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Development of Resistance Against Tafenoquine in the Blood Induced Infection of *Plasmodium yoelii* and Its Possible Simultaneous Effect on Sporozoite Induced Tissue Stage

Jayanti Neerja, Rajeev Gaur* and Anurag Singh
Department of Microbiology, Dr. R.M.L. Avadh University
Faizabad,Ayodhya-224001, (U.P.),India

ABSTRACT

Nearly about 50 % of the population is fighting the adversity of the malarial infections although there has been a huge researches as well as remedy development to bring down the incidence of malarial infections significantly. Moreover the emergence and transmission of resistance to antimalarial treatment continues to hamper malaria elimination efforts. The proportion of antimalarial use within the population and the presence of residual drug concentrations were identified to be the main predictors of the emergence and transmission of resistance. The present investigation focuses on whether the experimental resistance developed in the blood stage for Tafenoquine and Primaquine using serial passage on the basis of 2% delay time is also reflected in the tissue stage of the parasites life cycle as well. The experimental transmission of sporozoite induced infection of a rodent parasite *Plasmodium yoelii nigeriensis* through hamsters by serial cyclic passage using *Anopheles stephensi* as vector has been a potential test system for major causal prophylactic and anti-relapse antimalarial. In the undermentioned research drugs at a dose of 30mg/Kg were curative against the blood induced infection and became resistant to maximum tolerated dose of 40mg/Kg of Primaquine after XVII passages over a period of 240 days and for 90mg/Kg of Tafenoquine after XIX consecutive passages over a period of 218 days.The experimental transmission of sporozoite induced infection of a rodent parasite *Plasmodium yoelii nigeriensis* through hamsters using *Anopheles stephensias* vector has been a potential test system for major causal prophylactic and anti-relapseantimalarial. This test system will help evaluate novel strategies as alternative to the frequent extensive use of safer and potential drugs like Tafenoquine.

KEY WORDS: MALARIA, P. YOELII NIGERIENSIS, TAFENOQUINE.

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*Corresponding Author: rajeevagaur@rediffmail.com
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INTRODUCTION

The investigations throughout the world has found out that about half of the world's populations are vulnerable to the risk of Malaria. There have been huge efforts made to bring down the incidence of malaria significantly. The capability of malaria causing microorganisms to emerge, transmit and show multidrug resistance has been a hurdle in the treatment of the malarial infections.Moreover researches are on board, recently an 8 aminoquinoline,

Tafenoquine (WR238605), jointly developed by Walter Reed Army Institute of Research and Glaxosmithcline pharmaceuticals has shown its efficacy as 10 times more potent and safer with better bioavailability as blood and tissue schizontocidal compared to Primaquine (Geoffrey et al., 2007) Our present investigation focuses on whether the experimental resistance developed in the blood stage for tafenoquine and Primaquine using serial passage on the basis of 2% delay time (Walsh et al., 2009) is also reflected in the tissue stage of the parasite's life cycle as well which will also give insight into the mode of action of the drug at two different stages of parasite's life cycle (Peters et al., 1993Yehenewet al., 2016).

Any chemo-prophylactic drug, taken infrequently to improve compliance, should be well tolerated and highly effective against all stages of malaria. In contrast to the drugs active only against blood stages Primaquine has been the mainstay therapy for several years for clearing parasites from the liver (Peters et al., 1993). Primaquine has a low therapeutic index with serious side effects like methaemoglobin formation in G6PD deficient individuals (Walsh et al., 2009). In view of the avoidance of certain side effects a far safer prophylactic as well as blood schizontocidal 8 aminoquinoline WR238605 (Tafenoquine) with better bioavailability and longer half-life, has been synthesized (World Health organization, 2014; 2014; 2015 Geoffrey and Bryan 2017). However the efficacy of newest and safest drugs is challenged by ever increasing emergence of resistance (Ponsa et al., 2003; Shanks et al., 2001). Till date, emergence of resistance has been described for all the available drugs (Looareesuwanet al., 1999).The aim of present study is to investigate that the extensive use of the drug as blood schizontocidal might lead to a rapid emergence of resistance for the drug which can also hamper its efficacy as transmission blocker by being reflected at tissue stage as well. If it would so, the mode of action of the drug will be the same at both of the parasitic stages.

