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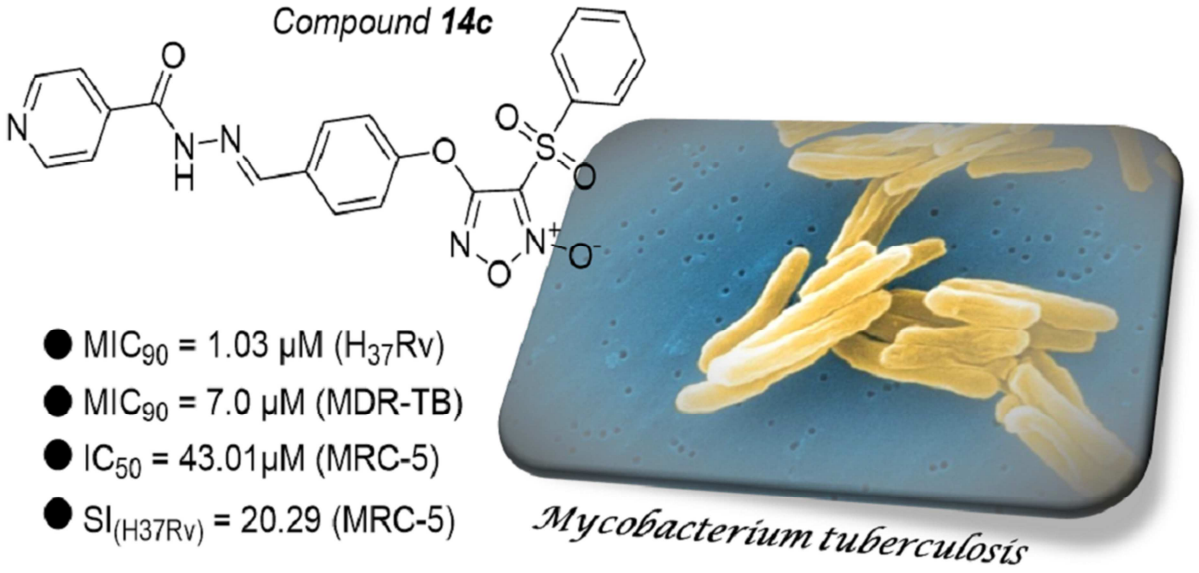

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Synthesis and biological activity of furoxan derivatives against *Mycobacterium tuberculosis*

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Abstract

Tuberculosis (TB) remains a serious health problem responsible to cause millions of deaths annually. The scenario becomes alarming when it is evaluated that the number of new drugs does not increase proportionally to the emergence of resistance to the current therapy. Furoxan derivatives, known as nitric oxide (NO) donors, have been described to exhibit antitubercular activity. Herein, a novel series of hybrid furoxan derivatives (1,2,5-oxadiazole 2-*N*-oxide) (compounds **4a-c**, **8a-c** and **14a-c**) were designed, synthesized and evaluated *in vitro* against *Mycobacterium tuberculosis* (MTB) $H_{37}Rv$ (ATCC 27294) and a clinical isolate MDR-TB strain. The furoxan derivatives have exhibited MIC_{90} values ranging from 1.03 to 62 µM ($H_{37}Rv$) and 7.0 to 50.0 µM (MDR-TB). For the most active compounds (**8c**, **14a**, **14b** and **14c**) the selectivity index ranged from 3.78 – 52.74 (MRC-5 cells) and 1.25 – 34.78 (J774A.1 cells). In addition, it was characterized for those compounds log$P_{o/w}$ values between 2.1 – 2.9. All compounds were able to release NO at levels ranging from 0.16 – 44.23%. Among the series, the phenylsulfonyl furoxan derivatives (compounds **14a-c**) were the best NO-donor with the lowest MIC_{90} values. The most active compound (**14c**) was also stable at different pHs (5.0 and 7.4). In conclusion, furoxan derivatives were identified as new promising compounds useful to treat tuberculosis.

Keywords: furoxan; tuberculosis; phenotypic screening; *Mycobacterium tuberculosis*; antituberculosis agents.

1. Introduction

Tuberculosis, caused mainly by *Mycobacterium tuberculosis* (MTB), is the infectious disease responsible for the largest number of deaths in the world, exceeding even human immunodeficiency virus (HIV). The latest surveys conducted by World Health Organization (WHO) in 2014 showed 9.6 million of new cases around the world and 1.5 million of deaths annually [1]. The emergence of drug resistant strains, including multidrug resistant (MDR), extremely drug resistant (XDR) and the recently cases of totally drug resistant (TDR) increase the challenges to eliminate TB worldwide. Furthermore, WHO estimates that one third of the world population are infected by latent TB [2], whose treatment is unavailable due to the lack of new drugs [3–5].

The current treatment against MTB have shown limitations which include: high toxicity [6–10], drug-drug interactions [11], long-term therapy and low efficacy against resistant strains. After a gap of 50 years without any new antitubercular drugs, bedaquiline (SIRTURO®; Janssen, Beerse, Belgium) was approved by the United States Food and Drug Administration (FDA) for the treatment of MDR-TB; however, resistant strains to this drug are already reported [12]. After bedaquiline, there was a noteworthy increase in the number of papers describing compounds with potent antitubercular activity [13–16].

In order to find new antitubercular drugs, we have established a phenotypic-based screening program with more than five thousand compounds present in our current library. From these data, we have identified (hydroxybenzylidene)isonicotinohydrazide derivatives active against MTB. Specifically, the compound (*E*)-*N*'-(4-hydroxybenzylidene)isonicotinohydrazide (**I**) (**Fig.1**) have exhibited MIC_{90} value of 1.0 µM against MTB $H_{37}Rv$ and selective index against VERO and J774A.1 cell lines

71 superior to 100. Notwithstanding, this molecule did not show antitubercular activity
72 against MDR strains, presenting MIC_{90} values superior to 62 µM.
73 In this work, using the molecular hybridization approach, we designed new analogues of
74 (*E*)-*N*'-(4-hydroxybenzylidene)isonicotinohydrazide (**I**) containing the furoxan moiety
75 [17] (**Fig. 1**). Furoxan derivatives represent an important class of compounds that exhibit
76 a variety of biological activities, such as, antimycobacterial [18], antichagasic [19] and
77 antileishmanicidal [20]. The wide spectrum of biological activities of furoxan derivatives
78 have been associated to its ability to generate nitric oxide after biotransformation [21,22].
79 NO is an important mediator produced by macrophages during MTB infection and has an
80 essential role to eliminate MTB [23]. It has been demonstrated that NO can disrupt
81 bacterial DNA, proteins, signaling mediators, and/or induction of macrophage apoptosis
82 [24]. Nitric oxide is also increased in macrophages during the infection and its inhibition
83 promotes MTB growth [25]. MTB infected mice treated with nitric oxide synthase
84 inhibitors exhibited higher mortality rates and pathological tissue damages compared to
85 control group without treatment [26].
86 Not only endogenous, but also exogenous sources of NO have demonstrated effectiveness
87 to reduce the number of bacilli. Some works have demonstrated that low levels of NO-
88 donors can kill the mycobacteria [27–29]. These data suggested that strategies aiming to
89 raise NO levels seem to be promising as antitubercular therapy. Therefore, in a
90 continuing effort to develop new drug candidates to treat TB infection, we report herein
91 the synthesis, NO-donor release, experimental logP values, antitubercular and cytotoxic
92 activities of furoxan derivatives (**4a-c**, **8a-c**, and **14a-c**) (**Fig. 1**). The antimycobacterial
93 activity against a clinical isolate of MDR strain (resistant to isoniazid, rifampicin,

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streptomycin and ethambutol) was characterized for the most active compounds. Moreover, for the most potent compound, we also studied the chemical stability at different pHs (1.0; 5.0; 7.4 and 9.0).

