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CONTRACT NO: DAMD17-90-C-0131

TITLE: STUDY OF COMPOUNDS FOR ACTIVITY AGAINST LEISHMANIA

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REPORT DATE: October 27, 1991

92-01555

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No 0704-0188	
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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 1991 October 27	3. REPORT TYPE AND DATES COVERED Annual Report (9/28/90 - 10/27/91)	
4. TITLE AND SUBTITLE Study of Compounds for Activity Against <u>Leishmania</u>			5. FUNDING NUMBERS Contract No. DAMD17-90-C-0131 62787A 3M162787A870.AM.037 WUDA335539	
6. AUTHOR(S) W.L. Hanson V.B. Waits W.L. Chapman, Jr.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Georgia Research Foundation Athens, Georgia 30602			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick Frederick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) During the period covered by this report, a total of 75 compounds were tested for efficacy against <u>Leishmania donovani</u> infections in hamsters. A computer analysis of the data from the study of approximately 6000 compounds tested since this project was initiated in 1974 suggested a number of compounds with sufficient promise to warrant further study during this contract period. The 8-aminoquinolines were the most active compounds studied, one of which was 100% suppressive at dosage levels as low as 13mg/kg. Six phenanthrene methanols as well as two dibenzopyrroles selected for study were not highly active against <u>L. donovani</u> , and some were toxic.				
14. SUBJECT TERMS <u>Leishmania donovani</u> ; phenanthrene methanols; chemotherapy; dibenzopyrroles; 8-aminoquinolines; trifluralin; oligonucleo- tides			15. NUMBER OF PAGES	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

19. ABSTRACT (Continued)

The antitubulin herbicide, trifluralin, and several of its derivatives were found to be inactive against L. donovani in hamsters.

In addition, a total of 20 oligonucleotides, consisting of both sense and antisense, were studied for inhibition of multiplication of promastigotes of L. donovani in vitro. The data suggested little or no inhibition of L. donovani in vitro by these oligonucleotides as studied.



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ACKNOWLEDGEMENT

For technical assistance in carrying out this work, we wish to thank Mrs. Barbara Harris, Mr. Mark Komorowski, and Miss Shannon Waits. The contribution of Ms. Nell Landers in the typing of this report is gratefully acknowledged.

FOREWORD

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PI Signature William L. Hanson Date Oct. 27, 1991

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INTRODUCTION

Protozoan parasites of the genus *Leishmania* are widespread throughout the world where they cause considerable disease in human beings as well as some animals including dogs in numerous tropical and sub-tropical countries (1, 2, 3). Since the leishmaniases commonly exist as zoonoses, these diseases pose a significant potential threat to military personnel as well as military dogs throughout endemic areas. Recent publicity regarding infection of personnel involved in Operation Desert Storm has reemphasized the military significance of the leishmaniases.

Better drugs are needed for the treatment of the leishmaniases since those currently available are often not satisfactorily effective and are potentially toxic to man and animals. A total of 6,591 compounds have been studied in this laboratory under previous contracts for *in vivo* antileishmanial activity prior to the initiation of the current project. Among the most promising active compounds found during these studies is the 8-aminoquinoline, WR06026. This compound is now being field tested.

It is believed that continued screening for potentially active antileishmanial compounds is justifiable in the event that WR06026 does not perform in the field as expected due to unforeseen host and/or parasite factors. To this end, all previous data has been analyzed by Dr. Craig Canfield (Pharmaceutical Systems, Inc., 927B North Russell Avenue, Gaithersburg, Maryland 20879) who has identified five classes of compounds which warrant further study. In addition to compounds of these classes, studies have been carried out on a variety of other compounds from other sources such as anti-tubulin compounds (4) and antisense nucleotides (Dr. Rich Meyer, Microprobe, Inc., working under contract SBRI DAMD 17-88-8201-Phase II).

This report summarizes the results of studies conducted for this contract during the period September 28, 1990 - September 27, 1991.

MATERIALS AND METHODS

A. Primary Visceral Test System

A Khartoum strain of *L. donovani* (WR378) was used and the golden hamster (*Mesocricetus auratus*), 50-70 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 (5, 6, 7) as modified by Hanson et al. (8). 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 and nature of the compound.

