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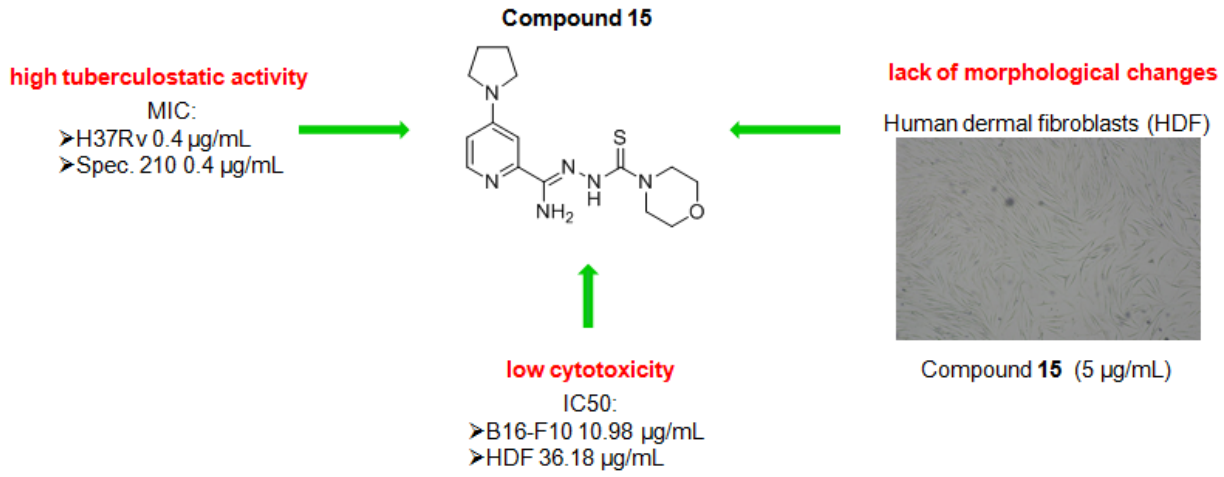

# 4-Substituted picolinohydrazonamides as a new class of potential antitubercular agents

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**Abstract**

The series of new 4-substituted picolinohydrazonamides were synthesized (**6-25**) and evaluated for tuberculostatic activity. Compounds having a hydrophilic cyclic amine such as morpholine and pyrrolidine at the end of the thiosemicarbazide chain, exhibited the highest antimycobacterial activity. The antimycobacterial activity of compounds **6**, **11**, and **15** (MIC 0.4-0.8 µg/mL) was higher than that of reference drugs. Moreover, derivative **15** exhibited lower activity against other tested microorganism such as bacteria gram-positive, gram-negative or fungi. Thus, this compound is characterized by the selectivity of antimicrobial activity. Antiproliferative study conducted against human dermal fibroblasts (HDF) and mouse melanoma cell line (B16-F10) revealed low cytotoxicity of compound **15**. Conducted research allowed to identify compound **15** as leading for further research.

**Keywords:** synthesis; tuberculosis; thiosemicarbazide; cytotoxic activity; microscopic observation

## 1. Introduction

Tuberculosis (TB) is an infectious disease caused by the bacteria *Mycobacterium tuberculosis* complex[1,2]. It seemed that the discovery of streptomycin, isoniazid, and rifampicin and the development of combination therapy will eliminate TB[3–6]. However, the growing resistance of *M. tuberculosis* and the emergence of strains resistant to first- and second-line drugs have increased the incidence of TB, thereby increasing mortality caused by this disease[7–12]. TB is the ninth leading cause of death in the world and leading cause from a single infectious agent[13]. The World Health Organization estimates that 558 000 people developed drug-resistant TB in 2017. Moreover, 1.7 billion people have a latent TB infection and the risk of developing TB during their lifetime[14,15]. Although there is a dramatic increase in bacterial resistance to the antibiotics used, the number of newly approved antibacterial drugs has declined[16–20]. Drug development for TB has stagnated for decades, until 2012, when the FDA approved bedaquiline as a new antitubercular drug[21–23]. The current situation could be called a crisis of antibiotic therapy. It is necessary to urgently obtain novel drugs with a new mechanism of action[24–26].

Thiosemicarbazides, which due to the presence of sulfur and nitrogen atoms can easily form hydrogen bonds with proteins are in the focus of scientists[27]. Compounds of this structure are known for their wide biological activity: antibacterial, antiviral, cytotoxic, anticonvulsant, analgesic[28–30]. Thiosemicarbazides are also known as compounds active against tuberculosis. These include vanadium and manganese complexes, conjugates with 4-nitropyrrole or hybrids with an imidazole and piperazine moiety[31–33].

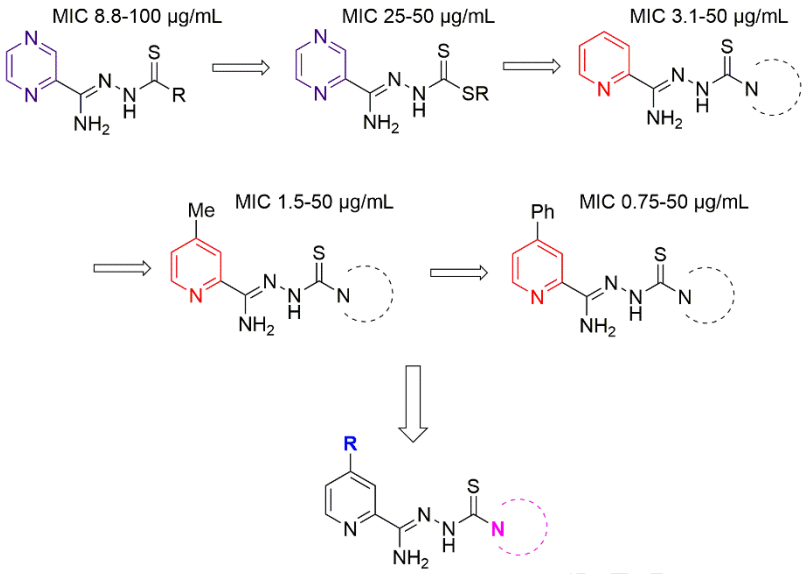



Figure 1. Project concept

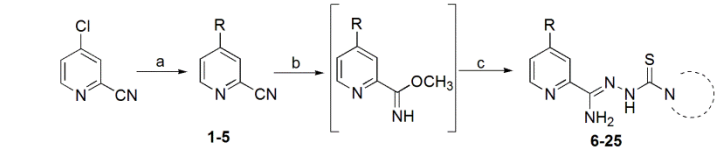
Our team's long-term study of heterocyclic compounds exhibiting antitubercular activity has also established thiosemicarbazides as promising tuberculostatic agents (Figure 1). First, thiosemicarbazides containing a ring of pyrazine, which is also present in pyrazinamide first-line drug, were obtained. The activity of these derivatives was varied with minimum inhibitory concentrations (MICs) in the range 8.8–100 µg/mL. Substituents belonging to the group of secondary cyclic amines (morpholine, hexamethyleneimine or phenylpiperazine) increased tuberculostatic activity while substituents being primary alkylamines (aniline, 4-aminomorpholine or allylamine) caused a decrease in activity. Subsequently, derivatives with the thioester moiety were obtained, but their activity was negligible (MIC 50–100 µg/mL)[34,35]. Those results prompted us to convert pyrazine to pyridine ring, which is found in isoniazid, another first-line chemotherapeutic. The obtained derivatives inhibited the growth of mycobacteria over two times more strongly than their pyrazine analogs[36]. The substitution of the pyridine ring at the C4 position with a methyl or phenyl group resulted in an increase in activity. Some of the obtained compounds were characterized by good antitubercular activity with MICs in the range 0.75–3.1 µg/mL. It is noteworthy that the morpholine substituent is essential for antitubercular activity because the derivatives possessing this moiety exhibited the highest activity. Based on the obtained results, we synthesized cycloalkylaminothiosemicarbazides to check the effect of other substituents on the activity of this group of compounds.

