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Original article

Furoxan derivatives demonstrated *in vivo* efficacy by reducing *Mycobacterium tuberculosis* to undetectable levels in a mouse model of infection

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ABSTRACT

Objectives: The most recent survey conducted by the World Health Organization described Tuberculosis (TB) as one of the top 10 causes of death and the leading cause of death from a single infectious agent. The increasing number of TB-resistant cases has contributed to this scenario. In light of this, new strategies to control and treat the disease are necessary. Our research group has previously described furoxan derivatives as promising scaffolds to be explored as new antitubercular drugs. **Results:** Two of these furoxan derivatives, **(14b)** and **(14c),** demonstrated a high selectivity against *Mycobacterium tuberculosis*. The compounds **(14b)** and **(14c)** were also active against a latent *M. tuberculosis* strain, with MIC_{90} values of 6.67 μM and 9.84 μM, respectively; they were also active against monoresistant strains (MIC_{90} values ranging from 0.61 to 20.42 μM) and clinical MDR strains (MIC_{90} values ranging from 3.09 to 42.95 μM). Time-kill experiments with compound **(14c)** showed early bactericidal effects that were superior to those of the first- and second-line anti-tuberculosis drugs currently used in therapy. The safety of compounds **(14b)** and **(14c)** was demonstrated by the Ames test because these molecules were not mutagenic under the tested conditions. Finally, we confirmed the safety, and high efficacy of compounds **(14b)** and **(14c)**, which reduced *M. tuberculosis* to undetectable levels in a mouse aerosol model of infection. **Conclusion:** Altogether, we have identified two advanced lead compounds, **(14b)** and **(14c),** as novel promising candidates for the treatment of TB infection.

1. Introduction

Mycobacterium tuberculosis is the primary causative agent of tuberculosis (TB) in humans. In 2018, 10.0 million people developed TB worldwide. In the same year, TB caused an estimated 1.2 million deaths among HIV-negative people and 251,000 deaths among HIV-positive people [1]. Furthermore, over the past decade, there has been an alarming increase in multidrug-resistant (MDR), extensively drug-resistant (XDR), and totally drug-resistant (TDR) cases of TB [2–4]. There is also an alarming concern regarding the high incidence of TB-HIV co-infection, in which treatment with first-line drugs such as rifampicin presents a great challenge because of drug-drug interactions with anti-HIV drugs [5]. As noted by the World Health Organization (WHO) new drugs that could simplify and/or shorten the treatment of TB would considerably improve TB control programs [6]. Hence, there is an urgent need to develop compounds that have good safety profiles and are active against drug-resistant strains of *M. tuberculosis*.

Bedaquiline (BDQ), approved by the U.S. Food and Drug Administration in 2012, was the first drug available for treatment of MDR-TB after more than five decades [7]. Since then, two others nitroimidazole drugs delamanid and pretomanid have been exhibited activity against MDR-TB [8]. Specifically, the combination of pretomanid with BDQ and linezolid for the treatment of a specific type of highly treatment-resistant tuberculosis have shown high efficacy [9]. In

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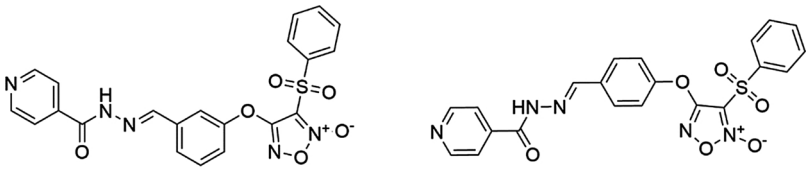

14b 14c

Fig. 1. Chemical structure of compounds 14b and 14c.

addition, several drug candidates have advanced from preclinical stages to clinical trials, including sutezolid, SQ109, PBTZ-169, Q203, TBA-7371 and GSK 070 [10–12]. Nevertheless, there is still a troubling discrepancy between the number of new compounds discovered and their advance to preclinical development stages [13].

Heterocyclic *N*-oxides have emerged as a promising class of agents to treat a plethora of diseases and disorders, especially infectious diseases [14]. We have identified and characterized furoxan and benzofuroxan derivatives as new classes of antitubercular bactericidal agents that are active against drug-sensitive and drug-resistant TB strains with confirmed *in vivo* efficacy in murine models [15–17]. These compounds were identified using the phenotypic whole-cell screening approach [18–20].

In the present work, the anti-*M. tuberculosis* of these furoxan derivatives (**14b**) and (**14c**) (Fig. 1) was investigated against a wide variety of drug-resistant clinical isolates. Moreover, the *in vivo* efficacy in a murine model of acute *M. tuberculosis* infection was characterized in order to comprehend the potential of these compounds as drug candidates useful to treat TB infection.

2. Materials and methods

Chemistry. The compounds **(14b)** and **(14c)** were obtained according to previously described methods [15,17,20].

