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New lipophilic isoniazid derivatives and their 1,3,4-oxadiazole analogues: synthesis, antimycobacterial activity and investigation of their mechanism of action

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Abstract

The development of novel drugs is essential for the treatment of tuberculosis and other mycobacterial infections in future. A series of *N*-alkyl-2-isonicotinoylhydrazine-1-carboxamides was synthesized from isoniazid (INH) and then cyclized to *N*-alkyl-5-(pyridin-4-yl)-1,3,4-oxadiazole-2-amines. All derivatives were characterised spectroscopically. The compounds were screened for their *in vitro* antimycobacterial activity against susceptible and multidrug-resistant *Mycobacterium tuberculosis* (*Mtb.*) and nontuberculous mycobacteria (NTM; *M. avium*, *M. kansasii*). The most active carboxamides were substituted by a short *n*-alkyl, their activity was comparable to INH with minimum inhibitory concentrations (MICs) against *Mtb.* of 0.5-2 μM. Moreover, they are non-toxic for HepG2, and some of them are highly active against INH-resistant NTM (MICs ≥4 μM). Their cyclization to 1,3,4-oxadiazoles did not increase the activity. The experimentally proved mechanism of action of hydrazine-1-carboxamides consists of the inhibition of enoyl-ACP-reductase (InhA) in a way similar to INH, which is blocking the biosynthesis of mycolic acids. *N*-Dodecyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine as the most efficacious oxadiazole inhibits growth of both susceptible and drug-resistant *Mtb.* strains with uniform MIC values of 4-8 μM with no cross-resistance to antitubercular drugs including INH. The mechanism of action is not elucidated but it is different from INH. Obtained results qualify these promising derivatives for further investigation.

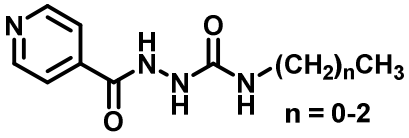
Highlights

•2-Isonicotinoylhydrazine-1-carboxamides and 1,3,4-oxadiazoles were synthesised.
•Activity against *M. tuberculosis* and nontuberculous mycobacteria (MIC ≥0.5 μM).
•Low or no cytotoxicity for HepG2 cells.
•2-Isonicotinoyl-*N*-methylhydrazine-1-carboxamide targets InhA identical to isoniazid.
•5-(Pyridin-4-yl)-1,3,4-oxadiazol-2-amines inhibit multidrug-resistant strains.

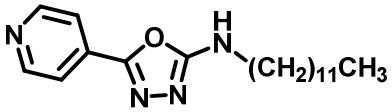
Keywords

antimycobacterial activity; isoniazid; 2-isonicotinoylhydrazine-1-carboxamide; *Mycobacterium tuberculosis*; 1,3,4-oxadiazole; tuberculosis

Graphical abstract



MIC for *M. tuberculosis* $H_{37}Rv$ of 0.5-2 μM
activity against atypical mycobacteria
(n=2; MIC 4-16 μM)
InhA inhibitors



MIC for *M. tuberculosis* $H_{37}Rv$ of 4-8 μM
identical activity against multidrug- and
extensively drug-resistant *M. tuberculosis*
mechanism of action is not related to isoniazid

1. Introduction

Genomic revolution optimism in tuberculosis (TB) drug research remains unfulfilled; results did not bring a new therapeutic intervention and the whole effort returned to the cell screening.[1] The treatment of drug sensitive TB involves isoniazid (INH), rifampicin (RIF), pyrazinamide and ethambutol (EMB) for the first two months followed generally by 4 months treatment with INH and RIF. Beside the need to shorten and simplify the therapy of susceptible TB, the treatment of multidrug- (MDR) and extensively drug-resistant (XDR) TB requires the regimens based on *in vitro* drug susceptibility testing results and needs to be treated much longer with other drugs, usually more toxic and with unpleasant side effects. These drugs should be also compatible with antiretroviral therapy due to HIV/AIDS co-infection. Thus, the development and implementation of new drugs overcoming resistance is a major goal for future control of TB.[2]

Isoniazid (INH, isonicotinic hydrazide, pyridine-4-carbohydrazide) is still one of the most efficient and useful first line anti-TB drug, which plays the most important role in the treatment of TB all over the world. In fact it has been used since 1952 as a prodrug, which must be activated by the multifunctional mycobacterial enzyme catalase-peroxidase (KatG). The main target of INH is the inhibition of the synthesis of mycolic acids, very specific building blocks in the mycobacterial cell wall, responsible for its highly lipophilic character. The activated molecule of INH is thought to suppress mycolic acid biosynthesis by the inhibition of enoyl-ACP reductase (InhA), an enzyme involved in fatty acid biosynthesis in the presence of NADH or NAD^+.[3] This makes InhA one of the best-validated targets for the treatment of TB. Most cases of INH-resistance are mediated by mutations in *katG* gene, leading to the inability to activate the drug; the second most common resistance is caused by mutations in the *inhA* gene. These two mutations among others are responsible for approximately 75 % of all cases of *Mycobacterium tuberculosis* (*Mtb.*) resistance in clinical setting.[4] For example, the mutation rate responsible for INH resistance is 100 times greater in comparison with resistance to RIF.[5]

A long duration of the treatment and other facts have induced an increase of resistance to all anti-TB drugs and to the key molecule INH. A growing awareness of the increasing drug-resistance and a great need for therapy shortening together with killing also latent forms of *Mtb.*, lead to the discovery of more efficient and less toxic treatment regimens. The need to overcome the resistance to INH led to a huge number of structure modifications.[6] Hydrophobic moieties were introduced into the basic structure of INH to enhance penetration into a highly lipophilic cell wall and avoid toxicity[7]. To protect INH molecule against *N*-arylaminoacetyl transferases at N^2 and thus combat the rise of resistance functionalization of hydrazine group [8],[9],[10],[11], incorporation of one nitrogen[12],[13] or both nitrogen atoms[14] of hydrazide group into another heterocycle ring and other modifications were performed and then summarized in many review articles.[15],[16],[17],[18]

We have already described several synthetic modifications of the isoniazid molecule linked with another active part through a methine bridge[19] by conjugation with an aniline group with electron-withdrawing substituents[20], *via* a carbonyl group[21] and by the preparation of a new type of “double” active molecules based on the fluorinated hydrazides of a benzoic acid scaffold as an isoniazid isostere[22]. The current report deals with an introduction of lipophilic aliphatic chains into the hydrazide part of INH molecule and with a cyclization of these derivatives into the 1,3,4-oxadiazoles, which have been published as very efficient antimycobacterial agents.[23],[24],[25],[26]

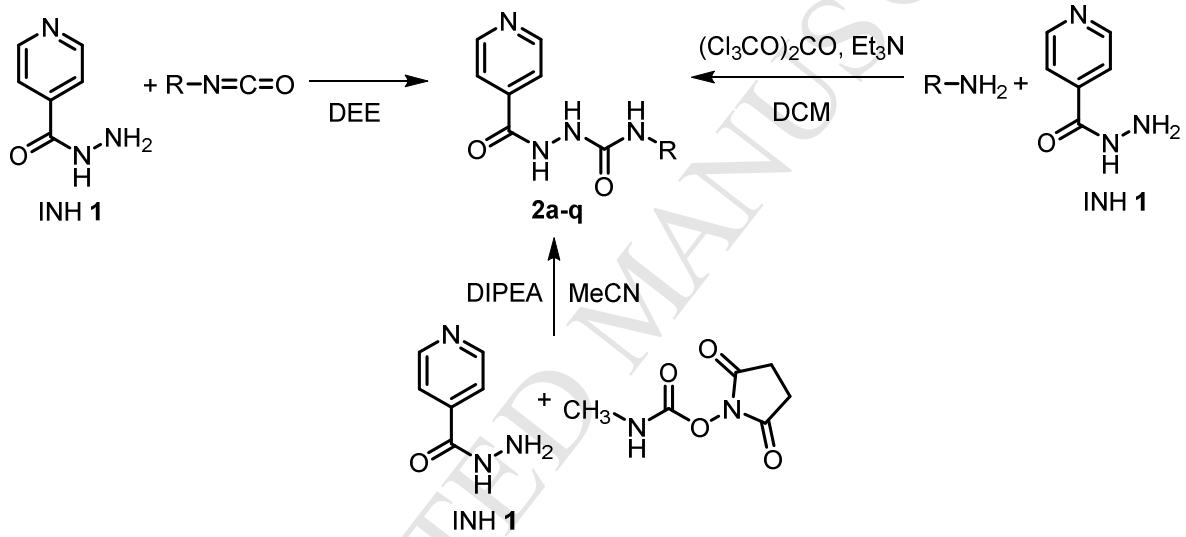
Hence, an attempt to improve the INH molecule by introducing chemical modifications in its core structure in order to enhance biological responses against *Mtb.*, to reduce hepatotoxicity, to circumvent resistance phenomena for both drug-resistant *Mtb.* and nontuberculous mycobacteria (NTM) continues to be an interesting scientific challenge. The goal of the new effort in

development is to identify compounds that are able to attain the same clinical efficacy as isoniazid and avoid much of the current resistance to INH by bypassing the requirement for KatG activation and directly inhibiting InhA.

2. Results and Discussion

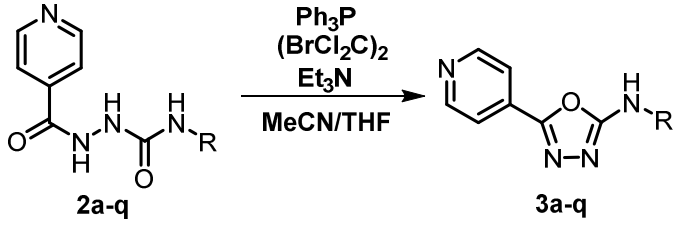
2.1. Chemistry

The majority of *N*-alkyl-2-isonicotinoylhydrazine-1-carboxamides (**2b-i**, **2k**, **2l**, **2n**, **2p**, **2q**) were synthesised from isoniazid **1** and appropriate commercially available isocyanate *via* a previously described method[21] (Method A; yields 72-92 %). 2-Isonicotinoyl-*N*-methylhydrazine-1-carboxamide **2a** was obtained from *N*-succinimidyl *N*-methylcarbamate as a methyl isocyanate substitute and isoniazid **1** in presence of *N*,*N*-diisopropylethylamine (DIPEA; Method B with 87% yield). Three carboxamides (**2j**, **2m**, **2o**) were prepared from appropriate amines, triphosgene (bis(trichloromethyl)carbonate) and INH **1** under the nitrogen atmosphere (Method C) with yields of 65-76 %. The synthetic approach is depicted in Scheme 1.



Scheme 1. Synthesis of *N*-alkyl-2-isonicotinoylhydrazine-1-carboxamides **2a-q** (R: *n*-alkyl from C_1 to C_{18}; DEE: diethyl ether; DIPEA: *N*,*N*-diisopropylethylamine; MeCN: acetonitrile; Et_3N: triethylamine; DCM: dichloromethane; $(Cl_3CO)_2CO$: triphosgene.

N-Substituted 5-(pyridin-4-yl)-1,3,4-oxadiazole-2-amines **3** were obtained by cyclization of the 2-isonicotinoylhydrazine-1-carboxamides **2** with triphenylphosphine in the presence of 1,2-dibromo-1,1,2,2-tetrachloroethane in anhydrous solvent[21] (Scheme 2). Their yields ranged from 52 to 68 %.