## 2. Results and discussion

### 2.1. Chemistry

Derivatives with methylenehydrazinecarbothioamide moiety at the C2 position and the nucleophilic substituent at the C4 position were synthesized. The desired derivatives possessed cycloalkylamine rings (morpholine, piperidine, pyrrolidine, and phenylpiperazine) in thiosemicarbazide moiety. The synthetic route used in this study is outlined in Scheme 1. The starting material for the synthesis was commercially available 4-chloropicolinonitrile. In the first step, the chlorine atom was substituted in a nucleophilic reaction. The process was carried out in dioxane with the addition of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) at reflux for 1 h. Phenol, thiophenol, morpholine, pyrrolidine, and phenylpiperazine were used as nucleophilic agents. Subsequently, the nitrile group was converted into

the methyl imidate group. The reaction was performed in the presence of methanol and a catalytic amount of DBU. The reaction mixture was refluxed for 4 h and monitored by TLC (thin layer chromatography). To obtain final products, the corresponding methyliminoesters were condensed with various cycloalkylamino-1-carbothiohydrazides by heating for 15–60 minutes at boiling temperature. Carbohydrazides were obtained in the reaction between cyclic amines and methyl hydrazinecarbodithioate in alcohol or water. The synthesis method of cycloalkylamino-1-carbothiohydrazides is described by Klayman et al[37]. From the synthesis process, 20 new final derivatives were obtained. Syntheses of the desired cycloalkylaminethiosemicarbazide derivatives **6–25** were achieved with 36–96% yields. All the obtained compounds were purified by crystallization. All the newly synthesized compounds were characterized by IR, $^{1}$H NMR, and $^{13}$C NMR spectra and elemental analysis. The results of the spectral analysis were in accordance with the assigned structures.




| Compd. | R | N | Compd. | R | N | Compd. | R | N |
|---|---|---|---|---|---|---|---|---|
| 6 |  |  | 14 |  |  | 22 |  |  |
| 7 | |  | 15 |  |  | 23 |  |  |
| 8 | |  | 16 | |  | 24 | |  |
| 9 | |  | 17 | |  | 25 | |  |
| 10 |  |  | 18 |  |  | | | |
| 11 | |  | 19 |  |  | | | |
| 12 | |  | 20 | |  | | | |
| 13 | |  | 21 | |  | | | |

Scheme 1. Synthesis of cycloalkylaminethiosemicarbazide derivatives **6-25**. a) dioxane, DBU, reflux, 1 h; b) methanol, DBU, reflux, 4 h; c) cycloalkylamino-1-carbothiohydrazides (1 eq), reflux, 15–60 min

## 2.2. Biological activity

All the obtained compounds were evaluated for their *in vitro* tuberculostatic activity against *M. tuberculosis* $H_{37}$Rv strain and "wild-type" strain isolated from patients with TB (Spec. 210) resistant to *p*-aminosalicylic acid (PAS), isonicotinic acid hydrazide (INH), ethambutol (ETB), and rifampicin (RMP). MICs were determined as the minimum concentration inhibiting the growth of tested TB strains in relation to the probe with no tested compound. INH and PZA were used as reference drugs.

Extensive microbiological tests were conducted for five compounds with the highest antitubercular activity (**6**, **7, 11, 15,** and **19**). This experiment aimed to verify the selectivity of tested compounds. The study was performed on gram-positive bacteria *Staphylococcus epidermis, Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Bacillus cereus, Streptococcus pyogenes, Streptococcus pneumonia*, and *Streptococcus mutans*; gram-negative bacteria *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa*, and *Proteus mirabilis*; and fungi *Candida albicans* and *Candida parapsilosis*. Ciprofloxacin (CIP), vancomycin (VAN), and fluconazole (FCZ) were used as reference drugs.

The most potent compounds were also tested for cytotoxic activity against human dermal fibroblasts (ATCC PCS-201-010) and mouse melanoma cells (ATCC CRL-6375). The obtained results allowed

to determine whether the tested compounds in a concentration showing antimycobacterial activity also show a toxic effect against the selected cell lines. MAP and bFGF were used as positive controls. All values are means of at least two independent experiments, each performed in duplicate. Microscopic images of cell lines B16-F10 and HDF showing changes caused by the addition of compounds **1** and **10** at a concentration of 5 µg/mL were also obtained. The bioactive data are summarized in Tables 1–4 and Figure 3.

2.2.1. Tuberculostatic activity

The obtained compounds exhibited very good tuberculostatic activity with MICs in the range of 0.4–25 µg/mL (Table 1). All compounds exhibited the same activity against sensitive and resistant strains. The presence of the highest hydrophilic cycloamines such as pyrrolidine and morpholine in the thiosemicarbazide chain showed a positive effect on antitubercular activity. In particular, derivatives having a morpholine ring (**7, 11, 15,** and **19**) showed the highest tuberculostatic activity among the tested compounds with MICs in the range of 0.4–3.1 µg/mL. Their antimycobacterial activity was equal or higher than that of reference drugs. Excellent antitubercular potency (MIC 0.4 µg/mL) was also observed for compound **6,** which had pyrrolidine in the thiosemicarbazide chain and phenoxy group at the C4 position of the pyridine ring. The presence of highly lipophilic phenylpiperazine moiety (**9, 13, 17, 21,** and **25**) significantly decreased the antitubercular potency (MIC 6.25–25 µg/mL). Similarly, the presence of phenylpiperazine at the pyridine ring resulted in a decrease in antitubercular activity. The phenylpiperazine substituent negatively affected tuberculostatic activity probably as a result of high lipophilicity and high molecular weight. The piperidine substituent in the thiosemicarbazide chain did not have a significant effect on the antimycobacterial activity. Presumably, the basicity of the substituent occurring at the C4 position of the pyridine ring influenced the tuberculostatic activity of the tested compounds. Derivatives having a pyrrolidine moiety at the pyridine ring (**14–17**), characterized by the highest basicity, exhibited high antitubercular potency (MIC 0.4–6.25 µg/mL). In contrast, derivatives having a thiophenoxy group (**10–13**) of low basicity showed significantly lower activity (MIC 0.8–25 µg/mL).

Table 1. *In vitro* tuberculostatic activity of compounds **6–25.**

| | Minimal inhibitory concentration (µg/mL) | |
|---|---|---|
| Compound | $H_{37}R_v$ | Spec. 210 |
| **6** | **0.4** | **0.4** |
| **7** | **3.1** | **3.1** |
| 8 | 12.5 | 12.5 |
| 9 | 25 | 25 |
| 10 | 6.25 | 6.25 |
| **11** | **0.8** | **0.8** |
| 12 | 12.5 | 12.5 |
| 13 | 25 | 25 |
| 14 | 6.25 | 6.25 |
| **15** | **0.4** | **0.4** |
| 16 | 6.25 | 6.25 |
| 17 | 6.25 | 6.25 |
| 18 | 6.25 | 6.25 |
| **19** | **3.1** | **3.1** |
| 20 | 6.25 | 6.25 |
| 21 | 6.25 | 6.25 |
| 22 | 12.5 | 12.5 |
| 23 | 6.25 | 6.25 |

| | | |
|---|---|---|
| 24 | 25 | 25 |
| 25 | 25 | 25 |
| INH | 3.1 | 12.5 |
| PZA | 25 | >400 |

2.2.2. Antimicrobial activity

Table 2. Antimicrobial activity against gram-positive bacteria

| | MIC (µg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| Compound | **6** | **7** | **11** | **15** | **19** | **CIP** | **VAN** |
| Microorganism | | | | | | | |
| **Gram-positive bacteria** | | | | | | | |
| *S. aureus* ATCC 25923 | 0.49 | 15.6 | 0.98 | 15.6 | 15.6 | 0.49 | 0.98 |
| *S. aureus* ATCC 6538 | 0.98 | 7.8 | 3.9 | 7.8 | 15.6 | 0.24 | 0.49 |
| *S. aureus* ATCC 43300 | 0.24 | 125 | 15.6 | 31.3 | 15.6 | 0.24 | 0.49 |
| *S. epidermidis* ATCC 12228 | 0.12 | 3.9 | 0.49 | 1.95 | 15.6 | 0.49 | 0.98 |
| *M. luteus* ATCC 10240 | 1.95 | 0.49 | 0.24 | 0.49 | 7.8 | 0.98 | 0.12 |
| *B. subtilis* ATCC 6633 | 0.49 | 7.8 | 1.95 | 0.98 | 31.3 | 0.03 | 0.24 |
| *B. cereus* ATCC 10876 | 0.98 | 31.3 | 7.8 | 1.95 | 31.3 | 0.12 | 0.98 |
| *S. pyogenes* ATCC 19615 | 1.95 | 7.8 | 3.9 | 7.8 | 1000 | - | 0.24 |
| *S. pneumoniae* ATCC 49619 | 7.8 | 31.3 | 31.3 | 250 | >1000 | - | 0.24 |
| *S. mutans* ATCC 25175 | 0.98 | 7.8 | 1.95 | 1.95 | 1000 | - | 0.98 |

Compound **6** showed a higher bacteriostatic activity against *S. aureus* ATCC 43300 (MIC 0.24 µg/mL) and *S. epidermidis* ATCC 12228 (MIC 0.12 µg/mL) than against the tested *M. tuberculosis* strains (MIC 0.4 µg/mL) (Table 2). The potency of *S. epidermidis* ATCC 12228 growth inhibition was higher than for reference drugs ciprofloxacin and vancomycin. The MIC of this compound for all tested bacteria of the genus *Bacillus* and *Staphylococcus* was below 1 µg/mL. Moreover, this compound was characterized by very good activity against *S. mutans* ATCC 25175 with MIC at the same level as that of vancomycin (0.98 µg/mL). Bacteriostatic and bactericidal activity against gram-positive bacteria of compound **7** was at a moderate level. Only for the *M. luteus* strain, MIC was lower (0.49 µg/mL) than for the tested *M. tuberculosis* strains (3.1 µg/mL). Compound **11** showed higher bacteriostatic activity than tuberculostatic activity against *M. luteus* ATCC 10240 and *S. epidermidis* ATCC 12228 strains. The bacteriostatic activity of compounds **15** and **19** was not greater than their tuberculostatic activity for all the tested gram-positive bacteria strains. Compound **15** exhibited good antimicrobial activity against *M. luteus* ATCC 10240 and *B. subtilis* ATCC 6633 strains with MICs below 1 µg/mL. All tested compounds had very weak or no bacteriostatic activity against gram-negative bacteria (Table 3). The antifungal activity of the tested derivatives was much lower than the reference drug fluconazole. The most sensitive strains on the test group of compounds were *S. epidermidis* ATCC 12228 and *M. luteus* ATCC 10240.