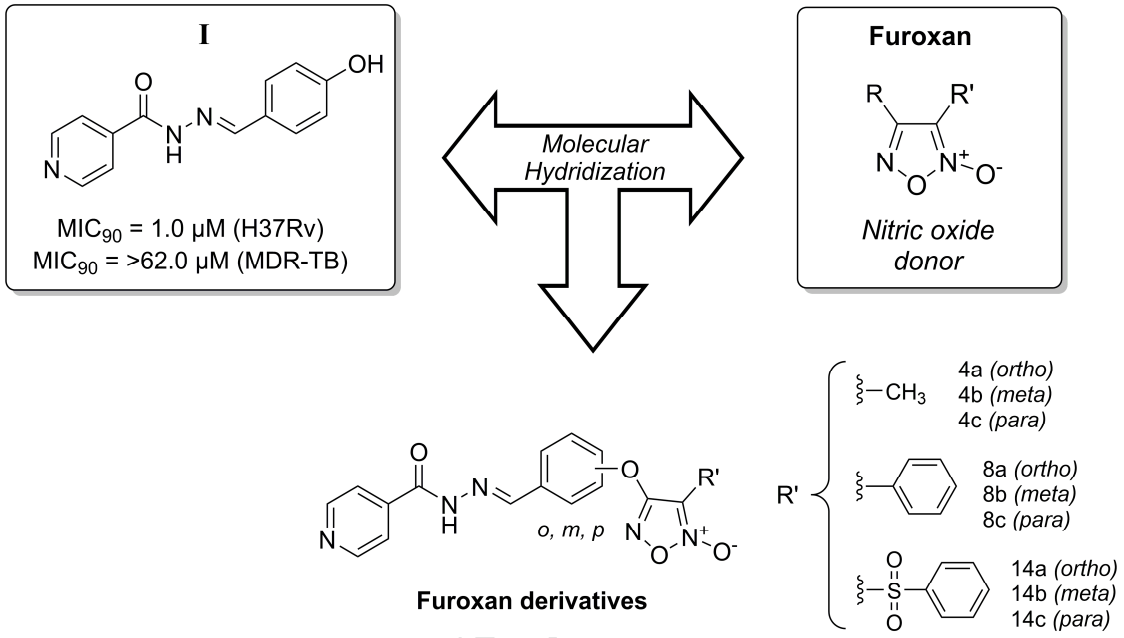


Fig. 1. Design of the hybrid furoxanyl *N*-acylhydrazone derivatives.

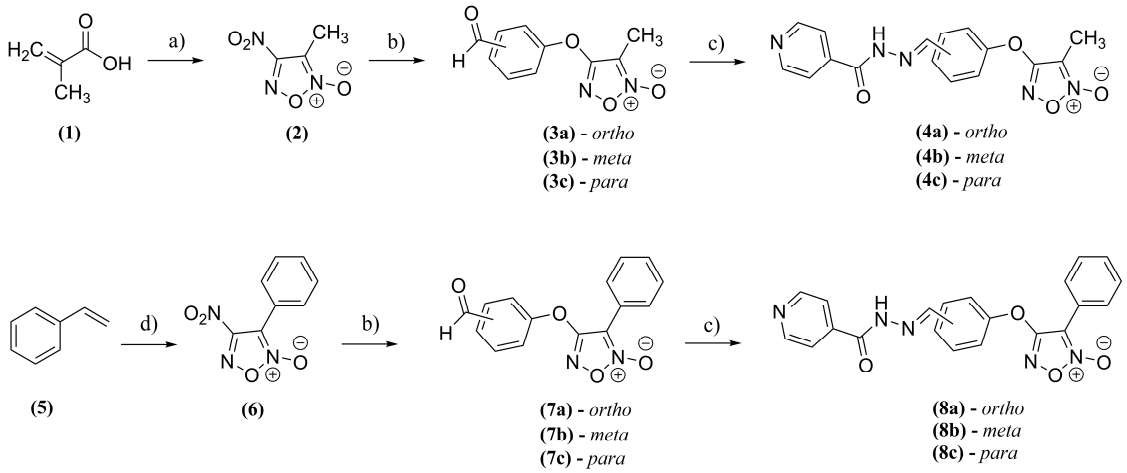
2. Results

2.1. Chemistry

The synthetic routes for the preparation of furoxan derivatives (**4a-c**, **8a-c**, and **14a-c**) derivatives are summarized in **Scheme 1** and **2**.

Compounds **2**, **6**, and **12** were synthesized according to a previously described methodology [20,30–32]. The 2-, 3- or 4-hydroxybenzaldehyde was reacted with compounds **2**, **6**, and **12** in dichloromethane medium, using 1,8-

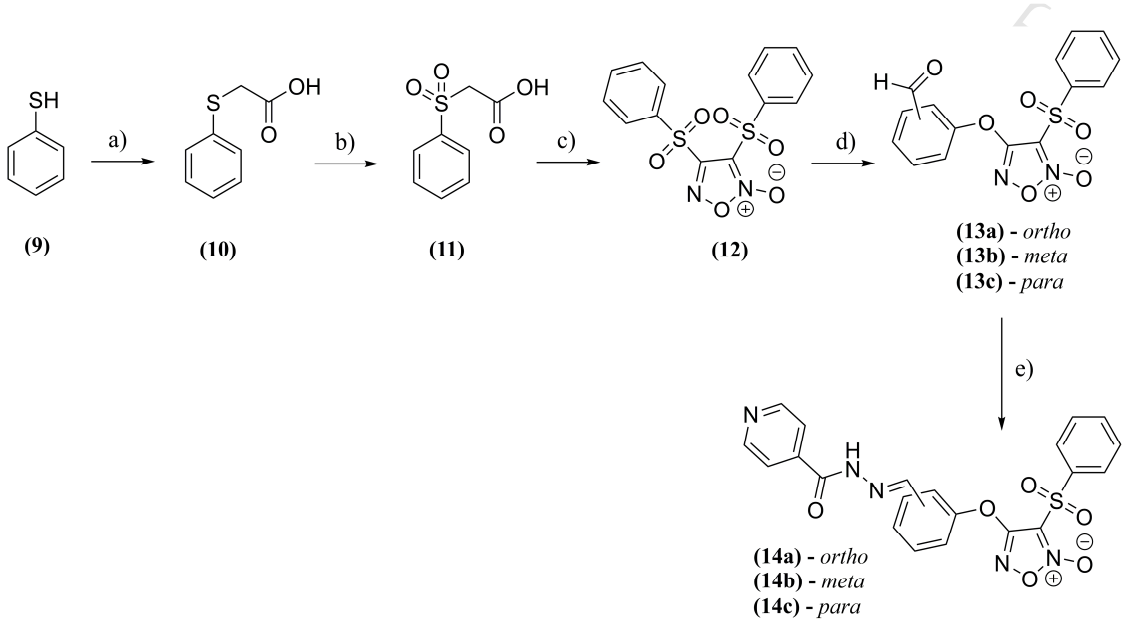
diazabicyclo[5.4.0]undec-7-ene (DBU) as base, to provide the furoxan derivatives **3a-c**, **7a-c**, and **13a-c**, in yields varying between 20% and 67 %.



Scheme 1. Reagents and conditions: **(a)** 1,2-dichloroethane, H_2SO_4 60%, $NaNO_2$, 50 °C, 30 min; **(b)** 2, 3 or 4- hydroxybenzaldehyde, 1,8-diazabicycloundec-7-ene (DBU), anhydrous dichloromethane, r.t., 2 h; **(c)** isonicotinic hydrazide, ethanol, acetic acid, r.t., 12 h; (**d**) acetic acid, hydrochloric acid, dichloromethane, $NaNO_2$, r.t, 12 h.

The last step to obtain all furoxan derivatives (**4a-c**, **8a-c**, and **14a-c**) involves the coupling reaction between aldehyde function present in the furoxan derivatives and isonicotinic hydrazide in order to obtain the target compounds in excellent yields varying between 83% and 95% (**Scheme 1** and **2**). All chemical structures were established by infrared (IR) spectroscopy, elemental analysis and ^{1}H and ^{13}C nuclear magnetic resonance (NMR). The analysis of ^{1}H NMR spectra of all acyl hydrazone derivatives (compounds **4a-c**, **8a-c**, and **14a-c**) have shown a single signal referring to ylidenic hydrogen attributed to the *E*-diastereomer [33–36]. All compounds were also analyzed by

high-performed liquid chromatography (HPLC), and their purity was confirmed to be greater than 98.5%.



Scheme 2. Reagents and conditions: **(a)** monochloroacetic acid, NaOH, H_2O, 110 °C, 3 h; **(b)** hydrogen peroxide 30%, acetic acid, r.t., 24 h; **(c)** fuming nitric acid, acetic acid, 110 °C, 1 h; **(d)** 2, 3 or 4- hydroxybenzaldehyde, 1,8-diazabicycloundec-7-ene (DBU), anhydrous dichloromethane, r.t., 2 h; **(e)** isonicotinic hydrazide, ethanol, acetic acid, r.t., 12 h**.**

2.2. Antitubercular activity

The antitubercular activity of hybrid furoxan derivatives (**4a-c**, **8a-c**, and **14a-c**) and intermediates (**3a-c**, **7a-c**, and **13a-c**) were determined against *Mycobacterium tuberculosis* H_{37}Rv ATCC 27294 and a clinical isolate MDR strain resistant to isoniazid, rifampicin, streptomycin and ethambutol. Among the furoxan intermediates, only compounds from the phenylsulfonyl (**13a-c**) series were active against MTB; the MIC_{90}

for these compounds ranged from 2.89 to 26.01 μM, while the methyl (**3a-c**) and phenyl (**7a-c**) series presented MIC_{90} values superior to 88 μM.

In the assays, hybrid furoxan derivatives (**4a-c**, **8a-c**, and **14a-c**) showed similar biological activity than those exhibited for intermediates (**3a-c**, **7a-c**, and **13a-c**). Phenylsulfonyl (**14a-c**) series were the most activity compounds with MIC_{90} ranging from 1.03 to 8.60 μM. Furthermore, the *para* isomer (compound **8c**) from the phenyl series was also active against MTB with MIC_{90} value of 11.82 μM. Interestingly, in the presence of a nitric oxide scavenger (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide - PTIO) all compounds have shown MIC_{90} superior to 62 μM. This data demonstrates the importance of NO to antitubercular activity for these derivatives.

