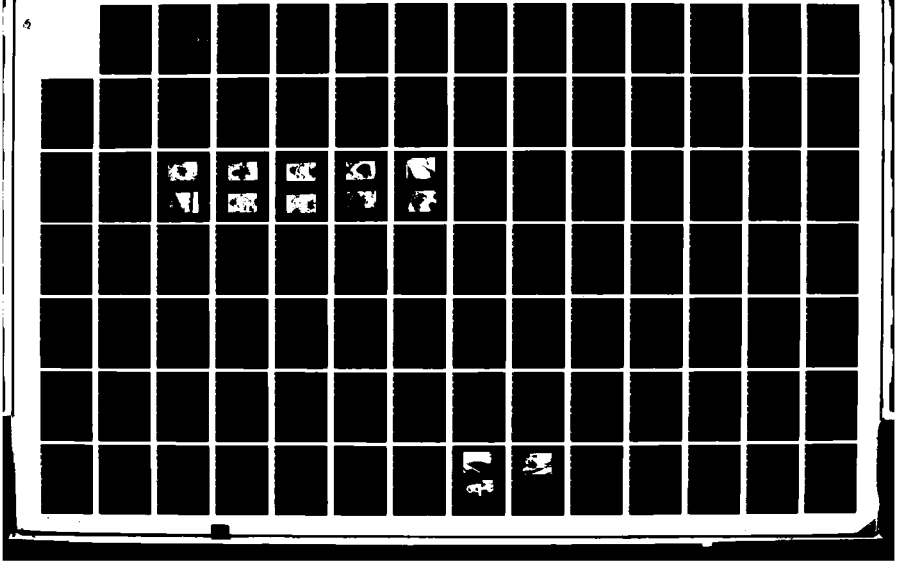
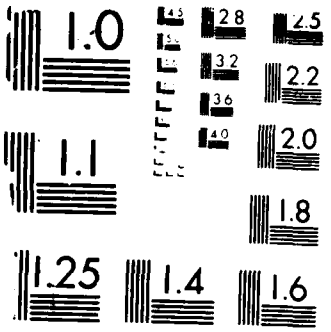
AD-A187 950 TESTING OF COMPOUNDS FOR EFFICACY AGAINST SCHISTOSOMIASIS(U) LOWELL UNIV MA CENTER FOR TROPICAL DISEASES J I BRUCE 25 AUG 87 04-4267/2 1/2

UNCLASSIFIED DAMD17-85-C-5209 F/G 6/15 NL





MICROCOPY RESOLUTION TEST CHART

AD_____

"Testing of Compounds for Efficacy Against Schistosomiasis"

Annual Summary Report

John I. Bruce, Ph.D.

25 August 1987

Supported By

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

Contract No. DAMD17-85-C-5209

Center for Tropical Diseases
University of Lowell
Lowell, Massachusetts 01854

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE

AD-A187950

REPORT DOCUMENTATION PAGE

Form Approved OMB No 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): 04-4267/2	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Lowell, Center for Tropical Diseases; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): 450 Aiken Street, Lowell, MA 10854	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION: US Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-85-C-5209
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 63750A; PROJECT NO. 3M46. 37500808; TASK NO. BC; WORK UNIT ACCESSION NO 072

11. TITLE (Include Security Classification)
"Testing of Compounds for Efficacy Against Schistosomiasis"

12. PERSONAL AUTHOR(S)
Bruce, John I.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Summary Report	FROM 86/08/15 TO 87/08/15	87/08/25	103

16. SUPPLEMENTARY NOTATION

17 COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	03	
06	13	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Antipenetrant; Antischistosomal drug; Schistosomiasis; Prophylactic
(Continued)

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

A total of 19 candidate compounds were tested for prophylactic activity in mice and hamsters. Two of these compounds (ZM65703 and BC78878) were studied at oral and subcutaneous regimens against S. mansoni and S. japonicum in mice and hamsters and against S. haematobium in hamsters. Moderate levels of protection which varied from species to species and regimen used were found, however, there was no complete protection. In addition gross dermal toxicity was also observed. These two compounds are not recommended for further evaluation as prophylactic candidate drugs.

The remaining 17 compounds were tested for prophylactic activity at the oral regimen against S. mansoni in mice. One of these compounds (BL23702) was found to be highly active, with a protection level of 99.5%. Only 2 worms (1 male and 1 female found in separate mice) were found during perfusion of the group of 10 treated mice which received a single dose of 100 mg/kg of drug for 5 consecutive days. No gross toxicity was observed for this compound. This drug is now being studied at an advanced test level in primates.
(Continued)

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT: ☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION: Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mrs. Virginia M. Miller	22b. TELEPHONE (Include Area Code): 301/663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

Unclassified

Subject Terms Continued: Block 18

Schistosoma mansoni	Cercariae
Schistosoma japonicum	Snails
Schistosoma haematobium	Schistosome Drug Resistance

Abstract Continued: Block 19

Three other compounds (BL23695, BL23677 and BL23510) gave protection levels against S. mansoni (Kenyan strain) ranging from 91.0% to 94.9% respectively. For each of the mice treated with these three compounds, mature adult male and female worms capable of depositing eggs in tissue, and thus causing pathology, were found. At the present time, no further studies on these compounds are contemplated.

Three protocols concerned with schistosomiasis were developed and initiated. Protocol WRHN-IIIMa was initiated to study the efficacy of a final formulation of the newly developed topical antipenetrant drug in monkeys. Protocol WRHN-IIIMb was develped and initiated to study the oral prophylactic activity of compound BL23702 in primates. Protocol WRHN-IIIMc was established to study the use of Marmoset monkeys as antipenetrant models. The monkeys used in each of these protocols have been exposed and no data is available at this time.

Investigation of the non-responsiveness of the Kenyan strain of S. mansoni to the positive reference drug control revealed that it is naturally resistant to this drug. This increases to six the number of drug-resistant strains of schistosomes maintained in this laboratory.

AD_____

"Testing of Compounds for Efficacy Against Schistosomiasis"

Annual Summary Report

John I. Bruce, Ph.D.

25 August 1987

Supported By

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

Contract No. DAMD17-85-C-5209

Center for Tropical Diseases
University of Lowell
Lowell, Massachusetts 01854

SUMMARY

A total of 19 candidate compounds were tested for prophylactic activity in mice and hamsters. Two of these compounds (ZM65703 and BC78878) were studied at oral and subcutaneous regimens against S. mansoni and S. japonicum in mice and hamsters and against S. haematobium in hamsters. Moderate levels of protection which varied from species to species and regimen used were found, however, there was no complete protection. In addition, gross dermal toxicity was also observed. These two compounds are not recommended for further evaluation as prophylactic candidate drugs.

The remaining 17 compounds were tested for prophylactic activity at the oral regimen against S. mansoni in mice. One of these compounds (BL23702) was found to be highly active, with a protection level of 99.5%. Only 2 worms (1 male and 1 female found in separate mice) were found during perfusion of the group of 10 treated mice which received a single dose of 100 mg/kg of drug for 5 consecutive days. No gross toxicity was observed for this compound. This drug is now being studied at an advanced test level in primates.

Three other compounds (BL23695, BL23677 and BL23510 gave protection levels against S. mansoni (Kenyan strain) ranging from 91.0% to 94.9% respectively. For each of the mice treated with these three compounds, mature adult male and female worms capable of depositing eggs in tissue, and thus causing pathology, were found. At the present time no further studies on these compounds are contemplated.

Three protocols concerned with schistosomiasis were developed

and initiated. Protocol WRHN-IIIMa was initiated to study the efficacy of a final formulation of the newly developed topical antipenetrant drug in monkeys. Protocol WRHN-IIIMb was developed and initiated to study the oral prophylactic activity of compound BL23702 in primates. Protocol WRHN-IIIc was established to study the use of Marmoset monkeys as antipentrant models. The monkeys used in each of these protocols have been exposed and no data is available at this time.

Investigation of the non-responsiveness of the Kenyan strain of S. mansoni to the positive reference drug control revealed that it is naturally resistant to this drug. This increases to six the number of drug-resistant strains of schistosomes maintained in this laboratory.

FOREWARD

1. Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

2. In conducting the research described in this report, the investigator(s) 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 Animals Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised, 1978).

TABLE OF CONTENTS

INTRODUCTION

The parasitic disease schistosomiasis is endemic in 74 countries in Africa, Asia, the Middle East, South America and islands of the Carribean (1). There are approximately 200-300 million infected persons with nearly 600 million constantly at risk in endemic areas (1). The disease has shown remarkable and substantial increases due in part to the creation of new water resources such as dams, lakes and irrigation schemes needed to meet increased energy and food demands. In addition, the introduction of the new water resources has caused dramatic ecological changes to occur in many of the endemic areas for schistosomiasis. Another recently occurring problem in the fight against schistosomiasis has been the emergence of drug-resistant strains of the parasite in Brazil (2, 3) and more recently in Kenya (4). To further complicate the drug-resistant picture, strains of S. mansoni which have been maintained for long periods in the laboratory without having any contact with drugs have been found to be resistant to one or more schistosomicides. Two strains used in this program have been found to be resistant. One strain from Kenya has been found to be resistant to niridazole (used as the reference drug in this program) and the other, a strain from Puerto Rico, was found to be resistant to oxamniquine, a drug which is included in the arsenal for use by the Army against S. mansoni infections. This emerging problem may compromise the success which has been made against the disease by use of chemotherapy.

An example of the spread of schistosomiasis into an area heretofore free of the disease is the country of Jordan. Up until 1984, Jordan was one of only a few countries in the Middle East free from both susceptible snails and the parasite. But during the past 10 years the threat of the disease becoming

established has been growing due to the presence of the vector-snail intermediate host which has defied attempts by the Ministry of Health to prevent its spread by use of chemicals. Indigenous cases have now been discovered nine years after finding the snail intermediate host and eight years after the influx of foreign infected migrant workers (5, 6) thus indicating active transmission.

From the military prospectus there are three stages of the schistosomiasis disease process in which medical casualties could be expected to occur: 1) skin penetration which is associated with penetration of the infective larva (cercariae), 2) Katayama fever, associated with the initial stages of egg deposition during the chronic phase of the disease and 3) chronic schistosomiasis, associated with granuloma formation in liver or urinary bladder after the third to fourth month of infection. Currently, there are no infective larva (cercariae) repellents, and neither prophylactic or suppressive drugs nor vaccines are available for use by the military of the United States. Even if it were possible to recognize early stages of the schistosome parasite in exposed personnel, there are no drugs available to treat early infections. Three drugs are currently available to treat mature infections in humans. These are praziquantel, active against all species of schistosomes infective to man; oxamniquine, active against one species of schistosome infective to man, namely Schistosoma mansoni (most effective against new world S. mansoni; and metrifonate, active against urinary schistosomiasis, namely S. haematobium (7). Strains of S. mansoni resistant to oxamniquine (3, 4) have been isolated from patients in Brazil and Kenya. It also appears that strains of S. haematobium resistant to metrifonate have emerged (8). This leaves only one antischistosomal agent, praziquantel, with

minimal side effects for use in treatment of schistosomiasis at the present. Studies to determine if praziquantel is capable of causing drug-resistant schistosomes to occur are in progress.

The lack of specific preventative measures for use by military personnel poses a significant potential problem for military operations which may occur in areas of strategic interest to the United States. Casualties have occurred during previous operations to British, French, Canadian and American forces.

During the past several decades many compounds have been tested in an effort to find a formulation which would afford protection against invasion of the skin by Schistosoma sp. infective larva (cercariae). This subject has been amply reviewed by several authors (9, 10, 11, 12, 13).

Evaluation (14, 15) of chemical preparations for their topical prophylactic antischistosomal activity is an ongoing program of the United States Medical Research and Development Command whose overall goal is to develop a substance which will protect personnel unable to avoid water contact when operating in areas where schistosomiasis is endemic.

The objective of the program as supported at the Center for Tropical Diseases, University of Lowell is to conduct secondary and definitive test evaluations of compounds showing superior antipenetration prophylactic efficacy against S. mansoni, S. japonicum and S. haematobium in rodents and/or primates. In addition, these evaluations may be made against other schistosome species and/or drug-resistant forms if requested. Curative and/or suppressive evaluation of compounds can also be carried out when requested.

During this funding period several protocols were initiated and completed or were initiated and are still in progress at the time of this report. These protocols are as follows:

I. Protocols Completed.

WRHN-IIa. Three compounds (two experimental and one reference compound) were evaluated in mice and hamsters.

II. Protocols Paritally Completed.

WRHN-IIb. Nineteen compounds (seventeen experimental and two reference compounds - both niridazole) were evaluated in mice. There are still nine compounds to be evaluated in this series.

III. Protocols in Progress.

a. WRHN-IIIMa. Topical antipenetrant primate study. This test used a final formulation against S. mansoni in Cebus apella monkeys.

b. WRHN-IIIMb. Prophylactic drug study. This test used a compound found to be highly active against S. mansoni in rodents. The compound is now under evaluation against S. mansoni in Cebus apella monkeys.

c. WRHN-IIIMc. Penetration model study. This test is being conducted in the Marmoset monkeys using S. mansoni as the schistosome species.

MATERIALS AND METHODS

Techniques for Cultivation and Maintenance of Snail Intermediate Host Species.

The procedures used for cultivating and maintaining the species and strains of schistosomes and their respective snail intermediate hosts are described and presented in Appendix I.

Animals.

The mice used were male ICR (outbred) weighing between 17-25 grams. The golden hamsters used were males weighing between 75-90 grams.

Schistosome Species.

The species of schistosomes used in this study were Schistosoma mansoni of Puerto Rican and Kenyan origin, S. haematobium of Egyptian and Nigerian origin, S. japonicum of Philippine origin. The snail intermediate host for S. mansoni is Biomphalaria glabrata from Puerto Rico and B. sudanica from Kenya, for S. haematobium the snail intermediate host is Bulinus truncatus truncatus from Egypt and B. truncatus rohlfsi from Nigeria, and for S. japoncium the snail intermediate host is Oncomelania hupensis quadrasi from the Philippine Islands.

Compounds

Protocol WRHN-IIa and WRHN-IIb.

A total of 31 compounds were received for evaluation in the secondary prophylactic test system. Twenty-eight of these drugs were designated as experimental compounds and three as the positive reference control drug (niridazole). These compounds were selected for advanced testing from the test results of the Brazil schistosomiasis primary prophylactic test and Walter Reed Institute of Research, Division of Experimental Therapeutics in-house testing program. In Table I, the compounds are presented by bottle number, Walter Reed accession number and quantities received at this institution.

In regard to the 31 compounds received for testing during this funding period there are no known adverse effects involved in handling these substances. However, good laboratory practice

has been used. All compounds are stored according to the labeling instructions: Red label = hygroscopic, Blue label = refrigerate, and Green label = compound in liquid form. No label indicates that no special storage conditions are required.

TABLE I

BOTTLE NUMBER	WALTER REED NUMBER	QUANTITY
ZM65703	007930	13 grams
BC78878	102796	20 grams
BL23953	169898 AC	4 grams
BL23962	147178 AE	4 grams
BL23695	169894 AE	4 grams
BL23677	140719 AH	4 grams
BL23686	149883 AJ	0.66 grams
BL23702	249313 AD	4 grams
BL28592	255750 AB	4 grams
BL26749	234927 AC	4 grams
BE19575	199385 AB	4.6 grams
BK21070	178460 AB	4 grams
BL28494	190266 AB	3.5 grams
BL28510	187445 AB	1.5 grams
BL28501	188225 AB	3.4 grams
BL28485	251901 AB	5 grams
BL26785	256075 A	3.75 grams
BL26776	256076 A	3.75 grams
BL35319	248310 AD	3 grams
*AQ21946	69504 DO	0.720 grams
*BH30042	234928 AA	0.875 grams
*BK98839	253817 AA	0.595 grams
*BL07459	254583 AA	0.260 grams
*BL07468	254574 AA	0.318 grams
*BL07566	254581 AA	0.278 grams
*BL24503	255967 AA	0.595 grams
*BL21459	255751 AA	0.295 grams
*BL21468	255752 AA	0.294 grams
AG63908 (niridazole)	005950	20 grams
BL26758 (niridazole)	005950 AK	10 grams
BL26758 (niridazole)	005950 AL	3.500 grams

* Compounds not yet tested.

Vehicles

Two vehicles were used. Pharmaceutical grade peanut oil for subcutaneous injections and Tween 80-methyl cellulose-saline (TMCS) for *per os*. TMCS is made as indicated in Table II.

