Hypothesis

Design, Synthesis, and *In Vitro* Antituberculosis Activity of 2(5*H*)-Furanone Derivatives

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Abstract

A series of 2(*5H*)-furanone-based compounds were synthesized from commercially available mucohalic acids. From the first-generation compounds, three showed inhibitory activity (10 μg/mL) of at least 35% against *Mycobacterium smegmatis* mc^2 155 growth (Bioscreen C system). In screening the active first-generation compounds for growth inhibition against *Mycobacterium tuberculosis* H37Rv, the most active compound was identified with a minimum inhibitory concentration (MIC_{99}) of 8.07 μg/mL (15.8 μM) using BACTEC 460 system. No cross-resistance was observed with some current first-line anti-TB drugs, since it similarly inhibited the growth of multidrug resistant (MDR) clinical isolates. The compound showed a good selectivity for mycobacteria since it did not inhibit the growth of selected Gram-positive and Gram-negative bacteria. It also showed synergistic activity with rifampicin (RIF) and additive activity with isoniazid (INH) and ethambutol (EMB). Additional time-kill studies showed that the compound is bacteriostatic to mycobacteria, but cytotoxic to the Chinese

Additional Supporting Information may be found in the online version of this article.

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Hamster Ovarian (CHO) cell line. From a second generation library, two compounds showed improved anti-TB activity against *M. tuberculosis* H37Rv and decreased CHO cell cytotoxicity. The compounds exhibited MIC values of 2.62 μg/mL (5.6 μM) and 3.07 μg/mL (5.6 μM) respectively. The improved cytotoxicity against CHO cell line of the two compounds ranged from $IC_{50} = 38.24$ μg/mL to $IC_{50} = 45.58$ μg/mL when compared to the most active first-generation compound ($IC_{50} = 1.82$ μg/mL). The two second generation leads with selectivity indices (SI) of 14.64 and 14.85 respectively, warrant further development as anti-TB drug candidates. © 2016 IUBMB Life, 68(8):612–620, 2016

Keywords: *synthetic; furanone; antimycobacterial; selective; synergy; cytotoxicity*

Introduction

Tuberculosis (TB) is one of the most common infectious diseases and continues to be a major cause of morbidity and mortality worldwide (1). The emergence of HIV infection, decline of socioeconomic standards, and a reduced emphasis on tuberculosis control programs have contributed to the disease's resurgence in industrialized countries (2,3). The lethal synergy of the tuberculosis and human immunodeficiency virus (HIV) epidemics have complicated the global fight against TB (4,5). Resistance of *Mycobacterium tuberculosis* strains to current antitubercular agents, leading to the emergence of multidrug-resistant (MDR) *M. tuberculosis* strains, is an increasing problem worldwide (5,6). These trends are predicted to continue for decades and warrant the urgent need for new TB therapies (7).

The furanone ring system, also known as butyrolactone or butenolide, is a widely recognized component of natural products, exhibiting an extensive spectrum of pharmacological activities (8). In particular, compounds bearing the 2-furanone backbone have exhibited diverse biological activities, such as antiprotozoal, antibacterial (including inhibition of biofilm formation), antimycobacterial, antitumoral, antifungal, and cyclooxygenase-2 inhibition (8–14). The efficacy of several butyrolactone derivatives against mycobacteria suggests that these structures be a new template for tuberculosis drug development (14,15).

Our research effort towards the development of novel antitubercular agents encompasses the search for new classes of compounds, which are structurally distinct from known antitubercular drugs in order to target other mechanisms of action. Within the context of medicinal chemistry, the furanones are chemically tractable as they lend themselves to synthesis of a varied range of analogues (16). The furanones do however possess the potential liability of cytotoxicity as previously reported by Lattman et al. (17).

In an effort to develop novel drugs to treat TB, a series of furanone-based compounds were chemically synthesized from commercially available mucohalic acids. The current work describes the synthesis and biological screening of novel 2(5*H*)-furanone derivatives. From a first generation library of synthetic 2(*5H*)-furanone compounds, that were screened for antimicrobial activity, one compound was identified that showed antituberculosis activity and specificity for mycobacteria over other reference bacterial strains. From a second generation library of furanone derivatives, two compounds were identified which showed improved activity and cytotoxicity. Here we present data that suggest the two active second-generation compounds offer a synthetic backbone that could be further developed and optimised as TB antibiotic leads.

Materials and Methods

Ethical approval for this study was obtained from the Health Research Ethics Committee of Stellenbosch University (reference no: N08/05/159).

Synthetic Chemistry

All starting materials used were commercially available (Sigma-Aldrich, St. Louis) and were used as obtained from the supplier without further purification. Column chromatography was performed using Merck Kielselgel 60 (particle size 0.040–0.063 mm), while thin layer chromatography was done on Merck aluminum-supported silica gel 60 F_{254}. NMR spectra were recorded at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR on a Varian Unity INOVA 400 NMR as a solution in either $CDCl_3$ or DMSO-d_6. Chemical shift data (δ-values) are recorded in parts per million (ppm) relative to tetramethylsilane as an internal standard, coupling constants are quoted in hertz (see Supporting Information file for representative NMR spectra). All melting points were determined on a Stuart SMP10 melting point apparatus and are uncorrected.

General Procedure for the Preparation of Mucohalic Acid Carbonates 3-8. Methyl, ethyl, or benzyl chloroformate (1.05 equiv) was added to a cold (−10 to −5 °C) solution of mucobromic **1** or mucochloric **2** acid (0.019 mol) in dry dichloromethane (75 mL). Diisopropylethylamine (1.1 equiv) was then added over 10 min and stirring at −10 to −5 °C was continued for 1.5 h. The reaction mixture was quenched with water (30 mL) and diluted with dichloromethane (30 mL). The phases were separated and the organic phase was concentrated under reduced pressure. The residue was purified by silica gel column chromatography.

