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Original Article

Evaluation of New Antibacterial Drugs and their Combinations in a Murine Model to Identify Short Duration Alternative Chemotherapy for Leprosy

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The objective of the research is to test the efficacy of new drugs and drug combinations in mice infected with *Mycobacterium leprae* (*M. leprae*) as alternative to current WHO MDT. Individual drugs tested were Rifampicin (RMP), Rifapentine (RPT) and Moxifloxacin (MOXI). Drug combinations were RMP, Clarithromycin (CLARI), Minocycline (MINO) and RMP, MINO and Ofloxacin (OFLO). RPT drug combinations were RPT, CLARI, MINO and RPT, OFLO, MINO. Both the drugs and drug combinations were used as daily regimen and intermittent regimen. WHO MB MDT served as a positive control. Mice pre-inoculated with *M. leprae* were allotted to daily and intermittent groups and administered selected drugs and drug combinations. At the end of 12 months post sub-inoculation, mice were sacrificed and the proportion % of viable bacilli were counted using Spearman and Karber method. It was noted that RMP, RPT and Moxifloxacin indicated a range of 89.99% to 99.99% bactericidal effect when used in daily or intermittent doses in both normal and TR mice. Drug combinations showed bactericidal effect comparable to that of WHO MDT. From the study it was concluded that if the present duration of MDT has to be shortened then daily dose regimen with RMP/MINO/OFLO or RPT/CLARI/MINO are recommended for a clinical trial.

Keywords: *Mycobacterium leprae,* Rifampicin, Rifapentine, Drug sensitivity, Multibacillary, Multi-Drug Therapy

Introduction

Leprosy is a chronic infectious disease which is caused by an obligate intracellular pathogen-*Mycobacterium leprae*. Disease is treated by multi drug therapy (MDT) which is a combination of Dapsone, Rifampicin and Clofazimine (Prasad 1998).

Despite targeting the reservoir of infection with MDT, transmission of the disease continues to occur in leprosy endemic countries as repre-

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sented by a stable ratio of annual new case detection rate (ANCDR) by prevalence rate (PR) (Desikan 2012). The standard WHO regimen of MDT for multibacillary leprosy contains three important drugs each of which has an individual role to play in controlling the dissemination of *M. leprae* infection in the host. The duration of the therapy is for a period of 12 months in multibacillary disease and for 6 months in paucibacillary leprosy. Dapsone is a bacteriostatic drug which blocks the enzyme dihydropteroate synthase in *M. leprae* leading to inhibition of folic acid synthesis by the bacteria (Cambau et al 2006). Rifampicin is a bactericidal drug which inhibits the effect of RNA polymerase β subunit of *M. leprae* thereby arresting bacterial transcription (Bullock 1983). Clofazimine acts as an anti-inflammatory drug controlling host inflammatory responses and is also involved in arresting bacterial replication (Degang et al 2012).

The duration of treatment with MDT is very long, increasing possibilities for defaulting and/or incomplete therapy. An effective short duration alternative therapy with bactericidal drugs and their combinations need to be investigated for effectual control of dissemination of infection and attenuation of bacterial effect. RPT, CLARI, Moxifloxacin (MOXI), MINO and OFLO as individual drugs and their combinations have been studied extensively in leprosyon *M. leprae* to identify their efficacy as shorter duration alternative therapy for leprosy (Gelber 1987, Ji and Grosset 1991, Ji and Grosset 2000, Ji et al 1991, Ji et al 1992, Ji et al 1994, Ji et al 1996a, Ji et al 1996 b, Ji et al 1998).

In the current study, RPT, CLARI, MINO, MOXI and OFLO and their combinations are tested against in intermittent and daily dose regimens in sensitive strains of *M. leprae* through mouse foot pad experiments. Loss in viability of *M. leprae* in response to these drugs and their combinations are assessed for efficacy of drugs.

Methodology

The study was conducted following the ethical guidelines of the Indian Council of Medical Research and was approved by the Institutional Ethical Committee of Schieffelin Institute of Health-Research and Leprosy Centre and the Animal Ethical Committee.

1. **Identification of Sensitive strains of *M. leprae:***

   M. leprae were extracted from the lesional skin biopsies of leprosy cases at SIH-R&LC Karigiri who were diagnosed with bacteriological index of $\geq$ 3 at both the ear lobes and at the site of the skin lesions. DNA was extracted from the bacilli and genome sequencing confirmed that both the strains are sensitive to drugs in MDT.

2. **Selection of Alternative Drugs and Dosage Regimens:**

   The alternative drugs which include CLARI, RPT, MINO, MOXI and OFLO were chosen from the earlier studies in murine models, (Ji et al 1991, Ji and Grosset 2000) which proved as effective alternatives to MDT in leprosy. The proportional bacte-ricidal technique was employed in the establishment of efficacy of drug regimens based on the dosage (Colston et al 1978). The intermittent and the daily dose regimens were followed as per the earlier experiments (Ji B et al 1996 a, b).

3. **Mice used in the Experiments:**

- **Normal Mice**: Cross bred albino (CBA mice) were used to multiply the identified strains of M. leprae to prepare sufficient inoculum for the experiments.
- **Immunocompromised (Thymectomized Irradiated (TR)) Mice:** The mice were thymectomized at 6-8 weeks and then subjected to radiation of 900 rads after a further 3 weeks. The mice were then inducted into the experiments after a further period of 6 weeks.

4. **Preparation of Initial Inoculum :** *M. leprae* strains were extracted from the biopsy tissues of relapse patient using manual homogenization protocol in normal saline and injected into foot pads of 5 TR mice. Nine months later the TR mice were euthanized and *M. leprae* was extracted from the hind footpads. Suspensions were then pooled and an inoculum prepared to yield 2.5×10^6 bacilli per ml with a solid ratio of 1%.