The four more active compounds (**8c** and **14a-c**) were also evaluated against a clinical isolate MDR strain and have showed MIC_{90} values ranging from 7.0 to 50.0 μM (**Table 2**). Compounds **4a-c** and **8a-b** showed MIC_{90} superior to 62 μM (**Table 1**) and were not considered promising for the determination of cytotoxicity.

2.3. Determination of cytotoxicity

Cytotoxicity studies were performed using two different cell lines: MRC-5 and J774A.1. The selectivity index (SI) represents the ratio between IC_{50} and MIC_{90}. For this assay, the furoxans intermediates (**7c** and **13a-c**) have exhibited cytotoxic effect and low selectivity index against J774A.1 cell line. On the other hand, hybrid furoxan (**8c** and **14a-c**) have shown IC_{50} values ranging from 34.4 to 623.4 μM (MRC-5) and 4.30 to 408.97 μM (J774A.1), respectively. For these compounds, SI values ranged from 3.78 to 52.74 (MRC-5) and 1.25 to 34.78 (J774A.1) (**Table 1**).

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164 *2.4. Nitric oxide release*

165 The nitrite production resulted from the oxidative reaction of nitric oxide, oxygen and
166 water for the hybrid compounds (**4a-c**, **8a-c**, and **14a-c**) was quantified through Griess
167 reaction [37–39]. The results, expressed as percentages of nitrite (NO_2^-; mol/mol), are
168 summarized in **Table 1**. Isosorbide dinitrate (DNS), used as a positive control, induced
169 7.5% of nitrite formation. All furoxan derivatives (**4a-c**, **8a-c**, and **14a-c**) were able to
170 induce nitrite formation at levels ranging from 0.16% to 43.55%.

171

172 *2.5. Partition Coefficient study*

173 The partition coefficients were characterized by HPLC method [40] for all hybrid
174 furoxan derivatives. The log$P_{o/w}$ values of the furoxan derivatives were positive and the
175 values ranged from 1.2 to 2.9 (**Table 1**).

176

177 *2.6. In vitro stability study*

178 Chemical hydrolysis was performed for the most active compound (**14c**) in order to
179 characterize chemical stability at different pHs (1.0; 5.0; 7.4 and 9.0). At extreme pHs
180 (1.0 and 9.0) the compound was unstable. After 1 hour, at pH 1.0, compound **14c** have
181 undergone 90% of degradation; while, for pH 9.0 the compound was reduced by 50%.
182 However, at pH 5.0 and 7.4, the compound **14c** have shown better stability. After 6h, it
183 was not detected significant chemical degradation at pH 5.0; while a reduction of 15% at
184 pH 7.4 was observed. After 24h, a reduction of 20% of compound **14c** was quantified at
185 pHs 5.0 and 7.4 (**Fig. 2**).

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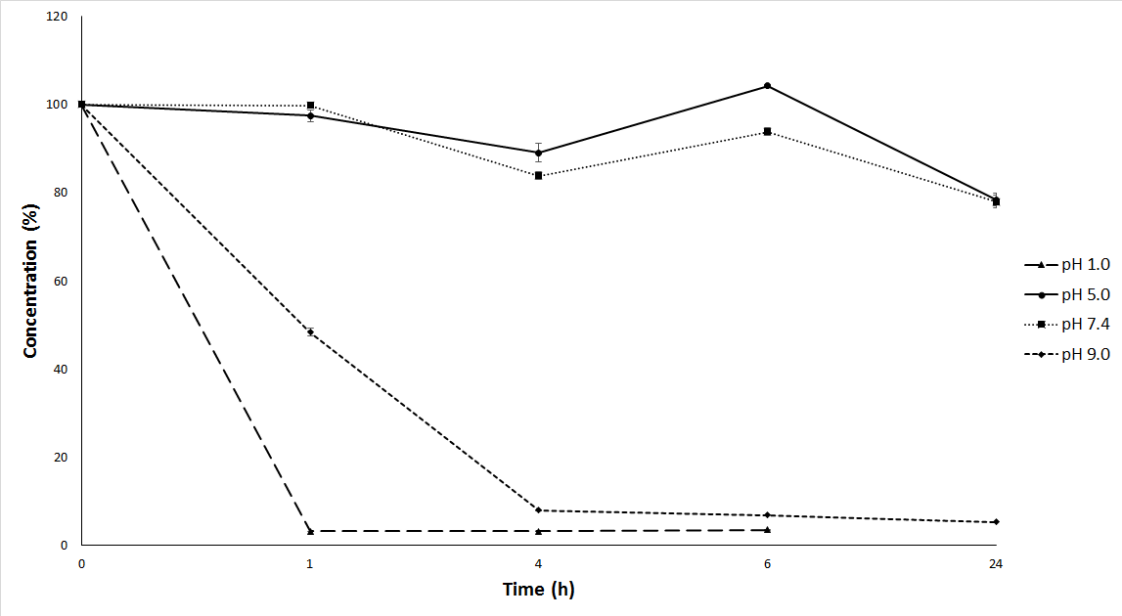

187

188 **Fig. 2.** *In vitro* chemical stability. Hydrolytic profile of compound **14c** in buffer (pH 1.0;

189 5.0; 7.4 and 9.0) (data are represented as means ± SEMs and expressed as %).

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Table 1 Antitubercular activity of compounds against *Mycobacterium tuberculosis* $H_{37}Rv$; cytotoxicity against MRC-5 and J774A.1 cell lines (IC_{50}); selectivity index (SI); NO release data and experimental LogP [d].

Compounds	MIC_{90} (μM) – $H_{37}Rv$	MIC_{90} (μM), [a] $H_{37}Rv$, PTIO	IC_{50} (μM) for MRC-5	SI[1]	IC_{50} (μM) for J774A.1	SI[2]	% NO_2^- (mol/mol), [b, c] $_L$-Cys, 50×10^{-4} M	LogP [d]
Intermediate furoxans								
3a	> 62.0	-	-	-	-	-	0	-
3b	> 62.0	-	-	-	-	-	0	-
3c	> 62.0	-	-	-	-	-	0	-
7a	> 62.0	-	-	-	-	-	25.10 ± 0.07	-
7b	> 62.0	-	-	-	-	-	23.10 ± 0.40	-
7c	> 62.0	-	-	-	-	-	19.44 ± 0.70	-
13a	20.23	-	-	-	7.4	0.4	21.19 ± 4.12	-
13b	20.89	-	-	-	5.6	2	27.05 ± 3.83	-
13c	26.01	-	-	-	2.2	0.1	24.45 ± 3.94	-
Hybrid furoxans								
4a	> 62.0	-	-	-	-	-	0.35 ± 1.71	1.4
4b	> 62.0	-	-	-	-	-	0.16 ± 2.13	1.3
4c	> 62.0	-	-	-	-	-	2.02 ± 1.36	1.3
8a	> 62.0	-	-	-	-	-	11.22 ± 0.5	2.7

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8b	> 62.0	-	-	-	-	-	6.87 ± 0.66	2.9
8c	11.82	> 62.0	623.44	52.74	408.97	34.78	7.33 ± 1.77	2.9
14a	8.60	> 62.0	34.40	3.78	10.75	1.25	44.23 ± 0.81	2.2
14b	1.61	> 62.0	30.10	14.13	4.30	3.00	38.49 ± 4.05	2.3
14c	1.03	> 62.0	43.01	20.29	10.75	11.98	43.55 ± 4.26	2.1
RIF	0.5	-	-	-	-	-	0	-
INH	0.11	-	-	-	-	-	0	-
DNS	-	-	-	-	-	-	7.17 ± 0.54	-

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194 [a] Determined using the REMA methodology [41] in the presence of an equimolar
195 concentration of the PTIO reagent (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-
196 oxide), a nitric oxide scavenger.

197 [b] Mean ± standard error of the mean.

198 [c] Determined by Griess reaction, after incubation for 1 h at 37 °C in pH 7.4 buffered
199 water, in the presence of a 1:50 molar excess of L-cysteine.

200 [d] Determined by partition coefficient (*n*-octanol/water), HPLC method [40].

201 [e] Abbreviations: DNS, isosorbide dinitrate (DNS possesses two ONO_2 groups that may
202 release NO); SI^1, ratio between IC_{50} for MRC-5 and MIC_{90}; SI^2, ratio between IC_{50} for
203 J774A.1 and MIC_{90}; RIF, rifampicin and INH, isoniazid (reference drugs); dash (-) means
204 not determined.

205

206 **Table 2** Antitubercular activity of the most activity compounds against a clinical isolate
207 MDR-TB strain (MIC_{90}).