MATERIAL AND METHODS

Blood and sporozoite induced infections with rodent malaria parasite *P.yoelii nigeriensis* (strain N 67) in swiss mice maintained at this institute, have been used in the present study. Outbred Swiss mice of either sex weighing 22-25 g were procured from the animal facilities at the institute and maintained on commercial pellet diet and water ad libitum under standard housing conditions. Ethical guidelines on handling and use of experimental animals were followed during the conduct of the study.

Drugs: Doses were calculated as base mg /Kg.Pure samples of the drugs tafenoquine were procured from Walter Reed Army Institute of Research, Washington DC, U.S.A. were used for the evaluation.

Drug preparation: Suspension of Primaquine was prepared by simply dissolving it's disulphate salt in the water.For Tafenoquine, a paste prepared with 2-3 drops of tween 80 was suspended in water to make a suspension.

Drug administration: Drugs were administered orally through a modified round ended 18 gauge needle in 0.1-0.5 ml volume.

2% Delay time: The immune system of a vertebrate host is enhanced when the parasitaemia reaches beyond 2%. For determining the 2 % delay time, the drug is administered for single day or 4 days from 0-3 days and the time in attaining 2% for drug treated as well as control group is monitored (Merkliet al., 1976).The 2% delay time was calculated by subtracting the time for control group to reach 2% parasitaemia from the time in drug treated animals in 2% parasitaemia. Higher delay time is indicative of the drug's capacity to suppress the growth of the parasite.

Determination of curative dose level and the sequential selection of resistance for the drugs: The evaluation of blood schizontocidal activity in rodent model, mice were inoculated intraperitoneally with $1x10^6$ *P.yoelii nigeriensis* and treated with the drugs. The dose of 10mg/kg of Tafenoquine was on day 6,7 and 8 during 1st passage and a total of 30 mg/kg was administered followed by an increased dose of 50 mg/kg in 2nd passage shown in the table 3 and so on. Giemsa stained blood smears from the experimental animals were microscopically observed upto day 28 post inoculation. The animals fail to develop infection till day 28 were considered as cured. The parasitaemia levels from animals that developed patent infection were sequentially recorded to determine the time to patency in every serial passage. The two criteria for the assessment of development of resistance were: The gradual decline in sensitivity with serial passages showing early patency is indicative of development of resistance and the patency established before 28 days after the treatment with curative dose of the drug.

Cyclic transmission of the parasite: usually 3-5 days old *Anopheles stephensi* mosquitoes reared in the insectorium were allowed to engorge blood from an infected hamster carrying appropriate gametocytes numbers. The infected hamster was restrained to allow undisturbed mosquito feeding by injecting 50 mg/Kg of thiopentone which immobilizes the animal for 30-40 min.

Causal prophylactic activity: The treatment drug was administered in three doses on day -1,0, ±1 and animals were challenged intravenously with $1x10^5$ *P.yoelii nigeriensis* sporozoites on day 0.Blood smears were observed from day 3 to day 28 to record the day of patency (Puri, 2000).

Procedure for determining the mosquito infectivity:

- Ookinete determination by examining the partially digested blood meal within 16-20 hrs after blood meal recovered from the lumen of the midgut.
- Oocyst numbers monitored between day 4-7 after blood meal by dissection of midguts of infected mosquitoes.
- Sporozoite determinations made by examining the salivary glands on day 10-12 post blood meal.A minimum of 20 mosquitoes for each batch were

dissected and midgut infections with oocysts were quantified with regard to following parameters: percentage of mosquitoes positive for oocyst and mean number of oocysts / positive mosquitoes.

Dissections for salivary gland sporozoites: For *P.yoelii nigeriensis* transmission. The salivary gland dissections are usually carried out between 10-14 days post infective blood meal.

Harvesting and preparation of sporozoite inoculum: The mosquito thoraces ground and centrifuged in 1:3 serum saline mixture contains sporozoite.

Sporozoite inoculation to vertebrate host: A volume of 0.2 ml containing 1X 10^4 of the sporozoite suspension was inoculated intravenously using a 26 gauge needle and animals were observed from day 3 onwards to monitor the infectivity.

RESULTS AND DISCUSSION

Blood schizontocidal response of Primaquine and Tafenoquine was evaluated at three dose levels in 10 mice each after the 4 days regimen. The results (table 1) showed that animals treated at 30mg/Kg for both of the drugs (Primaquine and tafenoquine) did not develop patent infection till day 28 and hence were cured. Treatment at lower doses showed patency.