5. **Inoculation of Mice and Induction into treatment schedules:**

i. **Inoculation into experimental mice:** The inoculum mentioned in the above section was then diluted to 1×10^5 bacilli per 0.03ml and was injected into hind foot pads of 275 normal and 265 TR mice with each of the sensitive strains respectively. The number of mice were calculated based on the treatment regimens in Table 1 taking into account the 10% possibility of failure to develop swollen footpads and 20% mortality rate during experiments. The mice were grown on normal diet for 9 months for the footpads to be swollen with *M. leprae*.

ii. **Treatment Schedules:** After the end of 9 months, at least 253 out of 265 TR mice (94.4%) and 253 out of 275 normal mice (92.0%) developed swollen footpads. The count was estimated in a representative set of two mice and was identified to be on an average of 3×10^6 bacilli per each hind foot pad. These mice were then allocated into the treatment schemes as shown in Table 1. The combinations and the dosages were administered as represented in Table 1. WHO- MB MDT was used as a positive control in the intermittent dosage and untreated mice were used as negative controls.

6. **Sub inoculations:** At each time point of harvest, bacterial counts were estimated in hind foot pads of two mice as mentioned in Table 1. The bacterial harvest suspensions from each of the two mice were then pooled, the bacterial counts were estimated and 10-fold serial dilutions were made to form 10^2, 10^3, 10^4 and $>10^5$ bacilli/ml (undiluted) suspensions. Each dilution was then sub-inoculated in 2 normal mice and 1 TR mice except for undiluted suspensions which is performed in 2 normal and 2 TR mice. The mice were maintained on normal diet for 12 months. At the end of 12 months, the mice were sacrificed, footpads harvested and the bacterial counts estimated from each hind footpad.

   After hind footpads were harvested from all the groups with different dilutions that are sub-inoculated, the bacterial count of $>10^5$ bacilli in each footpad is considered as positive which indicates that the organisms have multiplied and also include viable organisms. Besides estimating the bacterial load in terms of bacillary count, the solid ratios (Shepard and Mcrae 1965) of the bacilli were also estimated during each count. The solid bacillus is defined as the organism that was stained adequately by Ziehl Nielsen staining and whose length is approximately four times its width. The solid ratio is the number of solid bacilli by total number of organisms per milliliter of suspension.

7. **Statistical Methods and Assessment of treatment efficacy:** The bactericidal effect in each of the regimens was estimated by measuring and comparing the proportion of viable organisms in each groups using Spearman and Karber calculations (Shepard 1982). The calculations employ a median infective dose (ID50) and the percentage of viable *M. leprae* organisms remaining after treatment was derived from the equation: %

Table 1 : Schedule of Experiments for Sensitive strains

Groups	Drugs* (mg/kg/dose)	No of Mice	Duration of treatment	Frequency of harvests**	Number of Mice in sub inoculations*** At each Harvest#		Total	
	Intermittent Dosage:				**N**	**TR**	**N**	**TR**
1	RMP (10) + CLARI (100) + MINO (50)	9	24 Wks.	W4, W12, W24	8	5	24	15
2	RMP (10) + MINO (50) + OFLOX (150)	9	24 Wks.	W4, W12, W24	8	5	24	15
3	RPT (10) + CLARI (100) + MINO (50)	9	24 Wks.	W4, W12, W24	8	5	24	15
4	RPT (10) + MINO (50) + OFLOX (150)	9	24 Wks.	W4, W12, W24	8	5	24	15
5	WHO MDT	9	24 Wks.	W4, W12, W24	8	5	24	15
6	RPT (10)	9	24 Wks.	W4, W12, W24	8	5	24	15
7	MOXI (150)	9	24 Wks.	W4, W12, W24	8	5	24	15
8	RMP (10)	9	24 Wks.	W4, W12, W24	8	5	24	15
	Daily Dosage:				**N**	**TR**	**N**	**TR**
9	RMP (10)	23	6 Days	D1,D3^{W0},D3^{W12},D3^{W24},D60,D6^{W12},D6^{W24}	8	5	56	35
10	RPT (10)	23	6 Days	D1,D3^{W0},D3^{W12},D3^{W24},D6^{W0},D6^{W12},D6^{W24}	8	5	56	35
11	MOXI (150)	23	6 Days	D1,D3^{W0},D3^{W12},D3^{W24},D60,D6^{W12},D6^{W24}	8	5	56	35
12	RMP (10) +CLARI (50)+ MINO (20)	23	6 Days	D1,D3^{W0},D3^{W12},D3^{W24},	8	5	56	35
13	RMP (10)+MINO (25)+ OFLOX (150)	23	6 Days	D1,D3^{W0},D3^{W12}, D3^{W24},D60,D6^{W12},D6^{W24}	8	5	56	35
14	RPT (10) +CLARI (50) + MINO (25)	23	6 Days	D1,D3^{W0},D3^{W12}, D3^{W24},D60,D6^{W12},D6^{W24}	8	5	56	35
15	RPT (10)+ MINO (25)+OFLOX (150)	23	6 Days	D1,D3^{W0},D3^{W12}, D3^{W24},D60,D6^{W12},D6^{W24}	8	5	56	35
	Untreated Control:				**N**	**TR**	**N**	**TR**
16	Control Mice on Normal Diet	20		D0,D1,D3,D6, W4,W8,W12,W24	-	2	-	16

*Moxi = Moxifloxacin, Mino = Minocycline, Clari= Clarithromycin, Oflox= Ofloxacin, RMP= Rifampicin, RPT= Rifapentine. **W= Week, D= Day. ***N = normal mice; TR =Thymectomized radiated mice

† =RMP at 10 mg/kg of body weight once every 4 weeks plus 0.01% DDS (Dapsone) 1 0.005% CLO (Clofazimine) in daily diet.

= At each harvest, the inoculum was diluted into 102, 103, 104, and undiluted (>105) and injected into 2 normal mice and 1 TR mice per dilution totaling to 8 normal mice and 5 TR mice (one additional mice inoculation of >105).

**The superscripts 0, 12wk, and 24wk indicate that treatment was ceased after day 3 or 6 and that three each were sacrificed at the next day, 12 weeks, and 24 weeks later, respectively, for subinoculation.

viable *M. leprae* = 0.69/50% infectious dose. A two tailed *p* value of *<0.05* was considered statistically significant.