TABLE II

Ingredient	Amount
Tween	0.40 ml
Methyl cellulose	0.20 gr
Sodium chloride	0.85 gr
Distilled water	98.55 ml

The sodium chloride was dissolved into distilled water and warmed to 70°C. Five ml of the warm saline solution was added to the Tween 80 and stirred until dissolved. The rest of the saline solution was then added. The solution was allowed to come to room temperature and the methyl cellulose added. The mixture was placed in a refrigerator overnight to facilitate dissolution of the methyl cellulose.

Drug Solutions

All animals were given 100 mg/kg of body weight subcutaneous and *per os* treatments. To obtain the proper concentrations of drug in a reasonable volume of carrier the following formula was used:

1) 10 mg of compound per ml of carrier (weight per volume).

2) Animal weight (in grams) X 0.01 = ml of solution per animal per day.

To calculate the volume of drug solution needed for each day of treatment the formula below was used:

Number of Animals	X	Avg. Weight plus 10%	X	0.01 ml per gm body wt.	=	Total vol. used for treatment

Example: 1) 10 mice X (25 gm + 2.5 gm) X 0.01 ml = 2.75 ml of solution

2) 10 hamsters X (90 gm + 9 gm) X 0.01 ml = 9.9 ml of solution

To example 1 above, 27.5 mg of compound is added to 2.75 ml of carrier soluiton (weight/volume) and to example 2, 99.0 mg of compound is added to 9.9 ml of carrier solution (weight/volume).

Treatment and Exposures

Treatment

Subcutaneous: Each animal was injected under the skin in the anterior - dorsal area just posterior to the neck for five days.

Per os: Each animal was administered the appropriate volume by gavage needle for five days.

Exposures

Mice and hamsters were anesthetized with sodium pentobarbital and exposed percutaneously to numbers of cercariae as shown in Table III. (See Appendix I.)

For protocol WRHN-IIa two experimental compounds (ZM65703 and BC78878) and the positive reference control compound (AG639078, niridazole) were studied. The experimental design for this protocol is presented in Table III.

TABLE III

Exp. No.	Animal Host	Parasite Species	Cercariae per Animal	Route	Perfusion Day After Infec.
1	Hamster	S. haematobium (Egy)	250	ORAL	90
2	Hamster	S. haematobium (Egy)	250	SQ	90
3	Mouse	S. japonicum	35	ORAL	49
4	Hamster	S. japonicum	75	ORAL	49
5	Hamster	S. mansoni	200	ORAL	49
6	Hamster	S. haematobium (Nig)	250	ORAL	90
7	Mouse	S. mansoni	150	ORAL	49

The experiments were conducted (Table III) using 10 animals for each compound, 10 animals as vehicle controls, and 10 animals for infection controls (no treatment). Each experiment used 50 animals per parasite species using the two experimental compounds and niridazole. The animals were treated on Monday, Tuesday, Wednesday, Thursday and Friday. All animals (including the infection controls) were infected Wednesday, but prior to treatment. Treatment consisted of 100 mg/kg of drug for five days.

For protocol WRHN-IIb, seventeen compounds and the positive reference control drug (niridazole) were studied for prophylactic activity against only S. mansoni in mice using an oral treatment regimen and a cercarial dosae of 150. These animals were sacrificed and perfused for worms at 49 days after exposure.

Adult Worm Recovery.

After the worms had a chance to mature (49 days for both S. mansoni and S. japonicum, and 90 days for S. haematobium) but before the egg burden caused mortality hamsters were sacrificed by injection with 0.5 ml of sodium pentobarbital (65 mg/ml sodium pentobarbital). The animals were necropsied and perfused using a method similar the the Perf-O-Suction method of Radke et al.

(16). The number of male, female and immature worms were counted and recorded for each animal. Worm burdens from mice were determined by hepatoportal perfusion of the animals. All animals were killed by intraperitoneal injection of 0.02 ml/gm/bw of heparinized (100 units per ml) of pentobarbital (65 mg/ml) solution. Worms were collected by whole body perfusion according to a modified method of Radke et al. (16) using heparinized (10 units per ml) 0.9 percent saline solution following the protocol which is currently being used at Lowell University. The worms from each perfused animal were counted as to male, female and maturity.

The number of worms recovered from the vehicle control animals was recorded and used to calculate the relative protection of drugs using the following formula (17):

$$\text{Relative Protection} = \frac{x - y}{x} \times 100$$

Where x = avg. number of worms recovered from control animals
Where y = avg. number of worms recovered from protected animals

RESULTS

The number of worms recovered at necropsy for animals treated with the test compound and those of respective control groups is shown in Tables IV - IX. Appendix II includes calculations showing mean worm burden calculations used in computing the test compound efficacy for each experimental group of animals.

The two experimental compounds (ZM65703 and BC78878) studied

in protocol WRHN-IIa did not provide complete protection against cercarial invasion of any of the species of schistosomes studied (Tables I, II and III). The partial protection which was observed varied from species to species and according to the route of administration of the drug (Tables I, II and III). Mature active male and female worms and viable eggs in tissue were observed for all groups of mice infected with either S. mansoni, S. japonicum or S. haematobium and treated with either of the two test compounds. The positive reference compound (niridazole) was very active against S. haematobium (99.7%) when administered subcutaneously and against S. japonicum (100%) when given orally.

Intense gross dermal toxicity was observed with compound ZM65703. The abnormalities observed were in the form of blebs, lesions and/or bumps and are shown in photographs 2 - 7. The toxicity observed with compound BC78878 was characterized by blebs and bumps, but no lesions were observed for this compound (photographs 8, 9). Abnormalities associated with subcutaneous injections of the positive reference control compound were also observed (photograph 10). No such abnormalities (lesions, blebs or bumps) were observed for animals receiving the vehicle used for suspending the drugs (photograph 1).

Of the compounds evaluated for prophylactic activity against S. mansoni (Kenyan strain) in mice, four of them showed levels of protection above 90% or more (Table VII and VIII). One of these compounds BL23702 gave nearly complete protection (99.5%) with only 2 worms (1 male and 1 female) found among the 10 animals examined. Protection levels observed for the other three compounds were as follows: BL23695 at 96.5%, BL23677 at 94.9% and BL23510 at 91.0% (87.2% for S. mansoni - Puerto Rican strain).

TABLE IV

PROPHYLACTIC EFFECT OF TEST COMPOUNDS AGAINST SCHISTOSOMA HAEMATOBIUM - EGYPTIAN STRAIN IN HAMSTERS EXPOSED EXPERIMENTALLY TO 250 CERCARIAE

EXP. #	ROUTE	CONTROL/ DRUG GROUP	WORM BURDENS AFTER PERFUSION										
			COLLECTION FILTER*				TISSUE EXAMINATION**				TOTAL		EFFICACY
			M	F	SM[1]	SF[2]	M	F	SM	SF	WORMS	ANIMALS	(%)
1	Oral	Untreated	157	128	-	-	40	43	-	-	368	8	-
		TMCS (veh)	366	300	1	1	25	24	-	-	687	9	-
		AG63908	213	101	3	1	12	11	-	-	341	10	55.3
		BC78878	164	127	2	-	9	7	-	-	309	10	59.5
		ZM65703	152	109	2	4	35	35	-	-	337	9	51.0
2	SQ	Untreated	238	137	4	1	24	25	-	-	429	10	-
		Peanut Oil (veh)	283	216	5	1	46	47	-	-	598	10	-
		AG63908	1	-	1	-	-	-	-	-	2	10	99.7
		BC78878	139	81	8	2	9	8	-	-	247	10	58.7
		ZM65703	129	99	1	1	9	9	-	-	248	10	58.5

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

TABLE V

EFFICACY OF ORAL DOSES OF TEST COMPOUNDS AGAINST *SCHISTOSOMA JAPONICUM* AND *S. MANSONI* IN MICE

EXP. #	SCHISTO. SPECIES (STRAIN)	CONTROL/ DRUG GROUP	WORM BURDENS AFTER PERFUSION										
			COLLECTION FILTER*				TISSUE EXAMINATION**				TOTAL WORMS	TOTAL ANIMALS	EFFICACY (%)
			M	F	SM[1]	SF[2]	M	F	SM	SF			
3	Sj+ (Phi+)	Untreated	133	115	2	14	2	2	1	-	269	10	-
		TMCS (veh)	159	116	-	5	1	1	-	-	282	10	-
		AG63908	-	-	-	-	-	-	-	-	0	10	100.0
		BC78878	151	117	5	9	4	5	-	-	291	10	-3.2
		ZM65703	55	20	15	-	-	-	-	-	90	10	68.1
7	Sm++ (Ken++)	Untreated	278	261	3	10	-	-	-	-	552	10	-
		TMCS (veh)	258	211	3	2	7	10	-	-	491	10	-
		AG63908	133	81	12	-	-	-	-	-	226	10	54.0
		BC78878	252	201	10	1	1	1	-	-	466	10	5.1
		ZM65703	29	28	-	12	-	-	-	-	69	10	85.9

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

Sj: *Schistosoma japonicum* Sm: *Schistosoma mansoni*
Phi: Philippine strain Ken: Kenyan strain

\+ *S. japonicum* infected animals were exposed to 35 cercariae.
++ *S. mansoni* infected animals were exposed to 150 cercariae.

TABLE VI

EFFICACY OF ORAL DOSES OF TEST COMPOUNDS AGAINST SCHISTOSOMA JAPONICUM, S. MANSONI AND S. HAEMATOBIUM IN HAMSTERS

EXP. #	SCHISTO. SPECIES (STRAIN)	CONTROL/ DRUG GROUP	WORM BURDENS AFTER PERFUSION										
			COLLECTION FILTER*				TISSUE EXAMINATION**				TOTAL		EFFICACY
			M	F	SM1	SF2	M	F	SM	SF	WORMS	ANIMALS	(%)
4	Sj (Phi+)	Untreated	60	60	-	132	9	10	-	-	271	10	-
		TMCS (veh)	122	120	-	109	24	26	-	-	401	10	-
		AG63908	85	85	-	51	4	5	-	-	230	10	44.0
		BC78878	53	53	-	82	29	34	-	-	251	10	38.9
		ZM65703	109	109	-	184	8	7	-	-	417	10	-1.5
5	Sm (Ken++)	Untreated	517	447	3	53	60	68	-	-	1148	10	-
		#	263	278	1	27	1	6	-	-	576	6	-
		TMCS (veh)	623	547	2	21	35	34	-	-	1262	10	-
		AG63908	324	305	5	6	7	4	-	-	651	7	26.3
		#	477	451	2	6	23	26	-	-	985	10	21.9
		BC78878	535	474	7	12	15	15	-	-	1058	10	16.2
		ZM65703	548	475	11	129	3	3	-	-	1169	10	7.4

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

Sj: Schistosoma japonicum Sm: Schistosoma mansoni

Phi: Philippine strain Ken: Kenyan strain

\+ S. japonicum infected animals were exposed to 75 cercariae.

++ S. mansoni infected animals were exposed to 200 cercariae.

\# 2 infection control groups were necessary since the AG63908 group was repeated to reduce group size.

(Continued on next page)

TABLE VI (CONTINUED)
EFFICACY OF ORAL DOSES OF TEST COMPOUNDS AGAINST *SCHISTOSOMA JAPONICUM*, *S. MANSONI* AND *S. HAEMATOBIUM* IN HAMSTERS

EXP. #	SCHISTO. SPECIES (STRAIN)	CONTROL/ DRUG GROUP	WORM BURDENS AFTER PERFUSION										
			COLLECTION FILTER*				TISSUE EXAMINATION**				TOTAL WORMS	TOTAL ANIMALS	EFFICACY (%)
			M	F	SM[1]	SF[2]	M	F	SM	SF			
6	Sh (Nig^{+++})	Untreated	163	144	8	8	8	7	-	-	388	10	-
		TMCS (veh)	181	161	4	3	11	8	-	-	368	10	-
		AG63908	64	40	3	1	2	1	-	-	111	10	69.8
		BC78878	93	106	2	6	19	18	-	-	244	10	33.7
		ZM65703	154	145	4	5	7	3	-	-	318	10	13.6

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

Sh: *Schistosoma haematobium* Nig: Nigerian strain

+++ *S. haematobium* infected animals were exposed to 250 cercariae.

TABLE VII

EFFICACY OF ORAL DOSES OF TEST COMPOUNDS AGAINST SCHISTOSOMA MANSONI - KENYAN STRAIN IN MICE EXPOSED EXPERIMENTALLY TO 150 CERCARIAE

EXP. #	CONTROL/ DRUG GROUP	WORM BURDENS AFTER PERFUSION										
		COLLECTION FILTER*				TISSUE EXAMINATION**				TOTAL		EFFICACY
		M	F	SM[1]	SF[2]	M	F	SM	SF	WORMS	ANIMALS	(%)
8a	Untreated	196	196	1	75	-	1	-	-	469	10	-
	TMCS (veh)	146	164	4	38	16	19	-	-	387	8	-
	BL26758	116	48	8	6	-	-	-	-	178	10	63.2
	BL23962	170	158	7	32	-	-	-	-	367	10	24.2
	BL23953	110	115	4	66	2	2	-	-	299	10	38.2
	BL23695	4	6	-	3	1	1	-	-	15	9	96.5
8b	Untreated	344	259	8	2	-	-	-	-	613	10	-
	TMCS (veh)	230	188	7	9	-	-	-	-	434	10	-
	BL26758	253	110	10	4	-	-	-	-	377	10	13.1
	BL23702	1	-	1	-	-	-	-	-	2	10	99.5
	BL23677	18	1	2	1	-	-	-	-	22	10	94.9
	BL23686	43	28	9	3	-	-	-	-	83	10	80.9

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

(Continued on next page)

TABLE VII (CONTINUED)
EFFICACY OF ORAL DOSES OF TEST COMPOUNDS AGAINST SCHISTOSOMA MANSONI - KENYAN STRAIN IN MICE EXPOSED EXPERIMENTALLY TO 150 CERCARIAE

EXP. #	CONTROL/ DRUG GROUP	WORM BURDENS AFTER PERFUSION										
		COLLECTION FILTER*				TISSUE EXAMINATION**				TOTAL		EFFICACY
		M	F	SM1	SF2	M	F	SM	SF	WORMS	ANIMALS	(%)
	Untreated	366	258	8	4	-	-	-	-	636	10	-
	#	365	285	14	2	2	2	-	-	670	10	-
	TMCS (veh)	353	259	10	1	4	4	-	-	631	10	-
12	BL26758	265	217	7	4	1	1	-	-	495	10	21.5
	BL28510	39	24	5	5	-	-	-	-	73	9	87.2
	BL28501	184	164	7	12	-	1	-	-	368	10	41.7
	BL28485	342	268	1	1	1	-	-	-	613	10	2.8
	BK21070	147	95	7	5	-	-	-	-	254	8	49.8
	Untreated	331	284	10	5	1	1	-	-	632	10	-
	TMCS (veh)	267	272	26	17	-	-	-	-	582	10	-
13	BL26758	181	155	27	7	1	1	-	-	372	9	29.0
	BL28494	310	284	3	6	-	-	-	-	603	10	-3.6
	BL28592	292	273	15	22	-	-	-	-	602	10	-3.4
	BE19575	256	245	4	24	-	-	-	-	529	10	9.1
	BL26749	211	203	5	23	-	-	-	-	442	10	24.0

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.
** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.
1: Denotes stunted female worms. 2: Denotes stunted male worms.
Two infection control groups were necessary since the TMCS group and the BK21070 group had to be repeated. (The original TMCS and BK21070 groups were discarded.)