WR 238,605, a novel 3-phenoxy-substituted 8-aminoquinoline, possesses causal prophylactic, blood schizontocidal and gametocytocidal activity against rodent malaria parasites (Sinha et al., 2014). It has an established fact that this 8 aminoquinoline drug has shown its superiority in terms of efficacy as well as safety over primaquine against multidrug resistant lines of *P.yoelii* and *P.berghei* (Peters et al., 1993). In combination with chloroquine (CQ), WR 238,605 display a synergistic or 'resistance-reversing' action against CQ-resistant *P. yoelii* NS parasites (Llanos et al., 2014). In view of the present scenario with escalating resistance to available drugs and their safety issues Tafenoquine is a good candidate compound for clinical trials against multiresistant *P.falciparum* strain as well as a causal prophylactic and ant relapsing agent against *Plasmodium vivax* infections.

Table 1. Blood schizontocidal response of Tafenoquine against *P.yoelii nigeriensis* -67 in Swiss mice based on delay time in attaining 2% parasitaemia

Drug	Dose mg/Kg	No.of mice	Time to reach 2% parasitaemia	2% delay time relative to control group	Mean ±SE	Cure Rate%
	30	6	All protected			100
Tafenoquine	10	6	6,9,9,9,10,11	3.6,3.6,3.6,6.6,7.6,8.6	5.6 ±0.58	0
(WR238605)	5	6	3.8, 4,5,6.1,7,7	1.4,1.6,2.6,3.7,4.6,4.6	3.1 ± 0.58	0
Control		4	1.7,2,2.9,3 Mean ±SE2.4±0.39			

Table 2. Blood schizontocidal response of Primaquine against *P.yoelii nigeriensis* -67 in Swiss mice based on delay time in attaining 2% parasitaemia.

Drug	Dose mg/Kg	No.of mice	Time to reach 2% parasitaemia	2% delay time relative to control group	Mean ±SE	Cure Rate%
	30	5	All protected			100
Primaquine	10	5	6.7,7.3,7.5,7.6,7.9	4.0,4.6,4.8,4.9,5.2	4.7 ±0.19	0
	5	5	3.8, 5.02,5.4,5.5	1.1,2.3,2.7,2.8,3.1		
Control		4	1.7,2.6,2.9,3.6	Mean ±SE 2.7 ±0.39		

In our study also It has shown its blood schizontocidal as well as causal prophylactic efficacy against Chloroquine resistant *P.yoelii nigeriensis* (table1 and 7).Owing to the situation our study is aimed to investigate whether the frequent clinical use of this antimalarial would lead to development of resistance at blood induced stages and if so, would it be reflected at tissue stage as well. In our experimental study we tried to develop resistance for the drugs at blood stage which did not reflect at tissue stage of the parasite as the causal prophylactic activity assessed remained the same as before and after the development of resistance (table 8). The outcome of the study reveals that the site of action of the drugs are different against both the blood and the tissue induced infections as it were the same the resistance developed at blood stage would have reflected at tissue stage at the same time

with the altered causal prophylactic activity after the development of resistance.

No alteration in causal prophylactic activity is indicative of the different sites of targets for the drug. WR 238,605, a novel 3-phenoxy-substituted 8-aminoquinoline, possesses causal prophylactic, blood schizontocidal and gametocytocidal activity against rodent malaria parasites (Yehenew et al., 2016). It has an established fact that this 8 aminoquinoline drug has shown its superiority in terms of efficacy as well as safety over primaquine against multidrug resistant lines of *P.yoelii* and *P.berghei* (Yehenew et al., 2016). In combination with chloroquine (CQ), WR 238,605 display a synergistic or 'resistance-reversing' action against CQ-resistant *P. yoelii* NS parasites (Llanos et al., 2014). In view of the present scenario with escalating resistance to available drugs and their safety issues Tafenoquine is a good candidate compound for clinical trials against multiresistant *P.falciparum* strain as well as a causal prophylactic and antirelapsing agent against *Plasmodium vivax* infections. In our study also it has shown its blood schizontocidal as well as causal prophylactic efficacy against chloroquine resistant *P.yoelii nigeriensis* (table 1 and 2).