Scheme 2. Synthesis of *N*-alkyl-5-(pyridin-4-yl)-1,3,4-oxadiazole-2-amines **3** (Ph_3P: triphenylphosphine; $(BrCl_2C)_2$: 1,2-dibromo-1,1,2,2-tetrachloroethane; MeCN: acetonitrile; Et_3N: triethylamine; THF: tetrahydrofuran)

All of the compounds **2**-**3** (Table 1) were characterized by ^{1}H and ^{13}C NMR, IR spectra and melting points. Their purity was checked by TLC and elemental analysis.

2.2. Antimycobacterial Activity

Both series, 2-isonicotinoylhydrazine-1-carboxamides **2** and their cyclic analogues 5-(pyridine-4-yl)-1,3,4-oxadiazole-2-amines **3** were evaluated for their *in vitro* antimycobacterial activity against *Mtb.* 331/88 ($H_{37}Rv$) and three NTM strains: *Mycobacterium avium* 330/88 and two strains of *Mycobacterium kansasii*: 235/80 and a clinical isolate, 6509/96. The first-line antituberculosis drug and synthetic precursor, isoniazid (INH), was used as the reference compound (Table 1).

All of the *N*-alkyl-2-isonicotinoylhydrazine-1-carboxamides **2** and *N*-alkyl-5-(pyridin-4-yl)-1,3,4-oxadiazole-2-amines **3** displayed a significant antimycobacterial activity with minimum inhibitory concentrations (MICs) starting from 0.5 μM; a limited solubility in the testing medium prevents determination of all exact MIC values of two derivatives (**2q**, **3n**) bearing a long alkyl chain. An identical complication was present partly for **3l** and **3m** in the case of NTM.

Mtb. was the most susceptible species with MICs ≥0.5 μM for 2-isonicotinyolhydrazine-1-carboxamides **2** and ≥4 μM for 5-(pyridin-4-yl)-1,3,4-oxadiazole-2-amines **3**. Contrarily, *Mycobacterium avium* showed the highest rate of tolerance (MIC values ≥32 μM and ≥8 μM for **2** and **3**, respectively). Compounds **2** inhibited the growth of *M. kansasii* at the concentrations of 4 μM and higher, derivatives **3** with MICs ≥8 μM with no significant difference between the collection and the isolated strain.

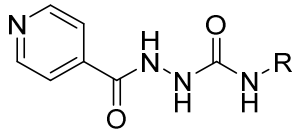
Analysing structure-activity relationships in the group of carboxamides **2**, the optimal activity for *Mtb.* is conferred by a small alkyl (methyl, ethyl, propyl **2a**-**c**, MICs ≤2 μM). Pentyl and butyl (**2d**-**e**) led to a moderately increased MIC of 8 μM. Thus, hydrophilicity (log*P* <1) modulates anti-TB properties positively. An additional elongation of the alkyl chain decreases potency up to 125 μM (octyl and nonyl, **2h**-**i**). *N*-Decyl-2-isonicotinyolhydrazine-1-carboxamide **2j** exhibited the best suppression of *Mtb.* cells among derivatives with a long aliphatic chain (16/32 μM), MIC values of its higher homologues are uniform of 32-62.5 μM. For *M. avium*, the most potent carboxamides are those substituted from C_{11} to C_{16} alkyls (MICs ≤125 μM) with C_{12} superiority (**2l**, 32-62.5 μM). Contrarily to *Mtb.*, the introduction of shorter alkyls resulted in very low active or inactive compounds (**2a**-**b**, **2d**-**e**, MICs ≥1000 μM), the propyl derivative **2c** being an exception (250/500 μM). 2-Isonicotinoyl-*N*-propylhydrazine-1-carboxamide **2c** was also identified to be the most potent agent against both *M. kansasii* strains with MIC values of 4-16 μM. Other favourable substituents are undecyl (**2k**), tridecyl (**2m**) and ethyl (**2b**). Generally, an optimum is associated with C_2-C_3 plus eight-membered and longer chains.

In contrast to **2**, their cyclized counterparts **3** with C_1-C_3 alkyls showed only a limited antimycobacterial action (MICs ≥500 μM) including a complete resistance of *M. avium*. This strain tolerates also **2d**-**e** at the concentration of 1000 μM. Butyl, hexyl, heptyl, tridecyl, pentadecyl, hexadecyl and octadecyl derivatives (**3d**, **3f**-**g**, **3m**, **3o**-**q**) exhibited moderate MIC values (≥125 μM), while *N*-pentylamine **3e** was slightly superior against *Mtb.* (62.5 μM). The excellent antimycobacterial properties are connected with ten, eleven and twelve-membered alkyl chains (**3j**-**k**). *N*-Dodecyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine **3l** is the most efficient and selective in the suppression of *Mtb.* (4/8 μM, i.e., 8 times higher concentration than obtained for INH) and *M. avium* (8/16 μM), while *N*-decyl derivative **3j** blocks both strains of *M. kansasii* at the lowest concentration observed (8-16 μM).

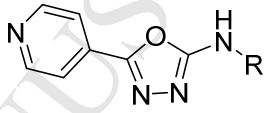
Predominantly, the cyclization of the INH-based carboxamides **2** to the more lipophilic oxadiazoles **3** did not improve antimicrobial potency. For *Mtb.*, the majority of carboxamides (bearing the

shortest, shorter and the longest alkyls **2a-g**, **2m-p**) exceeded their analogous oxadiazoles **3**, four pairs are comparable (± one dilution; **2h-k**) and there is only one but a very important example of an improved anti-TB activity: *N*-dodecyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine **3l**, which is 4-8 times more efficient than its counterpart **2l**. Oxadiazoles **3h**, **3j** and **3l** share an enhanced action against *M. avium* when compared to carboxamides **1** (up to 15.63 times). In the inhibition of *M. kansasii*, only decyl derivative **3j** improved sharply the activity of **2j** (up to 15.63 times), three other adjacent pairs with even-membered carbon chains produced a comparable inhibition (**3f**, **3h**, **3k**). When compared to parent INH, three derivatives led to an identical *in vitro* inhibition of *Mtb.* (**2a-c**), overall majority was superior against *M. avium* (**2c**, **2g-i**, **2k-p**, **3h-j**, **3l-m**, **3o-p**). INH, **2c** and **3j** exhibited a comparable activity for the clinical isolate of *M. kansasii* 6509/96, remaining novel molecules **2** and **3** are inferior. Carboxamides **2b-c**, **2e-p** as well as oxadiazoles **3f-l** and **3o-q** produced MIC values against *M. kansasii* 235/80 lower than INH.

Table 1. Antimycobacterial activity of *N*-alkyl-2-isonicotinoylhydrazine-1-carboxamides **2** and *N*-alkyl-5-(pyridin-4-yl)-1,3,4-oxadiazole-2-amines **3**



2



3

Code	R	MIC [μM]										Clog*P*
		Mtb. 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96			
		14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d	
2a	Methyl	**0.5**	**1**	>1000	>1000	1000	>1000	>1000	500	500	1000	-1.03
3a		500	500	>1000	>1000	500	1000	>1000	1000	1000	1000	0.36
2b	Ethyl	**1**	**2**	1000	>1000	32	62.5	62.5	32	62.5	62.5	-0.69
3b		>1000	>1000	>1000	>1000	1000	>1000	>1000	1000	>1000	>1000	0.69
2c	Propyl	**1**	**1**	250	500	**4**	**8**	**16**	**4**	**8**	**8**	-0.20
3c		500	500	>1000	>1000	500	1000	1000	1000	1000	1000	1.18
2d	Butyl	8	8	>1000	>1000	500	1000	1000	250	250	500	0.22
3d		250	500	>1000	>1000	500	1000	1000	1000	1000	1000	1.60
2e	Pentyl	8	8	>1000	>1000	62.5	125	125	125	125	125	0.63
3e		62.5	62.5	>1000	>1000	500	1000	1000	500	1000	1000	2.02
2f	Hexyl	32	32	500	500	250	250	250	250	500	500	1.05
3f		250	250	500	500	250	500	500	125	250	500	2.43
2g	Heptyl	32	62.5	250	250	62.5	125	125	125	250	250	1.47
3g		125	125	500	500	250	500	500	250	500	500	2.86
2h	Octyl	62.5	125	250	250	32	62.5	125	62.5	125	250	1.89
3h		62.5	62.5	62.5	125	62.5	125	125	62.5	125	125	3.27
2i	Nonyl	62.5	125	250	250	32	62.5	125	62.5	125	125	2.30
3i		62.5	62.5	125	125	125	250	250	125	250	250	3.68
2j	Decyl	16	32	500	500	32	62.5	125	125	250	250	2.72
3j		16	16	32	62.5	**8**	**16**	**16**	**16**	**16**	**16**	4.10
2k	Undecyl	32	62.5	125	125	16	32	62.5	62.5	125	125	3.14
3k		32	32	500	500	**8**	**16**	32	125	125	250	4.52
2l	Dodecyl	32	32	32	62.5	32	62.5	125	62.5	62.5	62.5	3.55
3l		**4**	**8**	**8**	**16**	125	125*	125*	125*	125*	125*	4.94
2m	Tridecyl	32	62.5	125	125	16	32	62.5	32	62.5	62.5	3.97
3m		250	250	250	250	250*	250*	250*	125	250	250*	5.35

2n	Tetradecyl	32	62.5	125	125	32	62.5	62.5	32	62.5	62.5	4.39
3n		250*	250*	250*	250*	250*	250*	250*	250	250*	250*	5.77
2o	Pentadecyl	32	62.5	125	125	32	62.5	62.5	62.5	62.5	125	4.81
3o		125	125	250	500	62.5	125	250	125	250	250	6.19
2p	Hexadecyl	32	62.5	125	125	32	62.5	62.5	32	62.5	62.5	5.22
3p		250	250	250	250	250	500	500	250	500	500	6.61
2q	Octadecyl	250*	250*	250*	250*	250*	250*	250*	250*	250*	250*	6.06
3q		125	250	500	500	250	250	250	250	500	500	7.44
INH		0.5	1	>250	>250	>250	>250	>250	8	8	8	-0.64

INH: isoniazid; the best values for each strain are provided in bold. *: at presented concentration grow of strain was observed, at duplex concentration there was present precipitate and/or turbidity; the determination of exact MIC value was not possible.

The most active compounds **2** (i.e., with MIC against *Mtb.* ≤1 μM) and the most efficient 1,3,4-oxadiazole **3l** underwent an advanced screening against four MDR-TB strains and one XDR-TB strain with different resistance patterns (Table 2). MDR strain is a *Mtb.* strain that is resistant concomitantly to INH and RIF. XDR involves MDR in addition to resistance to any of the fluoroquinoles and at least one of the three parenteral second-line drugs (amikacin, kanamycin, capreomycin).

Although all of the evaluated carboxamides **2** showed a growth inhibition of drug-resistant strains independently of the resistance pattern, their MIC values are significantly higher than those obtained for drug-susceptible *Mtb.* strain $H_{37}Rv$ (up to 125 times). The highest activity was observed for 2-isonicotinoyl-*N*-propylhydrazine-1-carboxamide **2c** (a uniform value of 16 μM), followed by *N*-methyl derivative **2a** (32-62.5 μM). These results indicate the cross-resistance to parent INH **1**. The described phenomena is common for the majority of "simple" INH modifications but not inevitable.[16]

Importantly, MIC values of *N*-dodecyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine **3l** for both drug-susceptible and resistant TB are identical (4-8 μM) thus indicating INH-independent mechanism of action and/or the ability to circumvent the mechanism(s) of INH resistance. Our findings indicate that **3l** does not share any cross-resistance with conventionally used drugs (INH, rifamycines, EMB, streptomycin, ofloxacin, clofazimine, aminoglycosides) and it may be a prospective and perspective agent for combating drug-resistant TB. Importantly, the drug-likeness of **3l** was checked using traditional Lipinski´s rule of five. It defines four physicochemical parameter ranges (molecular weight ≤500, log*P* ≤5, number of H-bond donors ≤5 and number of H-bond acceptors ≤10) that are associated with an acceptable aqueous solubility and intestinal permeability required for oral bioavailability.[27] Notwithstanding the presence of C_{12} alkyl, the oxadiazole fits this rule without any violation.

