Experimental and Clinical Studies on Rifacinna® - The New Effective Antituberculous Drug (Review)

Dimova Velichka[1,*], Atanasova Ivana[1], Tomioka Haruaki[2], Sato Katsumasa[2], Reddy Venkata[3], Geeta Nadadhur[3], Daneluzzi Donna[3], Gangadharam Patisapu[3], Kantardjiev Todor[4], Dhople Arvind[5], Feshchenko Yurii[6], Yashina Ljudmila[6], Toumanov Andrei[6], Zhivkova Zvetana[7] and Sano Chiaki[2]

[1]Res. Inst. Pharmacology, Bulgaria; [2]Shimane Univ. School Med., Izumo, Japan; [3]Illinois University, Chicago; [4]National Centerof Infectious and Parasitic Diseases, Bulgaria; [5]Florida Inst. Technology; [6]National. Institute of Pulmology and Tuberculosis- Kiev, Ukraine; [7]Medical University, Faculty Pharmacy, Bulgaria

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Abstract: A new rifamycin derivative 3-(4-cinnamyl-piperazinyl iminomethyl) rifamycin SV (T_9) and its sodium salt ($T_{11,}$ Rifacinna®) were *in vitro, in vivo,* toxicologically and clinically investigated in comparison with rifampicin, rifapentine, rifabutin, rifalazil. Our experiments showed that Rifacinna exhibits excellent *in vitro* activity against Gram (+), Gram (-) aerobic, anaerobic pathogens and mycobacteria. Rifacinna is active against *Staphylococcus, Streptococcus* sp. including MRSA, with MIC_{90}- 0.06 - 0.5mg/L; against Gram (+), Gram (-) anaerobes with MIC_{90} 0.5 - 1mg/L; against *Mycobacterium tuberculosis* (MTB) with MIC_{90} 0.062 mg/L; against MDR resistant MTB (25% - 30%) and *Mycobacterium avium* complex (MAC) strains with MICs 0.6-1.0mg/L. It shows high intraphagocytic activity against MAC strains (0.06-0.125mg/L). Single daily dose 10mg/kg provides complete erradication of mycobacteria in experimental generalized tuberculosis. Pharmacological studies established: excellent pharmacokinetic profile - following single oral dose 10mg/kg T_{max} 5 - 6h, C_{max} 5-9mg/L, $T_{1/2}$ 33-34 h; low toxicity; no teratogenic and embryotoxic effects. The clinical study of rifacinna shows higher therapeutic efficacy than rifampicin in patients with infiltrative, disseminated and cavitary form of pulmonary tuberculosis, good tolerability and safety profile. Some of the recent patents related to the treatment of tuberculosis are discussed in this review article.

Keywords: Tuberculosis, rifamycins, rifacinna, T_9, $T_{11,}$ *Mycobacterium tuberculosis*, *in vitro*, *in vivo*, pharmacokinetics, experimental tuberculosis, toxicology, clinical study.

INTRODUCTION

Tuberculosis (TB) remains the most frequent and important infectious disease to cause morbidity and death. Throughout the developed world there is public and governmental concern about the increasing prevalence of resistance of *Mycobacterium tuberculosis* to antimicrobial chemotherapy and few effective treatments exist [1]. The number of mycobacterial strains resistant to available drugs is continually increasing. New antimicrobial agents and improved methods are therefore needed for the treatment and prevention of infection [2-4]. We are entering the new millenium with TB being an even greater global problem than it was at the beginning of the twentieth century. Recent outbreaks of multidrug-resistant TB (MDR-TB) created the urgency to discover new antituberculosis drugs. Unfortunately, no new drugs except rifabutin (RBT) and rifapentine (RPT) have been approved for TB treatment during the 35 years after release of rifampin (RMP). Besides random screening of several new compounds, investigations of structural analogs of existing drugs will be a useful approach. Rifampin (RMP) has been one of the most effective drugs for the treatment of tuberculosis; however, in the recent past RMP-resistant strains have been on the increase. Moreover, RMP is not effective against *Mycobacterium avium* complex (MAC) strains. The creation of new chemotherapeutic regimens effective against *Mycobacterium tuberculosis* (MTB) and MAC strains that permit shortening the duration of treatment is a major priority for antituberculosis drug development. A shorter regimen, which could be possible with new drugs, should lead to improved patient compliance, decrease toxicity, helping to maximize cure and limit the emergence of new, resistant strains. Shorter regimens are expected to reduce the burden of therapy for patients and health care delivery systems, leading to increased global coverage with directly observed therapy, higher completion rates, and improved control of TB [5]. TB was perhaps the first field in which it was appreciated that combination therapy was critical [6]. Current regimens require combined treatment of tuberculosis with multiple drugs, including isoniazid, rifampin, ethambutol and pyrazinamide for the full dosing period of 6 months (American Thoracic Society Documents Am J Respir Crit Care Med 2003;167:603-662). It is difficult to comply completely with this complex and prolonged regimen, and consequently, there is a substantial rate of treatment failure, even among patients with drug-sensitive disease. Thus, the availability of a more potent antibiotic that could clear infection more rapidly would be very valuable. Due to the complexity of the pathology of *M. tuberculosis* it is unlikely that a single new drug will be enough. In any case, the key tasks for an antituberculosis regimens are: 1. the development of long-acting drugs with large dosage intervals in order to facilitate the Directly

*Address correspondence to this author at the Medical Department, Actavis EAD, 1407 Sofia, 29, At.Dukov str., Bulgaria;
Tel: ++35929321554/++35928741026; Fax: ++35929321888;
Mobile: ++359888240526; E-mail: vdimova@actavis.bg;
veldimova@mail.bg

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Observed Treatment, Short-Course (DOTS) and enhance patient's compliance, 2. the prevention of drug-resistant MTB strains using drugs exhibiting potently early microbicidal activity, and 3. the eradication of slowly metabolizing and, if possible, dormant populations of MTB organisms that cause relapse [7]. Favorable pharmacokinetic properties, low incidence of side effects and low cost are the characteristics that would make antimycobacterial drugs suitable for extenisive use.

The research and development time of any new anti-TB drugs will be long, slow and difficult, and involve huge expenses. The challenge in TB pharmacotherapy is to devise well-tolerated, efficacious, short-duration regimens that can be used successfully against drug-resistant and drug-resistant TB in a heterogeneous population of patients [8, 9]. Strategies to search for new anti-tuberculosis drugs involve: screening libraries of small molecules and natural products or the previous identification of targets crucial to the microorganism and the subsequent design of new molecules. Development of new drugs from known compounds having already shown safety and efficacy is an attractive strategy from the economical, pharmaceutical and clinical point of view. Several derivatives of known molecules and new compounds with different targets have been studied with promising preliminary results [10, 11].

Rifamycins are a group of anti-bacterial agents characterized by a chromophoric naphthohydroquinone group spanned by a long aliphatic bridge, which belong to the family of ansamycins antibiotics [12]. Derivatives of rifamycin SV are known to exhibit antibiotic activity against various pathogenic bacteria (especially mycobacteria) by inhibiting the RNA polymerase, thereby inhibiting synthesis of mRNA. Rifampicin (INN) is the most important drug of this group in TB treatment with better activity against Gram-positive and negative bacteria, especially against mycobacteria, with excellent oral bioavailability [12, 13].

