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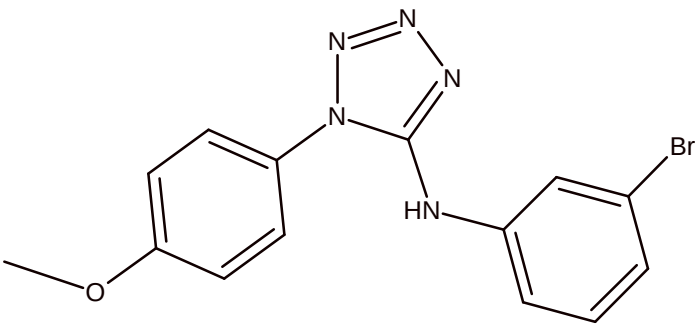
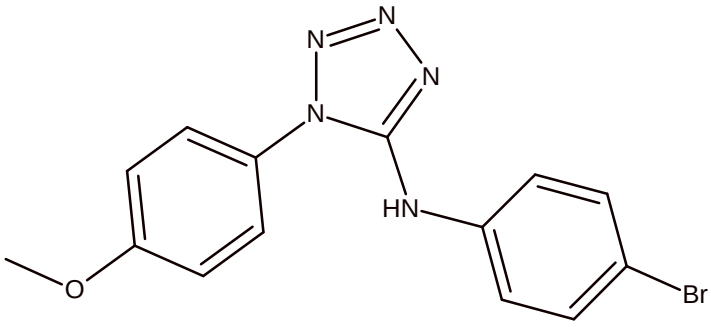


8a



9a

MIC 2 µg/ml (Mtb H_{37}Rv, Mtb Spec. 192, Mtb Spec. 210)

FICI 0.375 µg/ml (9a/Streptomycin synergism; Mtb H_{37}Rv, Mtb Spec. 192, Mtb Spec. 800)

IC_{50} 103.3 – 125.5 µM (normal V79 and HaCaT cell lines)

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Research article

Development of (4-methoxyphenyl)-1*H*-tetrazol-5-amine regioisomers as a new class of selective antitubercular agents

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Keywords: *Mycobacterium tuberculosis;* Tetrazole; Antitubercular activity; Tuberculosis; Regioisomers

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Abstract:

A series of halogenated (4-methoxyphenyl)-1*H*-tetrazol-5-amine regioisomers (**1a-9a**, **1b-9b**) were synthesized from their corresponding thiourea analogues (**1-9**). The synthesis pathway was confirmed by an X-ray crystallographic studies of **1a**, **1b** and **5a**. Title derivatives were tested for their *in vitro* antitubercular activity against standard, "wild-type" and atypical mycobacteria. The highest therapeutic potential was attributed to isomeric N-(bromophenyl)tetrazoles **8a** and **9a**. Their growth-inhibitory effect against multidrug-resistant *Mycobacterium tuberculosis* Spec. 210 was 8-16-fold stronger than that of the first-line tuberculostatics. Other new tetrazole-derived compounds were also more or equally effective towards that pathogen comparing to the established pharmaceuticals. Among non-tuberculous strains, *Mycobacterium scrofulaceum* was the most susceptible to the presence of the majority of tetrazole derivatives. The synergistic interaction was found between **9a** and streptomycin, as well as the additivity of both **8a** and **9a** in pairs with isoniazid, rifampicin and ethambutol. None of the studied compounds displayed antibacterial or cytotoxic properties against normal and cancer cell lines, which indicated their highly selective antimycobacterial effects.

1. Introduction

Despite recent approaches in diagnostics and treatment opportunities, and general certain decrease in overall incidences, tuberculosis (TB) remains one of the major global health diseases. The leading factors of TB incidence are country of birth (such as Asia, Africa, South America), HIV coinfection, prolonged exposure to a case of sputum smear-positive TB and use of some immunosuppressants, *e.g.* anti-tumor necrosis factor (TNF) agents [1]. Among all occurrences, pediatric cases constitute 10% of all incidents of the illness [2].

Effective therapy of TB consists of several bactericidal drugs administrated in a combination for a dozen of months [3]. First-line treatment of drug-sensitive TB is reliant of a four-drug cure, comprising of isoniazid, rifampicin, ethambutol and pyrazinamide, applied for at least six months [1, 3, 4]. However, although efficient in around 90% cases [5], this long therapy is not always well-tolerated or correctly dosed, and may cause a range of adverse effects, including hepatitis, anemia, neural or gastrointestinal symptoms [1]. What is more, there are increasing incidences of multidrug-resistant TB cases (MDR-TB), defined as resistance to isoniazid and rifampicin, and extensively drug – resistant TB (XDR-TB), covering MDR isolate combined resistance to fluoroquinolone and one injectable second-line

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antituberculostatic [1, 3, 6]. Treatment of both M/XDR-TB requires a longer cure (up to 24 months) and more expensive antimicrobials with a higher toxicity risk, as compared to the drug-susceptible disease. Standards of resistant mycobacteria therapy comprise at least five agents, including an antibacterial fluoroquinolone, that shorten the length of treatment [1, 7]. Nonetheless, according to WHO details, the recovery rate of XDR-TB is only 26% [8]. It has been also estimated that one third of the population could be latently infected with TB (LTBI), and has up to 10% danger of progressing to an active tuberculosis form [9].

Development of shorter and cheaper TB regiments combining new and established drugs, especially to resistant *Mycobacterium* isolates, is still the greatest global health challenge. Over the last dozen of years, many natural or (semi-)synthetic chemicals have been evaluated against mycobacteria to discover new agents to replace or at least to complement currently used tuberculostatics. Antitubercular pharmaceuticals of natural origin are these produced by *Streptomyces* (e.g. streptomycin, cycloserin, rifampicin) [10]. On the other hand, marine-derived potential anti-TB compounds include derivatives of various biosynthetic classes, such as alkaloids, terpenoids, quinolones, pyrenes, sterols or lactones [11]. Despite many efforts, only two substances have been recently approved for the treatment of TB in the past four decades: delamanid, a representative nitroimidazole and one of the dihydoquinolines – diarylquinoline. Other heterocycles developed specifically for the therapy of MDR-TB infections come from a group of oxazolidinones, macrolidine antibiotics and etylenediamines. Their mode of action is based on inhibition of cell wall synthesis (nitroimidazoles, etylenediamines), cell respiration (nitroimidazoles), ATP (dihydoquinolines) or protein synthesis (oxazolidinones, macrolides) [7].

The large potential of 1*H*-tetrazol-5-yl derivatives as a new type of antimicrobial [12-15], antifungal [16], antihypertonic [17-19], cardiotonic [20, 21], anti-inflammatory [22, 23], analgesic [24] and cytotoxic [25-27] agents was recently proved. Current investigations of selective, highly efficient 1*H*-tetrazole-based antituberculars have focused on modification of the structures of dinitrophenyl derivatives with selanyl-, oxa- or sulfanylalkyl linker [28-31]. Roh *et al.* [32] synthesized a series of water-soluble 2*H*-terazole compounds bearing N-benzylpiperazine moiety with high activity against drug-sensitive and MR strains of bacilli. New thiazolone piperazinyl-tetrazole derivatives bearing organometallic substituents were found to be more potent against standard *M. tuberculosis* H_{37}Rv strain than currently used antitubercular drugs [33]. It was established that combinations of N-phenyltetrazole compounds with antibacterials (chloromycin, norfloxacin) or antifungal fluconazole gave synergistic effects with a lower dosage and broader antimicrobial spectrum than their

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separate administration [34]. On the other hand, fluorinated 2-aryltetrazole analogues of nitroimidazooxazines exerted *in vivo* a strong antimycobacterial effect, retaining high microsomal stability [35]. Antitubercular potency was also denoted for 5-phenyltetrazoles with *para*-methoxyphenyl substituent on a β-lactam core [36].

In view of above considerations, this work reports the synthesis of a series of halogen-containing N-[4-(methoxy)phenyl]-1*H*-tetrazol-5-amine derivatives and the *in vitro* evaluation of their antitubercular and cytotoxic properties. The interactions of the most active tetrazoles with first-line anti-TB drugs were also assessed.

2. Experimental

2.1 Chemistry

2.1.1 General procedure

The reagents were supplied from Alfa Aesar or Sigma Aldrich. Organic solvents (acetonitrile, DMF, chloroform and methanol) were supplied from POCh (Polskie Odczynniki Chemiczne). All chemicals were of analytical grade. Before use, dried DMF and acetonitrile were kept in crown cap bottles over anhydrous phosphorus pentoxide (Carl Roth). The NMR spectra were recorded on a Bruker AVANCE spectrometer operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. The spectra were measured in DMSO-d_6 and are given as δ values (in ppm) relative to TMS. Mass spectral ESI measurements were carried out on Waters ZQ Micro-mass instruments with quadruple mass analyzer. The spectra were performed in the negative ion mode at a declustering potential of 40-60 V. The sample was previously separated on a UPLC column (C18) using UPLC ACQUITYTM system by Waters connected with DPA detector. TLC analyses were performed on silica gel plates (Merck Kiesegel GF_{254}) and visualized using UV light or iodine vapour. Column chromatography was carried out at atmospheric pressure using Silica Gel 60 (230-400 mesh, Merck).

The X-ray measurements of compounds **1a** and **1b** were performed at 130 (2) K, whereas of the derivative **5a** at 100 (2) K on a Bruker D8 VENTURE diffractometer with TRIUMPH monochromator and MoKα fine-focus sealed tube (0.71073 Å). The crystals were positioned 70 mm (**1a**), 40 mm (**1b**) and 34 mm (**5a**) from the PHOTON II detector with CPAD technology; 464 frames were measured at 1° intervals with a counting time of 1s, 317 frames were measured at 1.3° intervals with a counting time of 20s, 1106 frames were measured at 0.5° intervals with a counting time of 20 for **1a**, **1b** and **5a**, respectively.

Data collection, cell refinement and data reduction for **1a**, **1b** and **5a** were carried out with Bruker SAINT software package [37]. The data were corrected for Lorentz and

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polarization effects. Absorption correction was applied using the multi-scan method (SADABS) [38]. The structures were solved with the ShelXT [39] structure solution program using Intrinsic Phasing and refined with the ShelXL [39] refinement package and Olex2 program [40] using Least Squares minimization. The atomic scattering factors were taken from the International Tables [41]. Crystal structures and refinement are specified in Table 1. The structure of **1a** is disordered, with fluorine atom occupying two alternative sites with 50% occupancy each. The figures were generated with Mercury (ver. 4.1.0) [42]. Molecular geometries for **1a** and **1b** were optimized at the HF/3-21G* level of theory, using the Gaussian 03 program [43]. CCDC 1942278-1942280 contains the supplementary crystallographic data for this paper. These data can be obtained *via* http://www.ccdc.cam.ac.uk/conts/retrieving.html.

2.1.2 General procedure for the synthesis of 3-(4-methoxyphenyl)thiourea derivatives (1-9)

A solution of commercially available (4-methoxyphenyl)aniline (0.003 mol, 0.40 g) in anhydrous acetonitrile (6 ml) was treated with a corresponding isothiocyanate (0.003 mol). The mixture was stirred at room temperature for 12 h. After that, the solvent was evaporated. The solid residue was crystallized from acetonitrile or purified by column chromatography (chloroform: methanol; 9.5:0.5 vol.).

