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Guilherme Felipe dos Santos Fernandes,[a] Paula Carolina de Souza,[a] Leonardo Biancolino Marin,[a] Konstantin Chegaev,[b] Stefano Guglielmo,[b] Loretta Lazzarato,[b] Roberta Fruttero,[b] Man Chin Chung,[a] Fernando Rogerio Pavan,[a] Jean Leandro dos Santos[a,*]

[a] School of Pharmaceutical Sciences, UNESP e Univ Estadual Paulista, Araraquara, 14800903, Brazil

[b] Dipartimento di Scienza e Tecnologia del Farmaco, Universita degli Studi di Torino, Turin, 10124, Italy

[*] Corresponding author.

E-mail address: santosjl@fcfar.unesp.br (J.L. dos Santos).

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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) H37Rv (ATCC 27294) and a clinical isolate MDR-TB strain. The furoxan derivatives have exhibited MIC90 values ranging from 1.03 to 62 μM (H37Rv) and 7.0-50.0 μM (MDR-TB). For the most active compounds (8c, 14a, 14b and 14c) the selectivity index ranged from 3.78 to 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 and 2.9. All compounds were able to release NO at levels ranging from 0.16 to 44.23%. Among the series, the phenylsulfonyl furoxan de-rivatives (compounds 14a-c) were the best NO-donor with the lowest MIC90 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.

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 Orga-nization (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 re-ported [12]. After bedaquiline, there was a noteworthy increase in the number of papers describing compounds with potent antitu-bercular activity [13-16].

In order to find new antitubercular drugs, we have established a phenotypic-based screening program with more than five thou-sand compounds present in our current library. From these data, we have identified (hydroxybenzylidene)isonicotinohydrazide de-rivatives active against MTB. Specifically, the compound (E)-N0-(4-hydroxybenzylidene)isonicotinohydrazide (I) (Fig. 1) have exhibited MIC90 value of 1.0 μM against MTB H37Rv and selective index against VERO and J774A.1 cell lines superior to 100. Notwithstanding, this molecule did not show antitubercular activity against MDR strains, presenting MIC90 values superior to 62 μM.

In this work, using the molecular hybridization approach, we designed new analogues of (E)-N'-(4-hydroxybenzylidene)iso-nicotinohydrazide (I) containing the furoxan moiety [17] (Fig. 1). Furoxan derivatives represent an important class of compounds that exhibit a variety of biological activities, such as, anti-mycobacterial [18], antichagasic [19] and antileishmanicidal [20]. The wide spectrum of biological activities of furoxan derivatives have been associated to its ability to generate nitric oxide after biotransformation [21,22]. NO is an important mediator produced by macrophages during MTB infection and has an essential role to eliminate MTB [23]. It has been demonstrated that NO can disrupt bacterial DNA, proteins, signaling mediators, and/or induction of macrophage apoptosis [24]. Nitric oxide is also increased in mac-rophages during the infection and its inhibition promotes MTB growth [25]. MTB infected mice treated with nitric oxide synthase inhibitors exhibited higher mortality rates and pathological tissue damages compared to control group without treatment [26].

Not only endogenous, but also exogenous sources of NO have demonstrated effectiveness to reduce the number of bacilli. Some works have demonstrated that low levels of NO-donors can kill the mycobacteria [27-29]. These data suggested that strategies aiming to raise NO levels seem to be promising as antitubercular therapy. Therefore, in a continuing effort to develop new drug candidates to treat TB infection, we report herein the synthesis, NO-donor release, experimental logP values, antitubercular and cytotoxic activities of furoxan derivatives (4a-c, 8a-c, and 14a-c) (Fig. 1). The antimycobacterial activity against a clinical isolate of MDR strain (resistant to isoniazid, rifampicin, 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).

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 Schemes 1 and 2.Compounds 2, 6, and 12 were synthesized according to a previously described methodology [20,30e32]. 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%. 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% (Schemes 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%.

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 H37Rv 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 MIC90 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 in-termediates (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 4.30-408.97 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).

2.4. Nitric oxide release

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

2.5. Partition coefficient study

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

2.6. In vitro stability study

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

Table 1

Antitubercular activity of compounds against Mycobacterium tuberculosis H37Rv; 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	IC50 (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
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	-

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

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

[b] Mean ± standard error of the mean.