Table 2. MIC values of selected carboxamides **2** and oxadiazole **3l** against MDR- and XDR-TB

	MIC [μM]									
	Mtb. 9449/2006		*Mtb.* Praha 1		*Mtb.* Praha 4		*Mtb.* 234/2005		*Mtb.* Praha 131 (XDR-TB)	
	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d
2a	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	32	62.5
2b	125	125	125	125	125	125	125	125	125	125
2c	16	16	16	16	16	16	16	16	16	16
3l	**4**	**4**	**4**	**8**	**4**	**8**	**4**	**8**	**4**	**8**

The lowest MIC values are given in bold. Resistance patterns for MDR-TB strains: 9449/2006 is resistant to INH, RIF, rifabutine, and streptomycin (STM); 234/2005 resistant to INH, RIF, rifabutine, STM, and EMB; Praha 1 resistant to

INH, RIF, rifabutine, STM, EMB, and clofazimine; Praha 4 resistant to INH, RIF, rifabutine, STM, EMB, ofloxacin (OFX), and clofazimine. XDR-TB strain Praha 131 is resistant to INH, RIF, rifabutine, STM, EMB, OFX, gentamicin, and amikacin.

Among 2-substituted 5-(pyridin-4-yl)-1,3,4-oxadiazoles, 2-(4,5-dibromo-1*H*-pyrrol-2-yl)-5-(pyridin-4-yl)-1,3,4-oxadiazole (Fig. 1, compound **a**) showed a growth inhibition of *Mtb.* H_{37}Rv with MIC of 3.5 µg/mL (i.e., 8.75 times higher than those determined for INH in the study). Regrettably, INH-resistant strains were not involved.[28] Interestingly, Navarette-Vázquez et al.[23] synthesized 5-(pyridin-4-yl)-2-substituted-1,3,4-oxadiazoles that are active against *Mtb.* H_{37}Rv and INH-susceptible clinical isolates. However, these derivatives lack amino group. In contrast to here reported results, the highest anti-TB potential for an INH-susceptible strain was observed in the presence of 2-pentadecyl and heptadecyl moieties (Fig. 1, structure **b**; MIC of 0.35 and 0.65 µM, respectively). The replacement by methyl or substituted methyl reduced dramatically activity, 2-(substituted)aryls produced intermediate MIC values. Other alkyl chains were not investigated. Unfortunately, their MIC values against isolates that are, *i.a.*, resistant to INH, are sharply higher (up to 63.9 times). Authors concluded that an increased steric hindrance at position 2 of the oxadiazole concomitantly with an escalated lipophilicity is required for the excellent biological activity. A follow-up study brought an evidence that 2-pentadecyl-5-(pyridin-4-yl)-1,3,4-oxadiazole (Fig. 1, structure **b**, n = 14) had a significant therapeutic effect *in vivo* in a murine model of progressive pulmonary TB when it is administered in liposomal form.[29] 1,5-Dimethyl-2-phenyl-4-({[5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl]methyl}amino)-1,2-dihydro-3*H*-pyrazol-3-one (Fig. 1, compound **c**) exhibited a comparable activity for both INH-susceptible and resistant *Mtb.* strains.[30] Described derivative containing 5-(pyridine-4-yl)-(1,3,4-oxadiazol-2-yl)methylamino moiety can be considered as a higher homologue of **3**. Based on presented data, it seems that the presence of alkylamino group on the position 2 of 1,3,4-oxadiazole is required for the activity against INH- and multidrug-resistant *Mtb.*

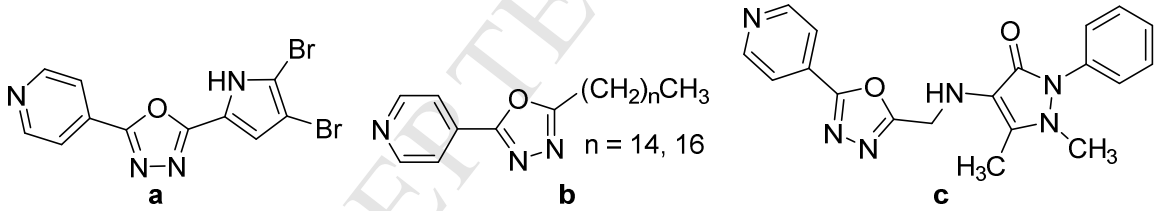


Figure 1. Related 2-substituted 5-(pyridine-4-yl)-1,3,4-oxadiazole-based antimycobacterial agents

2.3. *In vitro* Cytotoxicity Determination

Cytotoxicity of the tested compounds was measured using the standard hepatic *in vitro* model, cancer cell line HepG2. The used CellTiter 96 assay is based on the reduction of tetrazolium dye MTS in living cells to formazan, which is then determined colorimetrically. The reduction of the reagent is related to availability of NADH or NADPH. The decline in levels of these metabolically important compounds in the cell causes that the production of formazan is reduced.

The parameter IC_{50} allows the quantitative comparison of the toxicity among tested compounds. IC_{50} is the inhibitory concentration that reduces viability of the cell population to 50 % of the maximal viability. The cytotoxicity was determined for the majority of the substances (all carboxamides **2**, oxadiazoles **3j-l**; Table 3).

In general, the investigated carboxamides **2** and 1,3,4-oxadiazoles **3** share a substantially low cytotoxicity at concentrations in which are soluble in the testing medium. Only toxicity course of **3k**

did allow a valid IC_{50} value calculation; in other cases, we could not determine exact IC_{50} values due to their limited solubility. As expected, a longer alkyl led to a reduced solubility. None of the derivatives **2-3** was toxic at the concentration of 50 µM.

In conclusion, the substances **2a-f** as well as parent isoniazid **1** were found as *in vitro* certainly non-toxic compounds in HepG2 cells (IC_{50} >1000 µM), remaining compounds did not reduced growth of eukaryotic cells up to the fully soluble concentration. Obviously, the modification of INH **1** to *N*-alkyl-2-isonicotinoylhydrazine-1-carboxamides **2** did not enhance cytotoxicity.

We also calculated the selectivity indices (SI) as the ratio of IC_{50} to MIC, and values higher than 10 indicate rather acceptable toxicity (based on the analogy of the therapeutic index). Table 3 reports SI higher than 10, SI of **3l** and for the compound **3k** with the exactly known IC_{50} value. For *Mtb.* 331/88, SI of **2a-f** indicate a safety and a desired selectivity for tubercular bacilli. This distinct selectivity is also preserved for the drug-resistant strains in spite of the lower *in vitro* activity (SI >31.3). SI of **3k** is low (5.4) and for **3l**, it is higher than 12.5 and 6.25 after 2 and 3 weeks of incubation, respectively. Due to a low efficacy of INH analogues with shorter alkyls against *M. avium in vitro* and a limited solubility of longer alkyls bearing derivatives, no satisfactory SI was found. Regarding both strains of *M. kansasii*, three derivatives were identified as the selective verifiable: *N*-ethyl **2b**, *N*-pentyl **2e** and especially *N*-propyl **2c** carboxamides.

Table 3. Cytotoxicity of the tested substances **2-3** for HepG2 cells

Code	IC_{50} [µM]	Range of concentrations tested	SI for *Mtb.* 331/88	SI for MDR- and XDR-TB	SI for *M. kansasii*
2a	>5000*	0.5-5000	>5000	>80	ND
2b	>4000*	10-4000	>2000	>32	>64
2c	>5000*	5-5000	>5000	>312.5	>312.5
2d	>3000*	5-3000	>375	ND	ND
2e	>4000*	10-4000	>500	ND	>32
2f	>1000*	0.5-1000	>31.3	ND	ND
2g	>500**	0.5-750	ND	ND	ND
2h	>500**	0.5-750	ND	ND	ND
2i	>250**	0.5-500	ND	ND	ND
2j	>250**	0.5-500	ND	ND	ND
2k	>50**	0.5-500	ND	ND	ND
2l	>50**	0.5-350	ND	ND	ND
2m	>100**	1-300	ND	ND	ND
2n	>50**	1-300	ND	ND	ND
2o	>75**	0.3-186	ND	ND	ND
2p	>50**	0.5-200	ND	ND	ND
2q	>50**	0.5-200	ND	ND	ND
3j	>50**	0.5-350	ND	ND	ND
3k	~172.3	0.5-250	5.4	ND	5.4 (235/80)/0.7 (6509/96)
3l	>50**	0.5-100	>6.25 (>12.5)	>6.25 (>12.5)	ND
INH (1)	>5000*	0.5-5000	>5000	ND	>625 (6509/96)/ resistant (235/80)

***** IC_{50} is higher than tested concentration range

** measurement at higher concentration was unable due to the precipitation of the tested compound in the cell culture medium

Only SI higher than 10 and those obtained for **3k** and **3l** are reported.

2.4. Investigation of Mechanism of Action

The most active oxadiazole **3l** and the 2-isonicotinyol-*N*-methylhydrazine-1-carboxamide **2a** as the carboxamide that showed the lowest MIC values for *Mtb.*, cytotoxicity and the highest selectivity for *Mtb.* were subjected for the investigation of mechanism of action. In order to evaluate the enoyl-

ACP-reductase InhA as a target of **3l** and **2a**, we analyzed MICs of these compounds against *Mtb.* H_{37}Ra overproducing InhA protein. In principle, the strains overproducing drug target should be more resistant to the studied inhibitor due to an increased amount of the inhibited protein. Indeed, analysis of sensitivity of *Mtb.* H_{37}Ra carrying empty vector pMV261 and *Mtb.* H_{37}Ra pMV261-InhA by drop dilution method revealed that compared to the control strain InhA overproducer is a significantly more resistant to the compound **2a**. This result confirms InhA as molecular target of **2a** inside *Mtb.* cells (Fig. 2A). On the contrary, the sensitivity of InhA overproducer to the compound **3l** was identical to the control strain indicating another mode of action of this inhibitor (Fig. 2B).

