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INSIGHTS INTO MYCOBACTERIAL ACTIVITY AND CYTOTOXICITY OF SUBSTITUED ISOXAZOLE-4-CARBOHYDRAZIDE DERIVATIVES

PAULINA PŁOSZAJ[1], ADAM JUNKA[2], BOGUMIŁA SZPONAR[3], MARCIN MĄCZYŃSKI[1], STANISŁAW RYNG[1], MARZENNA BARTOSZEWICZ[2] and AGNIESZKA PIWOWAR[4]

[1]Chair and Department of Organic Chemistry, Wrocław Medical University, Borowska 211A, 50-556 Wrocław, Poland
[2]Institute of Pharmaceutical Microbiology and Parasitology, Wrocław Medical University, Borowska 211A, 50-556 Wrocław, Poland
[3]Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Weigla 12, 53-114 Wrocław, Poland
[4]Department of Toxicology, Wrocław Medical University, Borowska 211, 50-556 Wrocław, Poland

Abstract: The purpose of this study was to evaluate the antimycobacterial activity of novel derivatives of 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide **1**, isoniazid (INH) structural analogue. A set of 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide **1** derivatives **2a-j** have been obtained by condensation reactions with aldehydes and further transformed by cyclization with corresponding orthoesters to 5-amino-3-methylisoxazole[**5,4-d**]pyrimidin-4-one derivatives **3a-j** and **4a-j**. From the structural and functional point of view, all these products proved to be biologically important and could be used as substrates for further synthesis. 21 out of 31 structures were newly developed. Described compounds were screened against *Mycobacterium fortuitum* in MABA test. The most active compounds: **2e** (5-amino-3-methyl-N'-((E)-(4-nitrophenyl)methylidene)-1,2-oxazole-4-carbohydrazide) and **2g** (5-amino-N'-((E)-(2,4-dichlorophenyl)-methylidene)-3-methyl-1,2-oxazole-4-carbohydrazide) revealed minimum inhibitory concentration at 16 ěg/mL. These compounds have been screened for toxicity profile. Low cytotoxicity against lung (A549) and fibroblasts (L929) cell lines was determined. The results demonstrated the potential and importance of further development of 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide derivatives as a new class of antimycobacterial compounds and creates a possible direction in the basic research of medicinal chemistry.

Keywords: 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide, *Mycobacterium fortuitum*

Mycobacterium fortuitum is a rapidly growing mycobacterium that has been related with variety of human infections as an etiological agent (1). It is classified as nontuberculous mycobacteria (NTM), and belongs to the same class mycobacteria as the organisms causing tuberculosis and leprosy, but unlike those, NTM are widely spread in the environment (2). NTM have been isolated from such natural resources as water, soils, domestic and wild animals, milk and food products (3). Human diseases resulting from NTM infection are divided into four different clinical conditions: pulmonary disease, lymphadenitis, cutaneous disease, and disseminated disease, while pulmonary disease is the most common of them (3, 4). *Mycobacterium fortuitum* can be easily distinguished from other NTM by its ability to form colonies in less than 1 week and *in vitro* resistance to antimycobacterials (5). Although NTM are associated with a number of clinical diseases, only a few antitubercular agents are active against them (6). Therefore, there is an urgent need for the development of new antimycobacterial drug candidates displaying wide spectrum of antimycobacterial activity and with low toxicity profile.

Furthermore, unlike for the *Mycobacterium tuberculosis* infection, a notification of NTM cases is non-mandatory. This fact interferes with collection of data concerning the impact of NTM infections on public health. For the past few years, in western developed countries the spread of these infections is growing as tuberculosis follows the opposite trend (7). The complications of NTM infections has been notably severe in immune-compromised individuals as AIDS-positive or transplanted patients (8).

* Corresponding author: e-mail: paulina.ploszaj@gmail.com

During our ongoing search for antimycobacterial agents we synthesised a set of 5-amino-3-methyl-isoxazole-4-carbohydrazide derivatives and assessed their potential activity against nontuberculous mycobacteria *Mycobacterium fortuitum*. The value of the structures described in this paper may be further explored against other NTM species and *Mycobacterium tuberculosis* (MTB).

Hydrazides, derivatives of hydrazine, have been known for their antitubercular, antimicrobial, antiviral and antitumor activity (9-11). Due to synthetic and biological versatility, hydrazides are very useful lead-structures in medicinal chemistry. The most recognizable hydrazine derivative – isoniazide – is first-line treatment of MTB infections since 1950s. Isoniazid as well its derivatives revealed robust antitubercular activity and have been used for years in medicine (12, 13). Six other isoniazid-derived hydrazones (furonazide, glyconiazide, opioniazide, salinazid, streptonicozid and sulfoniazide) are registered as tuberculostatics, although they are not used nowadays (14). Moreover, similar structures built on other heterocyclic five-membered rings (oxadiazole, triazole) have proven strong activity against MTB (15, 16). 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide **1** is the main substrate for presented synthesis, the lead structure and chemically an analogue of isoniazid. And estimation of its potential activity is interesting.

Isoxazole hydrazide derivatives have never been reported as anitimycobacterial agents; however, they have been widely explored as immunomodulatory agents (17-20). In these circumstances, we decided to develop molecules through the combination of different pharmacophores in a single frame work to attempt of estimation of their as an effective control over mycobacteria.

Hydrazide **1** has been converted into 5-amino-3-methyl-N'-methylidene-1,2-oxazole-4-carbohydrazide derivatives **2a-j** by reaction of condensation with proper aromatic aldehydes. Substituents of aromatic ring have been chosen according to Topliss scheme, to provide diversity of chemical properties. In the second step, a set of structure **2a-j** is cyclized by orthoesters to obtain 5-amino-3-methylisoxazole (5,4-d)4-pyrimidinone derivatives **3a-j** and **4a-j**. Congenital bicyclic heterocycles have showed antitubercular activity (21, 22). Set of derivatives **3a-j** differ from derivatives **4a-j** with one methyl group in position 6 of pyrimidine ring. Methyl group would enhance lipophilicity of the molecule and makes it more bioavailable. Additionally, by providing this extra methyl group, rigid structures have been obtained. Stabilizing effect of methyl group can prevent from opening the ring in the metabolic pathway of the molecule. Similar effect has been reported for isoxazole ring in Leflunomide (23). If heterocyclic ring is not substituted, molecule is degraded into open structure – toxic metabolite. In case of Leflunomide only active metabolite is therapeutically effective.