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

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

Table 2

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

Compound	MIC90 (mM) e MDR-TB a
8c	24.3
14a	50.0
14b	21.3
14c	7.0
RIF	Res
INH	Res

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

3. Discussion

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 MIC90 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 MIC_{90} values below 8.6 μM. Moreover, a phenyl furoxan derivative (8c) also exhibited a promising MIC_{90} value of 11.82 μM. The MIC_{90} values of these four compounds (14a-c; 8a) were greater than several first and second line antitubercular drugs, such as pyrazinamide (>48 μM), cycloserine (245 μM) and kanamycin (3.4 μM) [49]. We also evaluated the furoxan intermediates (3a-c, 7a-c and 13a-c) against MTB $H_{37}Rv$, however, these compounds showed MIC_{90} values superior to 20.0 μM.

Our data suggest a direct effect in the pattern of substitution in the furoxan ring and the antitubercular activity. For phenylsulfonyl series (14a-c), the most active compounds were those in that N-acylhydrazone was substituted at para position (14c), followed by meta (14b) and ortho (14a) substitution. A similar effect can be observed in the phenyl series, wherein the para substituted derivative (8c) was the most potent compound among its regioisomers.

We also measured the levels of nitrite in the medium as an in-direct method to quantify NO release by compounds. The results demonstrated that NO release is dependent of the presence of a large excess of L-cysteine (1:50), since conditions without this aminoacid were not able to release NO (results not shown) [37]. All furoxan compounds were capable to generate nitrite in the medium at values ranging from of 0.16%-44.23%. Our findings appoint that the antitubercular activity seems to be related in part, to the ability to release nitric oxide by the furoxan subunit. It was observed that phenylsulfonyl series (14a-c) showed the best antitubercular activity and generated high levels of nitric oxide, while the methyl series (4a-c), with low NO-release profile, demonstrated inferior antitubercular activity. Moreover, we evaluated the anti-tubercular activity of these four 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 MIC90 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 rela-tionship 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 mM 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 MIC90 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 ac-tivity 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. Com-pound 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 h. After 24 h, 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 $logP_{o/w}$ (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 and 2014 (MIC_{90} inferior to 7), cLogP values ranged from 2 to 6 [16]. Hybrid furoxan derivatives reported here showed logPo/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 $logP_{o/w}$ 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, com-pounds (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 MIC90 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.

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 spec-trometer 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 aluminum sheets pre-coated 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 (Schemes 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 (83-95%).

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

White powder; yield, 83%; mp, 196-198 C. IR V_{max} (cm^{-1}; KBr pellets): 3.203 (N-H), 3.030 (C-H aromatic), 1.689 (C=O amide), 1639 (C=N imine), 1485 (N-O furoxan), 1448 (CH_3), 1284 (C-N aromatic), 1143 (C=O ether). ^1H NMR (300 MHz, DMSO-d6) d: 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-d6) d: 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.

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

White powder; yield, 89%; mp, 149-154 C. IR V_{max} (cm^{-1}; KBr pellets): 3.217 (N-H), 3.049 (C-H aromatic), 1.633 (C=O amide), 1548 (C=N imine), 1446 (N-O furoxan), 1413 (CH_3), 1305 (C-N aromatic), 1159 (C=O ether). ^1H NMR (300 MHz, DMSO-d6) d: 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-d6) d: 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, 214-217 C. IR V_{max} (cm^{-1}; KBr pellets): 3236 (N-H), 3078 (C-H aromatic), 1666 (C=O amide), 1604 (C=N imine), 1485 (N-O furoxan), 1408 (CH_3), 1305 (C-N aromatic), 1155 (C-O ether). ^1H NMR (300 MHz, DMSO-d6) d: 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-d6) d: 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.

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

White powder; yield, 85%; mp, 209e211 C. IR V_{max} (cm^{-1}; KBr pellets): 3.184 (N-H), 3.045 (C-H aromatic), 1.674 (C=O amide), 1600 (C=N imine), 1435 (N-O furoxan), 1.300 (C-N aromatic), 1.149 (C-O ether), 769 (aromatic). ^1H NMR (300 MHz, DMSO-d6) d: 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; 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 MHz, DMSO-d6) d: 162.31, 161.39, 150.50, 150.08, 142.69, 139.99, 131.72, 130.75, 128.91, 127.30, 127.23, 126.47, 125.57, 121.77, 121.43, 121.24, 107.92 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.