(Continued on next page)

TABLE VII (CONTINUED)
EFFICACY OF ORAL DOSES OF TEST COMPOUNDS AGAINST SCHISTOSOMA MANSONI - KENYAN STRAIN IN MICE EXPOSED EXPERIMENTALLY TO 150 CERCARIAE

EXP. #	CONTROL/ DRUG GROUP	WORM BURDENS AFTER PERFUSION										
		COLLECTION FILTER*				TISSUE EXAMINATION**				TOTAL		EFFICACY
		M	F	SM[1]	SF[2]	M	F	SM	SF	WORMS	ANIMALS	(%)
14	Untreated	630	574	2	5	21	19	-	-	1251	10	-
	TMCS (veh)	628	608	11	5	9	8	-	-	1269	10	-
	BL26758	445	438	19	11	3	1	-	-	917	10	27.7
	BL26776	488	503	2	14	5	4	-	-	1016	9	11.0
	BL35319	509	475	7	2	3	3	-	-	999	10	21.3
	BL26785	410	395	10	4	6	6	-	-	831	7	6.5

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

TABLE VIII

EFFICACY OF ORAL DOSES OF TEST COMPOUNDS AGAINST SCHISTOSOMA MANSONI - PUERTO RICAN STRAIN IN MICE EXPOSED EXPERIMENTALLY TO 150 CERCARIAE

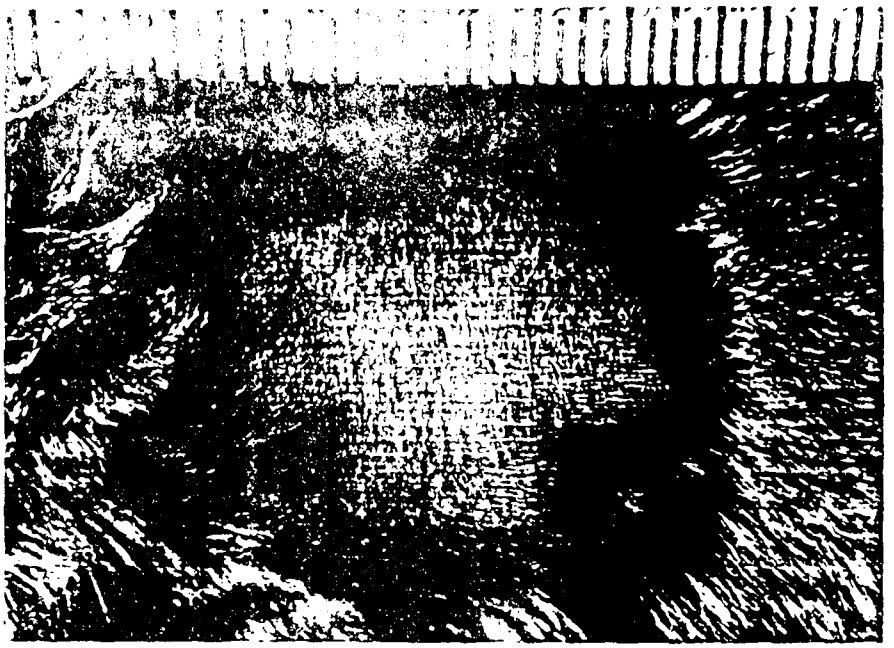
EXP. #	CONTROL/ DRUG GROUP	WORM BURDENS AFTER PERFUSION										
		COLLECTION FILTER*				TISSUE EXAMINATION**				TOTAL		EFFICACY
		M	F	SM[1]	SF[2]	M	F	SM	SF	WORMS	ANIMALS	(%)
10	Untreated	454	236	7	-	2	1	-	-	700	10	-
	TMCS (veh)	471	225	13	-	-	-	-	-	709	10	-
	BL26758	62	23	8	2	-	-	-	-	95	10	86.6
	BL28592	398	218	15	-	-	-	-	-	631	10	11.0
	BE19575	319	183	12	-	-	-	-	-	514	10	27.5
	BL26749	218	122	3	-	-	-	-	-	343	10	51.6
11	Untreated	369	285	7	1	1	1	-	-	664	10	-
	TMCS (veh)	294	204	2	-	-	-	-	-	500	8	-
	BL26758	27	16	-	7	-	-	-	-	50	10	92.0
	BL28494	268	199	6	-	-	-	-	-	473	9	15.8
	BK21070	53	35	3	-	-	-	-	-	91	8	81.8
	BL23510	28	9	2	-	-	-	-	-	39	7	91.0

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

Photograph 1



Skin surface after five days of subcutaneous injection with peanut oil (vehicle). No gross abnormalities such as bumps, clebs or lesions were observed.

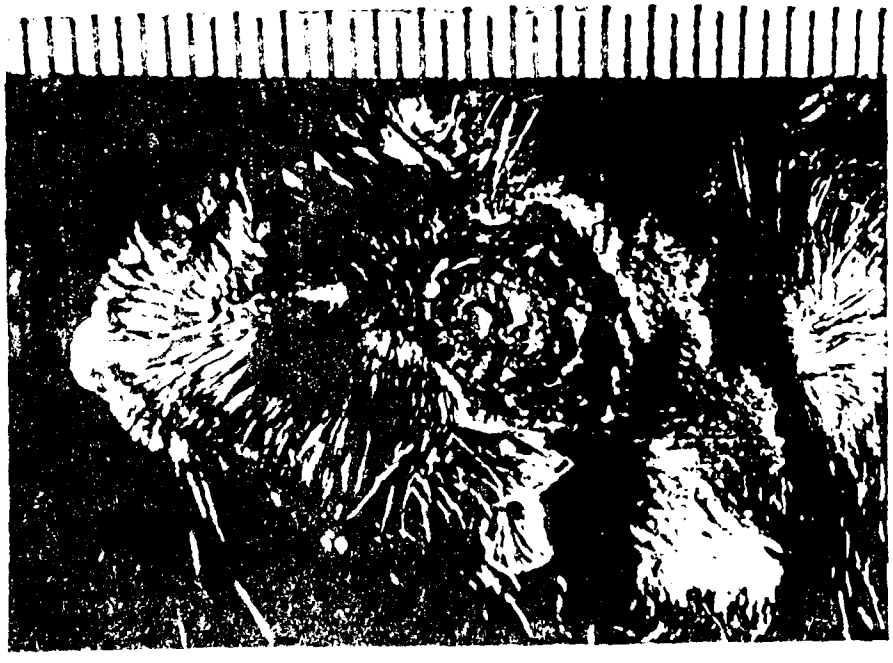
Photograph 2

[illegible] after five consecutive days [illegible] in peanut oil.

Photograph 3

Small lesion on a hamster injected for five consecutive days subcutaneously with compound ZM65703 in peanut oil.

Photograph 4

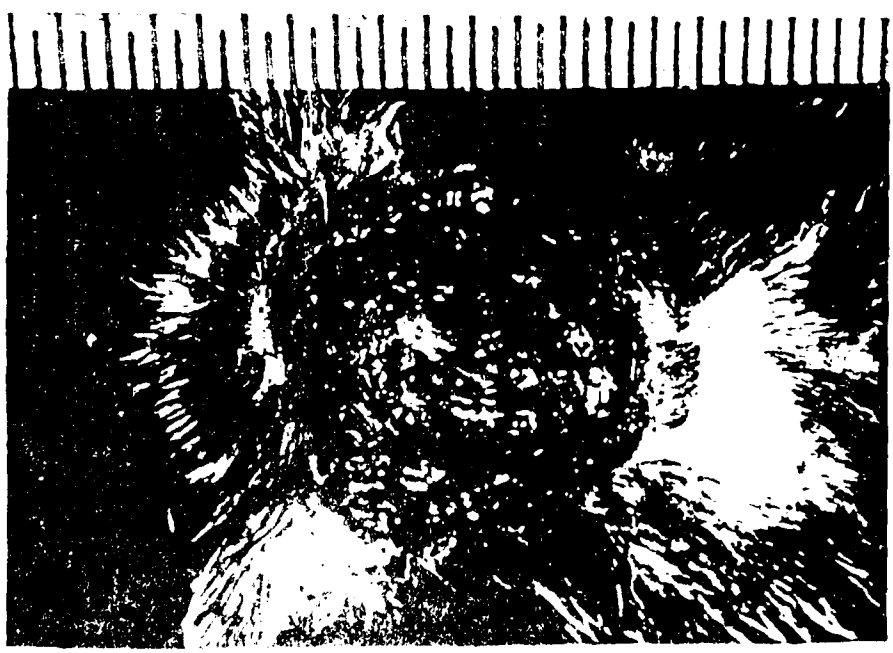


Medium [illegible] injected for five consecutive days [illegible] ZM65703 in peanut oil.

Photograph 5

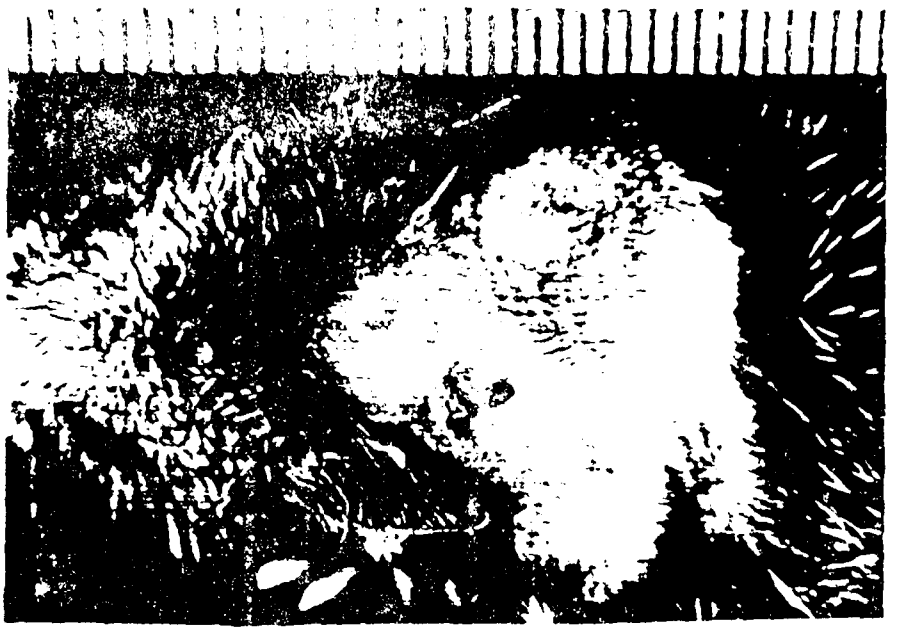
Large lesion on a hamster injected for five consecutive days subcutaneously with compound ZM65703 in peanut oil.

Photograph 6



Large lesion [illegible] Photograph 5 as seen on the following day.

Photograph 7

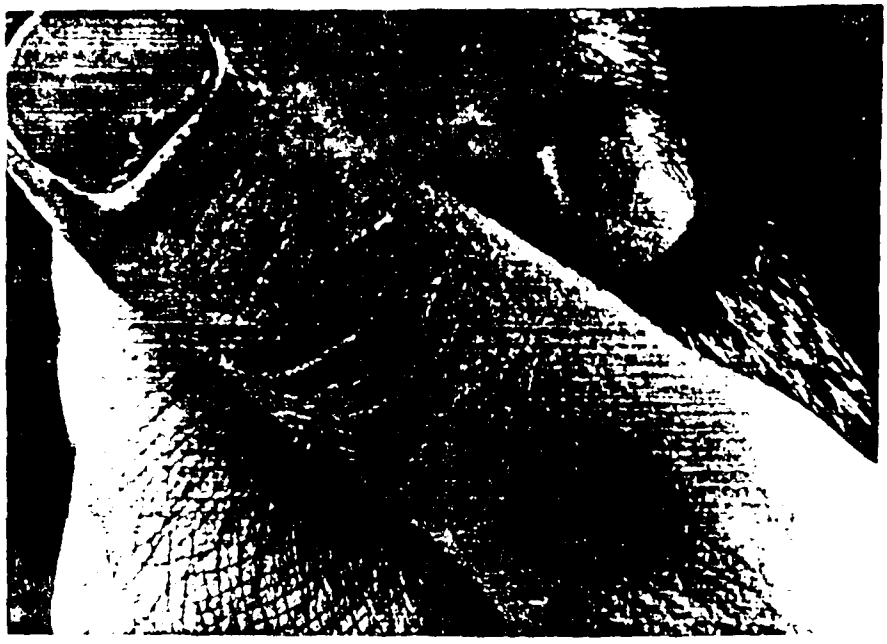


Firm bumps with small lesion on a hamster injected for five consecutive days subcutaneously with compound 2765703 in peanut oil.

Photograph 8

[illegible] five days [illegible] oil.

Photograph 9



Bleb on a hamster injected for f ve consecutive days subcutaneously with compound BC 8878 in peanut oil

Photograph 10

Bleb on [illegible] consecutive days subcutaneously [illegible] in peanut oil

TABLE IX

EFFICACY OF TWO DIFFERENT BATCHES OF REFERENCE COMPOUND (AG63908 AND BL26758) AGAINST SCHISTOSOMA MANSONI - PUERTO RICAN STRAIN AND S. MANSONI - KENYAN STRAIN

SCHISTOSOME SPECIES (STRAIN)	CONTROL/ DRUG GROUP	WORM BURDENS AFTER PERFUSION										
		COLLECTION FILTER*				TISSUE EXAMINATION**				TOTAL		AVERAGE NO. OF WORMS
		M	F	SM[1]	SF[2]	M	F	SM	SF	WORMS	ANIMALS	
S. mansoni (Kenyan)	Untreated	102	102	1	24	-	1	-	-	230	5	46
	BL26758	58	48	2	10	-	-	-	-	118	3	39
	AG63908	104	93	5	12	-	-	-	-	214	5	43
S. mansoni (Puerto Rican)	Untreated	63	62	-	53	7	5	-	-	190	4	48
	BL26758	12	7	1	-	-	-	-	-	20	5	4
	AG63908	28	26	-	2	-	-	-	-	56	6	9

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

We encountered problems in the response of the Kenyan strain of S. mansoni to the positive reference control drug (niridazole). A separate protocol (WRDR-IH) was established to investigate the reason for the tolerance of the Kenyan strain to the reference compound. The experiment consisted of comparison of the "old" batch of the reference drug with that of a "new" batch of the drug against the Kenyan strain of S. mansoni and a Puerto Rican strain of S. mansoni. Appropriate groups of untreated infected control mice were used for each S. mansoni strain. The results obtained (Table IX) indicate that the Kenyan strain was resistant to the does of the reference drug (niridazole) used (i.e. 100 mg/kg of drug for 5 consecutive days). Regardless of whether the batch of drug (niridazole) was "old" or "new" the Puerto Rican strain of S. mansoni used was very susceptible.

Protocol WRHN-IIIMa which deals with the evaluation of the final formulation of the topical antipenetrant drug has been initiated. When tested previously in rodent and primate test systems, this drug gave 100% protection against the skin invasion by infective larvae (cercariae) of three of the major schistosomes infective to man, namely, S. mansoni, S. japonicum and S. haematobium. The topical antipenetrant formulation is now being tested in Cebus apella monkeys against a drug-resistant (oxamniquine) strain of S. mansoni from Brazil. All test groups of monkeys (experimental and controls) in this study have been treated and exposed to infective larva (cercariae) and examination of fecal samples from each monkey to determine the efficacy of the drug treatments has begun. Description of this protocol is presented in Appendix III, Section I.

Protocol WRHN-IIIMb which deals with the evaluation of the

prophylactic properties of compound WR249313 (BL23702) found previously to be nearly 100% (99.5%) protective in the primary and secondary rodent test systems has been initiated. The tests in this protocol are being conducted in Cebus apella monkeys exposed to a drug-resistant (oxamniquine) strain of S. mansoni from Brazil. All monkeys (experimental and control) have been treated and exposed to infective larvae (cercariae). Examination of fecal samples to determine efficacy of the drug will begin within a few weeks. Description of this protocol is presented in Appendix III, Section II.

WRHN-IIIMc which deals with the study of the Marmoset penetration model study has been initiated. These animals were exposed to a drug-resistant (oxamniquine) strain of S. mansoni. Fecal examination to determine infectivity of the schistosome strain will begin within a few weeks. Description of this protocol is presented in Appendix III, Section III.

DISCUSSION

Compounds ZM65703 and BC78878 (Protocol WRHN-IIa) were priority prophylactic candidate drugs previously found to be highly active in the rodent primary prophylactic test. They were referred for study in the secondary prophylactic test and were studied extensively in oral and subcutaneous regimens against S. mansoni, S. japonicum and S. haematobium in mice and hamsters.

At the drug dosage (100 mg/kg X 5 days) used, complete protection was not obtained with either oral or subcutaneous regimen against any of the species of schistosomes studied. Mature adult male and female worms and viable eggs in tissue were found in all groups of animals exposed and treated with either drug. The presence of mature male and female worms capable of

depositing eggs which cause the major pathological manifestations for this disease (despite the presence of low worm burdens in some of the groups) indicate that these two drugs should not be a candidate for further prophylactic studies against schistosomiasis in rodents or higher mammalian species.

In addition, the dermal toxicity observed when the drugs were administered subcutaneously further indicate that these drugs should not be considered for further studies.

Testing of 17 compounds (WRHN-IIb) previously tested in the primary prophylactic test system and selected for priority testing in the secondary prophylactic system and a positive reference control drug (BL26758) were evaluated in mice against S. mansoni (Kenyan and/or Puerto Rican strains). One of these compounds (BL23702 - WR249313) was found to provide protection of 99.5% against the infective larvae (cercariae) when administered at a oral dosage regimen of 100 mg/kg X 5 days. Only two mature worms (one male and one female found in separate mice) were found among the group of 10 mice treated. The superior activity of this compound against S. mansoni in mice indicated that it should be studied further in the primate test system and against other species of schistosomes (i.e. S. japonicum and S. haematobium) in mice and hamsters.