In this paper, authors intend to asses antimycobacterial activity of 3 sets of derivatives and check if more rigid structures **4a-j** would act differently than those without stabilizing methyl group **3a-j**. Obtained results delineate further relationship between structure and activity of these derivatives and will be helpful in estimation their biological activity.

EXPERIMENTAL

Chemistry

The melting points were measured on the Barnstead Electrothermal IA9100 melting point apparatus (Electrothermal, UK) and were uncorrected. Elemental analysis made by Microlaboratory of the Department of Pharmacy, Wroclaw Medical University with Carlo Erba series NA 1500 elemental analyzer (Carlo Erba, Milan, Italy). 1H-NMR spectra (300.14 MHz) 1H-NMR spectra (300.14 MHz), 13C-NMR spectra (broadband full decoupling method: 75.47 MHz), ∅5mm tubes, Deutered solvent DMSO-d6 (ARMAR Chemicals, Dottingen, Switzerland) as an internal standard and using tetramethylsilane (TMS) as an internal reference (chemical shift in δ ppm). The course of reaction and the purity of products were checked by TLC (Slica gel on TLC-PET foils, 25 μm, 60 Å, SIGMA-ALDRICH, USA). IR spectra were recorded on FT-IR spectrophotometer Nicolet iS50 (ThermoScientific, USA) using attenuated total reflection (ATR) technique with frequencies expressed in cm^{-1}.

General procedure for the synthesis of 5-amino-3-methyl-N'-(substituted-methylidene)-1,2-oxazole-4-carbohydrazide (2a-j)

According to Ryng and Mączyński (24, 25) the hydrazide derivatives **2a-j** were prepared by reaction between the benzaldehyde (or substituted benzaldehyde to obtain derivatives) (1.0 equiv.) with 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide 1 (1.0 equiv.), initially dissolving the 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide in ethanol and adding the respective amount of benzaldehyde solution in ethanol (Scheme 1). After stirring for 0.5-5 h (TLC controlled) with heating below 80°C, the resulting mixture was concentrated under

reduced pressure. The residue was purified by crystallization (from ethanol/methanol). Compounds **1** and structures **2a, 2b, 2c, 2e, 2f**, **3a, 3b, 3c, 3f** have been previously described (24-26).

5-amino-3-methyl-N'-((E)-(4-methylphenyl) methylidene)-1,2-oxazole-4-carbohydrazide (2d)

White precipitate, yield 62%, m.p.: 187-190°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.28 s, 3H, CH_3 (position 3 in isoxazole ring); 2.33 s, 3H, CH_3 (substituted benzene ring); 7.50 s, 2H, NH_2 (broad signal), 7.26-7.30 d, 2H, Ar-H, J 7.95 Hz; 7.57-7.54 d, 2H, Ar-H, J 8.06 Hz; 8.19 s, 1H, N=C-H; 10.60 s, 1H, N-H, (acidic proton). C^{13}NMR (DMSO-d6, Bruker, 75.47 MHz) δ= 170.58, 168.92, 160.92, 158.18, 145.27, 131.67, 129.41 126.01, 87.22, 20.95, 11.79. IR ν_{max} (cm^{-1}): 3478 (N-H), 1658 (C=O). Analysis: calcd. for $C_{13}H_{14}N_4O_2$: C, 60.45; H, 5.46; N, 21.69%; found: C, 60.71; H, 5.50; N, 22.05%.

5-amino-N'-((E)-(2,4-dichlorophenyl)methylidene)-3-methyl-1,2-oxazole-4-carbohydrazide (2f)

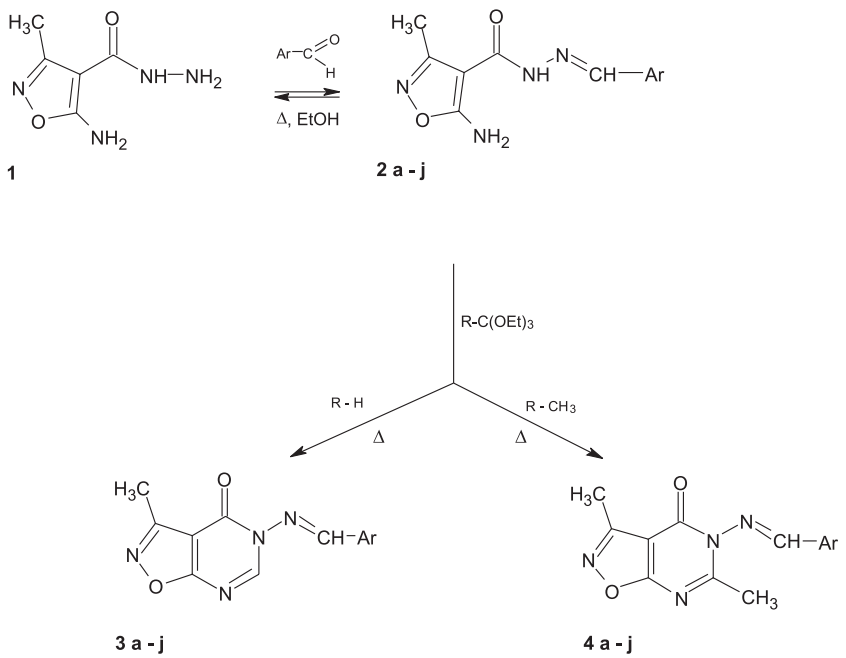
White precipitate, yield 72%, m.p.: 220-222°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.27 s, 3H, CH_3 (position 3 in isoxazole ring); 7.53 s, 2H, NH_2 (broad signal); 7.67-7.69 m, 2H, Ar-H, 7.86 m, 1H, Ar-H, 8.21 s, 1H, N=C-H; 10.84 s, 1H, N-H (acidic proton). C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 170.74, 161.27, 158.12, 145.64, 135.30, 131.83, 131.67, 131.04, 128.07, 126.56, 87.13, 11.70. IR ν_{max} (cm^{-1}): 3437 (N-H), 1627 (C=O). Analysis: calcd. for $C_{12}H_{10}Cl_2N_4O_2$: C, 46.03; H, 3.22; N, 17.89%; found: C, 45.80; H, 2.99; N, 17.53%.