The synthesis of thiourea derivatives **3-6** [44, 45], **7** [46] and **9** [47] under different conditions was published previously.

1-(2-fluorophenyl)-3-(4-methoxyphenyl)thiourea (1)

Yield 52%, white solid, m.p. 165°C. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 3.75 (s, 3H, CH_3O), 6.94-6.88 (m, 2H), 7.26-7.14 (m, 3H), 7.37-7.30 (m, 2H), 7.63 (t, J = 7.9 Hz, 1H), 9.30 (s, 1H, NH), 9.77 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7, 114.2, 116.1 (d, $^{2}J_{C-F}$ = 20.2 Hz), 124.5 (d, $^{3}J_{C-F}$ = 3.7 Hz), 126.5, 127.6, 127.6 (d, $^{2}J_{C-F}$ = 20.2 Hz), 129.0, 132.4, 157.1, 156.8 (d, $^{1}J_{C-F}$ = 244.5 Hz), 181.2. MS (ESI) calc. for $C_{14}H_{13}FN_2OS$ [M – H]$^{-}$: 276.33, found: 275.00.

1-(3-fluorophenyl)-3-(4-methoxyphenyl)thiourea (2)

Yield 50%, white solid, m.p. 145°C. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 3.75 (s, 3H, CH_3O), 6.95-6.89 (m, 3H), 7.26-7.22 (m, 1H), 7.38-7.30 (m, 3H), 7.54 (dt, J_1 = 2.2 Hz, J_2 = 2.1 Hz, 1H), 9.75 (d, 2H, J = 9.3 Hz, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7,

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110.3 (d, $^{2}J_{C-F}$ = 24.7 Hz), 111.0 (d, $^{2}J_{C-F}$ = 20.2 Hz),114.2, 119.3 (d, $^{4}J_{C-F}$ = 3.0 Hz), 126.5, 130.3 (d, $^{3}J_{C-F}$ = 9.7 Hz), 132.4, 141.9 (d, $^{3}J_{C-F}$ = 10.5 Hz), 157.1, 162.2 (d, $^{1}J_{C-F}$ = 240.0 Hz), 180.2. MS (ESI) calc. for $C_{14}H_{13}FN_2OS$ $[M - H]^-$: 276.33, found: 275.17.

1-(4-fluorophenyl)-3-(4-methoxyphenyl)thiourea (3)

Yield 81%, white solid, m.p. 165°C. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 3.75 (s, 3H, CH_3O), 6.95-6.90 (m, 2H), 7.39-7.21 (m, 4H), 7.49 (dd, J_1 = J_2 = 1.5 Hz, 1H), 7.64 (dd, J_1 = J_2 = 1.8 Hz, 1H), 9.23 (s, 1H, NH), 9.81 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7, 114.3, 126.6, 127.6 (d, $^{2}J_{C-F}$ = 15.7 Hz), 129.8, 130.2, 131.3 (d, $^{1}J_{C-F}$ = 150.0 Hz), 137.0, 157.2, 180.9. MS (ESI) calc. for $C_{14}H_{13}FN_2OS$ $[M - H]^-$: 276.33, found: 275.07.

1-(2-chlorophenyl)-3-(4-methoxyphenyl)thiourea (4)

Yield 67%, white solid, m.p. 128°C. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 9.74 (d, 2H, J = 10.5 Hz, NH), 7.70 (t, 1H, J = 1.95 Hz, Ar-H), 7.41-7.30 (m, 4H, Ar-H), 7.17-7.13 (m, 1H, Ar-H), 6.94-6.88 (m, 2H, Ar-H), 3.75 (s, 3H, OCH_3). ^{13}C NMR (75.4 MHz, DMSO) δ (ppm): 179.82, 156.69, 141.20, 132.40, 131.82, 129.88, 126.03 (2C), 123.76, 122.88, 121.83, 113.75 (2C), 55.21. MS (ESI) calc. for $C_{14}H_{13}ClN_2OS$ $[M - 2H]^-$: 292.78, found: 292.91.

1-(3-chlorophenyl)-3-(4-methoxyphenyl)thiourea (5)

Yield 83%, white solid, m.p. 169°C. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 9.79 (s, 1H, NH), 9.18 (s, 1H, NH), 7.65 (dd, 1H, J_1 = 1.5 Hz, J_2 = 1.2 Hz, Ar-H), 7.58 (dd, 1H, J_1 = 1.5 Hz, J_2 = 1.8 Hz, Ar-H), 7.39-7.34 (m, 3H, Ar-H), 7.20-7.14 (m, 1H, Ar-H), 6.95-6.90 (m, 2H, Ar-H), 3.75 (s, 3H, OCH_3). ^{13}C NMR (75.4 MHz, DMSO) δ (ppm): 179.87, 156.71, 141.19, 132.42, 131.73, 129.80, 126.09 (2C), 123.69, 122.85, 121.81, 113.73 (2C), 55.26. MS (ESI) calc. for $C_{14}H_{13}ClN_2OS$ $[M - 2H]^-$: 292.78, found: 292.82.

1-(4-chlorophenyl)-3-(4-methoxyphenyl)thiourea (6)

Yield 20%, white solid, m.p. 128°C. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 9.73 (d, 2H, J = 15.6 Hz, NH), 7.82-7.81 (m, 1H, Ar-H), 7.45-7.40 (m, 1H, Ar-H), 7.34-7.24 (m, 4H, Ar-H), 6.94-6.88 (m, 2H, Ar-H), 3.75 (s, 3H, OCH_3). ^{13}C NMR (75.4 MHz, DMSO) δ (ppm): 179.81, 156.70, 141.34, 131.80, 130.17, 126.65, 126.03 (2C), 125.76, 122.29, 120.79, 113.75 (2C), 55.22. MS (ESI) calc. for $C_{14}H_{13}ClN_2OS$ $[M - 2H]^-$: 292.78, found: 292.69.

1-(2-bromophenyl)-3-(4-methoxyphenyl)thiourea (7)

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Yield 64%, white solid, m.p. 190°C. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 9.68 (s, 2H, NH), 7.51-7.43 (m, 4H, Ar-H), 7.34-7.29 (m, 2H, Ar-H), 6.93-6.88 (m, 2H, Ar-H), 3.75 (s, 3H, OCH_3). ^{13}C NMR (75.4 MHz, DMSO) δ (ppm): 179.80, 156.62, 139.04, 131.92, 131.08 (2C), 125.98 (2C), 125.50 (2C), 116.15, 113.70 (2C), 55.21. MS (ESI) calc. for $C_{14}H_{13}BrN_2OS$ [M – H]$^-$: 337.23, found: 336.94.

1-(3-bromophenyl)-3-(4-methoxyphenyl)thiourea (8)

Yield 62%, white solid, m.p. 175°C. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 9.60 (d, 2H, *J* = 11.1 Hz, NH), 7.48-7.41 (m, 2H, Ar-H), 7.34-7.28 (m, 2H, Ar-H), 7.19-7.11 (m, 2H, Ar-H), 6.93-6.88 (m, 2H, Ar-H), 3.75 (s, 3H, OCH_3). ^{13}C NMR (75.4 MHz, DMSO) δ (ppm): 180.23, 159.05 (d), 156.59, 135.83 (d), 131.99, 126.21 (d, 2C), 126.05 (2C), 114.91 (d, 2C), 113.69 (2C), 55.21. MS (ESI) calc. for $C_{14}H_{13}BrN_2OS$ [M – H]$^-$: 337.23, found: 336.56.

1-(4-bromophenyl)-3-(4-methoxyphenyl)thiourea (9)

Yield 65%, white solid, m.p. 179 – 180°C. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 9.67 (s, 2H, NH), 7.53-7.48 (m, 2H, Ar-H), 7.39-7.29 (m, 4H, Ar-H), 6.94-6.88 (m, 2H, Ar-H), 3.75 (s, 3H, OCH_3). ^{13}C NMR (75.4 MHz, DMSO) δ (ppm): 179.89, 156.63, 138.59, 131.91, 128.17 (2C), 128.05, 126.00 (2C), 125.24 (2C), 113.71 (2C), 55.21. MS (ESI) calc. for $C_{14}H_{13}BrN_2OS$ [M – H]$^-$: 337.23, found: 336.72.

2.1.3 General procedure for the synthesis of N-(halogenophenyl)-1-(4-methoxyphenyl)-1H-tetrazol-5-amines (1a–9a) and 1-(halogenophenyl)-N-(4-methoxyphenyl)-1H-tetrazol-5-amines (1b–9b)

A suspension of an appropriate thiourea derivative **1-9** (0.001 mol), sodium azide (0.00375 mol) and mercury (II) chloride (0.00125 mol) in 5ml of dried DMF was prepared. Next 2-3 drops of triethylamine were added. The resulting mixture was stirred for maximum 6 h at room temperature or until TLC showed the end of the reaction. The reaction solution was filtered and then washed with $CHCl_3$. The filtrate was diluted with water and extracted with $CHCl_3$ (3x15ml). The combined organic fractions were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (chloroform: methanol; 9.5: 0.5 vol.).

N-(2-fluorophenyl)-1-(4-methoxyphenyl)-1H-tetrazol-5-amine (1a)

Yield 61%, white solid, m.p. 108°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.84 (s, 3H, CH_3O), 7.12-7.27 (m, 5H), 7.54-7.60 (m, 2H), 7.61-7.67 (m, 1H), 8.98 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.6, 114.9, 115.8 (d, ^{2}J = 18.7 Hz), 123.7 (d, ^{4}J = 1.5 Hz), 124.5 (d, ^{3}J = 3.7 Hz), 125.1 (d, ^{3}J = 7.4 Hz), 125.8, 126.7, 127.5 (d, ^{2}J = 11.2 Hz), 153.0, 154.3 (d, ^{1}J = 243.8 Hz), 160.1. MS (ESI) calc. for $C_{14}H_{12}FN_5O$ $[M - H]^-$: 285.28, found: 285.11.

1-(2-fluorophenyl)-N-(4-methoxyphenyl)-1H-tetrazol-5-amine (1b)

Yield 39%, white solid, m.p. 136-138°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.73 (s, 3H, CH_3O), 6.89-6.95 (m, 2H), 7.45-7.62 (m, 4H), 7.69-7.80 (m, 2H), 9.29 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.2, 114.0, 117.2 (d, ^{2}J = 18.0 Hz), 120.2, 120.5 (d, ^{2}J = 12.8 Hz), 125.7 (d, ^{3}J = 3.7 Hz), 129.6, 132.5, 133.0 (d, ^{3}J = 7.5 Hz), 153.4, 154.8, 156.8 (d, ^{1}J = 249.7 Hz). MS (ESI) calc. for $C_{14}H_{12}FN_5O$ $[M - H]^-$: 285.28, found: 285.34.

