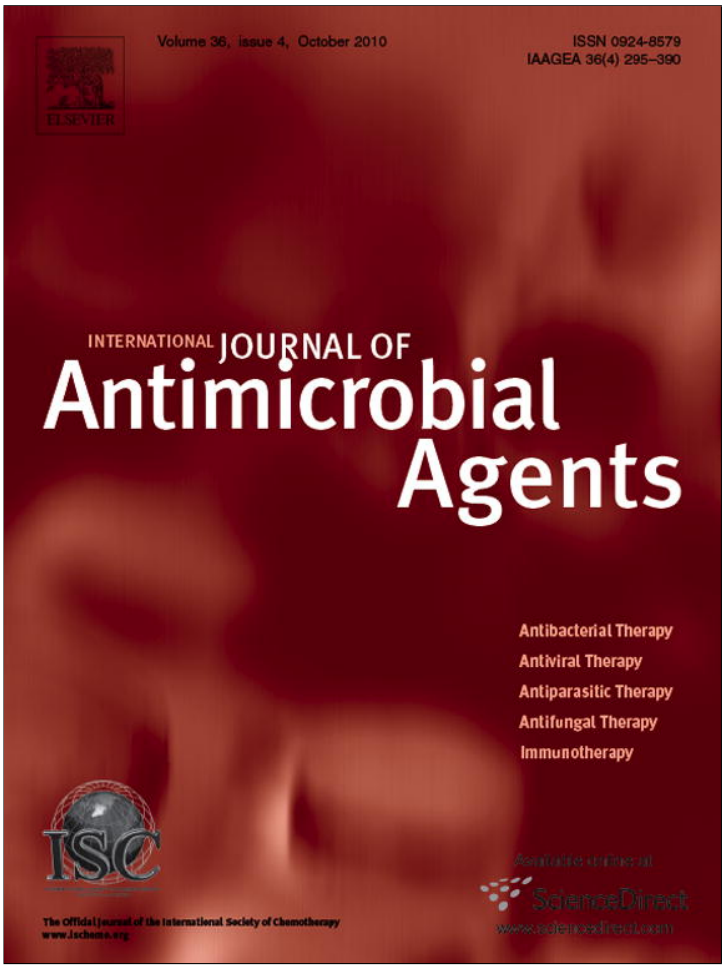

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Short communication

Antimycobacterial activity of UDP-galactopyranose mutase inhibitors

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ABSTRACT

The galactofuran region of the mycobacterial cell wall consists of alternating 5- and 6-linked β-D-galactofuranose (β-D-Gal*f*) residues, essential for viability. UDP-galactofuranose (UDP-Gal*f*), the donor for Gal*f*, is synthesised from UDP-galactopyranose (UDP-Gal*p*) by the enzyme UDP-galactopyranose mutase (UGM), which is not found in humans, rendering it a therapeutic target. The in vitro properties, i.e. enzymatic activity, antimycobacterial activity, cellular toxicity, activity in mycobacterial-infected macrophages and activity against non-replicating persistent mycobacteria, of (4-chlorophenyl)-[1-(4-chlorophenyl)-3-hydroxy-5-methyl-1H-pyrazol-4-yl]-methanone and 3-(4-iodophenyl)-2-[4-(3,4-dichlorophenyl)-thiazol-2-ylamino]-propionic acid were studied. The former compound, a pyrazole, was an inhibitor of UGM from *Mycobacterium tuberculosis* and *Klebsiella pneumoniae* and was effective against *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG and *M. tuberculosis* but ineffective against other bacterial strains tested. This compound showed potency against mycobacteria in infected macrophages but exhibited moderate cellular toxicity and was ineffective against non-replicating persistent mycobacteria. This is the first report of a compound both with UGM inhibitory properties and broad antimycobacterial activities. The latter compound, an aminothiazole, was active against UGM from *K. pneumoniae* and *M. tuberculosis* but was ineffective against *M. bovis* BCG or *M. tuberculosis* as well as demonstrating higher cellular toxicity. These data validate the choice of UGM as a target for active antimycobacterial therapy and confirm the pyrazole compound as a viable lead candidate.