Table 3. Sequential development of resistance to Tafenoquine (WR238605) in a strain of *P.yoelii* (N-67) in Swiss mice

Serial passage no.	Duration of passage	Drug administration On day	Drug dose on respective days mg/Kg	Total dose administered mg/Kg
I	0-10	3,5,7	10,10,10	30
II	10-22	3,5,9,10,11	10,10,10,10,10	50
III	23-36	3,4,5,6	10,20,20,20	70
IV	36-54	7,8,10,12,14,15,16	10,10,10,10,10,10,10	70
V	54-68	6,7,8,9	10,10,10,10	40
VI	69-79	3,4,6,7	10,10,10,20	50
VII	80-90	0-3	30,30,30,30	120
VIII	91-100	0-3	30,30,30,30	120
IX	100-105	6	30	30
X	106-112	0-3	45,45,45,45	180
XI	112-120	0-3	45,45,45,45	180
XII	171-180	0-3	60,60,60,60	240
XVII	181-195	0-3	60,60,60,60	240
XIX	206-218	0-3	90,90,90,90	360
XX	219-230	0-3	90,90,90,90	360

Owing to the situation our study is aimed to investigate whether the frequent clinical use of this antimalarial would lead to development of resistance at blood induced stages and if so, would it be reflected at tissue stage as well. A Primaquine resistant strain of *P.yoelii* was selected after sequential exposure to drug for 16 sequential passages over a period of 218 days. During first passage, a total dose of 30mg/kg was administered. Dose of 10mg/Kg was administered on day 6, 7 and 8. During second passage the dose of 10mg/Kg was continued but total dose was increased to 50mg/Kg. The schedule and dose of drug administration upto XX passage is presented in table (6). The induction of resistance to Primaquine was observed by standard 4 day test initially by the 12 th serial passage table(7). During the 15 th passage also 3 out of 5 mice showed decline in Primaquine sensitivity. Gradual many fold resistance was observed with increased duration of exposure upto XX passages and the strain became resistant to maximum tolerated dose of the drug. Cyclical transmission of the parasite was carried out with varying grades of resistant parasitic line.

It was found that only low to medium level of resistance developed through many passages was able to get transmitted and to produce sporozoite in the mosquito vector. Likewise Primaquine, Resistance to Tafenoquine was also achieved through serial passages and cyclical transmission was done intermittently with various levels of resistant lines and similar results were obtained showing only low to medium level resistant parasite being capable of producing sporozoites (Alanoet al., 1995; Sinha et al., 2014). The most successful transmission of the parasite to mosquitoes was achieved just after XIII th passage for Primaquine and VIII th passage for Tafenoquine lines. A twofold resistance for Tafenoquine against blood induced infection of *P.yoelii nigeriensis* was developed and sensitivity towards curative dose (30 mg/Kg in a 4 day regimen) was evaluated.

A total of XV serial blood passages within a period of 170 days resulted in the development of two fold resistance in the parasite for Tafenoquine. The parasite load was also kept free of the drug pressure intermittently. The stability of resistance after cryopreservation (123 days)

and drug free maintenance (150days) was evaluated and found to be constant. The drug was evaluated for its blood schizontocidal as well as for its causal prophylactic activity before and after the development of resistance.

Table 4. Evaluation of sensitivity to WR238605during selection of WR238605 (tafenoquine) resistant strain of *P.yoelli* in swiss mice.

Serial passage no.	Treatment Duration in days	No. of mice	Dose mg/Kg	Mice showing nill parasitaemia						Day of patency
				4	7	10	14	21	28	
VII	0-3	5	30	5	5	1	0	0	0	8,8,9,9,12
		5	45	5	5	5	5	5	5	-
		5	60	5	5	5	5	5	5	-
VIII	0-3	5	30	5	5	1	0	0	0	9,9,10,10,12
XII	0-3	5	30	5	1	0	0	0	0	5,5,6,6,9
XIII	0-3	5	45	5	2	1	0	0	1	5,6,6,8,11
XIV	0-3	5	45	5	2	0	0	0	0	5,6,6,8,10
		5	60	5	5	5	5	5	5	-
XV	0-3	5	45	4	0	0	0	0	0	5,5,6,6,8
		5	60	5	4	0	0	0	0	7,8,8,8,10
XVI	0-3	5	60	2	1	0	0	0	0	4,4,5,6,9
XVII	0-3	5	60	5	5	0	0	0	0	8,8,9,10,13
XIX	0-3	5	90	1	0	0	0	0	0	4,4,4,4,6
XX	0-3	5	90	5	2	0	0	0	0	5,6,6,8,9