N-(3-fluorophenyl)-1-(4-methoxyphenyl)-1H-tetrazol-5-amine (2a)

Yield 53%, white solid, m.p. 166°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.87 (s, 3H, CH_3O), 6.78-6.84 (m, 1H), 7.16-7.21 (m, 2H), 7.30-7.38 (m, 1H), 7.43-7.47 (m, 1H), 7. 56-7.61 (m, 3H), 9.45 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7, 104.8 (d, ^{2}J = 24.8 Hz), 108.3 (d, ^{2}J = 24.0 Hz), 113.9 (d, ^{4}J = 3.0 Hz), 115.0, 125.3, 127.6, 130.4 (d, ^{3}J = 9.7 Hz), 141.7 (d, ^{3}J = 11.2 Hz), 152.2, 160.5, 162.4 (d, ^{1}J = 241.0 Hz). MS (ESI) calc. for $C_{14}H_{12}FN_5O$ $[M - H]^-$: 285.28, found: 285.09.

1-(3-fluorophenyl)-N-(4-methoxyphenyl)-1H-tetrazol-5-amine (2b)

Yield 47%, white solid, m.p. 138°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.73 (s, 3H, CH_3O), 6.90-6.94 (m, 2H), 7.46-7.54 (m, 4H), 7.63-7.73 (m, 2H), 9.15 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.2, 113.4 (d, ^{2}J = 24.7 Hz), 114.0, 117.0 (d, ^{2}J = 20.2 Hz), 120.5, 122.0 (d, ^{4}J = 3.7 Hz), 131.7 (d, ^{3}J = 9.0 Hz), 132.7, 134.3 (d, ^{3}J = 11.2 Hz), 152.7, 154.8, 162.2 (d, ^{1}J = 244.5 Hz). MS (ESI) calc. for $C_{14}H_{12}FN_5O$ $[M - H]^-$: 285.28, found: 285.39.

N-(4-fluorophenyl)-1-(4-methoxyphenyl)-1H-tetrazol-5-amine (3a)

Yield 72%, white solid, m.p. 156-158°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.86 (s, 3H, CH_3O), 7.13-7.21 (m, 4H), 7.55-7.59 (m, 2H), 7.63-7.68 (m, 2H), 9.22 (s, 1H,

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NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7, 115.0, 115.3 (d, ^2J = 21.7 Hz), 119.9 (d, ^3J = 6.7 Hz), 125.5, 127.5, 136.2 (d, ^4J = 2.2 Hz), 152.6, 156.9 (d, ^1J = 237.0 Hz), 160.4. MS (ESI) calc. for $C_{14}H_{12}FN_5O$ $[M - H]^-$: 285.28, found: 285.21.

1-(4-fluorophenyl)-N-(4-methoxyphenyl)-1H-tetrazol-5-amine (3b)

Yield 28%, white solid, m.p. 156-158°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.73 (s, 3H, CH_3O), 6.88-6.93 (m, 2H), 7.46-7.53 (m, 4H), 7.71-7.75 (m, 2H), 9.07 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.2, 114.0, 116.8 (d, ^2J = 23.2 Hz), 120.3, 128.5 (d, ^3J = 5.2 Hz), 129.4 (d, ^4J = 2.2 Hz), 132.8, 152.9, 154.7, 158.9 (d, ^1J = 248.2 Hz). MS (ESI) calc. for $C_{14}H_{12}FN_5O$ $[M - H]^-$: 285.28, found: 285.53.

N-(2-chlorophenyl)-1-(4-methoxyphenyl)-1H-tetrazol-5-amine (4a)

Yield 57%, white solid, m.p. 100-102°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.84 (s, 3H, CH_3O), 7.13-7.20 (m, 3H), 7.31-7.37 (m, 1H), 7.48 (dd, J_1 = 5.1 Hz, J_2 = 1.5 Hz, 1H), 7.57-7.63 (m, 2H), 7. 67 (dd, J_1 = 5.1 Hz, J_2 = 1.5 Hz, 1H), 8.69 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7, 115.0, 123.8, 125.5, 125.7, 126.0, 126.1, 127.9, 129.7, 136.4, 152.8, 160.1. MS (ESI) calc. for $C_{14}H_{12}ClN_5O$ $[M - H]^-$: 301.73, found: 285.85.

1-(2-chlorophenyl)-N-(4-methoxyphenyl)-1H-tetrazol-5-amine (4b)

Yield 43%, white solid, m.p. 131-133°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.72 (s, 3H, CH_3O), 6.88-6.94 (m, 2H), 7.50-7.56 (m, 2H), 7.60-7.65 (m, 1H), 7.68-7.74 (m, 1H), 7. 78-7.83 (m, 1H), 9.19 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.2, 114.0, 120.1, 128.7, 130.3, 130.5, 130.6, 131.4, 132.6, 132.7, 153.2, 154.8. MS (ESI) calc. for $C_{14}H_{12}ClN_5O$ $[M - H]^-$: 301.73, found: 285.68.

N-(3-chlorophenyl)-1-(4-methoxyphenyl)-1H-tetrazol-5-amine (5a)

Yield 63%, white solid, m.p. 208°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.87 (s, 3H, CH_3O), 7.02-7.06 (m, 1H), 7.16-7.21 (m, 2H), 7.34 (t, J = 8.4 Hz, 1H), 7.55-7.61 (m, 3H), 7. 81 (t, J = 2.1 Hz, 1H), 9.42 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7, 115.0, 116.5, 117.4, 121.5, 125.3, 127.7, 130.4, 133.2, 152.1, 160.5. MS (ESI) calc. for $C_{14}H_{12}ClN_5O$ $[M - H]^-$: 301.73, found: 285.69.

1-(3-chlorophenyl)-N-(4-methoxyphenyl)-1H-tetrazol-5-amine (5b)

Yield 34%, white solid, m.p. 134-136°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.73 (s, 3H, CH_3O), 6.89-6.94 (m, 2H), 7.49-7.54 (m, 2H), 7.63-7.71 (m, 3H), 7.83-7.84 (m, 1H), 9.18 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.2, 114.0, 120.5, 124.6, 125.9, 130.0, 131.5, 132.7, 133.9, 134.2, 152.8, 154.8. MS (ESI) calc. for $C_{14}H_{12}ClN_5O$ [M – H]$^-$: 301.73, found: 285.81.

N-(4-chlorophenyl)-1-(4-methoxyphenyl)-1H-tetrazol-5-amine (6a)

Yield 69%, white solid, m.p. 161°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.86 (s, 3H, CH_3O), 7.16-7.21 (m, 2H), 7.34-7.40 (m, 2H), 7.55-7.60 (m, 2H), 7.66-7.71 (m, 2H), 9.36 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7, 115.0, 119.6, 125.4, 125.5, 127.6, 128.6, 138.9, 152.3, 160.4. MS (ESI) calc. for $C_{14}H_{12}ClN_5O$ [M – H]$^-$: 301.73, found: 285.77.

1-(4-chlorophenyl)-N-(4-methoxyphenyl)-1H-tetrazol-5-amine (6b)

Yield 31%, white solid, m.p.. 131°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.73 (s, 3H, CH_3O), 6.90-6.93 (m, 2H), 7.49-7.54 (m, 2H), 7.68-7.75 (m, 4H), 9.12 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.2, 114.0, 120.3, 127.7, 129.9, 131.9, 132.8, 134.6, 152.8, 154.7. MS (ESI) calc. for $C_{14}H_{12}ClN_5O$ [M – H]$^-$: 301.73, found: 285.58.

N-(2-bromophenyl)-1-(4-methoxyphenyl)-1H-tetrazol-5-amine (7a)

Yield 52%, white solid, m.p. 133-135°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.85 (s, 3H, CH_3O), 7.07-7.12 (m, 1H), 7.15-7.20 (m, 2H), 7.36-7.42 (m, 1H), 7.59-7.69 (m, 4H), 8.62 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7, 115.0, 117.0, 124.3, 125.6, 126.1, 126.4, 128.5, 132.9, 137.6, 152.9, 160.2. MS (ESI) calc. for $C_{14}H_{12}BrN_5O$ [M – H]$^-$: 346.19, found: 346.28.

1-(2-bromophenyl)-N-(4-methoxyphenyl)-1H-tetrazol-5-amine (7b)

Yield 48%, white solid, m.p. 136-138°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.72 (s, 3H, CH_3O), 6.88-6.94 (m, 2H), 7.51-7.57 (m, 2H), 7.59-7.69 (m, 2H), 7.75-7.79 (m, 1H), 7.93-7.96 (m, 1H), 9.15 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.2, 114.0, 120.1, 121.8, 129.3, 130.6, 131.9, 132.6, 132.9, 133.7, 153.1, 154.8. MS (ESI) calc. for $C_{14}H_{12}BrN_5O$ [M – H]$^-$: 346.19, found: 346.03.

N-(3-bromophenyl)-1-(4-methoxyphenyl)-1H-tetrazol-5-amine (8a)

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Yield 60%, white solid, m.p. 215-216°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.87 (s, 3H, CH_3O), 7.15-7.21 (m, 3H), 7.28 (t, *J* = 8.1 Hz, 1H), 7.55-7.59 (m, 1H), 7.62-7.65 (m, 1H), 7. 95 (t, *J* = 1.9 Hz, 1H), 9.40 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7, 115.0, 116.8, 120.3, 121.6, 124.4, 125.3, 127.7, 130.7, 141.5, 152.1, 160.5. MS (ESI) calc. for $C_{14}H_{12}BrN_5O$ $[M - H]^-$: 346.19, found: 346.31.

1-(3-bromophenyl)-N-(4-methoxyphenyl)-1H-tetrazol-5-amine (8b)

Yield 40%, white solid, m.p. 149-151°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.73 (s, 3H, CH_3O), 6.89-6.94 (m, 2H), 7.48-7.53 (m, 2H), 7.57-7.63 (m, 1H), 7.67-7.70 (m, 1H), 7.81-7.85 (m, 1H), 7.94-7.96 (m, 1H), 9.18 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 65.2, 124.0, 130.4, 132.2, 135.0, 138.6, 141.7, 142.7, 142.9, 144.3, 162.8, 164.8. MS (ESI) calc. for $C_{14}H_{12}BrN_5O$ $[M - H]^-$: 346.19, found: 346.16.

N-(4-bromophenyl)-1-(4-methoxyphenyl)-1H-tetrazol-5-amine (9a)

Yield 63%, white solid, m.p. 181-184°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.86 (s, 3H, CH_3O), 7.16-7.21 (m, 2H), 7.47-7.52 (m, 2H), 7.55-7.60 (m, 2H), 7.61-7.66 (m, 2H), 9.36 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.7, 113.4, 115.0, 120.0, 125.4, 127.6, 131.5, 139.3, 152.2, 160.4. MS (ESI) calc. for $C_{14}H_{12}BrN_5O$ $[M - H]^-$: 346.19, found: 346.42.

1-(4-bromophenyl)-N-(4-methoxyphenyl)-1H-tetrazol-5-amine (9b)

Yield 37%, white solid, m.p. 155-158°C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.73 (s, 3H, CH_3O), 6.89-6.94 (m, 2H), 7.49-7.54 (m, 2H), 7.61-7.66 (m, 2H), 7.83-7.88 (m, 2H), 9.13 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 55.2, 114.0, 120.3, 123.1, 127.8, 132.3, 132.8, 132.9, 152.7, 154.8. MS (ESI) calc. for $C_{14}H_{12}BrN_5O$ $[M - H]^-$: 346.19, found: 346.36.

