Design, synthesis, antitubercular and antiviral properties of new spirocyclic indole derivatives

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Abstract
A series of indole-based spirothiazolidinones have been designed, synthesized and evaluated, in vitro, for their antitubercular, antiviral, antibacterial, and antifungal activities. The structures of the new compounds were established by IR, 1H NMR, 13C NMR (proton decoupled, APT, and DEPT), electrospray ionization mass spectrometry, and microanalysis. Compounds bearing a phenyl substituent at position 8 of the spiro ring, exhibited significant antitubercular activity against Mycobacterium tuberculosis H37Rv ATCC 27294 at concentrations of 3.9 and 7.8 µM. Still, some of the tested compounds displayed activity on mycobacteria with MIC values of 16 and 31 µM. Four of the indole-spirothiazolidinone derivatives were found to be moderately active against Punta Toro virus, yellow fever virus or Sindbis virus in Vero cells. The antiviral EC50 values were in the range of 1.9–12 µM and the selectivity index (ratio of cytotoxic to antivirally effective concentration) was above 10 in some cases. The most potent effect was seen with the compound that is methylated at positions 2 and 8 of the spirothiazolidinone system.

Keywords Heterocycles · Spirothiazolidinone · Antitubercular activity · Antiviral activity · Cytotoxicity · Drug research

Introduction
Tuberculosis (TB) is a highly infectious disease caused by the bacillus Mycobacterium tuberculosis. For the past 5 years, it has been the leading cause of mortality from a...
single infectious disease, ranking above HIV/AIDS [1]. Major problems associated with the currently available TB treatment include long treatment duration, inadequate compliance, concurrent HIV infection, and increasing incidence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis [2–5]. This emergence of difficult to treat strains necessitates the discovery and development of novel antitubercular drugs. After this domain has been inactive for several decades, two new drugs became available, i.e., the mitochondrial ATP synthase inhibitor bedaquiline and mycolic acid biosynthesis inhibitor delamanid, which received accelerated approval for the treatment of MDR tuberculosis in 2012 and 2014, respectively [6]. Besides, diverse novel drug candidates are in preclinical or clinical development [6–8].

Indole-2-carboxamides incorporating an alicyclic system (Fig. 1a) have been extensively studied by different research groups [9–14]. This type of compounds was found to be highly active against both drug-susceptible and drug-resistant strains of Mycobacterium tuberculosis by acting on the MmpL3 transporter protein. In previous investigations, we have identified the indole-spirothiazolidinone system (Fig. 1b) as a promising scaffold against the Mycobacterium tuberculosis H37Rv strain [15, 16]. Some of these analogues exhibited in vitro antitubercular activity with GI (growth inhibition) values of 91–95% at a MIC (minimum inhibitory concentration) value of 6.25 µg/cm³. Recently, we reported on the synthesis of novel 5-fluoro-3-phenyl-1H-indole derivatives containing a 4-thiazolidinone nucleus (instead of spirothiazolidinone system) [17]. Two molecules (Fig. 1c) displayed notable antitubercular activity at concentrations tenfold lower than those that produced cytotoxicity in mammalian cell lines.

Furthermore, several spirothiazolidinone compounds synthesized in our laboratory were found to be efficient inhibitors of membrane fusion mediated by influenza virus hemagglutinin (HA) [18–20]. As demonstrated in Fig. 1d, these compounds have a similar backbone structure, consisting of an aromatic/polycyclic ring linked to a non-aromatic spiro system via an amide bridge. Some analogues displayed low micromolar activity against the influenza A/H3N2 subtype with a favorable selectivity index.

Based on these insights and our objective to optimize the antimicrobial activity of indolyl thiazolidinones and spirothiazolidinones, we here report the chemical synthesis, structural characterization and in vitro antitubercular, antiviral, antibacterial, and antifungal evaluation of new 5-chloro-3-phenyl-N-(2,7,8,9-substituted/nonsubstituted-3-oxo-1-thia-4-azaspiro[4.4]nonan/[4.5]decan-4-yl)-1H-indole-2-carboxamides 4a–4i, 5a–5h (Fig. 1e).

**Results and discussion**

**Chemistry and structural characterization**

The synthetic pathways for the preparation of the spirothiazolidinones 4a–4i and 5a–5h are illustrated in Scheme 1. Thus, the diazonium salt, formed by the reaction of 4-chloro-aniline with NaNO₂ and HCl, was reacted with ethyl 2-benzyl-3-oxobutanoate to obtain compound 1 [21] according

![Fig. 1 Structures of a, b, c indole-2-carboxamide-based antitubercular agents and d the spirothiazolidinone compounds acting as influenza A virus fusion inhibitors](image)
to the Japp–Klingemann reaction. The Fischer indole synthesis was carried out in acidic medium to cyclize 1 into ethyl 5-chloro-3-phenyl-1H-indole-2-carboxylate 2 [22]. Subsequent exposure of 2 to an excess of hydrazine hydrate afforded compound 3 [23]. The target spirocyclic compounds 4a–4i, 5a–5h were synthesized by treatment of the key intermediate 3 with appropriate cyclic ketones and mercapto acids in a one-pot reaction [15].