Results

The bacterial counts indicated a pre-treatment value of (mean Log_{10}) 7.04 + 0.04 at 9 months of inoculation for strain I and 8.17 + 0.15 for strain-II. The solid ratios were in the range of 1-3%. In the treated group, no change was noted in the bacterial counts with both intermittent and daily regimens at the end of 4 and 24 weeks indicating that the infection is well established and follow-up observations through sub-inoculations is feasible. These observations were in concordance with the earlier reports (Colston et al 1978).

Bacterial Counts in the Control Mice (Untreated Mice): In the untreated control mice, the bacterial counts were estimated in two mice from each group at different time points indicating 4 hind footpads for each dilution and at each time point of harvest. The positive foot pads are indicated by swollen foot pads with mean Log_{10} counts of 6.94. In the untreated control TR and normal mice, the sub-inoculations of the inoculum from treatment groups in various dilutions demonstrated positivity in all the foot pads and the proportion percent viable from all the harvests indicated a maximum value of 2.18197 as calculated using Spearman and Karber calculation (Shepard 1982).

Sensitive Strain - I

Intermittent Regimen – Normal Mice (Table 2) :

At the end of 4 weeks, among the single drugs, only RPT showed antibacterial effect (68.37%). Among the drug combinations, all combinations showed bactericidal effect including WHO MDT except RMP/CLARI/MINO. RPT/CLARI/MINO showed highest proportion of killing (89.99%) ($p < 0.05$).

At the end of 12 weeks, all single and drug combinations showed bactericidal effect. MOXI demonstrated the least proportion of killing (43.76%) ($p<0.05$) and RPT/CLARI/MINO showed the highest proportion of killing (99.98%). All other single and drug combinations also demonstrated substantial bactericidal effect.

At the end of 24 weeks, the bactericidal effect of single drugs and drug combinations had improved or was sustained. RMP and RPT showed the highest proportion of killing (99.98%) followed by RMP/CLARI/MINO and RMP/OFLO/MINO (99.94%). WHO MDT, RPT/CLARI/MINO and RPT/OFLO/MINO indicated (99.90%) of bacterial killing. MOXI showed the least proportion of killing at the end of 24 weeks (89.99%).

At the end of 24 weeks, comparisons between all combinations and single drugs indicated no statistically significant difference.

In normal mice, all the intermittent dose single drugs and drug combinations indicated higher bactericidal effect when compared to MOXI.

Intermittent Regimen – TR Mice (Table 2) :

At the end of 4 weeks, none of the single drugs or drug combinations showed any bactericidal effect.

At the end of 12 weeks, all combinations and individual drugs showed bactericidal effect except MOXI. RPT/CLARI/MINO showed the highest proportion of killing (99.98%). The bactericidal effect of RPT, RPT/OFLO/MINO and WHO MDT were comparable with RPT/CLARI/MINO. RMP showed the least proportion of killing (89.99%).

At the end of 24 weeks, all combinations and individual drugs showed good bactericidal effect except MOXI (89.99%). RPT and RMP showed the highest proportion of killing (99.98%). The proportion of killing in all other combinations including WHO MDT was comparable to RMP and RPT.

Table 2 : Rifampicin Sensitive Strain-I - Intermittent Dosage

Regimen (dose [mg/kg])[a]	Proportion % of Viable *M. leprae*			Proportion of viable *M. leprae* killed in %		
	4th week	12th week	24th week	4th week	12th week	24th week
Normal Mice:						
RMP (10)	2.18197	0.0218	0.00022	0	99.90	99.98
RPT (10)	0.69000	0.00218	0.00022	68.37	99.90	99.98
MOXI (150)	2.18197	1.22701	0.21820	0	43.76*	89.99
RMP (10) + CLARI (100) + MINO (50)	2.18197	0.03880	0.00123	0	98.22	99.94
RMP (10) + MINO (50) + OFLOX (150)	1.22701	0.02182	0.00123	43.76	98.99	99.94
RPT (10) + CLARI (100) + MINO (50)	0.21820	0.00022	0.00218	89.99*	99.98	99.90
RPT (10) + MINO (50) + OFLOX (150)	0.69000	0.00218	0.00218	68.37	99.90	99.90
WHO MDT	1.22701	0.00218	0.00218	43.76	99.90	99.90
TR Mice:						
RMP (10)	2.18197	0.21820	0.00022	0	89.99	99.98
RPT (10)	2.18197	0.00218	0.00022	0	99.90	99.98
MOXI (150)	2.18197	2.18197	0.21820	0	0	89.99
RMP (10) + CLARI (100) + MINO (50)	2.18197	0.06900	0.00218	0	96.83	99.90
RMP (10) + MINO (50) + OFLOX (150)	2.18197	0.06900	0.00218	0	96.83	99.90
RPT (10) + CLARI (100) + MINO (50)	2.18197	0.00022	0.00218	0	99.98	99.90
RPT (10) + MINO (50) + OFLOX (150)	2.18197	0.00690	0.00218	0	99.68	99.90
WHO MDT	2.18197	0.00218	0.00069	0	99.90	99.68
CONTROL	2.18197	2.18197	2.18197	0	0	0

*p<0.05 (Statistically Significant differences) (Z Test of Proportions)

At the end of 24 weeks, comparisons between all combinations and single drugs did not indicate any statistically significant difference.

In TR mice, all the intermittent drug combinations have a higher bactericidal effect except MOXI whose effect is moderate.

Daily Regimen – Normal Mice (Table 3) :

At the end of day 1, only RMP/OFLO/MINO showed weak antibacterial effect (43.76%). The rest of single drugs or drug combinations did not show bactericidal effect.

At the end of day 3, MOXI and RMP/CLARI/MINO showed bactericidal effect of 43.76% and 89.99% respectively. No other single drugs or drug combinations showed bactericidal effect.

At the end of day 6, all drug combinations and individual drugs showed equal bactericidal effect except MOXI and RMP/OFLO/MINO. RMP showed highest proportion of killing (99.90%).

At the end of 6th day, although the proportion of killing was highest for RMP, comparison with all the other single drugs and combinations (except MOXI and RMP/OFLO/MINO which showed no effect) did not demonstrate a statistically significant difference.

