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CHEMOTHERAPY OF MALARIA

FINAL REPORT

DORA S. RANE

For the period of June 1, 1975 to May 31, 1976

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P. berghei blood-induced malaria infection in mice - based on mortality.

T. rhodesiense blood-induced infection in mice - based on mortality.

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Primary screen - quantitative evaluation of potential antimalarial activity.

Primary screen - quantitative evaluation of potential trypanosomicidal activity.

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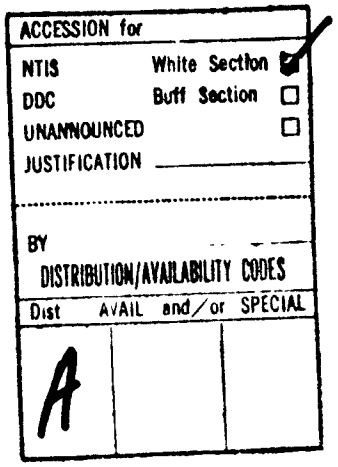
Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.



I. CONTINUATION OF THE SCREENING PROCEDURE FOR THE EVALUATION OF ANTIMALARIAL ACTIVITY OF CANDIDATE COMPOUNDS USING PLASMODIUM BERGHEI INFECTIONS IN MICE

This antimalarial screening program was initiated after the discovery that the Plasmodium falciparum parasite chiefly responsible for the high incidence of malaria in Vietnam was resistant to drugs such as chloroquine and quinine, generally recognized since World War II as satisfactory antimalarial agents. The urgent need for the screening operation was further emphasized when resistant strains were encountered in different malaria centers in Asia and in South America.

A total of approximately 256,198 compounds were tested from December 1, 1961 through May 15, 1976.

Table I summarizes the compounds tested and the mice used from December 1, 1961 through May 15, 1976.

The test system designed specifically for this operation is based on blood-induced Plasmodium berghei malaria infections in mice. It is a relatively simple and fast procedure. Its assessments of antimalarial effect and host toxicity are reproducible and reliable. It has introduced a new efficient method for screening large numbers of candidate compounds that have been successfully used as a model in the development of other large-scale screening operations.*

All compounds evaluated were obtained from the Department of Medicinal Chemistry at the Walter Reed Army Institute of Research and include:

(1) compounds structurally related to chemicals of known value as antimalarial agents;

(2) compounds structurally unrelated to compounds known to have antimalarial activity;

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* (1) A screening operation using blood-induced P. gallinaceum infections in chicks.
(2) A screening operation using sporozoite-induced P. gallinaceum infections in chicks.
(3) A screening operation based on blood-induced Trypanosoma rhodesiense infections in mice.

(3) structural analogues of compounds found active in our test system and representing several novel chemical groups.

Our own breeding colony of ICR/HA Swiss mice has continued to supply the animals used in our tests.

Evaluations of activity have been based on the responses to candidate compounds by P. berghei malaria in mice as expressed in comparisons of the maximum survival time of treated malaria-infected animals and the survival time of untreated malaria-infected controls.

Using young ICR/HA Swiss mice and a standard inoculum of P. berghei, it has been possible to produce a consistently uniform disease fatal to 100% of untreated animals within 6 to 7 days.

Since an established disease is less responsive to treatment than a disease in the early stages of development, treatment is withheld deliberately until a fairly high degree of parasitemia is evident. Test compounds are administered parenterally in a single dose on the third day post-infection at which time a 10-15% parasitemia has developed.

To be classified as active, a compound must suppress the disease and produce an unquestionably significant increase, 100% or more, in the life span of the treated animals over that of the untreated controls.

The severity of the challenges set up in our test system enhances the reliability of our evaluations and the antimalarial potential of the compounds selected for intensive preclinical studies.

METHOD*

ANIMAL HOSTS. The total supply of animals needed to screen candidate compounds has been obtained from our own breeding colony of ICR/HA Swiss mice. Test animals weigh from 18 to 20 grams, weight variations in any given experimental or control group being carefully limited to 2-3 grams. In any given test all animals are of a single sex and approximately the same age.

*Designed, developed and operated by Dr. Leo Rane until 1973, then operated by Mrs. Dora Rane until 1976.