5-amino-3-methyl-N'-((E)-pyridin-4-yl-methylidene)-1,2-oxazole-4-carbohydrazide (2h)

White/light brown solid, yield 81%, m.p.: 241-243°C; H^1NMR (300 MHz, DMSO, δ, ppm): 2.27 s, 3H, CH_3 (position 3 in isoxazole ring); 7.57 s, 2H, NH_2 (broad signal); 7.59-7.61 d, 2H, Ar-H, *J* = 7.74 Hz, 8.20 s, 1H, N=C-H; 8.63-8.61 d, 2H, Ar-H, *J* = 7.51 Hz; 10.95 s, 1H, N-H (acidic proton). C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 170.78, 161.39, 158.19, 150.22 (C-C), 142.70, 141.59, 120.66, 87.07, 11.73. IR ν_{max} (cm^{-1}): 3425 (N-H), 1651 (C=O). Analysis: calcd. for $C_{11}H_{11}N_5O_2$: C, 53.87; H, 4.52; N, 28.56%; found: C, 53.96; H, 4.74; N, 28.80%.

5-amino-N'-((E)-(3,4-dichlorophenyl)methylidene)-3-methyl-1,2-oxazole-4-carbohydrazide (2i)

White solid, yield 78%, m.p.:226-228°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.26 s, 3H,



Scheme 1. Synthetic route used for the preparation of derivatives.

Ar: **a** – C_6H_5, **b** – C_6H_4-4Cl, **c** – C_6H_4-4OH, **d** – C_6H_4-4Me, **e** – C_6H_4-4NO_2, **f –** C_6H_4-4OMe, **g** – C_6H_4-2,4diCl_2, **h** – C_6H_4-4C_5H_5N, **i** – C_6H_4-3,4diCl_2, **j –** C_6H_4-4CF_3

CH_3 (position 3 in isoxazole ring); 7.64-7.72 m, 2H, Ar-H; 7.89-7.90 m, 1H, Ar-H; 7.53 s, 2H, NH_2 (broad signal); 8.19 s, 1H, N=C-H; 10.78 s, 1H, N-H (acidic proton). C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 170.80, 161.43, 158.19, 142.65, 135.33, 131.82, 131.67, 131.08, 128.08, 126.56, 87.11, 11.70. IR ν_{max} (cm^{-1}): 3438 (N-H), 1621 (C=O). Analysis: calcd. for $C_{12}H_{10}Cl_2N_4O_2$: C, 46.03; H, 3.22; N, 17.89%; found: C, 46.29; H, 3.38; N, 18.11%.

5-amino-3-methyl-N'-{(E)-(4-(trifluoromethyl) phenyl)methylidene}-1,2-oxazole-4-carbo-hydrazide (2j)

White solid, yield 83%, m.p.: 161-163°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.26 s, 3H, CH_3 (position 3 in isoxazole ring); 7.52 s, 2H, NH_2 (broad signal); 7.76-7.79 d, 2H, Ar-H, *J* = 8.32 Hz; 7.88-7.85 d, 2H, Ar-H, *J* = 8.19 Hz; 8.27 s, 1H, N=C-H; 10.78 s, 1H, N-H (acidic proton). C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 170.72, 163.18, 158.16, 143.30, 138.52, 129.55, 129.13, 127.25, 125.70, 125.65, 122.27, 87.29, 11.96. IR ν_{max} (cm^{-1}): 3413 (N-H), 1656 (C=O). Analysis: calcd. for $C_{13}H_{11}F_3N_4O_2$: C, 50.00; H, 3.55; N, 17.94%; found: C, 49.73; H, 3.67; N, 18.14%.

3-methyl-5-((E)-(4-methylbenzylidene)amino) (1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (3d)

White precipitate, yield 53%, m.p.: 179-181°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.34 s, 3H, CH_3 (CH_3 attached to benzene ring); 2.51 s, 3H, CH_3 (position 3 in isoxazole ring); 7.24-7.22 d, 2H, Ar-H, *J* = 7.48 Hz; 7.54-7.53 d, 2H, Ar-H, *J* = 7.54 Hz; 8.18 s; 1H, N=C-H, 8.61 s; 1H, N=C-H (pyrimidine ring). C^{13}NMR (DMSO-d6, Bruker, 75.47 MHz) δ= 173.24, 161.35, 159.65, 143.12, 139.49, 131.68, 129.68, 129.18, 127.08, 126.71, 126.02, 87.21, 20.63, 11.07. IR ν_{max} (cm^{-1}): 1711 (C=O), 1538 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{14}H_{12}N_4O_2$: C, 62.68; H, 4.51; N, 20.88%; found: C, 62.39; H, 4.29, N, 20.71%

General procedure for the synthesis of 3-methyl-5-{(E)-(substituted)-amino} (1,2)oxazolo [5,4-d]pyrimidin-4(5H)-one (3a-j)

According to Mączyński (25) the hydrazide derivatives **3 a-j** were prepared by reaction between the appropriate (1.0 equiv.) compound **2** with triethyl ortoformate (4.0 equiv). After stirring for 1-4 h (TLC controlled) with constant boiling, the resulting mixture was filtered off under reduced pressure and rinsed with ethanol. The residue was purified by crystallization (from ethanol/methanol).

3-methyl-5-((E)-(4-nitrobenzylidene)amino)-(1, 2)-oxazolo(5,4-d) pyrimidin-4(5H)-one (3e)

Yellowish precipitate, yield 64%, m.p.: 218-220°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.50 s, 3H, CH_3 (position 3 in isoxazole ring); 8.21-8.19 d, 2H, Ar-H, *J* = 7.45 Hz; 8.43-8.40 d, 2H, Ar-H, *J* = 7.84 Hz; 8.86 s; 1H, N=C-H, 9.36 s; 1H, N=C-H (pyrimidine ring). C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 171.41, 165.45, 157.96, 154.13, 151.66, 149.71, 137.72, 129.99, 124.27, 101.66, 11.06. IR ν_{max} (cm^{-1}): 1706 (C=O), 1532 (corresponds to in-plane deformations of condensed rings). Analusis: calcd. for $C_{13}H_9N_5O_4$: C, 52.1; H, 3.03; N, 23.40%; found: C, 52.39; H, 3.39; N, 23.74%.