Compound	MIC_{90} (μM) – MDR-TB [a]
8c	24.3
14a	50.0
14b	21.3
14c	7.0
RIF	Res
INH	Res

208 [a] Resistance to isoniazid, rifampicin, streptomycin and ethambutol [42]. Res, resistant.

209

210 **3. Discussion**

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From a phenotypic-based screening containing more than five thousand compounds present in our current library, we have identified the (*E*)-*N*'-(4-hydroxybenzylidene)isonicotinohydrazide (**I**) (**Fig. 1**), (MIC_{90} = 1 μM), as a promising scaffold for molecular modifications (supplementary material). In our drug design, we have used the molecular hybridization between this compound and furoxan derivatives. The furoxan derivatives were selected due to its anti-mycobacterial effect [18], related in parts, to its ability to release NO after biotransformation [22].

In vivo, NO is produced as a result of cytokines and chemokines stimulation [43]. The antimycobacterial effects of NO were firstly demonstrated in murine macrophage infected with bacilli [44]. Accurately, iNOS -/- mutated mice have exhibited higher susceptibility to MTB infection and early death compared to non-mutated mice [45]. Exogenous NO has been shown as a useful strategy to kill the bacilli. It is established that compounds, such as pretomanid, can kill the MTB by induce NO intracellular after metabolism [46]. Furthermore, different NO-donors such as diethylenetriamine nitric oxide adduct (DETA/NO) [46] and *S*-nitrosothiols [47] are also examples of NO donors presenting antitubercular activity. Nitric oxide, as well as others reactive nitrogen intermediates, can alter mycobacterial DNA by generating abasic sites and strand breaks. Additional NO mycobacterial-induced toxicity include interaction with proteins resulting in enzymatic inactivation and/or structural modifications [48].

In this work, it was characterized that furoxan derivatives methyl (**4a-c**) and phenyl (**8a-b**) did not exhibit activity against MTB. For these compounds, it was found MIC_{90} values superior to 62 μM. However, three isomers from the phenylsulfonyl furoxan series: *ortho* (**14a**), *meta* (14b) and *para* (**14c**) have shown promising activity against MTB with

234 MIC_{90} values below 8.6 µM. Moreover, a phenyl furoxan derivative (**8c**) also exhibited a
235 promising MIC_{90} value of 11.82 µM. The MIC_{90} values of these four compounds (**14a-c;**
236 **8a**) were greater than several first and second line antitubercular drugs, such as
237 pyrazinamide (>48 µM), cycloserine (245 µM) and kanamycin (3.4 µM) [49]. We also
238 evaluated the furoxan intermediates (**3a-c**, **7a-c** and **13a-c**) against MTB $H_{37}Rv$, however,
239 these compounds showed MIC_{90} values superior to 20.0 µM.
240 Our data suggest a direct effect in the pattern of substitution in the furoxan ring and the
241 antitubercular activity. For phenylsulfonyl series (**14a-c**), the most active compounds
242 were those in that *N*-acylhydrazone was substituted at *para* position (**14c**), followed by
243 *meta* (**14b**) and *ortho* (**14a**) substitution. A similar effect can be observed in the phenyl
244 series, wherein the *para* substituted derivative (**8c**) was the most potent compound among
245 its regioisomers.
246 We also measured the levels of nitrite in the medium as an indirect method to quantify
247 NO release by compounds. The results demonstrated that NO release is dependent of the
248 presence of a large excess of L-cysteine (1:50), since conditions without this aminoacid
249 were not able to release NO (results not shown) [37]. All furoxan compounds were
250 capable to generate nitrite in the medium at values ranging from of 0.16% to 44.23%. Our
251 findings appoint that the antitubercular activity seems to be related in part, to the ability
252 to release nitric oxide by the furoxan subunit. It was observed that phenylsulfonyl series
253 (**14a-c**) showed the best antitubercular activity and generated high levels of nitric oxide,
254 while the methyl series (**4a-c**), with low NO-release profile, demonstrated inferior
255 antitubercular activity. Moreover, we evaluated the antitubercular activity of these four
256 promising compounds in the presence of an equimolar concentration of 2-phenyl-4,4,5,5-

tetramethylimidazoline-1-oxyl 3-oxide (PTIO), a nitric oxide radical scavenger [50], in order to verify the importance of nitric oxide for antitubercular activity. The results showed that in the presence of PTIO, the four promising compounds have shown MIC_{90} values higher than those found in the assay without the PTIO reagent, confirming the influence that nitric oxide plays in the antitubercular activity of these compounds.

The ability to release NO by furoxan derivatives is directly related to the substitution in the carbon atom at 3 position (C-3), neighboring to *N*-oxide function [20,39]. Furoxan derivatives with electron-withdrawing substituents at position C-3 (i.e., phenylsulfonyl-substituted (**14a-c**) derivatives) was able to release NO at high levels than methyl (**4a-c**) or phenyl (**8a-c**) series.

The most promising compounds (**8c**; **14a-c**) identified in the primary screening were also evaluated against MRC-5 and J774A.1 cell in order to characterize their respective cytotoxicity. These cells were selected because MRC-5 is widely used for phenotypic screening of drugs to be regarded as a normal cell derived from lung human and J774A.1 is a macrophage murine cell. Compounds (**8c**; **14a-c**) have demonstrated IC_{50} against MRC-5 at values ranging from 30.10 to 623.44 µM and SI values between 3.78 and 52.74. Regarding the J774A.1 cell line, it was found IC_{50} values ranged from 4.30 to 408.97 µM with SI values ranging from 1.25 to 34.78. Phenylsulfonyl derivatives (**14a-c**) were more cytotoxic than phenyl furoxan derivative (**8c**) against both cell lines; however, among the phenylsulfonyl derivatives it was not observed a relationship between NO-donor release and cytotoxic effect. The furoxan intermediates (**3a-c**, **7a-c** and **13a-c**) have shown cytotoxicity with IC_{50} ranging from 2.2 to 113.2 µM and SI values between 0.1 and 2 against J774A.1 cell.

After the initial screening, we selected the four promising compounds (**8c**; **14a-c**) to be evaluated against a clinical isolate MDR strain. This strain was phenotypically and genotypically characterized and it exhibited resistance to isoniazid, rifampicin, streptomycin and ethambutol [42]. Specifically, for this MDR strain it was characterized a mutation in the *inhA* gene, responsible for encode the NADH-dependent enoyl-ACP reductase of the FAS II system [51]. Compounds (**8c**; **14a-c**) showed MIC_{90} values ranging from 7.0 to 50.0 µM, being the compound **14c** (MIC_{90} 7.0 µM) the most promising among the series. These data suggest that furoxan moiety improved the antitubercular activity against MDR-TB, considering that compound (**I**) did not showed antitubercular activity against MDR-TB strains.

Therefore, we selected the most promising compound (**14c**) to analyze its chemical stability using an *in vitro* assay. We carried out the stability study under four conditions, (pHs 1.0, 5.0, 7.4 and 9.0) in order to mimic the acidic stomach (pH 1.0), the macrophage phagolysosome (pH 4.5 – 6.2) [52–54], the neutral plasma (pH 7.4) environments and a basic condition (pH 9.0), respectively. Compound **14c** were unstable at pH 1.0 and 9.0 being degraded around 90% and 50% after the first hour, respectively. Despite of that, it was not detected significant chemical degradation at pH 5.0 (0%) and 7.4 (15%) after 6 hours. After 24h, a reduction of 20% was observed at both pHs 5.0 and 7.4, showing a relative stability of compound **14c** in these pHs values. The degradation rates of compound **14c** were calculated by HPLC-MS/MS and the degradation products were not characterized (supplementary material).

Moreover, it is well established that the permeability through the peptidoglycan-arabinogalactan-mycolic core in the MTB is a great limitation for antitubercular drug

development [16]. Therefore, the lipophilicity, mostly expressed as log$P_{o/w}$ (the logarithm of the partition coefficient in a specific solvent ($P_{octanol}/P_{water}$)), is an important physico-chemical property that must be evaluated for new compounds during drug discovery [55,56]. Recently, we have identified that for the most active antitubercular compounds described in the literature between 2012-2014 (MIC_{90} inferior to 7), cLogP values ranged from 2 to 6 [16]. Hybrid furoxan derivatives reported here showed log$P_{o/w}$ values ranging from 1.3 to 2.9 (**Table 1**). We did not find a direct relationship between logP and antitubercular activity for all compounds; however, for those more active (**8c** and **14a-c**) we have observed log$P_{o/w}$ values superior to 2.1, comparable to those values reported in literature [16].