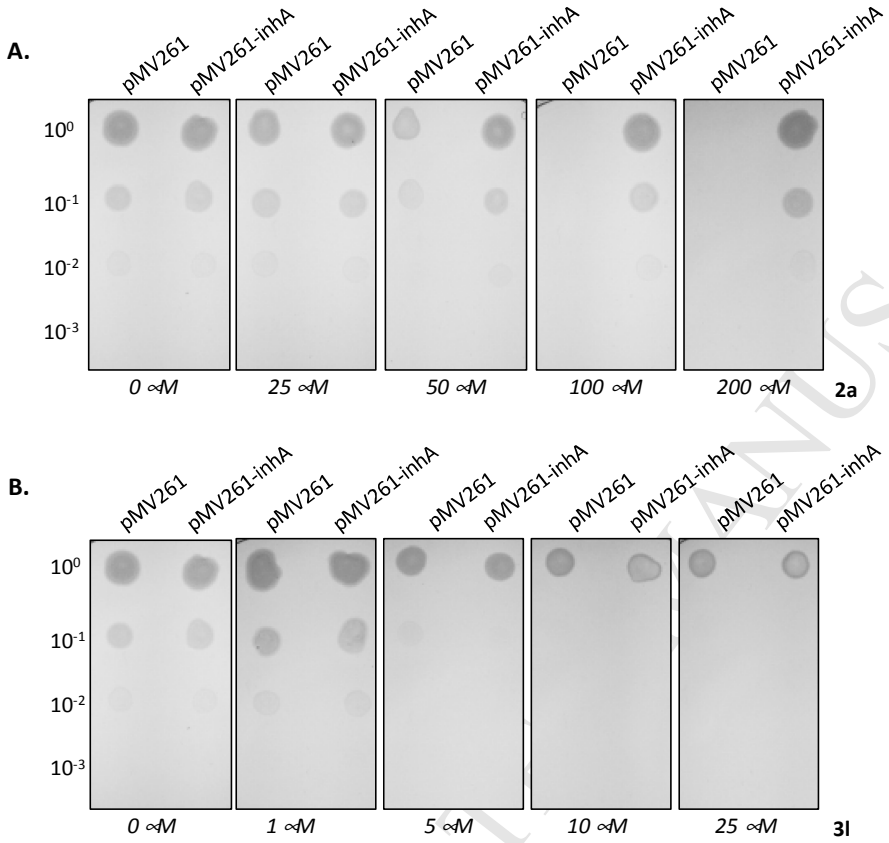


Figure 2. Determination of sensitivity of *Mtb.* H_{37}Ra pMV261 and *Mtb.* H37Ra pMV261-InhA to (A) **2a** and (B) **3l** by drop dilution method.

The inhibitory effect of **2a** on mycolic acids production was verified by ^{14}C metabolic labelling of *Mtb.* H_{37}Ra treated with this carboxamide derivative. Isoniazid was used as a control drug that inhibits InhA protein. The lipids were extracted from the labeled cells by the mixture of chloroform and methanol and separated by TLC (Fig. 3A). Clearly, the compound **2a** inhibited the synthesis of trehalose monomycolates and trehalose dimycolates similar to INH. Moreover, analysis of fatty and mycolic acids content of lipid fractions by TLC after their derivatization to corresponding methyl esters confirmed the inhibition of the synthesis of all types of mycolic acids by **2a**, while the production of short chain fatty acids was not affected (Fig. 3B).

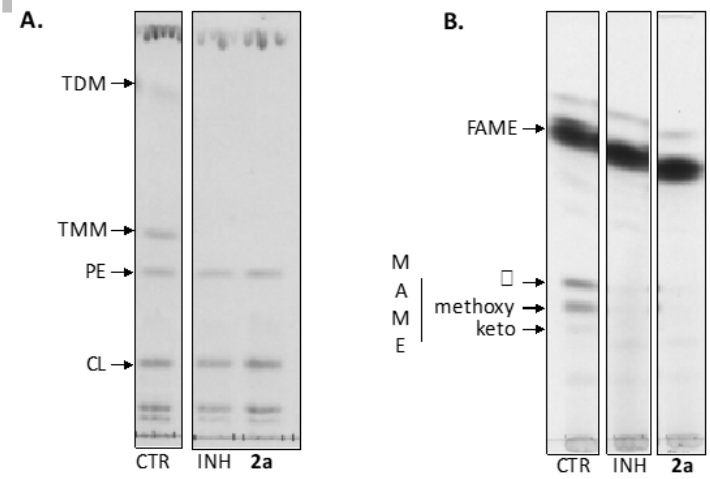


Figure 3. TLC analysis of (A) lipids and (B) corresponding methyl esters of fatty (FAME) and mycolic (MAME) acids isolated from ^{14}C labeled *Mtb.* $H_{37}Ra$ cells treated with 40 μM final concentration of **2a** and 36.5 μM of INH**.** Lipids were separated in chloroform: methanol: water [20:4:0.5] and detected by autoradiography. Different forms of methyl esters were separated in n-hexane: ethyl acetate [95:5; 3x] and detected by autoradiography. (TDM: trehalose dimycolates; TMM: trehalose monomycolates; PE: phosphatidylethanolamine; CL: cardiolipin; α-, methoxy- and keto- refer to the forms of MAMEs).

Next, we studied the mechanism of action of **3l** by ^{14}C metabolic labelling of *Mtb.* $H_{37}Ra$ treated with this compound in final concentrations 4 and 8 μM. Tested compound was added when O.D. of the culture reached 0.263 and after 24 h of further cultivation ^{14}C acetate as a metabolic tracer was added. Cells were harvested after next 24 h of cultivation. Comprehensive investigation of lipids, fatty and mycolic acids profiles by TLC using different solvent systems to separate major lipid components of mycobacterial envelope did not reveal any differences between the control and treated cultures suggesting that **3l** does not target the pathways leading to the synthesis of cell envelope. The mode of action of this compound thus remains to be elucidated.

Experimental Section

3.1. Synthesis

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Penta Chemicals (Prague, Czech Republic) and were used as received. Melting points were determined on a Büchi melting point B-540 apparatus (BÜCHI, Flawil, Switzerland) in open capillaries and the reported values are uncorrected. Elemental analyses (C, H, N) were performed with an automatic Fisons EA 1110 CHNS-O CE microanalyzer (FISONS EA 1110, Milano, Italy). Infrared spectra (ATR) were recorded on a Nicolet 6700 FTIR spectrometer in the range of 400–4000 cm^{-1}. NMR spectra (500 MHz for ^1H and 126 MHz for ^{13}C) were measured in DMSO-d_6 or $CDCl_3$ at ambient temperature using a Varian VNMR S500 instrument or a Varian Mercury-Vxbb 300 (300 MHz for ^1H and 75.5 MHz for ^{13}C). The chemical shifts (δ) are given in ppm, related to tetramethylsilane (TMS) as an internal standard. The coupling constants (J) are reported in Hz. The reactions and purity of the products were monitored by thin-layer chromatography using a mixture of dichloromethane and methanol (2:1, v/v) as the eluent; the plates were coated with 0.2 mm Merck 60 F254 silica gel (Merck Millipore, Darmstadt, Germany) and were visualised by UV irradiation (254 nm).

The calculated log*P* values (Clog*P*), which are the logarithms of the partition coefficients for octan-1-ol/water, were determined using the program CS ChemOffice Ultra version 15.0 (CambridgeSoft, Cambridge, MA, USA).

3.1.1. General procedure for synthesis of *N*-alkyl-2-isonicotinoylhydrazine-1-carboxamides **2**

Method A

Isoniazid **1** (274.2 mg, 2.0 mmol) was suspended in anhydrous diethyl ether (8 mL) and heated to initiate boiling of the mixture. The appropriate isocyanate (1.05 of equivalents, i.e., 2.1 mmol) was added in one volume. The reaction mixture was refluxed for 1 h, then let cool at the room temperature and stored for 24 hrs at -20 °C. Precipitate was filtered off and recrystallised from ethyl acetate when necessary.

Method B

Isoniazid **1** (137.0 mg, 1.0 mmol) was mixed with *N*,*N*-diisopropylethylamine (DIPEA, 348 µL, 2.0 mmol) and *N*-succinimidyl *N*-methylcarbamate (258.2 mg, 1.5 mmol) in acetonitrile (4 mL). The reaction mixture was stirred at the room temperature for 24 h, formed precipitate was filtered off and recrystallised from ethyl acetate.

Method C

Method C involved the generation of an appropriate isocyanate *in situ*. Triphosgene (240 mg, 0.8 mmol) was dissolved in anhydrous dichloromethane (7 mL) under nitrogen atmosphere and the appropriate amine (2.02 mmol) dissolved in anhydrous dichloromethane (4 mL) was added dropwise. The mixture was stirred for 30 min at room temperature, then treated with triethylamine (586 µL, 4.2 mmol) in anhydrous dichloromethane (4 mL). After 30 min, isoniazid **1** (277 mg, 2.02 mmol) was added. The reaction mixture was stirred for 10 hours at room temperature, then evaporated to dryness, treated with water (15 mL) and extracted with ethyl acetate (3 x 25 mL). The combined organic phase was dried over anhydrous sodium sulphate, filtered off and evaporated to dryness to give the final product, which was recrystallised from ethyl acetate when necessary.

3.1.1.1. 2-Isonicotinoyl-*N*-methylhydrazine-1-carboxamide (**2a**). Yield 87 % (method B); mp 203-205 °C (lit.[31] 224-225 °C). IR (ATR): 3248, 3091, 2928, 1665, 1556, 1532, 1490, 1413, 1305, 1223, 1151, 1064, 999, 848, 754, 670 cm^{-1}. ^{1}H NMR (DMSO, 300 MHz): δ 10.41 (1H, s, NH), 8.74 (2H, d, *J* = 5.6 Hz, H2, H6), 8.01 (1H, s, NH), 7.79 (2H, d, *J* = 5.7 Hz, H3, H5), 6.52 (1H, q, *J* = 4.6 Hz, NH), 2.57 (3H, d, *J* = 4.4 Hz, CH_3). ^{13}C NMR (DMSO, 75 MHz): δ 165.06, 158.83, 150.41, 139.98, 121.69, 26.46. Anal. Calcd for $C_8H_{10}N_4O_2$ (194.19): C, 49.48; H, 5.19; N, 28.85. Found: C, 48.85; H, 5.81; N, 28.91.

3.1.1.2. *N*-Ethyl-2-isonicotinoylhydrazine-1-carboxamide (**2b**). Yield 92 % (method A); mp 214-216 °C (lit.[32] 228-229 °C). IR (ATR): 3266, 3080, 2935, 1668, 1529, 1490, 1441, 1407, 1361, 1330, 1307, 1143, 1063, 998, 918, 852, 755, 712, 688 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.39 (1H, s, NH), 8.74 (2H, d, *J* = 5.9 Hz, H2, H6), 7.94 (1H, s, NH), 7.79 (2H, d, *J* = 6.1 Hz, H3, H5), 6.55 (1H, t, *J* = 5.5 Hz, NH), 3.09-3.02 (2H, m, CH_2), 1.01 (3H, t, *J* = 7.3 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.73, 157.88, 150.19, 139.76, 121.43, 34.04, 15.51. Anal. Calcd for $C_9H_{12}N_4O_2$ (208.22): C, 51.92; H, 5.81; N, 26.91. Found: C, 52.05; H, 5.98; N, 26.78.

3.1.1.3. 2-Isonicotinoyl-*N*-propylhydrazine-1-carboxamide (**2c**). Yield 85 % (method A); mp. 182-183°C. IR (ATR): 3261, 3079, 2928, 1671, 1556, 1532, 1488, 1408, 1385, 1303, 1246, 1148, 1064, 997, 916, 851, 757, 669 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.39 (1H, s, NH), 8.74 (2H, d, *J* = 5.8 Hz, H2, H6), 7.92 (1H, s, NH), 7.79 (2H, d, *J* = 6.3 Hz, H3, H5), 6.56 (1H, t, *J* = 5.5 Hz, NH), 3.02-2.95 (2H, m, $C^{1}H_2$), 1.55-1.45 (2H, m, $C^{2}H_2$), 0.83 (3H, t, *J* = 7.4 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.74, 158.23, 150.22, 139.77, 121.50, 41.23, 23.01, 11.23. Anal. Calcd for $C_{10}H_{14}N_4O_2$ (222.24): C, 54.04; H, 6.35; N, 25.21. Found: C, 53.85; H, 6.61; N, 24.96.