The structures of the new compounds were characterized by IR, 1H NMR, 13C NMR (proton decoupled, APT, and DEPT), electrospray ionization mass spectrometry (ESI-MS), and combustion analysis. The IR spectra of 4a–4i and 5a–5h exhibited the ring and the exocyclic C=O bands in the 1692–1713 cm⁻¹ and 1651–1670 cm⁻¹, respectively. The shifts observed in the amide bands when compared to that of 3 (1636 cm⁻¹) and the presence of additional lactam bands provided definite proof for the aimed cyclization. Observation of NH signals assigned to the indole NH (δ = 12.11–12.18 ppm) and amide NH (9.97–0.21 ppm) together with the absence of the NH₂ resonance of the intermediate hydrazide 3 in the 1H NMR spectra of 4 and 5, provided further evidence for the formation of new adducts.

The S–CH₂ (4b, 5b; R, R¹, R², R³ = H; 4c, 5c: νR = CH₃; R¹, R², R³ = H; 4d, 5d: R¹ = CH₃; R, R², R³ = H; 4e, 5e: R¹ = C₂H₅; R, R², R³ = H; 4f, 5f: R¹ = C₃H₇; R, R², R³ = H; 4g, 5g: R, R², R³ = CH₃; R¹ = H; 4h, 5h: R¹ = C₆H₅; R, R², R³ = H; 4i: R¹ = C(CH₃)₃; R, R², R³ = H) protons of the newly formed spiroalkane residue resonated at about 3.50–3.65 and 3.87–3.93 ppm, respectively. The S–CH₂ protons of 4a–4i appeared as singlets except for the methylene hydrogens of compound 4g which were observed as separate doublets with large coupling constants (J = 16.1 Hz) due to the geminal coupling resulting from the chiral centers of the thiazolidinone ring. The 1H NMR spectra of 5a–5h displayed the thiazolidinone S–CH protons as quartets or broad/distorted singlets and doublets. Assignment of the indole protons was achieved on the basis of the values and coupling constants reported for the 2,3,5-trisubstituted indole ring [15, 16, 24, 25]. The carbon resonances were assigned by chemical shifts and comparison with previously reported 13C NMR data for compounds having a similar backbone structure [15, 19, 26]. CH₃, CH₂, CH, and C signals were assigned by
Antitubercular activity

The antitubercular activity of compounds 4a–4i and 5a–5h was tested in vitro against M. tuberculosis H37Rv ATCC 27294 using the microdilution method. The lowest concentration of compound that inhibited 100% of mycobacterial growth in the culture was defined as the MIC. Rifampicin was used as the reference drug. Compounds were assayed using twofold dilutions starting at 1000 µM. As shown in Table 1, compounds 4h and 5h, bearing a phenyl substituent at position 8 of the spiro ring, exhibited the highest anti-TB activity at concentrations of 3.9 and 7.8 µM, respectively. Most of the compounds in series 4 (4b, 4c, 4d, 4f, and 4i) displayed some activity on mycobacteria with MIC values of 16 and 31 µM.

Looking at the chemical structures of the active compounds, it can be observed that the presence of a methyl group at position 2 of the spirocyclic system (series 5) led to a significant reduction in antitubercular activity. Introduction of a bulky aromatic substituent (C6H5) at position 8 of the ring, as in 4h and 5h, enhanced the antitubercular activity.

Antiviral activity

Compounds 4a–4i and 5a–5h were evaluated against a variety of DNA and RNA viruses in cell culture, namely: herpes simplex virus type-1 (HSV-1) and type-2 (HSV-2), an acyclovir-resistant thymidine kinase-deficient (TK−) mutant of HSV-1, vaccinia virus, human adenovirus-2, human coronavirus, vesicular stomatitis virus, Coxsackie B4 virus, respiratory syncytial virus, parainfluenza-3 virus, reovirus, Sindbis virus, Punta Toro virus, yellow fever virus, and influenza A and influenza B virus. The cytopathic effect reduction assays revealed that compounds 4b, 4c, 5b, and 5d were moderately active against Punta Toro virus, yellow fever virus or Sindbis virus in Vero cells (Table 2). The antiviral EC50 values were in the range of 1.9–12 µM and the selectivity index (SI: ratio of cytotoxic to antivirally effective concentration) was above 10 in some cases (see values between brackets in Table 2). The most potent effect was seen with compound 5d that is methylated at positions 2 and 8 of the spirothiazolidinone system. Of note, no antiviral activity was obtained for the analogues carrying a spiro-fused cyclopentane ring instead of cyclohexane (i.e., 4a and 5a) or a bulkier group than methyl on the cyclohexane residue. Introduction of a methyl group at position 2 of the ring system (e.g., compare compounds 4b and 5b) seemed to have a slightly positive effect on antiviral activity.

The test compounds did not exhibit activity against any of the other DNA- or RNA-viruses tested. Nevertheless, this broad antiviral testing allowed to determine the compounds’ cytotoxic activity in different mammalian cell lines (Table 3). In general, the compounds endowed with antiviral activity (4b, 4c, 5b, and 5d) tended to be less cytotoxic than the inactive derivatives.

Antibacterial and antifungal activity

The broad antibacterial and antifungal activity of compounds 4a–4i and 5a–5h was further assessed using Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 4352, Proteus mirabilis ATCC 14153, Staphylococcus aureus ATCC 29213, Staphylococcus epidermidis ATCC 12228, Enterococcus faecalis ATCC 29212, Candida albicans ATCC 10231, Candida parapsilosis ATCC 22019, and Candida tropicalis ATCC 1536
The compounds were assayed using twofold dilutions from 2500 to 156 µM. Neither of the test compounds produced antimicrobial activity below this concentration range.