P. berghei malaria in mice.

TABLE I

SUMMARY OF SCREENING LEVELS

DECEMBER, 1961 - MAY 15, 1976

	NUMBER OF COMPOUNDS	NUMBER OF MICE
DECEMBER, 1961 - MAY, 1964	6,915	250,000*
JUNE, 1964 - MAY, 1965	13,114	215,715
JUNE, 1965 - MAY, 1966	22,731	350,449
JUNE, 1966 - MAY, 1967	34,093	531,200
JUNE, 1967 - MAY, 1968	40,465	636,525
JUNE, 1968 - MAY, 1969	38,150	603,225
JUNE, 1969 - MAY, 1970	22,376	411,270
JUNE, 1970 - MAY, 1971	18,108	322,140
JUNE, 1971 - MAY, 1972	14,874	262,245
JUNE, 1972 - MAY, 1973	14,276	231,450
JUNE, 1973 - MAY, 1974	11,035	168,664
JUNE, 1974 - MAY, 1975	10,604	168,725
JUNE, 1975 - MAY 15, 1976	9,457	148,360
TOTAL	256,198	4,299,960

*Includes mice used in the development of the test.

Animals on test are housed in metal-topped plastic cages, fed a standard laboratory diet and given water ad lib. Once the infected mice are given the drug they are placed in a room maintained at 84° F (± 2° F) and a relative humidity of 66% (± 2%).

TEST PROCEDURE. Test animals receive an intraperitoneal injection of 0.5 cc of a 1:244 dilution of heparinized heart blood with a minimum of 90% parasitized cells, drawn from donor mice infected four days earlier with P. berghei. The donor strain is maintained by passing every four days in separate groups of mice inoculated with 0.5 cc of a 1:50 dilution of heparinized heart blood.

To check factors such as changes in the infectivity of our P. berghei strain or in the susceptibility of the host one group of mice is infected but not treated which serves as the negative control. In order to determine the effect a drug exerts on a malarial infection two parameters are measured; the first being an increase in survival time, and the second concerns curative action. For comparative purposes one standard compound, pyrimethamine, is administered at one level (120 mg/kg) to a group of 20 mice which serves as a positive control producing definite increases in survival time and curative effects. Another function of the positive control involves monitoring three procedures; the drug weighing, the preparation of drug solutions and suspensions, and the administration of drugs.

DRUG ADMINISTRATION. Test compounds are dissolved or suspended in peanut oil before they are administered.

Treatment consists of a single dose given subcutaneously 3 days post-infection. At the time of treatment a 10-15% parasitemia has developed. Although the disease is well established, it has not yet caused sufficient debility to affect an evaluation of the test compound's toxicity.

Deaths that occur before the 6th day, when untreated controls begin to die, are regarded as the result of a compound's toxic effects and not as the result of action by the infecting parasite.

In each experiment the compound on test is administered in graded doses (640, 160 and 40 mg/kg) to groups of 5 mice per dosage level. Increases in the dose levels of highly active compounds usually are followed by increases in the survival time of the treated mice.

If an active drug is toxic for the host, the toxicity of this compound may become a limiting factor to changes in dose levels.

Treated animals alive at the end of 60 days are considered as cured.

DRUG ACTIVITY. Acceptance of a drug as being sufficiently active for detailed studies is predicated on the margin between the maximum tolerated dose (MTD) and the minimum dose producing a significant effect (MED). A maximum tolerated dose is defined as the highest dose causing no more than one of five animals to die. The minimum effective dose is defined as the minimum dose increasing the life span of treated animals by 100% over the life span of untreated controls.

An increase of 100% in survival time is considered the minimum significantly effective response for a candidate compound.

Clearly inactive compounds are rejected after one test, borderline compounds after two tests. Active compounds are subject to a test to determine a dose-response curve (a 6 or 9 dosage level test) so that the spread between the maximum tolerated dose (MTD) and the minimum dose producing a signficant effect (MED) may be established. The total number of active compounds from June 1, 1970, to April 2, 1976, is summarized in Table II.