3.1.1.4. N-Butyl-2-isonicotinoylhydrazine-1-carboxamide (**2d**)[33]. Yield 79 % (method A); mp 175-177 °C. IR (ATR): 3293, 3089, 2933, 1669, 1555, 1518, 1487, 1436, 1407, 1375, 1292, 1221, 1140, 1063, 999, 902, 852, 757 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.39 (1H, s, NH), 8.74 (2H, d, *J* = 6.2 Hz, H2, H6), 7.91 (1H, s, NH), 7.88 (2H, d, *J* = 6.2 Hz, H3, H5), 6.58 (1H, t, *J* = 5.5 Hz, NH), 3.07-2.97 (2H, m, $C^{1}H_2$), 1.43-1.33 (2H, m, $C^{2}H_2$), 1.32-1.21 (2H, m, $C^{3}H_2$), 0.86 (3H, t, *J* = 7.7 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.73, 158.00, 150.19, 139.96, 121.43, 40.20, 31.95, 19.41, 13.71. Anal. Calcd for $C_{11}H_{16}N_4O_2$ (236.27): C, 55.92; H, 6.83; N, 23.71. Found: C, 55.84; H, 6.60; N, 23.92.

3.1.1.5. 2-Isonicotinoyl-*N*-pentylhydrazine-1-carboxamide (**2e**). Yield 91 % (method A); mp 175-177 °C. IR (ATR): 3293, 3090, 2933, 2860, 1663, 1556, 1518, 1437, 1406, 1376, 1350, 1288, 1219, 1142, 1062, 999, 908, 852, 758, 669 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.40 (1H, s, NH), 8.74 (2H, d, *J* = 6.0 Hz, H2, H6), 7.92 (1H, s, NH), 7.79 (2H, d, *J* = 5.8 Hz, H3, H5), 6.55 (1H, t, *J* = 5.5 Hz, NH), 3.06-2.96 (2H, m, $C^{1}H_2$), 1.47-1.32 (2H, m, $C^{2}H_2$), 1.32-1.16 (4H, m, $C^{3}H_2$, $C^{4}H_2$), 0.88 (3H, t, *J* = 7.6 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.94, 158.23, 150.41, 139.96, 121.45, 40.48, 29.53, 28.51, 21.91, 13.98. Anal. Calcd for $C_{12}H_{18}N_4O_2$ (250.30): C, 57.58; H, 7.25; N, 22.38. Found: C, 57.83; H, 7.60; N, 22.41.

3.1.1.6. N-Hexyl-2-isonicotinoylhydrazine-1-carboxamide (**2f**). Yield 86 % (method A); mp 175-177 °C. IR (ATR): 3286, 3085, 2927, 2858, 1671, 1549, 1516, 1485, 1436, 1406, 1374, 1341, 1300, 1220, 1139, 1064, 998, 913, 851, 756, 719, 695 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.40 (1H, s, NH), 8.75 (2H, d, *J* = 6.3 Hz, H2, H6), 7.92 (1H, s, NH), 7.79 (2H, d, *J* = 6.2 Hz, H3, H5), 6.55 (1H, t, *J* = 5.6 Hz, NH), 3.06-2.94 (2H, m, $C^{1}H_2$), 1.46-1.32 (2H, m, $C^{2}H_2$), 1.31-1.16 (6H, m, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$), 0.86 (3H, t, *J* = 6.7 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.73, 158.12, 150.18, 139.82, 121.37, 40.26, 31.06, 29.81, 25.98, 22.10, 13.94. Anal. Calcd for $C_{13}H_{20}N_4O_2$ (264.32): C, 59.07; H, 7.63; N, 21.20. Found: C, 59.24; H, 7.80; N, 22.33.

3.1.1.7. N-Heptyl-2-isonicotinoylhydrazine-1-carboxamide (**2g**). Yield 83 % (method A); mp 173-175 °C (lit.[21] 150-152.5 °C). IR (ATR): 3291, 3080, 2928, 2855, 1671, 1557, 1522, 1508, 1436, 1406, 1375, 1340, 1296, 1220, 1142, 1063, 997, 904, 850, 758, 677, 669 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.40 (1H, s, NH), 8.75 (2H, d, *J* = 5.8 Hz, H2, H6), 7.92 (1H, s, NH), 7.78 (2H, d, *J* = 5.7 Hz, H3, H5), 6.57 (1H, t, *J* = 5.6 Hz, NH), 3.04-2.93 (2H, m, $C^{1}H_2$), 1.46-1.16 (10H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$), 0.88 (3H, t, *J* = 6.4 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.95, 158.03, 150.42, 139.78, 121.66, 40.27, 31.26, 29.85, 28.75, 26.37, 22.11, 13.95. Anal. Calcd for $C_{14}H_{22}N_4O_2$ (278.36): C, 60.41; H, 7.97; N, 20.13. Found: C, 60.68; H, 7.71; N, 20.35.

3.1.1.8. 2-Isonicotinoyl-*N*-octylhydrazine-1-carboxamide (**2h**). Yield 82 % (method A); mp 168-170 °C. IR (ATR): 3292, 3080, 2923, 2855, 1671, 1632, 1557, 1522, 1508, 1487, 1436, 1406, 1375,

1340, 1295, 1219, 1141, 1063, 997, 907, 850, 758, 721, 674 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.39 (1H, s, NH), 8.74 (2H, d, *J* = 5.9 Hz, H2, H6), 7.92 (1H, s, NH), 7.79 (2H, d, *J* = 5.6 Hz, H3, H5), 6.57 (1H, t, *J* = 5.6 Hz, NH), 3.06-2.94 (2H, m, $C^{1}H_2$), 1.47-1.16 (12H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$), 0.88 (3H, t, *J* = 7.7 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.93, 158.24, 149.98, 140.02, 121.57, 40.34, 31.52, 30.01, 29.00, 28.93, 26.32, 22.24, 13.97. Anal. Calcd for $C_{15}H_{24}N_4O_2$ (292.38): C, 61.62; H, 8.27; N, 19.16. Found: C, 62.05; H, 8.09; N, 19.17.

3.1.1.9. 2-Isonicotinoyl-*N*-nonylhydrazine-1-carboxamide (**2i**). Yield 87 % (method A); mp 168-170 °C. IR (ATR): 3291, 3082, 2921, 2853, 1671, 1636, 1557, 1517, 1508, 1488, 1472, 1437, 1406, 1375, 1340, 1296, 1220, 1135, 1063, 998, 900, 851, 760, 720, 687 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.39 (1H, s, NH), 8.74 (2H, d, *J* = 6.5 Hz, H2, H6), 7.92 (1H, s, NH), 7.88 (2H, d, *J* = 6.5 Hz, H3, H5), 6.56 (1H, t, *J* = 5.8 Hz, NH), 3.06-2.93 (2H, m, $C^{1}H_2$), 1.50-1.15 (14H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$), 0.83 (3H, t, *J* = 7.4 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.83, 158.28, 150.21, 139.78, 121.46, 40.25, 31.42, 29.95, 29.21, 28.92, 28.83, 26.39, 22.18, 14.16. Anal. Calcd for $C_{16}H_{26}N_4O_2$ (306.41): C, 62.72; H, 8.55; N, 18.29. Found: C, 62.87; H, 8.83; N, 17.98.

3.1.1.10. N-Decyl-2-isonicotinoylhydrazine-1-carboxamide (**2j**). Yield 76 % (method C); mp 168-169 °C. IR (ATR): 3321, 3061, 2925, 2851, 1683, 1508, 1484, 1436, 1419, 1327, 1278, 1229, 1185, 1055, 1009, 905, 835, 754, 725, 653 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.46 (1H, s, NH), 8.79 (2H, d, *J* = 6.8 Hz, H2, H6), 7.95 (1H, s, NH), 7.87 (2H, d, *J* = 6.1 Hz, H3, H5), 6.54 (1H, t, *J* = 5.6 Hz, NH), 3.04-2.97 (2H, m, $C^{1}H_2$), 1.49-1.17 (16H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$), 0.85 (3H, t, *J* = 6.9 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.95, 158.14, 149.39, 139.65, 121.58, 40.20, 31.42, 29.88, 29.23, 29.15, 28.99, 28.88, 26.47, 22.23, 14.01. Anal. Calcd for $C_{17}H_{28}N_4O_2$ (320.44): C, 63.72; H, 8.81; N, 17.48. Found: C, 63.82; H, 8.61; N, 17.94.

3.1.1.11. 2-Isonicotinoyl-*N*-undecylhydrazine-1-carboxamide (**2k**). Yield 80 % (method A); mp 169-170 °C. IR (ATR): 3275, 3083, 2924, 2853, 1671, 1553, 1522, 1487, 1436, 1406, 1375, 1341, 1303, 1221, 1133, 1064, 997, 913, 852, 758, 721, 670, 657 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.39 (1H, s, NH), 8.74 (2H, d, *J* = 6.4 Hz, H2, H6), 7.91 (1H, s, NH), 7.79 (2H, d, *J* = 5.9 Hz, H3, H5), 6.53 (1H, t, *J* = 5.7 Hz, NH), 3.05-2.95 (2H, m, $C^{1}H_2$), 1.48-1.16 (18H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$), 0.86 (3H, t, *J* = 6.9 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.77, 158.21, 150.40, 139.96, 121.64, 40.44, 31.52, 30.04, 29.26, 29.23, 29.18, 29.03, 28.93, 26.50, 22.30, 14.15. Anal. Calcd for $C_{18}H_{30}N_4O_2$ (334.46): C, 64.64; H, 9.04; N, 16.75. Found: C, 64.86; H, 8.89; N, 16.97.

3.1.1.12. N-Dodecyl-2-isonicotinoylhydrazine-1-carboxamide (**2l**). Yield 83 % (method A); mp 167-169 °C. IR (ATR): 3291, 3088, 2923, 2853, 1663, 1554, 1509, 1486, 1436, 1407, 1376, 1348, 1296, 1220, 1136, 1063, 997, 901, 850, 751, 721, 692 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.38 (1H, s, NH), 8.76 (2H, d, *J* = 6.4 Hz, H2, H6), 7.90 (1H, s, NH), 7.80 (2H, d, *J* = 5.6 Hz, H3, H5), 6.52 (1H, t, *J* = 5.7 Hz, NH), 3.05-2.96 (2H, m, $C^{1}H_2$), 1.47-1.16 (20H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$, $C^{11}H_2$), 0.85 (3H, t, *J* = 6.6 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.85, 158.19, 150.37, 139.93, 121.61, 40.29, 31.48, 30.08, 29.25, 29.22, 29.19, 29.15, 29.01, 28.89, 26.48, 22.26, 14.11. Anal. Calcd for $C_{19}H_{32}N_4O_2$ (348.49): C, 65.48; H, 9.26; N, 16.08. Found: C, 65.82; H, 8.98; N, 15.95.

3.1.1.13. 2-Isonicotinoyl-*N*-tridecylhydrazine-1-carboxamide (**2m**). Yield 68 % (method C); mp 167-169 °C. IR (ATR): 3293, 3087, 2922, 2852, 1664, 1555, 1509, 1486, 1436, 1407, 1376, 1348, 1297, 1220, 1145, 1063, 997, 901, 850, 757, 721, 687 cm^{-1}. ^{1}H NMR (DMSO, 300 MHz): δ 10.38 (1H, s, NH), 8.75 (2H, d, *J* = 6.8 Hz, H2, H6), 7.92 (1H, s, NH), 7.77 (2H, d, *J* = 5.4 Hz, H3, H5), 6.55 (1H, t, *J* = 5.7 Hz, NH), 3.05-2.96 (2H, m, $C^{1}H_2$), 1.49-1.17 (22H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$, $C^{11}H_2$, $C^{12}H_2$), 0.85 (3H, t, *J* = 6.6 Hz, CH_3). ^{13}C NMR (DMSO, 75 MHz): δ 164.93, 158.23, 150.41, 139.97, 121.66, 40.28, 31.52, 30.05, 29.29, 29.25, 29.23, 29.21, 29.18, 29.06, 28.94, 26.51, 22.32, 14.17. Anal. Calcd for $C_{20}H_{34}N_4O_2$ (362.52): C, 66.26; H, 9.45; N, 15.46. Found: C, 66.51; H, 9.34; N, 15.73.