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 (208, 52, and 26 total mg/kg or 208 and 52 total mg/kg) 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, et al. (5-8).

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 the organ, and initial and final weights of the hamster, the raw data was evaluated with an IBM PC XT microcomputer using a program which calculates percent weight change, total numbers of parasites, mean numbers of parasites/organ, and percent parasite suppression. The computer program then performs linear and non-linear regression analysis and calculates a SD_{50} for each active compound from each of the analyses (drug dosage resulting in 50% suppression of amastigotes). The SD_{50} from the non-linear analysis is used for a comparison of the relative efficacy of the test compounds and the efficacy of test compounds relative to that of the reference compound, Glucantime. The linear regression analysis is included only for comparison with the non-linear analysis.

B. In Vitro Studies

Promastigotes of *L. donovani* were cultured from an infected hamster spleen in Schneider's Drosophila Medium (Hendricks, et al., 9) and quantitated using procedures described previously (Hanson and Roberson, 10).

Promastigotes from four-day cultures (fourth to twelfth subpassage) were used in this work. (Unpublished data indicates that this age culture is the best for establishing infections in hamsters.)

Cultures were harvested by centrifugation and resulting pellets were resuspended in Schneider's *Drosophila* Medium to a final concentration of 12.5×10^6 or 6.5×10^6 per ml. Using round bottom microtiter plates (Dynatech), 200 μ l of the parasite suspension was added to each well and plates incubated at 26°C (Day 0).

Approximately 24 hours later, compounds were added to appropriate wells at either 50 or 30 micromolar concentrations (Day 1). Triplicate cultures were used for each compound as well as untreated control wells. Cultures were again incubated until Day 4 at which time total numbers of promastigotes/ml for each well were determined using the procedures described by Hanson and Roberson (10).

Mean numbers of parasites per well for each treated well and for untreated wells were calculated. Percent suppression or inhibition of parasite growth was determined using the following formula:

Percent Suppression = mean number of parasites for the untreated controls minus the mean number of parasites for the test compound divided by the mean number of parasites for the untreated control times 100.

Negative percent suppression indicated enhanced growth of parasites in the treated wells as compared to growth in the untreated wells.

RESULTS

During this reporting period a total of 75 compounds were tested for efficacy against *Leishmania donovani* infections in hamsters. Of these, 56 compounds were tested at three dose levels and the remaining 19 were tested at two dose levels. The results from all compounds tested in this system are listed for reference in Table I.

Sixteen compounds from among those previously screened in this test system were retested at selected dose levels in order to more precisely define their SD_{50} s. These results were provided to Pharmaceutical Systems, Inc. where all data for each of the retested compounds were then simultaneously fit to a hyperbolic tangent equation for estimation of the SD_{50} for each compound using a computerized least square minimization procedure. Results from these tests indicated that two of these compounds warrant further attention. One 8-aminoquinoline (WR237937; BH67432) has given virtually 100% suppression at every dose level tested. The other compound of interest (WR1390809; AX64884) is a phenanthrene methanol (Figure I), with which complete suppression was never achieved due to apparent toxicity at 416 mg/kg. These results, however, suggested that other phenanthrene methanols should be investigated.

In addition to phenanthrene methanols and 8-aminoquinolines, a search of the entire computerized *Leishmania* data base indicated that several classes of compounds are of potential interest. These classes include C-nucleosides, dibenzopyrroles, disulfides, and aminothiols. The lack of availability of previously untested C-nucleosides in the WRAIR chemical inventory and the fact that none of the compounds of this class tested thus far has been more active than formycin B led to a decision to temporarily suspend further testing of C-nucleosides. Computer structure searches for available disulfides and aminothiols were not completed by the Department of Chemical Information during this reporting period; therefore, emphasis was placed upon testing of available phenanthrene methanols and dibenzopyrroles.

Table II summarizes the results from tests of six phenanthrene methanols that were selected for testing. None of the compounds was significantly more active at the dose levels used than was WR149809 (AX64884). Results from tests of the two dibenzopyrroles of greatest interest (BC10527 and AQ38003) are also included in Table II. Neither of these compounds was active at the dose levels tested.