5-Ethoxycarbonyloxy-3,4-dibromo-5*H*-furan-2-one **4**—Yield 48% (20% ethyl acetate: hexane), yellow solid, mp 84–86 °C. δ_H

(400 MHz, $CDCl_3$) 6.76 (1 H, s, CH), 4.34 (2 H, 2 q, $J = 7$ Hz, OCH_2), 1.38 (3 H, t, $J = 7$ Hz, OCH_2CH_3); δ_C (100 MHz, $CDCl_3$) 163.0, 152.5, 141.4, 119.2, 96.3, 66.0, 14.0.

General Procedure for the Etherification of Carbonates 9-12. Carbonate **3-8** (7.16 mmol) was combined with the desired phenol (1.1 equiv) and cesium fluoride (0.3 equiv, 1 equiv for biphenyl) in dichloromethane (65 mL) and stirred at room temperature overnight. The reaction was quenched with saturated aqueous ammonium chloride (50 mL) and partitioned between water (50 mL) and dichloromethane (70 mL). The organic extract was concentrated under reduced pressure and the resulting residue was purified by silica gel column chromatography.

5-(Biphenyl-4-yloxy)−3,4-dibromo-5*H*-furan-2-one **10**—Yield 72% (5% ethyl acetate: hexane) white crystals, mp 94–96 °C. δ_H (400 MHz, $CDCl_3$) 7.61–7.54 (2 H, m, phenyl), 7.50–7.42 (3 H, m, phenyl), 7.26-7.22 (2 H, m, phenyl), 7.07–7.04 (2 H, m, phenyl), 6.18 (1 H, s, CH); δ_C (100 MHz, $CDCl_3$) 163.3, 155.3, 154.8, 142.2, 140.1, 138.0, 132.9, 128.8, 128.6, 127.4, 127.0, 119.6, 119.4, 118.0, 117.6, 101.2.

General Procedure for the Amination of the Phenoxy or (Biphenyl-4-yloxy)-dihalide-5H-furan-2-ones 13-16. To a stirred solution of **9-12** (0.307 mmol) in dry tetrahydrofuran or *N*-methylpyrrolidinone (2 mL), the corresponding amine (2 equiv) was added at room temperature. After 2 h, the reaction mixture was diluted with water (10 mL) and left overnight in the fridge. The resulting solid was filtered, washed with water (10 mL), then heptane (5 mL), and dried. The product was purified by flash column chromatography.

5-(Biphenyl-4-yloxy)−3-bromo-4-[2-(7-chloroquinolin-4-ylamino)-ethylamino]-5*H*-furan-2-one **14**—Yield 78% (1–5% methanol: dichloromethane), yellow solid, mp 131–133 °C. δ_H (400 MHz, $CDCl_3$) 8.24 (1 H, br s, CQ), 8.04 (1 H, d, $J = 9$ Hz, CQ), 7.80 (1 H, d, $J = 2$ Hz, CQ), 7.54 (1 H, dd, $J = 9$ Hz and $J = 2$ Hz, CQ), 7.44–7.38 (5 H, m, phenyl), 7.07–7.05 (2 H, m, phenyl), 6.93–6.91 (2 H, m, phenyl), 6.54 (1 H, br s, CQ), 6.39 (1 H, br s, CH), 3.77 (2 H, br s, N-CH_2 or CQ-N-CH_2), 3.67 (2 H, br s, N-CH_2 or CQ-N-CH_2); δ_C (100 MHz, $CDCl_3$) 152.3 (2 C), 149.7, 138.2, 136.6, 133.7, 129.9, 129.3, 128.3 (3 C), 127.8 (2 C), 126.4, 124.2 (2 C), 119.8 (2 C), 118.1, 117.3, 97.0, 44.3. The furanone carbonyl does not appear on the ^{13}C NMR spectrum, this can be rationalized due to the slow relaxation basis.

General Procedure for the Preparation of 3,4-Dichloro-5-aryloxy-2(5H)-furanones 17-19. To a solution of mucochloric acid (1 equiv, 11.8 mmol) in tetrahydrofuran (20 mL), stirred at room temperature, was added the desired alcohol (0.75 equiv) and followed by drop-wise addition of concentrated sulfuric acid (2.00 mL). The reaction mixture, in a stoppered flask, was allowed to stir at room temperature for 48 h then poured into a separating flask containing water (100 mL) and diethyl ether (100 mL). The layers were separated, the organic layer washed with water (3 × 100 mL) and dried over magnesium sulfate. This was then filtered through a silica gel plug and the filtrate concentrated on a rotary evaporator. Residual solvent removal under high vacuum afforded the title compound.

3,4-Dichloro-5-octyloxy-5*H*-furan-2-one **19**—Yield 99% (100% diethyl ether), colorless oil. δ_H (400 MHz, $CDCl_3$) 5.77 (1 H, s, CH), 3.78–3.84 (1 H, m, OCH_2), 3.66–3.72 (1 H, m, OCH_2), 1.61–1.68 (2 H, m, OCH_2CH_2), 1.22–1.32 (10 H, m, 5 × CH_2), 0.86 (3 H, t, $J = 6$ Hz, CH_3); δ_C (100 MHz, $CDCl_3$) 163.2, 147.5, 124.3, 100.98, 70.4, 31.7, 29.2, 29.2, 29.1, 25.7, 22.6, 14.0.