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

White powder; yield, 92%; mp, 210-213 C. IR V_{max} (cm^{-1}; KBr pellets): 3.324 (N-H), 3.068 (C-H aromatic), 1.660 (C=O amide), 1604 (C=N imine), 1483 (N-O furoxan), 1.323 (C-N aromatic), 1.157 (C-O ether), 769 (aromatic). ^{1}H NMR (300 MHz, DMSO-d6) d: 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 = 5.7), 7.72 (1H; m), 7.62 (5H, m) ppm. ^{13}C NMR (75 MHz, DMSO-d6) d: 154.31, 145.39, 130.29, 123.46, 123.24, 121.39, 120.27, 119.00, 118.61, 113.94, 100.65 ppm. 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; N: 17,4.

5.2.6. (E)-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-phenyl-1,2,5-oxadiazole 2-oxide (8c)

White powder; yield, 88%; mp, 198-202 C. IR V_{max} (cm^{-1}; KBr pellets): 3.250 (N-H), 3.066 (C-H aromatic), 1.651 (C=O amide), 1610 (C=N imine), 1438 (N-O furoxan), 1.332 (C-N aromatic), 1.205 (C-O ether), 756 (aromatic). ^{1}H NMR (300 MHz, DMSO-d6) d: 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-d6) d: 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, 199-202 C. IR V_{max} (cm^{-1}; KBr pellets): 3.280 (N-H), 3.068 (C-H aromatic), 1.651 (C=O amide), 1645 (C=N imine), 1454 (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-d6) d: 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-d6) d: 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.

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

White powder; yield, 95%; mp, 202-204 C. IR V_{max} (cm^{-1}; KBr pellets): 3.182 (NeH), 3.003 (C-H aromatic), 1.680 (C=O amide), 1604 (C=N imine), 1444 (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-d6) d: 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-d6) d: 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, 194-197 C. IR V_{max} (cm^{-1}; KBr pellets): 3.238 (N-H), 3.068 (C-H aromatic), 1.664 (C=O amide), 1610 (C=N imine), 1450 (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-d6) d: 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-d6) d: 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 Middlebrook7H9 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 to 25 mg/mL. Rifampicin and isoniazid were used as a control drugs. A suspen-sion of the MTB H37Rv 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 2 105 UFC/mL and 100 mL of the inoculum was added to each well of a 96-well microtiter plate together with 100 mL of the com-pounds. Samples were set up in three independent assays. The plate was incubated for 7 days at 37 C. After 24 h, 30 mL 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 con-centration 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 routinely maintained in complete medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) plus amphotericin B (2 mg/L) and gentamicin (50 mg/ L) at 37°C, in a humidified 5% CO_2 atmosphere. After reaching confluence, the cells were detached, counted and adjusted to 1 105 cells/mL. The cells were seeded in 200 mL of complete medium in 96-well plates. The plates were incubated under the same conditions for 24 h to allow cell adhesion prior to drug testing. From the stock solutions described above the compounds were diluted using a Precision XS™ (BioTek®), to obtain final drug con-centration ranging from 0.09 to 250 mg/mL. Then, the cells were exposed to compounds for 24 h and 30 mL 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 IC_{50} value was defined as the highest drug concentration at which 50% of the cells are viable relative to the control. Samples were set up in three independent assays.

5.3.3. Nitric oxide release

Nitric oxide has a short half-life, therefore, the quantification of NO metabolites like nitrite and nitrate is a useful method to quantify this molecule in the medium [59,60]. The amount of NO released was indirectly detected by Griess reaction through the measurement of nitrites in the medium. A volume of 98 mL of a phosphate buffer (pH 7.4) solution containing 5 mM of L-cysteine was added in triplicate in a 96-well, flat-bottomed, polystyrene microtiter plate. After loading the plate with the phosphate buffer (98 mL), it was added 2 mL of solution containing the appropriate compound diluted in DMSO (3 mL). The final concentration of the compound in each well was $1\ 10^{-4}$ M. After 1 h of incubation at 37°C, it was added 100 mL of the Griess reagent (4 g of sulfanil-amide, 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 (0.5e100 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 detec-tor (model SPD-20A), quaternary pumping system mobile phase (model LC-20AT), solvent degasser (model DGU-20As) and a Agi-lent® Eclipse XDB C-18 column (250 mm 4,6 mm; 5 mm). For HPLC method it was used an isocratic flow [methanol:water (75:25)] at 1.0 mL/min. The volume injected was 20.0 mL and the wavelength in the detector was 210 nm. The following substances were used as standards to construct the curve log K log P: acetanilide, ben-zonitrile, nitrobenzene, toluene,