Table III contains a list of the most active compounds tested during this reporting period. The 8-aminoquinolines were by far the most active compounds and BH67432 was the most potent of this class. A single benzothiopyran (WR223619; ZN41968; Figure II) was also active, but this compound was much less potent than the 8-aminoquinolines.

In addition to testing of those compounds suggested by analysis of previous screening data, the antitubulin herbicide trifluralin and several of its derivatives were examined for *in vivo* antileishmanial activity. Neither trifluralin itself (AE73324) nor any of its derivatives were active (Table IV).

Table V summarizes the results of the *in vitro* testing of oligonucleotides for inhibition of promastigotes of *L. donovani*. The sequences of the oligonucleotides studied are presented in Table VI. None of the oligonucleotides were more than 50% inhibitive of the growth of *L. donovani in vitro*. As can be seen from Table V, two of these

oligonucleotides (LE004 ZV and LE504 ZV) were more active than the others. A more detailed examination of these results has been discussed by Dr. R. Meyer in his progress report for contact number DAMD 17-88-8201-Phase II.

DISCUSSION

Computer analysis of the results from testing of more than 6000 compounds screened since this project was initiated in 1974 resulted in the identification of 254 compounds that exhibited antileishmanial activity greater than or equal to that of pentavalent antimony. Of these, 224 compounds were found to be 8-aminoquinolines, while the remaining 30 were of diverse classes. Because WR06026, a compound currently undergoing field trials, is an 8-aminoquinoline, a decision was made to investigate the 30 compounds of other classes that had previously shown antileishmanial activity. After disregarding Sb and Sn containing compounds the following classes appeared to be of most interest: C-nucleosides, disulfides, aminothiols, phenanthrene methanols, and dibenzopyrroles. Computer structure searches to identify relevant compounds held in the WRAIR inventory were completed for all classes except disulfides and aminothiols.

Thirteen C-nucleosides had been previously tested, but none was more active than was formycin-B. The absence of additional compounds of this class in the inventory resulted in the decision to cease further examination of C-nucleosides for the immediate future, although compounds of this class remain of long term interest.

The phenanthrene methanol WR149809 (AX64884) had previously shown antileishmanial activity, while other phenanthrene methanols have shown antimalarial activity in rodents or primates. The three antimalarial phenanthrene methanols (AY91608, AX63172, AX67009) tested in our system failed to show significant antileishmanial activity. The lack of potency of these compounds combined with possible toxicity at higher dose levels has led us to conclude that further investigation of this class of compounds should be limited to any available phenanthrene methanols with an alkyl side chain similar to that of WR06026.

The dibenzopyrroles tested were inactive, but only a limited number of these compounds were available during the current reporting period. A computer structure search has identified several more compounds of this class and these will be tested during the coming contract year.

An overview of results from testing performed since 1974 as well as testing performed during the current reporting period continues to graphically demonstrate that 8-aminoquinolines are by far the most potent antileishmanial compounds yet examined by us. Recently, we have identified a group of these compounds that have shown activity against either visceral or cutaneous infection or both. In almost all cases these compounds had been previously administered intramuscularly at relatively high doses and had been virtually 100% suppressive at the lowest dose tested. It is possible that among these compounds there exists an 8-aminoquinoline that is active against both cutaneous and visceral leishmaniasis when administered orally, and that such a compound may provide a viable alternative to WR06026 should the latter fail clinical or field trials. With this possibility in mind, approximately thirty 8-aminoquinolines have been identified for additional testing in the coming contract year. It is anticipated that further investigations of such compounds will be emphasized in the immediate future along with testing of available dibenzopyrroles, specific phenanthrene methanol analogues, and xylosides that have previously demonstrated antimalarial activity. Also, disulfides and aminothiols identified by computer structure searches will be screened.

Despite the fact that the herbicide trifluralin had been reported to have antileishmanial activity *in vitro*, neither this compound nor any of its

analogues tested in our system showed *in vivo* activity. Such a disparity between *in vitro* and *in vivo* antiparasitic activity is not surprising and does not invalidate using reported cases in *in vitro* activity as leads for drug testing. It is anticipated that such leads will continued to be exploited in the future, in particular in instances where the compounds involved represent new classes of potential drugs, e.g., natural product derivatives.