In normal mice, at the end of 6th day except for MOXI as a single drug and RMP containing drug combinations, all other single drugs and drug combinations showed good bactericidal effect.

At the end of 12th week, the group with three daily doses of drugs, all single drugs and drug combinations showed bactericidal effect except for RMP and MOXI. RPT/CLARI/MINO and RPT/OFLO/MINO showed highest proportion of killing (99.98%).

At the end of 12th week, the group with six daily doses of drugs indicated that except for RMP, MOXI (43.79%) and RMP/CLARI/MINO, all other single drugs and drug combinations showed bactericidal effect with RPT/CLARI/MINO and RPT/OFLO/MINO showing the highest proportion of killing (99.89%).

At the end of 24 weeks, in the group with three daily doses of drugs, only RPT and RPT/CLARI/MINO showed good bactericidal effect with RPT/CLARI/MINO showing the best proportion of killing (98.99%) ($p < 0.05$).

At the end of 24 weeks, the group with six daily doses of drugs indicated that only RMP/CLARI/MINO and RPT/CLARI/MINO showed good bactericidal effect with RPT/CLARI/MINO having the best proportion of killing (99.90%) ($p < 0.05$).

In the normal mice when daily dose drug combinations were followed up for longer periods of time, combinations with CLARI/MINO either with RPT or RMP showed sustaining bactericidal effect.

Daily Regimen – TR Mice (Table 3) :

At the end of 1st and 3rd days, none of the single drugs or drug combinations demonstrated any bactericidal effect.

At the end of 6th day, all drug combinations and individual drugs showed equal bactericidal effect except for MOXI. RMP and RPT had the best proportion of killing (99.90% and 98.99%) ($p<0.05$).

At the end of 12th week, the group with three daily doses of drugs indicated that, except for MOXI, all other single drugs and drug combinations showed bactericidal effect with RPT/CLARI/MINO and RPT/OFLO/MINO being the highest (99.98%).

At the end of 12th week, the group with six daily doses of drugs showed that, except for RMP/CLARI/MINO, all other single drugs and drug combinations showed bactericidal effect with RPT/CLARI/MINO being the highest (99.99%) ($p<0.05$).

At the end of 24 weeks, the group with three daily doses of drugs showed that, except for RPT and

Table 3 : Rifampicin Sensitive Strain-I – Daily Dosage

Regimen	Proportion % of viable *M. leprae*							Proportion % of viable *M. leprae* killed						
(dose[mg/kg])[a]	D1	D3	D6	$D3^{Wk12}$	$D6^{Wk12}$	$D3^{Wk24}$	$D6^{Wk24}$	D1	D3	D6	$D3^{Wk12}$	$D6^{Wk12}$	$D3^{Wk24}$	$D6^{Wk24}$
Normal Mice:														
RMP (10)	2.18197	2.18197	0.00218	2.18197	2.18197	2.18197	2.18197	0	0	99.90	0	0	0	0
RPT (10)	2.18197	2.18197	0.01227	0.02182	0.12270	0.06900	1.22701	0	0	99.43	98.99	94.37	96.83	43.76
MOXI (150)	2.18197	1.22701	2.18197	2.18197	1.22701	2.18197	2.18197	0	43.76	0	0	43.76*	0	0
RMP(10)+ CLARI(100)+MINO (50)	2.18197	0.21820	0.69000	0.06900	2.18197	2.18197	0.06900	0	89.99*	68.37	96.83	0	0	96.83
RMP(10) + MINO(50)+OFLOX(150)	1.22701	2.18197	2.18197	0.12270	0.01227	2.18197	2.18197	43.76*	0	0	94.37	99.43	0	0
RPT (10)+ CLARI (100)+MINO (50)	2.18197	2.18197	0.01227	0.00022	0.00022	0.02182	0.00218	0	0	99.43	99.98	99.89	98.99*	99.90*
RPT (10)+ MINO (50)+OFLOX (150)	2.18197	2.18197	0.02182	0.00069	0.00022	1.22701	0.69000	0	0	98.99	99.96	99.89	43.76	68.37
TR Mice:														
RMP (10)	2.18197	2.18197	0.00218	0.06900	0.21820	2.18197	2.18197	0	0	99.90*	96.83	89.99	0	0
RPT (10)	2.18197	2.18197	0.02182	0.02182	0.69000	0.69000	2.18197	0	0	98.99*	98.99	68.37	68.37	0
MOXI (150)	2.18197	2.18197	2.18197	2.18197	0.69000	0.69000	2.18197	0	0	0	0	68.37	68.37	0
RMP(10) + CLARI(100)+MINO (50)	2.18197	2.18197	0.21820	0.21820	2.18197	2.18197	0.69000	0	0	89.99	89.99	0	0	68.37
RMP(10)+ MINO(50)+OFLOX(150)	2.18197	2.18197	0.21820	0.21820	0.00218	2.18197	2.18197	0	0	89.99	89.99	99.90*	0	0
RPT (10)+ CLARI (100)+MINO (50)	2.18 197	2.18197	0.21820	0.00022	0.00022	2.18197	0.00690	0	0	89.99	99.98	99.99*	0	99.96*
RPT (10) + MINO (50)+OFLOX (150)	2.18197	2.18197	0.21820	0.00123	0.00069	2.18197	2.18197	0	0	89.99	99.94	99.96*	0	0
CONTROL	2.18197	2.18197	2.18197	2.18197	2.18197	2.18197	2.18197	0	0	0	0	0	0	0

*p<0.05 (Statistically Significant differences) (Z Test of Proportions and Fischer Exact Tests)

MOXI all other single drugs and drug combinations did not show any bactericidal effect. The bactericidal effect of RPT and MOXI was poor (68.37%).