**Conclusion**

In the search for effective antimicrobial agents, we achieved the synthesis of novel spirothiazolidinone derivatives 4a–4i, 5a–5h with the 5-chloro-3-phenyl-1H-indole scaffold. Structures of the new compounds were characterized and confirmed by spectrometric methods (IR, 1H NMR, 13C NMR, and ESI-MS) and elemental analysis. Compounds 4a–4i and 5a–5h were evaluated for in vitro antitubercular, antiviral, antibacterial, and antifungal activity against various viral, bacterial, and fungal strains. Compounds 4h and 5h, bearing a bulky phenyl group at position 8 of the spiro ring, displayed appreciable anti-TB activity against *M. tuberculosis* H37Rv ATCC 27294 with MIC values of 3.9 and 7.8 µM, respectively. Compounds 4b, 4c, 5b, and 5d exhibited inhibitory effect on the replication of Punta Toro virus, yellow fever virus or Sindbis virus in Vero cells. The antiviral EC\textsubscript{50} values were in the range of 1.9–12 µM and the selectivity index (SI: ratio of cytotoxic to antivirally effective concentration) was above 10 in some cases. The most potent effect was seen with compound 5d that is methylated at positions 2 and 8 of the spirothiazolidinone system. Neither of the indole-spirothiazolidinone compounds showed activity against any of the bacterial or fungal strains tested, at concentrations below 2500–156 µM.

**Experimental**

All purchased solvents and chemicals were of analytical grade and used as received. Melting points were determined in open capillary tubes with a Buchi B-540 melting point apparatus. Microanalyses were performed on a Thermo
Table 3  Cytotoxic activity in diverse mammalian cell lines

| Compound | MCC50 [µM] | CC50 [µM] |
|----------|------------|-----------|
| HEL      | HeLa       | Vero      | MDCK | MDCK |
| 1        | > 100      | > 100     | > 100 | > 100 |
| 2        | 100        | 100       | 100   | 63   |
| 3        | > 100      | > 100     | > 100 | > 100 |
| 4a       | 20         | 20        | 4     | 2.3  |
| 4b       | 20         | 20        | 4     | 9.9  |
| 4c       | 20         | 20        | 4     | 2.4  |
| 4d       | 20         | 20        | 4     | 20   |
| 4e       | 20         | 20        | 4     | 2.3  |
| 4f       | 20         | 20        | 4     | 2.3  |
| 4g       | 20         | 20        | 4     | 18   |
| 4h       | 20         | 20        | 4     | 2.3  |
| 4i       | 20         | 20        | 4     | 2.3  |
| 5a       | > 100      | > 100     | > 100 | > 100 |
| 5b       | 100        | > 100     | > 100 | > 100 |
| 5c       | > 100      | > 100     | > 100 | > 100 |
| 5d       | 20         | 20        | > 20  | > 100 |
| 5e       | 20         | 20        | > 20  | > 100 |
| 5f       | 20         | 20        | > 20  | > 100 |
| 5g       | > 100      | > 100     | > 100 | > 100 |
| 5h       | 20         | 20        | > 20  | > 100 |
| DS-10,000d | ND     | > 100     | > 100 | ND   |
| Ribavirin | ND       | ≥ 250     | > 100 | > 100 |
| Ganciclovir | ND     | ND        | ND    | ND   |
| Brivudin  | > 250      | ND        | ND    | ND   |

ND not done

a HEL human embryonic lung fibroblast cells, HeLa human cervix carcinoma cells, Vero African green monkey kidney cells, MDCK Madin–Darby canine kidney cells
b MCC minimal cytotoxic concentration, or compound concentration causing minimal changes in cell morphology, as assessed by microscopy
c CC50 50% cytotoxic concentration, assessed by the spectroscopic MTS cell viability assay
d DS-10,000 dextran sulfate with MW 10,000; for this compound, data are expressed in µg per cm³

Finnigan Flash EA 1112 elemental analyzer and their results were found to be in good agreement (± 0.3%) with the calculated values. IR spectra were recorded in KBr discs (υ/cm⁻¹) on a Shimadzu IRAffinity-1 FTIR spectrophotometer.

1H NMR (DMSO-d₆). 13C NMR (APT, DMSO-d₆) were run on Varian Unity INOVA (500 MHz) instrument. 13C NMR (DEPT, DMSO-d₆) were run Bruker ARX (300 MHz).

General procedure for the synthesis of 5-chloro-3-phenyl-1H-indole-2-carboxylate (2) [22] A solution of 1 (0.02 mol) in 20 cm³ conc. HCl was heated under reflux on a water bath (70–80 °C) for 4 h. The crude product was filtered off, washed with water until tested neutral to litmus and used without further purification. Yield: 72.4%; m.p.: 90–93 °C (Ref. [21] 87–93 °C).

Ethyl 5-chloro-3-phenyl-1H-indole-2-carboxylate (2) [22] A solution of 1 (0.02 mol) in 20 cm³ conc. HCl was heated under reflux on a water bath (70–80 °C) for 4 h. The crude product was filtered off, washed with water until tested neutral to litmus and used without further purification. Yield: 67.8%; m.p.: 160–162 °C (Ref. [22] 158–160 °C).