4. Conclusion

In conclusion, a novel series of hybrid furoxan derivatives was synthesized and characterized. The furoxan derivatives have demonstrated nitric oxide release properties at levels ranging from 0.16% to 44.23%. Among the nine hybrid furoxan derivatives, compounds (**8c** and **14a-c**) showed MIC_{90} values ranging from 1.03 to 11.82 µM and SI ranging from 3.78 to 52.74 (MRC-5) and 1.25 to 34.78 (J774A.1). Moreover, the four selected compounds (**8c** and **14a-c**) presented activity against a clinical isolate MDR-TB strain with MIC_{90} values ranging from 7.0 to 50.0 µM. *In vitro* hydrolysis studies have demonstrated that compound **14c** is stable at pH 5.0 and 7.4 until 6 h. The results described here pointed out compounds **8c** and **14a-c** as novel lead compounds for the treatment of TB infection, including against resistant strain.

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5. Experimental section

5.1. Chemistry

Melting points (mp) were measured using an electrothermal melting point apparatus (SMP3; Bibby Stuart Scientific) in open capillary tubes. Infrared spectroscopy (KBr disc) were performed on an FTIR-8300 Shimadzu spectrometer, and the frequencies are expressed per cm^{-1}. The NMR for ^{1}H and ^{13}C of all compounds were scanned on a Bruker Fourier with Dual probe $^{13}C/^{1}H$ (300-MHz) NMR spectrometer and a Bruker Ascend (600-MHz) NMR spectrometer using dimethyl sulfoxide (DMSO-d_6) as solvent. Chemical shifts were expressed in parts per million (ppm) relative to tetramethylsilane. The signal multiplicities are reported as singlet (s), doublet (d), doublet of doublet (dd), and multiplet (m). Elemental analyses (C, H and N) were performed on a Perkin-Elmer model 2400 analyzer, and the data were within ± 0.4% of the theoretical values. The compounds were separated on a chromatography column with silica gel (60 Å pore size, 35-75-µm particle size) and the following solvents were used as mobile phase: dichloromethane, hexane, ethyl acetate and petroleum ether. The reaction progress of all compounds was monitored by thin-layer chromatography (TLC), which was performed on 2.0- by 6.0-cm^2 aluminum sheets precoated with silica gel 60 (HF-254; Merck) to a thickness of 0.25 mm and revealed under UV light (265 nm). All compounds were analyzed by HPLC, and their purity was confirmed to be greater than 98.5%. Reagents and solvents were purchased from commercial suppliers and used as received.

Compounds **2**, **6**, and **12** were synthesized according to a previously described methodology (**Scheme 1** and **2**) [20,30–32]. Isonicotinohydrazide were purchased commercially.

5.2. General procedure for the synthesis of compounds 4a-c, 8a-c, 14a-c and 23a-c

A solution of compound **3a-c**, **7a-c** or **13a-c** (0.87 mmol) in 10 mL of ethanol and 3 drops of hydrochloric acid was stirred at for 20 min at room temperature (r.t.). Next, isonicotinohydrazide (0.106 g, 0.87 mmol) was added, and the mixture was stirred at r.t. for 12 h. The reactions were monitored by TLC (98:2, ethyl acetate : methanol). The solvent was concentrated under reduced pressure, and 8 mL of ice water was added in order to precipitate the desired products. If necessary, the samples could be further purified through column chromatography (silica gel), using ethyl acetate-methanol (98:2) as the mobile phase to give the compounds **4a-c**, **8a-c** and **14a-c** with variable yields (83 to 95%).

*5.2.1. (E)-4-(2-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-methyl-1,2,5-oxadiazole 2-oxide (**4a**)*

White powder; yield, 83%; mp, 196 to 198°C. IR V_{max} (cm^{-1}; KBr pellets): 3.203 (N-H), 3.030 (C-H aromatic), 1.689 (C=O amide), 1,639 (C=N imine), 1,485 (N-O furoxan), 1,448 (CH_3), 1.284 (C-N aromatic), 1.143 (C-O ether). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 12.08 (1H; s), 8.78 (2H; d; *J* = 5.8), 8.60 (1H; s), 7.98 (1H; d; *J* = 8.8), 7.81 (2H; d; *J* = 6.0), 7.57 (2H; m), 7.47 (1H; t; *J* = 16.1), 2.24 (3H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 163.53, 161.69, 150.54, 150.39, 143.50, 140.27, 131.91, 128.29, 127.22, 125.46, 121.66, 121.55, 107.48, 7.07 ppm. Calculated analysis (%) for $C_{16}H_{13}N_5O_4$: C: 56.6; H: 3.8; N: 20.6. Found: C: 56.7; H: 3.8; N: 20.5.

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*5.2.2. (E)-4-(3-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-methyl-1,2,5-oxadiazole 2-oxide (**4b**)*

White powder; yield, 89%; mp, 149 to 154°C. IR V_{max} (cm^{-1}; KBr pellets): 3.217 (N-H), 3.049 (C-H aromatic), 1.633 (C=O amide), 1,548 (C=N imine), 1,446 (N-O furoxan), 1,413 (CH_3), 1.305 (C-N aromatic), 1.159 (C-O ether). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.78 (2H; d; *J* = 5.9), 8.49 (1H; s), 7.82 (3H; d; *J* = 5.9), 7.69 (1H; d; *J* = 7.6), 7.60 (1H; t; *J* = 15.7), 7.51 (1H; d; *J* = 8.4), 2.16 (3H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 163.20, 161.82, 153.01, 151.59, 150.40, 147.64, 140.35, 136.28, 130.78, 125.59, 121.59, 117.72, 107.61, 6.99 ppm. Calculated analysis (%) for $C_{16}H_{13}N_5O_4$: C: 56.6; H: 3.8; N: 20.6. Found: C: 56.5; H: 3.8; N: 20.5.

*5.2.3. (E)-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-methyl-1,2,5-oxadiazole 2-oxide (**4c**)*

White powder; yield, 85%; mp, 214 to 217°C. IR V_{max} (cm^{-1}; KBr pellets): 3.236 (N-H), 3.078 (C-H aromatic), 1.666 (C=O amide), 1,604 (C=N imine), 1,485 (N-O furoxan), 1,408 (CH_3), 1.305 (C-N aromatic), 1.155 (C-O ether). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 12.11 (1H; s), 8.78 (2H; d; *J* = 5.7), 8.50 (1H; s), 7.85 (2H; d; *J* = 8.7), 7.82 (2H; d; *J* = 5.9), 7.51 (2H; d; *J* = 8.6), 2.14 (3H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 162.76, 161.68, 154.06, 150.35, 147.80, 140.41, 131.96, 129.05, 121.53, 119.98, 107.59, 6.97 ppm. Calculated analysis (%) for $C_{16}H_{13}N_5O_4$: C: 56.6; H: 3.8; N: 20.6. Found: C: 56.7; H: 3.8; N: 20.6.

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394 *5.2.4. (E)-4-(2-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-phenyl-1,2,5-oxadiazole*
395 *2-oxide (**8a**)*

396 White powder; yield, 85%; mp, 209 to 211°C. IR V_{max} (cm^{-1}; KBr pellets): 3.184 (N-H),
397 3.045 (C-H aromatic), 1.674 (C=O amide), 1,600 (C=N imine), 1,435 (N-O furoxan),
398 1.300 (C-N aromatic), 1.149 (C-O ether), 769 (aromatic). ^{1}H NMR (300 MHz, DMSO-
399 d_6) δ: 12.06 (1H; s), 8.74 (2H; d; *J* = 6.0), 8.65 (1H; s), 8.14 (2H; d; *J* = 7.9), 8.03 (1H; d;
400 *J* = 7.8), 7.75 (2H; d; *J* = 6.0), 7.63 (5H; m), 7.50 (1H, t; *J* = 15.9) ppm. ^{13}C NMR (75
401 MHz, DMSO-d_6) δ: 162.31, 161.39, 150.50, 150.08, 142.69, 139.99, 131.72, 130.75,
402 128.91, 127.30, 127.23, 126.47, 125.57, 121.77, 121.43, 121.24, 107.92 ppm. Calculated
403 analysis (%) for $C_{21}H_{15}N_5O_4$: C: 62.8; H: 3.7; N: 17.4. Found: C: 62.9; H: 3.7; N: 17.3.
404

405 *5.2.5. (E)-4-(3-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-phenyl-1,2,5-oxadiazole*
406 *2-oxide (**8b**)*

407 White powder; yield, 92%; mp, 210 to 213°C. IR V_{max} (cm^{-1}; KBr pellets): 3.324 (N-H),
408 3.068 (C-H aromatic), 1.660 (C=O amide), 1,604 (C=N imine), 1,483 (N-O furoxan),
409 1.323 (C-N aromatic), 1.157 (C-O ether), 769 (aromatic). ^{1}H NMR (300 MHz, DMSO-
410 d_6) δ: 8.78 (2H; d; *J* = 5.6), 8.51 (1H; s), 8.10 (2H; d; *J* = 7.1), 7.94 (1H; s), 7.82 (2H; d; *J*
411 = 5.7), 7.72 (1H; m), 7.62 (5H, m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 154.31,
412 145.39, 130.29, 123.46, 123.24, 121.39, 120.27, 119.00, 118.61, 113.94, 100.65 ppm.
413 Calculated analysis (%) for $C_{21}H_{15}N_5O_4$: C: 62.8; H: 3.7; N: 17.4. Found: C: 62.8; H: 3.7;
414 N: 17,4.
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*5.2.6. (E)-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-phenyl-1,2,5-oxadiazole 2-oxide (**8c**)*

White powder; yield, 88%; mp, 198 to 202°C. IR V_{max} (cm^{-1}; KBr pellets): 3.250 (N-H), 3.066 (C-H aromatic), 1.651 (C=O amide), 1,610 (C=N imine), 1,438 (N-O furoxan), 1.332 (C-N aromatic), 1.205 (C-O ether), 756 (aromatic). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.78 (2H; d; *J* = 5.8), 8.51 (1H; s), 8.07 (2H; d; *J* = 6.6), 7.88 (2H; d; *J* = 8.7), 7.82 (2H; d; *J* = 5.9), 7.63 (5H; m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 153.62, 149.33, 126.38, 124.00, 123.26, 121.41, 118.97, 113.81, 112.77, 100.72 ppm. Calculated analysis (%) for $C_{21}H_{15}N_5O_4$: C: 62.8; H: 3.7; N: 17.4. Found: C: 62.9; H: 3.7; N: 17.3.