At the end of 24 weeks, in the group with six daily doses of drugs, except for RMP/CLARI/MINO and RPT/CLARI/MINO, all other single drugs and their combinations did not show any bactericidal

Table 4 : Rifampicin Sensitive Strain-II - Intermittent Dosage

Regimen (dose [mg/kg])[a]	Proportion % of Viable *M. leprae*			Proportion of viable *M. leprae* killed in %		
	4th week	12th week	24th week	4th week	12th week	24th week
Normal Mice:						
RMP (10)	0.12270	0.00218	0.00218	94.37	99.90	99.90
RPT (10)	0.69000	0.00218	0.02182	68.37	99.90	98.99
MOXI (150)	0.69000	0.00022	0.00022	68.37	99.98	99.98
RMP (10)+CLARI (100) + MINO (50)	0.12270	0.00218	0.00218	94.37	99.90	99.90
RMP (10) + MINO (50) + OFLOX (150)	0.02182	0.00218	0.00218	98.99	99.90	99.90
RPT (10)+CLARI (100)+MINO (50)	0.02182	0.00218	0.00218	98.99	99.90	99.90
RPT (10) + MINO (50)+OFLOX (150)	0.00218	0.00022	0.00218	99.90*	99.98	99.90
WHO MDT	0.02182	0.00069	0.00022	98.99	99.96	99.98
TR Mice:						
RMP (10)	0.21820	0.00218	0.00218	89.99	99.90	99.90
RPT (10)	2.18197	0.00218	0.02182	0	99.90	98.99
MOXI (150)	2.18197	0.00069	0.00039	0	99.96	99.98
RMP (10)+CLARI (100) + MINO (50)	2.18197	0.00218	0.00218	0	99.90	99.90
RMP (10) + MINO (50) + OFLOX (150)	0.69000	0.00218	0.00218	68.37	99.90	99.90
RPT (10)+CLARI (100)+MINO (50)	2.18197	0.00218	0.00218	0	99.90	99.90
RPT (10) + MINO (50)+OFLOX (150)	0.06900	0.00022	0.00218	96.83*	99.98	99.90
WHO MDT	0.69000	0.00022	0.00022	68.37	99.98	99.98
CONTROL	2.18197	2.18197	2.18197	0	0	0

*p<0.05 (Statistically Significant differences) (Z Test of Proportions and Fischer Exact Tests)

effect. RPT/CLARI/MINO had the best proportion of killing (99.96%) ($p < 0.05$).

In TR mice, when daily dose drug combinations were followed up for longer periods of time only RPT/CLARI/MINO showed sustaining bactericidal effect.

Sensitive Strain - II

Intermittent Regimen – Normal Mice (Table 4) :

At the end of 4 weeks, all single and drug combinations showed bactericidal effect. RPT/MINO/OFLO showed the highest proportion of killing (99.90%) (*p<0.05*). This was closely followed by RMP/CALRI/MINO, RMP/OFLO/MINO and WHO MDT (98.99%). Among the single drugs only RMP showed good antibacterial effect (94.37%). RPT and MOXI demonstrated poor effect (68.37%).

At the end of 12 weeks, all single and drug combinations showed bactericidal effect. MOXI and RPT/MINO/OFLO had the highest proportion of killing (99.90%). However results of all other single drugs and drug combinations were comparable to RPT/MINO/OFLO. At the end of 24 weeks, the bactericidal effect of single drugs and drug combinations improved or maintained. WHO MDT indicated highest proportion of killing (99.98%). Results of all other single drugs and drug combinations were comparable to WHO MDT.

At the end of 24 weeks, although WHO MDT indicated highest proportion of bacterial killing, this observation was not statistically significant. These results indicate that in normal mice, all the single drugs and drug combinations in the intermittent regimen have a good bactericidal effect with MOXI showing effect comparable to that of WHO MDT.

Intermittent Regimen – TR Mice (Table 4) :

At the end of 4 weeks, only RMP, RMP/MINO/OFLO, RPT/MINO/OFLO and WHO MDT showed bactericidal effect. RPT/MINO/OFLO had the highest proportion of killing (96.83%) (*p < 0.05*).

At the end of 12 weeks, all combinations and individual drugs showed bactericidal effect. RPT/CLARI/MINO and WHO MDT showed the highest proportion of killing (99.98%). However all other single drugs and drug combinations were comparable to RPT/CLARI/MINO and WHO MDT.

At the end of 24 weeks, all combinations and individual drugs showed good bactericidal effect. WHO MDT and MOXI had the highest proportion of killing (99.98%). However all other single drugs and drug combinations were comparable to WHO MDT and MOXI with no statistically significant difference.

In TR mice, all the intermittent drug combinations demonstrated a good bactericidal effect with MOXI showing effect comparable to that of WHO MDT.

Daily Regimen – Normal Mice (Table 5) :

At the end of 1st day, only RMP (68.37%) and RPT/OFLO/MINO (94.37%) showed bactericidal effect and rest of single drugs or drug combinations did not show any bactericidal effect.

At the end of 3rd day, except for MOXI, all other single drugs or drug combinations showed bactericidal effect. RMP/CLARI/MINO had the highest proportion of killing (99.90%).

At the end of 6th day, all drug combinations and individual drugs showed equal bactericidal effect. RPT showed the highest proportion of killing (99.68%) followed by RMP/OFLO/MINO and RPT/OFLO/MINO (99.43%).

At the end of 6th day statistical analysis revealed that RPT, RMP/MINO/OFLO and RPT/MINO/OFLO indicated a significant bacterial killing (*p<0.05*).

In normal mice, among the daily dose regimen, at the end of 6th day, all single drugs and drug combinations showed bactericidal effect with RPT, RMP/MINO/OFLO and RPT/MINO/OFLO showing highest bacterial killing.