5-((E)-(2,4-dichlorobenzylidene)amino)-3-methyl (1,2)oxazolo[5,4-d]pyrimidin-4(5H)-one (3g)

White precipitate, yield 56%, m.p.: 169-171°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.52 s, 3H, CH_3 (position 3 in isoxazole ring); 7.65-7.90 m, 2H, Ar-H; 8.15-8.16 m, 1H, Ar-H; 8.88s, 1H, N=C-H; 9.53 s, 1H, N=C-H (pyrimidine ring).C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 172.91, 162.35, 157.98, 152.06, 150.8, 138.23, 136.11, 129.96, 129.27, 128.60, 128.40, 101.75, 11.07. IR ν_{max} (cm^{-1}): 1711 (C=O), 1537 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{13}H_8Cl_2N_4O_2$: C, 48.32; H, 2.50; N, 17.34%; found: C, 48.56; H, 2.81; N, 17.12%.

3-methyl-5-((E)-(pyridin-4-ylmethylidene)amino) (1,2)oxazolo[5,4-d]pyrimidin-4(5H)-one (3h)

White precipitate, yield 54%, m.p.: 166-169°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.52 s, 3H, CH_3 (position 3 in isoxazole ring); 7.88-7.86 d, 2H, Ar-H, *J* = 5.95 Hz; 8.84-8.82 d, 2H, Ar-H, *J* = 5.86 Hz; 8.90 s, 1H, N=C-H; 9.28 s, 1H, N=C-H (pyrimidine ring). C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 173.01, 165.63, 157.96, 154.16, 151.75, 150.81, 139.01, 122.12, 101.68, 11.05. IR ν_{max} (cm^{-1}): 1700 (C=O), 1542 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{12}H_9N_5O_2$: C, 56.47; H, 3.55; N, 27.44%; found: C, 56.69; H, 3.84; N, 27.71%.

5-((E)-(3,4-dichlorobenzylidene)amino)-3-methyl(1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (3i)

White precipitate, yield 68%, m.p.: 213-215°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.51 s, 3H, CH_3 (position 3 in isoxazole ring); 7.92-7.89 d, 2H, Ar-H; 8.18 m, 1H, Ar-H; 8.87 s, 1H, N=C-H; 9.20 s, 1H, N=C-H, (pyrimidine ring). C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 173.28, 136.17, 157.93,

154.14, 151.68, 135.40, 132.64, 132.13, 131.58, 130.24, 128.63, 102.04, 11.06. IR ν_{max} (cm^{-1}): 1704 (C=O), 1536 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{13}H_8Cl_2N_4O_2$: C, 48.32; H, 2.50; N, 17.34%; found: C, 48.15; H, 2.77; N, 17.51%

3-methyl-5-{(E)-(4-(trifluoromethyl)benzylidene) amino}(1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (3j).

White precipitate, yield 61%, m.p.: 156-159°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.50 s, 3H, CH_3 (position 3 in isoxazole ring); 7.95-7.92 d, 2H, Ar-H, *J* = 8.26 Hz; 8.14-8.12 d, 2H, Ar-H, *J* = 8.11 Hz; 8.88 s, 1H, N=C-H, 9.26 s, 1H, N=C-H, (pyrimidine ring). C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 171.54, 162.41, 131.04, 158.65, 144.81, 135.12, 130.38, 129.27, 126.32 (C-C), 123.84, 100.22, 10.91. IR ν_{max} (cm^{-1}): 1703 (C=O), 1537 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{14}H_9F_3N_4O_2$: C, 52.18; H, 2.82; N, 17.39%; found C, 52.37; H, 2.99; N, 17.51%

General procedure for the synthesis of 5-((substituted)amino)-3,6-dimethyl(1,2)oxazolo (5,4-d)pyrimidin-4(5H)-one (4a-j)

The hydrazide derivatives **4 a-j** were prepared by reaction between the appropriate (1.0 equiv.) compound **2** with triethyl ortoacetate (4.0 equiv). After stirring for 1-5 h (TLC controlled) with constant boiling, the resulting mixture was filtered off under reduced pressure and rinsed with ethanol. The residue was purified by crystallization (from ethanol/methanol).

5-((E)-benzylideneamino)-3,6-dimethyl(1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (4a)

White precipitate, yield 63%, m.p.: 142-144°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.53 s, 3H, CH_3 (position 3 in isoxazole ring); 2.61 s, 3H, CH_3 (position 6 in pirymidine ring); 7.81-7.76, m, 5H, Ar-H; 8.72 s, 1H, N=C-H. C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 168.49, 166.16, 160.77, 158.16 (carbonyl), 156.58, 132.85, 131.88, 129.10, 127.45, 102.91, 23.28, 11.26. IR ν_{max} (cm^{-1}): 1701 (C=O), 1545 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{14}H_{12}N_4O_2$: C, 62.68; H, 4.51; N, 20.88%; found: C, 62.40; H, 4.55; N, 20.53%.

5-((E)-(4-chlorobenzylidene)amino)-3,6-dimethyl(1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (4b).

White precipitate, yield 62%, m.p.: 212-215°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.52 s, 3H, CH_3 (position 3 in isoxazole ring); 2.60 s, 3H, CH_3 (position 6 in pirymidine ring); 7.42 d, 2H, Ar-H, J=8.53 Hz; 7.74 d, 2H, Ar-H, *J* = 8.54 Hz; 8.73 s, 1H, N=C-H. C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 172.50, 166.86, 160.91, 158.11, 155.01, 139.41, 130.43, 130.19 (C-C), 129.51 (C-C), 100.35, 23.31, 11.43. IR ν_{max} (cm^{-1}): 1703 (C=O), 1540 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{14}H_{11}ClN_4O_2$: C, 55.55; H, 3.66; N, 18.51%; found: C, 55.32; H, 3.79; N, 18.87%.