1. Introduction

Mycobacterium tuberculosis is an intracellular human pathogen that targets alveolar macrophages and causes tuberculosis (TB). TB can be both active (replicating mycobacteria, resulting in disease) and latent (non-replicating mycobacteria, asymptomatic infection) [1]. In 2007 there were 9.3 million new cases of TB and an estimated 1.7 million deaths per year were reported [1,2]. The emergence of multidrug-resistant and extensively drug-resistant strains of *M. tuberculosis* impacts on current therapy [1]. Hence, there is a need for better, faster-acting and cheaper drugs [1].

The drugs isoniazid and ethambutol [1] are inhibitors of mycobacterial cell wall biosynthesis, suggesting the role of the cell wall. Mycobacteria have a cell wall composed of the mycolyl–arabinogalactan–peptidoglycan complex (MAPc) [3]. MAPc consists of peptidoglycan linked to a D-galactofuran and to D-arabinofuran. The D-galactofuran region consists of alternating 5- and 6-linked β-D-galactofuranose (β-D-Gal*f*) residues [3]. The precursor of Gal*f* is UDP-galactofuranose (UDP-Gal*f*), which is synthesised from UDP-galactopyranose (UDP-Gal*p*) by the enzyme UDP-galactopyranose mutase (UGM). Mutants of *Mycobacterium smegmatis*, a model for *M. tuberculosis*, show that the UGM gene is essential for bacterial viability [4]. Gal*f* is a common constituent of the glycans of prokaryotes and of eukaryotic parasites but is absent in higher eukaryotes, rendering it a target for inhibition by new drugs (UGM inhibitors) [5].

Two UGM inhibitors also inhibit bacterial growth [6,7]. Tangallapally et al. [6] identified inhibitors of mycobacteria only of the tuberculosis complex. Dykhuizen et al. [7] described aminothiazole compounds with activity tested only against one strain of the *M. smegmatis*, and other in vitro properties of the compound were not studied. A pyrazole compound with a similar structure to that of Dykhuizen's inhibitor and with reported activity against *M. tuberculosis* has been described by Castagnolo et al. [8], but its action against UGM as well as other in vitro properties have not been reported.

In this study, we synthesised the two abovementioned compounds, i.e. the pyrazole (4-chlorophenyl)-[1-(4-chlorophenyl)-3-hydroxy-5-methyl-1H-pyrazol-4-yl]-methanone (compound **1**; Fig. 1) [8] and the aminothiazole 3-(4-iodophenyl)-2-[4-(3,4-

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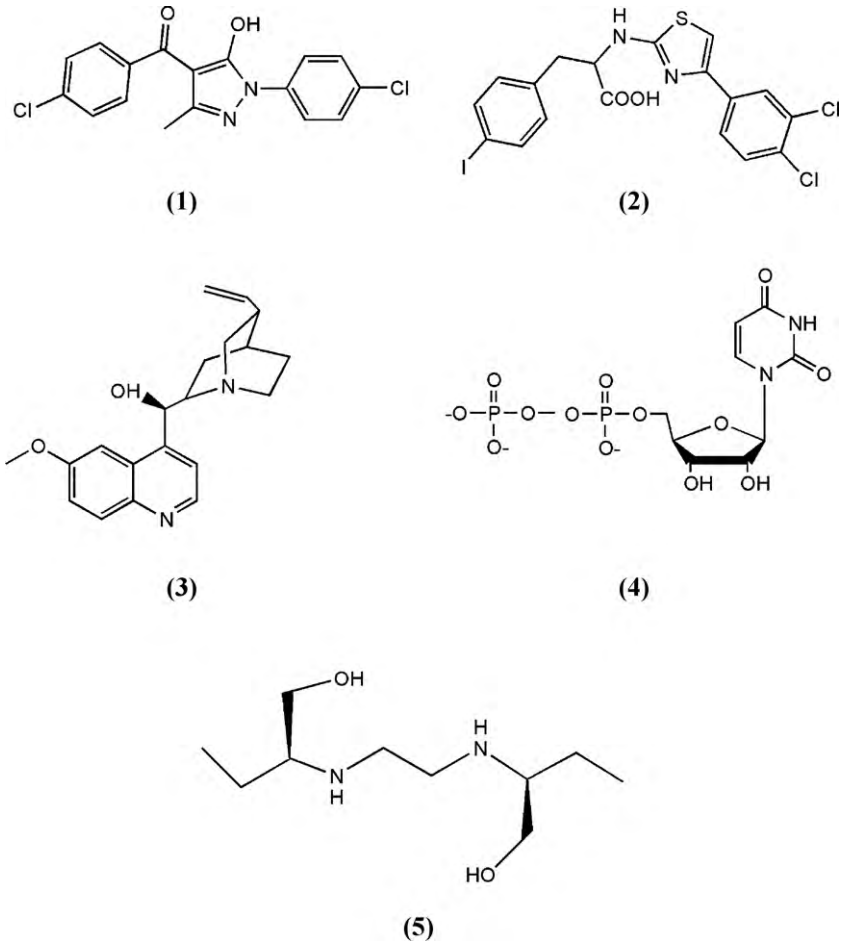