General Procedure for the Amination of 3,4-Dichloro-5-aryl- or alkyl-oxy-2(5H)-Furanones 20-22. To a solution of 3,4-dichloro-5-aryloxy-5*H*-furan-2-one **19** (1 equiv, 1.78 mmol) in *N*,*N*-dimethylformamide (10 mL) was added N^1-(7-chloroquinolin-4-yl)-ethane-1,2-diamine (1 equiv) and diisopropylethylamine (1 equiv), the stirred reaction mixture was heated at 80 °C for 24 h under a nitrogen atmosphere. The reaction mixture was allowed to cool down to room temperature, poured into a separating flask containing water (100 mL) and ethyl acetate (100 mL) and the layers separated. The organic layer was washed with water (4 × 100 mL), dried over magnesium sulfate, filtered through a Celite plug and the filtrate concentrated on a rotary evaporator. The desired product was obtained pure after column chromatography.

5-(Octyloxy)−3-chloro-4-[2-(7-chloroquinolin-4ylamino)-ethylamino]-5*H*-furan-2-one **22**—Yield 45% (50% ethyl acetate: hexane), cream white solid, mp 140 °C. δ_H (400 MHz, d_6-DMSO) 8.42 (1 H, d, $J = 6$ Hz, CQ), 8.22 (1 H, d, $J = 9$ Hz, CQ), 7.85 (1 H, t, $J = 6$ Hz, and $J = 12$ Hz, CQ), 7.80 (1 H, d, $J = 2$ Hz, CQ), 7.48 (1 H, dd, $J = 2$ Hz, and $J = 9$ Hz, CQ), 7.37 (1 H, br s, NH), 6.57 (1 H, d, $J = 6$ Hz, CH), 3.49-3.58 (6 H, m, OCH_2, 2 × NCH_2), 3.34 (1 H, br s, NH), 1.46 (2 H, quintet, $J = 7$ Hz, OCH_2CH_2), 1.14–1.18 (10 H, m, 5 × CH_2), 0.81 (3 H, t, $J = 6$ Hz and $J = 14$ Hz, CH_3); δ_C (100 MHz, d_6-DMSO) 151.8 (2C), 149.9, 149.0, 133.5, 127.5 (2 C), 124.2 (2 C), 123.9, 117.4, 98.7, 88.7, 68.2, 42.7, 31.1, 28.8, 28.6, 28.5, 25.3, 22.0, 13.8. The furanone carbonyl does not appear on the ^{13}C NMR spectrum, this can be rationalized due to the slow relaxation basis.

General Procedure for the Preparation of 3,4-Dichloro-5-(phenyl or 4′-biphenyl)−2(5H)-furanones 23-24. A solution was prepared of mucochloric acid (1 equiv, 11.5 mmol) and the desired phenyl (1 equiv) in dichloromethane (50 mL) and was cooled to 0 °C. To this cooled solution was added aluminum trichloride (1.5 equiv) in four portions over 30 min. The reaction mixture was initially stirred for 2 h at 0 °C and then stirred at room temperature for a further 18 h. The reaction mixture was poured onto ice-water and extracted with ethyl acetate (50 mL). The partially separated emulsion was filtered through celite, the phases were separated and the solvent was removed from the organic phase. The resulting residue was purified by silica gel column chromatography.

3,4-Dichloro-5-(4′-biphenyl)−2(5*H*)-furanone **24**—Yield 22% (0-20% ethyl acetate: hexane), off-white solid, mp 154–155 °C. δ_H (400 MHz, $CDCl_3$) 7.58 (2 H, d, $J = 8$ Hz, phenyl), 7.51 (2 H, d, $J = 8$ Hz, phenyl), 7.37–7.39 (2 H, m, phenyl), 7.29–7.30 (3 H,

m, phenyl), 5.81 (1 H, s, CH); δ_C (100 MHz, $CDCl_3$) 165.3, 152.2, 143.5, 139.9, 130.5, 128.9, 128.0, 127.9, 127.7, 127.2, 121.2, 83.5.

*General Procedure for the Preparation of 4-Amino-5-(phenyl or 4′-biphenyl)−2(5*H*)-furanones 25-26.* The corresponding amine (2.5 equiv) in 1,4-dioxane (0.4 mL) was added to a solution of 3,4-dichloro-5-(4'-biphenyl)−2(5H)-furanone (0.190 mmol) in 1,4-dioxane/chloroform (4 mL, 1: 1) and the resultant was stirred at 50 °C for 24 h. The material was poured into ethyl acetate (25 mL) and washed with dilute sodium carbonate solution (5%, 2 × 25 mL) and water (2 × 25 mL). The solvent was removed and the resultant oil was submitted to flash chromatography on silica gel.

5-(4′-Biphenyl)−3-chloro-4-[2-(7-chloroquinolin-4-ylamino)-hexylamino]-5*H*-furan-2-one **26**—Yield 22% (0–20% ethyl acetate: hexane), off-white solid, mp 222 °C. δ_H (400 MHz, 3 $CDCl_3$: 1 d_6-DMSO) 8.28 (1H, d, $J = 6$ Hz, CQ), 8.12 (1H, d, $J = 9$ Hz, CQ), 7.87 (1H, d, $J = 2$ Hz, CQ), 7.56–7.49 (4 H, m, phenyl), 7.43 (1 H, d, $J = 8$ Hz, CQ), 7.35 (2 H, dd, $J = 7$ Hz and $J = 8$ Hz, phenyl), 7.31–7.24 (3H, m, phenyl), 7.18 (1 H, br s, NH-CQ), 6.86 (1 H, br s, NH-C = C), 6.31 (1H, d, $J = 6$ Hz, CQ), 6.10 (1 H, s, CH), 3.32 (2 H, ddd, $J = 6$ Hz, $J = 8$ Hz, and $J = 14$ Hz, CH_2-NH-CQ), 2.92 (2 H, ddd, $J = 6$ Hz, $J = 8$ Hz, and $J = 14$ Hz, CH_2-NH-C = C), 1.59 (2 H, quintet, $J = 7$ Hz, CH_2), 1.52–1.10 (6 H, 3 × m, 3 × CH_2); δ_C (100 MHz, 3 $CDCl_3$: 1 d_6-DMSO) 166.9, 154.8, 151.4, 147.8, 145.1, 140.7, 139.4, 135.3, 134.6, 128.2, 128.1, 126.9, 126.3, 126.2, 126.1, 124.7, 123.0, 121.0, 116.2, 97.6, 91.9, 42.4, 41.9, 27.8, 27.3, 25.7, 25.6.

