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Synthesis and antitubercular activity of new *N*-[5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl]-(nitroheteroaryl)carboxamides

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Abstract: Nitro-substituted heteroaromatic carboxamides **1a-e** were synthesized and tested against three *Mycobacterium tuberculosis* cell lines. The activities can be explained in terms of the distribution of the electronic density across the nitro-substituted heteroaromatic ring attached to the amide group. 1,3,5-Oxadiazole derivatives **1c-e** are candidates for the development of novel antitubercular agents. Ongoing studies are focused on exploring the mechanism by which these compounds inhibit *M. tuberculosis* cell growth.

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Introduction

Tuberculosis (TB) is a one of the top causes of death worldwide, and in 2017 the World Health Organization reported 10 million new cases of TB, 1.6 million deaths due to TB and 0.3 million deaths resulting from co-infections by HIV [1]. Although the rate of decline in TB was 3.9 % from 2015 to 2017, there were 457,560 new cases of multidrug-resistant TB (MDR-TB) and 558,000 people with rifampicin-resistant TB (RR-TB). Therefore, TB poses a challenge to researchers searching for potent drugs that can control the growth of the bacillus *Mycobacterium tuberculosis* while minimizing side effects or the development of drug resistance [2]. Excellent reviews have been published covering the literature between 2000 and 2015 about compounds with activity against *M. tuberculosis* [3-5]. Notably, Kumar and co-workers grouped anti-TB compounds into 62 different molecular frameworks. In their most recent compilation (2005-2015), they only included compounds exhibiting minimum inhibitory concentration (MIC) values similar to or higher than those of standard drugs used in bioassays [6], with 12 different oxadiazoles meeting this criterion. The 1,3,4-oxadiazole ring system indeed exerts a wide variety of pharmacological activities such as anti-inflammatory [7], antiviral [8], antineoplastic [9], FAK inhibitory [10], adulticidal [11] and anti-Alzheimer properties [12]. A review of the literature up to 2017 includes 440 citations of 1,3,4-oxadiazoles with biological activity but only 34 of these citations describe 1,3,4-oxadiazoles with anti-TB activity [13]. A 5-nitrofuranyl scaffold is present in many derivatives with diverse biological activities including anti-inflammatory [14, 15], antibacterial [16], antileishmanial [17] and antitubercular agents [18-21]. It has been proposed that the presence of the nitro group increases the ability of a molecule to form hydrogen bonds with receptor [22].

We have recently reported the synthesis of caulerpin, a bis-indole alkaloid from the marine alga *Caulerpa sp*. that displays excellent biological activity [23]. Nevertheless, the conventional synthetic approach to medicinal chemistry is a time-consuming, complicated and expensive process that produces candidate compounds with low diversity. Although this approach is still valuable, it is unable to fulfill the increasing demand for new drugs. Therefore, experimental drug discovery is currently being aided with chemo-informatics approaches that are frequently used to identify active compounds, select candidates, and optimize leads. Chemo-informatic approaches has been promising over the past few years in finding pharmacologically active compounds across a broad range of therapeutic areas [24]. Katsuno and co-workers published a comprehensive summary of the criteria to consider when developing new drugs against infectious diseases such as TB [25], and in our work we consider criteria such as selectivity index (SI>10), MIC (lower than 10 μM) and activity against drug-resistant strains, to guide our screening of new compounds having potential use as anti-TB drugs. In the present study, we report a new compound active against *M. tuberculosis*, selected by carrying out a structural analysis of a major public database of compounds reported to have TB activity. We then used the bioisosterism concept [26] to generate a list of other possible compounds (Figure 1) which we subsequently synthesized and tested as inhibitors of the growth of *M. tuberculosis*.