Table 5. Sequential development of resistance to Primaquine in a strain of *P.yoelii* (N-67) in Swiss mice

Serial passage no.	Duration of passage	Drug administration On day	Drug dose on respective days mg/Kg	Total dose administered mg/Kg
I	0-11	6,7,8	10,10,10	30
II	11-30	5,6,9,10,17	10,10,10,10,10	50
III	31-41	-	-	
IV	42-53	5	10	10
V	54-62	3	10	10
VI	63-74	3,10	10,10	20
VII	77-89	4,6,7,8,	10,10,10,10	40
VIII	90-112	6,8,9,10,11,12	10,10,10,10,10,10	60
IX	113-126	4,5,9,10,13	10,20,10,10,10,10	70
X	127-139	3,4,8,9,	10,10,10,10	40
XI	140-151	5,6,9,10	10,10,10,20	50
XII	152-164	0-3	30,30,30,30	120
XIII	165-173	3,4,5,7,8	30,30,30,30,30	150
XIV	174-185	2,6,7	30,30,30	90
XV	219-228	0-3	30,30,30,30	120
XVI	219-228	0-3	30,30,30,30	120
XVII	229-240	0-3	40,40,40,40	160
XIX	249-257	0-3	40,40,40,40	160

Table 6. Sensitivity test during selection of Primaquine Resistant strain of *P.yoeliiin* Swiss mice.

Serial passage no.	No.of mice	Treatment Duration in days	Dose mg/Kg	Mice showing nill parasitaemia on day						Day of patency
				4	7	10	14	21	28	
XII	5	0-3	30	5	5	2	0	0	0	8,10,10,11, 11
XV	5	0-3	30	5	5	5	2	2	2	11,11,12,12,13
XVI	5	0-3	30	5	2	0	0	0	0	7,7,7,8,9
XVII	5	0-3	30	5	1	0	0	0	0	6,6,7,7,8
XVIII	5	0-3	30	5	1	0	0	0	0	6,7,7,7,9
XIX	5	0-3	40	5	0	0	0	0	0	5,5,6,6,7
XX	5	0-3	40	5	0	0	0	0	0	5,6,6,6,6

Table 7 Causal Prophylactic activity: Primaquine and Tafenoquine

Drug/dose A) Primaquine	Treatment days	No.of animals	Day of patency	Mean value ± SE	No. of mice cured	Cure Rate%
40 mg/Kg	-1,0, ±1	5	All protected	0	6/6	
40 mg/Kg	0 day	6	All protected	0	6/6	100
30 mg/Kg	-1,0, ±1	6	5,6,6,6,7 -ve	5 ± 0.94	1/6	100
20 mg/Kg	-1,0, ±1	5	5,5,6,6,7	5.8 ±0.54	0/6	16.6
Control		6	5,5,5,5,5	5.0 ±00		0
B) Tafenoquine			All protected			
40 mg/Kg	-1,0, ±1	6	All protected	0	6/6	100
20 mg/Kg	0 day	5	All protected	0	6/6	100
10 mg/Kg	-1,0, ±1	5	11,11,11,12,12	0	6/6	100
40 mg/Kg	-1,0, ±1	5	All protected	11.4 ±0.21	0/6	0
10 mg/Kg		5	4,4,6,6,7	0	6/6	100
05 mg/Kg		5		5.4 ± 0.53	0/6	0
Control		5	4,5,5,6,6	5.2 ± 0.33		

The causal prophylactic dose of the drug (10 mg/Kg in -1, 0, ±1) remained unaltered even after the development of resistance in a blood induced infection of Chloroquine resistant *Plasmodium yoelli*. (Vuonget al., 2015). The unaltered causal prophylactic activity of the drug at tissue stage is indicative of the two different mode of actions of the drugs at blood and tissue stage of infections. Previous investigations on pharmacodynamic and antimalarial activity reveal that degradation of hemoglobin by malarial parasite generates heme which is detrimental to the parasite and detoxified through its conversion into insoluble pigment, hemozoin by the parasite itself as a defence mechanismas also discussed by (Vennerstromet al., 1999).