Bacterial Strains and Culture Conditions

Mycobacterium smegmatis mc^2 155 was used as a surrogate strain for *M. tuberculosis* in primary screens of the first-generation synthetic compounds. Unless otherwise mentioned, *M. tuberculosis* H37Rv (ATCC 27294) was used in all secondary screens. Cultures for experimental treatment were initiated by diluting a frozen stock inoculum 1: 200 into fresh Middlebrook 7H9 supplemented with 10% v/v oleic acid-albumin-dextrose-catalase (OADC, Difco Laboratories, Detroit) and 0.05% v/v Tween 80 (Sigma-Aldrich, St. Louis) in vented, screw cap, tissue culture flasks (NUNC, Germany) and cultured to log phase (OD_{600} of 0.6) in a 5% CO_2 atmosphere at 37 °C.

Selected clinical isolates of *M. tuberculosis* were also used for the testing of antimycobacterial activity and synergistic effects of **14** with current anti-TB first line drugs. All isolates were obtained from a genotyped strain collection in the Department of Biomedical Sciences, University of Stellenbosch (18). The isolates were characterized as resistant or susceptible based on growth at critical concentrations of first line antibiotics. The strains included rifampicin mono-resistant (RIF^R) and isoniazid mono-resistant (INH^R) strains, resistant to 2 and 0.1 μg/mL (RIF^R and INH^R), respectively. The nature of the mutation conferring RIF^R was *rpo*B (+S/531TTG) and the mutation conferring INH^R was either *inh*A promoter (R1845) or katG (R1129 +S/315ACC) as determined by mutational analysis (18).

Selected Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC9027, *Klebsiella pneumoniae* ATCC13883) and Gram-positive bacteria (*Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 11778) were grown in Tryptone Soya Broth (Difco Laboratories, Detroit) and were used for selectivity testing. Both Gram-positive and Gram-negative strains will be referred as "bacteria" in general.

Antibiotics

Isoniazid, rifampicin, and ethambutol were purchased from Sigma-Aldrich (St Louis). Stock solutions of isoniazid and ethambutol were prepared in sterile distilled and deionized water at 10 mg/mL and sterilized by filtration (0.22 μm-pore-size polycarbonate filter). Stock solutions of rifampicin (10 μg/mL) and all synthetic furanone-based compounds were prepared in 100% dimethyl sulfoxide (DMSO) at 15 μg/mL. All synthetic compound and antibiotic stock solutions were stored at −80 °C until required.

Antibacterial Activity and Minimum Inhibitory Concentration (MIC) Determination

Primary screening of the first-generation synthetic compounds against *M. smegmatis* and selectivity screening against Gram-positive and Gram-negative bacteria were performed using the Bioscreen C automated biological growth reader system (Labsystems, Helsinki, Finland) as described (19) with minor modifications. Antimycobacterial activity against *M. smegmatis*, was tested at a base-line screen concentration (10 μg/mL) and for selectivity against bacteria at 3.125 to 50 μg/mL. Each well contained 100 μL of compound diluted at a specified concentration and 100 μL of bacteria (OD_{600} of 0.05) to a final volume of 200 μL per well. Rifampicin (6.25 μg/mL) and ciprofloxacin (0.1 to 2 μg/mL) were used for *M. smegmatis* and bacterial growth inhibition, respectively. Control wells contained 100 μL of compound solvent (without compound) plus 100 μL of bacteria (OD_{600} of 0.05) to a final volume of 200 μL per well. The cultures were incubated at 37 °C with constant shaking (moderate) and the OD_{600} measured at 30-min intervals over an incubation period of 48 h. Growth curves were generated and analysed using Research Express, version 1.00 (a software package of the Bioscreen C System). Growth changes were interpreted at 16 h of incubation (mid log phase, OD_{600} of 0.5) and the percentage inhibition was calculated relative to the untreated controls. All experiments were conducted in triplicate, with three independent experiments performed each time. Compounds that showed at least 35% growth inhibition against *M. smegmatis* were screened against *M. tuberculosis*.

Secondary screening of the three most active first-generation and all of the second-generation synthetic compounds against *M. tuberculosis* H37Rv was performed using the BACTEC 460 system (Becton Dickinson) (20). Clinical isolates of *M. tuberculosis* were identified as drug resistant by mutational analysis and genotyping (18). Multidrug-resistant (MDR) isolates were confirmed resistant at the critical concentrations of isoniazid (0.2 μg/mL), rifampicin (2.0 μg/mL) and