2.1.4 *In vitro* antitubercular activity

The starting thiourea compounds **1-9** were examined *in vitro* for their activity against the *M. tuberculosis* H_{37}Rv strain (ATCC 25618) and two 'wild-type' strains isolated from tuberculosis patients: one (Spec. 210) being resistant to *p*-aminosalicylic acid (PAS), isonicotinic acid hydrazide (Isoniazid, INH), ethambutol (EMB) and rifampicin (RMP) and the other (Spec. 192) being fully sensitive to the administrated tuberculostatics. Investigations were performed by a classical test-tube method of successive dilutions in Youmans'

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modification of the Proskauer and Beck liquid medium containing 10% of bovine serum [48, 49]. Bacterial suspensions were prepared from 14 days old cultures of slowly growing strains. Solutions of compounds in DMSO were tested. Stock solutions contained 10 mg of compounds in 1 milliliter. Dilutions (in geometric progression) were prepared in Youmans' medium. The medium containing one of the reference drug (INH, RMP, EMB or SM), without investigated substances, was used for comparison. Incubation was performed at 37 °C. The MIC values were determined as a minimum concentration inhibiting the growth of tested tuberculous strains in relation to the probe with no tested compound. The influence of the compound on the growth of bacteria at a certain concentration, 3.1, 6.25, 12.5, 25, 50 and 100 μg/ml, was evaluated.

The synthesized compounds **1a-9a** and **1b-9b** were tested *in vitro* for their tuberculostatic activity against typical strains (*M. tuberculosis* H_{37}Rv strain (ATCC 25618), *M. tuberculosis* Spec. 192, *M. tuberculosis* Spec. 210, *M. tuberculosis* Spec. 800) and atypical isolates (*M. bovis, M.scrofulaceum, M. fortuitum, M. avium, M. kansasii, M. xenopi, M. intracellulare*) using the MABA method (Microplate Alamar Blue Assay method) [50, 51]. Investigations were performed by two-fold serial microdilution method (in 96-well microliter plates) using Middlebrook 7H9 Broth medium (Beckton Dickinson) containing 10% of OADC (Beckton Dickinson). The inoculum was prepared from fresh LJ culture in Middlebrook 7H9 Broth medium with OADC, adjusted to a no. 1 McFarland tube, and diluted 1:20. The stock solution of a tested agent was prepared in DMSO. Each stock solution of a tested compound was diluted in Middlebrook 7H9 Broth medium with OADC by four-fold the final highest concentration to be tested. Compounds were diluted serially in a sterile 96-well microtiter plates using 100μl Middlebrook 7H9 Broth medium with OADC. Concentrations of tested agents ranged from 0.125 to 512 μg/ml. A growth control containing no antibiotic and a sterile control without inoculation were also prepared on each plate. The plates were incubated at 37°C for a week. After the incubation period, 30μl of Alamar blue solution was added to each well, and the plate was re-incubated for 24 h. The growth was indicated by the color change from blue to pink. The lowest concentration of a compound that prevented the color change was noted as its MIC. INH, RMP, SM and EMB as the reference drugs were used for comparison.

2.1.5 Drug interaction analysis

The combinations of the tested derivative and the reference drug were prepared in Middlebrook 7H9 medium (Becton-Dickinson, USA) containing 10% of OADC (Becton-

Dickinson, USA). Each derivative in a concentration ranging from 1 to 1/32 MIC was mixed with the reference drug in concentration equal to 1/2 MIC. Simultaneously, each of the reference drugs (INH, RMP, SM and EMB) in concentrations ranging from 1 to 1/32MIC was mixed with the tested derivative in concentration equal to 1/2MIC (MIC values of tested derivatives and reference drugs alone were determined as described above). Then, all samples (4 ml) were supplemented with an inoculum of tested strains (1°McFarland; 12 µl) and incubated for 14 days at 37 °C. MIC of the tested derivative in combination with the fixed concentration of the reference drug (MICD/comb with RD) was defined as the lowest concentration of the tested derivative combined with the reference drug, which inhibited the growth of microorganisms in a liquid medium. Similarly, MIC of the reference drug in a combination with the fixed concentration of the tested derivative (MICRD/comb with D) was defined as the lowest concentration of the reference drug combined with the tested derivative, which inhibited the growth of microorganisms in a liquid medium. FICI value was calculated according to the equation: FICI = ($MIC_{D/comb.\ with\ RD}/MIC_{D\ alone}$) + ($MIC_{RD/comb.\ with\ D}/MIC_{RD\ alone}$). The obtained FICI values were then used to determine whether synergism (FICI < 0.5), additivity (0.5 ≤ FICI ≥ 1), indifference (1 < FICI ≤ 4) or antagonism (FICI > 4) occurred between the tested agents [52].

2.1.6 Antimicrobial studies

The antimicrobial assays were conducted using reference strains of bacteria derived from international microbe collections: American Type Culture Collection (ATCC) and National Collection of Type Culture (NCTC). The group of reference strains included Gram-positive bacteria rods (*Staphylococcus aureus:* NCTC 4163, ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, ATCC 35984/RP62A) and Gram-negative bacteria isolates (*Pseudomonas aeruginosa* NCTC 6749, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10031, *Proteus vulgaris* NCTC 4635). Two "wild-type" strains were also evaluated (*S. epidermidis* 1457 and *P. aeruginosa* NCTC PAO 1).

The antimicrobial activity was expressed by minimum inhibitory concentration (MIC), according to CLCI reference procedures with some modification. MIC was tested by the twofold serial microdilution method (in 96-well microtiter plates) on MH II liquid medium. The final inoculum of all studied bacteria was 10^6 CFU/ml (colony forming unit per ml). The stock solution of tested compounds was prepared in DMSO and diluted in sterile medium (to maximum 1 % of solvent content). The concentrations of compounds varied from 0.03125 to

512 µg/ml. The MIC value was the lowest concentration of the tested compound, at which bacteria growth was no longer observed after 18 h of incubation at 35 ˚C [53].

2.1.7 Cytotoxicity studies

2.1.7.1 Cell culture

Chinese hamster lung fibroblast (V79) and human immortal keratinocytes (HaCaT) cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, USA) and cultured in DMEM (Dulbecco's Modified Eagle's Medium, Biowest SAS, France). Cells were seeded in 6 ml medium in a tissue culture flask (50 ml) in a 37 °C/5% CO_2 humidified incubator. Medium was supplemented with 10% heat-inactivated fetal bovine serum (Gibco Life Technologies, USA), penicillin (100 U/ml), streptomycin (100 µg/ml) and HEPES (20 mM). After reaching 80-90% confluence, cells were passaged using trypsin-EDTA (Gibco Life Technologies, USA) and seeded in 96-well plates (1×10^4 cells per well) for MTT assay.

2.1.7.2 MTT assay

The cell viability was assessed by using an enzymatic conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT) to insoluble formazan crystals by mitochondrial dehydrogenases, occurring in living cells. Cells were cultured in 96-well plates at a density of $lx10^4$ cells per well, and cultured to adhere for 24 hours at 37°C in a CO_2 incubator. After 24 hours of incubation, culture medium was replaced with a fresh medium, then cells were treated with various concentrations of the tested compounds (ranged from 140 µM to 20 µM), and incubated for 72 hours at 37°C in a CO_2 incubator. Untreated cells were used as the control. Subsequently MTT solution (0,5 mg/mL in free-serum medium) was added and samples were incubated for 4 hours at 37°C in a CO_2 incubator. Then the medium was aspirated and formed formazan crystals were solubilized by adding an isopropanol - DMSO mixture (1:1 vol). The optical density of the dissolved formazan crystals was measured using a UVM 340 reader (ASYS Hitech GmbH, Austria) at a wavelength of 570 nm. IC_{50} value was estimated using CompuSyn version 1.0.

2.1.7.3 LDH assay

Human immortal keratinocyte cell line from adult human skin (HaCaT), Human melanoma cell line (HTB-140), Human epithelial lung carcinoma cell line (A549) and Human

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colorectal adenocarcinoma (CaCo-2) were purchased from American Type Culture Collection (Rockville, USA), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) and Eagle's Minimum Essential Medium (EMEM), supplemented with 1% antibiotics (penicillin and streptomycin) and 10% heat-inactivated FBS-fetal bovine serum (Gibco Life Technologies, USA) at 37°C in a 5% CO_2 atmosphere. Cells were passaged using trypsin-EDTA (Gibco Life Technologies, USA) and cultured in 96-well plates (1×10^4 cells per well). Experiments were conducted in DMEM and EMEM with 2% FBS.

The determination was performed after 72 h incubation of cells in 96-well plates with chosen compounds as described before [25]. LDH Cytotoxicity Assay Kit was used to assess the activity of lactate dehydrogenase (LDH) released from cytosol of damaged cells to the supernatant and measured according to the protocol described by the manufacturer (Roche Diagnostics, Germany). An absorbance was measured at 490 nm using a microplate reader (Epoch microplate reader, BioTek Inc., USA) equipped with Gen5 software (BioTech Instruments, Inc., Biokom). Cytotoxicity of compounds was expressed as the LDH release (%) and counted by the following equation: [(A test sample − A low control)/(A high control − A low control)] × 100% (A-absorbance); where “low control” were cells in DMEM or EMEM with 2% FBS without tested compounds, and “high control” were cells incubated in DMEM or EMEM with 2% FBS and 1% Triton X-100 (100% LDH release).

3. Results and discussion

3.1 Chemistry

Our previous studies concerning the bioactivity of thiourea derivatives showed that 4-methoxyphenyl moiety is not responsible for the antimicrobial activity [54-57]. For this reason, a series of various halogenated 1-(4-methoxyphenyl)thioureas **1-9** were synthesized just for transformation to appropriate 1*H*-tetrazol-5-amines. These thiourea-derived compounds formed a group of close structural analogs bearing *ortho*-, *meta*- and *para*-halogenophenyl terminal fragments. Thus, the structures of the parent thiourea compounds and, in consequence, their tetrazole counterparts, allow to study the influence of the differently substituted electron-withdrawing element (fluorine, chlorine or bromine) on their antimicrobial and cytotoxic properties.

Tetrazoles can be obtained using four main methods [58, 59]. In our specific synthesis, 1,5-disubstituted tetrazoles were generated *via* oxidative desulfurization of 1,3-disubstituted

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thioureas, using sodium azide as an external nucleophile. Mercury (II) chloride was used as an desulfurization agent. The reaction was carried out at room temperature in DMF and in the presence of triethylamine (Scheme 1).

Assumptions based on the results of preliminary experiments have been confirmed, since for the designed reaction two isomeric products: the form A (**1a-9a)** and the form B (**1b-9b**), occurred simultaneously. However, one of the regioisomers should be considered as favorable (**1a-9a**), since it has been found in higher yields.