Table 3. Antimicrobial activity against gram-negative bacteria and yeasts

| | MIC (µg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| Compound | **6** | **7** | **11** | **15** | **19** | **CIP** | **FCZ** |
| Microorganism | | | | | | | |
| **Gram-negative bacteria** | | | | | | | |
| *E. coli* ATCC 25922 | 125 | 500 | 125 | >1000 | >1000 | 0.004 | - |
| *P. mirabilis* ATCC 12453 | 31.3 | 125 | 62.5 | >1000 | >1000 | 0.03 | - |
| *K. pneumoniae* ATCC 13883 | >1000 | 500 | >1000 | >1000 | 31.3 | 0.12 | - |
| *P. aeruginosa* ATCC 9027 | 250 | >1000 | >1000 | >1000 | >1000 | 0.49 | - |
| **Yeasts** | | | | | | | |
| *C. albicans* ATCC 2091 | 7.8 | 15.6 | 15.6 | 15.6 | 31.3 | - | 0.25 |
| *C. albicans* ATCC 102231 | 7.8 | 15.6 | 15.6 | 15.6 | 31.3 | - | 0.98 |
| *C. parapsilosis* ATCC 22019 | 7.8 | 15.6 | 15.6 | 15.6 | 31.3 | - | 1.95 |

2.2.3. Cytotoxic activity

The IC50 values for the examined derivatives were in the range of 2.56–38.83 µg/mL (Table 4). All tested compounds showed weaker antiproliferative activity on HDF cells than on tumor cells B16-F10. The determined IC50 values of obtained derivatives did not exceed the MIC against *M. tuberculosis*. IC50 for compound **19** for human dermal fibroblasts could not be calculated due to the weak antiproliferative activity of the compound across the tested concentrations. The selectivity index (IC50 HDF/IC50 B16-F10) indicated that compounds **6**, **7**, **11**, and **15** had efficacy against tumor cells greater than toxicity against normal cells. The calculated IC50-HDF/MIC-MT selectivity index for compounds **6, 7**, **11**, and **15** revealed their lack of cytotoxicity. The MIC of *M. tuberculosis* growth for compound **15** with the highest tuberculostatic activity was 90.45 times lower than the concentration causing antiproliferative effect against noncancer cells.

Table 4. Cytotoxic activity of compounds **6, 7, 11, 15,** and **19**

| | IC50 (µg/mL) | | SI IC50-HDF/ IC50-B16-F10 | SI' IC50-HDF/ MIC-MT |
|---|---|---|---|---|
| Cell line | B16-F10 | HDF | | |
| 6 | 2.56 | 26.91 | 10.39 | 67.27 |
| 7 | 9.38 | 38.83 | 4.13 | 12.52 |
| 11 | 11.10 | 32.65 | 2.91 | 40.38 |
| 15 | 10.98 | 36.18 | 3.29 | 90.45 |
| 19 | 17.85 | NA | - | - |

NA – not available in the range of concentrations used, IC50 could not be calculated due to the weak antiproliferative activity of compound across the tested concentrations.
The selectivity index (SI) was calculated for each compound using the formula SI = IC50 for normal cell line (HDF)/IC50 for the respective cancer cell line (B16-F10). A beneficial SI > 1.0 indicates a drug with efficacy against tumor cells greater than toxicity against normal cells. SI was also calculated using the formula SI = IC50 for normal cell line (HDF)/MIC against *M. tuberculosis*. An SI value > 1.0 indicates that the compound is nontoxic.

For two compounds with the highest tuberculostatic activity, cytotoxicity tests were extended by microscopic examination (Figure 2). Observations allowed us to see morphological changes in the

presence of tested compounds at a concentration of 5 μg/mL. In the image showing control, three-dimensional and compact growth of mouse melanoma cells was observed. After the addition of compounds **6** and **15,** drastic morphological changes were observed. Three-dimensional structures disappeared, while only necrotic cells remained. Compound **6** also caused morphological changes in human dermal fibroblasts. In the presence of derivative **15**, such changes, however, did not occur.

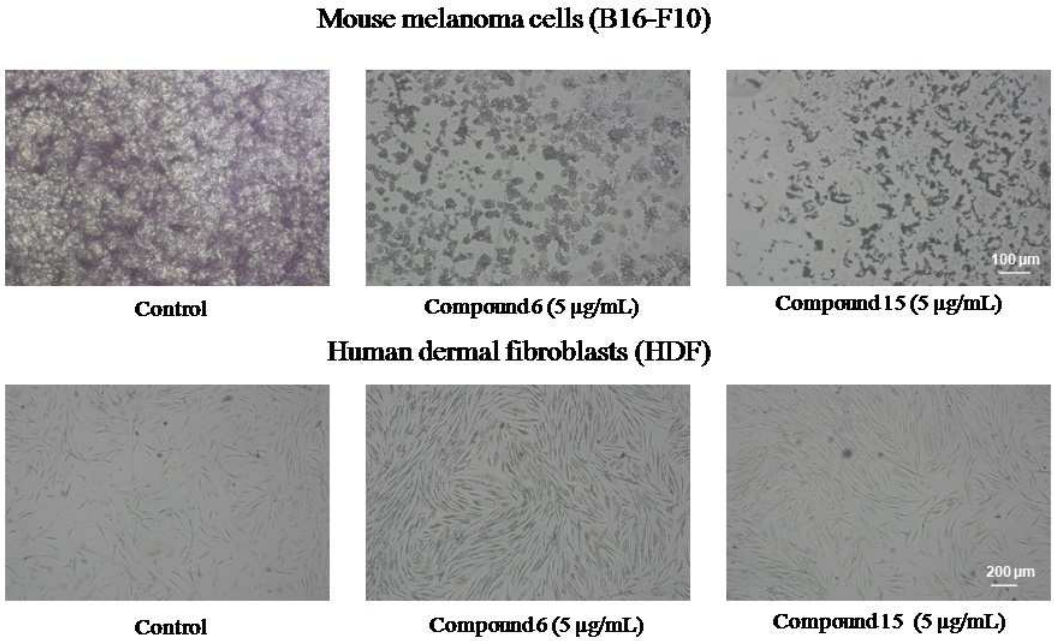



Figure 2. Microscopic pictures of the cell lines HDF and B16-F10.

## 3. Conclusions

In conclusion, a series of novel cycloalkylaminothiosemicarbazide derivatives were successfully synthesized. Their tuberculostatic activity *in vitro* was evaluated against *M. tuberculosis* H37Rv and Spec. 210 strains using the twofold serial dilution MIC method. Compounds **7**, **11**, **15**, and **19** possessing a morpholine ring at the end of the thiosemicarbazide chain exhibited the highest antitubercular activity. The most potent compound **15** exhibited antimycobacterial activity with a MIC 0.4 μg/mL, which is higher than that for reference drugs. Activity at the same level was also observed for compound **6**. Despite the same antimycobacterial potency, compound **15** exhibited greater selectivity than compound **6**. In addition, both compounds did not show a cytotoxic effect on HDF cells at concentrations exhibiting an antitubercular effect. However, extended microscopic studies showed that compound **6** caused morphological changes in fibroblast cells, whereas in the presence of compound **15,** such changes were not observed. From the results obtained, we can conclude that the synthesized derivatives have a promising anti-TB activity, and compound **15** may constitute a leading structure for further research on antitubercular drugs.