Results and discussion

We conducted a chemotype analysis of compounds in PubChem [27] with reported activity against *M. tuberculosis*. To this end, the chemotype methodology developed by Johnson and Xu [28] to identify promising molecular scaffolds as starting points for optimization was used. In the approach of Johnson and Xu, each chemotype is assigned

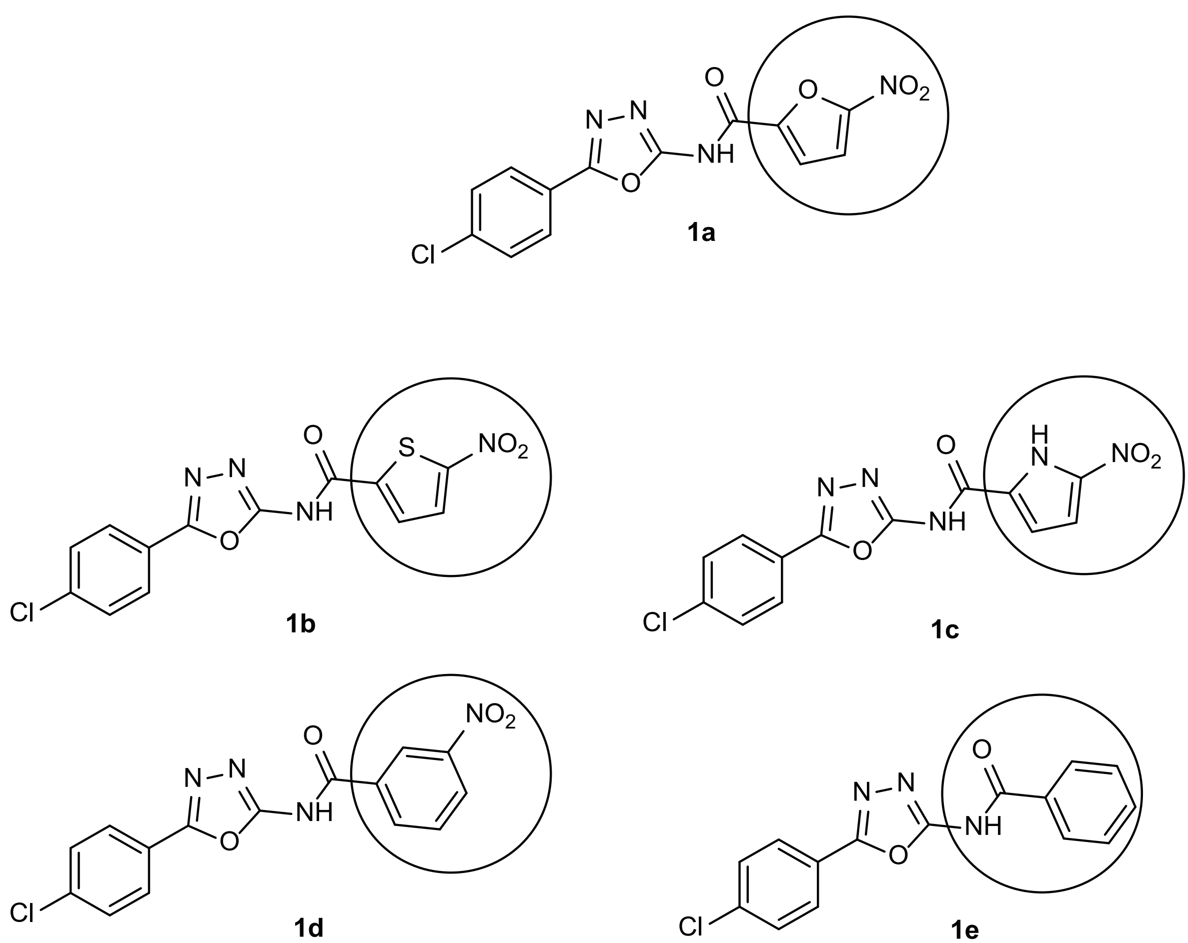


Figure 1 Designed compounds based on isosteric modification of lead compound **1a**.

a unique alphanumeric identifier of four characters [29]. The same approach was previously published to identify promising scaffolds for anti-AIDS activity [30]. Based on the substructure search, the molecular scaffold of compound **1a** (compound identifier, CID in PubChem 4225334) was selected (chemotype identifier BKQ4R). We also compared the newly designed compounds with the reference molecule **1a**. The high structure similarity suggested that their biological activity would be also comparable (Figure 1). This assumption was made considering that here are no activity cliffs, specifically, no similar compounds with very different biological activity [31]. Physical and spectroscopic data for compound **1a** have not yet been published.