3.1.1.14. 2-Isonicotinoyl-*N*-tetradecylhydrazine-1-carboxamide (**2n**). Yield 72 % (method A); mp 167-169 °C. IR (ATR): 3294, 3087, 2922, 2852, 1664, 1632, 1554, 1513, 1484, 1433, 1408, 1377, 1349, 1296, 1220, 1145, 1063, 998, 902, 850, 758, 721, 692, 665 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.37 (1H, s, NH), 8.72 (2H, d, *J* = 6.9 Hz, H2, H6), 7.90 (1H, s, NH), 7.77 (2H, d, *J* = 5.6 Hz, H3, H5), 6.53 (1H, t, *J* = 5.7 Hz, NH), 3.05-2.94 (2H, m, $C^{1}H_2$), 1.50-1.18 (24H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$, $C^{11}H_2$, ,$C^{12}H_2$, $C^{13}H_2$), 0.88 (3H, t, *J* = 6.4 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.90, 158.18, 150.37, 139.96, 121.62, 40.25, 31.47, 30.01, 29.25, 29.23, 29.22, 29.20, 29.17, 29.14, 29.01, 28.90, 26.48, 22.27, 14.12. Anal. Calcd for $C_{21}H_{36}N_4O_2$ (376.55): C, 66.99; H, 9.64; N, 14.88. Found: C, 66.72; H, 9.75; N, 14.52.

3.1.1.15. 2-Isonicotinoyl-*N*-pentadecylhydrazine-1-carboxamide (**2o**). Yield 65 % (method C); mp 168-170 °C. IR (ATR): 3319, 3024, 2921, 2849, 1645, 1555, 1481, 1408, 1382, 1338, 1287, 1065, 1005, 919, 846, 724, 672 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.47 (1H, s, NH), 8.73 (2H, d, *J* = 6.5 Hz, H2, H6), 7.93 (1H, s, NH), 7.74 (2H, d, *J* = 5.6 Hz, H3, H5), 6.58 (1H,t, *J* = 6.1 Hz, NH), 3.06-2.94 (2H, m, $C^{1}H_2$), 1.47-1.14 (26H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$, $C^{11}H_2$, ,$C^{12}H_2$, $C^{13}H_2$, $C^{14}H_2$), 0.85 (3H, t, *J* = 6.2 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.83, 158.02, 150.29, 139.89, 121.37, 40.28, 31.50, 30.06, 29.26, 29.24, 29.21, 29.20, 29.16, 29.12, 29.08, 29.03, 28.92, 26.54, 22.97, 14.08. Anal. Calcd for $C_{22}H_{38}N_4O_2$ (390.57): C, 67.66; H, 9.81; N, 14.35. Found: C, 67.98; H, 9.65; N, 14.63.

3.1.1.16. N-Hexadecyl-2-isonicotinoylhydrazine-1-carboxamide (**2p**). Yield 79 % (method A); mp 169-170 °C. IR (ATR): 3314, 3024, 2920, 2849, 1647, 1557, 1508, 1481, 1409, 1338, 1280, 1233, 1065, 994, 846, 755, 724, 661 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.38 (1H, s, NH), 8.72 (2H, d, *J* = 6.5 Hz, H2, H6), 7.90 (1H, s, NH), 7.75 (2H, d, *J* = 5.8 Hz, H3, H5), 6.53 (1H, t, *J* = 5.9 Hz, NH), 3.07-2.95 (2H, m, $C^{1}H_2$), 1.48-1.08 (28H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$, $C^{11}H_2$, ,$C^{12}H_2$, $C^{13}H_2$, $C^{14}H_2$, $C^{15}H_2$), 0.87 (3H, t, *J* = 6.3 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.68, 157.97, 150.23, 139.68, 121.40, 40.20, 31.46, 30.05, 29.27, 29.25, 29.23, 29.21, 29.19, 29.17, 29.15, 29.12, 29.05, 28.93, 26.48, 22.23, 14.01. Anal. Calcd for $C_{23}H_{40}N_4O_2$ (404.60): C, 68.28; H, 9.97; N, 13.85. Found: C, 68.33; H, 9.78; N, 13.99.

3.1.1.17. 2-Isonicotinoyl-*N*-octadecylhydrazine-1-carboxamide (**2q**). Yield 71 % (method A); mp 171-172 °C (lit.[32] 173-175 °C). IR (ATR): 3291, 2920, 2851, 1671, 1557, 1521, 1508, 1487, 1436, 1406, 1375, 1340, 1296, 1221, 1136, 1063, 998, 901, 850, 756, 721, 692 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 10.39 (1H, s, NH), 8.78 (2H, d, *J* = 6.6 Hz, H2, H6), 7.93 (1H, s, NH), 7.72 (2H, d, *J* = 5.5 Hz, H3, H5), 6.54 (1H,t, *J* = 6.1 Hz, NH), 3.09-2.93 (2H, m, $C^{1}H_2$), 1.47-1.09 (32H, m, $C^{2}H_2$, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$, $C^{11}H_2$, ,$C^{12}H_2$, $C^{13}H_2$, $C^{14}H_2$, $C^{15}H_2$, $C^{16}H_2$,

$C^{17}H_2$), 0.86 (3H, t, *J* = 6.4 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.70, 158.07, 150.30, 139.49, 121.57, 40.22, 31.48, 30.05, 29.25, 29.23, 29.22, 29.20, 29.18, 29.17, 29.15, 29.13, 29.11, 29.08, 29.05, 28.92, 26.49, 22.30, 13.99. Anal. Calcd for $C_{25}H_{44}N_4O_2$ (432.65): C, 69.40; H, 10.25; N, 12.95. Found: C, 68.51; H, 10.01; N, 12.76.

3.1.2. Cyclization to 5-(pyridin-4-yl)-1,3,4-oxadiazole-2-amines **3**

Triphenylphosphine (393.4 mg, 1.5 mmol) was added to the stirred suspension of 1,2-dibromo-1,1,2,2-tetrachloroethane (271.1 mg, 0.83 mmol) and the appropriate *N*-alkyl-2-isonicotinoylhydrazine-1-carboxamide **2** (0.75 mmol) in anhydrous acetonitrile or tetrahydrofuran (4 mL) at room temperature. The reaction mixture was stirred at room temperature for 10 min and cooled to 0 °C. Then, triethylamine (459 µL, 3.3 mmol) was added dropwise, and the stirring was continued for an additional 10 hours. A solid phase was filtered off. The resulting liquid was evaporated until dryness and dissolved in ethyl acetate. Undissolved solid was filtered off and recrystallized from acetonitrile, if necessary, giving the pure oxadiazole products **3**. Alternatively, they were purified using column chromatography and the mixture toluene/ethyl acetate 2:1 as the eluent.

3.1.2.1. N-Methyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3a**). Yield 57 %; mp 146-147 °C. IR (ATR): 3174, 2930, 1651, 1604, 1578, 1493, 1417, 1380, 1057, 1030, 824, 748, 731, 681. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.71 (2H, d, *J* = 5.9 Hz, H2, H6), 7.74 (2H, d, *J* = 6.1 Hz, H3, H5), 5.85 (1H, q, *J* = 4.8 Hz, NH), 3.12 (3H, d, *J* = 4.1 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 164.66, 156.95, 150.46, 131.49, 119.19, 29.83. Anal. Calcd for $C_8H_8N_4O$ (176.18): C, 54.54; H, 4.58; N, 31.80. Found: C, 54.32; H, 4.69; N, 31.62.

3.1.2.2. N-Ethyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3b**). Yield 66 %; mp 147-148 °C (lit.[34] 131-132 °C). IR (ATR): 3191, 3139, 2977, 2866, 1654, 1628, 1579, 1494, 1463, 1451, 1421, 1377, 1061, 1038, 830, 756, 730, 681. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.72 (2H, d, *J* = 6.0 Hz, H2, H6), 7.73 (2H, d, *J* = 6.1 Hz, H3, H5), 5.84 (1H, t, *J* = 6.0 Hz, NH), 3.55-3.47 (2H, m, CH_2), 1.33 (3H, t, *J* = 7.2 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 163.97, 156.82, 150.46, 131.52, 119.15, 38.49, 14.96. Anal. Calcd for $C_9H_{10}N_4O$ (190.21): C, 56.83; H, 5.30; N, 29.46. Found: C, 56.77; H, 5.60; N, 29.49.

3.1.2.3. N-Propyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3c**). Yield 58 %; mp 112-113 °C. IR (ATR): 3212, 2967, 1638, 1614, 1578, 1508, 1486, 1458, 1417, 1394, 1048, 827, 753, 721, 696. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.71 (2H, d, *J* = 6.1 Hz, H2, H6), 7.78 (2H, d, *J* = 5.7 Hz, H3, H5), 5.82 (1H, t, *J* = 5.6 Hz, NH), 3.48-3.36 (2H, m, C^1H_2), 1.69-1.60 (2H, m, C^2H_2), 0.92 (3H, t, *J* = 7.1 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 164.27, 156.52, 150.38, 131.46, 119.18, 45.86, 22.98, 11.34. Anal. Calcd for $C_{10}H_{12}N_4O$ (204.23): C, 58.81; H, 5.92; N, 27.43. Found: C, 58.78; H, 5.73; N, 27.13.

3.1.2.4. N-Butyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3d**). Yield 64 %; mp 106-108°C. IR (ATR): 3240, 2936, 1627, 1606, 1576, 1458, 1422, 1357, 1027, 830, 754, 722, 696 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.70 (2H, d, *J* = 6.1 Hz, H2, H6), 7.76 (2H, d, *J* = 5.9 Hz, H3, H5), 5.68 (1H, t, *J* = 5.4 Hz, NH), 3.50-3.39 (2H, m, C^1H_2), 1.58-1.50 (2H, m, C^2H_2), 1.44-1.29 (2H, m, C^3H_2), 0.91 (3H, t, *J* = 6.9 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 164.38, 156.53, 150.32, 131.58,

118.96, 43.38, 25.65, 19.20, 13.64. Anal. Calcd for $C_{11}H_{14}N_4O$ (218.26): C, 60.53; H, 6.47; N, 25.67. Found: C, 60.85; H, 6.32; N, 25.44.

3.1.2.5. N-Pentyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3e**). Yield 52 %; mp 105-106 °C. IR (ATR): 2958, 2930, 2859, 1625, 1605, 1574, 1437, 1414, 1373, 1044, 828, 749, 722, 695 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 8.74 (2H, d, *J* = 6.1 Hz, H2, H6), 7.74 (2H, d, *J* = 5.7 Hz, H3, H5), 5.56 (1H, t, *J* = 5.8 Hz, NH), 3.49-3.38 (2H, m, C^1H_2), 1.76-1.63 (2H, m, C^2H_2), 1.45-1.27 (4H, m, C^3H_2, C^4H_2), 0.91 (3H, t, *J* = 7.0 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.12, 156.58, 150.49, 131.68, 119.21, 43.68, 29.25, 28.75, 22.26, 13.94. Anal. Calcd for $C_{12}H_{16}N_4O$ (232.29): C, 62.05; H, 6.94; N, 24.12. Found: C, 62.28; H, 6.67; N, 24.50.