Latent *M. tuberculosis* minimum inhibitory concentrations (MIC_{90}) - LORA (Low Oxygen Recovery assay). The cultures were thawed, diluted in Middlebrook 7H12 broth using 7H12 medium (Middlebrook 7H9 broth, 1 mg/mL Casitone, 5.6 g/mL palmitic acid, 5 mg/mL bovine serum albumin, and 4 g/mL filter-sterilized catalase), and sonicated for 5 s. Two-fold serial dilutions of compounds **(14b)**, **(14c)**, and standard drugs were prepared in a volume 100 µL in white 96-well microtiter plates, and 100 µL of the cell suspension (5×10^5 CFU/mL) was added. The microplate cultures were placed under anaerobic conditions (oxygen concentration less than 0.16 %) by using an Anoxomat model WS-8080 (MART Microbiology) to complete two cycles of evacuation followed by filling with N_2 (10 % H_2, 5% CO_2). An anaerobic indicator strip was placed inside the chamber to confirm the anaerobic conditions. The plates were incubated at 37 °C for 10 days and then transferred to an ambient gaseous condition (5% CO_2-enriched air) incubator for 28 h. On day 11, we determined the luminescence [21]. The MIC_{90} values were defined as the lowest concentration causing a reduction in luminescence of 90 % relative to that of the controls. Samples were analyzed in three independent assays.

Activity of compounds against *M. tuberculosis* H37Rv isogenic monoresistant strains and clinical isolates (CI). MIC_{90} values were determined against *M. tuberculosis* H37Rv isogenic strains monoresistant to rifampin (ATCC 35,838); isoniazid (ATCC 35,822); streptomycin (ATCC 35,820); capreomycin, moxifloxacin, and bedaquiline (*University of Illinois at Chicago - Institute for Tuberculosis Research*) *via* microdilution technique REMA [22]. The MIC_{90} values were determined against four multi-drug resistance (MDR-TB) or extensively-drug resistance (XDR-TB) clinical isolates [23] by the same methodology. The plates were covered, sealed, and incubated at 37 °C and a 5% CO_2 atmosphere. After 7 days of incubation, 30 µL of resazurin solution was added to each well, incubated overnight at 37 °C and a 5% CO_2 atmosphere, and assessed for color development. The MIC_{90} values were defined as the lowest concentration causing a reduction in 90 % of fluorescence relative to that of controls. Samples were analyzed in three independent assays.

Spectrum of activity. The furoxan derivative compounds **(14b)** and **(14c)** as well as standard drugs were diluted in specific culture media according the microorganism. The maximum concentration of furoxan derivative compounds was 200 µM. The plates were covered, sealed in plastic bags, and incubated at 37 °C and a 5% CO_2 atmosphere. MIC_{90} values against *Escherichia coli* (ATCC 25,922) and *Staphylococcus aureus* (ATCC 29,213) were determined by measuring the optical density at 570 nm (OD_{570}) after 16 h of incubation in Mueller-Hinton II broth (Becton Dickinson, Sparks, MD, USA) and against *Candida albicans* (ATCC 10,231) at 570 nm after 48 h in Cellgrow RPMI 1640 medium (Mediatech Inc., Manassas, VA, USA). The MIC was defined as the lowest concentration resulting in 90 % reduction in absorption relative to that of untreated control cultures.

Mutagenicity (Ames test). The Ames test was performed according to the preincubation methodology developed by Maron & Ames (1983) [24] with different concentrations of the compounds **(14b)** and **(14c)** solubilized in DMSO. Concentrations ranged from 6.25 µg – 50 µg per plate. These concentrations were selected based on preliminary toxicity tests. A total of 0.5 mL of 0.2 M phosphate buffer and 0.1 mL bacterial culture were added to the compounds and then incubated at 37 °C for 20 – 30 min. Then, 2 mL of top agar supplemented with histidine and biotin traces was added to the mixture. It was homogenized lightly and plated on minimal glycoside medium. After solidification of the top agar, the plates were incubated at 37 °C for 48 h. After this period, the revertant colonies were counted with the aid of the Synbiosis ProtoCOL. The mutagenic index (MI) was also calculated for each concentration tested, and this was defined as the average number of revertants per test plate divided by the average number of revertants per negative (solvent) control plate. A sample was considered mutagenic when a dose–response relationship was detected and MI ≥ 2 at one or more concentrations [25]. The assay was performed in triplicate.

Time-Kill assay. Time-kill experiments were performed for up to 15 days to evaluate the bactericidal profile of compound **(14c)**. A *M. tuberculosis* H37Rv (ATCC27294) inoculum (6.73 ± 0.18 $Log_{10}CFU.mL^{-1}$) was challenged with 2 x MIC of the antibiotics and **(14c)** compound (Concentrations were: 0.72 µM for Isoniazid (INH), 0.01 µM for rifampicin (RMP), 0.88 µM for moxifloxacin (MOX), and 2.06 µM for compound **(14c)**). Every 48 h, an aliquot was collected, diluted, and seeded in solid medium to count $CFU.mL^{-1}$ for 15 days [26].