Fig. 1. Structures of the compounds: the putative UDP-galactopyranose mutase (UGM) inhibitor pyrazole (**1**); the UGM inhibitor control aminothiazole (**2**); quinine (**3**); uridine diphosphate (UDP) (**4**); and the arabinosyltransferase inhibitor ethambutol as a positive antimycobacterial control (**5**).

dichlorophenyl)-thiazol-2-ylamino]-propionic acid (compound **2**; Fig. 1) [7], and report their UGM inhibitory activity by capillary electrophoresis (CE) analysis. The antimycobacterial activity of the compounds against *M. smegmatis*, *Mycobacterium bovis* BCG and *M. tuberculosis* strain H37Rv was characterised. In vitro cellular toxicity against the human monocytic cell line THP-1 was examined. The ability of the compounds to kill replicating mycobacteria within macrophages in a model of BCG-infected macrophages was determined. Finally, the activity of the compounds against non-replicating persistent *M. smegmatis* was tested [9].

2. Materials and methods

2.1. Bacterial strains, cell lines and culture conditions

M. smegmatis ATCC 607 and ATCC 700084 (MC^2 155), *M. bovis* BCG ATCC 35734, *M. tuberculosis* strain H37Rv, The Gram-negative strains *Escherichia coli* ATCC 11303 and *Moraxella* (*Branhamella*) *catarrhalis* ATCC 25238 and the Gram-positive strains *Micrococcus luteus* ATCC 4698 and *Bacillus subtilis* ATCC 6051 were maintained in stock culture at −70 °C and were grown before each assay. *Mycobacterium smegmatis* was grown in brain–heart infusion agar (Difco Laboratories, Detroit, MI), Mueller–Hinton broth or agar (Difco) or Dubos broth base or Dubos oleic agar base (Difco). *Mycobacterium bovis* BCG and *M. tuberculosis* strain H37Rv were grown in Middlebrook 7H9 (Difco) liquid medium or Middlebrook 7H10 agar (Difco). Gram-positive and Gram-negative strains were grown on Muller–Hinton broth or agar. The monocytic cell line THP-1 ATCC 202 was cultured in RPMI medium (HyClone®; Thermo Fisher Scientific, Logan, UT) [10].