P. berghei malaria in mice

TABLE II

SUMMARY OF ACTIVE COMPOUNDS

JUNE 1, 1970 - MAY 15, 1976

YEAR	NUMBER OF COMPOUNDS TESTED	NUMBER OF COMPOUNDS ACTIVE
JUNE 1, 1970 - MAY 31, 1971	18,108	805
JUNE 1, 1971 - MAY 31, 1972	14,874	593
JUNE 1, 1972 - MAY 31, 1973	14,276	771
JUNE 1, 1973 - MAY 31, 1974	11,035	394
JUNE 1, 1974 - MAY 31, 1975	10,604	616
JUNE 1, 1975 - MAY 15, 1976	8,997	294
TOTAL	77,394	3,473

CONTINUATION OF THE SCREENING PROCEDURE FOR THE EVALUATION OF TRYPANOSOMICIDAL ACTIVITY OF CANDIDATE COMPOUNDS USING TRYPANOSOMA RHODESIENSE INFECTIONS IN MICE

The test system described herein was developed specifically to evaluate the trypanosomicidal activity of large numbers of candidate compounds.* Based on blood-induced Trypanosoma rhodesiense infections in mice, it performs as a primary screen or as a secondary screen and confirmatory test and gives precise quantitative evaluations of chemical compounds that demonstrate potentially useful therapeutic and/or prophylactic activity in T. rhodesiense infections. Consequently, it is also a helpful guideline in the synthesis of new active agents.

These agents include: (1) chemicals structurally related to compounds of known value in the treatment or prevention of T. rhodesiense infections; (2) chemicals structurally unrelated to compounds of known value in the treatment or prevention of T. rhodesiense infections and; (3) structural analogues of compounds that have demonstrated activity in our screening procedure and represent novel chemical groups.

All candidate compounds were obtained from the Department of Medicinal Chemistry at the Walter Reed Institute of Research.

Table I summarizes the number of compounds tested and the number of mice used from August 1, 1972, through May 15, 1976.

Our own colony of ICR/HA Swiss mice provided all the test animals needed in this operation. Using mice of a given age, sex and weight and a standard inoculum of the Wellcome CT strain of T. rhodesiense, it has been possible to produce a consistently uniform disease fatal to 100 percent of untreated animals within 4-6 days.

Test compounds were administered either parenterally or orally in a single dose on the day of infection.

Activity was determined by responses to candidate compounds by T. rhodesiense infections in mice as expressed in comparisons of the maximum survival time of the treated trypanosome-infected animals and the survival time of the untreated trypanosome-infected controls. To be classified as active, a compound must suppress the disease and produce an increase of at least 100% in the life span of the treated animals over that of the untreated controls.

*Designed, developed and operated by Dr. Leo Rane until 1973, then operated by Mrs. Dora Rane until 1976.

TABLE I

COMPOUNDS TESTED AND MICE UTILIZED

AUGUST 1, 1972 - MAY 15, 1976

YEAR	NUMBER OF COMPOUNDS	NUMBER OF MICE
AUGUST 1, 1972 - MAY 31, 1973	3,030	51,405
JUNE 1, 1973 - MAY 31, 1974	1,581	25,360
JUNE 1, 1974 - MAY 31, 1975	1,826	33,850
JUNE 1, 1975 - MAY 15, 1976	1,610	29,605
TOTAL	8,047	140,220

Acceptance of a test compound's activity was also predicated on the margin between the maximum tolerated dose (MTD) and the minimum dose producing a significant effect (MED). A maximum tolerated dose is defined as the highest dose causing no more than one of five animals to die and a minimum effective dose as the min' um dose is defined as the highest dose causing no more than one of five animals to die and a minimum effective dose as the minimum dose increasing the life span of treated animals 100% over the life span of untreated controls.

METHODS

Animal Hosts. Our own breeding colony of ICR/HA Swiss mice has supplied all the animals used in this screening procedure. Test animals weigh 30-32 grams, weight variations in any given experimental or control group being carefully limited to 3 grams. In all tests animals have been of the male sex and approximately of the same age.

Animals on test are housed in metal topped plastic cages, fed a standard laboratory diet and given water ad lib.

Once the mice have been given a drug they are kept in a room maintained at 84°F (± 2° F) and a relative humidity of 66% (± 2%).