REMA (*Resazurin Microtiter Assay)* under three different culture media conditions. The MIC_{90} values were determined based on the REMA protocol [22] with the adjustment of providing three different conditions in each experiment. These conditions included: (a) adjusting the culture medium to pH 6.0, (b) including 4% bovine serum albumin (BSA), and (c) supplementing with 10 % fetal bovine serum (FBS). The slightly acidic pH (pH 6.0) was selected because the pathogen can replicate and survive inside macrophages, and the interiors of macrophage phagolysosomes are pH 6.0. Albumin (synthesized in the liver) is the primary protein responsible for the transportation of poorly soluble molecules (both endogenous and exogenous) in the body [27]. In addition, albumin binding constitutes an essential pharmacological parameter that affects the mechanism of action (MOA) of antibiotics in

humans. We utilized FBS because it serves as a growth factor for mammalian cells and might interfere with the antitubercular action of some compounds.

Nanostructured lipid system (ME) preparation. Based on previous data [15] about the low chemical stability of these furoxan derivatives at low pH values (pH = 1.2; mimicking stomach pH); in order to ensure greater stability, compounds **(14b)** and **(14c)** were incorporate in a microemulsion system (ME). The system was prepared from a mixture of soy phosphatidylcholine, sodium oleate, and Eumulgin HRE 40 (polyoxyl 40 castor oil-hydrogenated) in proportion 3:6:8, as surfactant, cholesterol as oil phase, and 50 mmol L^{-1} phosphate buffer 7.4 as aqueous phase. The point chosen for the present study consisted of 10 % oil phase, 10 % surfactant, and 80 % aqueous phase as described as previously [28]. The mix was sonicated with the aid of a sonicator (Q700 from QSonica) with a power of 700 W (operating discontinuously) and amplitude of 15 % for 10 min. A 30 s break was allowed every 2 min, and an ice bath was applied throughout the sonication process. Then, the MEs were centrifuged at 11,180 (x g) for 15 min for removing the titanium residues released during the sonication process. The compounds **(14b)** and **(14c)** as well as a standard drug (RMP) were incorporated in ME at the desired concentrations for the *in vivo* experiments by mass solubilization at the respective volume and sonicated for 3 min in the batch mode with 15 % amplitude.

***In vivo* assays.** The experiments were approved by the Ethics Committee Protocol of UNESP (protocols: 09/2014 and 10/2014) and all experiments were performed in accordance with relevant guidelines and regulations. Female BALB/c mice (6–8 weeks; 20 g) were maintained in polycarbonate cages at 23 ± 2 °C, under a 12 -h light/dark cycle in specific pathogen-free conditions. They were provided with food and water *ad libitum*.

Tolerability test in BALB/c mice and toxicological analysis. The tolerability and toxicological analyses of furoxan derivative compounds **(14b)** and **(14c)** were performed with female BALB/c mice (6–8 weeks; 20 *g*) using the following eight experimental groups (n = 4 animals/group): ME group: microemulsion control group; RMP-ME group: standard drug in microemulsion vehicle; CMC group: CMC (carboxymethylcellulose) (Sigma) vehicle; RMP-CMC group: RMP was administered with CMC as the vehicle; Placebo Group: this group received filtered water; Compound **(14b)** group: compound **(14b)** was administered with the vehicle as the ME; and Compound **(14c)** group: compound **(14c)** was administered with the vehicle as the ME. All groups were treated by gavage. Compounds **(14b)** and **(14c)** were administered in 200 μL at a daily oral dose of 200 mg/kg body weight, and the RMP was administered at a concentration of 20 mg/kg body weight. The animals were monitored for 10 days, and the behavioral parameters (Hippocratic screening) were evaluated during these 10 days. At the end of the 10 consecutive days, the animals were weighed, and blood was collected *via* the submandibular vein [29]. The blood was collected in heparinized collecting tubes, and the tubes were centrifuged at 10,000 rpm for 10 min. The plasma was separated and stored at −70 °C. The plasma was evaluated for differences in the levels of liver transaminases (alanine aminotransferase and aspartate aminotransferase) and alkaline phosphatase between the treated and control groups. Then, mice were euthanized in a CO_2 chamber. After euthanasia, the kidneys and liver were surgically removed and placed in cassettes that were stored in 10 % formalin solution for histological analysis.

Histopathology analysis. For histological analysis, the biopsies in cassettes were immediately immersed in 10 % formalin. Then, biopsies in cassettes were kept in a room temperature for 72 h and submitted to routine processing. Serial 6-mm-thick histological sections obtained with a rotary microtome (820 Spencer Microtome, Spencer Products Co., Carson, CA, USA) and samples were stained with hematoxylin and eosin and then evaluated under a light microscope (Olympus BX51, Olympus Optical do Brasil Ltda., São Paulo, SP, Brazil). Histopathology analysis were evaluated by a pathologist blinded for all groups. A descriptive analysis of the histological characteristics of the tissue was carried out, and representative micrographs (64x and 250x magnifications) were obtained for every experimental and control group.