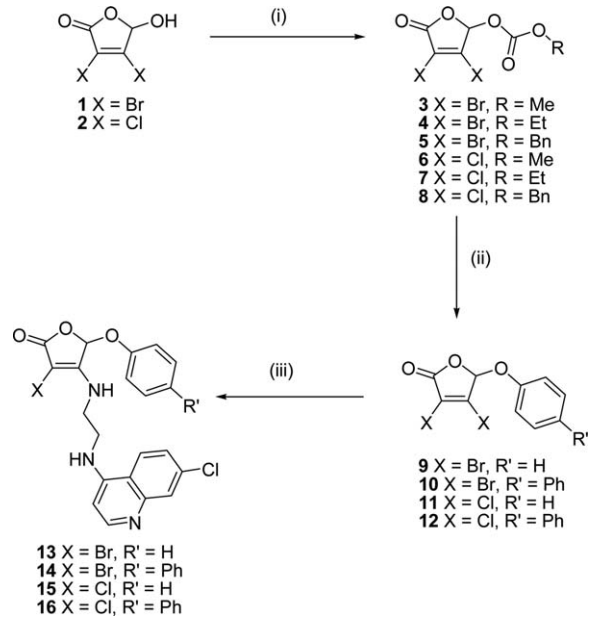
ethambutol (2.5 μg/mL) (21). *M. tuberculosis* H37Rv reference strain or *M. tuberculosis* clinical isolates were grown on 7H11 agar plates (Difco Laboratories, Detroit) and bacterial inocula were prepared from 4- to 7-week-old plates by transferring bacteria into capped plastic tubes containing 7H9 medium and 4 mm glass beads (Lasec, Johannesburg, South Africa). Bacterial clumps were broken-up by vortexing. Suspensions of *M. tuberculosis* (1 McFarland standard) (22), were inoculated into BACTEC 12B (7H12 Middlebrook) medium when the vials reached a growth index (GI) of 500. BACTEC 12B medium vials containing various combinations of test compounds and antibiotics (**7, 13**, **14**, all second-generation compounds, isoniazid, rifampicin, and ethambutol) were then inoculated with primary culture. The growth of the bacilli, expressed as GI, was monitored daily by measuring the release of $^{14}CO_2$ (*20*). Untreated controls (the undiluted and the 1:100 diluted bacterial inocula) were included in each experiment. When the GI of the 1:100 inoculum vial reached ΔGI [GI (current day) – GI (previous day)] of 30 or greater, the experiment was stopped (approximately 5–8 days incubation) (20). The MIC_{99} of a given drug was defined as the lowest concentration at which the GI of the vial in which the compound or the antibiotic was added was less than or equal to the GI of the 1:100 control (20).

Time-Kill Studies in Relation to Mycobacterial Growth

M. tuberculosis cultures at 4.2×10^6 colony forming units (CFU)/mL were treated with **14** at twofold increasing concentrations (range 2–128 μg/mL) for 21 days at 37 °C to establish the bacteriostatic as well as the bactericidal effect of the antimycobacterial agents. To determine the bactericidal or bacteriostatic effect after 1, 2, 5, 10, and 21 days of exposure, the bacterial suspensions (200 μL) were centrifuged at 11,000*g* for 4 min at room temperature, washed in 7H9 broth supplemented with OADC and centrifuged again, as above. Washed cells were 10-fold serially diluted and plated onto 7H11 agar supplemented with OADC. After 15 to 21 days of incubation at 37 °C, CFUs were counted.

Chequerboard Synergy Assay

The chequerboard titration was used in which serial dilutions of two antibiotics are studied for the effects on bacterial growth inhibition at all possible concentration combinations. The maximum and minimum concentrations of each diluted compound or antibiotic were at least fourfold above or below their MIC. The effects of synthetic compounds or antibiotics in combination were evaluated using GI values obtained from the BACTEC 460 system previously described (23). Synergy is defined as $x/y < 1/z$, where x is the GI obtained when the two antibiotics are in combination, y is the data for the lowest GI value obtained when the two agents are tested separately at the same concentration, and z is the number of drugs combined. In order to translate the experimental data into the classification of drug-drug interactions, the quotient x/y can be used. If the x/y value is equal to 1, it is interpreted that one of the drugs in the combination is inactive. If x/y is less than 0.5 for a two-drug combination, it implies that the two drugs together are more effective than when they are used individually, suggesting synergistic effects. If x/y values fall between 0.5 and 0.75, an additive effect may exist between the two drugs, suggesting a weak enhancement between them. Where x/y values are greater than 2, it suggests antagonistic interaction between the two drugs. When the window between x/y is greater than 1 and less than 2, this may be interpreted as the transition from no effect to antagonistic.

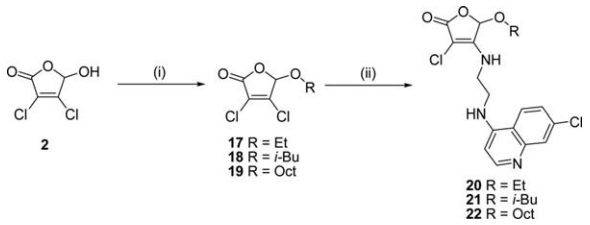


SCH 1

Reagents and conditions: (i) 1.05 equiv methyl/ethyl/benzyl chloroformate, 1.1 equiv diisopropylethylamine, CH_2Cl_2, −10 °C, 1.5 h, 48–50%; (ii) 1.1 equiv phenol, 0.3–1.0 equiv CsF, CH_2Cl_2, rt, overnight, 72 to 80%; (iii) 2.0 equiv amine, THF, or N*-methylpyrrolidine, rt, 2 h, 55 to 78%.*

Cytotoxicity Assay

All synthetic first and second-generation compounds, including **14, 22,** and **26** were tested for *in vitro* cytotoxicity against the Chinese Hamster Ovarian (CHO) cell line using the 3-(4,5-dimethiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (24). The test samples were prepared as 2,000 μg/mL solutions in 100% DMSO and were stored at −20 °C until use. The highest concentration of DMSO to which the cells were exposed to had no measurable effect on the cell viability. Emetine (Sigma-Aldrich, St. Louis) was used as a positive control in all cases. The initial concentration of emetine was 100 μg/mL, which was serially diluted in complete medium with 10-fold dilutions to give six concentrations, the lowest being 0.001 μg/mL. The same dilution technique was applied to all test samples with an initial concentration of 100 μg/mL to give five concentrations, with the lowest concentration being 0.01 μg/mL. The concentration of the test sample that inhibited 50% of the cells (IC_{50} value) was obtained from a dose–response curve, prepared using a nonlinear dose–response curve fitting analysis available in GraphPad Prism v. 2.01 software.