In this endeavour, in addition to searching for new compounds, investigating structural modifications of the existing lead drugs is an useful alternative approach [14, 15]. With the objective of developing an effective drug for the treatment of mycobacterial diseases, several analogs of RMP were synthesized and investigated for their activity against MTB and MAC (rifalazil - RLZ, formerly known as KRM-1648, rifametane- SPA-S-565, CGP40/469A), R-76-1, FCE-22250, FCE22807, CGP-7040, CGP-27557, CGP-29861 [10, 11]. RPT possesses 2 to 4 times higher *in vitro* antimycobacterial activity and considerably better pharmacokinetic properties than RMP; RBT is about 4 to 8 times more active than RMP against MTB and it possesses favorable pharmacokinetic features such as a long half-life and good tissue penetration [16] Both RBT and RPT are now included in drug regimens for the treatment of TB and MAC infections, particularly in HIV patients [17-19]. RLZ has the unique chemical structure and possesses about 100 times more potent *in vitro* activity than RMP against drug-susceptible isolates of slow-growing mycobacteria, including MTB and MAC, and exhibits markedly increased therapeutic efficacy against mycobacterial infections induced in mice [20, 21]. The pharmacokinetics of RLZ in human beings limits its dosages in patients with TB [22].

The novel inventive rifamycin compounds exert their antimicrobial activity through multiple antibacterial mechanisms targeting bacterial RNA polymerase, DNA gyrase and DNA topoisomerase IV, and, therefore, they exhibit reduced frequency of resistance, and slow or eliminate development of drug resistance.

The rifamycin derivatives are mainly designed with activity against drug-resistant microorganisms, and with reduced frequency of developing mutational resistance, designed to address drug resistance to both the rifamycin and quinolone class of antibiotics by chemically linking rifamycin and quinolone antibacterial pharmacophores together [23-25], substitution of amino alcohol [26], substitution of ethylene diamines [27], compound for treating vascular diseases and controlling cell proliferation [28].

In a similar manner there has been developed a new rifamycin analogue, 3-(4-cynnamyl-piperazinyl-iminomethyl)- rifamycin SV. After *in vitro* (mainly according Microbroth dilution method as per NCCLS guidelines) and *in vivo* screening of numerous promising compounds, sinthesized with drug-design strategy with rifamycin SV as a target compound, 3-(4-cinnamyl-piperazinyl iminomethyl) rifamycin SV (T_9) and its sodium salt ($T_{11,}$ Rifacinna®) were selected for pharmacological and chemotherapeutic studies [29].

Investigations conducted by the developpers of drug have indicated good therapeutic activity, lack of toxicity, encouraging pharmacokinetics and bioavailability in experimental animals. Sodium salt of cynnamyl-rifamycin (T_{11}), water soluble, retains the antibacterial and antituberculous activity *in vitro* and *in vivo* of T_9 and provides a huge advantages - possibilities for parenteral administration (Fig. (**1**)).

In this review, we highlight the antimycobacterial activity (against MTB and MAC), pharmacology (toxicology, pharmacokinetic) and clinical efficacy [30] of the final product - Rifacinna [3-4(-cinnamyl-1-piperazinyl) iminomethyl rifamicin SV sodium].

The antibacterial activity of T_{11} against gram positive and gram negative aerobic bacteria in comparison with RMP and RBT as determined by their MICs are briefly in order: against *Enterococcus* sp. T_{11} > RMP> RFB; against *Staphylococcus* sp. esp in case of MRS is $T_{11,}$RFB> RMP ; against *Heamophilus influenzae* is T_{11} >RMP and RBT [31-33]. Against obligatory anaerobic bacteria the activity of T_{11} in comparison with RMP and RBT as determined by their MICs generally is in order RBT> RMP.> T_{11} [31-34].

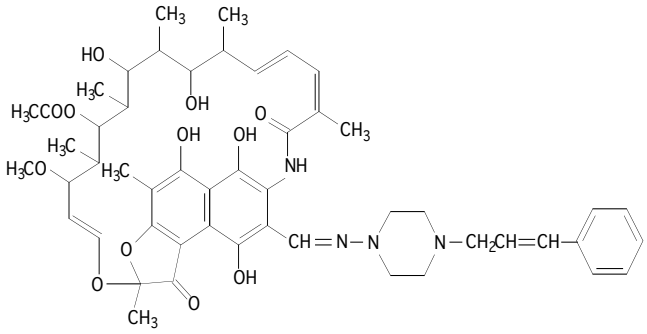
I. *IN VITRO*

I.1. Activity Against *Mycobacteria*

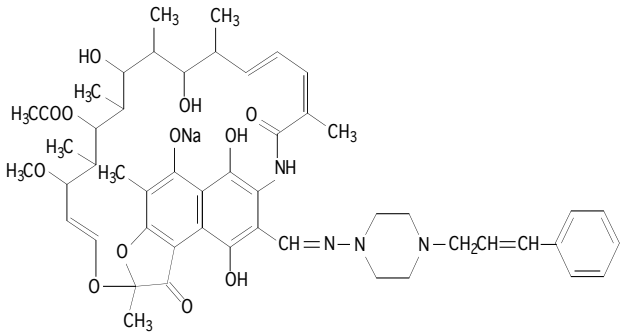
In Table **1**. the activity of T_{11} against RMP sensitive, INH and ETB resistant strains, as well against some MDR strains of *Mycobacterium tuberculos* is higher that that of RMP.

In Table **2** [31, 32, 35, 36] the activity of T_{11} against *M. avium* strains (MICs < 0.01- 0.25 mg/L) is greater than the activity of RMP (MICs 0.15-1- > 8 mg/L) [37].

In Table **3**, the MICs of T_9 and RMP against *M. avium* are respectively 2.0- 4 mg/L and 4 > 8 mg/L and against MTB H37Rv respectively 0.12- 0.25mg/L and 0.50- 1mg/L



T_9



T_{11}

Fig. (1). Chemical structure of 3-4(-cinnamyl-1-piperazinyl) iminomethyl rifamicin SV -(T_9), and sodium salt (T_{11})

Table 1. MICs of T_9 and RMP against *M. tuberculosis* (Sensitive & Resistant Strains)[a][31, 32]

M. Tuberculosis Strains	Susceptibility or Resistance to Anti TB Drugs[a]	MIC (mg/L) of:	
		T_9	RMP
RMP -SUSCEPTIBLE			
H37Rv	RMP-S	0.03	0.12
2237	RMP-S	0.015	0.12
2242	RMP-S	0.015	0.12
2257	RMP-S	0.03	0.25
MTB14	RMP-S	< 0.06	0.25
RESISTANT to drugs other than RMP			
MTB1	INH-R	0. 03	0.50
MTB2	INH-R	0. 25	0.06
MTB11	EMB-R	< 0. 06	0.25
MTB13	INH-R	< 0. 06	< 0.06
MTB36	INH-R	< 0. 06	0.50
2218	MDR	0. 5	0.06
RMP -RESISTANT			
MTB9	RMP- R	> 8.00	> 8.00
MTB29	MDR	> 8.00	> 8.00
MTB31	MDR	> 8.00	> 8.00
MTB33	RMP- R	1.00	2.00
Mtb37	RMP- R	0.25	> 8.00
2219	MDR	> 8.00	8.00
2225	MDR	8.0	> 8.00
2227	MDR	1.0	4.00
2230	MDR	4.0	> 8.00

a INH - Ioniazid; **EMB -** Ethambutol; **S** - susceptible; **R -** resistant.

Table 2. MICs of T_9 and RMP against *M. Avium* Strains[a] [31, 32, 35, 36]

MIC (mg/L) of:		
***M. avium* strains**	**T_9**	**RMP**
101	0.06	0.50
5718	0.06	0.50
PLR3	0.03	0.50
LR25	< 0.015	0.25
LR541	0.015	0.03
LR545	< 0.015	0.015
LR549	< 0.015	0.12
LR562	< 0.015	0.015
SK12	0.06	0.151.0
SK18	0.06	8.0
SK22	0.06	0.50
SK32	0.06	0.50
SK40	0.06	1.0
SK46	0.06	0.50
SK47	0.12	> 8.0
CH1	0.12	> 8.0
CH2	0.25	> 8.0
CH3	0.25	> 8.0
CH4	0.06	0.06
CH5	0.06	0.25

a MICs determined radiometrically (by the BACTEC method) [35].