2.2. Compounds and UDP-galactopyranose mutase enzymes

The pyrazole (**1**) and the aminothiazole (**2**) (Fig. 1) were synthesised [7,8]. Quinine (**3**), uridine diphosphate (UDP) (**4**) and ethambutol (**5**) (Fig. 1) were obtained from Sigma Chemical Co. (St Louis, MO). Quinine (**3**) and ethambutol (**5**) comprised the toxicity and antimycobacterial positive controls, respectively; UDP (**4**) was the negative control. Isoniazid and rifampicin (Sigma) were used as controls for the *M. smegmatis* in vitro oxygen depletion assay [9]. Solutions (10 mg/mL) of the compounds were made in H_2O (compounds **3**, **4**, **5** and isoniazid) or dimethyl sulphoxide (DMSO) (compounds **1**, **2** and rifampicin) and used as stock solutions. UDP-Gal*f* was provided by Dr T.L. Lowary (University of Alberta, Edmonton, Canada). UGM was obtained from *Klebsiella pneumoniae* (*kp*UGM) and *M. tuberculosis* (*tb*UGM) [11].

2.3. Determination of UDP-galactopyranose mutase 50% inhibitory concentration (IC_{50}) values by capillary electrophoresis

Enzyme activity was measured by CE analysis. The extent of UDP-Gal*f* conversion to UDP-Gal*p* by UGM was determined [11,12] and was analysed by CE on a ProteomeLab™ PA 800 Protein Characterization System (Beckman-Coulter. Fullerton, CA) [13]. The IC_{50}

of pyrazole **1** and aminothiazole **2** against *tb*UGM and *kp*UGM was determined [12].

2.4. Antimicrobial susceptibility

2.4.1. Disk diffusion assay

Paper disks (Fisher Scientific Co., Ottawa, Ontario, Canada) were impregnated with 20 μg or 50 μg of compound. *Mycobacterium smegmatis* strains ATCC 700084 and ATCC 607, *E. coli*, *B. subtilis*, *M. luteus* and *M. catarrhalis* strains were grown and the culture was diluted and spread on plates. Plates were allowed to dry before applying dried disks and were incubated until inhibition zones were observed.

2.4.2. Microbroth dilution assay

The minimum inhibitory concentration (MIC) of the compounds against *M. smegmatis* strains ATCC 700084 and ATCC 607, *E. coli*, *M. catarrhalis*, *B. subtilis* and *M. luteus* was determined by microbroth assay [14]. The MIC was determined for wells with >99% inhibition of growth using ethambutol as a control.

2.4.3. Agar proportion method

The MIC of the compounds against *M. bovis* BCG was determined by the agar proportion method [14]. The MIC was determined for wells with >95% inhibition of growth using ethambutol as a control.

2.4.4. Disk diffusion assay with Mycobacterium tuberculosis

Paper disks were impregnated with 2.5, 5, 10 and 20 μg of pyrazole **1** and 6.25, 12.5, 25, 50 and 100 μg of aminothiazole **2** in a DMSO solution and dried. The rest of the procedure was as described above.

2.5. Toxicity assay

The toxicity of the compounds was determined by fluorescence-activated cell sorting (FACS) analysis of propidium iodide (PI)-stained THP-1 cells exposed to the compounds (400, 200, 100 and 50 μg/mL) for 24 h [10]. The positive controls for PI-stained THP-1 cells were 1% and 0.5% H_2O_2.

2.6. Macrophage infection assay

Phorbol myristate acetate-differentiated THP-1 macrophages were infected with *M. bovis* BCG [15]. Inhibitors at a final concentration of 5 μg/mL (for 24 h) and 10 μg/mL (for the following 24 h) were added and incubated for 24 h and 48 h, respectively. Cells were lysed and counting was performed [15].

2.7. Mycobacterium smegmatis in vitro oxygen depletion assay

The activity of the pyrazole **1** and aminothiazole **2** against non-replicating persistent *M. smegmatis* ATCC 700084 [9] was evaluated. Compounds, DMSO or controls were added by injection through the septa. Drug exposure lasted for 96 h and the bacterial suspension was then counted [9].

2.8. Statistical analysis

Significant differences were established with Student's *t*-test. *P*-values of <0.05 were considered significant.