Even though levels of protection above 90% were observed for three other compounds (BL23695, BL23677 and BL23510), mature male and female worms with associated viable eggs in tissue were found. The presence of mature male and female worms capable of depositing eggs which cause the major pathological manifestations (despite the presence of low worm burdens in mice of some groups) indicate that at the dosages used these compounds are ineffective and that further studies at this time are not warranted. Further

studies however may be warranted at higher dosage regimens and against S. japonicum and S. haematobium in mice and hamsters.

The discovery that a strain of schistosome (S. mansoni from Kenya) is resistant to treatment with the positive reference drug (niridazole) used in all of our drug evaluations points out again that emerging problem of drug-resistance in schistosomiasis therapy. The occurrence of drug-resistant strains in areas considered of stategic to the military poises a particular problem as regards the treatment of schistosomiasis. This problem needs immediate addressing within the framework of the current drug evaluation program over a long period of time.

CONCLUSIONS

Because of its nearly complete protection (99.5%) against invasion of mouse skin by S. mansoni cercariae compound BL23702 is a candidate for advanced studies in monkeys as a oral prophylactic agent against S. mansoni infections. Further studies are also recommended against S. japonicum and S. haematobium in mice and monkeys with oral and subcutaneous regimens.

The absence of complete protection against invasion of mouse skin by S. mansoni larvae for 16 compounds and dermal toxicity observed for 2 other compounds suggests that no further testing is warranted for any of these compounds.

The finding that the Kenyan strain of S. mansoni is resistant to the positive reference drug (niridazole) indicates that another strain of S. mansoni from Kenya should be obtained which is not resistant to this drug for use in the prophylactic test systems.

APPENDIX I*

Techniques for Cultivation and Maintenance of Snail and Schistosome Species and Safety Practices

* Appendix I includes Figures 1 - 5 and Tables 1 - 6

SECTION I

Techniques for Cultivation of Biomphalaria glabrata and Maintenance of Schistosoma mansoni

I. Units for Snail Maintenance

Two types of units are used.

A. A mobile unit similar to that described by Davis (1971) is used for holding large numbers of breeder and stock (Figure 1). The overall dimensions of this unit are 52 x 175 x 243 cm. This unit is capable of holding 32 glass aquaria (20-liter), 64 plastic trays or 288 petri dishes. There are two types of set-ups for this unit. One is equipped with air lines and fluorescent lights and is used to accommodate glass aquaria for stock snail cultures. Its air manifold system is connected to an oil extractor and air pressure gauge for removing harmful oil droplets from the air and measuring the air pressure, respectively. There are 2 of these units available in this laboratory, which is more than sufficient to meet any Biomphalaria culture requirements. The second type of set-up is one in which only fluorescent lights are used for accommodating the type of aquaria (plastic trays and petri dishes) which do not require aeration. This mobile unit is also used for cultivating algae needed as food for snails.

B. A mobile unit, constructed of heavy-duty steel (Figure 2) is used for holding pre-patent snails, patent snails and algal cultures. The overall dimensions of this unit are 61 x 122 x 188 cm. It is capable of holding 50 plastic trays or 180 petri dishes. This unit is set up in two

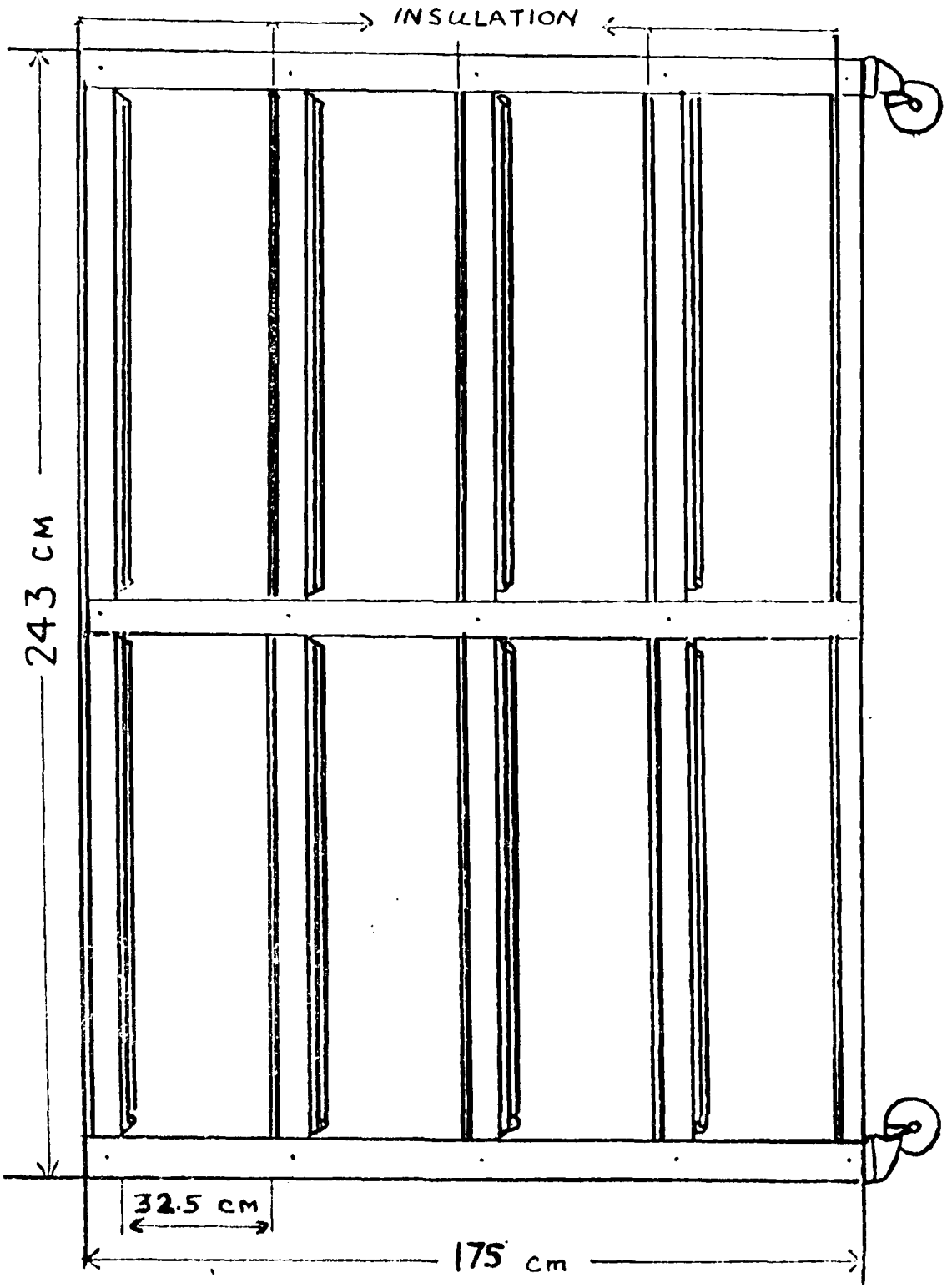


Figure 1 Snail Maintenance Unit (wooden, mobile type)

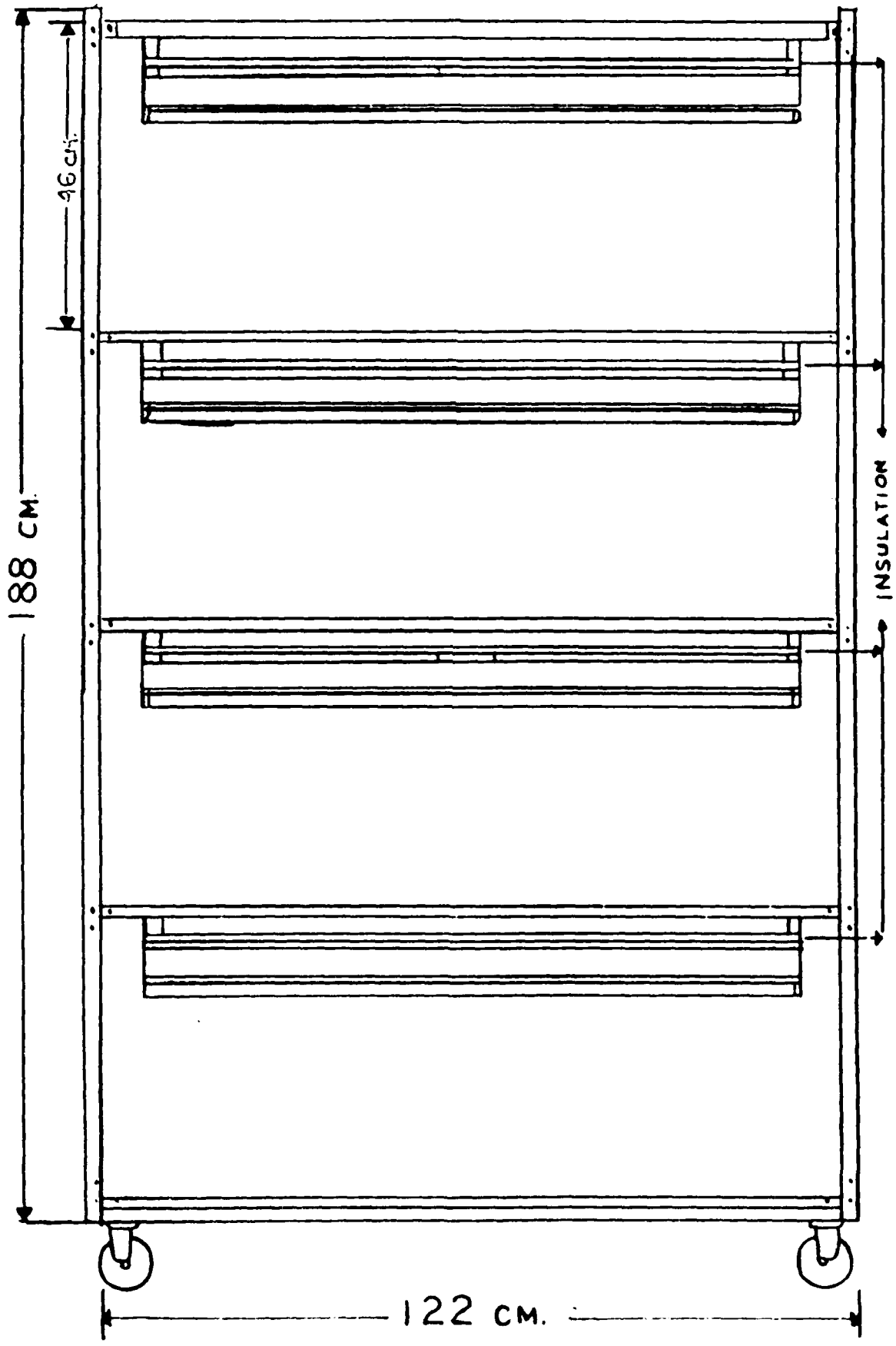


Figure 2 Snail Maintenance Unit (steel, mobile type)

types, neither of which is equipped with air lines. The first type of set-up employs fluorescent light. Four of these are available for accommodating plastic trays for pre-patent snails and petri dishes for algal cultures. The second type of set-up does not employ fluorescent lights. Two of these are available for accommodating plastic trays containing patent snails.

Three kinds of aquaria are used: 1) twenty-liter glass aquaria are used for rearing and maintaining stock snails; 2) plastic trays with a holding capacity of 1.5 liters of water are used as aquaria for breeder snails as well as for both pre-patent and patent snails; 3) petri dishes with a holding capacity of 40 ml of water are used as aquaria for newborn snails.

II. Mouse Restraining Chambers and Exposure Boards

The mouse restraining chambers used for exposing mice to cercarial suspensions by tail immersion technique is a modification of the one described by Stirewalt and Bronson (1955). The exposure boards used for holding the restraining chambers during exposure of mice to cercariae was designed by Loyd (Bruce and Radle, 1971) and is capable of accommodating 100 animals. This board has proven to be most effective for exposing large numbers of animals by tail immersion method to S. mansoni cercariae.

III. Environmental Paremeters

A. Light. In both types of snail maintenance units

described above, 40-w "cool" white fluorescent tubes are suspended 12 inches and 18 inches respectively above each shelf. The 20-liter glass aquaria used for maintaining stock snails are held under 12-hour light and 12-hour darkness. The plastic trays (when used for breeding snails and for maintaining pre-patent snails) are placed under constant light.

B. Water. Tap water is conditioned by passing it through a "Diamond" filter (Model #131-1575) containing layers of activated charcoal and sand. This water is then aerated for one day prior to use. The pH of the water is initially 7.1. The pH of the water in glass aquaria and plastic trays is monitored weekly.

C. Aeration. The water of all glass aquaria pans is continuously aerated. The air is supplied by a centrally located air compressor and passes through a water-oil extractor into the culture units. The water contained in plastic trays and petri dishes is not aerated.

D. Temperature. Temperature is monitored daily. A central air conditioning unit maintains the temperature in the snail cultivation rooms between 25°C and 27°C.

E. Food. Romaine lettuce is used as a basic food source. In addition, one or two dishfuls of mud, on which blue-green algae (Nostoc muscorum) has been grown (Liang, 1974) is placed in all glass aquaria, and plastic trays to serve as an additional food source. Snails in petri dishes are given only blue-green algae with mud. In order to improve the growth rate of

snails, an agar mud paste, has been formulated. This paste is found to be consumed very well by Biomphalaria, Bulinus and Oncomelania snails. It contains in addition to the mud, soya bean powder, rice powder, oyster shell powder, cerophyl (dehydrated cereal grass leaves), fish food, yeast and sodium chloride (modified from Moore et al. 1953; Madsen and Frandsen, 1980; and Basch, personal communication, 1984).

IV. Breeding

Snails measuring 12-15 mm are removed from glass aquaria and placed in plastic trays (10 per tray) containing 1.5 liters of aerated tap water and pieces of styrofoams (3 x 5 in.). Trays are placed under continuous light and supplied with food. After one week the trays are changed and the egg masses are removed from the trays and styrofoams, placed in petri dishes with aerated tap water and incubated under ceiling light for about one week until hatching occurs. Newborn snails (0.6-0.8 mm in shell diameter) are transferred with a pipette to petri dishes containing blue-green algae, mud and water and kept at a density of 50 snails per dish. Dishes are maintained under continuous light. At the end of one week, snails are transferred to new dishes with a density of 25 snails per dish. In 14 days, young snails 3-5 mm in size are available for use in either initiating new cultures or for eventual exposure to S. mansoni miracidia.

V. Rearing and Maintenance

For initiating new cultures, snails are removed from petri

dishes in groups of 250 and placed in 20-liter glass aquaria with aerated tap water. For snails which are exposed to miracidia, they are maintained in groups of 50 in plastic trays containing 1.5 liters of water. Patent snails are maintained in plastic trays, 25 snails per tray.

VI. Maintenance of *S. mansoni* Life Cycle

A. Collecting miracidia. Eggs and miracidia are collected according to the method of Liang and Kitikoon (1980). Liver and/or feces-free intestines, excised from mice 8-12 weeks after exposure to *S. mansoni* are used as the sources of eggs. Tissues are cut into pieces and placed into a 200-ml stainless steel Eberbach container with 20 ml of 0.85% saline. The container is placed on a single speed Waring blender (No. 700) connected to a variable autotransformer and tissues are homogenized for 5-10 sec. at very low speed (30 volts). The suspension is poured into a tiered column of sieves arranged in descending order of mesh openings (420 μ, 177 μ, 105 μ, and 45 μ). The eggs are washed through to the bottom sieve with 100 ml of 0.85% saline. Large pieces of tissues, which had been trapped in the top sieves, are re-homogenized in 20 ml of 0.85% saline. The procedure is repeated three times at low (50 volts), intermediate (70 volts) and finally high speed (100 volts). A volume of 100 ml of aerated tap water is poured into the sieve column to rinse eggs free from saline. The eggs are washed from the bottom sieve into a petri dish (2 x 10 cm) with 40 ml of aerated tap water and concentrated to the center of the dish by gentle rotation. The eggs are then pipetted into a small petri dish (1.5 x 6 cm) and

the dish is placed under ceiling illumination for hatching miracidia.

The entire procedure takes approximately 15 min. and miracidia usually appear within 5 min. To insure the high percentage of infectivity, the snails are exposed to miracidia which are less than one hour old.