Antisense RNA's have been exploited with varying success to block the activity of specific genes to inhibit the replication of viruses as well as various human cancer cells (11, 12). Dr. R. Meyer, Microprobe, Inc., under a separate contract (DAMD 17-88-C-8201) developed the idea to apply this technology against *Leishmania* and has synthesized a number of antisense oligonucleotides for possible inhibition of the growth of *Leishmania*. These oligonucleotides were supplied to our laboratory for testing. Thus far this approach has not appeared to be especially promising although some suggestion of inhibition of growth of *Leishmania donovani in vitro* was observed. One possible explanation for the lack of inhibition observed in these experiments is the fact that it is sometimes difficult to get the compounds into cells at the right time to block messenger RNA activities (11). Work in this area is continuing. (See report by Dr. Meyer for additional details.)

CONCLUSIONS

1. Selected aminothiols and disulfides, classes of compounds identified by computer analysis as being of potential interest should be tested. In addition, specific xylosides that have previously shown antimalarial activity should be screened.
2. Additional dibenzopyrroles must be tested in order to reach a final conclusion regarding this class of compounds. Testing of additional phenanthrene methanols should be limited to those with WR06026-like alkyl side chains if such compounds are available.
3. Since 8-aminoquinolines remain the most active class of antileishmanial compounds screened to date, it would be worthwhile to continue to examine this class in regard to activity against both visceral and cutaneous infection and effects of administration route and regimen.
4. Novel compounds as suggested by published *in vitro* antileishmanial studies or by reports of activity against other organisms should be investigated.
5. Deoxyoligonucleotides that have shown the greatest activity *in vitro* should be tested *in vivo*.

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Appendix 1

TABLE I. Summary of compounds studied for suppressive activity against *Leishmania donovani* during the period 28 September 1990 - 27 September 1991.

BN	DOSE1	SUPPRES1	DOSE2	SUPPRES2	DOSE3	SUPPRES3
BE20354	3.25	16	13	64	52	83
BG56256	0.05	26	0.2	2	0.8	46
BH50802	0.8	23	3.25	53	13	98
BH72317	0.2	- 3	0.8	26	3.25	74
BL20649	.80	17	3.25	22	13	45
AX64884	13	17	104	43	416	31
BH67432	13	100	52	100	3.25	98
ZN41968	13	9	52	50	208	99
ZN42812	.05	20	.2	16	.8	13
ZP39981	.05	- 12	.2	- 7	.8	- 1
ZP40699	.2	18	.8	14	3.25	42
ZP40868	.8	13	1.6	16	3.25	13
BJ04583	13	- 7	52	0	208	44
BE20274	26	- 20	104	21	416	86
BJ04636	13	- 15	52	30	104	29
BK74375	13	- 23	52	16	104	41
AE73324	13	21	52	- 2	208	3
AS72596	13	8	52	19	208	13
AU14450	13	15	52	16	208	23
AX17250	13	16	52	9	208	26
ZP41749	13	- 17	52	- 3	208	39
AY14763	13	- 18	52	- 38	208	- 30
BE75019	13	- 43	52	- 15	208	- 1
BK13925	13	- 27	52	- 38	208	- 19
BE20943	52	51	104	80	208	100
AU59100	52	78	104	90	208	100
AG09379	52	1	208	- 50	NDNDN	NDN
AG33437	52	- 43	208	- 30	NDNDN	NDN
AL73520	52	- 29	208	- 5	NDNDN	NDN
AM10000	52	18	208	1	NDNDN	NDN
AM10162	52	10	208	22	NDNDN	NDN
AT51074	52	- 12	208	3	NDNDN	NDN
ZE67962	52	2	208	4	NDNDN	NDN
ZE96721	52	9	208	18	NDNDN	NDN
ZN66045	52	5	208	- 14	NDNDN	NDN
BH72317	0.20	- 3	0.80	- 4	3.25	16
BL20649	0.80	6	3.25	- 1	13	- 11
ZN44003	0.1	- 2	0.4	19	1.6	5
ZP25914	0.20	- 6	0.80	- 1	3.25	12