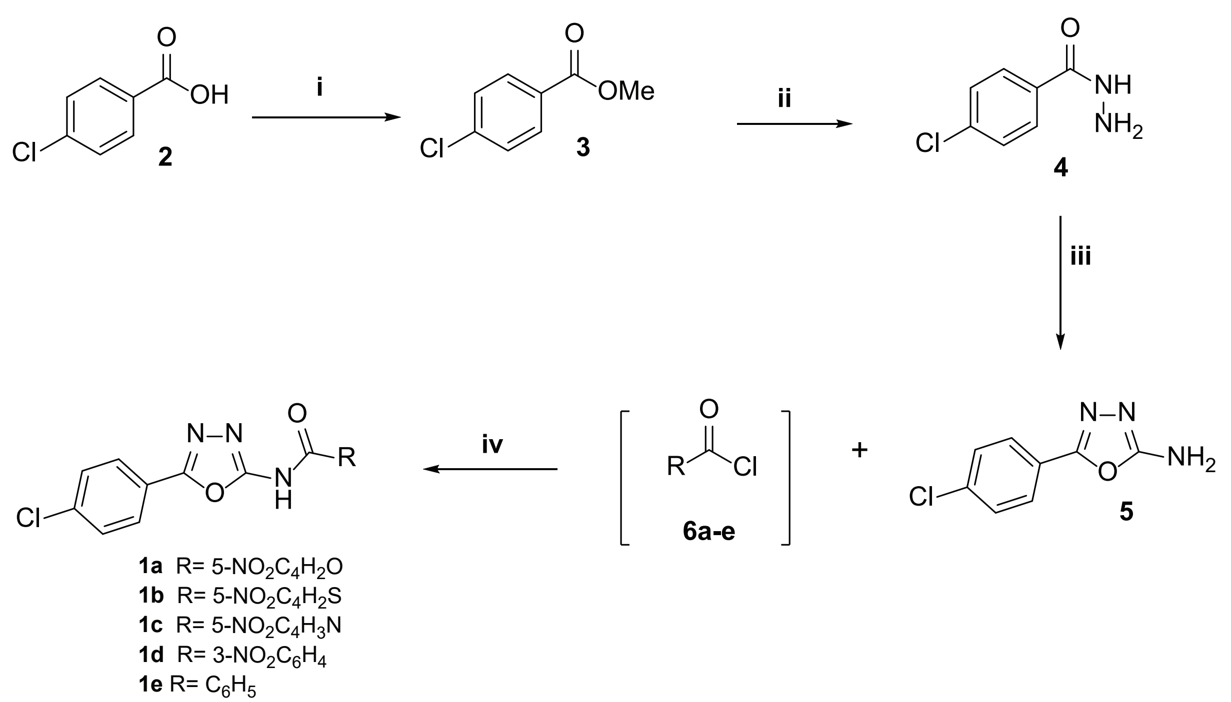
The preparation of compounds **1a-e** started with esterification of 4-chlorobenzoic acid (**2**, Scheme 1), with MeOH in the presence of sulfuric acid to provide methyl 4-chlorobenzoate (**3**). Next, the 4-chlorophenylhydrazide **4** was prepared from **3** [32]. Treatment of **4** with cyanogen bromide (CNBr) in MeOH afforded 5-(4-chlorophenyl)-1,3,4-oxadiazol-2-amine (**5)** in 62% yield [33]. The coupling reaction between **5** and crude acyl chlorides **6a-e** was achieved in the presence of sodium hydride in dry THF to afford the desired products **1a-e** in 30-62% yield [34].