5-((E)-(4-hydroxybenzylidene)amino)-3,6-dimethyl(1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (4c).

White precipitate, yield 64%, m.p.: 185-187°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.48 s, 3H, CH_3 (position 3 in isoxazole ring); 2.53 s, 3H, CH_3 (position 6 in pirymidine ring); 7.13-7.10, d, 2H, Ar-H, J=9.00 Hz; 7.88-7.91 d, 2H, Ar-H, *J* = 8.70 Hz; 8.68 s, 1H, N=C-H. C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 172.13, 170.45, 162.48, 160.53, 157.62, 154.20, 130.98, 124.12, 115.04, 99.50, 22.72, 10.96. IR ν_{max} (cm^{-1}): 2982 (O-H), 1705 (C=O), 1546 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{14}H_{12}N_4O_3$: C, 59.15; H, 4.25; N, 19.71%; found: C, 59.43; H, 4.59; N, 19.89%.

3,6-dimethyl-5-((E)-(4-methylbenzylidene)amino)(1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (4d)

White precipitate, yield 48%, m.p.: 220-223°C. H^1NMR (300 MHz, DMSO, δ, ppm): 1.91 s, 3H, CH_3 (4-methyl group in benzene ring); 2.38 s, 3H, CH_3 (position 3 in isoxazole ring); 2.50 s, 3H, CH_3 (position 6 in pirymidine ring); 7.37 d, 2H, Ar-H, *J* = 7.89 Hz; 7.39 d, 2H, Ar-H, *J* = 8.14 Hz; 9.12 s, 1H, N=C-H. C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 171.14, 169.55, 162.13, 158.80, 152.24, 140.82, 132.56, 129.79, 122.84, 110.11, 15.62, 14.15, 11.66. IR ν_{max} (cm^{-1}): 1706 (C=O), 1549 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{15}H_{14}N_4O_2$: C, 63.82; H, 5.0; N, 19.85%; found: C, 63.51, H, 5.14; N, 19.64%.

5-((E)-(4-nitrobenzylidene)amino)-3,6-dimethyl (1,2)xazolo(5,4-d)pyrimidin-4(5H)-one (4e)

Yellow precipitate, yield 71%, m.p.: 216-218°C; H^1NMR (300 MHz, DMSO, δ, ppm): 2.50 s, 3H, CH_3 (position 3 in isoxazole ring); 2.59 s, 3H, CH_3 (position 6 in pirymidine ring); 8.26-8.23, d, 2H, Ar-H, *J* = 8.84 Hz; 8.44-8.41 d, 2H, Ar-H, *J* = 8.77 Hz; 9.07 s, 1H, N=C-H. C^{13}NMR (DMSO-d_6,

Bruker, 75.47 MHz) δ = 172.18, 168.78, 160.84, 157.74, 153.92, 149.86, 137.52, 130.07, 124.26, 99.59, 22.80, 10.96. IR ν_{max} (cm^{-1}): 1705 (C=O), 1547 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{14}H_{11}N_5O_4$: C, 53.68; H, 3.54; N, 22.36%; found: C, 53.41; H, 3.74, N 22.67%.

5-((E)-(4-methoxybenzylidene)amino)-3,6-dimethyl(1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (4f)

White precipitate, yield 67%, m.p.: 174-176°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.48 s, 3H, CH_3 (position 3 in isoxazole ring); 2.53 s, 3H, CH_3 (position 6 in pirymidine ring); 3.87 s, 3H, O-CH_3; 7.15-7.12 d, 2H, Ar-H, *J* = 8.80 Hz; 7.93-7.90 d, 2H, Ar-H, *J* = 8.82 Hz; 8.69 s, 1H, N=C-H. C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 172.14, 170.47, 163.18, 160.53, 157.63, 154.20, 130.98, 124.28, 114.68, 99.50, 55.57, 22.73, 10.97. IR ν_{max} (cm^{-1}): 1711 (C=O), 1539 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{15}H_{14}N_4O_3$: C, 60.40; H, 4.73; N, 18.78%; found: C, 60.65; H, 4.53; N, 19.04%.

5-((E)-(2,4-dichlorobenzylidene)amino)-3,6-dimethyl(1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (4g)

White precipitate, yield 55%, m.p.: 209-211°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.48 s, 3H, CH_3 (position 3 in isoxazole ring); 2.57 s, 3H, CH_3 (position 6 in pirymidine ring); 7.86-7.98 m, 2H, Ar-H; 8.22 m, 1H, Ar-H (position 3 in benzene ring); 8.89 s, 1H, N=C-H. C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 172.28, 168.42, 160.80, 157.70, 154.06, 135.63, 132.47, 132.19, 131.57, 130.32, 128.73, 99.64, 22.79, 10.95.IR ν_{max} (cm^{-1}): 1706 (C=O), 1540 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{14}H_{10}Cl_2N_4O_2$: C, 49.87; H, 2.99; N, 16.62%; found: C, 49.71; H, 3.21; N 16.96%.

5-((E)-(pyridin-4-ylmethylidene)amino)-3,6-dimethyl (1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (4h)

Yellowish precipitate, yield 74%, m.p.: 198-200°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.28 s, 3H, CH_3 (position 3 in isoxazole ring); 2.51 s, 3H, CH_3 (position 6 in pirymidine ring); 7.58-7.55 m, 2H, Ar-H; 8.04-8.01 m, 2H, Ar-H; 8.67 s, 1H, N=C-H. C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 178.50, 173.29, 157.75, 152.59, 150.56, 135.32, 132.15, 128.70, 127.72, 119.51, 101.61, 17.13, 11.08. IR ν_{max} (cm^{-1}): 1701 (C=O), 1545 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{13}H_{11}N_5O_2$: C, 57.99; H, 4.12; N, 26.01%; found C. 57.62, H; 4.34, N 26.29%.