*5.2.7. (E)-4-(2-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (**14a**)*

White powder; yield, 85%; mp, 199 to 202°C. IR V_{max} (cm^{-1}; KBr pellets): 3.280 (N-H), 3.068 (C-H aromatic), 1.651 (C=O amide), 1,645 (C=N imine), 1,454 (N-O furoxan), 1.354 (C-N aromatic), 1.161 (S=O sulfone), 1.083 (C-O ether), 744 (aromatic). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.78 (2H; d; *J* = 5.1), 8.50 (1H; s), 8.07 (2H; d; *J* = 7.9), 7.93 (1H; t; *J* = 7.3), 7.85 (4H; m), 7.78 (1H; t; *J* = 7.7), 7.71 (1H; d; *J* = 7.5), 7.60 (1H; t; *J* = 7.9), 7.51 (1H; d; *J* = 8.9) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 154.08, 150.83, 145.10, 139.80, 132.57, 129.15, 128.56, 123.07, 122.31, 120.90, 118.31, 113.97, 113.84, 110.05, 103.58 ppm. Calculated analysis (%) for $C_{21}H_{15}N_5O_6S$: C: 54.2; H: 3.2; N: 15.1. Found: C: 54.3; H: 3.2; N: 15.0.

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*5.2.8. (E)-4-(3-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (**14b**)*

White powder; yield, 95%; mp, 202 to 204°C. IR V_{max} (cm^{-1}; KBr pellets): 3.182 (N-H), 3.003 (C-H aromatic), 1.680 (C=O amide), 1,604 (C=N imine), 1,444 (N-O furoxan), 1.357 (C-N aromatic), 1.166 (S=O sulfone), 1.083 (C-O ether), 742 (aromatic). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.79 (2H; d; *J* = 6.0), 8.48 (1H; s), 8.07 (2H; d; *J* = 7.3), 7.95 (1H; t; *J* = 13.8), 7.83 (4H; m), 7.77 (1H; m), 7.71 (1H; d; *J* = 7.6), 7.61 (1H; t; 15.7), 7.51 (1H; d; *J* = 9.3) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 154.08, 150.87, 145.12, 142.66, 139.79, 132.59, 129.16, 128.27, 123.08, 122.32, 120.94, 118.33, 113.99, 113.85, 110.08, 103.61 ppm. Calculated analysis (%) for $C_{21}H_{15}N_5O_6S$: C: 54.2; H: 3.2; N: 15.1. Found: C: 54.1; H: 3.2; N: 15.1.

*5.2.9. (E)-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (**14c**)*

White powder; yield, 92%; mp, 194 to 197°C. IR V_{max} (cm^{-1}; KBr pellets): 3.238 (N-H), 3.068 (C-H aromatic), 1.664 (C=O amide), 1,610 (C=N imine), 1,450 (N-O furoxan), 1.359 (C-N aromatic), 1.165 (S=O sulfone), 1.082 (C-O ether), 750 (aromatic). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.78 (2H; d; *J* = 6.0), 8.50 (1H; s), 8.05 (2H; d; *J* = 8.6), 7.88 (2H; d; *J* = 8.8), 7.82 (2H, d; *J* = 6.0), 7.77 (3H, t; *J* = 15.6), 7.53 (2H, d; *J* = 8.7) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 153.98, 150.42, 146.23, 142.65, 139.97, 132.66, 129.12, 128.58, 124.62, 122.33, 121.32, 120.89, 113.83, 112.44, 103.63 ppm. Calculated analysis (%) for $C_{21}H_{15}N_5O_6S$: C: 54.2; H: 3.2; N: 15.1. Found: C: 54.3; H: 3.2; N: 15.0.

5.3. Biological activity

5.3.1. Determination of Minimal Inhibitory Concentration (MIC_{90})

The antitubercular activity of all compounds was determined through the REMA methodology according procedures described by Palomino and coworkers [57]. Stock solutions of the tested compounds were prepared in DMSO and diluted in Middlebrook 7H9 broth (Difco) supplemented with 10% OADC enrichment (dextrose, albumin, and catalase) using a Precision XS™ (BioTek®), to obtain final drug concentration ranging from 0.09 – 25 μg/mL. Rifampicin and isoniazid were used as a control drugs. A suspension of the MTB H_{37}Rv ATCC 27294 or the clinical isolate MDR-TB strain was cultured in Middlebrook 7H9 broth supplemented with 10% OADC and 0.05 % Tween 80. The culture was frozen at −80 °C in aliquots. The concentration was adjusted to 2 x 10^5 UFC/mL and 100 μL of the inoculum was added to each well of a 96-well microtiter plate together with 100 μL of the compounds. Samples were set up in three independent assays. The plate was incubated for 7 days at 37 °C. After 24 h, 30 μL of 0.01 % resazurin in distilled water was added. The fluorescence of the wells was read using a Cytation™ 3 (BioTek®) in which were used excitations and emissions filters at wavelengths of 530 and 590 nm, respectively. The MIC_{90} value was defined as the lowest drug concentration at which 90 % of the cells are infeasible relative to the control.

5.3.2. Cytotoxicity assay

In vitro cytotoxicity assays (IC_{50}) were performed on MRC-5 (ATCC® CCL-171) and J774A.1 (ATCC® TIB-67), as described by Pavan and colleagues [58]. The cells were

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484 routinely maintained in complete medium (DMEM) supplemented with 10 % of fetal
485 bovine serum (FBS) plus amphotericin B (2 mg/L) and gentamicin (50 mg/L) at 37 °C, in
486 a humidified 5 % CO_2 atmosphere. After reaching confluence, the cells were detached,
487 counted and adjusted to 1 x 10^5 cells/mL. The cells were seeded in 200 μL of complete
488 medium in 96-well plates. The plates were incubated under the same conditions for 24 h
489 to allow cell adhesion prior to drug testing. From the stock solutions described above the
490 compounds were diluted using a Precision XS™ (BioTek®), to obtain final drug
491 concentration ranging from 0.09 – 250 μg/mL. Then, the cells were exposed to
492 compounds for 24 h and 30 μL of 0.01 % resazurin in distilled water was added. The
493 fluorescence of the wells was read using a Cytation™ 3 (BioTek®) in which were used
494 excitations and emissions filters at wavelengths of 530 and 590 nm, respectively. The
495 IC_{50} value was defined as the highest drug concentration at which 50 % of the cells are
496 viable relative to the control. Samples were set up in three independent assays.
497
498 *5.3.3. Nitric oxide release*
499 Nitric oxide has a short half-life, therefore, the quantification of NO metabolites like
500 nitrite and nitrate is a useful method to quantify this molecule in the medium [59,60]. The
501 amount of NO released was indirectly detected by Griess reaction through the
502 measurement of nitrites in the medium. A volume of 98 μL of a phosphate buffer (pH
503 7.4) solution containing 5 mM of L-cysteine was added in triplicate in a 96-well, flat-
504 bottomed, polystyrene microtiter plate. After loading the plate with the phosphate buffer
505 (98 μL), it was added 2 μL of solution containing the appropriate compound diluted in
506 DMSO (3 mL). The final concentration of the compound in each well was 1 x 10^{-4} M.

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After 1 h of incubation at 37 °C, it was added 100 μL of the Griess reagent (4 g of sulfanilamide, 0.2 g of *N*-naphthylethylenediamine dihydrochloride, 85 % phosphoric acid [10 mL] in distilled water [final volume, 100 mL]). After 10 min at room temperature, the absorbance was measured at 540 nm using a BioTek® microplate reader spectrophotometer. Standard sodium nitrite solutions (0.5 to 100 nmol/mL) were used to construct the calibration curve. The yields of nitrite are expressed as % NO_2^- (mol/mol). No production of nitrite was observed in the absence of $_L$-cysteine [37–39].