The activities of compounds **1a-e** as inhibitors of the growth of the *M. tuberculosis* strains H37Rv and 209 were evaluated using 250 µg/mL-0.98 µg/mL serial two-fold dilutions. Rifampin (RIF) is usually used for the treatment of mycobacterial infections, including TB, and the anti-TB activities of the synthesized compounds **1a-e** were compared with the activity of RIF. As shown in Table 1, RIF shows the MIC_{100} value of 0.06 µg/mL and is hence more active than compounds **1a-e**. Of compounds **1a-e, 1a** shows the most activity, with the MIC_{100} value of 7.8 µg/mL (23.45 µM), followed by **1b**, **1d** and **1e,** each of them with MIC_{100} of 15.6 µg/mL, followed by **1c**. According to the criteria for

Table 1 Anti-*M. tuberculosis* effects and cytotoxicity levels of compounds **1a-e**.

Compound	R	MIC_{100} (µg/mL)	MBC (µg/mL)	IC_{50}, in Vero cells (µg/mL)	Selectivity index (SI)
1a	5-$NO_2C_4H_2O$	7.80	2.00	106.0	13.59
1b	5-$NO_2C_4H_2S$	15.60	15.60	36.0	4.61
1c	5-$NO_2C_4H_3N$	31.25	31.25	68.3	2.18
1d	3-$NO_2C_6H_4$	15.60	3.90	61.1	3.92
1e	5-C_6H_5	15.60	31.25	44.1	2.83
Rifampin	-	0.06	ND	>1000	>16,666

M. tuberculosis H37Rv ATCC 27294 reference strain: *M. tuberculosis*. MIC: minimal inhibition concentration determined by using REMA. IC_{50}: inhibitory concentration at 50% determined by carrying out an MTT assay in Vero cells. MBC: minimal bactericidal activity. SI = IC_{50}/MIC_{100}.



Scheme 1 Synthetic route to compounds **1a-e**. Reagents and conditions: (i) MeOH, H_2SO_4, reflux; (ii) NH_2NH_2, MeOH; (iii) CNBr, MeOH; (iv) NaH, anhydrous THF, reflux.

anti-TB hits and leads, the MIC_{100} value of **1a** is not competent under replicating growth conditions (< 10 μM) but compounds **1b-e** demonstrate a greater SI and, therefore, can be considered as anti-TB leads. Minimal microbicidal activity measurements showed **1a** has the highest bactericidal activity, followed by **1d** and **1b**, and **1e,** with **1c** being the least active. The values of minimum bactericidal activity show that **1a** also has the highest bactericidal activity followed by **1d** and **1b**, with compounds **1c** and **1e** being the least active [35].

Another criterion used to determine whether a compound can be considered as an early lead for an anti-TB drug is *in vitro* activity against *M. tuberculosis* strains that are resistant to a single TB drug, such as isoniazid or RIF, indicating a new mechanism of action. We also evaluated the compounds with non-virulent bacteria *M. tuberculosis* H37Ra. Non-virulent bacteria are susceptible to lower concentrations of drugs than virulent strains. Specifically, the activities of the synthesized compounds **1a**–**1e** as inhibitors of the growth of bacteria of the *M. tuberculosis* strains H37Ra and 209 (RIF resistant-strain) using 250 μg/mL- 0.98 μg/mL serial two-fold dilutions were evaluated (Table 2). Also listed in Table 2 are the MIC_{100} values of RIF. All compounds are active against *M. tuberculosis* H37Ra (non-pathogenic) and 209 (resistant strain) with compound **1a** being the most active. This result shows that compound **1a** is an anti-TB hit with a new mechanism of action that should be explored.

It can be seen that bioisosteric modifications of the 5-nitrofuran moiety of compound **1a** to give compounds **1b-e** preserves anti-TB activity, albeit with inhibitory activities less than that of the lead compound **1a.** The data for compounds **1b-e** suggest that the anti-TB effect depends on the aromaticity of the ring attached to the amide group, particularly if this ring has low aromaticity (phenyl > thiophene > pyrrole > furan) [36]. The relatively low anti-TB activity of the 5-nitropyrrole compound **1c,** relative to the activities of **1b, 1d** and **1e**, may be due to the pyrrole-NH group of **1c** forming a hydrogen bonding interaction with the active site.