SCH 2 *Reagents and conditions: (i) excess ethanol/sec-butyl alcohol, 98% H_2SO_4 cat., rt, 24 h, quantitative or (i) 0.75 equiv* n*-octanol, 98% H_2SO_4 cat., THF, rt, 48 h, quantitative; (ii) 1.0–2.0 equiv amine, 1.0 equiv diisopropylethylamine,* N,N*-dimethylformamide, 70 to 90 °C, 24 h, 60 to 70%.*

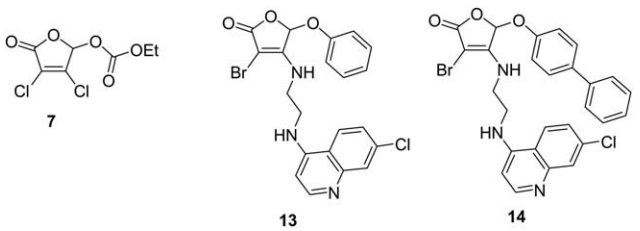


FIG 1 *Chemical structures of the three most active first-generation compounds against* M. smegmatis *mc² 155.*

Results and Discussion

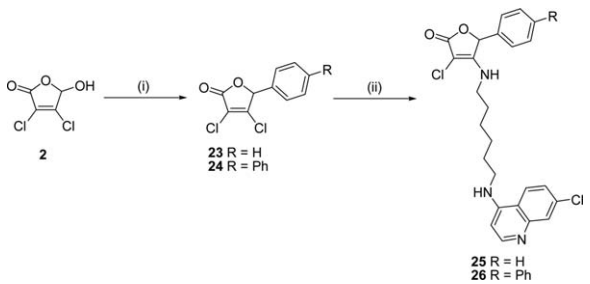
Synthetic Chemistry and Study Design

A library of related first-generation compounds were chemically synthesized from commercially available mucohalic acid furanones as depicted in Scheme 1. 3,4-Dibromo-5-hydroxy-2(5*H*)-furanone **1** and 3,4-dichloro-5-hydroxy-2(5*H*)-furanone **2** were transformed to their respective methyl, ethyl, and benzyl carbonates, giving a labile group at carbon C-5, by the treatment of the mucohalic acids with either methyl, ethyl, or benzyl chloroformate in the presence of diisopropylethylamine at a low temperature (25). The carbonates **3-8** were obtained in average yields of 50%. Two sequential nucleophilic substitution reactions of the carbonate and β-halo groups by selected phenols and 4-aminoquinoline diamine, respectively, delivered the target compounds **13-16** in moderate to high unoptimized yields (Scheme 1).

A second generation of furanone derivatives with improved activity and cytotoxicity were synthesized from the same commercially available mucochloric acid **2**. The mucochloric acid was protected on the alcohol (position C-5) with various branched and unbranched alkanes under basic conditions (Scheme 2). Following this, a nucleophilic substitution of the β-halo group was performed using the same conditions described in Scheme 1, to deliver target compounds **20-22** in moderate to high unoptimized yields. In synthesizing second-generation derivatives, the bromide in the C-3 position of the furanone-ring was exchanged for a chlorine atom as the bromide present in our first-generation compounds was proposed as one initiator of mammalian cell toxicity. Laboratory experience with the first-generation compounds, led us to observe that the 3-bromo-furanone derivatives decomposed when exposed to air, light and/or heat, suggesting possible instability *in vivo* as well. The second structural modification shows the biphenyl group in compound **14** being replaced with an octyl group (**19** and **22**), retaining lipophilic character of the molecule while assessing spatial effects on compound efficiency.

We also postulated that the observed cytotoxicity of **14** could be attributed to the acetal moiety which is labile under acidic conditions. The masked hemiacetal so formed would then be effective in generating covalent linkages to nucleophilic hydroxyl and amino substituents through its aldehyde form. In order to overcome this liability, a series of derivatives were synthesized containing a direct carbon-carbon linkage in place of the acetal. To this end, compounds **23** and **24** were prepared by Friedel-Crafts acylation of phenyl/biphenyl with mucochloric acid **2**. This was followed by the conjugate addition of various amines, 4-(6-aminohexyl)-amino-7-chloroquinoline (**25** and **26**) is used as a representative example in Scheme 3.

Antibacterial Activity. Based on a preliminary screen of the first-generation synthetic furanone-based compounds at 10 μg/mL against *M. smegmatis* mc² 155, three compounds (**7**, **13,** and **14**) were found to be active (chemical structures



SCH 3 *Reagents and conditions: (i) 1 equiv phenyl/biphenyl, 1.5 equiv $AlCl_3$, CH_2Cl_2, 0 °C – rt, 20 h, 20 to 22%; (ii) 2.5 equiv amine, 1,4-dioxane, $CHCl_3$, 50 °C, 24 h, 10 to 60%.*

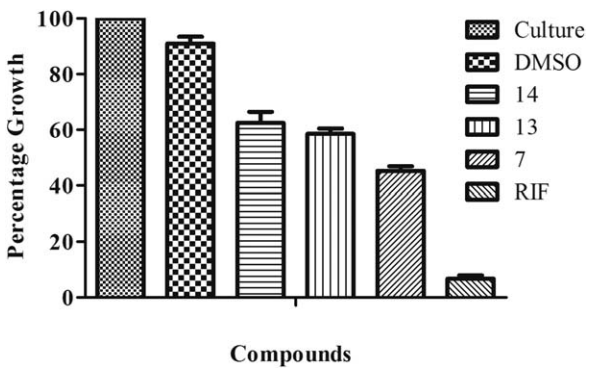


FIG 2 *The percentage growth of* M. smegmatis *in the presence of the compounds **7**, **13,** and **14** (10 μg/mL) that showed most activity. Compounds were tested in duplicate in three independent experiments.*