5-((E)-(3,4-dichlorobenzylidene)amino)-3,6-dimethyl(1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (4i)

White precipitate, yield 58%, m.p.: 210-212°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.49 s, 3H, CH_3 (position 3 in isoxazole ring); 2.57 s, 3H, CH_3 (position 6 in pirymidine ring); 7.87-7.99 m, 2H, Ar-H; 8.23 m, 1H, Ar-H; 8.90 s, 1H, N=C-H. C^{13}NMR (DMSO-d_6, Bruker, 75.47 MHz) δ = 172.17, 168.44, 160.82, 157.71, 153.94, 135.64, 132.47, 132.10, 131.46, 130.33, 128.70, 99.41, 22.47, 10.95. IR ν_{max} (cm^{-1}): 1708 (C=O), 1549 (corresponds to in-plane deformations of condensed rings). Analysis for $C_{14}H_{10}Cl_2N_4O_2$; calculated: C, 49.87; H 2.99; N, 16.62%; found: C, 49.76; H, 2.89; N, 16.50%.

3,6-dimethyl-5-{(E)-(4-(trifluoromethyl)benzylidene)amino}(1,2)oxazolo(5,4-d)pyrimidin-4(5H)-one (4j)

White precipitate, yield 59%, m.p.: 177-179°C. H^1NMR (300 MHz, DMSO, δ, ppm): 2.47 s, 3H, CH_3 (position 3 in isoxazole ring); 2.56 s, 3H, CH_3 (position 6 in pirymidine ring); 7.96-7.93 d, 2H, Ar-H, *J* = 8.24 Hz; 8.19-8.16 d, 2H, Ar-H, *J* = 8.04 Hz; 8.99 s, 1H, N=C-H. C^{13}NMR (DMSO-d6, Bruker, 75.47 MHz) δ = 169.67, 166.51, 161.19, 160.50, 157.65 (carbonyl), 153.90, 135.63, 132.12, 129.49, 126.05, 99.46, 22.76, 10.76. IR ν_{max} (cm^{-1}): 1709 (C=O), 1537 (corresponds to in-plane deformations of condensed rings). Analysis: calcd. for $C_{15}H_{11}F_3N_4O_2$: C, 53.58; H, 3.30; N, 16.66%; found: C, 53.81; H, 3.23; N, 16.32%.

Microbiological assays

***In vitro* evaluation of antibacterial activity against *Mycobacterium fortuitum* (MABA test)**

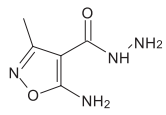
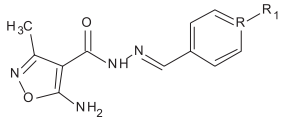
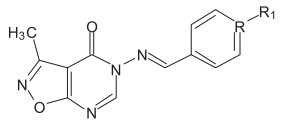
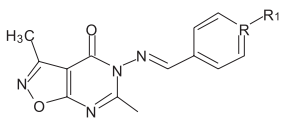
Mycobacterium fortuitum PCM 672 used throughout the study was obtained from the Polish Collection of Microorganisms (PCM) at the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław. Bacteria have been cultivated on liquid 79 medium for 24 h at 37°C with 5% CO_2 atmosphere.

The antibacterial activity of compounds has been determined using microplate Alamar Blue assay according to procedure presented by Ahmed et al (27). Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO) and were diluted with appropriate media on a 96-well microtitration plate. To the wells containing 100 µL

of PBS and 100 µL tested compound, aliquots of 100 µL of the diluted suspension has been added. Sealed plates were incubated at 37°C for 24 h and after that 20 µL of Alamar Blue has been added. Incubation continued at 37°C for 2 h. Fluorescence has been measured using Victor apparatus (Wallac, Perkin Elmer). Each compound has been tested in triplicates and the experiment was repeated twice. The minimal inhibitory concentration (MIC) was established as the lowest drug concentration inhibiting the bacterial growth by 90%. Values calculated and presented using Graph Pad Prism and STATISTICA.

Log P values calculated using Virtual Computational Chemistry Laboratory program ALOGPS v.2.1

Table 1. In vitro activity expressed by MIC in µg/mL of compounds.

Structure	Compound	R_1	R	MIC (µg/mL)	log P
	Izoniazid			0.2	- 0.71
	**1**			> 100	- 0.96
	**2a**	- H	C	> 100	1.91
	2b	- 4 Cl	C	> 100	2.44
	2c	- OH	C	> 100	1.87
	2d	- Me	C	> 100	2.12
	2e	- NO_2	C	16	2.12
	2f	- OMe	C	62.5	2.12
	2g	- 2,4 di Cl	C	16	2.98
	2h		N	31.2	0.98
	2i	- 3,4 di Cl	C	62.5	2.89
	2j	- CF_3	C	62.5	2.12
	**3a**	- H	C	> 100	1.46
	3b	- 4 Cl	C	> 100	2.08
	3c	- OH	C	> 100	1.37
	3d	- Me	C	> 100	1.89
	3e	- NO_2	C	62.5	1.89
	3f	- OMe	C	> 100	1.89
	3g	- 2,4 di Cl	C	62.5	3
	3h		N	> 100	0.76
	3i	- 3,4 di Cl	C	> 100	3.02
	3j	- CF_3	C	> 100	1.89
	**4a**	- H	C	> 100	1.97
	4b	- 4 Cl	C	> 100	2.51
	4c	- OH	C	> 100	1.68
	4d	- Me	C	> 100	2.28
	4e	- NO_2	C	62.5	2.28
	4f	- OMe	C	> 100	2.12
	4g	- 2,4 di Cl	C	> 100	3.19
	4h		N	> 100	1.04
	4i	- 3,4 di Cl	C	> 100	3.17
	4j	- CF_3	C	> 100	2.28

Cytotoxicity assay on A549 lung cells and L929 fibroblast cells

The A549 (ATCC® CCL-185™) lung cells and L929 (ATCC® CCL1™) fibroblast cells were used to evaluate cytotoxic potential of the tested compounds. The standard cytotoxicity test, utilizing ability of Neutral Red dye to accumulate inside of living cells was applied (28). Briefly, the cells were incubated in 96-well plates overnight in the presence of 0.01 or 0.005 mole of **2e**, **2h**, **2g** compounds. After 24 or 48 h of incubation, medium was removed and 100 µL of Natural Red medium (40 µg/mL) was introduced to wells of the plate. Cells with dye-containing medium were incubated for 2 h at 37°C. After incubation, dye was removed, wells were rinsed with PBS and left to dry at room temperature. Subsequently, 150 µL of de-stain solution (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid) was introduced to each well. The plate was vigorously shaken in a microtiter plate shaker for 30 min. until neutral red has been extracted from the cells and formed a homogeneous solution. Next, the value of neutral red absorbance was measured spectrometrically using 530 nm wavelength. The absorbance value of cells untreated with compounds tested was considered 100% of potential cellular growth (positive control). The de-stain solution served for blank assay. All compounds tested were DMSO-solved. Because some concentrations of this reagent are considered cytotoxic, additional control sample was performed. It consisted of cells incubated in medium containing 1% DMSO. Positive control was also applied in the test, however not displayed in results section. After 24 h incubation with 10% ethanol solution, cell viability was reduced with 75.9% and 71.8% respectively in A549 and L929.