Infection and treatment. Groups of 7 female BALB/c mice (6–8 weeks; 20 g) were infected by aerosol with a low dose (5×10^6 CFU/mL) of *M. tuberculosis* Erdman [30] in an aerosol infection chamber (Glas-Col, Terra Haute, IN). The compounds and RMP group were prepared in microemulsions, and the other RMP control group was prepared by suspension in 0.5 % (wt/vol) carboxymethylcellulose (CMC) such that the target dosages were obtained by once-daily dosing by oral gavage of a 200-μL suspension. Groups of 7 mice were dosed for 5 consecutive days each week. Compound **(14c)** was administered at a daily oral dose (gavage) at a concentration of 200 mg/kg body weight, and the RMP was administered at a concentration of 20 mg/kg. Mice were sacrificed 3 days after the final dose to minimize carryover from the lung homogenates to the plating medium. Both lungs were homogenized and diluted in Hanks' balanced salt solution (HBSS)-Tween, and aliquots were plated on Middlebrook 7H11 medium. CFUs were determined after 3 weeks of incubation at 37 °C.

3. Results

Minimum inhibitory concentration (MIC_{90}) in latent *M. tuberculosis* - LORA (Low Oxygen Recovery Assay). The minimum inhibitory concentration in latent *M. tuberculosis* of **(14b)** and **(14c)** compounds were determined in a microdilution technic after 10 days under anaerobic conditions and then transferred to an ambient gaseous condition. Compounds **(14b)** and **(14c)** were active against latent *M. tuberculosis* strain in a concentration values of 6.67 μM and 9.84 μM (MIC_{90}), respectively. Both compounds were more active than INH (isoniazid; MIC_{90} = > 100 μM) and MTZ (metronidazole; MIC_{90} = > 100 μM). The minimum inhibitory concentration (MIC_{90}) against latent *M. tuberculosis* is shown in Table 1.

Activity of compounds against *M. tuberculosis* H37Rv isogenic monoresistant strains and clinical isolates (CI). We tested both compounds against monoresistant strains (Table 1) and four genetically and phenotypically resistant strains to determine the activity against MDR-TB clinical isolate strains (Table 1). Compounds **(14b)** and **(14c)** were active against INH monoresistant strain with MIC_{90} values of 10.13 μM and 12.77 μM, respectively. RMP monoresistant strain exhibited MIC_{90} values of 1.21 μM and 1.53 μM, respectively. For MOX monoresistant strain the MIC_{90} values found were 1.09 μM and 1.61 μM, respectively). For BDQ monoresistant strain MIC_{90} values were 4.37 μM and 0.61 μM, respectively. For CAP monoresistant strain MIC_{90} values were 8.33 μM and 14.02 μM, respectively. For SM monoresistant strain MIC_{90} values were 11.47 μM and 20.42 μM, respectively. For MOX monoresistant strain MIC_{90} values were 1.09 μM and 1.61 μM, respectively. Compound **(14c)** was also potent against BDQ monoresistant strain with MIC_{90} values of 0.61 μM. Regarding activity of compounds against clinical isolates (CI), compounds **(14b)** and **(14c)** were active against CI 110 with MIC_{90} values of 30.85 μM and 14.74 μM, respectively; CI 104 with MIC_{90} values of 39.08 μM and 13.15 μM, respectively; CI 97 with MIC_{90} values of 7.20 μM and 3.09 μM, respectively and CI 85 with MIC_{90} values of 42.95 μM and 32.81 μM, respectively. For all clinical isolates, compound **(14c)** was more active than compound **(14b)**, which can be evidenced by the results of inhibitory concentration compared to clinical isolate CI104.

Spectrum activity. We selected three different microorganisms: *Escherichia coli* – ATCC 25,922 (Gram-negative), *Staphylococcus aureus* – ATCC 29,213 (Gram-positive), and *Candida albicans* – ATCC 10,231 (fungus) to determine the spectrum activity of furoxan derivatives compounds. Furoxan derivatives **(14b)** and **(14c)** were tested in a maximum concentration of 200 μM. Both compounds were not active for the tested microorganisms. These results indicate a low activity spectrum of these compounds.

Mutagenicity (Ames test). The mutagenicity induced by

Table 1
Overview of the profile of furoxan compounds against *M. tuberculosis* in different conditions. Results are presented in μM.