Table 3. MBCs of T_9 and RMP against *M. avium* and MTB [31, 32, 35]

Strains	MBC (mg/L)	
	T_9	**RMP**
M. avium		
SK22	2.0	8.0
SK18	2.0	> 8.0
101	2.0	> 8.0
5718	2.0	> 8.0
SK32	4.0	4.0
MTB H37Rv		
H37Rv	0.12	0.50
2237	0.50	1.00
MTB11	0.12	0.50
2257	0.12	0.50
	0.25	0.25

In Table **4**, the activity of T_{11}, RMP and RBT determined by their MICs against *M. avium* is T_{11}> RBT >RMP and against *M. intracellulare* RBT > T_{11}> RMP.

Table **5** [35, 38] shows that both T_9 and T_{11} exhibit significantly higher activity than RMP, when tested for RMP-resistant MTB strain and MAC isolates. In such cases, their activities were comparable to that of RFB. On the other hand, both T_9 and T_{11} exerted only the same levels of the activities against drug-susceptible MTB strain (H37Rv and Kurono). In this case, their activity was weaker than that of rifalazil (RLZ).

The results in Table **6** show that all tested drugs, including T_9 and T_{11} added at 1 and 10 mg/L caused bacterial killing of intramacrophage MAC.in the order RLZ > RBT > T_{11}, T_9 > RMP. Both T_9 and T_{11} added at 1 and 10 mg/L caused weak bactericidal activity against intracellular MAC residing within A-549 cells in the order RLZ > RBT, T_9, T_{11} > RMP. It is noteworthy that the antimicrobial activities of T_9 and T_{11} against intracellular MAC within A-549 cells were in the comparable levels as that of RBT [20]. In Table **7** [39, 40] T_9 showed better activity than RMP even after a single pulsed exposure (24h) of MTB to the drugs.

I.2. Activities of T_9, Rifampicin (RMP), Rifabutin (RFB), against *Mycobacterium leprea*

in vitro Activity (using an *in vitro* cell-free culture system) of a rifamycin derivatives RBT, rifapentine, T_9, KRM 1648, KRM 1657, KRM 1668, and KRM 2312. KRM 1648 (RLZ) against *Mycobacterium leprae* is found to be more bactericidal at lesser dose and is effective against RMP-resistant strains. KRM 1648 (RLZ) and others like KRM 2312, T_9 and RBT are also found to show synergism with ofloxacin. All these drugs are potential implements for MDT. Comparative activities of various rifamycin analogues against leprosy were studied by monitoring ATP concentrations and ^{3}H-thymidine uptake during the *in vitro* growth of *M. leprae* in DH medium.

Among the 7 analogues studied, KRM-1648 (RLZ) was found to be the most potent in inhibiting the growth of RMP sensitive strains of *M. leprae*, with MIC 0.05mg/L, followed by RLZ and T_9 (MIC of each being 0.1mg/L) and RBT (MIC of 0.2mg/L). MICs of T_9 against RMP-S and RMP-R strains of *M. leprae* were 0.1mg/L and 0.4mg/L, respectively. Furthermore, in common with RBT, but not with rifamycin, T_9 demonstrated synergy with ofloxacin against both RMP-S and RMP-R strains of *M. Leprae* [41-43].

Rifampicin, along with KRM-1657 and RLZ were least effective, with MIC for each of 0.4 mg/L. The effects of each of the rifamycin analogues were bactericidal at their respective MICs. The results were similar for RMP-R strains of *M. leprae*, but the MICs were higher than those obtained with RMP-S strains. It is concluded that rifamycin analogues are more potent than RMP against both RMP-S and RMP-R strains of *M. leprae* [42-44].

II. *IN VIVO* STUDIES

II.1.Tuberculous Infections in Experimental Animals

II.1.1. Mice

The therapeutic activity of T_9 against experimental tuberculosis in C57BL/6 mice Table **8** is very encouraging.

Table 4. ***in Vitro*** **Activity of T_{11}, RMP and RBT against** ***Mycobacterium*** **sp., Susceptible and Resistant Strains (mg/L) [31-33]**

Mycobacterium sp. (n=82)	T_{11}			RMP			RBT		
	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
MBTB (n = 61)	0.015 - 5.0	0.06	5.0	0.06 - 5.0	0.125	5.0	0.007 - 5.0	0.06	2.5
M.avium (n = 8)	≤ 0. 015 - 0. 06	≤ 0.015	0. 06	≤ 0. 015 - 0.5	0.031	0.5	≤ 0. 031 - 0.12	0.03	0.12
M. intracellulare (n = 12)	0. 06 - 0. 25	0. 06	0. 25	0. 06 - 5.0	1.0	> 5.0	0. 03 - 0.25	0.06	0.25
M.celatum (n = 1)	-	> 1.0	> 1.0	-	> 1.0	> 1,0	-	> 1.0	> 1.0

Table 5. MICs of Antimycobacterial Drugs against MTB and MAC[a] [35, 38]

Strains	MIC (mg/L)				
	RMP	RBT	RLZ	T_9	T_{11}
MTB H37Rv	0.2	0.05	0.025	0.1	0.2
MTB Kurono	0.2	0.05	0.025	0.1	0.2
MTB Watanabe	>100	12.5	5.0	25	25
MAC N-260 (*M. intracellulare*)	12.5	0.78	0.2	0.78	0.78
MAC N-444 (*M. avium*)	12.5	0.78	0.2	0.78	0.78

a) Determined by the agar dilution method with 7H11 medium.

Table 6. Antimicrobial Activities of T_9, T_{11}, RMP, RBT, RLZ against Intracellular MAC[a]

Incubation Time (day)	Drug (mg/L)	Log CFU/well (mean ± SEM, n = 3)	
		THP-1 Mφps	A-549 cells
0	None	4.90 ± 0.03	4.75 ± 0.01
5	None	5.39 ± 0.01[b]	5.37 ± 0.01[b]
5	RMP - 1 mg/L	4.73 ± 0.03[b]	4.23 ± 0.02
5	RMP- 10 mg/L	3.99 ± 0.03[b]	3.65 ± 0.03[b]
5	RBT - 1 mg/L	3.96 ± 0.05	3.68 ± 0.02
5	RBT - 10 mg/L	2.13 ± 0.07[b]	2.38 ± 0.01
5	RLZ - 1 mg/L	2.50 ± 0.09	2.23 ± 0.27[b]
5	RLZ - 10 mg/L	1.40 ± 0.01	1.30 ± 0.10[b]
5	T_9 - 1 mg/L	4.26 ± 0.04[b) d)]	3.84 ± 0.03[d)]
5	T_9 - 10 mg/L	3.24 ± 0.11[b)c)d)]	2.12 ± 0.12[b)d)]
5	T_{11} - 1 mg/L	4.26 ± 0.04[b)d)]	3.85 ± 0.01[d)]
5	T_{11} - 10 mg/L	3.13 ± 0.02[b)c)d)]	2.48 ± 0.07[b)d)]

a) Each cell infected with MAC N-444 was incubated in RPMI culture medium containing 5% FBS (THP-1 macrophages) or 1% FBS (A-549 cells) in the presence of indicated drugs for 5 days **b, c, d)** Significantly different from corresponding CFU values of RMP (**b**) RBT (c) and RLZ(**d**) the values of T-9 and T-11 ($p < 0.05$ by Bonferroni's multiple *t* test). The significant difference was not recognized between value of T-9 and value of T-11.