3.1.2.6. N-Hexyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3f**). Yield 58 %; mp 105-106 °C. IR (ATR): 3333, 2953, 2923, 2861, 1611, 1573, 1500, 1480, 1415, 1389, 1057, 1032, 829, 740, 726, 696 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ 8.71 (2H, d, *J* = 6.1 Hz, H2, H6), 7.73 (2H, d, *J* = 6.1 Hz, H3, H5), 5.70 (1H, t, *J* = 5.9 Hz, NH), 3.49-3.41 (2H, m, C^1H_2), 1.74-1.63 (2H, m, C^2H_2), 1.45-1.25 (6H, m, C^3H_2, C^4H_2, C^5H_2), 0.88 (3H, t, *J* = 7.2 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 164.20, 156.75, 150.41, 131.54, 119.19, 43.66, 31.51, 30.33, 26.29, 22.49, 13.95. Anal. Calcd for $C_{13}H_{18}N_4O$ (246.31): C, 63.39; H, 7.37; N, 22.75. Found: C, 63.54; H, 7.17; N, 22.52.

3.1.2.7. N-Heptyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3g**). Yield 61 %; mp 102-103 °C. IR (ATR): 3333, 2955, 2922, 2857, 1612, 1573, 1500, 1481, 1417, 1392, 1058, 1040, 829, 754, 748, 721, 696 cm^{-1}. ^1H NMR (DMSO, 500 MHz): δ 8.75 (2H, d, J = 6.7 Hz, H2, H6), 7.91 (1H, t, *J* = 6.1 Hz, NH), 7.70 (2H, d, *J* = 6.5 Hz, H3, H5), 3.33-3.21 (2H, m, C^1H_2), 1.57-1.48 (2H, m, C^2H_2), 1.40-1.22 (8H, m, C^3H_2, C^4H_2, C^5H_2, C^6H_2), 0.84 (3H, t, *J* = 7.3 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.88, 155.16, 150.35, 131.94, 120.60, 44.74, 31.44, 29.76, 28.63, 26.41, 22.21, 14.12. Anal. Calcd for $C_{14}H_{20}N_4O$ (260.34): C, 64.59; H, 7.74; N, 21.52. Found: C, 64.48; H, 7.92; N, 21.33.

3.1.2.8. N-Octyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3h**). Yield 66 %; mp 96-98 °C. IR (ATR): 3285, 2954, 2922, 2850, 1611, 1573, 1480, 1470, 1417, 1389, 1053, 1034, 829, 741, 730, 721, 695 cm^{-1}. ^1H NMR (DMSO, 500 MHz): δ 8.74 (2H, d, *J* = 6.7 Hz, H2, H6), 8.05 (1H, t, *J* = 5.8 Hz, NH), 7.64 (2H, d, *J* = 6.5 Hz, H3, H5), 3.30-3.23 (2H, m, C^1H_2), 1.59-1.51 (2H, m, C^2H_2), 1.38-1.21 (10H, m, C^3H_2, C^4H_2, C^5H_2, C^6H_2, C^7H_2), 0.84 (3H, t, *J* = 7.1 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.68, 155.95, 150.83, 131.63, 118.88, 42.78, 31.40, 29.72, 28.92, 28.85, 26.41, 22.25, 14.09. Anal. Calcd for $C_{15}H_{22}N_4O$ (274.37): C, 65.67; H, 8.08; N, 20.42. Found: C, 65.42; H, 8.25; N, 20.35.

3.1.2.9. N-Nonyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3i**). Yield 65 %; mp 95-96 °C. IR (ATR): 3334, 2921, 2853, 1613, 1573, 1482, 1415, 1044, 829, 752, 741, 721, 697 cm^{-1}. ^1H NMR (DMSO, 500 MHz): δ 8.72 (2H, d, *J* = 6.4 Hz, H2, H6), 8.01 (1H, t, *J* = 6.1 Hz, NH), 7.63 (2H, d, *J* = 6.4 Hz, H3, H5), 3.28-3.19 (2H, m, C^1H_2), 1.58-1.51 (2H, m, C^2H_2), 1.35-1.21 (12H, m, C^3H_2, C^4H_2, C^5H_2, C^6H_2, C^7H_2, C^8H_2), 0.83 (3H, t, *J* = 7.0 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.32, 155.97, 150.80, 131.52, 118.87, 42.73, 31.26, 29.69, 29.19, 28.96, 28.85, 26.39, 22.16, 13.95. Anal. Calcd for $C_{16}H_{24}N_4O$ (288.40): C, 66.64; H, 8.39; N, 19.43. Found: C, 66.39; H, 8.12; N, 19.55.

3.1.2.10. *N*-Decyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3j**). Yield 55 %; mp 97-98 °C. IR (ATR): 3275, 2954, 2918, 2849, 1613, 1573, 1493, 1471, 1416, 1390, 1058, 1049, 829, 741, 733, 720, 695 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 8.78 (2H, d, *J* = 6.4 Hz, H2, H6), 8.05 (1H, t, *J* = 6.2 Hz, NH), 7.70 (2H, d, *J* = 6.4 Hz, H3, H5), 3.26-3.19 (2H, m, $C^{1}H_2$), 1.59-1.50 (2H, m, $C^{2}H_2$), 1.38-1.22 (14H, m, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$), 0.82 (3H, t, *J* = 7.0 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.37, 156.01, 150.90, 131.63, 118.93, 42.75, 31.48, 29.70, 29.20, 29.14, 28.91, 28.86, 26.36, 22.28, 14.06. Anal. Calcd for $C_{17}H_{26}N_4O$ (302.42): C, 67.52; H, 8.67; N, 18.53. Found: C, 67.48; H, 8.98; N, 18.59.

3.1.2.11. 5-(Pyridin-4-yl)-*N*-undecyl-1,3,4-oxadiazol-2-amine (**3k**). Yield 63 %; mp 102-103 °C. IR (ATR): 3335, 2953, 2923, 2859, 1611, 1573, 1501, 1480, 1468, 1413, 1390, 1056, 1031, 828, 740, 725, 696 cm^{-1}. H NMR (DMSO, 500 MHz): δ 8.73 (2H, d, *J* = 6.5 Hz, H2, H6), 8.03 (1H, t, *J* = 6.2 Hz, NH), 7.65 (2H, d, *J* = 6.4 Hz, H3, H5), 3.28-3.20 (2H, m, $C^{1}H_2$), 1.59-1.51 (2H, m, $C^{2}H_2$), 1.39-1.22 (16H, m, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$), 0.83 (3H, t, *J* = 6.9 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.33, 155.96, 150.84, 131.44, 118.88, 42.74, 31.02, 29.74, 29.18, 29.14, 29.06, 28.93, 28.86, 25.97, 22.19, 13.99. Anal. Calcd for $C_{18}H_{28}N_4O$ (316.45): C, 68.32; H, 8.92; N, 17.71. Found: C, 68.12; H, 8.76; N, 17.65.

3.1.2.12. *N*-Dodecyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3l**). Yield 68 %; mp 105-107 °C. IR (ATR): 3331, 2955, 2918, 2851, 1612, 1573, 1501, 1481, 1471, 1414, 1389, 1053, 829, 741, 730, 719, 697 cm^{-1}. ^{1}H NMR (DMSO, 500 MHz): δ 8.75 (2H, d, *J* = 6.5 Hz, H2, H6), 8.03 (1H, t, *J* = 6.0 Hz, NH), 7.72 (2H, d, *J* = 6.5 Hz, H3, H5), 3.28-3.19 (2H, m, $C^{1}H_2$), 1.60-1.51 (2H, m, $C^{2}H_2$), 1.38-1.20 (18H, m, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$, $C^{11}H_2$), 0.84 (3H, t, *J* = 7.0 Hz, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 164.38, 156.18, 150.78, 131.58, 119.11, 42.82, 31.38, 29.75, 29.21, 29.17, 29.13, 29.08, 28.96, 28.87, 26.23. 22.16, 14.10. Anal. Calcd for $C_{19}H_{30}N_4O$ (330.48): C, 69.05; H, 9.15; N, 16.95. Found: C, 69.14; H, 9.22; N, 16.72.

3.1.2.13. 5-(Pyridin-4-yl)-*N*-tridecyl-1,3,4-oxadiazol-2-amine (**3m**). Yield 54 %; mp 105-107 °C. IR (ATR): 3325, 2955, 2920, 2851, 1613, 1573, 1507, 1481, 1470, 1417, 1388, 1060, 1035, 830, 754, 721, 696 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.69 (2H, d, *J* = 5.9 Hz, H2, H6), 7.70 (2H, d, *J* = 6.3 Hz, H3, H5), 5.63 (1H, t, *J* = 5.8 Hz, NH), 3.49-3.41 (2H, m, $C^{1}H_2$), 1.71-1.66 (2H, m, $C^{2}H_2$), 1.44-1.19 (20H, m, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$, $C^{11}H_2$, $C^{12}H_2$), 0.88 (3H, t, *J* = 6.7 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 164.11, 156.82, 150.51, 131.55, 119.16, 43.69, 31.87, 29.64, 29.62, 29.59, 29.57, 29.53, 29.49, 29.31, 29.19, 26.65, 22.64, 14.07. Anal. Calcd for $C_{20}H_{32}N_4O$ (344.50): C, 69.73; H, 9.36; N, 16.26. Found: C, 69.55; H, 9.16; N, 16.43.

3.1.2.14. 5-(Pyridin-4-yl)-*N*-tetradecyl-1,3,4-oxadiazol-2-amine (**3n**). Yield 59 %; mp 109-111 °C. IR (ATR): 3275, 2955, 2920, 2850, 1614, 1574, 1472, 1418, 1390, 1059, 830, 741, 732, 720, 697 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.73 (2H, d, *J* = 6.4 Hz, H2, H6), 7.75 (2H, d, *J* = 6.4 Hz, H3, H5), 5.55 (1H, t, *J* = 5.8 Hz, NH), 3.50-3.42 (2H, m, $C^{1}H_2$), 1.72-1.66 (2H, m, $C^{2}H_2$) 1.43-1.23 (22H, m, $C^{3}H_2$, $C^{4}H_2$, $C^{5}H_2$, $C^{6}H_2$, $C^{7}H_2$, $C^{8}H_2$, $C^{9}H_2$, $C^{10}H_2$, $C^{11}H_2$, $C^{12}H_2$, $C^{13}H_2$), 0.88 (3H, t, *J* = 6.8 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 164.07, 156.84, 150.51, 131.54, 119.16, 43.70, 31.87, 29.64, 29.62, 29.60, 29.57, 29.56, 29.53, 29.48. 29.30, 29.18, 26.64, 22.64, 14.07. Anal. Calcd for $C_{21}H_{34}N_4O$ (358.53): C, 70.35; H, 9.56; N, 15.63. Found: C, 70.11; H, 9.75; N, 15.83.

3.1.2.15. N-Pentadecyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3o**). Yield 60 %; mp 108-109 °C. IR (ATR): 3327, 2955, 2919, 2850, 1613, 1573, 1481, 1471, 1417, 1390, 1057, 829, 740, 731, 719, 696 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ 8.73 (2H, d, *J* = 5.7 Hz, H2, H6), 7.78 (2H, d, *J* = 6.3 Hz, H3, H5), 5.50 (1H, t, *J* = 5.9 Hz, NH), 3.50-3.42 (2H, m, C^1H_2), 1.73-1.67 (2H, m, C^2H_2), 1.42-1.21 (24H, m, C^3H_2, C^4H_2, C^5H_2, C^6H_2, C^7H_2, C^8H_2, C^9H_2, $C^{10}H_2$, $C^{11}H_2$, $C^{12}H_2$, $C^{13}H_2$, $C^{14}H_2$), 0.88 (3H, t, *J* = 6.8 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 164.06, 156.80, 150.35, 131.67, 119.21, 43.71, 31.88, 29.65, 29.64, 29.62, 29.61, 29.59, 29.57, 29.53, 29.49, 29.31, 29.20, 26.64, 22.65, 14.08. Anal. Calcd for $C_{22}H_{36}N_4O$ (372.56): C, 70.93; H, 9.74; N, 15.04. Found: C, 70.72; H, 9.53; N, 15.27.