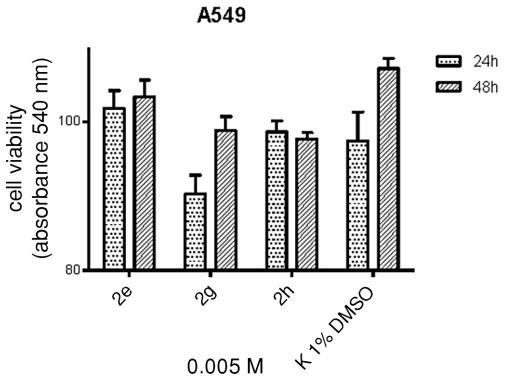


Figure 1a

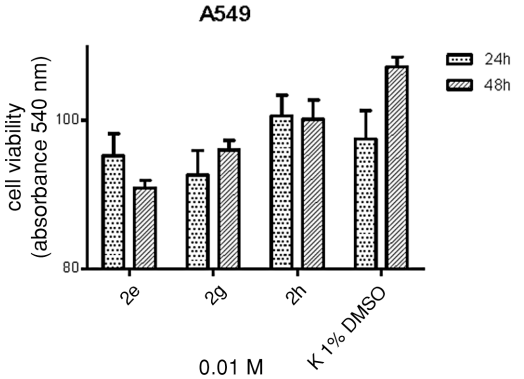


Figure 1b

Figure 1. Cell viability measured as a difference in absorbance for A549 cell line incubated with compounds 2e, 2g, 2h in concentration 0.005 M (a) and 0.01 M (b) for 24 and 48 hours, respectively

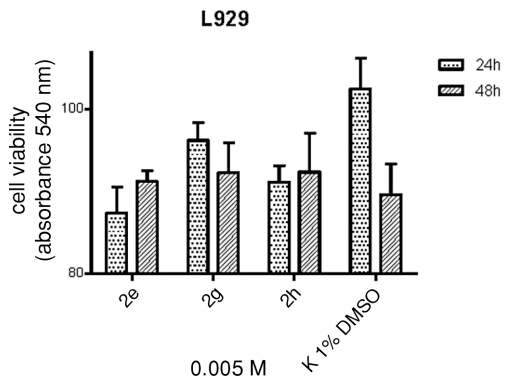


Figure 2a

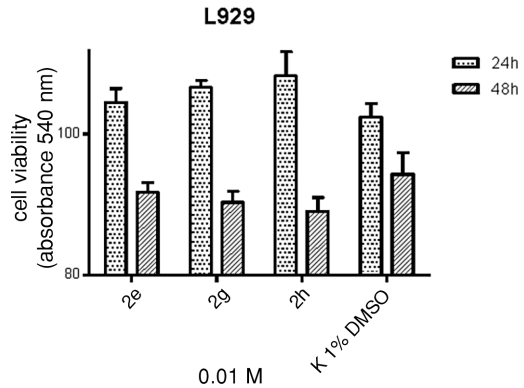


Figure 2b

Figure 2. Cell viability measured as a difference in absorbance for L29 cell line incubated with compounds 2e, 2g, 2h in concentration 0.005 M (a) and 0.01 M (b) 24 and 48 hours, respectively

RESULTS AND DISCUSSION

The synthesis of the 30 derivatives of 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide **1** was performed with good yields from commercially available materials. Among all synthetized compounds 21 are novel and unpublished (**2d**, **2g**, **2h**, **2i**, **2j**, **3d**, **3e**, **3g**, **3h**, **3i**, **3j**, **4a-j**). 9 compounds (**2a, 2b, 2c, 2e, 2f, 3a, 3b, 3c, 3f**) have been previously described by Ryng (24) and Mączyński (25). Hydrazide **1** has been condensed with aromatic aldehydes, to create 5-amino-3-methyl-N'-methylidene-1,2-oxazole-4-carbohydrazide derivatives **2a-j**. In the next step structures **2a-j** are cyclized by orthoesters triethyl ortoformate and triethyl ortoacetate to obtain 5-amino-3-methylisoxazole [5,4-*d*]4-pyrimidinone derivatives respectively **3a-j** and **4a-j.** Synthetic route used for the preparation of derivatives is presented on Scheme 1 below. 5-amino-3-methyl-N'-methylidene-1,2-oxazole-4-carbohydrazides **2a-j** have been obtained as white/yellow solids with yields ranging from 72% to 83%. Structures **2a-j** were furtherly converted into 5-amino-3-methylisoxazole [5,4-*d*]-4-pyrimidinone white/yellow solids **3a-j** with yields 53-68% and white/yellow solids **4a-j** with 48-74%.