## 4. Experimental

4.1. Chemistry

All materials and solvents were of analytical reagent grade. Thin-layer chromatography (TLC) was performed on Merck silica gel $60F_{254}$ plates and visualized with UV light. The results of elemental analyses (%C, H, N) for all the obtained compounds that were determined on Perkin-Elmer PE 2400 Series II CHNS analyzer (Perkin-Elmer, Shelton, CT) were in agreement with the calculated values within ±0.4% range. $^{1}H$ and $^{13}C$ NMR spectra in $CDCl_3$ or DMSO-$d_6$ were recorded on Varian Unity Plus (500 MHz) and Varian Gemini (200 MHz) instruments. IR spectra (KBr) were determined as KBr pellets of the solids on a Satellite FT-IR spectrophotometer. Melting points were determined on a Stuart SMP30 apparatus and retained without any corrections. Confirmation of compounds identification was performed with the use of 6224 LC-TOF/MS system (Agilent Technologies, Waldronn, Germany) equipped with electrospray ionisation source (ESI). 2 µl of sample was injected directly to the Mass spectrometry detector. The mobile phase consisted of 0.1 % formic acid in methanol (Sigma-Aldrich, USA) and 0.1 % formic acid in deionized water (70:30, v/v) from Milli-Q water system (Millpore Inc., Bedford, MA, USA). The analysis was carried out under isocratic condition with a flow rate at 0.3 ml/min at ambient temperature. The study was performed in positive polarity in a scan mode in a 60-1200 m/z mass range. Ion source parameters were as follows: temperature of drying gas (nitrogen), its flow rate and nebulizer pressure were set at 350 ºC, 11 L/min and 45 psi, respectively. The capillary voltage was set at 3500 V. Besides, skimmer and octopole voltages were 65 V and 750 V, respectively. Together with analysed sample reference masses were analysed simultaneously (121.0509 m/z and 922.0098 m/z; Agilent Technologies, Waldbronn, Germany). The reference masses were measured due to control accuracy of the measurement of masses detected in the analysed sample. All compounds were analysed in 3 different fragmentor voltages: 120, 150 and 200 V, in order to monitor compounds fragmentation pattern.

4.1.1. General procedure for the synthesis of nitriles 1–5

In a round-bottom flask, 40 mmol of 4-chloropicolinonitrile, 48 mmol of an appropriate nucleophilic agent, 25 mL of dioxane, and 6 mL of DBU were added. The mixture was heated at reflux for 1 h. Then, the solvent was evaporated, and ice was added. Precipitated products were filtered and recrystallized from cyclohexane or methanol.

*4-phenoxypicolinonitrile (**1**)*

Starting from 4-chloropicolinonitrile (5.6 g) and phenol (4.51 g), the title compound **1** was obtained as beige crystals (7.23 g, 88%): mp 90–92 °C (cyclohexane); IR (KBr): 3086, 3060 (υ $C_{Ar}$-H); 2234 (υ CN); 1647 (υ C=N); 1580, 1489, 1413 (υ C=C); 1279, 1206 (δ C-H) 957, 849, 781, 697 (γ C-H) $cm^{-1}$; $^{1}H$ NMR (500 MHz, DMSO-$d_6$): δ 7,15 (dd, 1H, Pyr, $J_1$=3 Hz, $J_2$=3 Hz); 7,23 (d, 2H, ArH, J=8 Hz); 7,34 (t, 1H, ArH, J=7 Hz); 7,51 (t, 2H, ArH, J=8 Hz); 7,67 (d, 1H, Pyr, J=8 Hz); 8,57 (d, 1H, Pyr, J=6 Hz) ppm; Anal. Calcd for $C_{12}H_8N_2O$ (196.06): C, 73.46; H, 4.11; N, 14.28; Found: C, 73.14; H, 4.07; N, 14.16.

*4-phenylthiopicolinonitrile (**2**)*

Starting from 4-chloropicolinonitrile (5.6 g) and thiophenol (4.91 g), the title compound **2** was obtained as light yellow crystals (8.67 g, 97.5%): mp 51–53 °C (cyclohexane); IR (KBr): 3047 (υ $C_{Ar}$-H); 2234 (υ CN); 1647 (υ C=N); 1570, 1437 (υ C=C); 835, 759, 691 (γ C-H) $cm^{-1}$; $^{1}H$ NMR (500 MHz, DMSO-$d_6$): δ 7,19 (dd, 1H, Pyr, $J_1$=2 Hz, $J_2$=3 Hz); 7,54-7,63 (m, 5H, ArH); 7,68 (d, 1H, Pyr, J=2 Hz); 8,47 (d, 1H, Pyr, J=5 Hz) ppm; Anal. Calcd for $C_{12}H_8N_2S$ (212.04): C, 67.90; H, 3.80; N, 13.20; Found: C, 67.73; H, 3.79; N, 13.02.

*4-pyrrolidinopicolinonitrile (**3**)*

Starting from 4-chloropicolinonitrile (5.6 g) and thiophenol (4 mL), the title compound **3** was obtained as white crystals (6.55 g, 90%): mp 132–133 °C (methanol); IR (KBr): 2979-2856 (υ C-H); 2230 (υ CN); 1602, 1530 (υ C=C); 1290, 1235, 1212, 1116 (δ C-H); 850, 829 (γ C-H) $cm^{-1}$; $^1H$ NMR (500 MHz, DMSO-$d_6$): 1.95-1.97 (m, 4H, $CH_2$); 3.28-3.30 (m, 4H, $CH_2$); 6,65 (d, 1H, Pyr, J=4 Hz); 7.05 (s, 1H, Pyr); 8.15 (d, 1H, Pyr J=5 Hz) ppm; Calcd for $C_{10}H_{11}N_3$ (173.10): C, 69.34; H, 6.40; N, 24.26; Found: C, 68.98; H, 6.38; N, 23.99.

*4-morpholinopicolinonitrile (**4**)*

Starting from 4-chloropicolinonitrile (5.6 g) and morpholine (4.18 mL), the title compound **4** was obtained as white crystals (7.92 g, 89%): mp 206-208 °C (methanol); IR (KBr): 3055 (υ $C_{Ar}$-H); 2975, 2855 (υ C-H); 2234 (υ CN); 1597 (υ C=C); 1258, 1116 (δ C-H); 990 (γ C-H) $cm^{-1}$; $^1H$ NMR (500 MHz, DMSO-$d_6$): δ 3,36 (t, 4H, $CH_2$, J=5 Hz); 3,68 (t, 4H, $CH_2$, J=5 Hz); 7,05 (dd, 1H, Pyr, $J_1$=3 Hz, $J_2$=3 Hz); 7,47 (d, 1H, Pyr, J=7 Hz); 8,24 (d, 1H, Pyr, J=7 Hz) ppm; Anal. Calcd for $C_{10}H_{11}N_3O$ (189.09): C, 63.48; H, 5.86; N, 22.21; Found: C, 63.39; H, 5.80; N, 22.17.

*4-(4-phenylpiperazin-1-yl)picolinonitrile (**5**)*

Starting from 4-chloropicolinonitrile (5.6 g) and 4-phenylpiperazine (6.4 mL), the title compound **5** was obtained as beige crystals (9.80 g, 92%): mp 179-183 °C (methanol); IR (KBr): 3074-2847 (υ C-H), 1647 (υ C=N), 1594, 1504, 1452 (υ C=C), 1390, 1271, 1236 (δ C-H), 837, 762 (ɣ C-H) $cm^{-1}$; $^1H$ NMR (500 MHz, DMSO-$d_6$): δ 3.22-3.24 (m, 4H, $CH_2$); 3.56-3.58 (m, 4H, $CH_2$); 6.79 (t, 1H, ArH, J=7 Hz); 6.96 (d, 2H, ArH, J=8 Hz); 7.10 (d, 1H, Pyr, J=5 Hz); 7.22 (t, 2H, ArH, J=7 Hz); 7.53 (s, 1H, Pyr); 8.24 (d, 1H, Pyr, J=6 Hz) ppm; Anal. Calcd for $C_{16}H_{16}N_4$ (264.14): C, 72.70; H, 6.10; N, 21.20; Found: C, 73.05; H, 5,93; N, 20,83.

4.1.2. General procedure for the synthesis of cycloalkylamine-1-thiosemicarbazides **6–25**

**Method A (6–13, 15, 18, 20, 21, 23)**

To a solution of nitrile (2 mmol) in methanol (15 mL), DBU (2.7 mmol, 0.4 mL) was added, and the mixture was refluxed for 4 h. After this time, 2 mmol of cycloalkylamino-1-carbothiohydrazides was added and heated at reflux for 15–60 min. Then, the mixture was poured onto 40 g of ice and acidified with acetic acid to give a precipitate. The resulting precipitate was filtered, dried, and recrystallized from an appropriate solvent.

**Method B (14, 16, 17, 19, 22, 24, 25)**

To a solution of nitrile (2 mmol) in methanol (15 mL), DBU (2.7 mmol, 0.4 mL) was added, and the mixture was refluxed for 4 h. After this time, 2 mmol of cycloalkylamino-1-carbothiohydrazides was added and heated at reflux for 15–60 min. After cooling, the precipitate was filtered, dried, and recrystallized from an appropriate solvent.