3.1.2.16. N-Hexadecyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3p**). Yield 63 %; mp 109-111 °C. IR (ATR): 3330, 2955, 2918, 2851, 1612, 1572, 1501, 1481, 1471, 1415, 1389, 1052, 1042, 830, 741, 730, 718, 697 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ 8.71 (2H, d, *J* = 6.4 Hz, H2, H6), 7.75 (2H, d, *J* = 6.6 Hz, H3, H5), 5.49 (1H, t, *J* = 5.9 Hz, NH), 3.49-3.40 (2H, m, C^1H_2), 1.73-1.67 (2H, m, C^2H_2), 1.45-1.21 (26H, m, C^3H_2, C^4H_2, C^5H_2, C^6H_2, C^7H_2, C^8H_2, C^9H_2, $C^{10}H_2$, $C^{11}H_2$, $C^{12}H_2$, $C^{13}H_2$, $C^{14}H_2$, $C^{15}H_2$), 0.88 (3H, t, *J* = 6.7 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 164.07, 156.86, 150.53, 131.53, 119.17, 43.71, 31.89, 29.68, 29.66, 29.63, 29.62, 29.60, 29.59, 29.57, 29.54, 29.50, 29.32, 29.19, 26.65, 22.65, 14.08. Anal. Calcd for $C_{23}H_{38}N_4O$ (386.58): C, 71.46; H, 9.91; N, 14.49. Found: C, 71.59; H, 10.04; N, 14.53.

3.1.2.17. N-Octadecyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine (**3q**). Yield 65 %; mp 114-115 °C. IR (ATR): 3293, 2954, 2917, 2850, 1611, 1572, 1507, 1481, 1471, 1414, 1388, 1050, 829, 741, 730, 717, 696 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ 8.72 (2H, d, *J* = 6.3 Hz, H2, H6), 7.80 (2H, d, *J* = 6.3 Hz, H3, H5), 5.48 (1H, t, *J* = 5.9 Hz, NH), 3.41-3.32 (2H, m, C^1H_2), 1.74-1.68 (2H, m, C^2H_2) 1.45-1.20 (30H, m, C^3H_2, C^4H_2, C^5H_2, C^6H_2, C^7H_2, C^8H_2, C^9H_2, $C^{10}H_2$, $C^{11}H_2$, $C^{12}H_2$, $C^{13}H_2$, $C^{14}H_2$, $C^{15}H_2$, $C^{16}H_2$, $C^{17}H_2$), 0.88 (3H, t, *J* = 6.5 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 164.08, 156.83, 149.84, 131.41, 118.98, 43.73, 31.91, 29.73, 29.69, 29.68, 29.66, 29.64, 29.63, 29.61, 29.59, 29.58, 29.55, 29.50, 29.33, 29.19, 26.66. 22.58, 14.08. Anal. Calcd for $C_{25}H_{42}N_4O$ (414.64): C, 72.42; H, 10.21; N, 13.51. Found: C, 72.64; H, 10.33; N, 13.28.

4.2. Biological Activity

4.2.1. *In vitro* Antimycobacterial Susceptibility Determination

Both series were evaluated for their *in vitro* antimycobacterial activity against *M. tuberculosis* 331/88 (i.e., $H_{37}Rv$; dilution of this strain was 10^{-3}), *Mycobacterium avium* 330/88 (resistant to INH, RIF, ofloxacin (OFX) and EMB; dilution 10^{-5}) and two strains of *M. kansasii*: 235/80 (dilution 10^{-4}) and the clinically isolated strain 6509/96 (dilution 10^{-5}). The used method is described in ref. [35]. The following concentrations were used: 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 μM. MIC (reported in μM) was the lowest concentration at which the complete inhibition of mycobacterial growth occurred. Isoniazid (INH) as both a first-line oral antimycobacterial drug and the synthetic precursor was used as the reference compound.

The most active derivatives (**2a**-**c**, **3l**) were evaluated under similar conditions against four MDR-TB strains and one XDR-TB strain (dilution 10^{-3}) with different resistance patterns. All strains were resistant to INH, RIF, rifabutine, and streptomycin (STM); an additional resistance was present in some cases: 234/2005 resistant additionally to EMB; Praha 1 resistant additionally to EMB, and clofazimine; Praha 4 to EMB, OFX, and clofazimine; and Praha 131 with an additional resistance to

EMB, OFX, gentamicin and amikacin (*i.e.*, XDR-TB strain). The following concentrations were used: 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, and 1 μM.

4.2.2. Cytotoxicity Evaluation (HepG2 cells)[35]

All of the carboxamides **2** and the selected 1,3,4-oxadiazoles (**3j-l**) were tested for their cytotoxicity in the human hepatocellular liver carcinoma cell line HepG2 (passage 2-4; ECACC, Salisbury, UK) using a standard colorimetric method that involves measuring a tetrazolium salt reduction (CellTiter(R) 96 AQueous One Solution Assay, Promega G3580, Fitchburg, USA).

The cells were routinely cultured in Eagle's minimum essential media supplemented with 10% foetal bovine serum, 1% L-glutamine solution, and a non-essential amino acid solution. The investigated compounds were dissolved in a very small volume of DMSO, and a small volume was added to the cell culture. The tested compounds were prepared in triplicate at eight incubation concentrations. The following types of controls were included: determination of 100% viability and 0% viability (the cells treated by 10% DMSO), no cell control, control for the determination of possible interaction of tested compounds with reagents, control of the setting of incubation medium and the control of the toxicity of DMSO.

The results are expressed as the inhibitory concentration that reduces cell viability to 50% of the maximal (control) viability (IC_{50}). IC_{50} was calculated in each of the tested substances using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA) and Microsoft Excel 2010.

4.2.3. Analysis of sensitivity of *Mtb.* H_{37}Ra strain overproducing InhA to **2a** and **3l**

InhA protein was overproduced in *Mtb.* H37Ra using pMV261-InhA construct as already described. Sensitivity of InhA overproducing strain, as well as a control strain carrying an empty vector to compounds **2a** and **3l** were analysed by drop dilution method. Both cultures grown in 7H9 broth supplemented with albumin-dextrose-catalase and 0.05% Tween 80 were adjusted to O.D.(600) 0.5 and 4 μl aliquots of 10^0, 10^{-1}, 10^{-2} and 10^{-3} dilutions were dropped on 7H11 agar supplemented with oleic acid-albumin-dextrose-catalase and different concentrations of tested compounds dissolved in DMSO (2 % final concentration). Plates were incubated for 25 days at 37 °C.

4.2.4. Determination of mode of action of **2a** and **3l** in *Mtb.* H_{37}Ra

The mechanism of action of **2a** was analyzed by metabolic labeling of *Mtb.* H_{37}Ra strain with ^{14}C glucose. *Mtb.* H_{37}Ra culture was grown in GAS media supplemented by 0.025% tyloxapol at 37 °C till O.D. (600) reached 0.307. Then tested compound dissolved in DMSO and INH were added in final concentrations 40 μM and 36.5 μM. The final concentration of DMSO was kept at 1%. ^{14}C glucose (specific activity 290 mCi/mmol, ARC) in the final concentration of 1 μCi/mL was added to each of the cultures after 1 h of cultivation with drugs and the cells were cultivated for next 24 h. Cells harvested from 18 ml cultures were 15 min incubated with 98% ethanol at 70 °C. Samples were centrifuged for 10 min at 10 000 x g and resulting pellets were delipidated by two 1 h extractions with chloroform: methanol (2:1, v/v) at 56 °C. These extracts were combined with ethanol extracts, dried under the stream of N_2 and subjected to biphasic Folch washing. The bottom organic phase was dried and dissolved in chloroform: methanol (2:1) – 0.2 ml per 40 mg of harvested cells. 10 μL of the lipid extracts were loaded on thin-layer chromatography (TLC) silica gel plates F254 (Merck) and the lipids were separated in the mixture of chloroform: methanol: water (20: 4: 0.5) and visualized by autoradiography. Fatty acid methyl esters (FAME) and mycolic

acids methyl esters (MAME) were prepared from 100 µl of lipid fractions as previously described[36]. Dried extracts were dissolved in chloroform/methanol (2:1) and loaded on TLC plates. FAME and different forms of MAME were separated by three runs in n-hexane: ethyl acetate (95:5) and detected by autoradiography.

For investigation of the mechanism of action of **3l** *Mtb.* $H_{37}Ra$ culture was grown in 7H9 broth supplemented with albumin-dextrose-catalase and 0.05% Tween 80. When O.D. (600) reached 0.263, compound **3l** was added in final concentrations 4 and 8 µM. After 24 h of cultivation with shaking (120 rpm) ^{14}C acetate (specific activity 106 mCi/mmol, ARC) in the final concentration of 0.5 µCi/mL was added to each of the cultures and the cells were cultivated for next 24 h. Lipids were extracted and analyzed as described above. Following solvent systems were used for their separation: chloroform: methanol: water (20:4:0.5); chloroform: methanol: ammonium hydroxide: water (65:25:0.5:4) and petroleum ether: ethyl acetate (98:2, three runs). FAME and MAME were prepared from whole cells and analyzed as described above, alternatively on TLC plates impregnated in 5% $AgNO_3$ and activated at 100 °C for 1h.

5. Conclusions

In summary, a series of seventeen *N*-alkyl-2-isonicotinoylhydrazine-1-carboxamides and their cyclic analogues, *N*-alkyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amines, was synthesized and evaluated against drug-susceptible and multidrug-resistant *Mycobacterium tuberculosis* strains and three strains of nontuberculous mycobacteria. The most active compounds against *Mtb.* comparable to isoniazid were 2-isonicotinoylhydrazine-1-carboxamides with a shorter alkyl. They also were found to be active against NTM as well as *in vitro* non-toxic compounds in HepG2 cells. 2-Isonicotinoyl-*N*-methylhydrazine-1-carboxamide as the most active and selective anti-TB agent was chosen for an investigation of mechanism of action. It targets InhA and inhibits the synthesis of mycolic acids inside *Mtb.* cells similarly to INH.

N-Decyl and dodecyl alkyls represent optimal substituents for 5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine scaffold. *N*-Dodecyl-5-(pyridin-4-yl)-1,3,4-oxadiazol-2-amine affects the growth of both susceptible, MDR- and XDR-TB strains at an identical concentration with no cross-resistance to established antimycobacterial drugs. Its mechanism of action is different from those of INH. It was proved that this oxadiazole is definitely not a prodrug of INH, thus constituting a new potentially promising group of antimycobacterial agents.

Conflicts of interest

None of the authors has declared any conflict of interest.

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Highlights

- 2-Isonicotinoylhydrazine-1-carboxamides and 1,3,4-oxadiazoles were synthesised.
- Activity against *M. tuberculosis* and nontuberculous mycobacteria (MIC ≥0.5 μM).
- Low or no cytotoxicity for HepG2 cells.
- 2-Isonicotinoyl-*N*-methylhydrazine-1-carboxamide targets InhA like isoniazid.
- 5-(Pyridin-4-yl)-1,3,4-oxadiazol-2-amines inhibit multidrug-resistant strains.