Class	Compound	Replicant state*	Latency state	Media conditions			Clinical resistant strains				Isogenic monoresistant strains					
				Acid pH	FBS	BSA	CI110	CI104	CI97	CI85	INHr	RMPr	MOXr	BDQr	CAPr	SMr
Furoxan	**14b**	1.61	6.67	0.74	3.54	13.92	30.85	39.08	7.20	42.95	10.13	1.21	1.09	4.37	8.33	11.47
	14c	1.03	9.84	1.20	3.07	9.07	14.74	13.15	3.09	32.81	12.77	1.53	1.61	0.61	14.02	20.42
Standard	RMP	0.5	0.1	–	–	–	0.24	60.75	60.75	2.59	0.01	> 1.00	0.10	0.04	0.21	0.03
Drugs	INH	0.11	> 100	–	–	–	71.46	> 100	> 100	22.77	> 5.0	0.35	0.28	0.23	> 5.00	> 5.00
	LIZ	–	–	–	–	–	–	–	–	–	0.94	0.45	1.06	1.08	3.06	6.9
	AMK	–	–	–	–	–	5.38	2.13	1.19	0.97	0.23	0.12	> 8.00	0.26	0.35	0.36
	PA-824	–	0.97	–	–	–	–	–	–	–	0.05	0.12	0.3	0.38	3.16	1.33
	BDQ	–	0.42	–	–	–	–	–	–	–	0.01	0.01	0.06	1.70	0.06	0.06
	CAP	–	–	–	–	–	–	–	–	–	–	–	–	–	60.46	1.72
	SM	–	–	–	–	–	–	–	–	–	–	–	–	–	2.55	> 100
	MET	–	> 100	–	–	–	–	–	–	–	–	–	–	–	–	–
	AMK	–	–	–	–	–	5.38	2.13	1.19	0.97	–	–	–	–	–	–
	MOX	–	–	–	–	–	7.75	3.21	6.8	2.22	–	–	–	–	–	–
	OFLO	–	–	–	–	–	36.94	61.63	> 100	2.52	–	–	–	–	–	–
	GAT	–	–	–	–	–	4.31	1.01	7.35	0.67	–	–	–	–	–	–

Abbreviations: (INHisoniazid; (RMPrifampicin; (MTZmetronidazole; (BDQBedaquiline; AMK (amikacin; GAT (gatifloxacin; (RMPrifampicin; KAN kanamycin; OFLO (ofloxacin; (MOXmoxifloxacin; (CAPcapreomycin; (INHrisoniazid resistant; (RMPrrifampicin resistant; (MOXrmoxifloxacin resistant; (BDQrBedaquiline resistant; (CAPrcapreomycin resistant and (SMr) streptomycin resistant. Acid pH: 6.0. FBS: 10 % fetal bovine serum. BSA: 4% bovine serum albumin. Dash (-) means not determined. *previous result from Fernandes et al., 2016 [15].

Table 2
Mutagenic activity expressed as the mean and standard deviation of the number of revertants and mutagenicity index (MI) (in parentheses) of strains TA98, TA100, TA102, and TA97a of *Salmonella typhimurium* exposed to different concentrations of compound **(14b)** with (+S9) and without (-S9) metabolic activation.

Treatment Revertant Colonies Number (M ± SD)/plate e MI								
μg/plate	TA 98		TA 100		TA 102		TA 97[a]	
	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9
0.0[a]	19 ± 1	16 ± 4	91 ± 18	82 ± 11	255 ± 24	228 ± 38	139 ± 11	143 ± 19
31.2	19 ± 3 (1.00)	21 ± 4 (1.33)	114 ± 19 (1.25)	109 ± 27 (1.34)	267 ± 30 (1.05)	205 ± 30 (0.90)	166 ± 25 (1.19)	168 ± 23 (1.17)
62.5	19 ± 2 (1.00)	21 ± 4 (1.28)	103 ± 16 (1.13)	113 ± 33 (1.39)	295 ± 26 (1.16)	154 ± 21 (068)	153 ± 13 (1.10)	157 ± 38 (1.09)
125	17 ± 4 (0.89)	21 ± 3 (1.31)	99 ± 11 (1.08)	100 ± 24 (1.23)	200 ± 23 (0.78)	–	133 ± 32 (0.96)	117 ± 34 (0.82)
C+	772 ± 122[b]	687 ± 35 [e]	1996 ± 53[c]	1884 ± 91[e]	2267 ± 95[d]	2421 ± 103[f]	654 ± 66[b]	867 ± 22 [e]

*P < 005 (ANOVA); **P < 001 (ANOVA), M ± SD = mean and standard deviation;
[a] Negative Control: dimethyl sulfoxide (DMSO - 100 μL/plate); C+ = Positive Control:
[b] 4 -nitro-o-phenylenediamine (NOPD – 100 μg/ plate – TA98, TA97a).
[c] Sodium Azide (125 μg/ plate – TA100).
[d] Mitomycin (0,5 μg/plate – TA102), S9 absence and.
[e] 2-anthramine (125 μg/plate – TA97a, TA98, TA100);
[f] 2-aminofluoren (100 μg/ plate – TA102), in the presence of S9.

Table 3
Mutagenic activity expressed as the mean and standard deviation of the number of revertants and mutagenicity index (MI) (in parentheses) of strains TA98, TA100, TA102, and TA97a of *Salmonella typhimurium* exposed to different concentrations of compound **(14c)** with (+S9) and without (-S9) metabolic activation.