*4-phenoxy-N'-(pyrrolidine-1-carbonothioyl)picolinohydrazonamide (**6**)*

Starting from 4-phenoxypicolinonitrile (0.392 g) and pyrrolidine-1-carbothiohydrazide (0.290 g), the title compound **6** was obtained as yellow crystals (0.370 g, 54%): mp 99–101 ˚C (ethanol); IR (KBr): 3420 (υ N-H); 2965, 2868 (υ C-H); 1581 (δ N-H); 1432 (δ C-H); 1232 (υ C-N) $cm^{-1}$; $^1H$ NMR (500 MHz, DMSO-$d_6$): δ 1,72-1,81 (m, 4H, $CH_2$); 3,53-3,54 (m, 4H, $CH_2$); 6,75 (s, 1H, NH); 7,07 (d, 1H, Pyr; J=3 Hz); 7,19-7,34 (m, 3H, ArH); 7,50 (t, 2H, ArH, J=8 Hz); 7,82 (s, 1H, Pyr); 8,44 (d, 1H, Pyr,

J=5 Hz); 9,34 (s, 1H, NH); 12,57 (s, 1H, NH) ppm; Anal. Calcd for $C_{17}H_{19}N_5OS$: C, 59.80; H, 5.61; N, 20.51; Found: C, 59.73; H, 5.41; N, 20.83; LC-MS *m/z*: 342.1385 $[M+H]^+$.

*N'-(morpholine-4-carbonothioyl)-4-phenoxypicolinohydrazonamide (**7**)*

Starting from 4-phenoxypicolinonitrile (0.392 g) and morpholine-4-carbothiohydrazide (0.322 g), the title compound **7** was obtained as yellow crystals (0.332 g, 62%): mp 159–160 ˚C (methanol-water 1:1); IR (KBr): 3419 (υ N-H), 2922, 2852 (υ C-H), 1671 (υ C=N), 1580 (δ N-H), 1463, 1417 (δ C-H), 1238 (υ C-N), 1112 (υ C-O-C) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 3.56-3.58 (m, 4H, $CH_2$); 3.81-3.85 (m, 4H, $CH_2$); 7.01 (s, 1H, NH); 7.23-7.25 (m, 2H, 1xArH+1xPyr); 7.33 (t, 1H, ArH, J=7 Hz); 7.51 (t, 3H, ArH, J=7Hz); 7.86 (s, 1H, Pyr); 8.60-8.61 (m, 2H, 1xPyr+1xNH); 12.60 (s, 1H, NH) ppm; $^{13}$C NMR (175 MHz, DMSO-$d_6$): δ 47.17 (2C), 66.69 (2C), 110.96, 113.99, 121.11, 121.40, 126.39, 131.06 (2C), 144.76, 146.60, 152.18, 153.78, 166.02; 179.32 ppm; Anal. Calcd for $C_{17}H_{19}N_5O_2S$: C, 57.12; H, 5.36; N, 19.59; Found: C, 57.13; H, 5.02; N, 19.28; LC-MS *m/z*: 358.1334 $[M+H]^+$.

*4-phenoxy-N'-(piperidine-1-carbonothioyl)picolinohydrazonamide (**8**)*

Starting from 4-phenoxypicolinonitrile (0.392 g) and piperidine-1-carbothiohydrazide (0.318 g), the title compound **8** was obtained as yellow crystals (0.326 g, 46%): mp 74-75 ˚C (ethanol); IR (KBr): 3393, 3276, 3144 (υ N-H); 3050 (υ $C_{Ar}$-H); 2971, 2924 (υ C-H); 1671 (υ C=N); 1576 (δ N-H); 1471 (δ C-H); 1233 (υ C-N) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.21-1.57 (m, 6H, $CH_2$); 3.40-3.83 (m, 4H, $CH_2$); 6.81 (s, 1H, NH); 6.99 (d, 1H, Pyr, J=3 Hz); 7.13-7.53 (m, 5H, ArH); 7.84 (s, 1H, Pyr) 8.59 (d, 1H, Pyr, J=5 Hz); 9.21 (s, 1H, NH); 12.79 (s, 1H, NH) ppm; Anal. Calcd for $C_{18}H_{21}N_5OS$: C, 60.82; H, 5.95; N, 19.70; Found: C, 60.76; H, 5.70; N, 19.32; LC-MS *m/z*: 356,1538 $[M+H]^+$.

*4-phenoxy-N'-(4-phenylpiperazine-1-carbonothioyl)picolinohydrazonamide (**9**)*

Starting from 4-phenoxypicolinonitrile (0.392 g) and 4-phenylpiperazine-1-carbothiohydrazide (0.472), the title compound **9** was obtained as yellow crystals (0.836 g, 96%): mp 147-150 °C (ethanol-water 1:1); IR (KBr): 3410 (υ N-H); 3056 (υ $C_{Ar}$-H); 2926 (υ C-H); 1645, 1581 (δ N-H); 1448, 1462 (δ C-H); 1225 (υ C-N) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 3.11-3.13 (m, 4H, $CH_2$); 3.97-3.99 (m, 4H, $CH_2$); 6.78-7.02 (m, 5H, 3xArH+1x$NH_2$); 7.20-7.34 (m, 6H, 5xArH+1xPyr); 7.50-7.51 (m, 2H, ArH); 7.87 (s,1H, Pyr); 8.60 (d, 1H, Pyr, J=4 Hz); 12.65 (s,1H, NH) ppm; $^{13}$C NMR (175 MHz, DMSO-$d_6$): δ 46.30 (2C), 49.01 (2C), 110.95, 113.99, 116.22 (2C), 119.51, 121.11 (2C), 126.38, 129.41 (2C), 131.06 (2C), 144.64, 146.64, 151.68, 152.18, 153.79, 166.02, 179.09 ppm; Anal. Calcd for $C_{23}H_{24}N_6OS$: C, 63.87; H, 5.59; N, 19.43; Found: C, 63.66; H, 5.62; N, 19.44; LC-MS *m/z*: 433.1805 $[M+H]^+$.

*4-(phenylthio)-N'-(pyrrolidine-1-carbonothioyl)picolinohydrazonamide (**10**)*

Starting from 4-phenylthiopicolinonitrile (0.424 g) and pyrrolidine-1-carbothiohydrazide (0.290 g), the title compound **10** was obtained as yellow crystals (0.285 g, 40%): mp 160-162 °C (ethanol); IR (KBr): 3417, 3228, 3130 (υ N-H); 3018(υ $C_{Ar}$-H); 2961, 2863 (υ C-H); 1665 (υ C=N); 1570 (δ N-H); 1422 (δ C-H); 1277 (υ C-N) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.75-1.77 (m, 4H, $CH_2$); 3.42-3.54 (m, 4H, $CH_2$); 6.73 (s, 2H, $NH_2$); 7.42 (s, 1H, Pyr); 7.55-7.61 (m, 5H, ArH); 8.34 (d, 1H, Pyr, J=5Hz); 9.24 (s, 1H, Pyr); 12.52 (s, 1H, NH) ppm; $^{13}$C NMR (175 MHz, DMSO-$d_6$): δ 115.83, 121.49, 128.54, 130.64 (2C), 130.76 (3C), 130.89, 135.27, 135.76 (2C), 148.31 (2C), 150.61, 151.40, 177.81 ppm; Anal. Calcd for $C_{17}H_{19}N_5S_2$: C, 57.11; H, 5.36; N, 19.59; Found: C, 57.17; H, 5.12; N, 19.29; LC-MS *m/z*: 358.1155 $[M+H]^+$.

*N'-(morpholine-4-carbonothioyl)-4-(phenylthio)picolinohydrazonamide (**11**)*

Starting from 4-phenylthiopicolinonitrile (0.424 g) and morpholine-4-carbothiohydrazide (0.322 g), the title compound **11** was obtained as yellow crystals (0.311 g, 41%): mp 74-76 °C (ethanol); IR (KBr): 3422 (υ N-H); 3052 (υ $C_{Ar}$-H); 2923, 2852 (υ C-H); 1575 (δ N-H); 1439, 1416 (δ C-H); 1112 (υ C-O-C) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 3.55-3.80 (m, 8H, $CH_2$); 6.95 (s, 1H, Pyr); 7.55-7.63 (m, 6H, 5xArH+1xNH); 8.04 (s, 1H, Pyr); 8.49 (d, 1H, Pyr, J=5Hz); 8.60 (s, 1H, NH); 12.56 (s, 1H, NH) ppm; Anal. Calcd for $C_{17}H_{19}N_5OS_2$: C, 54.67; H, 5.13; N, 18.75; Found: C, 54.72; H, 5.03; N, 18.75; LC-MS *m/z*: 374.1104 $[M+H]^+$.