B. Exposure of Snails. B. glabrata snails (3-5 mm in size) are recovered from petri dish cultures and exposed to miracidia with a dose of 5-10 miracidia per snail, either individually or en masse. Individual snail exposures are carried out using 15 x 17 mm glass vials. En masse exposures are carried out using 1.5 x 6 cm-size petri dishes. Each dish contains 5 ml of aerated tap water, 1 cm^2 of blue-green algae and 50 snails. Unless otherwise stated, en masse exposures are used routinely. The temperature for exposure is 25°C - 27°C and the snails are left for 3 - 5 hours in containers under ceiling illumination. After exposure, snails are placed into plastic trays containing 1.5 liters of aerated tap water supplied with lettuce, blue-green algae and mud, and agar mud paste at a density of 50 snails per tray. Snails are maintained under continuous light and they are changed weekly until screening for patency. A total of 1200 snails are exposed monthly. Practically no mortality is to be encountered before patency. From these 1000 to 1100 are to become positive, giving an infection rate of 85% to 90%.

C. Screening Snails for Infection. In order to improve the efficiency in identifying patency of snails, a simple detection method has been devised (Liang and Bruce, in

preparation). Snails exposed 3-4 weeks previously are placed in a petri dish with enough aerated tap water to barely cover their bodies. Under a dissecting microscope, snails are individually examined for the presence of daughter sporocysts. In positive snails the sporocysts, which appear as a whitish mottling in the hepatopancreas, are readily visible through the shells. This method permits selection of 93%-100% of the total snails which ultimately will become positive or negative and is carried out at the rate of 150-200 snails per hour. Subsequently, the positive snails are placed in plastic trays without illumination, and the negative snails are destroyed. Data which support the efficiency and fidelity of this screening method are shown in Table 1. When an early determination of infection is required, it is made by examining the presence of mother sporocysts in the foot muscles and tentacles of the snails at 14 days after exposure to miracidia.

D. Collecting cercariae. Approximately 1200 positive snails are maintained routinely in this laboratory. From these, two types of cercarial collections are made. Type 1 cercarial collection is performed once every two weeks to infect laboratory mice with 150-200 cercariae per mouse for the routine maintenance of S. mansoni cycle. Approximately 100 snails are set out for this purpose. Type 2 cercarial collection is carried out for the purpose of exposing animals for experimental purposes. The number of infected snails to be used will depend on the number of infected mammals to be exposed. Data collected previously on shedding of cercariae from snails provided us with information in predicting the number of snails required for collecting desired numbers of cercariae. Such a prediction model is shown in Table

2. The volume of water required to collect cercariae depends on the dose desired for exposure of mammals. An improved procedure for collection of cercariae has been devised (Table 3). When cercariae are needed, patent snails are removed from the plastic trays and placed in a beaker without water, but with moisture maintained. The beaker is then allowed to stand for 15-20 min. under ceiling illumination at room temperature (25°C-27°C). After this period of time, a small volume of water is added to the beaker and the snails are gently rinsed to remove feces and other debris. This water is then discarded and a second volume of water (100 ml per 100 snails) is added. The beaker is placed uncovered under a 15-w white fluorescent light (18 inches above beaker) for a period of 10-20 min. After this period, the water containing cercariae is decanted into another beaker allowing the snail feces, mucus and other debris to remain in the original vessel. The density of cercariae is then adjusted as follows. The entire procedure takes less than one hour. This procedure dramatically reduces the mortality among snails due to prolonged shedding time.

E. Estimating Cercarial Densities. While mixing the cercarial suspension continuously with a magnetic stirring bar, ten 0.1 ml aliquots are removed from the center of the suspension by a Cornwall continuous pipetting syringe and placed into a 10-cell Boerner counting slide. Before counting under a dissecting microscope, one drop of Lugol's iodine solution is added to each cell. The counts from 10 cells are averaged and the cercarial density is determined. The suspension is subsequently diluted to the desired number of cercariae

per 0.1 ml of suspension before exposure of mammals. For requests which require extremely large number of cercariae per dose, the suspension is passed through a hand-made nylon sieve with 20μ opening to concentrate the cercariae.

F. Exposure of Mammals. Outbred albino mice are used. Three methods of exposures of mammals are available. They are: 1) tail immersion exposure; 2) abdominal skin exposure; and 3) intraperiotoneal injection of cercariae. To insure material for life cycle maintenance, 10 mice will be exposed bi-weekly.

VII. Source and Strain of Snail and Parasite

A *S. mansoni* parasite and albino *Biomphalaria glabrata* snail from Puerto Rico and from Brazil (one susceptible to drugs and one resistant to oxamniquine are maintained).

Table 1 Efficacy of detecting daughter sporocysts in hepatopancreas 1638 Biomphalaria glabrata snails (Puerto Rican strain) exposed to miracidia of Schistosoma mansoni (Puerto Rican strain)

Group No.	Days post-exposure	No. of positive and negative snails confirmed microscopically	No. of positive and negative snails confirmed by crushing		% accuracy *
			positive	negative	
1	22	positive 367[a]	367[b]	0	100
		negative 22[c]	0	22[d]	100
2	23	positive 245[a]	245[b]	0	100
		negative 42[c]	3	39[d]	93
3	24	positive 232[a]	232[b]	0	100
		negative 54[c]	4	50[d]	93
4	27	positive 321[a]	321[b]	0	100
		negative 66[c]	2	64[d]	97
5	28	positive 269[a]	269[b]	0	100
		negative 20[c]	5	15[d]	75
Total	-	positive 1434[a]	1434[b]	0	100
		negative 204[c]	14	190[d]	93

* % accuarcy of microscopical method compared to crushing method $= \frac{b}{a} \times 100$ or $\frac{d}{c} \times 100$.

Table 2 Number of Snails Required to Collect Desired Number of Cercariae

Number of snails	Cercarial yield (thousands)
30 - 40	100 - 150
60 - 80	200 - 300
90 - 100	300 - 400
120 - 150	400 - 750

Table 3 Weekly cercarial production of 14 Biomphalaria glabrata(Puerto Rican) snails infected with Schistosoma mansoni(Puerto Rican) under 24-hour lighting

Week No.	No. of cercariae produced by individual snails														Toatl	Mean
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1	3027	5236	11418	6243	5999	7886	6405	4306	5102	6577	5821	566	4065	2307	74955	5354
2	5575	5658	5496	6091	7383	7120	7229	7095	7470	4565	4827	2601	2575	2830	76515	5466
3	4608	4906	4831	8393	5101	4746	3110	5368	6359	3385	3448	4382	2421	3272	64330	4595
4	1390	2923	2476	4071	1028	3427	3746	4717	3304	2304	1849	3426	1387	2519	38567	2755
5	575	3518	3479	1461	859	4227	3317	4582	2429	2177	3815	4676	4523	3246	42884	3064
6	2246	9942	9597	6449	3009	4304	4961	5818	6512	8503	6812	8650	3616	4502	84921	6066
7	2281	5030	6640	2249	5188	4814	3703	2342	2008	4208	4409	5354	4566	4772	57564	4112
8	2511	5321	7447	4475	2121	6988	8104	5090	3209	7362	9954	6673	5159	5180	79594	5686
9	1185	1054	5255	1449	1076	4435	6713	9214	3069	5132	3002	4386	1947	5177	53094	3793
Total	23398	43588	56636	40881	31764	47947	47288	48532	39462	44213	43937	40714	30259	33805	572424	40888
Mean	2600	4844	6293	4543	3530	5328	5255	5393	4385	4913	4882	4524	3363	3757	63603	4544
Snail sex	♂	♂	♂	♀	♂	♂	♀	♀	♂	♂	♀	♀	♀	♀	-	-

Snails were mono-miracidially exposed except Snail No. 4 which had a bisexual infection. The snails were maintained in Nostoc muscorum algal dishes, one snail per dish. Dishes were changed once a week when cercarial shedding was made. Covering of snails under darkness was not made. Snails were dried for 30 min. prior to shedding.

SECTION II

Techniques for Cultivation of *Bulinus truncatus truncatus* (Egyptian Strain) and *Bulinus truncatus rohlfsi* (Ghanian Strain) and the Maintenance of *Schistosoma haematobium* (Egyptian and Ghanian Strains)

I. Unit for Snail Maintenance

Two types of units are used.

A. A mobile unit similar to that designed by Davis (1971) is used for accommodating large numbers of snails (Figure 1). The overall dimensions of this unit are 52 x 175 x 243 cm. It is equipped with air lines and fluorescent lights and is used to accommodate glass aquaria holding stock *Bulinus* snail cultures. This unit is capable of holding 32 glass aquaria (20-liter size), 64 plastic trays or 288 petri dishes.

B. A mobile unit constructed of heavy-duty steel (Figure 2). The overall dimensions of this unit are 61 x 122 x 188 cm. It is equipped either with or without fluorescent lights. The unit equipped with fluorescent lights is used for accommodating the type of aquaria (plastic trays and petri dishes) which do not require aeration. This type of mobile unit is also used for cultivating algae needed as food for snails. The unit without fluorescent lights is used for accommodating patent snails. Each of these units is capable of holding 50 plastic trays or 180 petri dish cultures. There are five of these units available in this

laboratory, which is more than sufficient to meet the requirements for this proposal.

Three kinds of aquaria are used: 1) twenty-liter glass aquaria are used for maintaining stock snail cultures; 2) plastic trays with a holding capacity of 1.5 liters of water are used as breeding aquaria for collecting eggs and for maintaining pre-patent and patent snails; 3) petri dishes with a holding capacity of 40 ml of water are used for incubating and hatching eggs. Newborn snails are also kept in the petri dishes until they reach 3-4 mm in size.

II. Environmental Parameters

A. Light. The 20-liter glass aquaria used for maintaining stock snails are held under 12-hour light and 12-hour darkness in Type 1 units. The fluorescent light is suspended 12 inches above. The plastic trays (when used for breeding and for maintaining pre-patent snails) are placed in continuous artificial light provided by a 40-w "cool" white fluorescent tube suspended 18 inches above each shelf. Likewise, the petri dishes containing newborn snails are placed under similar light. Plastic trays with patent snails are maintained without illumination. Petri dishes containing egg masses are placed under ceiling light.

B. Water. Tap water is conditioned before use by passing it through a "Diamond" filter (Model #131-1575) containing layers of activated charcoal and sand. The water is then aerated for at least one day prior to use.

The pH of the water is approximately 7.1. The pH of the water in the aquaria and plastic trays is monitored weekly.

C. Aeration. Air supplied to glass aquaria is passed from a centrally located air compressor through a water-oil extractor into culture units. The water contained in plastic trays and petri dishes is not aerated.

D. Temperature. Temperature is monitored daily. It is maintained at 25°C-27°C.

E. Food. Romaine lettuce supplemented with blue-green algae (Nostoc muscorum) and mud forms the diet of stock snails in glass aquaria as well as those kept in plastic trays. The agar mud paste recipe described as an additional food source for Biomphalaria snails is also used for these species of snails.

III. Breeding

Adult snails are transferred to plastic trays containing 1.5 liters of aerated tap water and maitained at a density of ten snails per tray. Trays are placed under continuous light and supplied with food as described above. Trays are changed weekly, at which time egg masses are scraped from the wall of the trays as well as from the surface of lettuce.

IV. Hatching of Eggs and Maintenance of Newborn

Egg masses are placed in petri dishes with aerated tap water and incubated under ceiling light for one week until hatching. Approximately 50 newborn (0.6-0.8 mm in shell length) are transferred with a pipette to petri dishes with blue-green algae and mud, and are placed under continuous illumination. At the end of one week, snails are transferred to new dishes, 25 per dish. Within 14 days, young snails will reach 3-4 mm in size which is suitable for exposure to miracidia.

V. Maintenance of S. haematobium Life Cycle

A. Collecting Miracidia. The large intestines are excised from golden hamsters (Cricetus auratus) with S. haematobium infections of at least 120 days duration. The portion of the intestine having masses of egg nodules is severed and rendered free of feces by rinsing with 0.85% saline. The intestine is cut into pieces approximately 1 cm long and placed into a 200-ml stainless steel Eberbach container with 20 ml of 0.85% saline. The container is placed on a single-speed Waring blender (No. 700) connected to a variable autotransformer and the tissues are homogenized for 5-10 sec. at very low speed (30 volts). The suspension is poured into a tiered column of sieves arranged in descending order of mesh openings (420 μ, 177 μ, 105 μ, and 45 μ). The eggs are washed through to the bottom sieve with 100 ml of 0.85% saline. Large peices of tissues which had been trapped in the top sieves are

re-homogenized in 20 ml of 0.85% saline. The procedure is repeated three times at low (50 volts), intermediate (70 volts) and finally high speed (100 volts). A volume of 100 ml of aerated tap water is poured into the sieve column to rinse eggs free from saline. The eggs are washed from the bottom sieve into a petri dish (2 x 10 cm) with 40 ml of aerated tap water and concentrated to the center of the dish by gentle rotation. The eggs are then pipetted into a small dish (1.5 x 6 cm) and the dish is placed under ceiling illumination for hatching miracidia.

The entire procedure takes approximately 15 min. and miracidia usually appear within 10 min. Snails are exposed to miracidia which are less than one hour old.

B. Exposure of Snails. Snails (3-4 mm in size) are recovered from petri dish cultures and are exposed either individually or en masse. For individual exposures, snails are placed in glass vials (15 x 17 mm) containing 0.2-0.4 ml of aerated tap water. Each snail is exposed to 5-10 S. haematobium miracidia. En masse exposure is carried out using a petri dish (1.5 x 6 cm) containg 5 ml of aerated tap water and 1 cm^2 of blue-green algae at a density of 50 snails per dish. An average of 5-10 miracidia per snail is used. Unless otherwise requested by investigators, en masse exposures are used. The exposure temperature is 25°C-27°C and the snails are left for 3-5 hours in the exposure dishes under ceiling illumination.

After exposure, the snails are maintained for 3 to 4 additional days in new petri dishes with blue-green

algae and mud, 25 per dish. This step reduced the mortality of snails to nil. Thereafter, snails are maintained in groups of 50 per plastic tray containing 1.5 liters of aerated tap water and supplied with lettuce, blue-green algae, mud, and agar mud paste. They are placed under continuous light and the trays are changed weekly until they are screened for infection.

C. Screening snails for Infection. The technique used for screening Bulinus snails for the presence of infection is essentially the same as that described for screening Biomphalaria snails. Snails exposed to miracidia 4 weeks previously are placed in a petri dish with aerated tap water sufficient to just cover snail bodies. Under a dissecting microscope, snails are individually examined for the presence of daughter sporocysts visible through the snail shells. The sporocysts appear as whitish mottling in the hepatopancreas of snails. This method permits selection of 81%-100% of the total snails which ultimately will become positive or negative and is carried out at the rate of 50-75 snails per hour. The positive snails are then placed in plastic trays without illumination. The remaining snails, which show no signs of infection by this method, are re-examined 2 weeks later by the shedding method. Snails are kept in darkness for a period of 16 hours prior to inducing them to shed. The snails are then placed individually in glass vials (20 x 30 mm) without water for a period of 30 min. under ceiling illumination at room temperature ($25^{o}C$-$27^{o}C$). After this period, 5 ml of aerated tap water is added to each vial and the vials are then placed under 15-w white fluorescent lights (18 inches above vials) for 2 hours. With the aid of a dissecting

microscope, vials are examined for the presence of cercariae. Snails found to be negative are destroyed. Data which support the efficiency of this screening method is shown in Table 4. Early detection of infection is made by examining the presence of mother sporocysts in the foot muscles of the snails. Unlike those found in Biomphalaria snails, the mother sporocysts of S. haematobium (Egyptian strain) are found exclusively in foot muscles. Snails 14 days after exposure are placed on a moist petri dish (2 x 10 cm) and allowed to attach their feet on the surface of the dish. The dish is then inverted and the presence of the mother sporocysts is examined through the glass of the dish under a dissecting microscope by adjusting the microscope light. The sporocysts appear as opaque white specks embedded in the foot muscle.

D. Collecting Cercariae. Approximately 150 positive snails of each snail species infected with each S. haematobium strain are routinely maintained. Two types of cercarial collections are performed from these snail populations. Type 1 cercarial collection is performed monthly in order to infect hamsters with 400 cercariae for the routine maintenance of S. haematobium. About 50-70 snails are set out for this purpose. Type 2 cercarial collection is carried out for infection of animals for experimental purposes.

For collection of S. haematobium cercariae, the method used for Biomphalaria snails is also used, except that the patent snails are held in darkness in plastic trays for a period of 16 hours or more prior to shedding. They are then transferred from the plastic trays into a

Table 4 Efficacy of detecting daughter sporocysts in hepatopancreas of 445 *Bulinus* snails exposed to miracidia of *Schistosoma haematobium*

Species and strain of snail*	No. of positive and negative snails confirmed microscopically	No. of positive and negative snails confirmed by crushing		% accuracy **
		positive	negative	
B.t. truncatus (Egyptian)	positive 13[a]	13[b]	0	100
	negative 42[c]	0	42[d]	100
B. guernei (Gambian)	positive 98[a]	98[b]	0	100
	negative 43[c]	8	35[d]	81
B.t. rohlfsi (Ghanian)	positive 171[a]	169[b]	2	99
	negative 78[c]	4	74[d]	95

* Snails used were 4 weeks post-exposure.