Treatment Revertant Colonies Number (M ± SD)/plate e MI								
μg/plate	TA 98		TA 100		TA 102		TA 97[a]	
	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9
0.00[a]	13 ± 4	22 ± 4	133 ± 27	133 ± 26	398 ± 12	474 ± 26	102 ± 4	86 ± 10
6.25	18 ± 4 (1.4)	27 ± 3 (1.2)	159 ± 13 (1.2)	150 ± 35 (1.1)	399 ± 5 (1.0)	530 ± 19 (1.1)	119 ± 19 (1.2)	106 ± 22 (1.2)
12.5	16 ± 2 (1.3)	27 ± 4 (1.2)	160 ± 8 (1.2)	136 ± 47 (1.0)	400 ± 31 (1.0)	555 ± 22 (1.2)	120 ± 13 (1.2)	86 ± 12 (1.0)
25	16 ± 6 (1.4)	24 ± 6 (1.1)	173 ± 39 (1.3)	187 ± 53 (1.4)	403 ± 20 (1.0)	466 ± 25 (1.0)	119 ± 20 (1.2)	100 ± 8 (1.1)
37.5	15 ± 2 (1.2)	22 ± 5 (1.0)	159 ± 29 (1.2)	159 ± 14 (1.2)	400 ± 13 (1.0)	464 ± 12 (1.0)	111 ± 10 (1.1)	82 ± 27 (0.9)
50	14 ± 9 (1.1)	22 ± 3 (1.0)	152 ± 27 (1.1)	132 ± 21 (1.0)	363 ± 38 (0.9)	415 ± 18 (0.9)	108 ± 16 (1.0)	81 ± 21 (0.9)
C+	1183 ± 71[b]	1248 ± 94[e]	1462 ± 22[c]	1504 ± 29[e]	1067 ± 74[d]	1126 ± 90[f]	1294 ± 54[b]	1532 ± 75[e]

*P < 005 (ANOVA); **P < 001 (ANOVA), M ± SD = mean and standard deviation.
[a] Negative Control: dimethyl sulfoxide (DMSO - 100 μL/plate); C+ = Positive Control.
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[f] 2-aminofluoren (100 μg/ plate – TA102), in presence of S9.

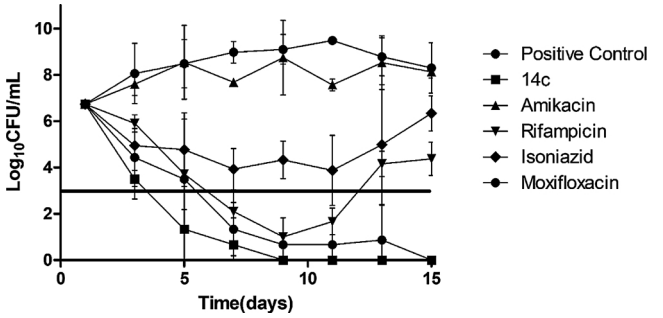

Fig. 2. Time − kill curves of compound **(14c)**, rifampicin, isoniazid, and moxifloxacin. Results are in $\log_{10}$ CFU/mL of *M. tuberculosis* H37Rv (ATCC 27,294) according to time (days). The CFU count was determined as the mean of three independent assays. The concentrations of the compounds were 0.72 μM for isoniazid, 0.01 μM for rifampicin, 0.88 μM for moxifloxacin, and 2.06 μM for compound **(14c)**. Bars: mean ± SD. The line indicates the compounds with bactericidal activity below 3 logs of reduction.

compounds **(14b)** and **(14c)** were determinate through Ames test at concentrations ranging from 6.25 μg – 50 μg per plate, according previous studies. The mutagenic potential of compounds **(14b)** and **(14c)** was characterized using strains TA98, TA100, TA102, and TA97a of *Salmonella typhimurium* exposed to different concentrations of compounds with (+S9) and without (-S9) metabolic activation. The Ames test is one of the mutagenic assay preconized by regulatory agencies such as FDA (Food and Drug Administration), which aims to ensure the safety of compounds. A compound is considered mutagenic when values of MI ≥ 2 at one or more concentrations [25]. In our experiment, both compounds did not demonstrate mutagenic potential (Table 2 and Table 3).

Time-Kill assay. Time − kill experiments were performed in order to evaluate the bactericidal profile of compound **(14c)** during 15 days. Compound **(14c),** at concentration of 2.06 μM, decreased the CFU about 5 Log_{10} compared to initial inoculum concentration after 5 days of exposure. For this compound an early-bactericidal activity was observed. After 15 days of exposure, compound **(14c)** at concentration of 2.06 μM, showed bactericidal activity similar to that of MOX at 0.88 μM (Fig. 2).

REMA (*Resazurin Microtiter Assay)* in three different culture media conditions. The MIC_{90} values were determined under three different conditions in each experiment according REMA protocol [22]. In normal conditions, compounds **(14b)** and **(14c)** showed MIC_{90} values of 2.05 μM and 1.63 μM respectively. When the culture media were modified to pH 6.0 and 10 % fetal bovine serum (FBS), no changes in MIC were observed. However, when 4% bovine serum albumin (BSA) was add, MIC decreased, probably due a high affinity for binding to albumin [27].