5.3.4. Partition coefficient (n-octanol/water) measured by HPLC method

The partition coefficient was characterized using the HPLC method according procedures described by OECD *Guidelines for the Testing of Chemicals* [40]. The equipment used was a Shimadzu HPLC model CBM 20-A (Shimadzu®) equipped with UV-VIS detector (model SPD-20A), quaternary pumping system mobile phase (model LC-20AT), solvent degasser (model DGU-20As) and a Agilent® Eclipse XDB C-18 column (250mm x 4,6mm; 5μm). For HPLC method it was used an isocratic flow [methanol:water (75:25)] at 1.0 mL/min. The volume injected was 20.0 μL and the wavelength in the detector was 210 nm. The following substances were used as standards to construct the curve log K x log P: acetanilide, benzonitrile, nitrobenzene, toluene, naphthalene, biphenyl and phenanthrene. The capacity factor (log*K*) of the hybrid compounds was determined from their retention times and interpolated in linearity curve log K x log P.

5.3.5. In vitro stability study

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In vitro hydrolysis was performed by HPLC method. The compound 14c was separated using a Phenomenex Luna reverse-phase C_{18} (2)-HTS column (2.5-μm particle, 2 by 50 mm). The isocratic flow was 50:50 (water : 0,1 % formic acid-acetonitrile, v/v) and the flow rate was 0.25 mL/min. The HPLC was coupled to a API 2000 triple quadrupole mass spectrometer equipped with a heated electrospray ionization interface (H-ESI) operated in the positive ionization mode at capillary voltage 5200 V, source temperature 350 °C, nitrogen gas flow 65 units.

For hydrolysis, an appropriate solution of compound 14c was diluted in acetonitrile at 1000 μM. Then, this solution was diluted to 10 μM using four different PBS buffer (phosphate-buffered saline) in order to provide the following pHs: 1.0; 5.0; 7.4 and 9.0. During the assay, all samples were maintained at constant agitation using a shaker (400 rpm) at 37 °C. Aliquots were taken from the solution at the following times: 0, 1, 4, 6, and 24 h. The injection volume in the HPLC was 5 μL. All analyses were conducted in triplicate, and the results were expressed as the averages of the concentrations in percentages (± standard error of the mean [SEM]).

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553 **References**

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555 [1] World Health Organization, Global tuberculosis report 2014
556 (WHO/HTM/TB/2014.08), 2014.
557 [2] World Health Organization, Global tuberculosis report 2013, 2013.
558 [3] K. Patel, S.S. Jhamb, P.P. Singh, Models of latent tuberculosis: Their salient
559 features, limitations, and development, J. Lab. Physicians. 3 (2011) 75–79.
560 [4] T.R. Rustad, M.I. Harrell, R. Liao, D.R. Sherman, The enduring hypoxic response
561 of Mycobacterium tuberculosis., PLoS One. 3 (2008) e1502.
562 [5] D.G.N. Muttucumaru, G. Roberts, J. Hinds, R. a Stabler, T. Parish, Gene
563 expression profile of Mycobacterium tuberculosis in a non-replicating state.,
564 Tuberculosis. 84 (2004) 239–246.
565 [6] A.R. Frydenberg, S.M. Graham, Toxicity of first-line drugs for treatment of
566 tuberculosis in children: review, Trop Med Int Heal. 14 (2009) 1329–1337.
567 [7] S.A. Tasduq, P. Kaiser, S.C. Sharma, R.K. Johri, Potentiation of isoniazid-induced
568 liver toxicity by rifampicin in a combinational therapy of antitubercular drugs
569 (rifampicin, isoniazid and pyrazinamide) in Wistar rats: A toxicity profile study,
570 Hepatol Res. 37 (2007) 845–853.
571 [8] M. Singh, P. Sasi, G. Rai, V.H. Gupta, D. Amarapurkar, P.P. Wangikar, Studies on
572 toxicity of antitubercular drugs namely isoniazid, rifampicin, and pyrazinamide in
573 an in vitro model of HepG2 cell line, Med. Chem. Res. 20 (2011) 1611–1615.
574 [9] J.J. Saukkonen, D.L. Cohn, R.M. Jasmer, S. Schenker, J. a. Jereb, C.M. Nolan, et
575 al., An official ATS statement: Hepatotoxicity of antituberculosis therapy, Am J
576 Respir Crit Care Med. 174 (2006) 935–952.
577 [10] D. Yee, C. Valiquette, M. Pelletier, I. Parisien, I. Rocher, D. Menzies, Incidence of
578 Serious Side Effects from First-Line Antituberculosis Drugs among Patients
579 Treated for Active Tuberculosis, Am J Respir Crit Care Med. 167 (2003) 1472–
580 1477.
581 [11] V. Sahasrabudhe, T. Zhu, A. Vaz, S. Tse, Drug Metabolism and Drug Interactions:
582 Potential Application to Antituberculosis Drugs, J Infect Dis. 211 (2015) S107–
583 S114.
584 [12] E. Segala, W. Sougakoff, A. Nevejans-Chauffour, V. Jarlier, S. Petrella, New
585 mutations in the mycobacterial ATP synthase: New insights into the binding of the
586 diarylquinoline TMC207 to the ATP synthase C-Ring structure, Antimicrob.
587 Agents Chemother. 56 (2012) 2326–2334.
588 [13] P.R. Baldwin, A.Z. Reeves, K.R. Powell, R.J. Napier, A.I. Swimm, A. Sun, et al.,
589 Monocarbonyl analogs of curcumin inhibit growth of antibiotic sensitive and
590 resistant strains of Mycobacterium tuberculosis, Eur J Med Chem. 92 (2015) 693–
591 699.
592 [14] P.S. Ng, U.H. Manjunatha, S.P.S. Rao, L.R. Camacho, N.L. Ma, M. Herve, et al.,
593 Structure activity relationships of 4-hydroxy-2-pyridones: A novel class of
594 antituberculosis agents, Eur J Med Chem. 106 (2015) 144–156.

[15] S. Saxena, G. Samala, J.P. Sridevi, P.B. Devi, P. Yogeeswari, D. Sriram, Design and development of novel Mycobacterium tuberculosis l-alanine dehydrogenase inhibitors, Eur J Med Chem. 92 (2015) 401–414.

[16] G.F.S. Fernandes, D.H. Jornada, P.C. Souza, C. Man Chin, F.R. Pavan, J.L. Santos, Current Advances in Antitubercular Drug Discovery: Potent Prototypes and New Targets, Curr. Med. Chem. 22 (2015) 3133–3161.

[17] C. Viegas-Junior, A. Danuello, V. da Silva Bolzani, E.J. Barreiro, C.A.M. Fraga, Molecular hybridization: a useful tool in the design of new drug prototypes, Curr Med Chem. 14 (2007) 1829–1852.

[18] P. Hernández, R. Rojas, R.H. Gilman, M. Sauvain, L.M. Lima, E.J. Barreiro, et al., Hybrid furoxanyl N-acylhydrazone derivatives as hits for the development of neglected diseases drug candidates, Eur J Med Chem. 59 (2013) 64–74.

[19] R.A. Massarico Serafim, J.E. Gonçalves, F.P. de Souza, A.P. de Melo Loureiro, S. Storpirtis, R. Krogh, et al., Design, synthesis and biological evaluation of hybrid bioisoster derivatives of N-acylhydrazone and furoxan groups with potential and selective anti-Trypanosoma cruzi activity, Eur. J. Med. Chem. 82 (2014) 418–25.

[20] L.A. Dutra, L. de Almeida, T.G. Passalacqua, J.S. Reis, F.A.E. Torres, I. Martinez, et al., Leishmanicidal Activities of Novel Synthetic Furoxan and Benzofuroxan Derivatives, Antimicrob Agents Chemother. 58 (2014) 4837–4847.

[21] H. Cerecetto, W. Porcal, Pharmacological properties of furoxans and benzofuroxans: recent developments, Mini Rev Med Chem. 5 (2005) 57–71.

[22] A. Gasco, R. Fruttero, G. Sorba, A. Di Stilo, R. Calvino, NO donors: Focus on furoxan derivatives, Pure Appl. Chem. 76 (2004) 973–981.

[23] M.I. Voskuil, D. Schnappinger, K.C. Visconti, M.I. Harrell, G.M. Dolganov, D.R. Sherman, et al., Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program, J Exp Med. 198 (2003) 705–713.

[24] E.D. Chan, J. Chan, N.W. Schluger, What is the role of nitric oxide in murine and human host defense against tuberculosis?, Am J Respir Cell Mol Biol. 25 (2001) 606–612.

[25] C. Jagannath, J.K. Actor, R.L. Hunter, Induction of nitric oxide in human monocytes and monocyte cell lines by Mycobacterium tuberculosis., Nitric Oxide. 2 (1998) 174–186.

[26] J. Chan, K. Tanaka, D. Carroll, J. Flynn, B.R. Bloom, Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis, Infect Immun. 63 (1995) 736–740.