** % accuracy of microscopical method compared to crushing method

$= \frac{b}{a} \times 100$ or $\frac{d}{c} \times 100$.

beaker without water, but with moisture maintained. The beaker is then allowed to stand for 30 min. under ceiling illumination at room temperature (25°C-27°C). After this period of time, a small volume of water is added to the beaker and the snails are gently rinsed to remove feces and other debris. This water is then discarded and a second volume of water is added (50 ml/50 snails) and the beaker is placed under 15-w white fluorescent lights (18 inches above beaker) for a period of 2 hours.

E. Exposure of Mammals. The golden hamster (Cricetus auratus) is used as the definitive host for maintaining S. haematobium in the laboratory and is the mammal provided to investigators for S. haematobium infection. The cercarial suspension obtained as described previously is decanted into a petri dish (2 x 10 cm) and placed under a dissecting microscope. Unlike S. mansoni exposure, S. haematobium cercariae are counted individually. Using an extra-finely drawn-out capillary pipette, individual cercariae are transferred onto the moistened shaven abdomen of a hamster which has been anesthetized with sodium pentobarbital and held on a large watch glass (125 mm in diameter). The hamster is exposed for at least 30 minutes. With this technique, the exact number of cercariae used for exposure is determined. Unless requested otherwise, the dose employed is 400 cercariae per hamster. Hamsters used for life cycle are sacrificed and autopsied approxmately 120 days later. For maintenance of the respective S. haematobium cycles, 6-7 hamsters are exposed monthly.

VI. Source and Strain of Snail and Parasite

The Egyptian strain of *Bulinus truncatus truncatus* snail and *Bulinus guernei* (Gambian) snail and *Schistosoma haematobium* (Egyptian strain) originated from the University of Michigan stock. A more recent isolate of *Bulinus truncatus truncatus* and *S. haematobium* from the field in Egypt (1980 and 1981, respectively) is now being maintained and will gradually replace the old stock (snail and parasite) if warranted for use in this program. The Ghanian and Voltan strains of *Bulinus truncatus rohlfsi* and *Schistosoma haematobium* (Ghanian strain) also originated from the University of Michigan stock.

SECTION III

Techniques for Cultivation of the 4 Subspecies of *Oncomelania hupensis* and the Maintenance of the 4 Geographic Strains of *Schistosoma japonicum*

I. Unit for Snail Maintenance

A mobile unit, constructed of heavy-duty steel (Figure 2) is used to accommodate large numbers of snails and cercariae for requesting investigators. The overall dimensions of the unit are 61 x 122 x 188 cm. Two types of units are used, neither of which is equipped with air lines. The unit with lighting is used for accommodating petri dish cultures containing patent, pre-patent, breeder snails and algal cultures. The unit without lighting is used for incubating snail eggs.

Three kinds of aquaria are used: 1) a petri dish (2 x 10 cm) containing peripherally placed mud, blue-green algae (*Nostoc muscorum*) and water is used for breeding and obtaining eggs (French, 1974 and 1977); 2) a petri dish containing a centrally placed mud mount, blue-green algae (*Nostoc muscorum*) and water is used for rearing young snails and maintaining both pre-patent and patent snails (Liang, 1974); 3) a petri dish containing a small amount of blue-green algae (*Nostoc muscorum*), mud and water is used for newborn snails (Liang, 1975).

II. Environmental Parameters

A. Light. Breeder, pre-patent and patent snails are maintained under a 40-w "cool" white fluorescent light with 12-hour light and 12-hour darkness. Eggs collected from breeding dishes are placed in clean petri dishes with aerated tap water and placed on shelves without direct illumination.

B. Water. Tap water is conditioned before use by passing it through a "Diamond" filter (Model #131-1575) containing layers of activated charcoal and sand. The water is then aerated for at least one day prior to use. The pH of the water is approximately 7.1.

C. Temperature. Temperature is monitored daily and maintained at $25^{\circ}C$-$27^{\circ}C$.

D. Food. Blue-green algae (Nostoc muscorum) grown in petri dishes with mud (Liang, 1974) is used exclusively as the food source. Newly formulated agar mud paste is used as an additional diet.

III. Breeding

A modification of the culture method described by French (1974 and 1977) is used for cultivating all subspecies of Oncomelania snails. Adult snails (5 males and 5 females per aquarium) are introduced into petri dish aquaria containing peripherally placed mud. After the surface of the mud is washed several times, a small amount of blue-green algae is added. The aquaria are checked weekly

for eggs which are then removed into a petri dish containing aerated tap water. At the same time, snail feces and soiled water are removed and fresh water and algae (if necessary) are added. A small amount of agar mud paste is also added.

IV. Hatching of Eggs and Maintenance of Newborn

Eggs previously placed in petri dishes with aerated tap water are placed on shelves without direct illumination for incubation and hatching. Eggs usually hatch within 18 days. New born snails are then placed in newly established aquaria containing a small amount of blue-green algae and allowed to grow for 2-3 weeks. The snails are maintained under 12-hour light and 12-hour darkness. After reaching 2-3 mm in shell length, the snails are used for exposure to miracidia or for establishing new rearing cultures by placing snails in petri dishes containing centrally placed mud with blue-green algae. A small amount of the newly formulated agar mud paste, as described for use in Biomphalaria cultivation, is also provided for these snails.

V. Maintenance of S. japonicum Life Cycle

A. Collecting Miracidia. Livers and/or feces-free intestines excised from mice exposed to S. japonicum cercariae (pertains to all 4 geographic strains) 8-12 weeks previously are used as the source of eggs to hatch miracidia. Tissues are cut into pieces and placed into a 200-ml stainless steel Eberbach container with 20 ml

of 0.85% saline. The container is placed on a single-speed Waring blender (No. 700) connected to a variable autotransformer and the tissues are homogenized for 5-10 sec. at very low speed (30 volts). The suspension is poured into a tiered column of sieves arranged in descending order of mesh openings (420 μ, 177 μ, 105 μ, and 45 μ). The eggs are washed through to the bottom sieve with 100 ml of 0.85% saline. Large pieces of tissues which had been trapped in the top sieves are re-homogenized in 20 ml of 0.85% saline. The procedure is repeated three times at low (50 volts), intermediate (70 volts) and finally high speed (100 volts). A volume of 100 ml of aerated tap water is poured into the sieve column to rinse eggs free from saline. The eggs are washed from the bottom sieve into a petri dish (2 x 10 cm) with 40 ml of aerated tap water and concentrated to the center of the dish by gentle rotation. The eggs are then pipetted into a small petri dish (1.5 x 6 cm) and the dish is placed under ceiling illumination for hatching miracidia. The entire procedure takes approximately 15 min. and miracidia usually appear within 15 min. If eggs do not hatch within this time period, the eggs are washed again with aerated tap water to facilitate hatching. Snails are exposed to miracidia which are less than one hour old.

B. Exposure of Snails. Oncomelania snails (pertains to all sub-species) 2-3 mm in shell length are recovered from rearing aquaria and exposed to 5-10 miracidia either individually or en masse. Individual snail exposures are carried out using glass vials (15 x 17 mm). En masse exposures are carried out using a petri dish (1.5 x 6 cm) containing 5 ml of aerated tap water and 1 cm^2

of blue-green algae. The temperature for exposures is 25°C-27°C and the snails are left for 3-5 hours in the exposure containers under ceiling illumination.

C. Screening Snails for Infection. Although it is possible to check Oncomelania snails for evidence of infection by placing them in water to shed, the results are often discouraging and this procedure is not routinely practiced in this laboratory. Instead, snails are checked for evidence of patency by using the same basic techniques as described for Biomphalaria and Bulinus snails. Shells of the snails are cleaned and are examined for the presence of daughter sporocysts visible through the shells. The sporocysts appear as whitish mottling in the hepatopancreas of snails. This method permits selection of 85-90% of the total snails which ultimately will become positive or negative and is carried out at the rate of 30-50 snails per hour. These visually-positive snails are cultured until patency. They are then either supplied to investigators or used for maintenance of life cycle. Those visually-negative snails are cultured separately until patency date. They are then crushed at the time the visually-positive snails are used and examined. Data which supports the efficiency of this screening method is shown in Table 5.

D. Collecting Cercariae. Cercariae are obtained by crushing snails in a small petri dish (1.5 x 6 cm) to which is added a small volume (about 10 ml) of water. Unlike the cercariae of S. mansoni and S. haematobium, most S. japonicum (all strains) cercariae swim to the surface of the water and usually remain there. To collect these cercariae a hair-loop is used. The

Table 5 Efficacy of detecting daughter sporocysts in hepatopancreas of 241 Oncomelania hupensis quadrasi (Leyte strain) snails exposed to miracidia of Schistosoma japonicum (Philippine strain)

Group No.	Days post-exposure	No. of positive and negative snails confirmed microscopically	No. of positive and negative snails confirmed by crushing		% accuracy *
			positive	negative	
1	120	positive 19[a]	19[b]	0	100
		negative 31[c]	1	30[d]	97
2	108	positive 61[a]	53[b]	8	86
		negative 79[c]	12	67[d]	85
3	106	positive 5[a]	5[b]	0	100
		negative 16[c]	2	14[d]	88
4	133	positive 10[a]	8[b]	2	80
		negative 20[c]	8	12[d]	60
TOTAL	-	positive 95[a]	85[b]	10	90
		negative 146[c]	23	123[d]	85

* % accuracy of microscopical method compared to crushing method = $\frac{b}{a} \times 100$ or $\frac{d}{c} \times 100$.

hair-loop is fastened to a very fine minute pin, which in turn is attached to a glass tube. The angle of the hair-loop is adjustable. To collect those cercariae which are submerged under the water surface, an extra finely drawn-out capillary pipette is used. For intraperitoneal injection, cercariae are collected by use of a syringe and needle under a dissecting microscope and the dose of cercariae specified by each investigator is injected into mice.

Two types of collections are made for S. japonicum cercariae (pertains to all strains). Type 1 collections are made for exposure of mice used for maintenance of life cycles of the 4 strains. For this purpose, 10-15 snails are used for collection of cercariae. Type 2 collections are carried out when animals are exposed for experimental purposes. The number of infected snails and cercariae to be used for exposure depends on the experimental conditions.

E. Exposure of Mammals. Outbred albino mice are used for maintaining life cycles as well as for providing infected animals to investigators. Two methods of mammal exposures are available. They are: 1) abdominal skin exposures; and 2) intraperitoneal injection of cercariae. An abdominal skin-exposure method is used for both maintaining the life cycles as well as for providing infected mice to investigators unless requested otherwise by the investigators. Exact numbers of cercariae are transferred to the moistened-shaven abdomen of the mouse, which has been anesthetized with sodium pentobarbital, and held on a small watch glass (100 mm diameter). The mouse is exposed for at least 30

min. For maintenance of each of the 4 strains of S. japonicum cycles, 8 mice are exposed monthly with the dose of 30-35 cercariae per mouse.

VI. Source and Strain of Snail and Parasite

Oncomelania hupensis hupensis (Vogel strain) and S. japonicum (Chinese-Vogel strain) are from the University of Michigan stock. O.h. hupensis (Shanghai strain) and S. japonicum (Chinese-Shanghai strain) were obtained through Dr. Mao from the People's Republic of China. O.h. nosophora (Kofu strain) and S. japonicum (Japanese strain) were supplied from Hamamatsu University direct to the University of Lowell. A more recent isolate, O.h. nosophora (Kofu strain) and S. japonicum (Japanese-YYA strain) have been received from the National Institute of Health, Japan, and will gradually replace the older strains.

O.h. formosana (Pu-yen strain) was obtained through Dr. Cross (NAMRU-2) direct from Taiwan and from this laboratory. S. japonicum (Formosan strain) originated from the University of Michigan stock.

O.h. quadrasi (Leyte strain) and S. japonicum (Philippine strain) originated from Leyte, Philippines through Dr. Sano, Hamamatsu University, Japan.

O.h. chiui (Alilao strain) was from the University of Michigan stock and from this laboratory. O.h. chiui (Linco strain) was from this laboratory.

SECTION IV

Routine Maintenance of Snail Laboratory
(applies for all snail species)

Daily checks are made of water levels and the temperature of glass aquaria, plastic trays and petri dishes. Snails which have climbed above the water level are returned to the water. Dead snails and decaying food matter are removed, and mortality among pre-patent and patent snail populations is recorded.

Aquaria containing cloudy water, protozoa, metazoa, etc. are changed immediately. The aquaria are thoroughly soaked with a 10% bleach solution, then thoroughly washed with hot running tap water followed by a final prolonged rinse with aerated tap water (Table 6).

Table 6 Routine laboratory tasks

Tasks	Daily	Weekly
Feed snails	x	
Check temperature	x	
Check level and condition of water in aquaria	x	
Check aeration and filtration system	x	
Check snail mortality; remove dead snails	x	
Record vital statistics	x	
Check pH of water		x
Collect egg masses		x
Set up breeding cultures		x
Set up rearing cultures		x
Change water of cultures holding pre-patent and patent snails		x

SECTION V

Maintenance of Records (applies for all species)

Three types of data recording forms are used to provide continuous monitoring of the production of stock snails and for maintenance of infected snails and mammals. They are designated as: Form 1 (rearing snails); Form 2 (snail infection); and Form 3 (mammal infection). Form 1 is used for recording data pertinent to snail rearing such as date of set-up, numbers of breeders, survivals, eggs laid, and the number and date of young snails produced as well as other pertinent data (Figure 3). Form 2 is used to record conditions of exposure of snails to miracidia, sources of miracidia, survival of snails and number of snails determined to be positive by shedding and crushing (Figure 4). Form 3 is used to record exposure of mammals to cercariae, source of cercariae, survival of mammals, and status of infection at the time the mammals are sacrificed (Figure 5).

With these forms one may monitor the infection parameters of schistosome species from the snail hosts to the experimental mammals. These records are bound periodically and maintained in a separate record room at this facility.

Species: No.

Source:

No. Snail: (♂: ♀:)

Age: Size:

Date Set-up:

Date	No. Alive	No. of				Remarks
		Mass	Egg	E/M	Young	

Figure 3 Form for Rearing Snails

Tray No.:
Date Inf.:
No. Exp.:
Method: en masse_____ ind._____
No. Mir.:
Definitive Host:
Date Inf.
Dose:

Parasite Strain:
Snail:
Age:
Size:
Exposure Time:

Date Examined	No. alive	% surv	Shed		% pos.	Crush		Total % pos.	Remarks
			+	-		+	-		

Date Remarks

Figure 4 Form for Snail Infection

MAMMAL INFECTION

Cage No. _____ Mammal Strain _____

Age _____ Weight _____

Date Exposed _____

Number Exposed _____

Exposure Method _____

Time Start Shed _____

Time Last Mammal Exp. _____

Age of Cercariae _____

Number of Cerc. Counts _____

Aver. Number Cercs. _____

Parasitic Strain _____

Snail Species _____

Date Snail Exp. _____

Number of Snails _____

Number of times previously shed _____

Date	Dead/ Sacrif	Per-fused	Worms Recovered					Hatch Method	Hatch Time + Results	Snails Exposed		Comments
			Total	Males	Females	Pairs	Imm.			No.	Species	

Figure 5 Form for Mammal Infection

REFERENCES

1. Bruce, J.I. and Radke, M.G., 1971. Culturing Biomphalaria and Oncomelania (Gastropoda) for large-scale studies of schistosomiasis: I. Cultivation of Biomphalaria glabrata and maintenance of Schistosoma mansoni in the laboratory. Bio-Med. Rep. 406th Med. Lab. 19:1-84.

2. Davis, G.M., 1971. Culturing Biomphalaria and Oncomelania (Gastropoda) for large-scale studies of schistosomiasis: II. Mass cultivation of Oncomelania (Prosobranchia: Hydrobiidae) for studies of Schistosoma japonicum. Bio-Med. 406th Med. Lab. 19:85-161.

3. French, J.R.P., III, 1974. Improved methods of culturing the subspecies Oncomelania hupensis: The snail hosts of Schistosoma japonicum, the oriental blood fluke. Sterkiana, 56:2-7.

4. French, J.R.P. III., 1977. A laboratory guide for culturing Oncomelania hupensis (Gastropoda: Hydrobiidae) Bull. Chin. Malacol. Soc., 4:47-54.

5. Liang, Y.S., 1974. Cultivation of Bulinus (Physopsis) globosus (Morelet) and Biomphalaria pfeifferi pfeifferi (Krauss), snail hosts of schistosomiasis. Sterkiana 53 and 54:7-52; 1-27.