naphthalene, biphenyl and phen-anthrene. The capacity factor (logK) of the hybrid compounds was determined from their retention times and interpolated in linearity curve log K log P.

5.3.5. In vitro stability study

In vitro hydrolysis was performed by HPLC method. The com-pound 14c was separated using a Phenomenex Luna reverse-phase C18 (2)-HTS column (2.5-mm 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 mM. Then, this solution was diluted to 10 mM 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 mL. 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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Appendix A. Supplementary data

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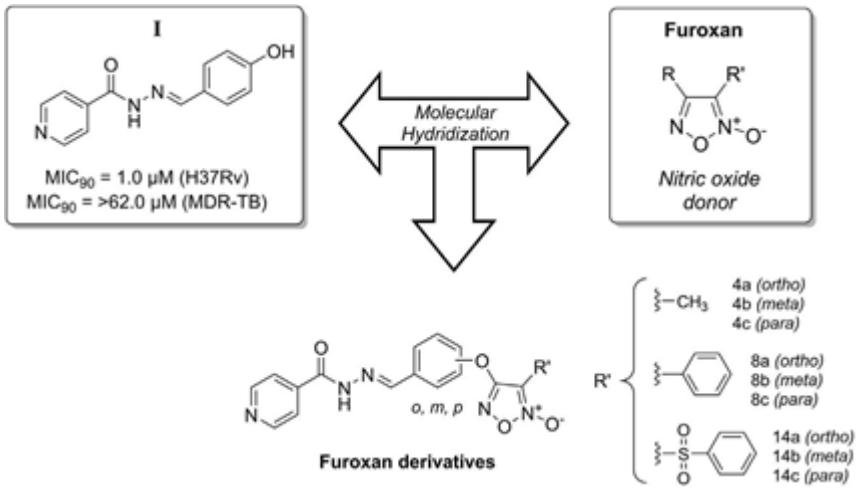
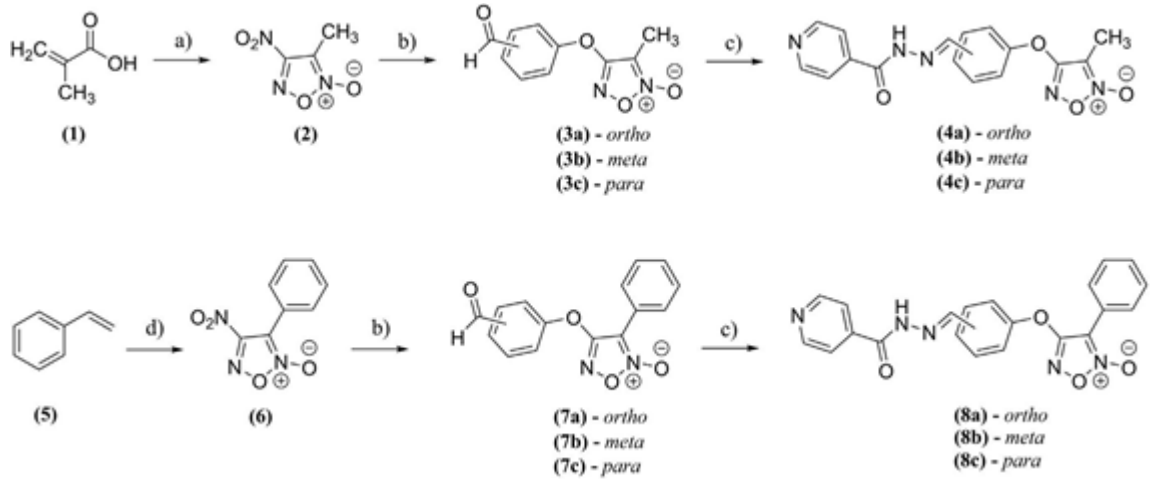
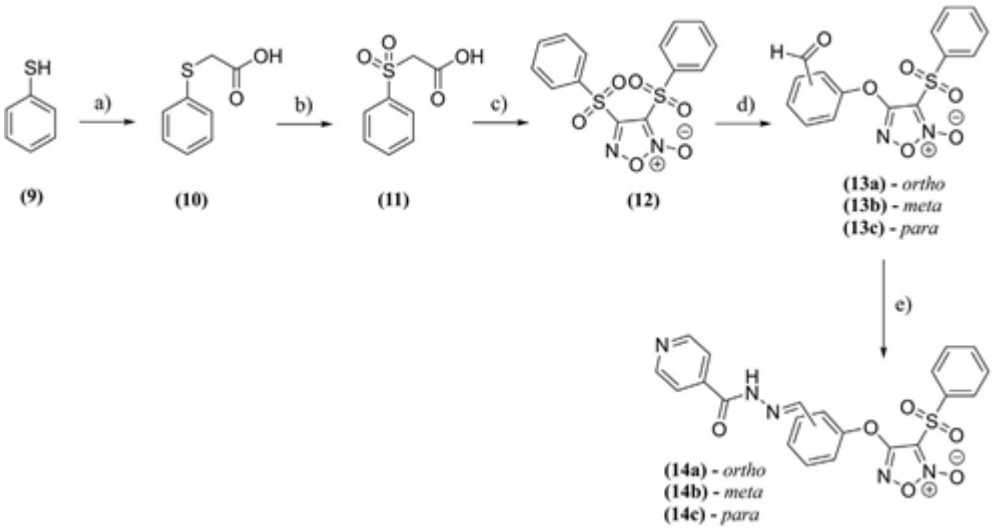