In the nuclear magnetic resonance spectra (^{1}H NMR) the signals of respective protons of the compounds were verified based on their chemical shifts, multiplicities and coupling constants. The spectra showed protons of methyl group (position 3 in isoxazole) in 5-amino-3-methyl-N'-(substituted-methylidene)-1,2-oxazole-4-carbohydrazides **2a-j** as a singlet at 2.26 – 2.28 ppm. Derivatives **3a-j** revealed the same singlet signal of methyl group in position 3 in isoxazole within 2.50 – 2.52 ppm and in derivatives **4a-j** was visible at 2.28 – 2.53 ppm. Additionally spectra of 5-((substituted)amino)-3,6-dimethyl(1,2)oxazolo[5,4-*d*]pyrimidin-4(5H)-ones **4a-j** showed singlet signals between 2.50 – 2.61 ppm corresponding to the methyl group substituted at position 6 of pyrimidine ring. Protons of amino group in position 5 of isoxazole ring in 5-amino-3-methyl-N'-(substituted-methylidene)-1,2-oxazole-4-carbohydrazides **2a-j** has been displayed as broad peak singlet at 7.50-7.57 ppm. The imine proton (N=C–H) was visible at 8.19 – 8.27 ppm in derivatives **2a-j**. The same group was displayed respectively at 8.18 – 8.90 ppm and 8.67 – 9.12 ppm in **3a-j** and **4a-j**. Protons attached in C6 of pirymidine ring in obtained 5-amino-3-methylisoxazole[5,4-*d*]4-pyrimidinone derivatives **3a-j** were displayed in the range 8.61 – 9.53 ppm. An acidic proton (N-H) characteristic for 5-amino-3-methyl-N'-(substituted-methylidene)-1,2-oxazole-4-carbo-hydrazides (**2a-j**) was observed at 10.60 – 10.95 ppm.

In the nuclear magnetic resonance spectra (^{13}C NMR) the signals of respective carbons have described in detailed within spectroscopic data section. Methyl group (position 3 in isoxazole) of 5-amino-3-methyl-N'-(substituted-methylidene)-1,2-oxazole-4-carbo-hydrazides **2a-j** was observed within range 11.73-11.96; **3a-j** and **4a-j** respectively 10.94-11.07 and 10.95-11.06. Spectra confirmed carbonyl carbon C=O at 170.58-170.58 in the set of derivatives **2a-j** and 154.16-158.80 in the **3a-j** and **4a-j**.

The chemical structures of compounds were confirmed by analysing products using FTIR. Analysis showed a medium absorption band at 3414-3478 cm^{-1}, referring to the stretch vibrations of the amine group N-H in 5-amino-3-methyl-N'-methylidene-1,2-oxazole-4-carbohydrazides **2a-j**. Strong absorption peak C=O was revealed between 1621-1658 cm^{-1} in compounds **2a-j,** 1700-1716 cm^{-1} in **3a-j** and 1701-1709 cm^{-1} in **4a-j**. Characteristic absorption seen within 1536-1549 cm^{-1} in spectra of 5-amino-3-methylisoxazole [5,4-*d*]4-pyrimidinone derivatives **3a-j** and **4a-j** corresponds to in-plane deformations of condensed rings.

Elementary analysis indicated by the symbols of the elements was within ± 0.4 % of the theoretical values.

All compounds were evaluated against *Mycobacterium fortuitum* with MIC results reported in Table 1. The most effective compounds (**2e**, **2g**, and **2h**) showed MIC 16-31.2 μg/mL, while isoniazide activity is 0.2 μg/mL. The remaining compounds were effective at high concentrations or ineffective (MIC > 100 μg/mL). Relatively, the set of derivatives with the highest activity is set **2a-j**. Cyclized derivatives **3a-j** and **4a-j** in particular, tend to be less active or inactive. This trend could be related with stabilizing effect of pyrimidine ring. Additional methyl group in derivatives 4a-j did not impact antimycobacterial activity significantly. For further development of 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide derivatives, carboxyhydrazide scaffold should be maintained to preserve mycobacterial activity. Summary results of compound tested against *Mycobacterium fortuitum* have been displayed in Table 1.

The cytotoxicity was tested *in vitro* against lung cells A549 and fibroblasts L929 using Neutral red uptake assay. The cytotoxic activity of 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide derivatives was assayed only on compounds with MIC = 31.2 μg/mL (**2e**, **2h**, **2g**). Despite incubation

time (24 or 48 h), compound **2e** did not diminished A549 cells viability in concentration 0.005 M. In concertation 0.01 M viability decreases to 95 and 91% respectively for incubation time. Opposite effect has been observed for the same compound within L929 cell line. In lower concentration cell viability falls back to 87% with 24 h incubation, however remain 100% in higher concentration. For reasonable explanation of this effect, more detailed toxicity tests should be performed. Lung cells exposed to **2h** retain up to 90% of viability regardless compound concentration and incubation time. Fibroblasts viability remain up to 96% in exposure to 2 h despite the conditions. There was almost no toxic effect observed in A549 with use of compound **2g** (99 and 98% cell viability in 24 and 48 incubation for 0.005 M; 101 and 100% cell viability in 24 and 48 incubation for 0.01 M). Tests with L929 cell line and **2g** varies from 91 up to 103 % viability.

Cell viability (%) has been determined in respect to K 1% DMSO. Results have been analysed with 2way ANOVA with Bonferoni post-hoc test to confirm statistical significance of the obtained results. Tested compounds revealed low toxic profile in lung cells A549 and fibroblasts L929. Results have been presented in Figures 1a,1b and 2a, 2b.

CONCLUSIONS

Nontuberculous mycobacteria are virulent pathogens whose importance and impact on human health has recently been increasing. Furthermore, NTM infections are emerging in previously unrecognized settings, with new clinical manifestations. Emerging global drug resistance and the fact that only a few agents are active against nontuberculous mycobacteria prompt the need to search for new, effective and non-toxic drug candidates. Here we revealed that derivatives of 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide **1** exhibited mild mycobacterial activity against *Mycobacterium fortuitum* in comparison to isoniazid. Aside from mycobacterial activity, selected compounds showed non-toxic effect *in vitro* on two cell line cultures. Taking into consideration obtained results, derivatives of 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide **1** are promising and should be further developed in both nontuberculous and tuberculous assays with structure modifications. Based on the structure–activity relationship information obtained from evaluating the antimycobacterial activities, we suggest that 4-nitro and 2,4-di-chloro substituents at the aromatic backbone might be a good scaffold in a isoxazole-4-carbohydrazide compound possessing potent activity. As structural analogues of isoniazide and Schiff bases, they are potentially good lead structures for future development of drug candidates with low toxicity profile. Biological activity of sets **3a-j** and **4a-j** was significantly lower that **2a-j**. This finding pointed out the possible direction in the basic research of antimycobacterial agents.

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