Table 5 : Rifampicin Sensitive Strain-II – Daily Dosage

Regimen	Proportion % of viable *M. leprae*							Proportion % of viable *M. leprae* killed						
(dose[mg/kg])[a]	D1	D3	D6	$D3^{Wk12}$	$D6^{Wk12}$	$D3^{Wk24}$	$D6^{Wk24}$	D1	D3	D6	$D3^{Wk12}$	$D6^{Wk12}$	$D3^{Wk24}$	$D6^{Wk24}$
Normal Mice:														
RMP (10)	0.69000	0.21820	0.69000	0.69000	0.06900	0.21820	2.18197	68.37	89.99	68.37	68.37	96.83	89.99	0
RPT (10)	2.18197	0.02182	0.00690	1.22701	0.00690	0.06900	2.18197	0	98.99	99.68*	43.76	99.68	96.83*	0
MOXI (150)	2.18197	2.18197	0.69000	0.69000	0.69000	1.22701	0.38802	0	0	68.37	68.37	68.37	43.76	82.2
RMP(10) + CLARI(100)+MINO (50)	2.18197	0.00218	0.12270	0.06900	0.01227	2.18197	1.22701	0	99.90	94.37	96.83	99. 43	0	43.76
RMP(10)+ MINO(50)+OFLOX(150)	2.18197	0.01227	0.01227	0.02182	0.00022	2.18197	0.00022	0	99. 43	99.43*	98.99	99.98	0	99.98*
RPT (10)+ CLARI (100)+MINO (50)	2.18197	0.38802	0.38802	0.03880	0.00218	2.18197	0.69000	0	82.21	82.21	98.22	99.90	0	68.37
RPT (10) + MINO (50) +OFLOX (150)	0.12270	0.03880	0.01227	0.06900	0.00218	0.00022	0.03880	94.37*	98.22	99.43*	96.83	99.90	99.98*	98.22
TR Mice:														
RMP (10)	2.18197	0.21820	2.18197	0.21820	2.18197	2.18197	2.18197	0	89.99	0	89.99	0	0	0
RPT (10)	2.18197	0.21820	2.18197	2.18197	0.00690	0.69000	2.18197	0	89.99	0	0	99.68	68.37	0
MOXI (150)	2.18197	0.21820	2.18197	2.18197	2.18197	0.69000	2.18197	0	89.99	0	0	0	68.37	0
RMP(10)+ CLARI(100)+MINO (50)	2.18197	0.02182	0.06900	2.18197	0.21820	2.18197	2.18197	0	98.99*	96.83	0	89.99	0	0
RMP(10)+ MINO(50)+OFLOX(150)	2.18197	0.06900	0.21820	0.06900	0.00022	2.18197	0.00022	0	96.83	89.99	96.83	99.98	0	99.98
RPT (10)+ CLARI (100)+MINO (50)	2.18197	2.18197	2.18197	0.21820	0.21820	2.18197	2.18197	0	0	0	89.99	89.99	0	0
RPT (10) + MINO (50)+OFLOX (150)	2.18197	0.06900	0.06900	0.21820	0.00218	0.00022	0.00690	0	96.83	96.83	89.99	99.90	99.98*	99.68
CONTROL	2.18197	2.18197	2.18197	2.18197	2.18197	2.18197	2.18197	0	0	0	0	0	0	0

*p<0.05 (Statistically Significant differences) (Z Test of Proportions)

At the end of 12th week, in the group with three daily doses of drugs, all single drugs and drug combinations showed bactericidal effect with RMP/MINO/OFLO being highest (98.99%).

At the end of 12th week, in the group with six daily doses of drugs, all single drugs and drug combinations showed bactericidal effect. RMP/MINO/OFLO showed highest proportion of killing (99.98%). All other single drugs and drug combinations showed comparable results with RMP/MINO/OFLO except RMP and MOXI.

At the end of 24 weeks, in the group with three daily doses of drugs, only RMP, RPT, MOXI, RPT/MINO/OFLO showed bactericidal effect. RPT/MINO/OFLO showed the highest proportion of killing (99.98%).

At the end of 24 weeks, in the group with six daily doses of drugs, except for RMP and RPT, all other single drugs and drug combinations showed bactericidal effect. RMP/OFLO/MINO showed the highest proportion of killing (99.98%) ($p < 0.05$).

In the normal mice when daily dose drug combinations were followed up for longer periods of time, combinations with MINO/OFLO either with RPT or RMP showed sustaining bactericidal effect.

Daily Regimen – TR Mice (Table 5) :

At the end of 1st day, all single drugs and drug combinations did not show any bactericidal effect. At the end of 3rd day, except for RPT/CLARI/MINO, all other single drugs or drug combinations demonstrated bactericidal effect. RMP/CLARI/MINO showed the highest proportion of killing (98.99%).

At the end of 6th day, only RMP/CLARI/MINO, RMP/MINO/OFLO and RPT/MINO/OFLO showed bactericidal effect. Both these combinations indicated the best proportion of killing (96.83%) ($p<0.05$).

In TR mice, among the daily dose regimen, at the end of 6th day, only RMP/CLARI/MINO, RPT/MINO/OFLO and RMP/MINO/OFLO showed bactericidal effect.

At the end of 12th week, in the group with three daily doses of drugs, RMP, RMP/MINO/OFLO, RPT/CLARI/MINO and RPT/MINO/OFLO showed bactericidal effect. RMP/MINO/OFLO showed the highest proportion of killing (96.83%).

At the end of 12th week, in the group with six daily doses of drugs, except for RMP and MOXI all other single drugs and drug combinations showed bactericidal effect. RMP/MINO/OFLO showed the highest proportion of killing (99.98%).

At the end of 24 weeks, in the group with three daily doses of drugs, only RPT, MOXI, RPT/MINO/OFLO showed bactericidal effect. The bactericidal effect of RPT/MINO/OFLO was the highest (99.98%) ($p<0.05$).

At the end of 24 weeks, in the group with six daily doses of drugs, only RMP/MINO/OFLO and RPT/MINO/OFLO indicated 99.98% of bacterial killing.

In TR mice, when daily dose drug combinations were followed up for longer periods of time, combinations with MINO/OFLO and either with RPT or RMP showed sustaining bactericidal effect.

Sensitive Strain - I

Comparison of intermittent and daily dose drug combinations - Normal Mice: (Table 6)

When 12 weeks of intermittent dosage and three daily dose drug combinations were compared, it was noted that RPT containing drug combinations showed equal bactericidal effect in both the regimes. RMP containing drug combinations did not do as well as the RPT containing combinations.