Table 7. Intracellular activities of T_9 and RMP against MTB H37Rv[a] [*]

Treatment group Mean GI .[+] SD (drug concn [mg/L])	
Day 0 control, baseline	22.0 + 1.7
Day 4 control	176.0 + 21.1
T_9 - 0.125 mg/L	5.0 + 1.7
T_9 - 0.50 mg/L	3.3 + 0.5
RMP- 0.125 mg/L	102.6 + 4.0
RMP -0.5 mg/L	18.3 + 6 1.1

a Activities were determined after 24-h exposures to drugs in J774A.1 cells. GIs were determined by the BACTEC method. GI- growth index [39, 40].

No organisms could be detected both in lungs and spleens after treatment with 20 or 40mg/kg dose of T_9 for 8 weeks. The activity of T_9 was much superior to RMP even with 10 and 5mg/kg doses. Preliminary pharmacological studies with T_9 in mice showed that after a dose of 10 mg/kg, the plasma C_{max} was 9.33μg/ml with a T_{max} of 4.93h and $T_{1/2}$ of 34.75h [5]. After similar dose C_{max} of RMP in mice was 7.85 mg/L at Tmax 1hour [3]. Even at the 5mg/kg dose, T_9 caused significant reductions of CFU, compared with the 20mg/kg dose of RMP, in both lungs and spleens. After 8 weeks of treatment with RMP (20 mg/kg), there was a reduction of only 1.97log CFU/g in the lungs and 1.46 log CFU/g in the spleens; on the other hand, T_9 at the 5mg/kg dose caused reductions of 3.99 and 3.13log CFU/g in the lungs and spleens, respectively. In mice treated with 40 and 20mg/kg of T_9, tuberculosis bacilli could not be detected at 4 weeks in both lungs and spleens.

Pharmacokinetic studues by Tomioka *et al*. have shown C_{max} of 0.60, 12.73 and 0.92mg/L for KRM-1648, RMP and RBT respectively in mice with 20mg/kg dose [45, 46]. Comparison of the pharmacokinetics of some of the rifampin analogues with respect to their MICs against mycobacteria indicates that T_9 has good therapeutic value.

II.1.2.Guinea Pigs

In generalized experimental tuberculosis in guinea pigs daily oral administration of T_9 10 mg/kg provides complete microbial erradication and organ sterilization after 30-40-day treatment. Similar therapeutic effect was achieved with RMP at 80mg/kg [47].

III. INFLUENCE OF T_9 ON DRUG METHABOLIZING ENZYMES OF LIVER SYSTEM (DMELS).

The influence of T_9 10mg/kg on some parameters of DMELS was investigated - in single and repeated (4 consecutive days) doses administered orally. Rat liver microsomes isolated after Fouts were used.The level of microsomal cytochrome b_5 and P450 were determined after Omura and Sato [48], determination of heme- after Paul [49].The activity of ethylmorphine demethylase was determined after Davis; of aniline hydroxylase- after Karuzina [50]; of NADPH-cyt c - reductase- after Dallner [51] and UDPG-transferase- after Pogell [52, 53].

At single dose of T_9 10mg/kg decreases the activity of NADPH-cyt c- reductase. An induction of drug metabolizing enzymes was found after 4 days treatment with T_9 10mg/kg. An increase of the levels of cyt and heme was established. The activity of ethylmorphine demethylase, as well as of NADPH - cyt c - reductase were increased. The established induction was probably of phenobarbituric type. In UDPG-transferase no changes were observed.

The data obtained in this study show that the inducing effect of T_9 on rat liver drug metabolism is lower in comparison with that of RMP [54].

IV. PHARMACOKINETICS STUDIES

IV.1. Pharmacokinetics of T_9 and its Sodium Salt T_{11} in Mice, Rats and Rabbits

Neither the serum concentrations nor the C/t profile differ significantly when T_9 and T_{11} are applied in equal p.o doses in mice. The absorption of T_{11} (T_9) in mice is neither rapid nor complete. The maximum plasma levels are achieved at 8th hour, and the bioavailability is in the range 0.2-0.3.

Irrespective of the administration route the primary peak in the C/t curve is followed up by a long lasting plateau at a relative high concentration, resembling steady state after multiply dosing. The antibiotic is characterized by a long half life as it is confirmed by the presented in Table **9** values of the elimination parameters.

The concentration profile of both T_9 and T_{11} are analogues. The results in similar pharmacokinetic parameters, summarized in Table **9**.

The common features in the pharmacokinetic profile following single p.o. administration of equal dosis in mice (Table **9**, Fig. **3**) and rats (Table **10**, Fig. **4**) are complicated absorption, prolonged "plateau phase" at high concentration, and rather delayed elimination. In both animal species T_9 is characterized with a very low elimination. The serum half-life in rat is found to be approx. 20h - much longer as compared with RMP ($t_{1/2}$ of 4h) [12]. This delayed elimination process ensures still detectable, and microbial active serum level even 144 h after drug ingestion. Some quantitative interspecies differences are evident. This is in good agreement with the two fold differences reported in previous study on T_9 pharmacokinetics in both animal species [55].

IV.1.1. Pharmacokinetics of T_9 and T_{11} in Rabbit

Initially the serum levels rise rapidly and a broad area of maximal concentration within the range 8 - 24h after the treatment is obvious. In the next period of approx. twenty four h the serum levels decrease, and thereafter are settled up again at a relative constant value. The averaged values for maximal and steady state concentrations and the duration of the "plateau period" are shown in Table **11**.

The pharmacokinetics of a new cinamyl rifamycin derivative T_9 and its sodium salt T_{11} is studied in mice, rats and rabbits following single po intake (dose 20 mg/kg). The pharmacokinetic behaviour in mice and rats is very similar with common features: complicated absorption, long-lasting

Table 8. ***In Vivo*** **Activities of T_9 against MTB H37Rv In C57bl/6 Mice[a] [31, 32]**

Treatment Group (Dose)	Mean Log CFU/G of Tissue ± SD in Organ at Indicated Time					
	LUNG			SPLEEN		
	Day 1	Week 4	Week 8	Day 1	Week 4	Week 8
T_9 -10mg/kg		3.37± 0.04	2.49[c]		3.49± 0.15[b]	4.21± 1.44[d]
T_9 - 5mg/kg		4.67 ± 0.25	3.39± 0.85*		4.71 6 ±.25	3.58 ± 0.24*
RMP-20mg/kg		5.82± 0.63	5.41 ± 0.19**		5.50 ± 0.42	5.25 ± 0.42**
Control	7.38 ± 0.28	8.82 ± 0.94	10.20 ± 0.23	6.71 ± 0.12	7.64 ± 0.60	8.32 ± 0.46

a The sensitivities of detection were 1,000 and 100 CFUs per organ at weeks 4 and 8, respectively. No CFUs were detectable in either organ at any time point with T_9 doses of 40 and 20 mg/kg. *, *P* , 0.001, compared with RMP group; **, *P* , 0.001, compared with control group. ***b*** Determined from CFU counts from three mice.; ***c*** Determined from CFU count from one mouse; ***d*** -determined from CFU counts from two mice [35].