Test Procedure. Test animals receive an intraperitoneal injection of 0.5 cc of a 1:50,000 dilution of heparinized heart blood drawn from a donor mouse infected 3 days earlier.

The donor line is maintained by 3 day blood passes, each animal receiving 0.1 cc of a 1:500 dilution of heparinized heart blood drawn from a 3 day donor. Donors, like test animals, weigh 30-32 grams, weight variations for each pass being limited to 3 grams.

To check factors such as changes in the infectivity of our T. rhodesiense strain, or in the susceptibility of the host one group of infected untreated mice are included as negative controls. In order to determine the effect a drug exerts on a trypanosome infection two parameters are measured; the first being an increase in survival time, and the second concerns curative action. For comparative purposes standard compounds, stilbamidine isethionate and 2-hydroxystilbamidine isethionate are administered at one level each (26.5 mgs/kg) to separate groups of 10 mice which serve as positive controls producing definite increases in survival time and curative effects. Another function of these two positive controls involves a check on whether three procedures are performed correctly; the drug weighing, the preparation of drug solutions or suspensions and the administration of drugs.

Drug Administration. Test compounds are dissolved or suspended in peanut oil and prepared in three or more graded doses. At least three different doses of each test compound are included in an experiment. Groups of 5 mice per dose level of drug are utilized.

Treatment consists of a single dose administered subcutaneously or orally on the day of infection. Deaths that occur before the fourth day, when untreated controls begin to die, are regarded as the result of a compound's toxic effect and not as the result of action by the infecting parasite.

Increases in the dose levels of highly active compounds usually are followed by increases in the survival time of the treated mice. However, if an active drug is toxic for the host, the toxicity of this compound may become a limiting factor to changes in dose levels.

Treated animals alive at the end of 30 days are considered as cured.

Drug Activity. An increase of 100% in survival tin is considered the minimum significantly effective response for a candidate compound. Clearly inactive compounds are rejected after one test, borderline compounds after two tests.

Active compounds are subjected to a test to determine a dose response curve (6 or 9 different doses) so that the spread between the maximum tolerated dose (MTD) and the minimum dose producing a significant effect (MED) may be established.

COMPOUNDS WITH DEFINITE CHEMOTHERAPEUTIC ACTIVITY AGAINST TRYPANOSOMA RHODESIENSE INFECTIONS IN MICE

During the opening period of this project, June 1, 1972 - May 31, 1973, our screening procedure was developed and its reliability established. 3,030 selected compounds were screened, including a number of agents known to be effective in T. rhodesiense infections and drugs drawn from our antimalarial program. Of these 68 demonstrated a degree of activity sufficient to produce at least 100 percent increases in the survival time of treated T. rhodesiense infected mice.

1,581 compounds were tested in the period, June 1, 1973 - May 31, 1974. Of these 185 demonstrated a degree of activity sufficient to produce at least 100 percent increases in the survival time of treated T. rhodesiense infected mice, 92 were active subcutaneously and 93 orally.

1,826 compounds were tested in the period June 1, 1974 - May 31, 1975. Of the 298 recognized as active compounds, 225 were active subcutaneously and 73 orally.

1,426 compounds were tested in the period June 1, 1975 - March 17, 1976. Of the 237 recognized as active compounds, 191 were active subcutaneously and 46 orally.

This breakdown is significant since: (1) activity evaluations provided in our screening procedure are precise and quantitative; (2) dose response curves of active compounds administered subcutaneously show a wider spread between the maximum tolerated dose (MTD) and the minimum dose producing a significant effect (MED) than dose response curves of active compounds administered orally and; (3) these dose responses also reveal a wider spread of toxic effects when active compounds toxic for the host are administered subcutaneously rather than orally.

SPECIAL TRYPANOSOME EXPERIMENT

We had observed that, following the administration of a single curative dose of certain antitrypanosomal drugs to mice experimentally inoculated with T. rhodesiense, the cured mice could not be infected when rechallenged periodically with the organism. Whether the resistance to reinfection was immunologic or due to the persistence of the drug in the animals is the subject of a special investigation.

This year we continued to give repeated lethal challenges of T. rhodesiense to mice representing 100 percent cures in routine tests, and seventeen groups of cured mice were rechallenged at intervals of one month or more. Our negative controls were by necessity younger animals but their average weight approximated the average weight of the mice in a rechallenged group.