Tafenoquine accumulates within the food vacuoles and inhibits the detoxification of heme to hemozoin by the malarial parasite. It does so by inhibiting hematin polymerization by binding to its -oxo dimer, thus inhibiting the formation of hemozoin, which consists of cyclic heme dimers arranged in an ordered crystalline structure through intermolecular hydrogen bonding. Tafenoquine, via its hydroxylated metabolites, stimulates the hexose monophosphate shunt, increases methemoglobin production and decreases glutathione levels in the cells. The pro-oxidant properties of its metabolites correlate with its exoerythrocytic schizontocidal action and also contribute to its erythrocytic (Marcsisinet al., 2014) schizontocidal action.

Like PQ (Vuonget al., 2015) cytochrome P450 is supposed to be held responsible for the activation of TQ as it is metabolized by the enzyme, CYP2D6a (a liver microsomal enzyme) (Pybuset al., 2013) as was demonstrated by the CYP2D6 knockout mice lacking anti-malarial activity of TQ, This work could also be discussed with the works of Pradines and Brueckner (Pradines et al., 2006; Brueckneret al., 1998). This report from laboratory animals shows

Table 8. Causal Prophylactic activity of Primaquine and Tafenoquine after development of resistance

Drug/dose A) Primaquine	Treatment days	No.of animals	Day of patency	Mean value ± SE	No. Of mice cured	Cure Rate%
40 mg/Kg	0 day	6	All protected	0	6/6	100
30 mg/Kg	-1,0, ±1	6	5,6,7,6, 5	6 .0 ± 0.33	0/6	0
Control		6	5,6,5,6,5	5.4 ±00		
B) Tafenoquine						
	-1,0, ±1	5	All protected	0	6/6	100
10 mg/Kg	0 day	5	10,10,11,12,12	0	6/6	0
40 mg/Kg	±1 day	5	All protected	11.0 ±0.40	6/6	100
10 mg/Kg	-1,0, ±1	5	4,4,6,6,7	5.4 ± 0.53	0/6	0
05 mg/Kg						
Control		5	4,5,5,6,6	5.2 ± 0.32		

Note: With the sequential development of resistance, for Tafenoquine at 30, 45, 60 and 90 mg/kg and for Primaquine at 30 and 40 mg/Kg, the strain were tested intermittently for the gametocytes count and it's cyclical transmission, preservation being carried out in liquid nitrogen. The results obtained are shown in the table below. Preservation had no effect on cyclical transmission.

Table 9. cyclical transmission of the parasite at different resistance level for Primaquine and Tafenoquine resistant strain

Primaquine	Strain resistant to drug when given in 3 days regimen (0-3days)	Cyclical transmission of the parasite
Tafenoquine resistant strain	30 mg/Kg	± ± ± ±
	40mg/Kg	± ± ±
	30mg/Kg	± ± ± ±
	45mg/Kg	± ± ± ±
	60mg/Kg	± ±
	90mg/Kg	---

Note: In case of Primaquine resistant line only till XVIII passage, the cyclic transmission was satisfactory and went down further gradually whereas in case of Tafenoquine resistant line, the transmission slowed down after XII th passage. The strains were preserved at every level of resistance developed and tested for cyclical transmission and causal prophylactic activity.

the association between CYP2D6 metabolism and TQ pharmacokinetics. The follow up of the literature till date coincide with our findings and suggestive of the two different location of tafenoquine targets with two different tissue level organizations.

CONCLUSION

The study targeted the investigation on extensive usage of drugs as blood schizontocidal that has lead to the emergence in resistance of drugs which has hampered efficacies of treatment by being transmission blocker in tissues as well. The drug was evaluated for its blood schizontocidal as well as for its causal prophylactic activity before and after the development of resistance.In the above mentioned research drugs at a dose of 30mg/Kg were curative against the blood induced infection and became resistant to maximum tolerated dose of 40mg/Kg of Primaquine after XVII passages over a period of 240 days and for 90mg/Kg of Tafenoquine after XIX consecutive passages over a period of 218 days.The experimental transmission of sporozoite induced infection of a rodent parasite *Plasmodium yoelii nigeriensis* through hamsters using *Anopheles stephensias* vector has been a potential test system for major causal prophylactic and anti-relapseanti-malarial. This test system will help evaluate novel strategies as alternative to the frequent extensive use of safer and potential drugs like Tafenoquine.

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