3. Results and discussion

3.1. Synthesis and enzymatic activity

The pyrazole **1** and aminothiazole **2** were synthesised and tested in UGM inhibition assays by CE both against *tb*UGM and *kp*UGM (level of identity 40%). The pyrazole **1** was a UGM inhibitor, with calculated IC_{50} values of 62 ± 1 μM and 44 ± 1 μM against *tb*UGM and *kp*UGM, respectively. The aminothiazole **2** gave IC_{50} values of 37 ± 1 μM and 7.2 ± 1 μM against *tb*UGM and *kp*UGM, respectively.

3.2. Antimicrobial activity of the compounds

The pyrazole **1** and aminothiazole **2** had activity against both *M. smegmatis* strains tested. A disk sensitivity assay indicated greater activity of the pyrazole **1** (25 ± 1 mm for ATCC 700084 and 35 ± 2 mm for ATCC 607) than the aminothiazole **2** (9 ± 1 mm for ATCC 700084 and 15 ± 1 mm for ATCC 607). Compounds **1** and **2** did not inhibit the growth of *E. coli* but growth inhibition zones were observed for *B. subtilis* (12 ± 1 mm and 11 ± 1 mm), *M. luteus* (25 ± 1 mm and 23 ± 1 mm) and *M. catarrhalis* (25 ± 1 mm and 19 ± 1 mm). No activity was observed for DMSO, quinine (**3**) or UDP (**4**). The MICs for compounds **1**–**4** against *M. smegmatis* and *M. bovis* BCG were determined. MICs for the pyrazole **1** were 3.3 μg/mL and 6.7 μg/mL for the *M. smegmatis* tested and 6.5 μg/mL for *M. bovis* BCG. MICs for the aminothiazole **2** were 12.5 μg/mL for both *M. smegmatis* strains, whereas it was ineffective against *M. bovis* BCG at the higher concentration (50 μg/mL) tested. MICs for the pyrazole **1** were 100 μg/mL for *B. subtilis*, *M. luteus* and *M. catarrhalis* and >200 μg/mL for *E. coli*. MICs for the aminothiazole **2** were 50 μg/mL for *B. subtilis* and *M. luteus*, 25 μg/mL for *M. catarrhalis* and >200 μg/mL for *E. coli*.

Quinine (**3**) and UDP (**4**) were ineffective against *M. smegmatis* or *M. bovis* BCG at a concentration <200 μg/mL or <400 μg/mL, respectively. Ethambutol (**5**), an approved drug against *M. tuberculosis* and used as an antimycobacterial positive control, showed a MIC value of 0.1 μg/mL for *M. bovis* BCG and <0.2 μg/mL for *M. smegmatis*.

The pyrazole **1** had activity against *M. tuberculosis* H37Rv as evidenced by growth inhibition zones at 2.5 μg (9.0 ± 1.7 mm), 5 μg (14.5 ± 1.0 mm), 10 μg (20.7 ± 1.2 mm) and 20 μg (29.3 ± 1.2 mm) of compound. The aminothiazole **2** did not inhibit the growth of *M. tuberculosis* H37Rv at any of the concentrations tested up to 100 μg.

These results indicate that pyrazole **1** is the first UGM inhibitor with broad antimycobacterial activity reported to date and suggest that the primary mode of action is inhibition of UGM, since compound **1** was not effective at concentrations <100 μg/mL against the Gram-positive or Gram-negative strains tested. The data validate the choice of UGM as a target for the development of new antimycobacterial drugs.

3.3. Toxicity against the human monocytic cell line THP-1

Toxicity for mammalian cells was determined by exposing undifferentiated THP-1 monocytes to compounds **1**–**5** (Fig. 2A). The percentage of dead cells was detected by PI staining and FACS analysis and the 50% lethal dose (LD_{50}) was calculated. UDP (**4**) was the least toxic, with an LD_{50} of >400 μg/mL. The pyrazole **1** and quinine (**3**) were the next least toxic, with LD_{50} values of ca. 50 μg/mL and 70 μg/mL, respectively. The MIC for compound **1** both for *M. smegmatis* and BCG was 3.7–6.7 μg/mL (≥8-fold difference from LD_{50}), thus we conclude that the pyrazole **1** has moderate toxicity similar to that of quinine. Values for ethambutol (**5**) were an LD_{50} of ca. 350 μg/mL and a MIC of 0.1 μg/mL (ca. 3500-fold difference). The aminothiazole **2** was the most toxic, with an LD_{50} value <50 μg/mL (Fig. 2A), close to the *M. smegmatis* MIC. The pyrazole **1** thus appears