Three groups of mice did not survive a second challenge and one group did not survive a third. Table I records the number of challenges received by each of the remaining thirteen groups, the date of the original routine test and the date of the first re-challenge, the WRAIR bottle number of the drug that produced cures with a single treatment, and the present number of survivors.

A two-year test, started in October, 1974, will allow us to study the staying power of one compound, BG-00512*, and its effectiveness at various intervals. A brief outline of the complete work plan is submitted. The two positive control drugs used in our routine tests, stilbamidine isethionate (ZG-76354) and hydroxystilbamidine isethionate (AH-25296) at dose levels equimolar with the 424 mg/kg dose of BG-00512, were included to serve as a basis for comparison.

The 1200 animals in this test are males of the same age, and weigh 28-30 grams. 220 additional mice of the same sex, age and weight were put aside as a pool from which will be drawn the negative controls used on day zero and in each of the ten successive tests. All test animals have been kept in individual cages (one mouse/cage) to avoid unnecessary wounds and fatalities.

The 1200 animals were given a single treatment on day zero: 400 with ZG-76354 at a dose of 708 mg/kg; 400 with AH-25296 at a dose of 689 mg/kg; and 400 with BG-00512 at a dose of 424 mg/kg. 200 animals in each of these three groups also received a lethal challenge of T. rhodesiense. These infections and treatments were regarded as preliminary preparations.

*See Table II.

DATE OF ORIGINAL ROUTINE TESTS	BN OF CURATIVE DRUG	DATE OF FIRST CHALLENGE	NUMBER OF CHALLENGES	NUMBER OF SURVIVORS AT DOSE LEVEL OF TREATMENT		
				424	106	26.5
Jan. 8, 1975	BE-42056	Feb. 13, 1975	9*	3		
Feb. 5, 1975	BE-66663	Mar. 16, 1975	8*	3	2	
Dec. 11, 1974+	**BE-43697	Jan. 22, 1975	7*	2	1	
Mar. 12, 1975	BE-55900	Apr. 16, 1975		2		
Sept. 10, 1975	BA-62807	Oct. 15, 1975	3***	4	3	1
Aug. 27, 1975	BE-99055	Sept. 1, 1975		5	2	2
Aug. 27, 1975	BE-99126	Sept. 1, 1975		4	1	
Aug. 20, 1975	BG-01108	Oct. 1, 1975		5	3	2
Aug. 20, 1975	BE-96296	Oct. 1, 1975		5	5	5
Aug. 20, 1975	BE-80323	Oct. 1, 1975		3	2	1
Oct. 8, 1975	BE-98843	Nov. 5, 1975	2***	4	3	1
Dec. 3, 1975	BE-98665	Jan. 14, 1976	1	4	3	1
Dec. 23, 1975	BG-37635	Jan. 29, 1976		4		

+ - 3 month interval between 2nd and 3rd challenges.
2 month interval between 4th and 5th challenges.
* - Last challenge after an interval of 4 months, January 14, 1976.
** - 2 mice at the top dose and 1 at the 2nd dose were sacrificed after the 4th challenge for blood passes to normal mice. No parasites were evident microscopically during and at the end of a 30-day period.
*** - Last challenge after 2 months, January 21, 1976.

TABLE II

BN:BD-38691 (BG-00512) ORIGINAL TEST - 2/27/74

DOSE LEVELS OF ORIGINAL SINGLE TREATMENT: 424 mg/kg, 106 mg/kg, 26.5 mg/kg

#1 - Positive Control - Stilbamidine isethionate (26.5 mg/kg)
#2 - Positive Control - 2-hydroxy-stilbamidine isethionate (26.5 mg/kg)