The bactericidal effect shown at 12 weeks was sustained into 24th week in daily dose of

Table 6 : Comparison of intermittent and daily dose drug combinations Normal Mice - Sensitive Strain-I

Regimen	Dosage	Proportion of *M. leprae* killed		
		Week 4	Week 12	Week 24
RMP/CLARI/MINO	Intermittent	0	98.22	99.94
	Day 3	-	96.83	0
	Day 6	-	0	96.83
RMP/MINO/OFLO	Intermittent	43.76	98.99	99.94*
	Day 3	-	94.37	0
	Day 6	-	99.43	0
RPT/CLARI/MINO	Intermittent	89.99*	98.99	99.90
	Day 3	-	98.99	98.99
	Day 6	-	98.89	99.90
RPT/MINO/OFLO	Intermittent	68.37	99.90	99.90*
	Day 3	-	99.96	43.76
	Day 6	-	99.89	68.37
WHO MDT	Intermittent+ Daily	43.76	99.90	99.90

*p<0.05 (Statistically Significant differences) (Z Test of Proportions)

Table 7 : Comparison of intermittent and daily dose drug combinations TR Mice - Sensitive Strain-I

Regimen	Dosage	Proportion of *M. leprae* killed		
		Week 4	Week 12	Week 24
RMP/CLARI/MINO	Intermittent	0	96.83	99.90
	Day 3	-	89.99	0
	Day 6	-	0	68.37
RMP/MINO/OFLO	Intermittent	0	96.83	99.90*
	Day 3	-	89.99	0
	Day 6	-	99.90	0
RPT/CLARI/MINO	Intermittent	0	99.98	99.90
	Day 3	-	99.98	0
	Day 6	-	99.99	99.96
RPT/MINO/OFLO	Intermittent	0	99.68	99.90*
	Day 3	-	99.94	0
	Day 6	-	99.96	0
WHO MDT	Intermittent+			
Daily	0	99.90	99.68	

*p<0.05 (Statistically Significant differences) (Z Test of Proportions)

RPT/CLARI/MINO but not in RPT/MINO/OFLO (Table 6).

When 12 weeks of intermittent dosage and six daily dose drug combinations were compared, all drug combinations showed good bactericidal effect except for RMP/CLARI/MINO. Only RPT/CLARI/MINO sustained the effect into the 24th week (Table 6).

In normal mice at 12 weeks the bactericidal effect of all drug combinations was comparable between the intermittent dose and daily dose with no statistical significance between them. However at 24 weeks, only daily dose RPT/CLARI/MINO showed bactericidal effect comparable with its intermittent regimen and was also comparable to WHO MDT. This effect was also statistically significant among the daily dose drug combinations at 24 weeks ($p<0.05$).

Thus both intermittent and daily dose of RPT/CLARI/MINO showed good bactericidal effect comparable to that of WHO MDT in normal mice in sensitive strain I.

Comparison of intermittent and daily dose drug combinations - TR Mice : (Table 7)

When intermittent and daily dose regimen were compared in TR mice of sensitive strain I, at 12 weeks, three daily dose drug combinations with RPT showed good a bactericidal effect as the intermittent dosage. Three daily dose regimen drug combinations with RMP did not do as well. The bactericidal effect shown at 12 weeks with RPT combinations was not sustained to 24 weeks (Table 7).

At 12 weeks and six daily drug dose combinations, three of the four combinations showed good bactericidal effect except RMP/CLARI/MINO. Only RPT/CLARI/MINO sustained the effect into the 24th week (Table 7). At 12 weeks the bactericidal effect of all drug combinations was comparable between the intermittent dose and daily dose with no statistically significant difference between them.

However at 24 weeks only six daily dose RPT/CLARI/MINO showed bactericidal effect comparable with its intermittent regimen which was also comparable to WHO MDT. This effect was also statistically significant among the daily dose drug combinations at 24 weeks ($p<0.05$).

Thus both intermittent and daily dose of RPT/CLARI/MINO showed good bactericidal effect comparable to that of WHO MDT in TR mice in sensitive strain I.

Sensitive Strain-II

Comparison of intermittent and daily dose drug combinations - Normal Mice : (Table 8)

When intermittent and daily dose regimen were compared in normal mice of sensitive strain II, at 12 weeks, three daily dose, all drug combinations showed comparable bactericidal effect as the intermittent dosage. The bactericidal effect shown at 12 weeks was sustained to 24 weeks in daily dose of RPT/MINO/OFLO but not in all other three regimen (Table 8).

At 12 weeks and six daily dose drug combinations, all four combinations showed comparable bactericidal effect as the intermittent dose. Only RMP/MINO/OFLO and RPT/MINO/OFLO sustained the effect into the 24th week (Table 8). At 12 weeks the bactericidal effect of all drug combinations was comparable between the intermittent dose and daily dose with no statistical significance between them.

However at 24 weeks only six daily dose RMP/MINO/OFLO and three/six daily dose of RPT/MINO/OFLO showed effect comparable with its intermittent regimen and also comparable to WHO MDT. This effect was also statistically significant among the daily dose drug combinations at 24 weeks ($p<0.05$).

Table 8 : Comparison of intermittent and daily dose drug combinations - Normal Mice - Sensitive Strain-II

Regimen	Dosage	Proportion of *M. leprae* killed		
		Week 4	Week 12	Week 24
RMP/CLARI/MINO	Intermittent	94.37	99.90	99.90
	Day 3	-	96.83	0
	Day 6	-	99.43	43.76
RMP/MINO/OFLO	Intermittent	98.99	99.90	99.90
	Day 3	-	98.99	0
	Day 6	-	98.98	99.98
RPT/CLARI/MINO	Intermittent	98.99	99.90	99.90
	Day 3	-	98.22	0
	Day 6	-	99.90	68.37
RPT/MINO/OFLO	Intermittent	99.90	98.99	99.90
	Day 3	-	96.83	99.98
	Day 6	-	99.90	98.22
WHO MDT	Intermittent+ Daily	98.99	99.96	99.98

*p<0.05 (Statistically Significant differences) (Z Test of Proportions)