Tolerability test in BALB/c mice and toxicological analysis. The tolerability and toxicological analyses were determinate after oral administration of furoxan derivatives **(14b-ME)** and **(14c-ME)** at 200 mg/Kg. RMP, used as drug control, was administered by oral route at 20 mg/Kg. The animals, monitored over 10 days, did not exhibit modifications in the behavioral parameters (Hippocratic screening). The levels of liver transaminases (alanine aminotransferase and aspartate aminotransferase) and alkaline phosphatase was monitored in the treated and control groups after blood collected *via* the submandibular vein [29]. It was not observed differences between both groups (Results in ***Supplemental Material***). After euthanasia, kidneys and liver were surgically removed, processed and the tissues analyzed microscopically. Through histopathology analysis, it was not found differences between both groups (Results in ***Supplemental Material***).

Infection and treatment. The *in vivo* anti-*M. tuberculosis* efficacy for compounds **(14b-ME)** and **(14c-ME)** were shown in Fig. 3. After only 15 doses (5 consecutive days each week), compounds **(14b-ME)**

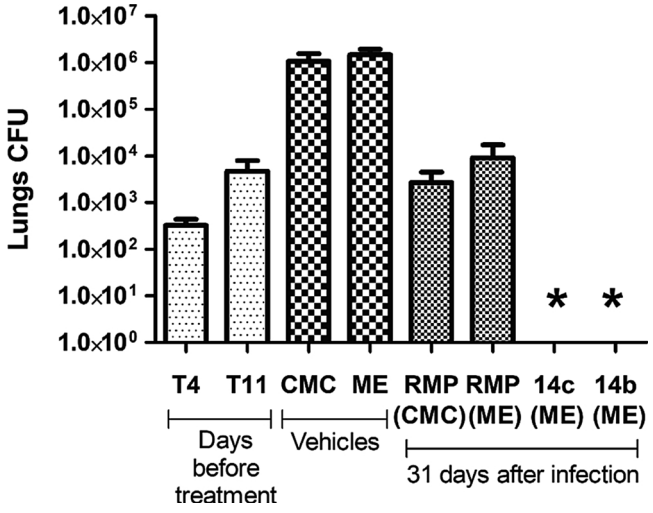

Fig. 3. Efficacy of compounds **(14b)** and **(14c)** against acute TB mice model. Compounds were incorporated in microemulsion (Dose = 200 mg/kg) and controls administrated once-daily by oral gavage of a 200 μL suspension. Female 20 g BALB/c mice were infected by aerosol with a low dose (5×10^6 CFU/mL) of *M. tuberculosis* Erdman. The treatment started at 10 days post infection and terminated at 29 days post infection. Groups of 7 mice were dosed for 5 consecutive days per week. CFU were determined, after 3-day washout period, at day 31 post infection. The lungs were homogenized and diluted in Hanks' balanced salt solution (HBSS)-Tween, and aliquots were plated on Middlebrook 7H11 medium. Bars: S.D. *undetectable mycobacteria levels.

and **(14c-ME)** were able to decrease CFU to undetectable numbers in the lungs of mice at 200 mg/Kg. The standard drug RMP were administered in the preconized dose of 20 mg/Kg body weight and decreased the CFU in only 2 $\log_{10}$. Among the drugs used to treat tuberculosis, none show this rapid bactericidal action or decrease the number of bacilli in the lungs of *M. tuberculosis* infected mice to undetectable levels as demonstrated by the compounds **(14b-ME)** and **(14c-ME).**

4. Discussion

Currently, TB is a public health problem. Although the present treatment regimen for drug-sensitive TB is highly effective, the increase in MDR-TB and XDR-TB cases is contributing to the difficulty of controlling the disease [31].

The ability of *M. tuberculosis* to remain in a latent state is likely a major factor precluding the efficacy of antibiotic therapy and contributing to the development of resistance [32,33]. In this present work, the tested compounds were active against latent *M. tuberculosis* with MIC_{90} values of 6.67 μM and 9.84 μM, for compounds **(14b) and (14c)**, respectively. The search for active compounds against mycobacteria in latent states is important, once it provides the possibility of treating patients who are carriers of *M. tuberculosis*. These furoxan derivative compounds were more potent when tested against latent *M. tuberculosis* than: indolcarboxamide ($\text{MIC}_{90} > 27.00$ μM) [34], a potential preclinical candidate for the treatment of TB. Also, both derivatives furoxans were more active in latency state than ethambutol and INH ($\text{MIC}_{90} > 1000$) μM [35].