5-Chloro-3-phenyl-1H-indole-2-carboxylic hydroxide (3) [23] A mixture of 2 (0.02 mol), 20 cm³ ethanol, and 8 cm³ H₂NNH₂·H₂O (98%) was heated under reflux on a water bath (70–80 °C) for 6 h. The resulting brown solid was filtered off and recrystallized from ethanol. Yield: 53.7%; m.p.: 227–230 °C (Ref. [23] 234–236 °C).

Ethyl 5-chloro-3-phenyl-1H-indole-2-carboxylic acid or 2-mercaptopropionic acid (0.01 mol) in 20 cm³ conc. HCl was heated under reflux on a water bath (70–80 °C) for 4 h. The resulting brown solid was filtered off and recrystallized from ethanol or ethyl acetate.

5-Chloro-3-phenyl-N-(2,7,8,9-substituted/ nonsubstituted-3-oxo-1-thia-4-azaspiro[4.4]nonan/ [4.5]decan-4-yl)-1H-indole-2-carboxamides 4a–4i, 5a–5h

A mixture of 3 (0.0025 mol), an appropriate cyclohexanone/cyclopentanone (0.003 mol), and mercaptoacetic acid or 2-mercaptopropionic acid (0.01 mol) in 20 cm³ dry toluene was heated to reflux with a heating mantle for 5–6 h using a Dean–Stark water separator. Excess toluene was evaporated in vacuo. The resulting residue was treated with saturated NaHCO₃ solution until CO₂ evolution ceased and was allowed to stand overnight or in some cases refrigerated until solidification. The solid thus obtained was washed with water, dried, and recrystallized from ethanol or ethyl acetate.

5-Chloro-N-(3-oxo-1-thia-4-azaspiro[4.4]nonan-4-yl)-3-phenyl-1H-indole-2-carboxamide (4a, C₂₂H₂₀ClN₃O₂S) White crystals; yield: 87.7%; m.p.: 227–230 °C (Ref. [23] 234–236 °C).
5-Chloro-N-(3-oxo-1-thia-4-azaspiro[4.5]decan-4-yl)-3-phenyl-1H-indole-2-carboxamide (4b, C_{23}H_{22}ClN_{3}O_{2}S) White crystals; yield: 76.9%; m.p.: 229–232 °C; Rf = 0.48 (EtOAc/CHCl_{3} 1:1); IR (KBr): ̃ν = 3279 (N–H), 1711, 1651 (C=O) cm⁻¹; 1H NMR (DMSO-d_{6}, 500 MHz): δ = 0.89–0.95 (3H, 2d, J = 7.3 Hz, 8-CH_{2} CH_{3}-sp.), 1.08–1.11 (2H, d, J = 6.4 Hz, 7-CH_{3}-sp.), 1.24–1.70 (8H, m, CH/CH_{2}-sp.), 3.58, 3.59 (2H, 2 s, S–CH_{2} -sp.), 7.29, 7.30 (1H, dd, J = 7.8, 2.0 Hz, H6-ind.), 7.37 (1H, tt, J = 7.8 Hz, 3-C_{6}H_{5}(H_{3},H_{5})-ind.), 7.45 (2H, t, J = 7.3 Hz, 3-C_{6}H_{5}(H_{4},H_{5})-ind.), 7.51-7.55 (4H, m, H4, H7, 3-C_{6}H_{5}(H_{2},H_{6})-ind.), 10.05, 10.06 (1H, 2 s, CONH), 12.11, 12.12 (1H, 2 s, NH) ppm; 13C NMR (proton decoupled and DEPT, DMSO-d_{6}): δ = 12.14 (1H, s, CONH), 12.11, 12.12 (1H, 2 s, NH) ppm; MS (ESI−): m/z (%) = 466.8 ([M-H]−, 100), 454.4 ([M-H+2]−, 34.3).

5-Chloro-N-(7-methyl-3-oxo-1-thia-4-azaspiro[4.5]decan-4-yl)-3-phenyl-1H-indole-2-carboxamide (4c, C_{24}H_{23}ClN_{3}O_{2}S) White powder; yield: 83.4%; m.p.: 268–270 °C; R_{f} = 0.52 (EtOAc/CHCl_{3} 1:1); IR (KBr): ̃ν = 3231 (N–H), 1711, 1651 (C=O) cm⁻¹; 1H NMR (DMSO-d_{6}, 500 MHz): δ = 0.76–0.85 (1H, m, CH/CH_{2}-sp.), 0.87 (3H, d, J = 6.4 Hz, 7-CH_{3}-sp.), 1.24–1.70 (8H, m, CH/CH_{2}-sp.), 3.58 (2H, s, S–CH_{2} -sp.), 7.29 (1H, dd, J = 8.8, 2.0 Hz, H6-ind.), 7.37 (1H, tt, J = 7.3, 1.5 Hz, 3-C_{6}H_{5}(H_{4})-ind.), 7.45 (2H, t, J = 7.3 Hz, 7-CH_{3}(H_{5},H_{3})-ind.). 7.49 (1H, d, J = 2.0 Hz, H4-ind.), 7.52-7.54 (3H, m, H7, 3-C_{6}H_{5}(H_{2},H_{6})-ind.), 10.06 (1H, s, CONH), 12.14 (1H, s, NH) ppm; 13C NMR (APT, DMSO-d_{6}, 125 MHz): δ = 22.42 (7-CH_{3}-sp.), 22.88, 28.36 (CH_{2}-sp.), 29.97 (C_{5}-sp.), 32.98, 36.54, 45.20 (CH_{2}-sp.), 72.59 (C_{5}-sp.), 114.56, 114.63 (CH-ar.), 118.50 (C-ar.), 119.28, 119.37 (CH-ar.), 124.54 (CH-ar.), 124.55 (C-ar.), 127.42 (CH-ar.), 127.85 (C-ar.), 128.25 (C-ar.), 128.91 (C-ar.), 130.51 (CH-ar.), 133.43 (C-ar.), 134.62 (C-ar.), 162.18 (CO–NH), 167.66 (CO-sp.) ppm.