Table 2 REMA-determined MIC_{100} values of **1a-e** against virulent, nonvirulent and RIF-resistant *M. tuberculosis* bacteria.

Compound	R	MIC_{100} (μg/mL) in H37Rv ATCC 27294	MIC_{100} (μg/mL) in H37Ra	MIC_{100} (μg/mL) in Mtb-209 (resistant)
1a	5-NO2C4H2O	7.80	1-2.00	7.8
1b	5-NO2C4H2S	15.60	15.60	15.60
1c	5-NO2C4H3N	31.25	7.8	7.8
1d	3-NO2C6H4	15.60	31.30	15.60
1e	5-C6H5	15.60	62.50	31.25
Rifampin	-	0.06	0.008	>64

M. tuberculosis H37Rv ATCC 27294 reference strain: Mtb. *M. tuberculosis* H37Ra: non-virulent strain. Mtb-209: RIF-resistant clinical isolate of *M. tuberculosis*.

Conclusions

Compounds **1a-e** were synthesized using two convergent pathways with the hydrazide **2** as a common intermediate. The anti-TB activities of compounds **1a-e** were evaluated using three *Mycobacterium tuberculosis* cell lines. The results suggest that the anti-TB activity of compounds **1a-e** can be explained in terms of the distribution of electronic density across the ring attached to the amide group. Our ongoing studies are focused on exploring the mechanism by which these new compounds inhibit *M. tuberculosis* cell growth.

Experimental

Melting points were measured in open capillaries using a Mel-Temp apparatus. ^{1}H-NMR spectra were recorded in DMSO-d_6 on a Jeol Eclipse 300 spectrometer (300 MHz). ^{13}C-NMR spectra were recorded at 75 MHz under otherwise similar conditions. IR spectra were obtained in KBr pellets using a Magna-IR spectrometer. Mass spectra were recorded on a Jeol JEM-AX505HA spectrometer with electronic impact (EI) ionization at 70 eV for low-resolution measurements and a Jeol AccuTOF DART and Jeol JMS700 (FAB+) instruments, for high-resolution measurements. Flash column chromatography was carried out on silica gel 60 (230–400 mesh ASTM) from Macherey-Nagel GmbH. Progress of the reactions was monitored by using TLC. The compounds were visualized using a dual short-wavelength/long-wavelength UV lamp or staining with an ethanol solution of potassium permanganate, vanillin or *p*-anisylaldehyde. All reagents were used as purchased from Aldrich.

Synthesis of methyl 4-chlorobenzoate (3)

A solution of 4-chlorobenzoic acid (**2**, 33.07 mmol) and H_2SO_4 (1 mL) in anhydrous methanol (20 mL) was heated under reflux for 20 h. After consumption of starting material (monitored using TLC), the mixture was neutralized with a saturated $NaHCO_3$ solution (2 x 25 mL) and extracted with AcOEt (3 x 100 mL). The organic extracts were combined, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give **3** as a yellow oil (yield 80 %). The physical and spectral data of **3** were in accordance with previously reported data [37].

Synthesis of 4-chlorobenzohydrazide (4)

A solution of ester **3** (26.36 mmol) and hydrazine (61.85 mmol) in anhydrous methanol (20 mL) was heated under reflux for 12 h. After consumption of the starting material (as monitored using TLC), the reaction mixture was cooled, and the resulting solid was filtered using suction. The crude product was crystallized from hexane to give **4** as a white crystalline solid; yield 82%; mp 162-164°C. The physical and spectral data of **4** were in accordance with previously reported data [37].

Synthesis of 5-(4-chlorophenyl)-1,3,4-oxadiazol-2-amine (5)

A solution of **4** (5.86 mmol) and CNBr (10.65 mmol) in anhydrous methanol was heated under reflux for 5 h. After consumption of the starting material (as monitored using TLC), the mixture was neutralized with a saturated $NaHCO_3$ solution (2 x 25 mL) and the resulting solid was filtered using suction. The crude product was crystallized from methanol to give **5** as a white solid; yield 62%; mp 242-244°C. The physical and spectral data of **5** were in accordance with previously reported data [37].