Date of Reinfection		# of Survivors Reinfected	Dilution of Inoculum	Day of Death/ # of Mice	MST*
3/29/74	Positive Control #1	10	1:50,000	5/8, 6/1	4.4
	Positive Control #2	10		6/1	
		5 (424 mg/kg)			
		5 (106 mg/kg)			
		5 (26.5 mg/kg)		12/1	
4/13/74	Positive Control #1	1	1:500	6/1	3.0
	Positive Control #2	9		6/8, 7/1	
		5 (424 mg/kg)		11/1	
		5 (106 mg/kg)		7/1, 11/1	
		4 (26.5 mg/kg)		5/1, 6/2	
5/27/74		4 (424 mg/kg)	1:50,000		4.4
		3 (106 mg/kg)			
		1 (26.5 mg/kg)		7/1	
6/26/74		4 (424 mg/kg)	1:50,000		4.2
		3 (106 mg/kg)			
7/26/74		4 (424 mg/kg)	1:50,000		4.4
		3 (106 mg/kg)			
8/28/74		4 (424 mg/kg)	1:50,000		4.2
		3 (106 mg/kg)			

*Mean Survival Time of Negative Controls.

Beginning with Test #1 and in each following test, 10 animals in a section of the "1" columns of each compound will receive a single challenge, whereas the ten animals in a section of the "2" columns of each compound will be rechallenged in every test after the one in which they were first challenged. Thus after the tenth and final test, the animals in the "2" columns that were first challenged in Test #1 will have been challenged ten times, the animals in the "2" columns first challenged in Test #2 will have been challenged nine times, etc. A copy of our test schedule is included to indicate the intervals between each test.

Thus, the mice that received one challenge at different time intervals will provide a basis for comparing the staying power of our positive control drugs and BG-00512 while the mice that received multiple challenges will provide a basis for comparing the continued effectiveness of BG-00512 and the positive control drugs.

Since this study has not been completed, a final report cannot be submitted. Please note that the 6th of the ten challenges will be given on May 26th. Thereafter, the time intervals between challenges are increased.

	NG-76354 708 mg/kg				NH-55296 630 mg/kg				[illegible] 111 mg/kg				[illegible]
	ALONE		+ TRYP		ALONE		+ TRYP		ALONE		+ TRYP		
	1	2	1	2	1	2	1	2	1	2	1	2	
DAY ZERO	100	100	100	100	100	100	100	100	100	100	100	100	20
#1	* 10	********** 10	* 10	********** 10	* 10	********** 10	* 10	********** 10	* 10	********** 10	* 10	********** 10	20
#2	* 10	********* 10	* 10	********* 10	* 10	********* 10	* 10	********* 10	* 10	********* 10	* 10	********* 10	20
#3	* 10	******** 10	* 10	******** 10	* 10	******** 10	* 10	******** 10	* 10	******** 10	* 10	******** 10	20
#4	* 10	******* 10	* 10	******* 10	* 10	******* 10	* 10	******* 10	* 10	******* 10	* 10	******* 10	20
#5	* 10	****** 10	* 10	****** 10	* 10	****** 10	* 10	****** 10	* 10	****** 10	* 10	****** 10	20
#6	* 10	***** 10	* 10	***** 10	* 10	***** 10	* 10	***** 10	* 10	***** 10	* 10	***** 10	20
#7	* 10	**** 10	* 10	**** 10	* 10	**** 10	* 10	**** 10	* 10	**** 10	* 10	**** 10	20
#8	* 10	*** 10	* 10	*** 10	* 10	*** 10	* 10	*** 10	* 10	*** 10	* 10	*** 10	20
#9	* 10	** 10	* 10	** 10	* 10	** 10	* 10	** 10	* 10	** 10	* 10	** 10	20
#10	* 10	* 10	* 10	* 10	* 10	* 10	* 10	* 10	* 10	* 10	* 10	* 10	20
	100	100	100	100	100	100	100	100	100	100	100	100	20

+ - EACH STAR REPRESENTS ONE MILLILITRE.

Test #1	-	December 3, 1975	(1 month)
#2	-	January 7, 1976	(2 months)
#3	-	February 11, 1976	(3 months)
#4	-	March 17, 1976	(4 months)
#5	-	April 21, 1976	(5 months)
#6	-	May 26, 1976	(6 months)
#7	-	September 1, 1976	(9 months)
#8	-	December 1, 1976	(1 year)
#9	-	May 4, 1977	(18 months)
#10	-	November 9, 1977	(2 years)

DATE OF EACH TEST ADJUSTED FOR WEDNESDAY, OUR REGULAR TEST DAY.

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