TABLE I. (continued)

BN	DOSE1	SUPPRES1	DOSE2	SUPPRES2	DOSE3	SUPPRES3
ZP40868	0.20	- 3	0.80	14	3.25	20
AX64884	52	20	208	16	832	25
BE20274	52	- 8	104	19	208	61
BE20532	52	1	104	4	208	6
BE20943	52	68	104	85	208	100
BJ04583	52	41	104	65	208	56
BK74375	52	51	104	81	208	82
ZN41968	0.8	- 7	3.25	13	13	21
AH02786	52	- 7	208	- 5	NDNDN	NDN
BM08808	52	- 12	208	- 14	NDNDN	NDN
AX26839	13	16	52	12	208	16
ZM37665	13	9	52	19	208	37
ZP48113	13	33	52	8	208	6
ZP48122	13	8	52	18	208	24
AX76053	13	25	52	29	104	14
AX76062	13	32	52	29	104	42
AY91608	13	27	52	28	104	37
AX63172	13	17	52	26	104	43
AX67009	13	- 24	52	5	104	- 5
AQ38003	26	- 1	52	- 5	208	- 8
BC10527	26	- 22	52	13	208	- 8
BG46894	52	- 5	208	- 2	NDNDN	NDN
BC02936	52	- 13	416	9	NDNDN	NDN
BC86165	52	- 29	416	5	NDNDN	NDN
BK03947	52	- 19	416	1	NDNDN	NDN
BK13774	52	- 19	416	- 16	NDNDN	NDN
BK13989	52	- 12	416	- 15	NDNDN	NDN
BK14011	52	7	416	- 1	NDNDN	NDN

TABLE II. Summary of phenanthrene methanol and dibenzopyrrole compounds studied for suppressive activity against *Leishmania donovani*.

BN	DOSE1	SUPPRES1	DOSE2	SUPPRES2	DOSE3	SUPPRES3
AX64884	13	17	104	43	416	31
AX64884	52	20	208	16	832	25
AX26839	13	16	52	12	208	16
AX76062	13	32	52	29	104	42
AY91608	13	27	52	28	104	37
AX63172	13	17	52	26	104	43
AX67009	13 -	24	52	5	104 -	5
AQ38003	26 -	1	52 -	5	208 -	8
BC10527	26 -	22	52	13	208 -	8

TABLE III. Summary of the most active compounds studied in the primary visceral screen during the period 28 September 1990 - 27 September 1991.

BN	DOSE1	SUPPRESS1	DOSE2	SUPPRESS2	DOSE3	SUPPRESS3
BE20354	3.25	16	13	64	52	83
BH50802	0.8	23	3.25	53	13	98
BH72317	0.2	- 3	0.8	26	3.25	74
BH67432	13	100	52	100	3.25	98
ZN41968	13	9	52	50	208	99
BE20943	52	51	104	80	208	100
BE20943	52	68	104	85	208	100
BK74375	52	51	104	81	208	82

TABLE IV. Summary of trifluralin and derivative compounds studied for suppressive activity against *Leishmania donovani*.

BN	DOSE1	SUPPRES1	DOSE2	SUPPRES2	DOSE3	SUPPRES3
AE73324	13	21	52	- 2	208	3
AS72596	13	8	52	19	208	13
BE75019	13	- 43	52	- 15	208	- 1
BK13925	13	- 27	52	- 38	208	- 19
BC02936	52	- 13	416	9	NDNDN	NDN
BC86165	52	- 29	416	5	NDNDN	NDN
BK03947	52	- 19	416	1	NDNDN	NDN
BK13774	52	- 19	416	- 16	NDNDN	NDN
BK13989	52	- 12	416	- 15	NDNDN	NDN

TABLE V. Summary of results obtained from selected oligonucleotide compounds* studied for suppressive activity against *Leishmania donovani* promastigotes *in vitro*.