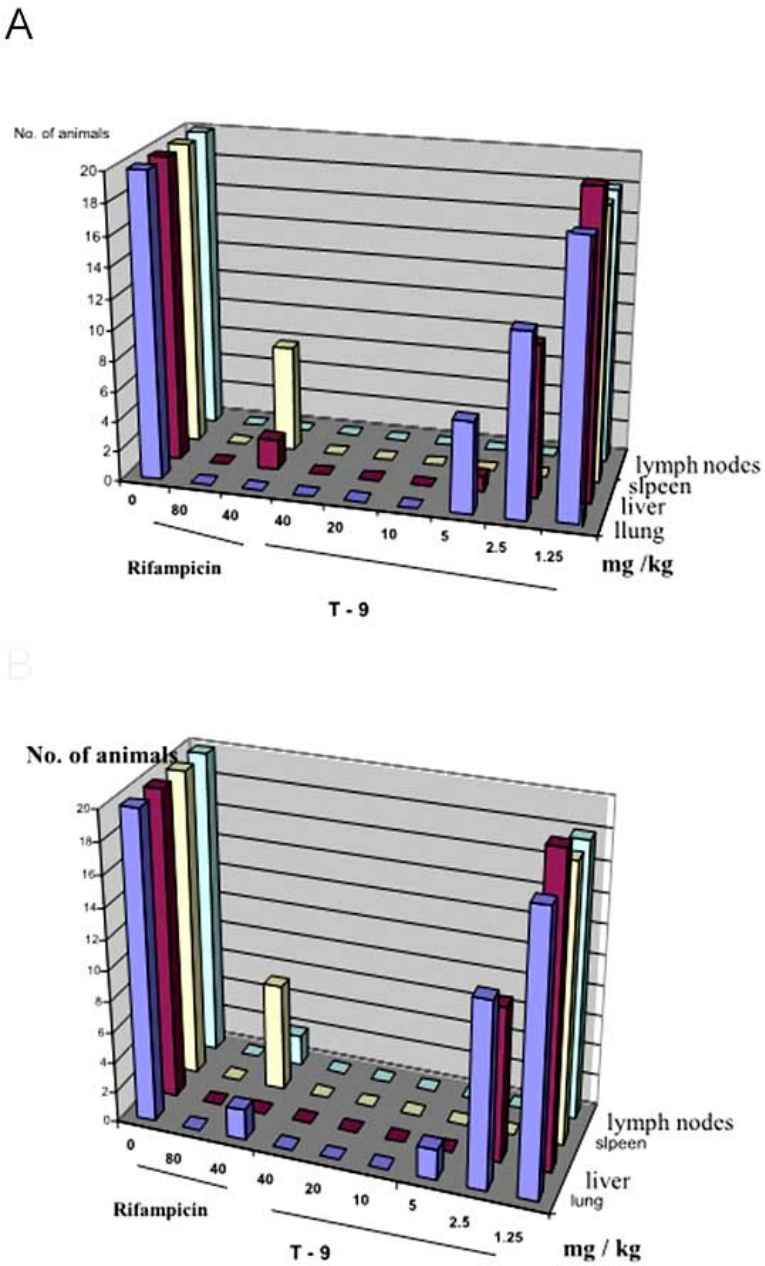


Fig. (2). (**a**, **b**) Therapeutic effect of T_9 on experimental TB infection in guinea pigs [47].

Table 9. Nonmodel Pharmacokinetic Parameters Following P.O. Administration of T_9 and T_{11} (20mg/kg) in Rat

Compound	C_{max}, mg/L	T_{max}, h	C^{ss}, mg/L	K, h^{-1}	$t_{1/2}$, h	AUC mg.h/L	R
T_9	8.20	6	6.08 (±0.86)	0.035	20.53	568.99	0.925
T_{11}	9.20	2	7.64 (±0.42)	0.038	19.73	681.32	0.894

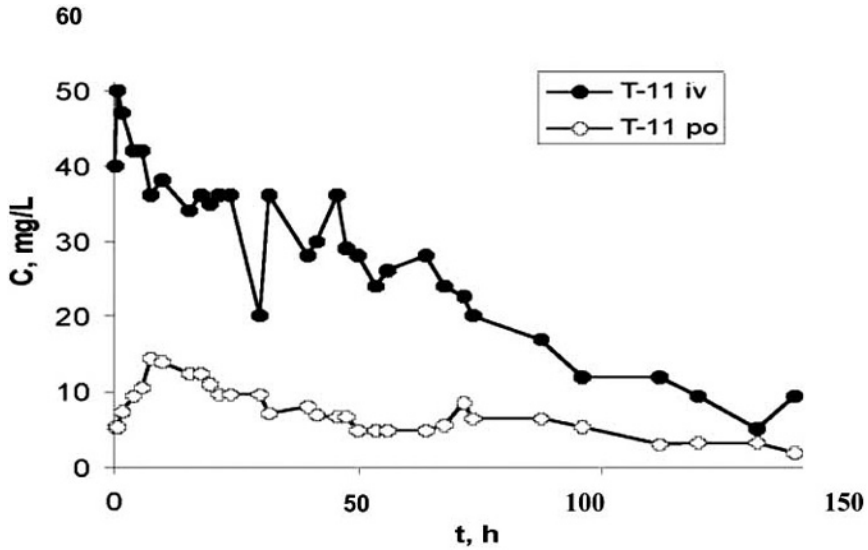


Fig. (3). Plasma levels following I.V. and P.O. Administration of T_{11} (20mg/kg) in Mice.

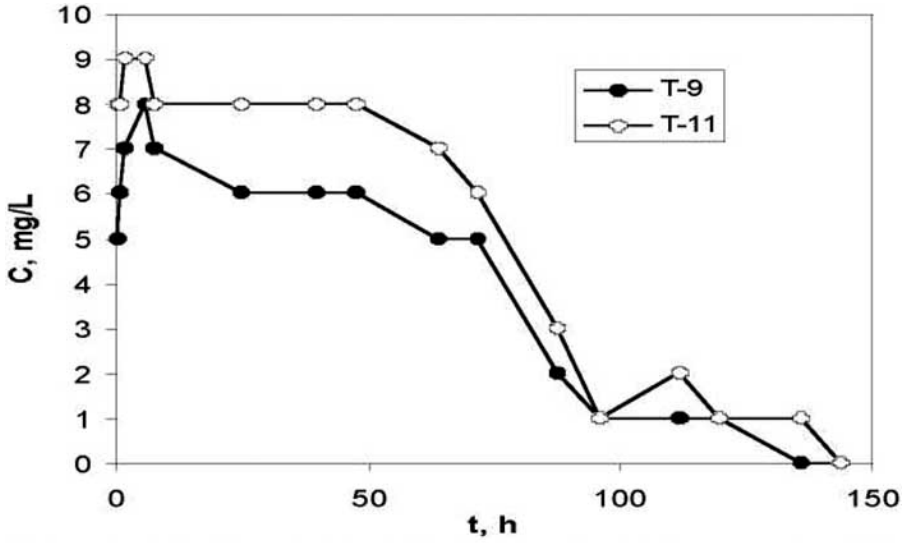


Fig. (4). Plasma levels following P.O. Administration of T_9 and T_{11} (20mg/kg) in Rat.