According to our knowledge, this is the first report showing simultaneous one-pot synthesis of isomeric tetrazole-based compounds with good yields, using mentioned reaction conditions. Our previous findings confirmed that the yield of each product form is directly related to the steric hindrance caused by substituents. We also presumed that physicochemical properties of substituents are of secondary importance and need to be evaluated individually. In this case the moieties attached to amine groups of thiourea are similar, therefore cyclization *via* oxidative desulfurization can be conducted in two ways, with the use of the one of the amine groups of the thiourea arrangement. Favored forms A (**1a-9a**) were obtained in a yield range of 52 – 72 %, whereas yields of forms B (**1b-9b**) equaled 28 – 48 %. The proposed mechanism of the reaction is shown on Scheme 1S (Supplementary material).

Since the cyclization reaction conducting oxidative desulfurization is well known, there is no need to provide theoretical fundamentals of the method. We have assumed that the rate of the preferable form A is related to the occurrence of halides attached to the terminal phenyl ring. The thiourea nitrogen atom bonding to the 4-methoxyphenyl scaffold should be considered as more attainable during formation of the tetrazole ring.

[Scheme 1]

3.2 X-ray crystallography studies

The compound **1a** crystallizes in a monoclinic system, space group $P2_1/c$, with one molecule in the asymmetric part of the unit cell (Fig. 1). There are four molecules in the unit cell forming pairs bound by N-H…N hydrogen bonds (2.143Å, see Fig. 1S, Supplementary material). The substituents are tilted with respect to the tetrazole ring. The angles between mean planes calculated for atoms forming rings are 87.2° (methoxyphenyl) and 44.5° (fluorophenyl moiety). Similarly, the derivative **1b** crystallizes in a monoclinic system, space group $P2_1/c$, with one molecule in the asymmetric part of the unit cell (Fig. 1). There are four

molecules in the unit cell forming pairs bound by N-H…N hydrogen bonds (2.060 Å, see Fig. 1S, Supplementary material), but arranged a bit unlike **1a**. Additionally, the molecules from adjacent pairs form dimers by C-H…O bonds (2.696 Å). This motif in turn forces substituents to be tilted differently with respect to the tetrazole ring. The angles between mean planes denoted for atoms building rings are 35.8° (methoxyphenyl) and 63.7° (fluorophenyl moiety). On the other hand, the product **5a** crystallizes in a monoclinic system, space group $P2_1$, with one molecule in the asymmetric part of the unit cell (Fig.1). There are two molecules in the unit cell (Fig. 1S, Supplementary material). They are weakly bound by C-H…N bonds (2.640 Å, see Fig. 2S, Supplementary material). Despite its structural similarity to **1a**, the substituents are tilted with respect to the tetrazole ring in a completely different way (their superpositions are shown in Fig. 2). The angles between mean planes calculated for atoms forming rings are 49.0° (methoxyphenyl) and 17.7° (chlorophenyl moiety). The tetrazole ring forms short contacts with the substituents C-H…N (2.567Å, 2.626 Å, chlorophenyl) and C-H…N (2.747 Å, methoxyphenyl) in the crystal lattice. Additionally, the substituents from adjacent molecules are bound by C-H…O hydrogen bonds (2.532 Å, Fig. 2S, Supplementary material). The calculations at HF/3-21G* level of theory showed that the optimized geometry of **1a** has lower energy than **1b** by 14.4 kcal/mol. This result indicates that the form A of compounds synthesized with higher yields should be considered as more stable.

[Fig.1.]

[Fig.2.]

3.3 In vitro antimycobacterial activity

All prepared compounds were tested for their *in vitro* antitubercular potency against *M. tuberculosis* H_{37}Rv strain and three "wild-type" mycobacteria isolated from tuberculosis patients: multidrug-resistant Spec. 210 (with resistance to p-aminosalicylic acid (PAS), INH, EMB and RMP), Spec. 192, fully susceptible to established tuberculostatics, and INH-monoresistant Spec. 800. The tests included also the group of non-tuberculous bacilli (*M. scrofulaceum, M. fortuitum, M. avium, M. kansasii, M. xenopi, M. intracellulare*). The antimycobacterial activities were expressed as minimum inhibitory concentrations (MICs), with the first-line anti-TB drugs: isoniazid (INH), rifampicin (RMP), streptomycin (SM) and ethambutol (EMB) used as standards.

The preliminary studies of antimycobacterial activity of parent 1-(halogenophenyl)-3-(4-methoxyphenyl)thiourea derivatives revealed that their tuberculostatic potency varied from

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moderate to weak (Table 2). Although none of the new thioureas reached the level of the references potential, 2-chloro- (**4**) and 4-bromophenylthiourea (**9**) exhibited the highest inhibitory effect against *M. tuberculosis* 192 strain, with MICs equaled 25 μg/ml. Compounds **4**, **6-8** exerted the same activity level towards the 210 isolate as the standards: RMP and EMB. It was noticed that the presence of bromine at any position of the phenyl ring (derivatives **7-9**) or the substitution of *ortho*- and *para*- position with chlorine (**4**, **6**), promoted antituberculostatic activity of studied phenylthiourea compounds.

The rationally designed structures of tetrazole compounds **1a-9a** and **1b-9b** allowed to study how the type of their structural isomerism influenced biological properties, specifically antimycobacterial activity. Thus, the multidrug-resistant *M. tuberculosis* 210 pathogen was found to be the most susceptible to the presence of synthesized 1,5-regioisomers of tetrazole (Table 3). All of them, except of the derivative **7a**, were more effective towards the bacilli than established pharmaceuticals. N-(bromophenyl)tetrazole-5-amines (**8a**, **9a**), with MIC values of 2 μg/ml, exerted the highest activity, being up to 16-fold more potent than the references RMP and EMB, and 8 times more active comparing to INH and SM. Additionally, other fluorophenyl- (**2a, 3b**) and chlorophenyl-based (**4a, 4b-6b**) tetrazoles expressed 2-4-fold higher inhibitory effect than the standard tuberculostatics. Remaining compounds of the series, N-substituted (**1a, 6a**) and 1-substituted (**1b, 2b, 7b-9b**) were equally active against mycobacteria as INH and SM (MIC = 16 μg/ml). The MIC values of halogen derivatives **3a** and **5a** were the same as these denoted for RMP and EMB (32 μg/ml). Moreover, the activity profile of two isomeric bromophenyltetrazoles **8a** and **9a** towards *M. tuberculosis* $H_{37}R$ and drug-sensitive Spec.192 strains was comparable to three tuberculostatics used: RMP, SM and EMB. The MIC for all these agents equaled 1-2 μg/ml. One-quarter of the referential EMB potency (*i.e.* 8 μg/ml) was established for tetrazoles derived from the N-substituted series (**1a, 4a**), and predominantly 1-substituted group (**3b-6b**). Other synthesized compounds presented moderate antitubercular activity, with MIC parameters corresponding to 16-64 μg/ml. None of the derivatives was as active as the most powerful INH. In contrary, INH-monoresistant *M. tuberculosis* Spec. 800 appeared the least sensitive to the treatment with new tetrazoles, comparing to the reference drugs. Selected halogen compounds (**8a, 9a, 3b-6b**) were effective just at MIC values of 128 μg/ml. Considering the structure-activity relationship, one can notice, that the antimycobacterial potency towards standard and "wild-type" pathogens depends on: 1) the type of positional isomer tested (N-halogenophenyls more effective than 1-substituted compounds); 2) the character of halogen in the benzene ring (the bioactivity

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decreases in a line: bromo >> chloro> fluoro); and 3) the place of its substitution (*meta*- and *para*- isomers more effective than *ortho*-).

Among atypical bacilli, *M. scrofulaceum* strain was the most sensitive to exposition to the tested tetrazoles (Table 4). The derivative of 1-(3-chlorophenyl)-1*H*-tetrazol-5-amine (**5b**) revealed the highest effectiveness (MIC = 2 μg/ml) and was 32 time more active than the standard INH. Halogenophenyl-substituted compounds **2a, 2b, 6b** exposed fourfold of isoniazid activity, whereas tetrazoles **3a, 4a, 1b, 4b, 8b** were similarly effective as the reference. The product **3b,** bearing 4-chlorophenyl moiety, was equally potent to INH. A strong inhibitory effect of N-halogenophenyl derivatives **3a, 6a, 2a** against *M. intracellulare* strain was also observed; they were several fold more or equally potent as the referential INH. Newly synthesized compounds were much more less effective against pathogens such as *M. avium, M. kansasii* and *M. xenopii* than antimycobacterials used; their MIC ranged from 64 to 512 μg/ml. On the contrary, only 2-chlorophenyl derivative **4b** had the same activity as INH towards *M. fortuitum* species, however only at the concentration of 128 μg/ml. To sum up, 1-halogenophenyl-substituted isomers were the most efficient against evaluated non-tuberculous mycobacteria. They were preferentially substituted with fluorine or chlorine at rather *meta*- and *para*- than *ortho*- position of the benzene ring. For these types of bacilli, an introduction of bromine element and the substitution of primary amine group with halogenophenyl core decreased the bioactivity.

As it was shown in Table 3, calculated logP values of all synthesized compounds were lower than 5, that intend them for eventual oral administration (see also Table 1S, Supplementary material). The lipophilicity factors of poorly active thiourea derivatives (**1-9)** were in the range of 3.23-3.61, whereas their cyclic, bioactive tetrazole analogues (**1a-9a**, **1b-9b**) appeared to be more hydrophilic with logP values varied from 2.69 to 3.07. Compounds **4a**, **8a** and **9a**, the most effective against typical *M. tuberculosis* strains, possessed the highest logP indexes among tested N-substituted regioisomers. On the other hand, 1-substituted tetrazoles, marked as the most potent towards standard and "wild-type" (**3b-6b**) or non-tuberculous (**5b**, **6b**) mycobacteria, were characterized by middle-ranged lipophilicity (2.71-2.97). Finally, heterocycles **2a**, **3a** with the lowest logP values, exerted the strongest inhibitory effect against atypical bacilli.

[Table 2]

[Table 3]

[Table 4]

3.4 Drug interaction analysis

The recommended tuberculosis treatment is based on a combination of several pharmaceuticals administrated for months, in order to kill mycobacteria in their various stages of the illness progress [6, 34, 60, 61]. Since the multidrug resistance to bacilli has become a problem, the use of new combinations including established anti-TB drugs and newly synthesized compounds needs to be assessed. For this aim, we have evaluated the potency of the most promising derivatives **8a** and **9a** in a combined therapy with one of the four reference tuberculostatics. Calculated FICI values (Tables 5-8) allowed to determine interactions of mixed substances as synergism (FICI < 0.5), additivity ($0.5 \leq$ FICI ≤ 1), indifference ($1 <$ FICI ≤ 4) or antagonism (FICI > 4) [52].