5-Chloro-N-(8-propyl-3-oxo-1-thia-4-azaspiro[4.5]decan-4-yl)-1H-indole-2-carboxamide (4f, C_{26}H_{28}ClN_{3}O_{2}S) Beige powder; yield: 75.7%; m.p.: 233–236 °C; R_{f} = 0.55 (EtOAc/CHCl_{3} 1:1); IR (KBr): ̃ν = 3291, 3217 (N–H), 1707, 1661 (C=O) cm⁻¹; 1H NMR (DMSO-d_{6}, 500 MHz): δ = 0.88 (3H, t, J = 7.3 Hz, 8-CH_{2} CH_{3}-sp.), 1.05–1.34 (7H, m, CH/CH_{2}-sp., 8-CH_{2} CH_{2} CH_{3}-sp.), 1.60–1.82 (6H, m, CH/CH_{2}-sp.), 3.58 (2H, s, S–CH_{2} -sp.), 7.29 (1H, dd, J = 8.8, 2.0 Hz, H6-ind.), 7.35 (1H, tt, J = 7.3, 1.5 Hz, 3-C_{6}H_{5}(H_{4})-ind.), 7.45 (2H, t, J = 7.3 Hz, 3-C_{6}H_{5}(H_{3},H_{5})-ind.), 7.51 (1H, d, J = 2.0 Hz, H4-ind.), 7.53–7.56 (3H, m, H7, 3-C_{6}H_{5}(H_{2},H_{6})-ind.), 10.10 (1H, s, CONH), 12.13 (1H, s, NH) ppm; 13C NMR (APT, DMSO-d_{6}, 125 MHz): δ = 14.58 (8-CH_{2} CH_{2} CH_{3}-sp.), 19.99, 28.30, 28.36, 29.77 (CH_{2}-sp., 8-CH_{2} CH_{2} CH_{3}-sp.), 35.25 (C_{8}-sp.), 36.85, 38.76 (CH_{2}-sp., 8-CH_{2} CH_{2} CH_{3}-sp.), 72.74 (C_{5}-sp.), 1539
5-Chloro-N-7,7,9-trimethyl-3-oxo-1-thia-4-azaspiro[4.5]-decan-4-yl)-3-phenyl-1H-indole-2-carboxamide (4g, \(\text{C}_{26}\text{H}_{28}\text{ClN}_{3}\text{O}_{2}\)) White crystals; yield: 85.1%; m.p.: 264-266.5 °C; \(R_f=0.56\) (EtOAc/CHCl\(_3\) 1:1); IR (KBr): \(\nu=3277\), 3219 (N-H), 1705, 1695, 1665 (C=O) cm\(^{-1}\); \(^1\)H NMR (DMSO-\(d_6\), 500 MHz): \(\delta=0.76\) (1H, t, \(J=12.7\) Hz, CH/CH\(_2\)-sp.), 0.90 (6H, s, \(7-\text{(CH}_3\)\(_2\)-sp.), 1.04 (3H, s, \(9-\text{CH}_3\)-CONH), 12.17 (1H, s, NH) ppm; \(^{13}\)C NMR (APT, DMSO-\(d_6\), 126 MHz): \(\delta=126.98\) (8-ar.(CH)-sp.), 127.09 (CH-ar.), 127.73 (8-ar.(CH)-sp.), 137.54 (C-ar.), 138.84 (C-ar.), 162.12 (CO–NH), 167.79 (CO-sp.) ppm.

5-Chloro-N-(7,7,9-trimethyl-3-oxo-1-thia-4-azaspiro[4.5]-decan-4-yl)-3-phenyl-1H-indole-2-carboxamide (4g, \(\text{C}_{26}\text{H}_{28}\text{ClN}_{3}\text{O}_{2}\)) White crystals; yield: 85.1%; m.p.: 264-266.5 °C; \(R_f=0.56\) (EtOAc/CHCl\(_3\) 1:1); IR (KBr): \(\nu=3277\), 3219 (N-H), 1705, 1695, 1665 (C=O) cm\(^{-1}\); \(^1\)H NMR (DMSO-\(d_6\), 500 MHz): \(\delta=0.76\) (1H, t, \(J=12.7\) Hz, CH/CH\(_2\)-sp.), 0.90 (6H, s, \(7-\text{(CH}_3\)\(_2\)-sp.), 1.04 (3H, s, \(9-\text{CH}_3\)-CONH), 12.17 (1H, s, NH) ppm; \(^{13}\)C NMR (APT, DMSO-\(d_6\), 126 MHz): \(\delta=126.98\) (8-ar.(CH)-sp.), 127.09 (CH-ar.), 127.73 (8-ar.(CH)-sp.), 137.54 (C-ar.), 138.84 (C-ar.), 162.12 (CO–NH), 167.79 (CO-sp.) ppm.

