.

2. The efficacy of WR06026 > sinefungin > amphotericin B > glucantime > 9-deazainosine > pentamidine against Leishmania donovani in hamsters.

3. The toxicity of the sinefungin and amphotericin B along with the much greater efficacy of WR06026 indicates that the latter remains the best candidate drug for possible future use in human beings.

4. Some metabolites of WR06026 are efficacious against L. donovani and the activity of one is similar to that of WR06026.

RECOMMENDATIONS

1. Continue the primary visceral and cutaneous test systems at a moderate level to identify new classes of compounds with antileishmanial activity.

2. Increase activity in endeavors such as the selection of compounds for *in vivo* testing which show considerable promise in various types of *in vitro* studies. Also included should be selected compounds currently used in humans for other infectious diseases.

3. Continue to study *in vivo* the metabolic products of WR06026 as they can be identified and synthesized by officials at WRAIR, investigate possible synergistic activities of these metabolites, and study the efficacy of metabolites in hamsters as well as monkeys.

4. Continue *in vivo* studies of drug combinations for activity against both visceral and cutaneous leishmaniases. Drugs for these studies should be selected on a rational basis from promising leads identified in this work as well as those identified by other investigators.

5. Perform secondary testing of especially promising compounds in the squirrel monkey as a part of the logical sequence in antileishmanial drug development.

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