Synergistic effect among the derivative **9a** and SM was observed in both INH-susceptible *M. tuberculosis* H_{37}Rv and Spec. 192 strains, as well as in INH-monoresistant 800 species. In all cases, the SM/**9a** combination showed a fourfold decrease in MIC value of the drug, when ½ MIC of tetrazole was applied. On the other hand, the RMP/**8a**, SM/**8a** and EMB/**8a** additive interactions against *M. tuberculosis* H_{37}Rv and 192 pathogens were denoted. Addition of the mentioned tetrazole allowed to reduce the tuberculostatic dose, expressed as MIC, 2 or even 4 times. The additivity between the compound **8a** and RMP towards multidrug-resistant 210 strain was also suggested. What is more, the combination of **8a** with each of the conventionally used drug was additive in inhibition of the growth of INH-monoresistant *M. tuberculosis* 800 pathogen. As for previously mentioned isolates, the effective MIC value of the tuberculostatic could be up to four-fold lower, when it was paired with that tetrazole derivative. Similarly, the additive interaction of the isomer **9a** with RMP or EMB against *M. tuberculosis* H_{37}Rv and Spec. 192 isolates was detected. Against the multi-resistant 210 species, for all drugs, except for EMB, additivity was denoted, with ½ MIC of the compound **9a** added. Interestingly, INH/**9a** and EMB/**9a** pairs exerted the additive inhibitory effect, when applied toward monoresistant Spec. 800 bacillus. Only some combinations of tested tetrazoles against both resistant isolates revealed indifference. Since the effect of pairing of investigated derivatives with INH was antagonistic towards both drug-sensitive mycobacteria strains (*M. tuberculosis* H_{37}Rv and Spec. 192), they must be excluded from a combination treatment. However, the established antagonism, along with very high MICs values of evaluated tetrazoles against INH-monoresistant *M. tuberculosis* Spec. 800 suggest that INH and derivatives **8a**, **9a** could hit the same molecular target.

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INH, the multi-targeted lead antituberculostatic, acts as a prodrug that is activated in a cell by the mycobacterial bifunctional catalase-peroxidase (KatG) [62]. Generated INH-derived free radicals form covalent adducts with NADH or NADPH, capable to disrupt the synthesis of both mycolic and nucleic acids [62, 63]. INH-NAD connections are powerful inhibitors of InhA, an enoyl acyl carrier protein reductase, involved in the formation of mycolic acids, major lipid components of the mycobacterial cells [64]. In turn, INH-NADP adducts restrain the activity of MabA, an NADPH-dependent β-ketoacyl-ACP reductase, also engaged in the biosynthesis of long-chain fatty acids. On the other hand, complexes of INH with $NADP^+$ were described as inhibitors of dihydrofolate reductase (DHFR), the key enzyme necessary for the folate biosynthesis pathway in the microbial cells [62]. Among mentioned molecular goals, direct InhA- targeted ligands, especially omitting the KatG-activating step, are considered as the most promising to combat tuberculosis [65].

Numerous polycyclic molecules with nitrogen-based heterocyclic rings, structurally related to INH and substituted tetrazoles presented in our study, have been found as effective InhA inhibitors. Guardia et al. described a series of halogenated benzamides bearing 1*H*-pyrazolyl and pyridin-2-yl fragments with micromolar activities against InhA [66], whereas the group of Shaikh found potent inhibitors among derivatives of carbazole, substituted with thioxothiazolidine-4-one moiety [67]. Additionally, piperazine indoleformamides, pyrazoles, pyrrolidine carboxamides, imidazopiperidines, thiadiazoles, triazoles, thiazoles and proline-derived hybrids were also identified as potent enoyl-ACP-reductase inhibitors [64, 68, 69]. Among them, halogen-containing pyrazol-3-yl- and pyridin-2-yl-thiadiazol-2-amines used in the low nanomolar range, acted as direct, reversible InhA inhibitors. The presence of both a (hetero)aryl moiety substituted with fluorine, chlorine and/or bromine, and nitrogen-rich rings have promoted inhibitory properties to the mycobacterial InhA.

The presented *in vitro* studies of antimycobacterial combinations of the title 1*H*-tetrazol-5-amines are a useful first step to predict their possible mechanism of action and to estimate their effectiveness in further clinical researches.

[Table 5]

[Table 6]

[Table 7]

[Table 8]

3.5 Antibacterial activity

In order to check the selectivity of the inhibitory potential of the studied derivatives against *M. tuberculosis* strains, the effect of thiourea-based compounds **1**-**9** and their tetrazoles towards 10 bacterial isolates (5 Gram-positive rods and 5 Gram-negative cocci) was examined (Table 9). None of the starting 4-methoxyphenylthioureas (**1-9**) possessed antibacterial activity. Only some tetrazole-containing regioisomers (**2a-4a**, **6a**, **1b**, **3b**, **4b**) exerted slight growth-inhibitory potency towards *S. epidermidis* and *S. aureus* strains, with MIC values ranging from 32 to 64 µM. Other synthesized tetrazoles were inactive towards representative bacterial isolates (MIC ≥ 128 µM). The obtained results indicated that the antimycobacterial profile of derivatives presented in this study is highly selective.

[Table 9]

3.6 Cytotoxicity evaluation

To get information concerning toxicity of newly synthesized tetrazoles (**1a**-**9a**, **1b**-**9b**) in mammalian cells, their *in vitro* effects on two normal cell lines, V79 (Chinese hamster lung fibroblast) and HaCaT (human immortal keratinocyte) were evaluated (Table 10). The results showed that compounds, applied at the concentration of 100 µM for 72 h, had negligible impact on the viability of non-neoplastic cells. The IC_{50} values of all derivatives were higher than 100 µM, that demonstrated their good safety and selectivity towards mycobacterial cells. What is more, they were found to be more than several dozen times less cytotoxic than the reference doxorubicin against both tested cell lines.

To confirm the safety of the most promising antitubercular agents (**8a**, **9a**), the LDH cytotoxicity assay on cancer cell lines: HTB-140 (human melanoma), A549 (human epithelial lung carcinoma), CaCo-2 (Caucasian colon adenocarcinoma), as well as on normal HaCaT cell line was performed (Fig.3.). Studied tetrazoles were used at the concentration of 100 µM, because of their high IC_{50} value. The level of cell damage in normal HaCaT cells caused by the presence of **8a** and **9a** was low, and accounted for 4.88 and 7.25 %, respectively. Both derivatives expressed also low cytotoxic influence on tumor cell lines. Although the LDH release was the strongest for **8a** in HTB-140 and CaCo-2 cells, it was still not significant, and did not exceed 17%.

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The obtained cytotoxicity data confirmed a minor influence of 1*H*-tetrazole derivatives on the growth and viability of normal and pathogenic cells.

[Table 10]

[Fig.3.]

4. Conclusions

In this work a set of isomeric (4-methoxyphenyl)-1*H*-tetrazol-5-amines, bearing differentially substituted halogen atom on the terminal benzene ring, was synthesized from their thiourea counterparts. These structural modifications led to an increase of hydrophilicity and activity of compounds against standard and drug-resistant *M. tuberculosis* strains. Among studied regioisomers, N-substituted halogenophenyl derivatives were found to be more effective than 1-substituted compounds. What is more, the bioactivity was the highest for bromophenyl agents, substituted at *meta*- or *para*- position.

Newly synthesized tetrazole-based derivatives exerted also a promising potential in the combined anti-TB therapy. The synergistic effect of **9a**/SM combination against three *M. tuberculosis* strains was denoted, and also the additivity of both **8a** and **9a** compounds when paired with INH, RMP or EMB was described. The antagonism of tetrazoles-INH combinations towards both drug-sensitive isolates (*M. tuberculosis* H_{37}Rv and Spec. 192), and the high resistance of Spec. 800 to these compounds suggest a possible inhibitory effect of newly synthesized derivatives against InhA. Regarding the cytotoxicity, the synthesized tetrazoles showed negligible impact on two normal and three cancer cell lines at a concentration of 100 µM, and did not noticeably inhibit the growth of Gram-positive and Gram-negative bacteria.

Although further changes in chemical structure of tetrazole-derived compounds are needed in order to develop their antimycobacterial profile and therapeutic safety, selective substances, such as **8a** and **9a**, are the most promising for the combined human mycobacteria treatment.

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Conflict of interest

The authors declare no conflict of interest.

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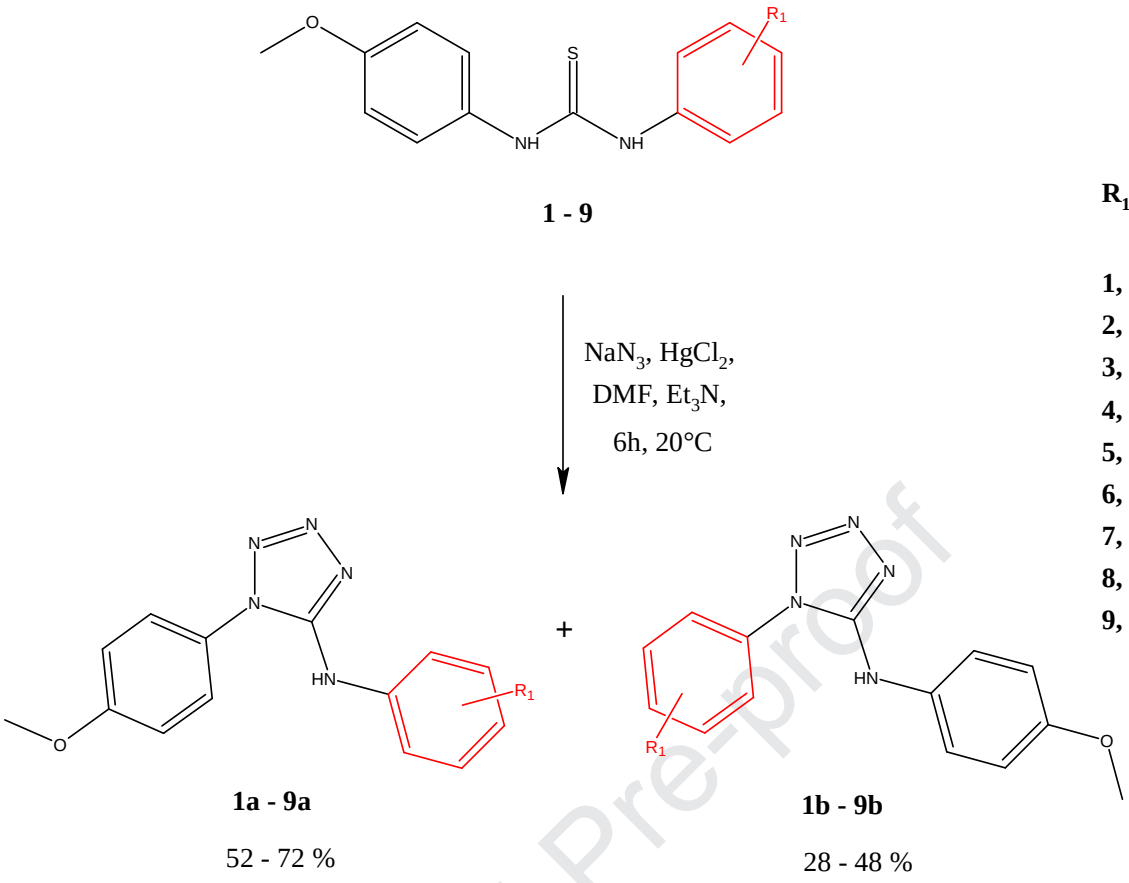

R_1:

1, 1a, 1b	2-F
2, 2a, 2b	3-F
3, 3a, 3b	4-F
4, 4a, 4b	2-Cl
5, 5a, 5b	3-Cl
6, 6a, 6b	4-Cl
7, 7a, 7b	2-Br
8, 8a, 8b	3-Br
9, 9a, 9b	4-Br

Scheme 1. Synthesis of 1,5-disubstituted tetrazoles **1a-9a** and **1b-9b**.