5-Chloro-N-(7,7,9-trimethyl-3-oxo-1-thia-4-azaspiro[4.5]-decan-4-yl)-3-phenyl-1H-indole-2-carboxamide (4g, \(\text{C}_{26}\text{H}_{28}\text{ClN}_{3}\text{O}_{2}\)) White crystals; yield: 85.1%; m.p.: 264-266.5 °C; \(R_f=0.56\) (EtOAc/CHCl\(_3\) 1:1); IR (KBr): \(\nu=3277\), 3219 (N-H), 1705, 1695, 1665 (C=O) cm\(^{-1}\); \(^1\)H NMR (DMSO-\(d_6\), 500 MHz): \(\delta=0.76\) (1H, t, \(J=12.7\) Hz, CH/CH\(_2\)-sp.), 0.90 (6H, s, \(7-\text{(CH}_3\)\(_2\)-sp.), 1.04 (3H, s, \(9-\text{CH}_3\)-CONH), 12.17 (1H, s, NH) ppm; \(^{13}\)C NMR (APT, DMSO-\(d_6\), 126 MHz): \(\delta=126.98\) (8-ar.(CH)-sp.), 127.09 (CH-ar.), 127.73 (8-ar.(CH)-sp.), 137.54 (C-ar.), 138.84 (C-ar.), 162.12 (CO–NH), 167.79 (CO-sp.) ppm.
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5-Chloro-N-(2,7-dimethyl-3-oxo-1-thia-4-azaspiro[4.5]decan-4-yl)-3-phenyl-1H-indole-2-carboxamide (5c, C25H26ClN3O2S) White crystals; yield: 86.0%; m.p.: 262–264 °C; Rf = 0.65 (EtOAc/CHCl3 1:1); IR (KBr): v = 3235 (N–H), 1694, 1661 (C=O) cm⁻¹; 1H NMR (DMSO-d6, 500 MHz): δ = 0.75–0.80 (1H, m, CH/CH2-sp.), 0.87 (3H, d, J = 6.0 Hz, 7-CH3-sp.), 1.23–1.68 (8H, m, CH/CH2-sp.), 1.41 (3H, d, J = 6.3 Hz, 2-CH3-sp.), 3.88 (1H, s*, S–CH-sp.), 7.29 (1H, dd, J = 8.6, 2.0 Hz, H6-ind.), 7.37 (1H, tt, J = 7.3, 1.5 Hz, 3-C6H5(H3,H5)-ind.), 7.50 (1H, d, J = 7.8 Hz, 8-CH2 CH2 CH3-sp.), 3.87 (1H, q*, J = 6.8 Hz, 2-CH3-sp.), 3.00, 3.05, 3.07, 3.11, 3.15, 3.19 (8CH2-sp.), 71.42, 71.71 (C5-sp.), 114.55, 114.64 (CH-ar.), 118.52 (C-ar.), 119.26, 119.32, 119.39 (CH-ar.), 124.56, 124.64 (CH-ar.), 127.85 (C-ar.), 128.24 (C-ar.), 128.88 (CH-ar.), 130.51 (C-ar.), 133.39 (C-ar.), 134.60 (C-ar.), 160.27 (CO–NH), 170.41 (CO-sp.) ppm.