TABLE 1 *Susceptibility testing of* **M. tuberculosis** *clinical isolates with 14 at MIC (8 μg/mL)*

Drug susceptibility	*No.* M. tuberculosis *isolates*	*Percentage inhibition (%)*
INH and RIF susceptible	20	>90
INH, RIF and EMB susceptible	1	>90
INH mono-resistant	2	>90
RIF mono-resistant	1	>90
INH and RIF resistant	1	>90
INH, RIF and EMB resistant	1	>90

shown in Fig. 1). Their activity was interpreted at 16 h of growth and the percentage inhibition was calculated relative to the untreated control (Fig. 2). The most active compounds **7**, **13,** and **14** showed 55%, 40%, and 38% inhibition, respectively. Secondary screening of these three compounds was carried out against *M. tuberculosis* H37Rv using the BACTEC 460 TB system. At the base-line testing concentration of 10 μg/mL, **7**, **13,** and **14** showed 36%, 20%, and 99% inhibition, respectively. The MIC_{99} for **14** was determined to be 8.07 μg/mL.

Clinical isolates including pan-susceptible and multidrug resistant (MDR) strains showed similar susceptibility when tested with **14** at the MIC_{99} of 8 μg/mL (Table 1). Specificity for antimycobacterial activity was determined by testing **14** against Gram-positive and Gram-negative bacteria for growth inhibition using the Bioscreen C system. There was no growth inhibition in any of the bacterial species tested, even at concentrations seven times higher than the *M. tuberculosis* MIC. Screening of the second-generation synthetic furanone-based compounds at 10 μg/mL against *M. tuberculosis*, identified compounds with improved activity (chemical structures shown in Fig. 3). The MIC_{99} for **22** and **26** were determined to be

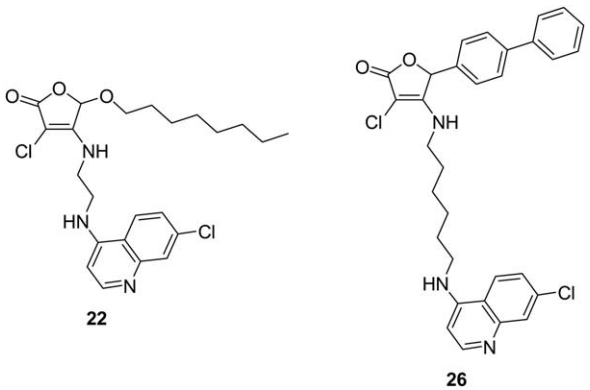


FIG 3 *Chemical structures for the two most active second-generation compounds against* M. tuberculosis *H37Rv.*

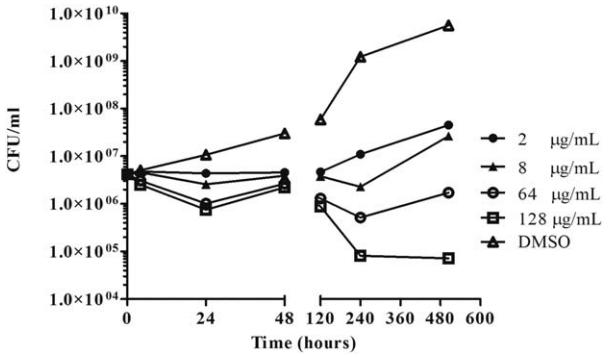


FIG 4 M. tuberculosis *H37Rv in the log phase (4.2 × 10^6 CFU/mL) of growth was exposed to* **14** *at various concentrations (μg/mL) for 21 days at 37 °C. After 4 h, 1, 2, 5, 10, or 21 days of drug exposure, viable cultures were determined by colony forming units (CFU) counting.*

2.62 μg/mL and 3.07 μg/mL against *M. tuberculosis* H37Rv, respectively.

Time-Kill Studies in Relation to Mycobacterial Growth. A bacteriostatic effect was defined as the lowest concentration that inhibited visible growth on an agar plate at day 21 as described (26). A bactericidal effect is defined as the killing capacity of the agent expressed as the lowest concentration that resulted in ≥99% killing at day 1, 2, 5, 10, or 21 (26). The minimum bactericidal concentration (MBC) for mycobacteria is defined as the minimal concentration effectively reducing bacterial counts by 99%. Compound **14** showed antimycobacterial activity at all concentrations tested using CFU as shown in Fig. 4. INH showed a bactericidal effect, reducing the bacterial count by more than 5 log units in 24 h (data not shown). We observed no further effect of the INH, substantiating previous

TABLE 2 *Synergy quotient for 14 tested in two-drug combinations with isoniazid (INH), rifampicin (RIF), or ethambutol (EMB) against* **M. tuberculosis** *H37Rv*

Synergy quotients (x/y) in drug combinations (drug + 0.5 × MIC ***14****)*

Drug	INH*	RIF**	EMB***
Dose	0.8 × MIC	0.5 × MIC	0.25 × MIC
Quotients (mean $x/y \pm$ SD)[a]	$0.65^b \pm 0.388$	$0.11^c \pm 0.098$	$0.75^b \pm 0.268$

MICs: INH = 0.05 μg/mL; RIF = 0.4 μg/mL; EMB = 1.6 μg/mL; ***14*** *= 8 μg/mL.*
[a]*Data obtained from two separate experiments. Please refer to the chequerboard synergy assay for x/y quotient definition.*
[b]*Additive effect. < 0.75 but > 0.5.*
[c]*Synergy was defined as x/y < 1/z.*
0.04 μg/mL; **0.2 μg/mL;*0.4 μg/mL.*