General procedure for synthesis of compounds 1a-e

A solution of 5-(4-chlorophenyl)-1,3,4-oxadiazol-2-amine (**5**, 0.51 mmol) in anhydrous THF (15 mL) was treated with NaH (1.53 mmol), and the resulting mixture was cooled to 0°C under a nitrogen atmosphere before addition of acyl chloride **6a-e** (0.51 mmol). The mixture was stirred at room temperature for 15 h, quenched with a saturated $NaHCO_3$ solution (30 mL) and extracted with AcOEt (3 x 50 mL). The organic extracts were combined, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified using flash column chromatography on silica gel eluting with hexanes/EtOAc (6:4) to furnish the 1,3,4-oxadiazole **1a-e.**

***N*-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)-5-nitrofuran-2-carboxamide (1a)**

Yield 30% of a yellow solid; mp 250-252°C; IR: 3139, 1607 cm^{-1}; ^1H NMR (DMF-d_7): δ 7.40 (1H, d, *J* = 4 Hz), 7.64 (2H, d, *J* = 8 Hz), 7.74 (1H, d, *J* = 4 Hz), 7.94 (2H, d, *J* = 8 Hz); ^{13}C NMR (DMF-d_7): δ 113.8, 116.5, 123.6, 127.1, 127.8, 129.8, 136.5, 152.2, 157.8, 162.6, 168.3. HRMS (DART). Calcd for $C_{13}H_8ClN_4O_5$, $[M+1]^+$: *m/z* 335.0183. Found: *m/z* 335.0186.

***N*-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)-5-nitrothiophene-2-carboxamide (1b)**

Yield 38% of a yellow solid; mp 260-262°C; IR: 3103, 1714 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 7.68 (2H, d, *J* = 8 Hz), 7.95 (3H, m), 8.15 (1H, d, *J* = 4 Hz). ^{13}C NMR (DMSO-d_6): δ 124.5, 128.9, 129.4, 130.0, 130.8, 131.4, 137.6, 143.9, 154.2, 159.8, 165.4. HRMS (DART). Calcd for $C_{13}H_7ClN_4O_4S$, $[M]^+$: *m/z* 350.9954. Found: *m/z* 350.9957.

***N*-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)-5-nitro-1*H*-pyrrole-2-carboxamide (1c)**

Yield 30% of a yellow solid; mp 278-280°C; IR: 3176, 1635 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 6.98 (1H, d, *J* = 4 Hz), 7.07 (1H, d, *J* = 4 Hz), 7.67 (2H, d, *J* = 8.5 Hz), 7.95 (2H, d, *J* = 8.5 Hz); ^{13}C NMR (DMSO-d_6): δ 111.5, 114.6, 122.9, 128.2, 130.1, 132.2, 136.7, 143.4, 158.8, 159.5, 160.0. HRMS (DART). Calcd for $C_{13}H_9ClN_5O_4$, $[M+1]^+$: *m/z* 334.0343. Found: *m/z* 334.0347.

***N*-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)-3-nitrobenzamide (1d)**

Yield 59% of a white solid; mp 264-266°C; IR: 3176, 1635 cm^{-1}; ^1H NMR (DMSO-d_6): δ 8.88 (1H, s), 8.49 (1H, d, *J* = 7.5 Hz), 8.39 (1H, d, *J* = 7.5 Hz), 7.95 (2H, d, *J* = 8 Hz), 7.78 (1H, m), 7.65 (2H, d, *J* = 8 Hz); ^{13}C NMR (DMSO-d_6): δ 122.1, 123.2, 123.7, 127.4, 128.0, 129.7, 130.5, 130.6, 134.8, 135.4, 136.7, 147.9, 165.6. HRMS (DART). Calcd for $C_{15}H_{10}ClN_4O_4$, $[M+1]^+$: *m/z* 345.0390. Found: *m/z* 345.0394.