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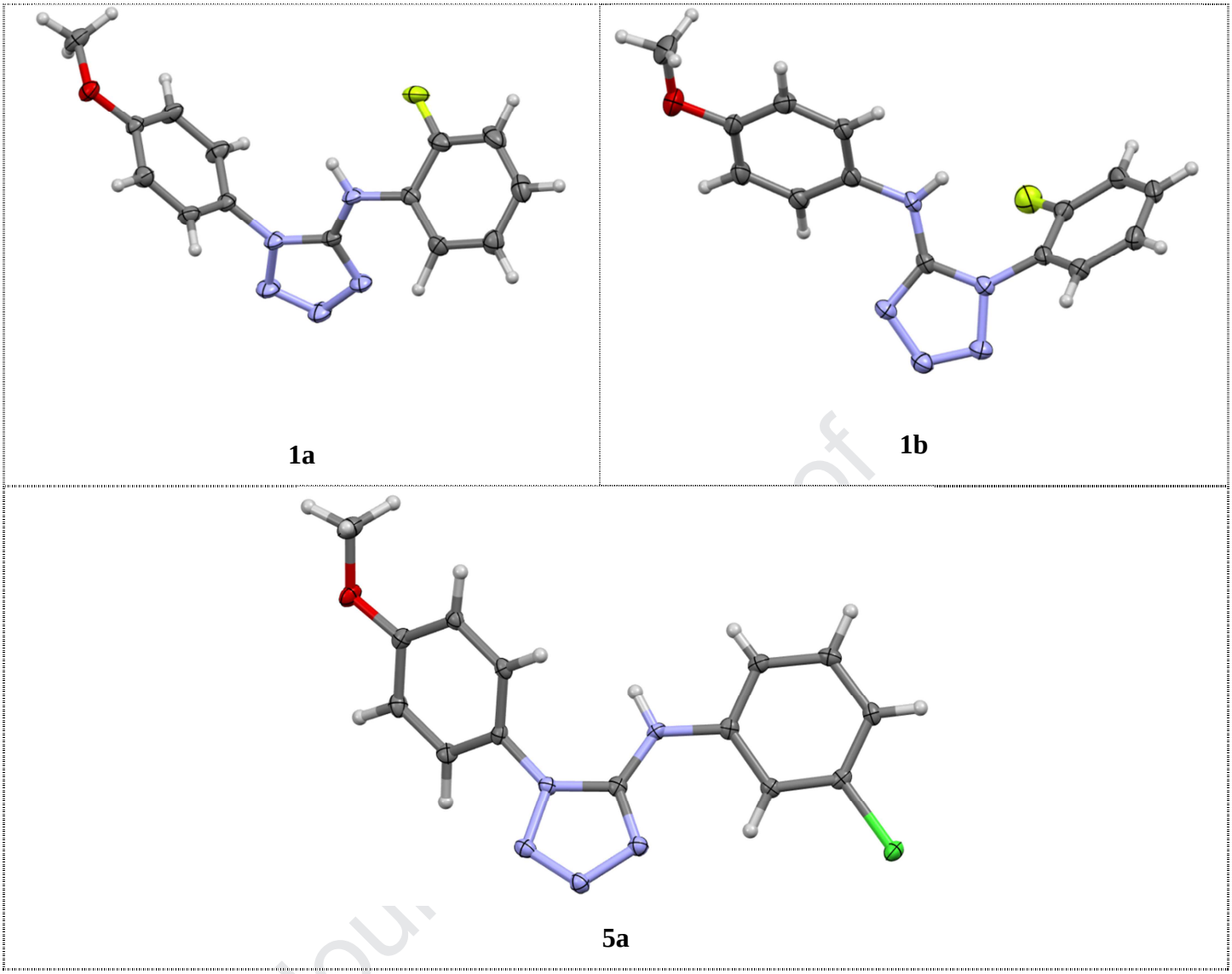

Fig.1. Crystal structure of compounds **1a, 1b** and **5a**. Thermal ellipsoids drawn at 50% probability level (disordered fluorine atom omitted for clarity).

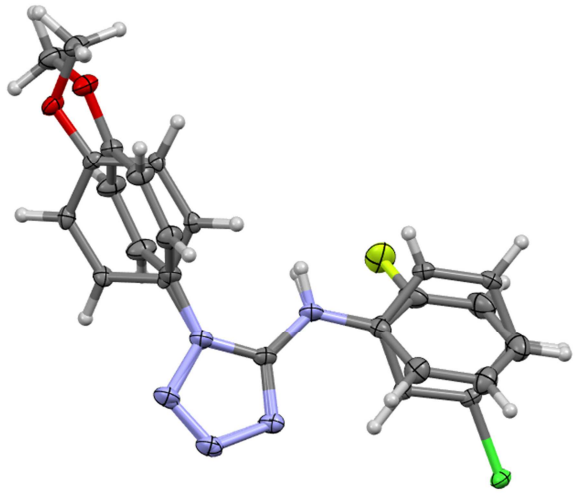

Fig.2. The superposition of molecules **1a** and **5a**.

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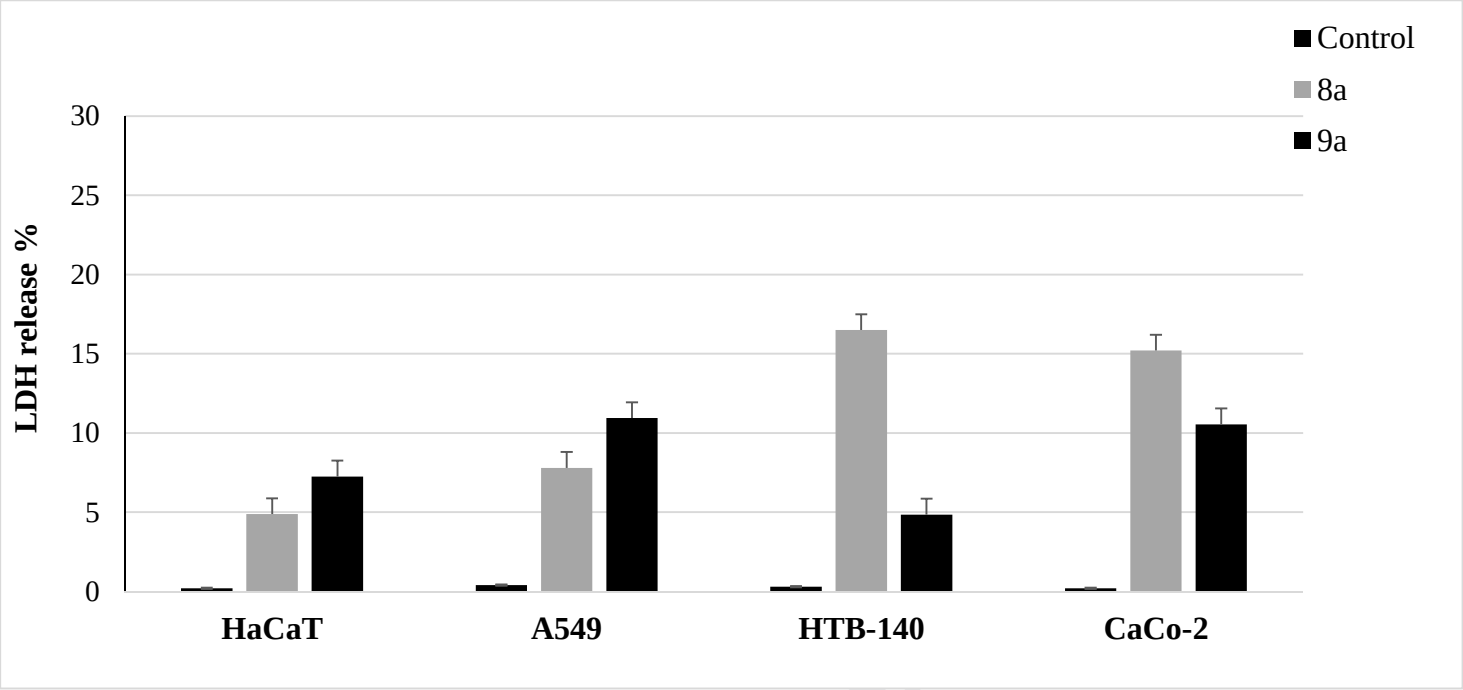

Fig.3. LDH release as a marker of cell death in HTB-140, A549, CaCo-2 and HaCaT cells, treated with compounds **8a** and **9a** at a concentration of 100 μM for 72 h. Data are expressed as the mean ± SD from three independent experiments performed in triplicate.

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Table 1. Crystal data and structure refinement for compounds **1a**, **1b** and **5a**.

Compound	**1a**	**1b**	**5a**
Empirical formula	$C_{14}H_{12}F_1N_5O_1$	$C_{14}H_{12}F_1N_5O_1$	$C_{14}H_{12}Cl_1N_5O_1$
Formula weight	285.29	285.29	301.74
Space group	*P2₁/c*	*P2₁/c*	*P2₁*
Unit cell dimensions			
a [Å]	19.371(4)	19.4612(15)	7.5550(6)
b [Å]	5.9526(11)	7.3902(5)	6.7847(5)
c [Å]	11.742(2)	9.3640(7)	13.0918(10)
α [°]	90.00	90.00	90.00
β [°]	101.905(5)	100.633(3)	102.494(3)
γ [°]	90.00	90.00	90.00
Volume *V* [$Å^3$]	1324.8(4)	1323.63(17)	655.17(9)
Z [molecules/cell]	4	4	2
$D_{calculated}$ [Mg m^{-3}]	1.430	1.432	1.529
Absorption coefficient μ/mm^{-1}	0.106	0.106	0.298
θ range for data collection [°]	3.22-30.02	2.96-25.24	2.76-25.48
Limiting indices	-24 < = *h* = > 27	-23 < = *h* = > 20	-9 < = *h* = > 9
	-8 < = *k* = > 8	-8 < = *k* = > 8	-8 < = *k* = > 8
	-16 < = *l* = > 16	-11 < = *l* = > 11	-15 < = *l* = > 15
Reflections collected/unique	15027/3863	14665/2387	11160/2424
Data/parameters	3863/209	2387/233	2424/238
Goodness of Fit	1.030	1.035	1.081
Final *R* index ($I > 2\sigma$)	0.0430	0.0421	0.0287
wR^2	0.0932	0.0938	0.0751
Largest diff. Peak and hole [$Å^{-3}$]	0.297 and -0.217	0.48 and -0.20	0.40 and -0.22
Flack parameter	-	-	0.013(16)

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Table 2. Activity of 1-(halogenophenyl)-3-(4-methoxyphenyl)thioureas **1-9** against selected *Mycobacterium tuberculosis* strains – minimal inhibitory concentrations (MIC, μg/ml).