*4-(phenylthio)-N'-(piperidine-1-carbonothioyl)picolinohydrazonamide (**12**)*

Starting from 4-phenylthiopicolinonitrile (0.424 g) and piperidine-1-carbothiohydrazide (0.318 g), the title compound **12** was obtained as yellow crystals (0.500 g, 67%): mp 150-152 °C (ethanol); IR (KBr): 3412, 3269, 3134 (υ N-H); 3031 (υ $C_{Ar}$-H); 2920, 2848 (υ C-H); 1668 (υ C=N); 1572 (δ N-H); 1473 (δ C-H); 1245 (υ C-N) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.36-1.56 (m, 6H, $CH_2$); 3.59-3.82 (m, 4H, $CH_2$); 6.77 (s, 1H, NH); 6.93 (d, 1H, Pyr, J=4 Hz); 7.51-7.62 (m, 5H, ArH); 8.02 (s, 1H, Pyr); 8.48 (d, 1H, Pyr, J=5 Hz); 9.17 (s, 1H, NH); 12.67 (s, 1H, NH) ppm; $^{13}$C NMR (175 MHz, DMSO-$d_6$): δ 25.28, 26.15 (2C), 47.36 (2C), 118.67, 122.36, 128.41, 130.73, 130.89 (2C), 135.30, 135.59, 143.71, 144.74, 150.30, 152.31, 178.81 ppm; Anal. Calcd for $C_{18}H_{21}N_5S_2$: C, 58.19; H, 5.70; N, 18.85; Found: C, 57.87; H, 5.58; N, 18.55; LC-MS *m/z*: 372.1311 $[M+H]^+$.

*N'-(4-phenylpiperazine-1-carbonothioyl)-4-(phenylthio)picolinohydrazonamide (**13**)*

Starting from 4-phenylthiopicolinonitrile (0.424 g) and 4-phenylpiperazine-1-carbothiohydrazide (0.472), the title compound **13** was obtained as yellow crystals (0.860 g, 95%): mp 164-166 °C (methanol); IR (KBr): 3413, 3232 (υ N-H); 3034 (υ $C_{Ar}$-H); 2926 (υ C-H); 1598, 1572 (δ N-H); 1225 (υ C-N) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 3.10 (bs, 4H, $CH_2$); 3.98 (bs, 4H, $CH_2$); 6.78-6.81 (m, 1H, ArH); 6.95-6.97 (m, 3H, 2xArH+1xPyr); 7.21 (t, 2H, ArH, J=8 Hz); 7.56-7.64 (m, 5H, ArH); 8.06 (s, 1H, Pyr); 8.38 (s, 1H, NH); 8.51 (d, 1H, Pyr, J=5 Hz); 9.50 (s, 1H, NH); 12.56 (s, 1H, NH) ppm; Anal. Calcd for $C_{23}H_{24}N_6S_2$: C, 61.58; H, 5.39; N, 18.73; Found: C, 61.24; H, 5.33; N, 18.54; LC-MS *m/z*: 449.1576 $[M+H]^+$.

*4-(pyrrolidin-1-yl)-N'-(pyrrolidine-1-carbonothioyl)picolinohydrazonamide (**14**)*

Starting from 4-pyrrolidinopicolinonitrile (0.346 g) and pyrrolidine-1-carbothiohydrazide (0.290 g), the title compound **14** was obtained as yellow crystals (0.230 g, 36%): mp 229-230 °C (ethanol); IR (KBr): 3446, 3287, 3114 (υ N-H); 3021 (υ $C_{Ar}$-H); 2966, 2866 (υ C-H); 1669 (υ C=N); 1608 (δ N-H); 1427 (δ C-H); 1287 (υ C-N) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.81-1.98 (m, 8H, $CH_2$); 3.32-3.54 (m, 8H, $CH_2$); 6.55-6.57 (m, 1H, Pyr); 7.08 (s, 1H, NH); 7.23 (s, 1H, Pyr); 8.17 (d, 1H, Pyr, J=6 Hz); 8.40 (s, 1H, NH); 12.41 (s, 1H, NH) ppm; Anal. Calcd for $C_{15}H_{22}N_6S$: C, 56.58; H, 6.96; N, 26.39; Found: C, 56.68; H, 6.73; N, 26.14; LC-MS *m/z*: 319.1703 $[M+H]^+$.

*N'-(morpholine-4-carbonothioyl)-4-(pyrrolidin-1-yl)picolinohydrazonamide (**15**)*

Starting from 4-pyrrolidinopicolinonitrile (0.346 g) and morpholine-4-carbothiohydrazide (0.322 g), the title compound **15** was obtained as yellow crystals (0.430 g, 64%): mp 208-210 °C (methanol); IR (KBr): 3399, 3157 (υ N-H); 2923, 2852 (υ C-H); 1672 (υ C=N); 1599 (δ N-H); 1459, 1418 (δ C-H); 1256 (υ C-N); 1111 (υ C-O-C) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.95 (t, 4H, $CH_2$, J=6 Hz); 3.32-3.33 (m, 4H, $CH_2$); 3.56 (t, 4H, $CH_2$, J=6 Hz); 3.78 (t, 4H, $CH_2$, J=5 Hz); 6.57-6.58 (m, 1H, Pyr); 7.25 (d, 1H, Pyr, J=2 Hz); 7.54 (s, 1H, NH); 8.18 (d, 1H, Pyr, J=6 Hz); 8.56 (s, 1H, NH); 12.44 (s, 1H,

NH) ppm; $^{13}$C NMR (175 MHz, DMSO-$d_6$): δ 25.27 (2C), 47.18 (2C), 47.59 (2C), 66.72 (2C), 105.33, 108.84, 144.20, 146.27, 149.47, 152.63, 178,85 ppm; Anal. Calcd for $C_{15}H_{22}N_6OS$: C, 53.87; H, 6.63; N, 25.13; Found: C, 54.10; H, 6.56; N, 24.78; LC-MS *m/z*: 335.1650 $[M+H]^+$.

*N'-(piperidine-1-carbonothioyl)-4-(pyrrolidin-1-yl)picolinohydrazonamide (**16**)*

Starting from 4-pyrrolidinopicolinonitrile (0.346 g) and piperidine-1-carbothiohydrazide (0.318 g), the title compound **16** was obtained as yellow crystals (0.309 g, 47%): mp 198-200 °C (ethanol); IR (KBr): 3377, 3259, 3135 (υ N-H); 3025 (υ $C_{Ar}$-H); 2926, 2850 (υ C-H); 1611, 1585 (δ N-H); 1421 (δ C-H); 1244 (υ C-N) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.44-1.57 (m, 6H, $CH_2$); 1.91-1.98 (m, 4H, $CH_2$); 3.29-3.32 (m, 4H, $CH_2$); 3.80-3.88 (m, 4H, $CH_2$); 6.54-6.56 (m, 1H, Pyr); 7.23 (s, 1H, Pyr); 7.38 (s, 1H, NH); 8.17 (d, 1H, Pyr, J=6 Hz); 8.35 (s, 1H, NH); 12.56 (s, 1H, NH) ppm; $^{13}$C NMR (175 MHz, DMSO-$d_6$): δ 25.27 (4C), 25.33, 26.13 (2C), 47.33, 47.58, 105.17, 108.65, 144.42, 145.28, 149.46, 152.63, 178.36 ppm; Anal. Calcd for $C_{16}H_{24}N_6S$: C, 57.80; H, 7.28; N, 25.28; Found: C, 57.45; H, 7.29; N, 24.96; LC-MS *m/z*: 333.1856 $[M+H]^+$.

*N'-(4-phenylpiperazine-1-carbonothioyl)-4-(pyrrolidin-1-yl)picolinohydrazonamide (**17**)*

Starting from 4-pyrrolidinopicolinonitrile (0.346 g) and 4-phenylpiperazine-1-carbothiohydrazide (0.472), the title compound **17** was obtained as yellow crystals (0.590 g, 72%): mp 200-202 °C (ethanol); IR (KBr): 3419, 3296 (υ N-H); 3081 (υ C-H); 2960, 2854 (υ C-H); 1646 (υ C=N); 1602 (δ N-H); 1420 (δ C-H); 1222 (υ C-N) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.97-1.98 (m, 4H, $CH_2$); 2.48-2.49 (m, 4H, $CH_2$); 3.33-3.35 (m, 4H, $CH_2$); 3.97-3.99 (m, 4H, $CH_2$); 6.57 (d, 1H, Pyr, J=6 Hz); 6.78 (t, 1H, ArH, J=7 Hz); 6.96-6.97 (m, 2H, ArH); 7.21 (t, 2H, ArH, J=7 Hz); 7.27 (s, 1H, Pyr); 7.60 (s, 1H, NH); 8.19 (d, 1H, Pyr, J=6 Hz); 8.58 (s, 1H, NH); 12.47 (s, 1H, NH) ppm; $^{13}$C NMR (175 MHz, DMSO-$d_6$): δ 46.10 (2C), 46.29 (2C), 49.01 (2C), 66.06 (2C), 106.06, 109.85, 116.22 (2C), 119.50, 129.41 (2C), 144.87, 145.80, 150.28, 151.70, 156.17, 178.73 ppm; Anal. Calcd for $C_{21}H_{27}N_7S$: C, 61.59; H, 6.64; N, 23.94; Found: C, 61.30; H, 6.70; N, 23.66; LC-MS *m/z*: 410.2120 $[M+H]^+$.