***N*-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)benzamide (1e)**

Yield 78% of a white solid; mp 224-226°C; IR: 3050, 1712 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 7.65 – 7.51 (3H, m), 7.69 (2H, d, *J* = 8.5 Hz), 7.98 (2H, d, *J* = 8.5 Hz), 8.04 (2H, d, *J* = 7 Hz), 12.2 (1H, br); ^{13}C NMR (DMSO-d_6): δ 122.2, 127.8, 128.3, 128.6, 129.6, 132.2, 133.0, 136.4, 158.0, 160.4, 164.9. HRMS (DART). Calcd for $C_{15}H_{11}ClN_3O_2$, $[M+1]^+$: *m/z* 300.0540. Found: *m/z* 300.0540.

***In vitro* antimycobacterial assay**

Stock solutions of all compounds were prepared in 100% dimethyl sulfoxide (DMSO) at 10 μg/mL. For the REMA assay, compounds were diluted in 7H9 medium without tyloxapol. For reference drugs, stocks of 64 μg/mL were prepared and filtered through a membrane with pore diameters of 0.22 mm (Millipore; Darmstadt, Germany). All working solutions were kept refrigerated at -20^{0} C until they were evaluated.

The cytotoxicity assay

The assay was carried out using Vero cell lines (kidney of African green monkey) from ATCC. The cells were cultured in RPMI 1640 medium supplemented with 10% FBS and nonessential amino acids.

Cytotoxicity assay

Ten-thousand Vero cells were placed in a 96 well-plate and incubated for 24 h in 100 µL of RPMI medium. After incubation, the plate was washed and new fresh medium with the compound at a various concentration was added. Each tested compound was incubated for 48h at 37°C in a 5% CO_2 atmosphere. Then, a volume of 10 µL of MTT (5 µg/mL in sterile PBS) was added to each well and incubation was continued for another 4 h. The medium was removed and a volume of 100 µL of DMSO was added to solubilize the formazan. Absorbance was determined at a wavelength of 570 nm and cytotoxicity was calculated as % toxicity = (1-(ABS problem/ABS control))*100. Controls were cells without treatment [38].

M. tuberculosis culture conditions

M. tuberculosis, H37Rv, H37Ra and 209 strains were cultivated in a 7H9-glycerol-10% ADC-0.01% tyloxapol medium at 37°C until an O.D. of 0.4 at a wavelength of 600 nm was reached. Working bacteria-solutions were obtained by dilution 1:25 in 7H9-ADC 10%.

Antimicrobial susceptibility test using the resazurin microtiter assay (REMA)

The assay used here was previously described by Collins and Franzablau [39]. Briefly, the outer wells of a 96-well plate were each filled with 200 µL of sterile PBS to prevent dehydration from occurring during the long-duration (8-day) incubation. RIF was used as a reference drug (16 -0.001 µg/mL serial two-fold dilutions) in each plate, and controls of DMSO, DMSO+Mtb, medium, media+Mtb, and compound only to validate the plate were also included. Compounds were evaluated at various concentrations from 0.98 µg/mL to 250 µg/mL, and in triplicate in independent assay experiments. Plates were incubated for six days; then, 30 µL of 0.01% resazurin (weight/volume) (Sigma Aldrich) were added to each well and the plates were incubated for two more days. Visual inspection was used to determine the colors of the contents of each well, with blue interpreted as no growth, pink as growth, and MIC as the last concentration in which blue color were observed. The minimum inhibitory concentration (MIC) is defined as the minimum concentration of tested compound to which there was not higher shift from resazurin (blue) to resorufin (pink) than that generated by control of a 1:100 dilution of the bacterial inoculum [36].

Minimal bactericidal concentration

The MBC values were determined for the well (s)+compound that did produce a shift in color and were then re-incubated in fresh medium. A volume of 5 µL of this well (s) was added to 195 µL of fresh medium and incubated for carrying out the REMA assay as described above. MBC corresponds to the minimum concentration of tested compound that does not produce a shift in cultures reincubated in fresh medium [36].

Selectivity indexes

The **SI** values were obtained using the formula SI = IC_{50} in Vero cells and the MIC_{100} values were determined using REMA.

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