Compound	logP*	*M. tuberculosis* $H_{37}R_v$	*M. tuberculosis* Spec 210 (multidrug-resistant)	*M. tuberculosis* Spec. 192 (sensitive to tuberculostatics)
1	3.28	50	100	50
2	3.30	50	100	100
3	3.23	50	100	100
4	3.44	25	50	25
5	3.52	100	100	100
6	3.53	50	50	50
7	3.52	50	50	50
8	3.61	50	50	50
9	3.61	25	>100	25
Isoniazid (INH)		6.25	12.5	6.25
Rifampicin (RMP)		<3.1	50	<3.1
Streptomycin (SM)		<3.1	25	<3.1
Ethambutol (EMB)		<3.1	50	<3.1

*logP values calculated using online software: http://www.swissadme.ch

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Table 3. Activity of synthesized tetrazoles against selected *Mycobacterium tuberculosis* strains – minimal inhibitory concentrations (MIC, μg/ml).

Compound	LogP*	R	*M. tuberculosis* $H_{37}Rv$	*M. tuberculosis* Spec. 192 (sensitive to tuberculostatics)	*M. tuberculosis* Spec 210 (multidrug-resistant)	*M. tuberculosis* Spec. 800 (INH-monoresistance resulting from mutation in InhA promotor part)
1a	2.75	2-F-Ph	16	16	16	ND
2a	2.71	3-F-Ph	8	8	8	256
3a	2.71	4-F-Ph	32	32	32	512
4a	2.97	2-Cl-Ph	8	8	8	256
5a	2.93	3-Cl-Ph	32	32	32	256
6a	2.95	4-Cl-Ph	16	16	16	256
7a	3.05	2-Br-Ph	64	64	64	512
8a	3.02	3-Br-Ph	2	2	2	128
9a	3.04	4-Br-Ph	2	2	2	128
1b	2.69	2-F-Ph	16	16	16	ND
2b	2.86	3-F-Ph	16	16	16	256
3b	2.71	4-F-Ph	8	8	8	128
4b	2.94	2-Cl-Ph	8	8	8	128
5b	2.97	3-Cl-Ph	8	8	8	128
6b	2.96	4-Cl-Ph	8	8	8	128
7b	3.01	2-Br-Ph	16	16	16	256
8b	3.07	3-Br-Ph	16	16	16	256
9b	3.05	4-Br-Ph	16	16	16	256
Isoniazid (INH)			0.125	0.125	16	2
Rifampicin (RMP)			1	1	32	1
Streptomycin (SM)			1	1	16	1
Ethambutol (EMB)			2	2	32	2

ND – not determined
*logP values calculated using online software: http://www.swissadme.ch

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Table 4. Activity of tetrazoles **2a-9a** and **1b-9b** against atypical mycobacteria – minimal inhibitory concentrations (MIC, μg/ml).

	M. avium	*M. intracellulare*	*M. kansasii*	*M. scrofulaceum*	*M. fortuitum*	*M. xenopi*
2a	128	64	512	16	256	512
3a	256	16	512	32	512	>512
4a	128	128	512	32	256	512
5a	256	128	512	512	256	>512
6a	>512	32	>512	>512	>512	512
7a	256	128	512	256	256	512
8a	256	128	512	512	256	>512
9a	512	128	512	512	>512	256
1b	256	128	256	32	256	128
2b	128	64	256	16	256	128
3b	>512	512	512	64	>512	256
4b	512	128	256	32	128	512
5b	64	128	512	2	512	512
6b	256	128	512	16	>512	128
7b	>512	256	256	128	>512	>512
8b	64	>256	512	32	512	512
9b	>512	256	512	256	>512	512
Isoniazid (INH)	32	64	32	64	128	64

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Table 5. Effects of combinations of compounds **8a** or **9a** with rifampicin (RMP), isoniazid (INH), streptomycin (SM) or ethambutol (EMB) against *M. tuberculosis* H_{37}Rv strain.

Compound (1 MIC - 1/32 MIC)/ Drug (1/2 MIC)	MIC Compound/ Drug	Drug (1MIC - 1/32 MIC)/ Compound (1/2 MIC)	MIC Drug/ Compound	FICI Compound/Drug	Effect
8a/ RMP	0.5	RMP/ **8a**	0.25	0.5	additive
8a / INH	0.25	INH/ **8a**	0.5	4.125	antagonistic
8a / SM	0.5	SM/ **8a**	0.25	0.5	additive
8a / EMB	0.5	EMB/ **8a**	1	0.75	additive
9a/ RMP	0.5	RMP/ **9a**	0.25	0.5	additive
9a / INH	0.25	INH/ **9a**	0.5	4.125	antagonistic
9a / SM	0.25	SM/ **9a**	0.25	0.375	synergistic
9a / EMB	0.25	EMB/ **9a**	1	0.625	additive

Table 6. Effects of combinations of compounds **8a** or **9a** with rifampicin (RMP), isoniazid (INH), streptomycin (SM) or ethambutol (EMB) against *M. tuberculosis* 192 strain.

Compound (1 MIC - 1/32 MIC)/ Drug (1/2 MIC)	MIC Compound/ Drug	Drug (1MIC - 1/32 MIC)/ Compound (1/2 MIC)	MIC Drug/ Compound	FICI Compound/Drug	Effect
8a / RMP	0.5	RMP/ **8a**	0.25	0.5	additive
8a / INH	0.25	INH/ **8a**	0.5	4.125	antagonistic
8a / SM	0.5	SM/ **8a**	0.25	0.5	additive
8a / EMB	0.5	EMB/ **8a**	1	0.75	additive
9a / RMP	0.5	RMP/ **9a**	0.25	0.5	additive
9a / INH	0.25	INH/ **9a**	0.5	4.125	antagonistic
9a / SM	0.25	SM/ **9a**	0.25	0.375	synergistic
9a / EMB	0.25	EMB/ **9a**	1	0.625	additive

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Table 7. Effects of combinations of compounds **8a** or **9a** with rifampicin (RMP), isoniazid (INH), streptomycin (SM) or ethambutol (EMB) against multidrug-resistant *M. tuberculosis* 210 strain.

Compound (1 MIC - 1/32 MIC)/ Drug (1/2 MIC)	MIC Compound/ Drug	Drug (1MIC - 1/32 MIC)/ Compound (1/2 MIC)	MIC Drug/ Compound	FICI Compound/Drug	Effect
8a / RMP	0.5	RMP/ **8a**	8	0.5	additive
8a / INH	0.25	INH/ **8a**	16	1.125	indifferent
8a / SM	0.5	SM/ **8a**	16	1.25	indifferent
8a / EMB	2	EMB/ **8a**	16	1.5	indifferent
9a / RMP	1	RMP/ **9a**	8	0.75	additive
9a / INH	0.25	INH/ **9a**	8	0.625	additive
9a / SM	1	SM/ **9a**	8	1	additive
9a / EMB	2	EMB/ **9a**	16	1.5	indifferent

Table 8. Effects of combinations of compounds **8a** or **9a** with rifampicin (RMP), isoniazid (INH), streptomycin (SM) or ethambutol (EMB) against INH-monoresistant *M. tuberculosis* 800 strain.

Compound (1 MIC - 1/32 MIC)/ Drug (1/2 MIC)	MIC Compound/ Drug	Drug (1MIC - 1/32 MIC)/ Compound (1/2 MIC)	MIC Drug/ Compound	FICI Compound/Drug	Effect
8a / RMP	64	RMP/ **8a**	0.25	0.75	additive
8a / INH	16	INH/ **8a**	1	0.625	additive
8a / SM	8	SM/ **8a**	0.5	0.56	additive
8a / EMB	32	EMB/ **8a**	1	0.75	additive
9a / RMP	128	RMP/ **9a**	0.125	1.125	indifferent
9a / INH	32	INH/ **9a**	0.5	0.5	additive
9a / SM	16	SM/ **9a**	0.25	0.375	synergistic
9a / EMB	32	EMB/ **9a**	1	0.75	additive

Table 9. Antibacterial activity of compounds **1-9**, **1a-9a** and **1b-9b**, expressed as MIC (μg/ml).

Comp.	*S. epidermidis ATCC 35984/ RP62A*	*S. epidermidis ATCC 12228*	*S. epidermidis 1457*	*S. aureus ATCC 6538*	*S. aureus NCTC 4163*	*P. aeruginosa NCTC 6749*	*P. aeruginosa PAO 1*	*E. coli ATCC 25922*	*K. pneumoniae ATCC 10031*	*P. vulgaris NCTC 4635*
1-9	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
1a	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
2a	128	32	256	64	128	>256	>256	>256	>256	>256
3a	128	64	128	128	128	128	>256	>256	>256	128
4a	256	128	64	32	32	>256	>256	>256	>256	64
5a	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
6a	128	64	128	128	128	>256	>256	>256	>256	128
7a	256	128	256	256	256	>256	>256	>256	>256	>256
8a	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
9a	>256	256	>256	>256	>256	>256	>256	>256	>256	>256
1b	64	64	64	64	64	256	256	256	256	256
2b	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
3b	128	64	128	64	64	256	256	256	>256	256
4b	64	64	128	64	64	256	256	256	256	256
5b	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
6b	256	256	>256	>256	256	>256	>256	>256	>256	>256
7b	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
8b	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
9b	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Ciprofloxacin	0.0625	0.125	0.125	0.125	0.125	0.125	0.125	<0.0625	<0.0625	<0.0625

Table 10. Cytotoxic activity (IC_{50}, µM) of studied compounds against normal V79 and HaCaT cell lines, estimated by the MTT assay[a]

Compound	**V79**[b]	**HaCaT**[c]
1a	115.12±3.4	111.34±3.2
2a	247.52±3.3	126.63±5.1
3a	236.45±2.8	155.23±4.1
4a	245.61±5.6	134.23±4.9
5a	242.54±4.9	141.23±3.5
6a	245.23±2.3	143.23±5.2
7a	239.43±3.8	137.23±4.3
8a	241.31±5.6	138.45±4.6
9a	212.46±5.8	131.26±3.8
1b	212.36±4.4	122.08±4.7
2b	122.78±4.2	101.21±2.8
3b	145.34±2.5	101.45±2.9
4b	101.57±3.8	100.13±3.7
5b	124.21±5.7	103.45±2.9
6b	114.51±4.1	102.37±1.8
7b	119.27±5.3	104.62±2.2
8b	124.34±3.7	107.29±4.9
9b	125.45±2.4	103.27±2.7
Doxorubicin	2.21±0.08	0.23 ± 0.03

[a]Data are expressed as mean±SD; IC_{50} (µM) - the concentration of the compound that corresponds to a 50% growth inhibition of a cell line (as compared to the control), after culturing the cells for 72 h with the individual compound. [b]Chinese hamster lung fibroblast cell line, [c]Human immortal keratinocyte cell line

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Highlights:

- A series of halogenated 1*H*-tetrazol-5-amine regioisomers were synthesized
- *In vitro* activity studies against typical and atypical mycobacteria were performed
- Synergism of **9a** with SM against three *M. tuberculosis* strains was denoted
- Additive effect of **8a** and **9a** with first-line drugs: EMB, INH and RMP was observed
- Compounds exerted no antibacterial and cytotoxic action.

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