5-Chloro-N-(2-ethyl-2-methyl-3-oxo-1-thia-4-azaspiro[4.5]decan-4-yl)-3-phenyl-1H-indole-2-carboxamide (5e, C26H28ClN3O2S) White needles; yield: 87.2%; m.p.: 198–200 °C; Rf = 0.64 (EtOAc/CHCl3 1:1); IR (KBr): v = 3383 (N-H), 1711, 1653 (C=O) cm⁻¹; 1H NMR (DMSO-d6, 500 MHz): δ = 0.89 (3H, t, J = 7.3 Hz, 8-CH2 CH2 CH3-sp.), 1.02–1.24 (5H, m, CH/CH2-sp., 8-CH2 CH3-sp.), 3.14 (3H, d, J = 6.8 Hz, 2-CH3-sp.), 1.62–1.88 (6H, m, CH/CH2-sp.), 3.87 (1H, q*, J = 6.3 Hz, S–CH-sp.), 7.30 (1H, dd, J = 8.8, 2.0 Hz, H6-ind.), 7.37 (1H, tt, J = 7.8, 1.5 Hz, 3-C6H5(H4)-ind.), 7.46 (2H, t, J = 7.8 Hz, 3-C6H5(H3,H5)-ind.), 7.51 (1H, d, J = 2.0 Hz, H4-ind.), 7.52–7.56 (3H, m, H7, 3-C6H5(H2,H6)-ind.), 10.13 (1H, s, CONH), 12.13 (1H, s, NH) ppm; 13C NMR (APT, DMSO-d6, 125 MHz): δ = 11.92 (8-CH2 CH3-sp.), 20.35 (2-CH3-sp.), 29.23, 29.67, 36.98 (CH2-sp., 8-CH2 CH3-sp.), 37.14, 37.27 (C2-sp.), 37.42 (C8-sp.), 37.86, 38.79 (CH2-sp., 8-CH2 CH2 CH3-sp.), 71.42, 71.71 (C5-sp.), 114.55, 114.61 (CH-ar.), 118.50 (C-ar.), 119.32, 119.39 (CH-ar.), 124.56, 124.64 (CH-ar.), 127.85 (C-ar.), 128.24 (C-ar.), 128.88 (CH-ar.), 130.51 (C-ar.), 133.39 (C-ar.), 134.60 (C-ar.), 160.27 (CO–NH), 170.41 (CO-sp.) ppm.
(6H, s*, 7-(CH3)3-sp.), 1.03 (3H, d, J = 6.4 Hz, 9-(CH2-)sp.), 1.24–1.42 (2H, m, CH/CH2-sp.), 1.38 (3H, d, J = 6.8 Hz, 2-(CH2-)sp.), 1.62–1.80 (4H, m, CH/CH2-sp.), 3.87 (1H, s*, S–CH-sp.), 7.29, 7.30 (1H, 2dd, J = 8.8, 2.0 Hz, H6-ind.), 7.38 (1H, t*, J = 7.3 Hz, 3-C6H5(H4)-ind.), 7.45–7.48 (3H, m, H4, 3-C6H5(H3, H5)-ind.), 7.50–7.54 (3H, m, H7, 3-C6H5(H2, H6)-ind.), 10.02 (1H, s, CONH), 12.13, 12.17 (1H, 2 s, NH) ppm; 13C NMR (APT, DMSO-d6, 125 MHz): δ = 20.05 (2-CH3-sp.), 22.23, 25.68, 26.21, 27.69 (7-(CH3)3, 9-(CH3)-sp.), 32.27 (CH2-sp.), 36.84, 36.96 (C9-sp.), 37.46, 37.57 (C2-sp.), 45.22, 46.80, 47.29 (CH2-sp., C7-sp.), 50.05 (2-CH3-sp.), 70.59 (C5-sp.), 114.36, 114.63 (CH-ar.), 118.52 (C-ar.), 119.29, 119.38 (CH-ar.), 125.42, 126.77 (CH-ar.), 128.18, 128.24 (C-ar.), 128.97 (CH-ar.), 133.45, 133.48 (C-ar.), 134.61 (C-ar.), 162.44 (CO–NH), 170.24 (CO-sp.) ppm; MS (ESI +): (M + H)+, 31.2, 426.3 [(M + H) + 2]+, 10.7.

5-Chloro-N-(2-methyl-8-phenyl-3-oxo-1-thia-4-azaspiro(4.5)-decan-4-yl)-3-phenyl-1H-indole-2-carboxamide (5H, C30H28ClN3O2S) Beige powder; yield: 82.3%; m.p.: 213–215 °C; Rf = 0.69 (EtOAc/CHCl3 1:1); IR (KBr): ̄ν = 3289 (N–H), 1705, 1663 (C=O) cm−1; 1H NMR (DMSO-d6, 500 MHz): δ = 1.45 (3H, d, J = 6.8 Hz, 2-CH3-sp.), 1.58–2.00 (8H, m, CH2-sp.), 2.45–2.51 (2H, m, CH-sp., DMSO-d6), 3.92 (1H, d*, J = 5.6 Hz, S–CH-sp.), 7.20 (1H, t*, J = 7.3, 1.5 Hz, 8-C6H5(H4)-sp.), 7.25 (2H, d*, J = 6.8 Hz, 8-C6H5-sp.), 7.30–7.34 (3H, m, H6-ind., 8-C6H5-sp.), 7.42 (1H, t*, J = 7.3, 1.5 Hz, 3-C6H5(H4)-ind.), 7.51–7.61 (6H, m, H4, H7, 3-C6H5(H2, H3, H5, H6)-ind.), 10.21 (1H, s, CONH), 12.18 (1H, s, NH) ppm; 13C NMR (APT, DMSO-d6, 125 MHz): δ = 20.03 (2-CH3-sp.), 30.76, 31.24 (CH2-sp.), 37.38 (C2-sp.), 38.10 (CH2-sp.), 41.95 (C8-sp.), 70.78 (C5-sp.), 114.57, 114.63 (CH-ar.), 118.61 (C-ar.), 119.34, 119.43 (CH-ar.), 124.59, 124.69 (CH-ar.), 126.49 (C-ar.), 126.97 (8-ar(CH)-sp.), 127.08 (CH-ar.), 127.43 (8-ar(CH)-sp.), 127.91 (C-ar.), 128.27 (C-ar.), 128.95 (CH-ar.), 130.63 (CH-ar.), 133.45 (C-ar.), 134.63 (C-ar.), 146.28 (8-ar(CH)-sp.), 162.14 (CO–NH), 170.47 (CO-sp.) ppm; MS (ESI+): m/z (%) = 497.1 [(M + H)+, 31.2], 426.3 [(M + H) + 2]+, 10.7.