Fig. 1. Design of the hybrid furoxanyl N-acylhydrazone derivatives.



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.



Scheme 2. Reagents and conditions: (a) monochloroacetic acid, NaOH, H2O, 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.

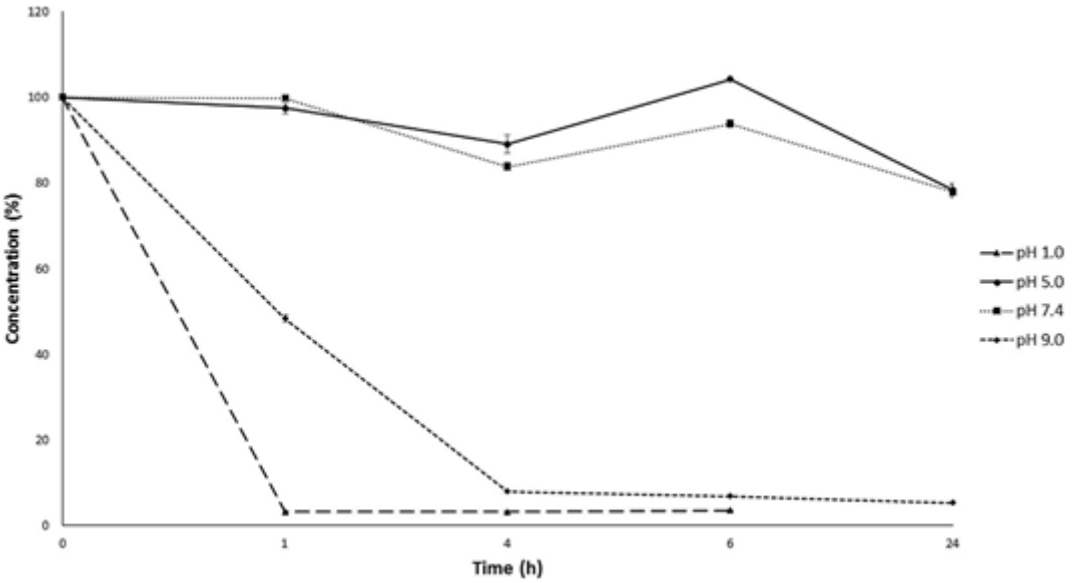


Fig. 2. In vitro chemical stability. Hydrolytic profile of compound 14c in buffer (pH 1.0; 5.0; 7.4 and 9.0) (data are represented as means ± SEMs and expressed as %).