Table 10. Nonmodel Pharmacokinetic Parameters Following P.O. Administration of T_9 and T_{11} (20mg/kg) in Mice

Administration Route	C_{max} mg/L	T_{max}, h	K h^{-1}	$t_{1/2}$ h	AUC, mg.h/L	f	CL ml.min/kg	V l/kg
T_{11} i.v	50	1	0.0177	39.08	3616.7	-	0.092	0.312
T_{11} p.o	14.4	8	0.0182	38.02	977.45	0.27	0.092	0.303
T_9 p.o	12.4	8	0.0206	33.70	734.69	0.20	0.092	0.270

Table 11. Peak and Steady State Concentrations and Time Intervals for Their Maintenance Following P.O. Administration of T_9 and T_{11} (20mg/kg) In Rabbit

Compound	$C_{max}(1)$, mg/L	$T_{max}(1)$, h	$C_{max}(1)$, mg/L	$T_{max}(1)$, h	C^{ss}, mg/L	Steady State Duration, h	AUC_{0-144}, mg/L
T_9	2.47 (± 0.55)	2	3.75 (± 0.25)	19.6 (± 2.9)	0.90 (± 0.19)	64-144	232.6 (± 34.2)
T_{11}	2.75 (± 0.88)	2	3.87 (± 0.21)	18.6 (± 2.7)	1.68 (± 0.35)	48-144	293.8 (± 29.7)

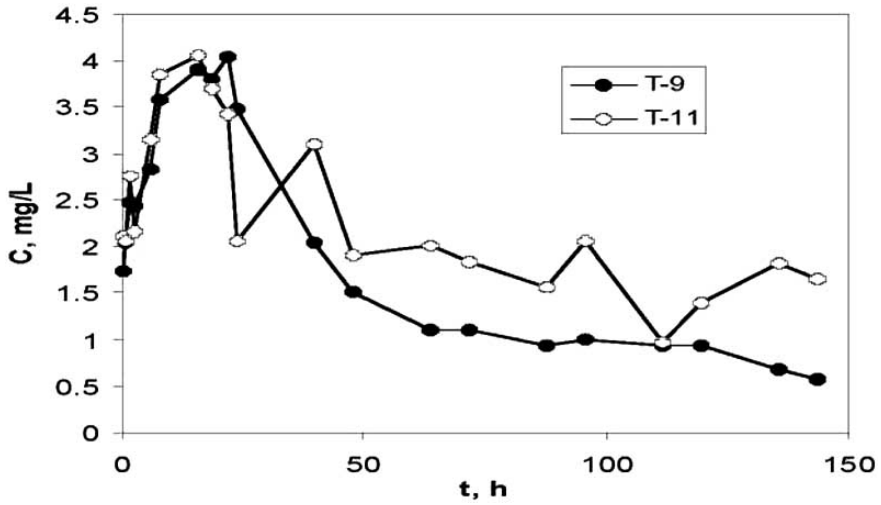


Fig. (5). Plasma levels following P.O. Administration of T_{11}(20mg/Kg) in Rabbit.

"plateau phase" at high concentration, and slow elimination. Higher extent of biotransformation and first- pass effect are plausible reason for the lower serum levels in rats determining also lower AUC and bioavailability. Rather different concentration profile is observed in rabbits (Table **11**, Fig. **5**). The initial sharp peak is followed up by a broad area with maximal concen-tration, and a "plateau phase" at level exceeding 25% of C_{max} lasting up to the end of the examined period without reaching terminal elimination phase. The solubility limited absorption and the enterohepatic circulation are considered as responsible for the unusual pharmacokinetic behaviour. By all tested experimental animals the serum levels are still antimicrobial active 144h after the treatment. Despite of certain species differences the observed common disposition patterns are favourable and are a reliably basis for further investigations.

V. TOXICOLOGICAL STUDIES [56]

The substance of 3-(4-cinnamyl-1-iminomethyl) piperazinyl rifamycin SV (T_9) and 3-(4-cinnamyl-1-iminomethyl) piperazinyl rifamycin SV sodium salt (T_{11}) were orally administered as 1% suspension with Tween-80. Doses - 0, 30, 40, 120, 240, 400mg/kg. Duration of treatment- 4 weeks, 90 days, 180 days.

V.1. Acute Toxicity- LD 50 [mg/kg]

The LD_{50} values after single oral application Table **12** strongly evidence that (T_9) and its sodium salt (T_{11}) might be classified as a low-toxic.

V.2. Subacute (4 Weeks) Toxicity

Rats Wistar .Oral administration of Rifacinna 400 mg/kg or 1 /6 of LD 50 - No statistically significant differences and changes in the behaviour, mortality, haematological tests, clinical biochemistry and urine test of the animals were found during the experiments.

V.3. Subacute (90 Days) Toxicity - Subchronic

Rats Wistar. Oral administration of Rifacinna on rats Wistare after dosing 400 mg/kg (1/6 of LD 50)- changes in serum transaminases (AST, ALT) were established. Liver fat degeneration in isolated animals without any changes in the other organs was found.

No statistically significant differences and changes in the behaviour, mortality, haematological tests, urine test of the animals were found during the experiments.

V.4. Chronic (180 Days) Toxicity

Rats Wistar. Dose 240 mg/ kg - moderate reversible changes localized in the liver. No statistically significant differences and changes in the behaviour, mortality, haematological tests, and urine test of the animals were found during the experiments. Pathomorphological study- moderate reversible liver changes after Rifacinna - 240 mg/kg.

V.5. Chronic (180 Days) Toxicity - Dog

Dose 30 mg/kg- moderate changes in the liver (isolate cases of fat dystrophy) and kidney. No pathological changes in body weight, behaviour, mortality, haematological tests,

Table 12. Acute Toxicity- LD 50 [mg/Kg] of T_{11} (Rifacinna) in Mice and Rats

Animals	Rout of Administration	T_9	T_{11} (Rifacinna)	RMP
mice	i.p.	760 (650-936)	225 (180-390)	640 (613-667)
	p.o.	990 (900-1085)	2406 (2120- 2480)	885 (855-916)
	i.m	850 (726-995)	-	
rats	i.p.	1000 (725-1380)	-	550 (531-570)
	p.o.	2300 (1870-2850)	-	1720 (1343-2200)

Index of absorption (IA) for - rats is 2.3, and 1.2 for mice.

clinical biochemistry, and urine test. There was marked sensitiveness related to the animal species.

VI. EMBRYOTOXICITY AND TERATOGENICITY ON RATS (WISTAR)

The lack of statistically significant differences in the studied indices for embryotoxicity shows that the 3-(4-cinnamyl-1-iminomethyl) piperazinyl rifamycin SV suspension has no embryotoxic effect in the studied laboratory animals species.

VII. TERATOGENICITY

In rat and rabbit doses of Rifacinna 200mg/kg (40 times higher that recommended for humans) show the lack of visual external, skeleton and visceral malformations. In rat after dosing 40mg/kg/ day Rifacinna causes isolated cases of skeleton malformations. In rabbits after dosing 80mg/ kg/day the raising of skeleton malformations were established [56].

No experimental data about Rifacinna mutagenic or carcinogenic effects on animals were available

VIII. RIFACINNA - CLINICAL STUDY [30]

Object of the study were 60 patients with infiltrative pulmonary TB in stage of disintegration (MBT+) and disseminated TB in stage of infiltration and disintegration (MBT+). The object of the clinical study were the effectiveness and tolerability of Rifacinna in the treatment of pulmonary TB.

Design- Open, comparative, parallel study in 60 tuberculous patients with infiltrative lung tuberculosis in cavitary phase (MBT+) or disseminated cavitary tuberculosis treated during the complex therapy with Rifacinna - capsules 150 and 300mg **(**production of company Ecopharm 2002, Bulgaria) (30 patients) and Rifampicin - capsules 150mg (production of company Borshchagivski HFZ, Ukraine) (30 patients) was carried out.The field of study- The Hospital for Tuberculosis, Kiev, Ukraine. Methods- clinical (Table **13**), laboratory (Table **14**), radiological (Table **15**), microbiological (Tables **16**, **17**) and instrumental.