[27] S. Sharma, M. Sharma, S. Roy, P. Kumar, M. Bose, Mycobacterium tuberculosis induces high production of nitric oxide in coordination with production of tumour necrosis factor-α in patients with fresh active tuberculosis but not in MDR tuberculosis, Immunol Cell Biol. 82 (2004) 377–382.

[28] R. Long, R. Jones, J. Talbot, I. Mayers, J. Barrie, M. Hoskinson, et al., Inhaled nitric oxide treatment of patients with pulmonary tuberculosis evidenced by positive sputum smears, Antimicrob Agents Chemother. 49 (2005) 1209–1212.

[29] R. Long, B. Light, J. a. Talbot, Mycobacteriocidal action of exogenous nitric oxide, Antimicrob Agents Chemother. 43 (1999) 403–405.

[30] A.D. Nikolaeva, Y.N. Matyushin, V.I. Pepekin, V.S. Smelov, V.V. Bulidorov, T.I. Bulidorova, et al., Synthesis and study of detonation properties of 3-methyl-4-

nitrofuroxan, Bull. Acad. Sci. USSR. 21 (1972) 927–928.

[31] A. Kunai, T. Doi, T. Nagaoka, H. Yagi, K. Sasaki, Stereoselective synthesis of (E)-2-hydroxyimino-2-phenylacetonitrile by photolysis of 4-azido-3-phenylfurazan 2-oxide, Bull. Chem. Soc. Jpn. 63 (1990) 1843–1844.

[32] W. V. Farrar, The 3,4-Bisarenesulphonylfuroxans, J Chem Soc. (1964) 904–906.

[33] G. Palla, G. Predieri, P. Domiano, C. Vignalli, W. Turner, Conformational behaviour and E/Z isomerization of N-acyl and N-aroylhydrazones, Tetrahedron. 42 (1986) 3649–3654.

[34] G.J. Karabatsos, J.D. Graham, F.M. Vane, syn-anti Isomer Determination of 2,4-Dinitrophenylhydrazones and Semicarbazones by N.M.R., J. Am. Chem. Soc. 84 (1962) 753–755.

[35] A.B. Lopes, E. Miguez, A.E. Kümmerle, V.M. Rumjanek, C.A.M. Fraga, E.J. Barreiro, Characterization of amide bond conformers for a novel heterocyclic template of N-acylhydrazone derivatives, Molecules. 18 (2013) 11683–11704.

[36] G.J. Karabatsos, R.A. Taller, Structural Studies by Nuclear Magnetic Resonance. V. Phenylhydrazones, J Am Chem Soc. 85 (1963) 3624–3629.

[37] G. Sorba, C. Medana, R. Fruttero, C. Cena, A. Di Stilo, U. Galli, et al., Water soluble furoxan derivatives as NO prodrugs., J Med Chem. 40 (1997) 463–9.

[38] D. Tsikas, Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: Appraisal of the Griess reaction in the l-arginine/nitric oxide area of research, J Chromatogr B Anal. Technol Biomed Life Sci. 851 (2007) 51–70.

[39] J.L. Santos, C. Lanaro, L.M. Lima, S. Gambero, C.F. Franco-Penteado, M.S. Alexandre-Moreira, et al., Design, synthesis, and pharmacological evaluation of novel hybrid compounds to treat sickle cell disease symptoms, J Med Chem. 54 (2011) 5811–5819.

[40] OECD, Test No. 117: Partition Coefficient (n-octanol/water), HPLC Method, in: OECD Guidel. Test. Chem., OECD Publishing, Paris, 2004: pp. 1–11.

[41] J. Palomino, A. Martin, M. Camacho, H. Guerra, J. Swings, F. Portaels, Resazurin Microtiter Assay Plate : Simple and Inexpensive Method for Detection of Drug Resistance in Mycobacterium tuberculosis, Antimicrob. Agents Chemother. 46 (2002) 2720–2722.

[42] M. Miyata, F.R. Pavan, D.N. Sato, L.B. Marino, M.H. Hirata, R.F. Cardoso, et al., Drug resistance in Mycobacterium tuberculosis clinical isolates from Brazil: phenotypic and genotypic methods, Biomed. Pharmacother. 65 (2011) 456–459.

[43] R.A. Robbins, P.J. Barner, Springall, J.B. Warren, O.J. Kwon, L.D.K. Buutery, et al., Expression of Inducible Ntiric Oxide in Human Lung Epithelial Cells, Biochem Biophys Res Commun. 203 (1994) 209–218.

[44] J. Chan, Y. Xing, R.S. Magliozzo, B.R. Bloom, Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages, J Exp Med. 175 (1992) 1111–22.

[45] C.A. Scanga, V.P. Mohan, K. Tanaka, D. Alland, J.L. Flynn, J. Chan, The inducible nitric oxide synthase locus confers protection against aerogenic challenge of both clinical and laboratory strains of Mycobacterium tuberculosis in mice, Infect Immun. 69 (2001) 7711–7717.

[46] R. Singh, U. Manjunatha, H.I.M. Boshoff, Y.H. Ha, P. Niyomrattanakit, R. Ledwidge, et al., PA-824 Kills Nonreplicating Mycobacterium tuberculosis by

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687 Intracellular NO Release, Science (80-.). 322 (2008) 1392–1395.
688 [47] T.R. Garbe, N.S. Hibler, V. Deretic, Response to reactive nitrogen intermediates in
689 Mycobacterium tuberculosis: Induction of the 16-kilodalton alpha-crystallin
690 homolog by exposure to nitric oxide donors, Infect Immun. 67 (1999) 460–465.
691 [48] C.-S. Yang, J.-M. Yuk, E.-K. Jo, The role of nitric oxide in mycobacterial
692 infections, Immune Netw. 9 (2009) 46–52.
693 [49] Global Alliance for TB Drug Development, Handbook of anti-tuberculosis agents,
694 Tuberculosis. 88 (2008) 85–170.
695 [50] S. Goldstein, A. Russo, A. Samuni, Reactions of PTIO and Carboxy-PTIO with
696 NO, NO2, and O2, J. Biol. Chem. 278 (2003) 50949–50955.
697 [51] C. Vilchèze, W.R. Jacobs, The mechanism of isoniazid killing: clarity through the
698 scope of genetics., Annu. Rev. Microbiol. 61 (2007) 35–50.
699 [52] D.J. Hackam, O.D. Rotstein, W. Zhang, S. Gruenheid, P. Gros, S. Grinstein, Host
700 resistance to intracellular infection: mutation of natural resistance-associated
701 macrophage protein 1 (Nramp1) impairs phagosomal acidification., J Exp Med.
702 188 (1998) 351–64.
703 [53] O.H. Vandal, C.F. Nathan, S. Ehrt, Acid resistance in Mycobacterium tuberculosis,
704 J Bacteriol. 191 (2009) 4714–4721.
705 [54] N.K. Dutta, P.C. Karakousis, Latent tuberculosis infection: myths, models, and
706 molecular mechanisms., Microbiol Mol Biol Rev. 78 (2014) 343–71.
707 [55] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and
708 computational approaches to estimate solubility and permeability in drug
709 discovery and developmental settings, Adv Drug Deliv Rev. 23 (1997) 3–25.
710 [56] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and
711 computational approaches to estimate solubility and permeability in drug
712 discovery and development settings, Adv Drug Deliv Rev. 46 (2001) 3–26.
713 [57] J. Palomino, A. Martin, M. Camacho, H. Guerra, J. Swings, F. Portaels, Resazurin
714 Microtiter Assay Plate: Simple and Inexpensive Method for Detection of Drug
715 Resistance in Mycobacterium tuberculosis Resazurin Microtiter Assay Plate :
716 Simple and Inexpensive Method for Detection of Drug Resistance in
717 Mycobacterium tuberculosis, Antimicrob Agents Chemother. 46 (2002) 2720–
718 2722.
719 [58] F.R. Pavan, P.I.D.S. Maia, S.R. a Leite, V.M. Deflon, A. a. Batista, D.N. Sato, et
720 al., Thiosemicarbazones, semicarbazones, dithiocarbazates and
721 hydrazide/hydrazones: Anti - Mycobacterium tuberculosis activity and
722 cytotoxicity, Eur J Med Chem. 45 (2010) 1898–1905.
723 [59] D. Tsikas, Methods of quantitative analysis of the nitric oxide metabolites nitrite
724 and nitrate in human biological fluids., Free Radic Res. 39 (2005) 797–815.
725 [60] L. Bellavia, D.B. Kim-Shapiro, S.B. King, Detecting and monitoring NO, SNO
726 and nitrite in vivo, Futur. Sci OA. 1 (2015) 1–16.
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Highlights

- Furoxan derivatives activity against MTB H_{37}Rv and MDR-TB strains.
- Antitubercular activity due to nitric oxide release.
- High selective index against MRC-5 and J774A.1 cell lines.
- Compound **14c** was stable at pH 5.0 and 7.4 and exhibited logP value of 2.1.

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