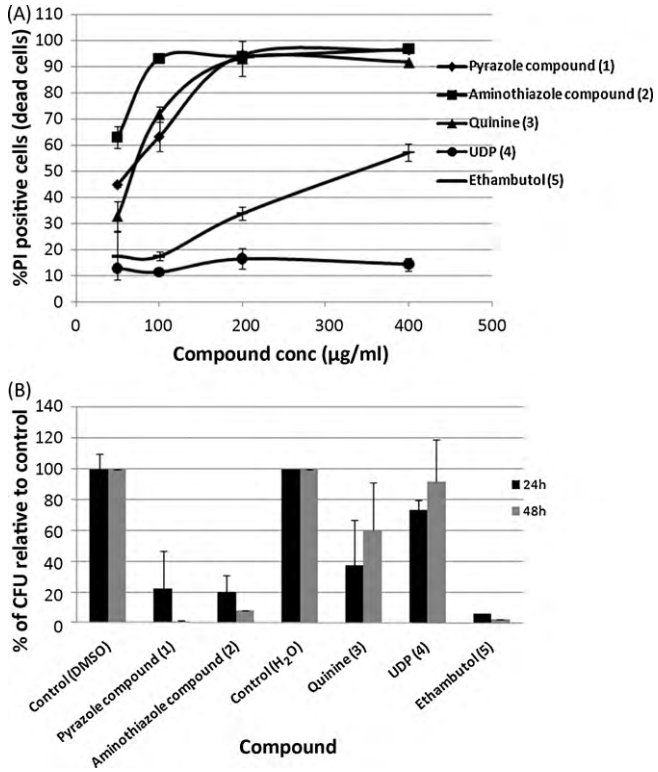

Fig. 2. (A) Toxicity assay in THP-1 cell line as determined by fluorescence-activated cell sorting (FACS) analysis of propidium iodide (PI)-stained cells. (B) Macrophage infection assay. Inhibitors were added at a concentration of 5 μg/mL for 24 h and 10 μg/mL for 48 h. Pyrazole **1** and aminothiazole **2** compounds were dissolved in dimethyl sulphoxide (DMSO). UDP, uridine diphosphate; CFU, colony-forming units.

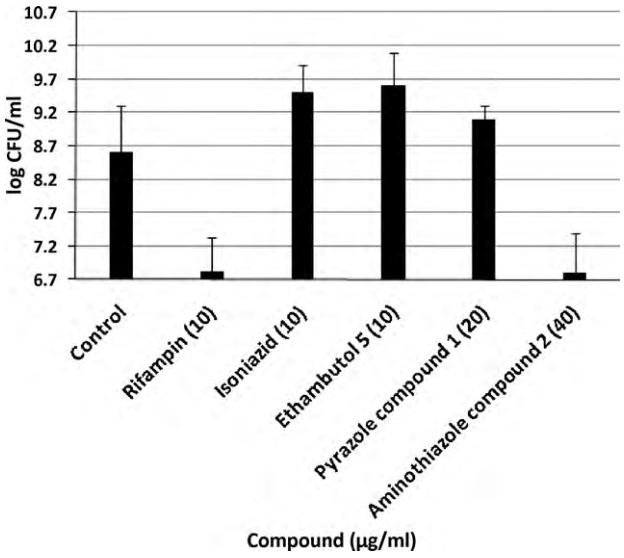

Fig. 3. Recovery of non-replicating persistent *Mycobacterium smegmatis* following treatment with pyrazole (**1**) at 20 μg/mL, aminothiazole (**2**) at 40 μg/mL and standard antimycobacterial drugs at 10 μg/mL. Cultures were grown under gradual oxygen depletion for 120 h and were then treated with compounds for 96 h. The bacterial suspension was then diluted, plated and colony-forming units (CFU) were counted. Values shown are the mean ± standard deviation of three experiments.

to be a more promising lead candidate for antimycobacterial therapy than the aminothiazole **2**.