*4-morpholino-N'-(pyrrolidine-1-carbonothioyl)picolinohydrazonamide (**18**)*

Starting from 4-morpholinopicolinonitrile (0.378 g) and pyrrolidine-1-carbothiohydrazide (0.290 g), the title compound **18** was obtained as yellow crystals (0.382 g, 57%): mp 228-230 °C (methanol); IR (KBr): 3390 (υ N-H); 3059 (υ $C_{Ar}$-H); 2964, 2858 (υ C-H); 1663 (υ C=N); 1602, 1579 (δ N-H); 1426 (δ C-H); 1234 (υ C-N); 1123 (υ C-O-C) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.81 (t, 4H, $CH_2$, J=6 Hz); 3.39-3.40 (m, 4H, $CH_2$); 3.55-3.57 (m, 4H, $CH_2$); 3.72 (t, 4H, $CH_2$, J=5 Hz); 6.94 (t, 1H, Pyr, J=4 Hz); 7.20 (s, 1H, NH); 7.55 (s, 1H, Pyr); 8.27 (d, 1H, Pyr, J=5 Hz); 8.40 (s, 1H, NH); 12.45 (s, 1H, NH) ppm; Anal. Calcd for $C_{15}H_{22}N_6SO$: C, 53.87; H, 6.63; N, 25.13; Found: C, 54.02; H, 6.52; N, 24.99; LC-MS *m/z*: 335.1651 $[M+H]^+$.

*N'-(morpholine-4-carbonothioyl)-4-morpholinopicolinohydrazonamide (**19**)*

Starting from 4-morpholinopicolinonitrile (0.378 g) and morpholine-4-carbothiohydrazide (0.322 g), the title compound **19** was obtained as yellow crystals (0.365 g, 52%): mp 223-224 °C (methanol); IR (KBr): 3397, 3234 (υ N-H); 3088 (υ $C_{Ar}$-H); 2960-2853 (υ C-H); 1662 (υ C=N); 1605 (δ N-H); 1412 (δ C-H); 1244 (υ C-N); 1124 (υ C-O-C) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 3.40 (t, 4H, $CH_2$, J=5 Hz); 3.56 (t, 4H, $CH_2$, J=4Hz); 3.73 (t, 4H, $CH_2$, J=4 Hz); 3.79 (t, 4H, $CH_2$, J=4 Hz); 6.98 (s, 1H, Pyr); 7.58-7,60 (m, 2H, 1xPyr+1xNH); 8.29 (d, 1H, Pyr, J=6 Hz); 8.54 (s, 1H, NH); 12.48 (s, 1H, NH) ppm; $^{13}$C NMR (175 MHz, DMSO-$d_6$): δ 46.09 (2C), 47.17 (2C), 66.05 (2C), 66.71 (2C), 106.06, 109.87,

144.82, 145.92, 150.28, 156.17, 178.97 ppm; Anal. Calcd for $C_{15}H_{22}N_6O_2S$: C, 51.41; H, 6.33; N, 23.98; Found: C, 51.46; H, 6.14; N, 23.90; LC-MS *m/z*: 351.1597 $[M+H]^+$.

*4-morpholino-N'-(piperidine-1-carbonothioyl)picolinohydrazonamide (**20**)*

Starting from 4-morpholinopicolinonitrile (0.378 g) and piperidine-1-carbothiohydrazide (0.318 g), the title compound **20** was obtained as yellow crystals (0.424 g, 61%): mp 208-210 °C (ethanol); IR (KBr): 3399 (υ N-H); 3069 (υ $C_{Ar}$-H); 2922, 2852 (υ C-H); 1666 (υ C=N); 1605 (δ N-H); 1427 (δ C-H); 1242 (υ C-N); 1111 (υ C-O-C) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.44-1.57 (m, 6H, $CH_2$); 3.31-3.40 (m, 4H, $CH_2$); 3.73-3.82 (m, 8H, $CH_2$); 6.95 (d, 1H, Pyr, J=4 Hz); 7.40 (s, 1H, NH); 7.56 (s, 1H, Pyr); 8.28 (d,1H, Pyr, J=6 Hz); 8.40 (s, 1H, NH); 12.60 (s, 1H, NH) ppm; Anal. Calcd for $C_{16}H_{24}N_6OS$: C, 55.15; H, 6.94; N, 24.12; Found: C, 55.34; H, 6.67; N, 23.78; LC-MS *m/z*: 349.1805 $[M+H]^+$.

*4-morpholino-N'-(4-phenylpiperazine-1-carbonothioyl)picolinohydrazonamide (**21**)*

Starting from 4-morpholinopicolinonitrile (0.378 g) and 4-phenylpiperazine-1-carbothiohydrazide (0.472), the title compound **21** was obtained as yellow crystals (0.600 g, 71%): mp 143-145 °C (methanol); IR (KBr): 3424, 3299, 3161 (υ N-H); 3051 (υ $C_{Ar}$-H); 2957, 2846 (υ C-H); 1676 (υ C=N); 1595 (δ N-H); 1426 (δ C-H); 1225 (υ C-N); 1113 (υ C-O-C**)** $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 3.11 (t, 4H, $CH_2$, J=4 Hz); 3.41 (t, 4H, $CH_2$, J=4 Hz); 3.73 (t, 4H, $CH_2$, J=4 Hz); 3.97 (t, 4H, $CH_2$, J=4 Hz); 6.78 (t, 1H, ArH, J=7 Hz); 6.96-6.97 (m, 3H, 2xArH+1xPyr); 7.22 (t, 2H, ArH, J=7 Hz); 7.59-7.61 (m, 2H, 1xPyr+1xNH); 8.30 (d, 1H, Pyr, J=6 Hz); 8.54 (s, 1H, NH); 12.51 (s, 1H, NH) ppm; Anal. Calcd for $C_{21}H_{27}N_7OS$: C, 59.27; H, 6.40; N, 23.04; Found: C, 59.18; H, 6.30; N, 23.06; LC-MS *m/z*: 426.2068 $[M+H]^+$.

*4-(4-phenylpiperazin-1-yl)-N'-(pyrrolidine-1-carbonothioyl)picolinohydrazonamide (**22**)*

Starting from 4-(4-phenylpiperazin-1-yl)picolinonitrile (0.528 g) and pyrrolidine-1-carbothiohydrazide (0.290 g), the title compound **22** was obtained as yellow crystals (0.602 g, 74%): mp 208-212 °C (ethanol); IR (KBr): 3437, 3310 (υ N-H), 2855 ( υ C-H), 1670 (υ C=N), 1646 (δ N-H), 1599 (υ C=C), 1580 (δ N-H), 1421, 1361 (υ C=C), 1231, 988 (δ C-H), 757, 692 (ɣ C-H) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.81-1.82 (m, 4H, $CH_2$); 3.27 (t, 4H, $CH_2$, J=4 Hz); 3.54-3.55 (m, 4H, $CH_2$); 3.59-3.60 (m, 4H, $CH_2$); 6.80 (t, 1H, ArH, J=7.3 Hz); 7.00 (d, 3H, 2ArH+Pyr, J=7 Hz); 7.18 (s, 1H, NH); 7.23 (t, 2H, ArH, J=7 Hz); 7.62 (s, 1H, Pyr); 8.28 (d, 1H, Pyr, J=5 Hz); 8.46 (s, 1H, NH); 12.47 (s, 1H, NH) ppm; Anal. Calcd for $C_{21}H_{27}N_7S$: C, 61.59; H, 6.64; N, 23.94; Found: C, 61.35; H, 6.68; N, 23.85; LC-MS *m/z*: 410.2123 $[M+H]^+$.

*N'-(morpholine-4-carbonothioyl)-4-(4-phenylpiperazin-1-yl)picolinohydrazonamide (**23**)*

Starting from 4-(4-phenylpiperazin-1-yl)picolinonitrile (0.528 g) and morpholine-4-carbothiohydrazide (0.322 g), the title compound **23** was obtained as yellow crystals (0.380 g, 45%): mp 150-152 °C (ethanol); IR (KBr): 3401 (υ N-H), 3088, 2922, 2850 (υ C-H), 1666 υ C=N), 1594 (υ C=C), 1540 (δ N-H), 1414, 1347 (υ C=C), 1230, 1112, 988 (δ C-H), 890, 760 (ɣ C-H) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 3.28 (t, 4H, $CH_2$, J=4 Hz); 3.56 (t, 4H, $CH_2$, J=4 Hz); 3.60 (t, 4H, $CH_2$, J=4 Hz); 3.79 (t, 4H, $CH_2$, J=4 Hz); 6.81 (t, 1H, ArH, J=7 Hz); 7.00 (d, 3H, 2ArH+1xPyr, J=8 Hz); 7.24 (t, 2H, ArH, J=7 Hz); 7.60 (s, 1H, NH); 7.64 (s, 1H, Pyr); 8.30 (d, 1H, Pyr, J=6 Hz); 8.60 (s, 1H, NH); 12.50 (s, 1H, NH) ppm; Anal. Calcd for $C_{21}H_{27}N_7OS$: C, 59.27; H, 6.40; N, 23.04; Found: C, 58.97; H, 6.40; N, 22.76; LC-MS *m/z*: 426.2070 $[M+H]^+$.