<u>Compound</u>	<u>Dosage</u>	<u>Percent Suppression</u>
LE001	50 μ m	7.81
LE501	50 μ m	- 0.95
LE002	50 μ m	5.17
LE502	50 μ m	- 4.76
LE003	50 μ m	-18.35
LE503	50 μ m	-20.38
LE004	50 μ m	20.78
LE504	50 μ m	32.07
LE005	50 μ m	11.92
LE505	50 μ m	14.14
LE001 ZV	30 μ m	37.31
LE501 ZV	30 μ m	6.62
LE002 ZV	30 μ m	- 3.92
LE502 ZV	30 μ m	-14.32
LE003 ZV	30 μ m	- 4.04
LE503 ZV	30 μ m	6.36
LE004 ZV	30 μ m	43.56
LE504 ZV	30 μ m	49.02
LE005 ZV	30 μ m	18.46
LE505 ZV	30 μ m	32.63

* Oligonucleotide compounds received directly from MicroProbe Corporation.

TABLE VI. Sequence of the oligonucleotides studied in vitro against L. donovani promastigotes.

NPC #	SEQUENCE
ANTISENSE	
LE001 ZV	CAA TAA AGT ACA GAA ACT GAT ACT TAT ATA GCG TT
LE002 ZV	ACT GAT ACT TAT ATA GCG TT
LE003 ZV	AT ACT TAT ATA GCG TT
LE004 ZV	T TAT ATA GCG TT
LE005 ZV	CAA TAA AGT ACA
SENSE	
LE501 ZV	AAC GCT ATA TAA GTA TCA GTT TCT GTA CTT TAT TG
LE502 ZV	AAC GCT ATA TAA GTA TCA GT
LE503 ZV	AAC GCT ATA TAA GTA T
LE504 ZV	AAC GCT ATA TAA
LE505 ZV	T GTA CTT TAT TG
ANTISENSE	
LE001 900617	CAA TAA AGT ACA GAA ACT GAT ACT TAT ATA GCG TT
LE002 900618-1	ACT GAT ACT TAT ATA GCG TT
LE003 900618	AT ACT TAT ATA GCG TT
LE004 900620	T TAT ATA GCG TT
LE005 900618	CAA TAA AGT ACA
SENSE	
LE501 900626	AAC GCT ATA TAA GTA TCA GTT TCT GTA CTT TAT TG
LE502 900618	AAC GCT ATA TAA GTA TCA GT
LE503 900618	AAC GCT ATA TAA GTA T
LE504 900618	AAC GCT ATA TAA
LE505 900618	T GTA CTT TAT TG

Appendix 2

FIGURE I. Structure of phenanthrene methanol WR149809.

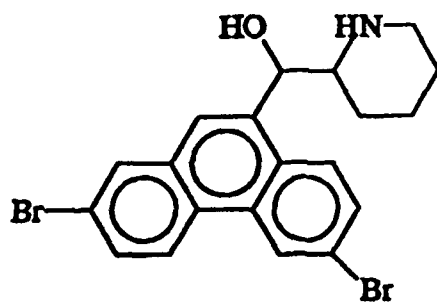
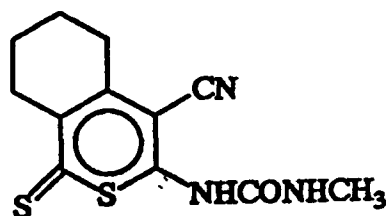


FIGURE II. Structure of WR223619.



Appendix 3

PERSONNEL EMPLOYED FROM THIS CONTRACT

<u>Position and Name</u>	<u>Percent Effort</u>	<u>Length of Employment</u>
Research Coordinator II Virginia B. Waits	100%	09/28/90 - Present
Laboratory Technician Mark Komorowski	50%	01/17/91 - 05/29/91
Laboratory Technician Barbara Harris	100%	09/23/91 - Present
Laboratory Technician Shannon Waits	10%	09/28/90 - Present

BIBLIOGRAPHY OF PUBLISHED WORK

Hanson, W. L., Chapman, W. L., Jr., Waits, V. B., and Lovelace, J. K.
1991. Development of *Leishmania* (Viannia) *panamensis* Lesions and
Relationship of Numbers of Amastigotes to Lesion Area on Antimony-
Treated and Untreated Hamsters. J. Parasit. 77(5): 780-783.

GRADUATE DEGREES RESULTING FROM THIS CONTRACT

None