Middlebrook 7H9 broth medium (Becton and Dickinson, USA) was used and the medium was adjusted to pH 7.0 at 25 °C. Each bottle was controlled for sterility before it was used. Resazurin purchased from Sigma-Aldrich (St Louis, USA) was dissolved in sterile distilled water to a final concentration of 0.02% and sterilized by filtration, then stored at 4 °C until use. Rifampicin purchased from Becton–Dickinson (BD, USA) was dissolved in sterile distilled water to a final concentration of 1 μg/cm3 (critical concentration). The synthesized compounds were dissolved in 100% dimethyl sulfoxide according to CLSI methods [27, 28]. Stock solutions were obtained by 40-fold dilution in DMSO followed by sterile filtration. From here, working stocks at 4000 μM were obtained by diluting 1/10 in MB7H9 medium. The final concentrations were 1000 μM to 0.49 μM for the synthesized compounds. For rifampicin, the critical concentration (1 μg/cm3) was used [27, 28].

Inoculum suspensions of mycobacteria were prepared according to the CLSI guidelines as described previously. The isolates were subcultured on Löwenstein Jensen medium and incubated at 37 °C for 20–25 days. A few colonies from freshly grown M. tuberculosis were suspended in Middlebrook 7H9 broth medium to obtain 1.0 McFarland turbidity, then diluted ten times with the same medium.

The broth microdilution test was performed in sterile 96-well U-shaped microdilution plates (LP Italiano SPA, Milano, Italy). Rows A–F contained 100 mm3 of the compounds, whereas rows G (positive control) and H (negative control) contained 100 mm3 medium. One hundred μm3 of the inoculum was added to all wells except for row H. The microplates were incubated at 35 °C for about 7–10 days, when mycobacterial growth was clearly visible as a white sediment in the positive control. Microbial growth was confirmed by Ehrlich–Ziehl–Neelsen acid-fast stain. Resazurin solution (30 mm3) was added to each well and the plates were incubated for one additional day. At that time, the first purple colored well in which no growth was visible, was defined as the compounds’ MIC value (Table 1).

### Antitubercular activity assays

The microdilution method was performed according to a standard protocol from the Clinical and Laboratory Standard Institute (CLSI) [27, 28]. The resazurin microtitre assay ( REMA) has been developed as a colorimetric and standard method for drug susceptibility testing. The minimum inhibitory concentrations (MICs) were determined according to color changes at the end of incubation [29–31]. The strain used, i.e., Mycobacterium tuberculosis H37Rv ATCC 27294 is susceptible to all common antitubercular drugs.

### Antiviral activity assays

Stock solutions of the test and reference compounds were prepared in 100% DMSO at 5–25 mM. During incubation with the cells, the highest test concentration was 100 μM (or 250 μM for ribavirin). The antiviral reference compounds were: ganciclovir, brivudin, zanamivir, amantadine, ribavirin, dextran sulfate-10,000, and mycophenolic acid. Antiviral evaluation was carried out with a broad panel of viruses using cytopathic effect (CPE) reduction assays. Human influenza A (H1N1 and H3N2) and B viruses were examined on Madin–Darby canine kidney (MDCK) cells. Respiratory syncytial virus, vesicular stomatitis virus and Coxackie B4 virus were evaluated on human cervix carcinoma.
HeLa cells. African Green Monkey Vero cells were used for parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie B4 virus, Punta Toro virus and yellow fever virus. Human embryonic lung (HEL) fibroblast cells were infected with herpes simplex virus types 1 and 2, vaccinia virus, human adenovirus-2, and human coronavirus 229E.

Semi-confluent cell cultures in 96-well plates were infected with virus at a multiplicity of infection of 100 CCID50 (50% cell culture infective dose) per well. Together with the virus, fourfold dilutions of the test or reference compounds were added. The plates were incubated at 37 °C (or 35 °C for influenza- and coronavirus) until far advanced CPE was visible, i.e., during 3–6 days or 10 days in the case of adenovirus-2. Then, microscopy was performed to score the CPE and calculate the 50% antivirally effective concentration (EC50). Microscopy was also done to assess cytotoxicity, expressed as the compound concentration causing minimal changes in cell morphology (minimal cytotoxic concentration; MCC).”}

**Antibacterial and antifungal activity assays**

Antimicrobial activity against *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, and *Candida tropicalis* ATCC 750 was determined by the microbroth dilutions technique using CLSI recommendations [32, 33]. Serial twofold dilutions ranging from 2500 μM to 1.22 μM were prepared in the test medium, i.e., Mueller–Hinton broth for bacteria and RPMI-1640 medium for yeast strains. The inoculum was prepared using a 4–6 h broth culture of each bacteria type and 24 h culture of yeast strains adjusted to a turbidity equivalent to 0.5 McFarland standard, diluted in broth media to give a final concentration in the test tray of $5 \times 10^5$ cfu/cm$^3$ for bacteria and $5 \times 10^5$ cfu/cm$^3$ for yeast. The trays were covered and placed into plastic bags to prevent evaporation. The bacteria trays were incubated at 35 °C for 18–20 h while the yeast-containing trays were incubated at 35 °C for 46–50 h. The MIC was defined as the lowest concentration of compound giving complete inhibition of visible growth. Amikacin and fluconazole were used as reference antibiotics for bacteria and yeast, respectively; their MIC values were within the accuracy range of the CLSI guidelines [34].

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