*4-(4-phenylpiperazin-1-yl)-N'-(piperidine-1-carbonothioyl)picolinohydrazonamide (**24**)*

Starting from 4-(4-phenylpiperazin-1-yl)picolinonitrile (0.528 g) and piperidine-1-carbothiohydrazide (0.318 g), the title compound **24** was obtained as yellow crystals (0.370 g, 44%): mp 168-170 °C (ethanol); IR (KBr): 3402, 3241 (υ N-H), 3058, 2928, 2848 (υ C-H), 1665 (υ C=N), 1958, 1541 (δ N-H), 1479, 1417 (υ C=C), 1339, 1231, 987 (δ C-H), 755 (ɣ C-H) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 1.43-1.44 (m, 4H, $CH_2$); 1.56-1.57 (m, 2H, $CH_2$); 3.26-3.27 (m, 4H, $CH_2$); 3.60-3.61 (m, 4H, $CH_2$); 3.82-3.83 (m, 4H, $CH_2$); 6.81 (t, 1H, ArH, J=7.3); 7.00 (d, 3H, 2ArH+Pyr, J=8 Hz); 7.23 (t, 2H, ArH, J=8 Hz); 7.42 (s, 1H, NH); 7.63 (s, 1H, Pyr); 8.29 (d, 1H, Pyr, J=5 Hz); 8.47 (s, 1H, NH); 12.61 (s, 1H, NH) ppm; Anal. Calcd for $C_{22}H_{29}N_7S$: C, 62.38; H, 6.90; N, 23.15; Found: C, 62.43; H, 6.73; N, 23.13; LC-MS *m/z*: 424.2277 $[M+H]^+$.

*4-(4-phenylpiperazin-1-yl)-N'-(4-phenylpiperazine-1-carbonothioyl)picolino hydrazonamide (**25**)*

Starting from 4-(4-phenylpiperazin-1-yl)picolinonitrile (0.528 g) and 4-phenylpiperazine-1-carbothiohydrazide (0.472), the title compound **25** was obtained as yellow crystals (0.370 g, 44%): mp 177-181 °C ethanol); IR (KBr): 3429, 3228 (υ N-H), 3139-2829 (υ C-H), 1674 (υ C=N), 1599, 1558 (δ N-H), 1497, 1425, 1350 (υ C=C), 1230, 988 (δ C-H), 753, 693 (ɣ C-H) $cm^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$): δ 3.11 (t, 4H, $CH_2$, J=4 Hz); 3.27 (t, 4H, $CH_2$, J=4 Hz); 3.61 (t, 4H, $CH_2$, J=4 Hz); 3.99 (t, 4H, $CH_2$, J=4 Hz); 6.77-6.83 (m, 2H, ArH); 6.96-7.03 (m, 5H, 4xArH+1xPyr); 7.20-7.26 (m, 4H, ArH); 7.42 (s, 1H, NH); 7.66 (s, 1H, Pyr); 8.31 (d, 1H, Pyr, J=6 Hz); 8.61 (s, 1H, NH); 12.53 (s, 1H, NH) ppm; Anal. Calcd for $C_{27}H_{32}N_8S$: C, 64.77; H, 6.44; N, 22.38; Found: C, 64.47; H, 6.46; N, 22.02; LC-MS *m/z*: 501.2545 $[M+H]^+$.

4.2. Biological assays

4.2.1. Antimycobacterial activity assay

The synthesized compounds were examined *in vitro* for their tuberculostatic activity against the *M. tuberculosis* $H_{37}$Rv strain and "wild-type" strain isolated from patients with TB: Spec. 210 resistant to *p*-aminosalicylic acid (PAS), isonicotinic acid hydrazide (INH), ethambutol (ETB), and rifampicin (RMP). Investigations were performed by a classical test tube method of successive dilution in Youmans' modification of the Proskauer and Beck liquid medium containing 10% of bovine serum[38,39]. Bacterial suspensions were prepared from 14-day-old cultures of slowly growing strains and from 48-h-old cultures of saprophytic strains[40,41]. Solutions of compounds in ethylene glycol were tested. Stock solutions contained 10 mg of compounds in 1 mL. Dilutions (in geometric progression) were prepared in Youmans' medium. The medium containing no investigated substances and containing isoniazid (INH), pyrazinamide (PZA), or rifampicin (RMP) as reference drugs was used for comparison. Incubation was performed at a temperature of 37 °C. MICs were determined as the minimum concentration that inhibits the growth of tested TB strains in relation to the probe with no tested compound. The influence of the compound on the growth of bacteria at concentrations 0.75, 1.5, 3.1, 6.2, 12.5, 25, 50, and 100 µg/mL was evaluated.

4.2.2. Antimicrobial assay

The compounds were screened for antibacterial and antifungal activities by the microdilution broth method using Mueller-Hinton broth and Mueller-Hinton broth with 5% lysed sheep blood for the growth of nonfastidious and fastidious bacteria, respectively, or Mueller-Hinton broth with 2% glucose for the growth of fungi. MICs of the tested derivatives were evaluated for the panel of reference microorganisms from American Type Culture Collection (ATCC), including gram-negative bacteria (*E. coli* ATCC 25922, *P. mirabilis* ATCC 12453, *K. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC 9027), gram-positive bacteria (*S. aureus* ATCC 25923, *S. aureus* ATCC 43300, *S.*

*aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *M. luteus* ATCC 10240, *B. subtilis* ATCC 6633, *B. cereus* ATCC 10876, *S. pyogenes* ATCC 19615, *S. pneumoniae* ATCC 49619, *S. mutans* ATCC 25175), and fungi (*C. albicans* ATCC 10231, *C. albicans* ATCC 2091, *C. parapsilosis* ATCC 22019).

The compounds dissolved in dimethyl sulfoxide (DMSO) were first diluted to a concentration of 1000 µg/mL in an appropriate broth medium recommended for bacteria or yeasts. Then, using the same media, serial twofold dilutions were made to obtain final concentrations of the tested derivatives that ranged from 0.98 to 1000 µg/mL. Sterile 96-well polystyrene microtitrate plates (Nunc, Denmark) were prepared by dispensing 200 µL of appropriate dilution of the tested derivatives in broth medium per well. The inocula were prepared with fresh microbial cultures in sterile 0.85% NaCl to match the turbidity of 0.5 McFarland standard, and 2 µL was added to wells to obtain a final density of $1.5 \times 10^6$ colony forming units (CFU)/mL for bacteria and $5 \times 10^4$ CFU/mL for yeasts. After incubation (bacterial strains at 35°C for 24 h and yeast strains at 30°C for 48 h), the MICs were visually assessed as the lowest concentration showing complete growth inhibition of the reference microbial strains. Appropriate DMSO control (at a final concentration of 10%), a positive control (containing inoculum without the tested derivatives), and a negative control (containing the tested derivatives without inoculum) were included on each microplate. Ciprofloxacin, vancomycin, and fluconazole were used as the standard drugs. Each experiment was performed in triplicate.

4.2.3. Cytotoxicity assay

Compounds were dissolved in DMSO at 20 mg/mL, and further dilutions were made in deionized double-distilled water. Samples were tested at 1, 5, 20, and 100 µg/mL. Magnesium ascorbyl phosphate (MAP, 100 µg/mL) and basic fibroblast growth factor (bFGF, 15 ng/mL) were used as positive controls. Human dermal fibroblasts (ATCC PCS-201-010) and B16-F10 mouse melanoma cells (ATCC CRL-6475, Manassas, VA) were plated at 6000 cells/well in a 96-well plate with phenol-free DMEM supplemented with 10% fetal bovine serum (FBS) and CnT-57 medium (Zen-Bio), and the samples were added immediately thereafter in triplicates. The culture was incubated for 72 h. Deionized double-distilled water was the negative control. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO) was added to cell cultures at the end of the 72-h incubation period, and incubation was pursued for additional 2.5 h. The culture media were then discarded, and the intracellular MTT reduction product formazan was solubilized in isopropanol.

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Highlights

- A series of 4-substituted picolinohydrazonamide derivatives were synthesized.
- Compounds were tested for their tuberculostatic and antimicrobial activity.
- Cytotoxic activity against human dermal fibroblasts and mouse melanoma cells was determined.
- Compound **15** is selected as good leading structure for further studies.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Gdansk 18th Nov 2019 Katarzyna Gołda