The results of the clinical study on therapeutic efficacy and tolerability of the Rifacinna after administration of single daily dose 450-600mg in 60 days course of treatment are as follow:

1. High therapeutic efficacy of rifacinna in combined anti-tuberculosis therapy in patients with cavitary pulmonary TB (MBT+) and disseminated pulmonary TB in stage of infiltration and cavitation (MBT+). 2. Pronounced positive dynamics of the clinical symptoms - reliable two weeks earlier than group of RMP fading away of the intoxication symptoms and decrease of cough and constriction. 3. Reliable positive radiographic dynamics in the group of rifacinna- one month earlier in comparison with the control group.

4. (RMP). Rifacinna produces culture negativity reliably two weeks earlier than that of the control group.

5. Rifacinna is well tolerated and does not provoke disturbances in the liver function.

On the basis of the results of the clinical study rifacinna caps. is recommended for further clinical investigations.

CURRENT & FUTURE DEVELOPMENTS

The last 10 years have seen resurgent industry activity in discovery and development of new drugs for the treatment of tuberculosis. Nowadays, there are different classes of compounds under research and development to obtain new drugs [57]. Various new antituberculous agents are currently being subjected to *in vitro*, *in vivo* studies and at least some of the potentially promising compounds are entered clinical trials [58-63]. There is a number of interesting compounds in development, all with unique properties and great potential to ultimately provide faster and more effective treatments for TB patients: fluoroquinolones Gatifloxacin (GAT) and Moxifloxacin (MXF); Nitroimidazoles - PA-824 and OPC-67683; Diarylquinoline - TMC207; Ethylene-diamine - SQ109; Pyrrole - LL-3858.

Some newer fluoroquinolones are effective against non-dividing bacteria as well; they do not have cross-resistance to other classes of TB drugs [64-66]. Among fluoroquinolones, GAT and MXF, approved for use in other bacterial infections, are one of the most active against *M. tuberculosis*. They specifically inhibit the DNA gyrase. *in vitro* Potency against *M. tuberculosis* H37Rv is with MIC_{50} 0.03- 0.12mg/L and MIC_{50} 0.06-0.5mg/L respectively [67]. Used in treatment of MDR TB MXF possesses long half-life (9h, plasma) and 2 mo. RMP+MXF+PZA followed by 4 mo RMP+MXF leads to sterilization and lower relapse rate. In Phase 2 EBA study MXF is less effective than INH. Two Phase 2 trials to substitute MXF for EMB showed no

Table 13. Clinical Indices in the Groups of Rifacinna (N=30) and RMP (N=30) [30]

SYMPTOMS		Before Treatment		After 2 Weeks		After 1 Month		After 2 Month	
		Rifacinna	RMP	Rifacinna	RMP	Rifacinna	RMP	Rifacinna	RMP
Constrictions	- there is not;	-	-	63.3	40.0	100.0	80.0	100.0	100.0
	- slight	40.0	36.7	36.7	33.3	-	20.0	-	-
	- moderate	46.6	46.7	-	26.7	-	-	-	-
	- strong	13.4	16.7	-	-	-	-	-	-
Temperature	- normal	-	-	100.0	86.7	100.0	100.0	100.0	100.0
	- sub-febril	90.0	86.7	-	13.3	-	-	-	-
	- febril	10.0	13.4	-	-	-	-	-	-
Cough	- there is not	-	-	93.3	83.8	100.0	90.0	100.0	100.0
	- slight	20.0	26.7	6.7	13.3	-	10.0	-	-
	- moderate	70.0	66.7	-	3.3	-	-	-	-
	- strong	10.0	6.7	-	-	-	-	-	-
Quantity of Sputa	- there is not	-	-	70.0	66.7	100.0	80.0	100.0	100.0
	- till 50 ml/day	70.0	66.7	30.0	23.3	-	20.0	-	-
	- > 50 ml/day	30.0	33.3	-	10.0	-	-	-	-
Sputa-characters	- mucous	6.7	13.3	60.0	63.3	-	-	-	-
	- mucous-suppurative	70.0	80.0	-	-	-	-	-	-
	- suppurative	23.3	6.7	-	-	-	-	-	-
Sputa with blood	- there is	667	66.7	10.0	10.0	-	-	-	-
	- there is not	33.3	33.3	90.0	90.0	100.0	100.0	100.0	100.0
Self-confidence	- satisfactory	6.7	10.0	56.7	50.0	100.0	80.0	100.0	100.0
	- slight weakness	46.7	40.0	30.0	26.6	-	20.0	-	-
	- moder. weakness	40.0	33.3	13.3	23.3	-	-	-	-
	-consid. weakness	6.7	16.7	-	-	-	-	-	-
Crepitations	- there is	40.0	40.0	-	-	-	-	-	-
	- there is not	60.0	60.0	100.0	100.0	100.0	100.0	100.0	100.0
Muted pulmo sounds	- there is;	20.0	20.0	-	86.7	-	-	-	-
	- there is not	80.0	80.0	100.0	13.3	100.0	100.0	100.0	100.0

In the group of Rifacinna, symptoms of intoxication decreased an average of 2 weeks earlier comparing the group of RMP.

Table 14. Blood Biochemical Analysis Before and After Treatment (M ± N) [30]

INDICES	Rifacinna (n=30)		RMP (n=30)	
	Before Treatment	After Treatment	Before Treatment	After Treatment
Total proteins (g/L)	7.0±0.22	7.3 ± 0.21	6.8 ± 0.13	6.9 ± 0.09
Bilirubin (mcmol/L)	13.8±0.02	12.4 ± 0.05	13.1 ± 0.10	12.6 ± 0.22
ALAT (mcmol/L)	0.52±0,01	0.49 ± 0.05	0.47 ± 0.05	0.52 ± 0.01
ACAT (mcmol/L)	0.41±0.02	0.47 ± 0.03	0.45 ± 0.01	0.42 ± 0.02
Residual nitrogene (mcmol/L)	21.3±0.2	21.4 ± 0.15	21.8 ± 0.12	20.4 ± 0.12
Urine (mcmol/L)	4.3±0.08	4.5±0,06	4.3±0.07	4.4±0.05
Sugar in blood(mcmol/L)	4.7±0.05	4.4±0.04	4.8±0.03	4.6±0.04

Note: No reliable difference between the indices of two groups.

Table 15. Radiographic Changes in the Groups of Rifacinna and RMP (% patients) [30]

Changes in the Lung	Before Treatment		After 1 Month		After 2 Month	
	Rifacinna	RMP	Rifacinna	RMP	Rifacinna	RMP
Foci	14.0 ± 0.02	12.0 ± 0.01	7.0 ± 0.03*	10.0 ± 0.02	5.0 ± 0.02	4.0 ± 0.01*
Infiltrative	16.0 ± 0.03	18.0 ± 0.01	6.0 ± 0.02*	12.0 ± 0,01	2.0 ± 0.02	3.0 ± 0.04*
cavity(cavern)	17.4 ± 0.02	16.6 ± 0.03	3.4 ± 0.05*	9.4 ± 0.04	0	0*

Note: *Reliable difference of the index (p<0,05) before and in the process of medication.