3.4. Macrophage infection assay

Mycobacterium tuberculosis replicates within macrophages [1]. To test the effect of the compounds on mycobacterial replication within macrophages, THP-1 macrophages were infected with *M. bovis* BCG and then exposed to compounds **1**–**5** for 24 h and 48 h at a concentration of 5 μg/mL and 10 μg/mL, respectively (Fig. 2B). The pyrazole **1** and aminothiazole **2** showed some effectiveness, resulting in a low percentage of colony-forming units (CFU) relative to the control both after 24 h and 48 h. Compounds **1** and **2** showed 80% inhibition after 24 h ($P = 0.03$ and $P = 0.02$) and 99% ($P = 0.001$) and 93% ($P = 0.001$) inhibition, respectively, after 48 h compared with the control CFU value. Ethambutol (**5**), which is active against BCG, displayed 95% ($P = 0.001$) inhibition after 24 h and 98% ($P = 0.001$) after 48 h compared with the control. Pyrazole **1** activity did not differ from that of ethambutol (**5**) after 24 h ($P = 0.43$) and 48 h ($P = 0.06$), suggesting that both compounds are active against BCG. However, the activity observed for the aminothiazole **2** differed from that for ethambutol (**5**) after 48 h ($P = 0.001$), but not after 24 h ($P = 0.36$). This correlates with the lack of activity of the aminothiazole **2** against BCG at 10 μg/mL. Quinine (**3**) showed 63% inhibition after 24 h ($P = 0.03$) and 40% ($P = 0.25$) after 48 h compared with the control CFU value. The pyrazole **1** percentage of CFU did not differ from that of quinine (**3**) after 24 h ($P = 0.5$) and 48 h ($P = 0.06$); both compounds showed similar cellular toxicity. Similar results were observed when the activity levels of the aminothiazole **2** were compared with those of quinine (**3**) after 24 h ($P = 0.4$) and 48 h ($P = 0.07$). This suggests that the pyrazole **1** has some potency in this model since it is effective against BCG despite its cellular toxicity. Results for the aminothiazole **2** activity within macrophages are presumably due to its high toxicity against THP-1 macrophages (Fig. 2A) since this compound has no activity against BCG at the concentrations tested.

3.5. Mycobacterium smegmatis in vitro oxygen depletion assay

Mycobacterium smegmatis in the non-replicating persistent state are resistant to killing by standard antimycobacterial drugs [9], similar to non-replicating persistent *M. tuberculosis* [1,9]. To test the effect of the pyrazole **1** and aminothiazole **2** on the non-replicating persistent state, *M smegmatis* strain ATCC 700084 was grown under oxygen depletion (Fig. 3). Pyrazole **1** at a concentration of 20 μg/mL did not kill non-replicating persistent *M. smegmatis* compared with the control ($P = 0.30$). Aminothiazole **2** at a concentration of 40 μg/mL was active in killing non-replicating persistent *M. smegmatis* compared with the control ($P = 0.04$) and had a similar activity to the control rifampicin ($P = 0.94$). The antimycobacterial drugs and cell wall inhibitors ethambutol (**5**) and isoniazid (both at 10 μg/mL) showed similar activities to the pyrazole **1** ($P = 0.27$ and $P = 0.29$, respectively) and the control ($P = 0.14$ and $P = 0.15$, respectively) but differed from aminothiazole **2** ($P = 0.006$ and $P = 0.008$, respectively) and rifampicin ($P = 0.005$ and $P = 0.007$, respectively). These data suggest that pyrazole **1** acts in a similar fashion to standard cell wall inhibitors and that there is a different mode of killing *M. smegmatis* between the two UGM inhibitors.

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Competing interests: None declared.

Ethical approval: Not required.

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