6. Liang, Y.S. and van der Schalie, H., 1975. Culturing Lithoglyphopsis aperta Temcharoen, a new snail host for Schistosoma japonicum, Mekong strain. J. Parasit. 61:915-919.

7. Liang, Y.S. and Kitikoon V., 1980. Cultivation of Lithoglyphopsis aperta, snail vector of Schistosoma mekongi. In: Bruce, J.I., Sornmani, S., Asch, H.L. and Crawford, K.A. (eds.), The Mekong Schistosome. Malacol. Rev., Suppl. 2, pp. 35-45.

8. Liang, Y.S. and Bruce, J.I. (in preparation). A manual for the cultivation of snail intermediate hosts of medically important molluscs.

9. Lo, C.T., 1972. Compatibility and host-parasite relationships between species of the genus Bulinus (Basommatophora: Planorbidae) and an Egyptian strain of Schistosoma haematobium (Trematoda: Digenea). Malacologia II:225:280.

10. Sandt, D.G., Bruce, H.I. and Radke, M.G., 1965. A system for mass producing the snail Australobis glabratus and cercariae of Schistosoma mansoni. J. Parasit. 51: 1012-1013.

APPENDIX II

Calculations for Mean Worm Burdens and Their Use in Computing Test Compound Efficacies

Calculations for Mean Worm Burdens and Their Use in Computing Test Compound Efficacies

Experiment I S. haematobium (Egyptian strain) Oral Route in Hamsters

Mean = 76.3

AG63908 $100 \times \frac{76.3 - 34.1}{76.3} = 55.3$

BC78878 $100 \times \frac{76.3 - 30.9}{76.3} = 59.5$

ZM65703 $100 \times \frac{76.3 - 37.4}{76.3} = 51.0$

Experiment II S. haematobium (Egyptian strain) SQ Route in Hamsters

Mean = 59.8

AG63908 $100 \times \frac{59.8 - 0.18}{59.8} = 99.7$

BC78878 $100 \times \frac{59.8 - 24.7}{59.8} = 58.7$

ZM65703 $100 \times \frac{59.8 - 24.8}{59.8} = 58.5$

Experiment III S. japonicum (Philippine strain) Oral Route in Mice

Mean = 28.2

AG63908 100.0

BC78878 $100 \times \frac{28.2 - 29.1}{28.2} = -3.2$

ZM65703 $100 \times \frac{28.2 - 9.0}{28.2} = 68.1$

Experiment IV *S. japonicum* (Philippine strain) Oral Route in Hamsters

Mean = 41.1

AG63908 $100 \times \frac{41.1 - 23.0}{41.1} = 44.0$

BC78878 $100 \times \frac{41.1 - 25.1}{41.1} = 38.9$

ZM65703 $100 \times \frac{41.1 - 41.7}{41.1} = -1.5$

Experiment V *S. mansoni* (Kenyan strain) Oral Route in Hamsters

Mean 126.2

AG63908 $100 \times \frac{126.2 - 93.0}{126.2} = 26.3$

BC78878 $100 \times \frac{126.2 - 105.8}{126.2} = 16.2$

ZM65703 $100 \times \frac{126.2 - 116.9}{126.2} = 7.4$

Experiment VI *S. haematobium* (Nigerian strain) Oral Route in Hamsters

Mean = 36.8

AG63908 $100 \times \frac{36.8 - 11.1}{36.8} = 69.8$

BC78878 $100 \times \frac{36.8 - 24.4}{36.8} = 33.7$

ZM65703 $100 \times \frac{36.8 - 31.8}{36.8} = 13.6$

Experiment VII *S. mansoni* (Kenyan strain) Oral Route in Mice

Mean = 49.1

AG63908 $100 \times \frac{49.1 - 22.6}{49.1} = 54.0$

BC78878 $100 \times \frac{49.1 - 46.6}{49.1} = 5.1$

ZM65703 $100 \times \frac{49.1 - 6.9}{49.1} = 85.9$

Experiment VIIIa *S. mansoni* (Kenyan strain) Oral Route in Mice

Mean = 48.4

BL26758 $100 \times \frac{48.4 - 17.8}{48.4} = 63.2$

BL23962 $100 \times \frac{48.4 - 36.7}{48.4} = 24.2$

BL23953 $100 \times \frac{48.4 - 29.9}{48.4} = 38.2$

BL23695 $100 \times \frac{48.4 - 1.7}{48.4} = 96.5$

Experiment VIIIb *S. mansoni* (Kenyan strain) Oral Route in Mice

Mean = 43.4

BL26758 $100 \times \frac{43.4 - 37.7}{43.4} = 13.1$

BL23702 $100 \times \frac{43.4 - 0.2}{43.4} = 99.5$

BL23677 $100 \times \frac{43.4 - 2.2}{43.4} = 94.9$

BL23686 $100 \times \frac{43.4 - 8.3}{43.4} = 80.9$

Experiment X *S. mansoni* (Puerto Rican strain) Oral Route in Mice

Mean = 70.9

BL26758 $100 \times \frac{70.9 - 9.5}{70.9} = 86.6$

BL28592 $100 \times \frac{70.9 - 63.1}{70.9} = 11.0$

BE19575 $100 \times \frac{70.9 - 51.4}{70.9} = 27.5$

BL26749 $100 \times \frac{70.9 - 34.3}{70.9} = 51.6$

Experiment XI *S. mansoni* (Puerto Rican strain) Oral Route in Mice

Mean = 62.5

BL26758 $100 \times \frac{62.5 - 5.0}{62.5} = 92.0$

BL28494 $100 \times \frac{62.5 - 52.6}{62.5} = 15.8$

BK21070 $100 \times \frac{62.5 - 11.4}{62.5} = 81.8$

BL23510 $100 \times \frac{62.5 - 5.6}{62.5} = 91.0$

Experiment XII *S. mansoni* (Kenyan strain) Oral Route in Mice

Mean = 63.1

BL26758 $100 \times \frac{63.1 - 49.5}{63.1} = 21.5$

BL28510 $100 \times \frac{63.1 - 8.1}{63.1} = 87.2$

BL28501 $100 \times \frac{63.1 - 36.8}{63.1} = 41.7$

BL28485 $100 \times \frac{63.1 - 61.3}{63.1} = 2.8$

BK21070 $100 \times \frac{63.1 - 31.7}{63.1} = 49.8$

Experiment XIII *S. mansoni* (Kenyan strain) Oral Route in Mice

Mean = 58.2

BL26758 $100 \times \frac{58.2 - 41.3}{58.2} = 29.0$

BL28494 $100 \times \frac{58.2 - 60.3}{58.2} = -3.6$

BL28592 $100 \times \frac{58.2 - 60.2}{58.2} = -3.4$

BE19575 $100 \times \frac{58.2 - 52.9}{58.2} = 9.1$

BL26749 $100 \times \frac{58.2 - 44.2}{58.2} = 24.0$

Experiment XIV *S. mansoni* (Kenyan strain) Oral Route in Mice

Mean = 126.9

BL26758 $100 \times \frac{126.9 - 91.7}{126.9} = 27.7$

BL26776 $100 \times \frac{126.9 - 112.9}{126.9} = 11.0$

BL35319 $100 \times \frac{126.9 - 99.9}{126.9} = 21.3$

BL26785 $100 \times \frac{126.9 - 118.7}{126.9} = 6.5$

Appendix III*

Protocols

Sections I, II and III

* Appendix III includes Photographs 1 - 3 and Tables 1 - 5

Section I
Protocol WRHN-IIIMa
Topical Antipenetrant Primate Study

BACKGROUND

The topical antipenetrant compound which is being evaluated in this protocol was previously evaluated in mice, hamsters and rhesus monkeys and provided virtually 100% protection against skin invasion by the cercariae of *S. mansoni*, *S. japoncium* and *S. haematobium*. As a result of the excellent results obtained previously, final formulation was prepared for possible studies in human volunteers. Before this phase of study can be initiated, the efficacy of the formulation must be tested in mice, hamsters and monkeys. This protocol covers the final evaluation of the folumations in *Cebus apella* monkeys.

MATERIALS AND METHODS

Test Compound.

The topical antipenetrant formulation (BL44970) and the placebo (BL44989) were prepared by Miles Laboratory through an agreement with the Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research. Both the test compound and the placebo were prepared in the same vehicle.

Animals.

Cebus apella were obtained from Worldwide Primates, Inc., Miami, Florida. Monkeys were kept under quarantine for 45 days at Worldwide Inc. facilities prior to shipment to Lowell. Upon arrival at Lowell University they were immediately placed in quarantine for 45 days during which time they were tested for tuberculosis by the skin test method and examined for helminthic infections. Both males and females weighing between 2.5 - 4.5 kg were used.

Animals were housed individually in standard regulation cages, fed monkey chow and water ad libitum. All animals are kept in a temperature/humidity controlled animal quarters in a 12 hr. light/dark cycle.

Animal Care.

Monkeys are maintained as outlined by the "Guide for the Care and Use of Laboratory Animals" (1986).

All animals were anesthetized with intramuscular injections of a mixture of ketamine hydrochloride to temporarily immobilize them during treatment applications, washing, and exposure to parasites. No painful or stressful treatments occurred.

Monkeys will be sacrificed at the termination of the study by giving an overdose of the anesthetic and exsanguination.

METHODS

Animals were treated, washed, exposed and examined according to the study design outlined in Table 1.

Drug Application.

Animals were anesthetized as described and animal clippers were used to clip the coarse hair from the entire right arm of each animal. The drug solution was applied to the clipped arms of the experimental animals using a 4 X 4" gauze pad (clamped by and wrapped around a hemostat). The entire arm was treated by wiping it from the shoulder and down the arm to include the hands. Vehicle control animals were treated in the same manner with the placebo.

Note: 20 ml aliquots of the drug solution and placebo were alotted per animal. The total volume was not used for each animal. The amount used for each animal was that volume required to cover the shaved area of the right arm.

Washing.

For all experimental animals, including the placebos, topically treated areas were washed 10 min post-treatment to remove excess compound which had not been topically absorbed. Each treated arm was placed in a 152 mm X 450 mm pipet washer and washed with filtered water at one cycle per minute for 30 minutes (one cycle is defined as the action of the water filling from the level of the finger tips to the shoulder and draining from the shoulder to the finger tips.

Exposure.

An oxamniquine-resistant strain of S. mansoni known as MAP which was obtained from Brazil in January, 1986 was used for all schistosome infections. This strain is maintained in laboratory bred Biomphalaria glabrata (BH- Belo Horizonte, State of Minas Gerais, Brazil) and outbred CD_1 albino male mice. Cercariae were shed from infected B. glabrata en masse as follows:

1. The evening prior to the days of exposure, the trays containing the infected snails were covered with a black cloth to simulate a dark period.

2. Infected B. glabrata were stressed by drying them on paper towels and exposing them to fluorescent light for a 1/2 hr.

3. Filtered, aerated water was then added to the beaker containing the infected snails to just cover the snails.

4. The beaker was observed for shedding.

5. The first addition of water was the discarded to remove dirt, fecal matter, etc. and a second addition of water was made to collect the cercariae.

6. The solution containing cercariae was poured into a perti dish and used for the infection of experimental animals.

7. The cercariae were counted by drawing them from the solution with a micro-pipet as observed through a dissecting microscope.

Note: When necessary, the cercarial solution was diluted with filtered, aerated water to facilitate ease in counting the correct number of cercariae.

The Cebus apella monkeys were exposed to a dose of 400 cercariae each for 45 min. Ten CD_1 swiss albino male mice were exposed to 100 cercariae each by the tail immersion method for 45 min in order to determine the viability of the cercariae. Five mice were exposed prior to the exposure of the experimental animals. The remaining five mice were exposed following the exposure of the experimental animals.

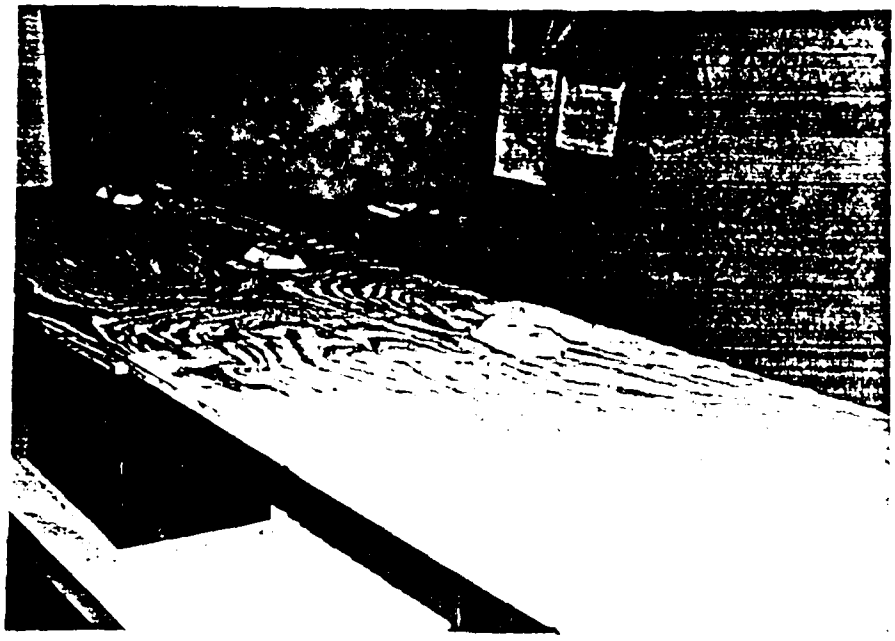
The monkeys were anesthetized as previously described for drug treatment. The treated arms of the monkeys were immersed into a 1.4 liter Rubbermaid refrigerator door pitcher as shown in photographs 1 - 3. The water level in the containers was such that it was below the upper most washed area of the forearm. The monkeys were exposed for 45 min and the arms were then drawn from the container and allowed to air dry. 100 ml aliquots of formalin were added to the infection solution. The contents of the containers were concentrated by pouring the infection solution through a 20 u sieve (3" in diameter). The recovered cercariae were washed off the sieve with filtered water into a petri dish. The cercariae were then stained with 4% Lugol's iodine for counting.

All equipment which may have been contaminated with cercariae was disinfected with bleach (5.25% sodium hypochlorite). All personnel required to handle cercariae were protected from accidental infection by disposable protective garments, disposable surgical gloves and full face visors. Bleach (5.25% sodium hypochlorite) and 95% ethanol were available at all times during the infection to decontaminate any accidental spills or equipment or personnel respectively.

Examination

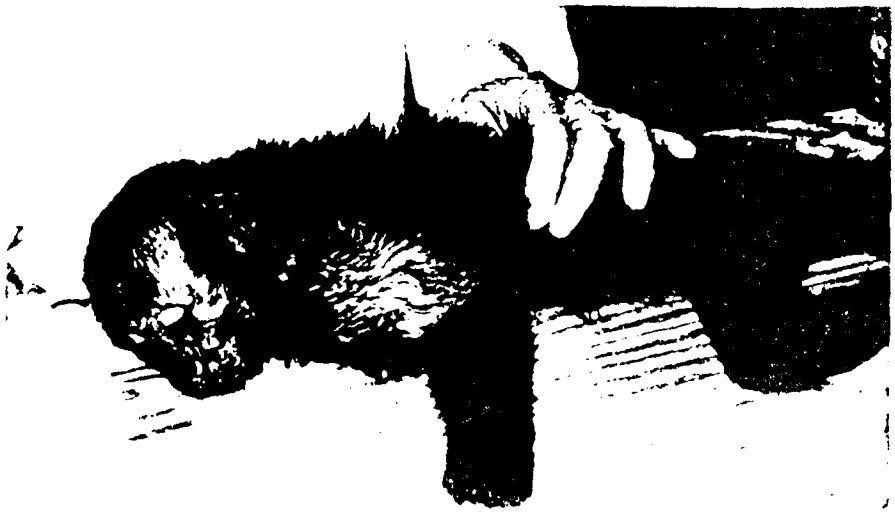
Prior to the beginning of experimentation, all monkeys were screened for parasites. Fecal samples were examined using the AMS-3 concentration technique.

Photograph 1



Setup of table and containers for topical antipenetrant study in Cebus apella monkeys.

Photograph 2



Exposure of Cebus apella monkeys to schistosome infection. Right arm is in container, hand extends through the container.

Photograph 3

Exposure of Cebus apella monkeys to schistosome infection. Note the hand extended on third monkey on the right. Protective clothing worn during entire procedure.

Beginning on day 28 post-exposure and continuing twice per week until the termination of the study, fecal samples from each animal will be examined for schistosome eggs using the AMS-3 concentration technique.