Another desirable feature of a new TB drug is activity against resistant bacteria [2]. Regarding activity of compounds against clinical isolates (CI), compounds **(14b)** and **(14c)** were active against CI 110 with MIC_{90} values of 30.85 μM and 14.74 μM, respectively and CI 97 with MIC_{90} values of 7.20 μM and 3.09 μM. For these isolates, in a previous study [23], no mutations were found in the genes *katG*, *inhA* and *ahpC*, we only found mutations in *rpoB* related to rifampicin with changes from C to G in codon 531 leading to a change from serine to tryptophan in isolate CI97. Clinical isolates CI104 with MIC_{90} values of

39.08 μM and 13.15 μM, respectively, and CI85 with MIC_{90} values of 42.95 μM and 32.81 μM, respectively, showed mutations in the *katG* gene with changes from G to C at codon 315 leading to a change from serine to tyrosine. In the CI104 isolate, the same mutation was found in rifampicin as in the CI97 isolate. First of all, it is noted that the two compounds were active in all isolates with similar MICs, different from the isoniazid that was not active in any concentration. It is very common for the activity against clinical isolates to be less than the standard $H_{37}Rv$, pan-sensitive strain, which was isolated in 1934. The behavior of the compounds in both isolates that had or did not mutate in *katG* (gene responsible for the production of the catalase enzyme that active isoniazid, which is a prodrug) was very similar. This fact added to the stability of the compounds as demonstrated in this work reinforces the idea that these compounds have a different mechanism of action than isoniazid.

Antimicrobials are classified depending on whether they possess a narrow, intermediate or broad spectrum of activity. Compounds **(14b)** and **(14c)** were tested against *S. aureus*, *E. coli*, and *C. albicans*, and no antimicrobial activity was detected.

One of the tests advocated by the FDA regarding the safety of a new drug is the Ames test. Both compounds, **(14b)** and **(14c),** showed no mutagenicity against TA 98, TA100, TA102, or TA97a *Salmonella typhimurium* strains with (+S9) or without (-S9) metabolically active conditions. Furoxan derivatives have been described as non-mutagenic compounds through AMES test [17,36–38]. These data were confirmed in our finds, which revealed that N-oxide moiety, differently of nitro compounds, did not exhibit mutagenic effects.

A time-kill assay of the **(14c)** compound showed a bactericidal effect, and **(14c)** performed better than first-line antibiotics (RMP and INH) already used in TB treatment. Previous experiments have shown that compound **14c** is chemically stable at pHs 7.4 and 9; however, certain instability is observed at acidic conditions (pH < 5), which motivated the formulation in microemulsion in order to protect against acid condition in the stomach after oral administration in this experiment [15]. Additionally, compound **(14c)** showed an effect comparable to moxifloxacin, a second-line antibiotic employed in MDR-TB treatment. Compound **(14c)** reduced the initial inoculum (6.73 ± 0.18 $Log_{10}UFC.mL^{-1}$) sharply until the seventh day and maintained 1 $Log_{10}UFC.mL^{-1}$ until the fifteenth day. This prolonged effect is advantageous; extrapolating to future *in vivo* applications, this new compound could be administered in a large dose range.

In the presence of FBS (fetal bovine serum), compound (**14c)** showed a slight decrease in activity compared to normal conditions. This decrease in activity was even greater in the presence of 4% albumin. Compared to normal conditions, the MIC value was higher. It is likely that albumin decreases activity against bacteria by interacting with compound (**14c).** These results suggested that it would be necessary to find a way to make this compound more available *in vivo*. Therefore, in *in vivo* assays, we incorporated the compound into a nanostructured lipid system (ME) to increase stability and to preserve these molecules to reach the bloodstream.

For toxicology studies, mice were monitored daily for 10 days. No significant variation in behavior was observed during this period. To probe for potential liver damage, levels of liver transaminases were tested in plasma. No significant differences were observed for alanine aminotransferase, aspartate aminotransferase, or alkaline phosphatase between treated and control groups. No significant differences were observed when compared to the control group (Results in ***Supplemental Material***).

Histology of the liver and kidneys revealed similar morphology when compared with controls groups; all results showed that the treatments did not cause lesions or abnormalities (Results in ***Supplemental Material***).

After developing a nanostructured lipid system (ME) to ensure greater stability and improve solubility, both compounds, **(14b)** and **(14c**) were highly active in an *in vivo* mouse model of infection. No detectable *M. tuberculosis* was observed in the lungs of treated mice. Previously, this level of activity has only been observed using a combination of standard TB drugs [36,39].

5. Conclusion

In conclusion, compounds **(14b)** and **(14c)** are promising lead compounds with MIC_{90} values of 6.67 and 9.84 μM, respectively, against a latent *M. tuberculosis* strain. Both compounds showed no mutagenicity and were active against monoresistant and MDR-TB strains (MIC_{90} = 0.61–32.81 μM). In addition, treatment led to undetectable levels of the bacterium in the lungs of mice. No standard drugs have shown this effect. Altogether, these findings highlight the furoxan derivatives **(14b)** and **(14c)** as novel lead compounds of antitubercular agents with potent activity in a mouse model of infection.

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Declaration of Competing Interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110592.

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