Table 9 : Comparison of intermittent and daily dose drug combinations - TR -Mice - Sensitive Strain-II

Regimen	Dosage	Proportion of *M. leprae* killed		
		Week 4	Week 12	Week 24
RMP/CLARI/MINO	Intermittent	0	99.90	99.90*
	Day 3	-	0	0
	Day 6	-	89.99	0
RMP/MINO/OFLO	Intermittent	68.37	99.90	99.90
	Day 3	-	96.83	0
	Day 6	-	99.98	99.98
RPT/CLARI/MINO	Intermittent	0	99.90	99.90*
	Day 3	-	89.99	0
	Day 6	-	89.99	0
RPT/MINO/OFLO	Intermittent	96.83	99.98	99.90
	Day 3	-	89.99	99.98
	Day 6	-	99.90	99.68
WHO MDT	Intermittent+ Daily	68.37	99.98	99.98

p<0.05 (Statistically Significant differences) (Z Test of Proportions)

Thus intermittent and daily dose of RPT/MINO/OFLO and RMP/MINO/OFLO showed good bactericidal effect comparable to that of WHO MDT in normal mice of sensitive strain II.

Comparison of intermittent and daily dose drug combinations - TR Mice: (Table 9)

When intermittent and daily dose regimen were compared in TR mice of sensitive strain II, at 12 weeks, of the three daily dose drug combinations, all showed reasonable bactericidal effect as the intermittent dosage except for RMP/CLARI/MINO. The bactericidal effect shown at 12 weeks was sustained to 24 weeks in daily dose of RPT/MINO/OFLO ($p<0.05$) (Table 9).

At 12 weeks and six daily drug dose combination, drug combination with MINO/OFLO either with RPT or RMP showed good bactericidal effect as compared to intermittent dose. Both these regimen sustained the bactericidal effect into the 24th week ($p<0.05$) (Table 9). In TR mice, at 12 weeks the bactericidal effect of all drug combinations was comparable between the intermittent dose and daily dose with no statistically significance between them.

However at 24 weeks only six daily dose RMP/MINO/OFLO and three/six daily dose of RPT/MINO/OFLO showed effect comparable with its intermittent regimen and also comparable to WHO MDT. This effect was also statistically significant among the daily dose drug combinations at 24 weeks ($p<0.05$).

Thus both intermittent and daily dose of RPT/MINO/OFLO and daily dose of RMP/MINO/OFLO showed good bactericidal effect ($p<0.05$) comparable to that of WHO MDT in TR mice in sensitive strain II.

Discussion

In this study, alternative single drugs and drug combinations were tested in two sensitive strains of *M. leprae* using 6 regimens in normal and TR mice.

Single drugs: With the intermittent dose the overall bactericidal effect of single drugs, RMP, RPT and MOXI were excellent both in normal mice and TR mice in both the sensitive strains. With the daily dosage also, all the three drugs exhibited excellent bactericidal effect both in normal and TR mice in both the sensitive strains, with very little to choose between the three. Thus RMP, RPT and MOXI are excellent and equal in their bactericidal effect against sensitive strains.

Drug combinations: With the intermittent dose, all four drug combinations did as well as WHO MDT, with excellent overall bactericidal effect in normal and TR mice in both the sensitive strains.

In sensitive strain I, daily dose, drug combinations of CLARI/MINO with either RMP or RPT showed good bactericidal effect which sustained into the 24th week in the normal mice. In TR mice, daily dose, drug combinations RPT/CLARI/MINO showed good bactericidal effect which sustained into the 24th week.

In sensitive strain I, when intermittent (3 doses) and daily (one, three or six doses) regimen are compared, intermittent and daily combinations of CLARI/MINO with RPT or RMP showed good bactericidal effect in normal and TR mice comparable to WHO MDT.

In sensitive strain II, daily dose, drug combinations of MINO/OFLO with either RMP or RPT showed good bactericidal effect which sustained into the 24th week in the normal mice. In TR mice, daily dose, drug combinations RPT/MINO/ OFLO showed good bactericidal effect which sustained into the 24th week.

In sensitive strain II, when intermittent (3 doses) and daily (one, three or six doses) regimen are compared, intermittent and daily combinations of MINO/OFLO either with RMP or RPT showed highest bactericidal effect in normal and TR mice comparable to WHO MDT.

Thus drug combinations of either CLARI/MINO or MINO/OFLO with RPM or RPT in daily or intermittent regimen can be alternatives to the present WHO MDT regimen against sensitive strains.

From the study it seems that WHO MDT is still the best treatment option for sensitive strains of *M. leprae*. Replacing RMP with RPT or MOXI does not seem to give any additional benefit. Replacing DDS/CLOFAZ with MINO/OFLO or CLARI/MINO is a possibility if the duration of treatment has to be shortened and dose made intermittent.

If the present duration of MDT has to be shortened then intermittent, monthly supervised dose with RMP/CLARI/MINO or RMP/OFLO/MINO should be tried.

Mouse foot pad studies by Ji et al (1996b), suggested that a single dose of CLARI/ MINO with or without OFLO displayed bacterial activity as great as 4 weeks of daily treatment with DDS and Clofazamine. A similar finding is seen in this study where daily and intermittent regimes containing RMP or RPT with CLARI/MINO or OFLO/MINO have demonstrated bacterial activity comparable to the present WHO MDT.

Earlier studies show that ROM has shown good results in PB leprosy and also in MB leprosy (Kumar et al 2015, Manickam et al 2012 and Villahermosa et al 2004). There have been calls for ROM for treating multi bacillary leprosy (Lockwood et al 2012).

While designing newer alternate short course regimens we need to consider :

1) Moxifloxacin may be needed later for Rifampicin resistance cases, so this need not be used in the shorter MDT to be evaluated.
2) In this study, in view of the fact that Rifapentine combinations seem to only do marginally better than Rifampicin combinations, Rifapentine need not replace Rifampicin.
3) A clinical trial using intermittent, monthly treatment with ROM can be tried for both PB and MB. The duration of therapy may be for 6 months or 12 months.
4) Given the cost and side effects of Clarithromycin this drug need not be used for the present

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