All animals will be sacrificed and examined 49-54 days post-exposure to S. mansoni. Any schistosome (mature and/or stunted) parasites present will be recovered from each animal by the Perf-O-Suction technique, counted and the efficacy of the drug computed.

Table 1

Experimental Design for the Schistosoma mansoni Antipenetrant Study (BL44970 in Cebus apella monkeys

Group No.	Days Treated Pre-Infection	Treatment Type	No. of Animals
I	7	1% BL44970	4
II	7	Placebo	2
III	3	1% BL44970	4
IV	3	Placebo	2
V	1	1% BL44970	4
VI	1	Placebo	2
VII	0	Infection Control	5

Total number of animals = 23

Table 2

Result of Examination of Exposure Containers for Cercaraie After Exposure of Monkeys

#	Cercarial Count	% of Cercarial in Exp. Containers	% of Intact Cercariae	Group
1	190	48	6	
2	274	69	75	7A
3	132	33	39	
4	100	25	58	
5	180	45	76	7B
6	140	35	81	
7	215	54	20	
8	186	47	61	3A
9	66	17	73	
11	266	67	63	
12	231	58	73	3B
14	105	26	94	
15	241	60	27	
19	303	76	75	1A
20	192	48	92	
21	367	92	72	
22	336	84	42	1B
23	83	21	89	
24	102	26	54	Infection
25	96	24	80	Control-A
30	117	29	84	Infection
31	81	20	73	Control
32	113	28	72	B

Note 1: All "A" groups were exposed on 7/16/87; all "B" groups were exposed on 7/17/87.

Note 2: On 7/16/87 the monkeys were exposed in the following order: 1, 24, 3, 7, 9, 15, 2, 8, 19, 20, 25.
On 7/17/87 the monkeys were exposed in the following order: 30, 4, 11, 14, 21, 23, 31, 5, 6, 12, 22, 32.

Note 3: All monkeys were exposed to 400 cercaraie each.

Table 3

Results for Mice Exposed on 7/16/87 and 7/17/87

#	Cercarial Count for Those Exposed 7/16/87	Cercarial Count for Those Exposed 7/17/87
1	0	0
2	0	0
3	0	0
4	1	0
5	2	0
6	0	0
7	3	0
8	4	0
9	4	1
10	5	4

Note 1: Animals 1-5 were exposed prior to exposure of the first monkey; animals 6-10 were exposed after exposure of the last monkey.

SECTION II

Protocol WRHN-IIIMb

Potential Oral Prophylactic Study in Monkeys

BACKGROUND

Compound BL23702 (WR249313) was found to be highly active as an oral prophylactic compound against Schistosoma mansoni in infected mice. The compound is now being evaluated in this protocol as an oral prophylactic in Cebus apella monkeys.

MATERIAL AND METHODS

Test Compound.

BL23702 (WR249313) was obtained from the Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research. The vehicle used for preparation of the compound was Tween 80-methyl cellulose saline (TMCS).

Animals.

Cebus apella were obtained from Worldwide Primates, Inc., Miami, Florida. Monkeys were kept under quarantine for 45 days at Worldwide Primates, Inc. facilities prior to shipment to Lowell. Upon arrival at Lowell University they were immediately placed in quarantine for 45 days during which time they were tested for tuberculosis by the skin test method and examined for helminthic infections. Both males and females weighing between 2.5 - 4.5 kg were used.

Animals are housed individually in standard regulation cages, fed monkey chow and water ad libitum. All animals are kept in temperature/humidity controlled animal quarters on a 12 hr light/dark cycle.

Animal Care.

Monkeys will be maintained as outlined by the Guide for the Care and Use of Laboratory Animals" (1986).

All animals were anesthetized with intramuscular injections of a mixture of ketamine hydrochloride to temporarily immobilize them during treatment and exposure to parasites. No painful or stressful treatments occurred.

Monkeys will be sacrificed at the termination of the study by giving an overdose of the anesthetic and exsanguination.

METHODS

Animals were treated and exposed according to the study design outlined in Table 4.

Drug Administration.

Monkeys were given the drug while under slight anesthesia via a stomach tube. Each animal to be treated received the drug orally at a dosage of 100 mg/kg for 5 days. The treated animals were observed for signs of gross toxicity up to at least 4 hours post-therapy.

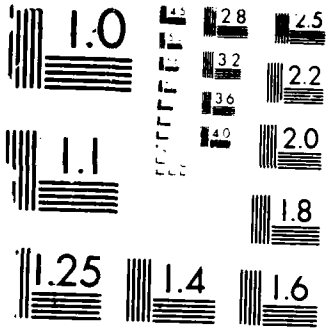
Exposure.

An oxamniquine-resistant strain of S. mansoni known as MAP which was obtained from Brazil in January, 1986 was used for all schistosome infections. This strain is maintained in laboratory bred Biomphalaria glabrata (BH- Belo Horizonte, State of Minas Gerais, Brazil) and outbred CD_1 albino male mice. Cercariae were shed from infected B. glabrata en masse as follows:

1. The evening prior to the day of exposure, the trays containing the infected snails were covered with a black cloth to simulate a dark period.

AD-A187 950 TESTING OF COMPOUNDS FOR EFFICACY AGAINST SCHISTOSOMIASIS(U) LOWELL UNIV MA CENTER FOR TROPICAL DISEASES J I BRUCE 25 AUG 87 84-4267/2 2/2

UNCLASSIFIED DAMD17-85-C-5209 F/G 6/15 NL


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0

2. Infected B. glabrata were stressed by drying them on paper towels and exposing them to fluorescent light for a 1/2 hr.

3. Filtered, aerated water was then added to the beaker containing the infected snails to just cover the snails.

4. The beaker was observed for shedding.

5. The first addition of water was the discarded to remove dirt, fecal matter, etc. and a second addition of water was made to collect the cercariae.

6. The solution containing cercariae was poured into a perti dish and used for the infection of experimental animals.

7. The cercariae were counted by drawing them from the solution with a micro-pipet as observed through a dissecting microscope.

Note: When necessary, the cercarial solution was diluted with filtered, aerated water to facilitate ease in counting the correct number of cercariae.

Monkeys were anesthetized with intramuscular injections of ketamine, and hamsters which were exposed as controls for the cercarial viability were immobilized by intraperitoneal injections of 20% sodium pentobarbital. The abdomens were shaved using animal clippers, then prewetted with conditioned water. Cercariae were counted by drawing them from a pool using a micro-pipet as observed through a dissecting microscope and transferred directly to the abdomens of the animals. In the case of the monkeys, the cercariae were concentrated in a specific area by placing a handmade glass tubing with a flared base (1.8 cm in diameter X 2.5 cm in height) on the abdomens and expelling the cercariae into it). All animals were exposed to cercariae for 30 min.

Examination.

Prior to the beginning of experimentation, all monkeys were screened for parasites. Fecal samples were examined using the AMS-3 concentration technique.

Beginning on day 28 post-exposure and continuing twice per week until the termination of the study, fecal samples from each animal will be examined for schistosome eggs using the AMS-3 concentration technique.

All animals will be sacrificed and examined at 49 - 54 days post-exposure to S. mansoni. Mature adult parasites will be recovered from each animal by the Perf-O-Suction technique, counted and the efficacy of the drug computed.

Table 4

Experimental Design for the Schistosoma mansoni Oral Prophylactic Study (BL23702) in Cebus apella

Group	Treatment Type	No. of Animals
I	Infection Control	4
II	Vehicle Control*	2
III	Reference Drug**	2
IV	Test Drug***	6

* Tween 80-methyl cellulose saline (TMCS)

** BL26758 (niridazole)

*** BL23702 (BL36450)

SECTION III

Protocol WRHN-IIIMc

Marmoset Penetration Model

BACKGROUND

The marmoset is being used as a model for the purpose of studying schistosome penetration dynamics. This species apparently has a natural skin barrier to schistosome cercariae, but when cercariae are injected subcutaneously some of the injected cercariae are able to survive and mature into adult worms. This study was designed to confirm these findings and thus provide the foundation for further indepth studies.

Animals.

Marmosets monkeys were obtained from Worldwide Primates, Inc., Miami, Florida. Monkeys were kept under quarantine for 45 days at Worldwide Primates, Inc. facilities prior to shipment to Lowell. Upon arrival at Lowell University they were immediately placed in quarantine for 45 days during which time they were tested for tuberculosis by the skin test method and examined for helminthic infections. Both male and female marmosets weigh about 1.1 kg.

Animals are housed individually in standard regulation cages, fed monkey chow and water ad libitum. All animals are kept in temperature/humidity controlled animal quarters on a 12 hr light/dark cycle.

Animal Care.

Monkeys will be maintained as outlined by the Guide for the Care and Use of Laboratory Animals" (1986).

All animals were anesthetized with intramuscular injections of a mixture of ketamine hydrochloride to temporarily immobilize

them during exposure to parasites. No painful or stressful treatments occurred.

Monkeys will be sacrificed at the termination of the study by giving an overdose of the anesthetic and exsanguination.

METHODS

Animals were treated and exposed according to the study design outlined in Table 5.

Exposure.

An oxamniquine-resistant strain of S. mansoni known as MAP which was obtained from Brazil in January, 1986 was used for all schistosome infections. This strain is maintained in laboratory bred Biomphalaria glabrata (BH- Belo Horizonte, State of Minas Gerais, Brazil) and outbred CD_1 albino male mice. Cercariae were shed from infected B. glabrata en masse as follows:

1. The evening prior to the day of exposure, the trays containing the infected snails were covered with a black cloth to simulate a dark period.
2. Infected B. glabrata were stressed by drying them on paper towels and exposing them to fluorescent light for 1/2 hr.
3. Filtered, aerated water was then added to the beaker containing the infected snails to just cover the snails.
4. The beaker was observed for shedding.
5. The first addition of water was then discarded to remove dirt, fecal matter, etc. and a second addition of water was made to collect the cercariae.
6. The solution containing cercariae was poured into a perti dish and used for the infection of experimental animals.
7. The cercariae were counted by drawing them from the solution with a micro-pipet as observed through a dissecting microscope.

Note: When necessary, the cercarial solution was diluted with filtered, aerated water to facilitate ease in counting the correct number of cercariae.

Monkeys were anesthetized with intramuscular injections of ketamine (10 mg/kg of body weight). Mice infectivity controls were anesthetized with intraperitoneal injections of 20% sodium pentobarbital (1 cc/100 gm of body weight). The abdomens of monkeys and mice were shaved using animal clippers, then prewetted with conditioned water. Cercariae were counted by drawing them from a pool using a micro-pipet as observed through a dissecting microscope and transferred directly to the abdomens of the mice, and to a 1 cc syringe for the monkeys. Metal rings (2 cm in diameter, 1 cm in height) were placed on the abdomens of the monkeys. The cercariae were then expelled from the syringe into the metal ring. All animals were exposed to cercariae for 30 min.

Examination.

Monkeys were paired for sex and weight for matched-pair analysis. Four monkeys were infected subcutaneously and 4 percutaneously with 150 S. mansoni cercariae each. Animals will be check for patency by stool concentration method starting at 6 wks post-infection. This is being done on a daily basis until patency occurs.

After patency, a weekly quantitative stool egg count for each monkey will be performed. At approximately 8 weeks after exposure 4 monkeys will be sacrificed (two from each group, Table 5).

At necropsy, portal pressure will be measured and microspheres will be injected in the portal vein. Then animals will be perfused by the Perf-O-Suction technique. Major organs will be taken and digested with KOH to obtain egg counts for organ egg load analysis. Worms will be counted, their sex and condition noted as well as gross pathology of the orgrans.

The remaining 4 monkeys will be maintained for another 4 months after which they will be sacrificed and the same procedure as described above will be performed.

Table 5

Experimental Design for Marmoset Experiment

Group No.	Method of Exposure	No. of Monkeys	Necropsy*
1	Subcutaneously	4	56 days
2	Percutaneously	4	120 days

* Two monkeys from each group will be necropsied at 56 days and two from each group at 120 days post-exposure.

REFERENCES

1. Mott, K.E., 1984. Schistosomiasis: New Goals. World Health, Geneva Switzerland, pp. 3-4.

2. Dias, L.C.S., Pedro, R.J., Rigo, E., Sotto, M.M.F. and Mafra, G.I., 1978. Linhagem humana, de Schistosoma mansoni resistente a esquistossomicidas. Revista de Saude Publica de Sao Paulo, 12:110.

3. Dias, L.C.S., Pedro, R.J., and Deberaldini, E.R., 1982. Use of praziquantel in patients with schistosomiasis mansoni previously treated with oxamniquine and/or hycanthone: Resistance of Schistosoma mansoni to schistosomicidal agents. Transactions of the Royal Society of Tropical Medicine and Hygiene. 76:652-654.

4. Coles, G.C., Bruce, J.I., Kinoti, G.H., Mutahi, W.T., Dias, E.R., and Katz, N. Drug resistance in schistosomiasis. Transaction of the Royal Society of Tropical Medicine and Hygiene. 80:347.

5. Saliba, E.K., Abdel-Hafey, S.K., and Tawfiq, M.R., and Mallett, J.C., 1980. The Threat of Schistosomiasis to Jordan. Jordan Medical Journal. 14:11-16.

6. Saliba, E.K., Abdel-Hafey, S.K., and Tawfiq, M.R., 1986. Schistosomiasis in Jordan: An Unwelcome Guest. Parasitology Today. 2:91-93.

7. Webbe, G. and Jordan, P., 1982. Schistosomiasis: Epidemiology, Treatment and Control. 361 pp. William Heineman Medical Books LTD, London.

8. Coles, G.C., Bruce, J.I., Kinoti, G.H. and Katz, N. Unpublished report.

9. Wright, W.H., Bauman, P.M. and Fry, N., 1948. The control of schistosomiasis, VII. Studies on the values of repellents and repellent ointments as a protection against schistosomiasis japonica. American Journal of Hygiene. 47:44-52.

10. Churchill, H.M., Nolan, M.O., Rogers, M.E. and Jones, M.F., 1945. Tests on fabrics and ointments for the control of schistosomiasis. National Research Council, Office of Scientific Research and Development, Tropical Disease Report. 59:7.

11. Chen, K.Y., Kwo, J.S., 1958. Protection experiments against the cercariae of Schistosoma japonicum. Chinese Medical Journal. 77:580.

12. Pellegrino, J., 1967. Protection against human schistosome cercariae. Experimental Parasitology. 21:112-131.

13. Greene, L.K. et al., 1983. Amoscanate as a topically applied chemical for prophylaxis against S. mansoni infections in mice. American Journal of Tropical Medicine and Hygiene. 32(6):1356-1363.

14. Grenan, M.M., Greene, L.K., Davidson, D.E., Jones, D.H., Shedd, T.R. and Hiestand, G., 1985. Hexarthorophene as a topically applied chemical for prophylaxos against Schistosoma mansoni infections in mice. Rev. Inst. Med. Sao Paulo. 27(4):190-196.

15. James, D.M. and Gilles, H.M., 1985. In: Human anti-parasitic drugs: pharmacology and usage. Chapter 11, The cestodes, pp. 232-246. John Wiley and Sons, New York, N.Y.

16. Radke, M.G., Berrios-Druan, L.A., and Moran, R., 1961. A perfusion procedure (Perf-O-Suction) for recovery of schistosome worms. Journal of Parasitology, 47:366-368.

17. Kemp, H.A., Hunter, G.W. III, Wilkins, O.P., Smalley, H., Dashiell, M.A., 1956. In: Studies of schistosomiasis, Part XI. Some ointments examined for protection against Schistosoma mansoni cercariae in preliminary tests. Military Medicine 119:1-10.

Forms

The data collected will be entered on the Walter Reed Army Institute of Research Anti-Schistosomiasis Drug Screen data forms which have been received along with a description of the codes to be used. The information will be entered onto a data disk and transmitted to WRAIR using a modem and the KERMIT program. If transmission cannot be completed, a copy of the data disk along with a copy of the data forms will be sent to the COTAR at WRAIR:

CPT. Robert E. Miller
ATTN: SGRD-WUM-B
Division of Experimental Therapeutics
WRAIR
Washington, DC 20307-5011

REPORT DISTRIBUTION LIST

12 Copies: Commander
US Army Medical Materiel Development Activity
ATTN: SGRD-UMB/MAJ Lightner
Fort Detrick
Frederick, MD 21701-5009

1 Copy: Commander
US Army Medical Research and Development Command
ATTN: SGRD-RMI-S
Fort Detrick
Frederick, MD 21701-5012

1 Copy: Dean, School of Medicine
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799

12 Copies: Administrator
Defense Technical Information Center
DTIC-DDAC
Cameron Station
Alexandria, VA 22304-6145

END

Feb.

1988

DTIC