TABLE 3 *Compound 14 tested in combination with rifampicin (RIF) against RIF mono-resistant* M. tuberculosis *isolate*

RIF-mono-resistant isolate	*RIF (μg/mL)*				
14 (2 μg/mL)	100	50	25	12.5	6.25
Quotients (mean $x/y \pm$ SD)	0.95 ± 0.328	0.74 ± 0.183	0.68 ± 0.087	0.012 ± 0.067	0.21 ± 0.095
Interpretations	No effect	Additive	Additive	Synergy	Synergy

findings that INH becomes less effective in nonreplicating organisms (27). However, treatment with **14** shows a 2 log growth reduction of bacterial count at 24 hours (Fig. 4). Compound **14** showed a bacteriostatic effect at the 8 μg/mL and a bactericidal effect at 128 μg/mL.

Chequerboard Synergy Assay. Synergistic, additive, or antagonistic effects of drugs in combination must be evaluated at concentrations that are below the level of their individual MICs for effects to be observed. Table 2 shows the MIC of each individual drug, as well as the quotient values for combinations of **14** with isoniazid, ethambutol, and rifampicin. The concentration of each of the drugs used in combination was expressed as a fraction of the MIC value. Table 2 includes only the combinations at which synergy or additive effects were observed. **14** at 0.5 × MIC showed synergy with rifampicin and an additive effect for both isoniazid and ethambutol when MIC fractions were used as indicated in Table 2. No antagonistic effect was observed for any of the two-drug combinations tested in this study.

To examine whether the synergistic interaction between **14** and rifampicin also facilitated inhibition of drug-resistant *M. tuberculosis* strains, we evaluated drug interaction with a RIF^R *M. tuberculosis* clinical isolate. The rifampicin MIC for the RIF^R *M. tuberculosis* R5182 strain was >100 μg/mL. The MIC for compound **14** was 8 μg/mL, the same concentration that was determined for rifampicin susceptible *M. tuberculosis* H37Rv (Table 2). The RIF^R strain showed minimal growth inhibition in the presence of 100 μg/mL rifampicin, indicative of resistance. Adding 0.25 × MIC **14** (2 μg/mL) to rifampicin (6.25 μg/mL) inhibited growth by more than 99%. Synergistic effects were observed at 12.5 and 6.25 μg/mL rifampicin ($x/y = 0.12$ and 0.21, respectively) in combination with 2 μg/mL **14** (Table 3). The synergy of **14** in combination with rifampicin decreases to an additive effect and then to no effect as the concentration of rifampicin approaches its MIC in the resistant strain. However, 0.5 × MIC of ethambutol in combination with rifampicin at the same concentrations did not show either additive or synergistic activity on a RIF^R strain (data not shown). These results suggest that the synergy between **14** and rifampicin function in both susceptible and resistant *M. tuberculosis* strains.

TABLE 4 *Activity versus cytotoxicity data of the furanone-based compounds*

Compound	*MIC (μg/mL) against H37Rv*	*Cytotoxicity (IC_{50}, μg/mL) in CHO cell-line*	*Selective index (SI) IC_{50}/MIC_{99}*
14	8.07 ± 0.073	1.82 ± 0.15	0.228
22	2.62 ± 0.08	38.24 ± 0.236	14.64
26	3.07 ± 0.09	45.58 ± 0.2	14.85

Cytotoxicity Assay. Toxicity tests are usually done in animal models, but in order to reduce the cost of drug development, preliminary *in vitro* toxicity assays are performed to ensure that the least toxic compounds are pursued. For this purpose **14** was tested for cytotoxicity against the CHO cells using the 3-(4,5-dimethiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. We show that **14** has an IC_{50} of 1.82 μg/mL, **22** has an IC_{50} of 38.24 μg/mL, and **26** has an IC_{50} of 45.58 μg/mL against CHO cells (Table 4). The selectivity index (SI) calculated as IC_{50} in CHO/MIC_{99} is 0.228 for **14**, 14.64 for **22,** and 14.85 for **26**. The improved selectivity index values of **22** and **26** are due to the structural modifications. For **22** the bromide in position C-3 of the furanone ring was exchanged for a chlorine atom, with the concomitant replacement of the biphenyl group with an octyl group, maintaining the lipophilicity of the molecule (calculated cLogP values: **14** 5.21 *vs*. **22** 5.26). For **26** the acid labile acetal moiety was replaced with a direct carbon-carbon linkage. From the observations it appears that the bromine plays a primary role in mammalian cell cytotoxicity and the acetal linkage a lesser role. The removal of these liabilities has no deleterious impact on antitubercular activity, suggesting that further development of the series is feasible.

Conclusion

In summary, we have been able to identify furanone-based compounds which have antimycobacterial activity with low cytotoxicity against a human cell line. Of the first-generation compounds tested, three active compounds were identified. **14** has a higher MIC than current anti-TB drugs; however, we were able to demonstrate that in combination with the current anti-TB drug rifampicin there is synergism of potency. **14** is

also active against MDR strains of *M. tuberculosis*, suggesting a different mechanism of action than the currently available TB drugs. **14** unfortunately showed some toxicity against the CHO cell line.

Of the second-generation compounds tested, two active compounds were identified (**22** and **26**). Compound **22** was found to have an SI of 14.64 indicating an improved cytotoxicity (64-fold) and mycobacterial growth inhibition (threefold) compared to the parental compound **14**. In addition, compound **26** was found to have an SI of 14.85 indicating an improved cytotoxicity (65-fold) and mycobacterial growth inhibition (2.5-fold) compared to the parental compound **14**. As such, both compounds **22** and **26** as our lead structures will be further investigated via time-kill and synergy studies for future drug development.

Acknowledgements

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