Table 16. Dynamics of Bacteria Secretion (% Patients) [30]. Negative Conversion of Sputum Smear Examination

Group	Secretion of Mycobacteria (Microscopic Examination of Sputa)			
	Before treatment	30th day of treatment	45th day of treatment	60th day of treatment
Rifacinna	**100**	51.1±2.6	**0**	**0**
RMP	**100**	40.2±2.0	**15.2±1.4***	**0**

Note: *Reliable difference of index (p < 0. 05) between two groups

Table 17. Secretion of MBT in the Groups of Rifacinna and RMP [30]

Group	Isolation of *Mycobacterium* TB (Day of Treatment)			
	Before Treatment	30th	45th	60th
Rifacinna	25.0 ± 1.2	8.2 ± 0.6	0	0
RMP	26.0 ± 1.1	12.3 ± 0.4	4.1 ± 0.2*	0

Note: *Reliable difference of index (p < 0.05) between two groups.

improvement. With ongoing clinical trials in Phase 3 GAT and MFX, should be able to place these fluoroquinolones in the context of an appropriate indication and appropriate treatment regimen in the next few years [68-71]. To determine their actual utility, and prevent indiscriminate induction of resistance, they should be prescribed only after the appropriate dose and multi-drug combinations are established in clinical trials, and never as monotherapy, as resistance is a class phenomenon.

Nitroimidazoles - PA-824 and OPC-67683- Two members of this chemical novel class of drugs are presently in phase II of clinical development: PA-824, a nitroimidazo-oxazine, being evaluated currently for drug sensitive TB, and OPC-67683, a nitroimidazo-oxazole, currently being studied in MDR-TB patients [69]. PA-824 was highly active against all the isolates tested, with an MIC < 1mg/L, and was equally active against the drug-sensitive and MDR isolates of *M. tuberculosis*, with MICs ranging from 0.039 to 0.531mg/L. The compound acts by a novel mechanism - both protein synthesis and lipid synthesis are substantially inhibited [72]. At a dose of 100mg/kg/day, PA-824 has substantial bactericidal activity during both the initial and the continuation phases of TB chemotherapy in the mouse model. The MED is 12.5 mg/kg /day and MBD is 100mg/kg/day [72, 73]. PA-824 at 100mg/kg was as active as MXF at 100mg/kg and INH at 25mg/kg and was slightly more active than RMP at 20 mg/kg. Because this activity is dose dependent and 100mg/kg is the lowest dose at which significant bactericidal activity could be demonstrated (i.e. the MBD), the equipotent dose in humans may be an important target dose for use for exploration of the activity of PA-824 in early clinical studies. The potential contribution of the compound to first-line chemotherapy regimens may be limited if a similar target exposure cannot be obtained in humans. The activity of PA-824 in bacterial mutagenesis assays still remains at considerably high levels and this will continue to be the major obstacle in its development as an anti-TB drug [74].

OPC-67683 is a markedly potent compound *in vitro* against *M. tuberculosis* with MIC 0.006-0.012mg/L. It is active against MDR TB and possesses long half-life (7.6hrs) and no cross-resistance or antagonistic activity with first-line anti-TB drugs. In a mouse model, OPC-67683 was estimated to have potential to shorten the standard 6-month treatment duration by approximately 2 months [69, 75]. Neither PA-824 nor OPC-67683 appears to have significant interactions with the cytochrome P450 enzyme system. Both compounds are orally bioavailable and have pharmacokinetic properties consistent with once daily or less frequent dosing.

Diarylquinoline - TMC207 (R207910) Diarylquinoline TMC207 (R207910) has a unique mode of action that targets mycobacterial ATP synthase subunit C. It shows significant *in vitro* potency against both drug sensitive and MDR strains of *M. tuberculosis* with MICs 0.03-0.12μg/ml. In the non-established infection mouse TB model, a single dose of

50mg/kg had a bacteriostatic effect, and a bactericidal effect was observed at 100mg/kg. It has a linear pharmacokinetics with a T_{max} of 5.5 h and a long terminal elimination half-life. It is metabolized by cytochrome P450 3A4, leading to 50% reductions in its plasma levels when coadministered with RMP. Oral once-daily administration has bactericidal activity at a dose of 400mg as monotherapy for 7 days in patients with pulmonary TB. Phase 2a EBA study was completed and the results show that TMC207 is less effective than RIF or INH. The compound is in phase 2 of clinical development, to evaluate it for safety and its ability to improve the efficacy of MDRTB treatment when combined with an optimized regimen of the second-line drugs [14, 69, 76]

Ethylene diamine SQ109 is a novel 1, 2-ethylene-diamine-based ethambutol analog. Its mode of action appears to involve inhibition of cell wall synthesis and it has a distinct mechanism compared with ethambutol [77]. SQ109 has excellent *in vitro* activity against both drug-sensitive, single-drug-resistant, and MDR *M. tuberculosis* strains with a MIC range of 0.16 to 0.64 mg/L. Moreover, SQ109 by itself at 10 mg/kg was able to reduce the number of lung CFU by over 1.5 to 2 $\log_{10}$ in a chronic mouse model of TB, activity that was similar to monotherapy with EMB at 100 mg/kg [74]. At 1 mg/kg and 10 mg/kg demonstrated activity similar to EMB at 100 mg/kg, but less activity than INH at 25mg/kg. In a mouse chronic infection model, INH, RMP, and SQ109 (SQ109 at 10 mg/kg) cleared lungs of bacilli faster than INH, RMP and EMB during the first 8 weeks of treatment. In pharmacokinetic studies C_{max} obtained by p.o. dosing of 100mg is 3.35± 2.35mg/L, after 200mg- 20±19.9 mg/L, after 300mg- 61.1± 29.4 mg/L [78-81]. The compound is metabolized by CYP2D6 and CYP2C19 in human liver microsome assays. To date, the drug has been evaluated in a phase 1 study [58, 72, 76].

Pyrrole- LL-3858 This agent is a pyrrole derivative with unknown action and demonstrated *in vitro* synergy with RMP. Its MIC *in vitro* against *Mycobacterium tuberculosis* H37Rv has been reported to be 0.12 - 0.25µg/ml. MIC90 0.125-0.25µg/ml. LL-3858 has *in vitro* synergy with RIFand is more effective than INH. Three months treatment with LL3858 as a monotherapy leads to culture negativity in some mice based on a recent publication about this drug, it is now in phase 1 trial in India [58, 69, 75].

Competitive Advantage The discovery of rifacinna as a promising drug is one good example of such attempts. Several studies with this drug have shown its value against *Mycobacterium tubercuosis* and members of the MAC. In pre-clinical studies, Rifacinna showed high activity *in vitro* (against Mycobacteria (*M. tuberculosis, M. Avium, M. leprae* with a hight MIC ranges (0.16-0.63mg/L). Rifacinna possesses high intraphagocytic activity against MAC strains (0.06-0.125 mg/L).

In *in vivo* studies the activity of T_9 was much superior to RMP even with 10mg/kg and 5mg/kg doses. Preliminary pharmacological studies in animals showed that Rifacinna has good oral bioavailability, long half-life - after a dose of 10mg/kg the plasma Cmax was 9.33 mg/L with a T_{max} of 4.93h and T1/2 of 34.75h [5]. As a monotherapy rifacinna demonstrates equivalent *in vivo* activity in mice at doses of 10mg/kg compared to that of RMP at 80mg/kg. Drug concentrations in serum maintain a level well above the MICs for mycobacteria for the entire time and provide substantial pharmacological effect. These data suggest that Rifacinna achieves a durable and effective drug concentration at several important sites of *M. tuberculosis* infection.

The first in-man clinical evaluation of rifacinna with substitution of RMP (10 mg/kg) with rifacinna (10 mg/kg) in a regimen containing ETB, INH and PZA results in better clinical efficacy, decreasing the time to standard of care effects when analysis is done at 1st or 2nd month.

The promising activity of rifacinna demonstrated in this series of studies warrants further evaluation in order to (i) test regimens that combine rifacinna with first-line and second- line antituberculosis drugs to determine whether it has such a potential to further shorten the duration of therapy, (ii) test regimens that combine Rifacinna with other drugs active against MDR TB, and (iii) test Rifacinna in combinations in a models of latent MDR TB infection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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