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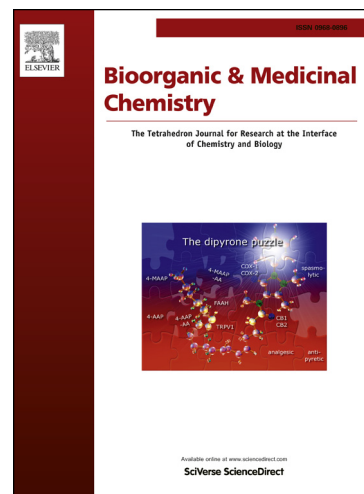
Novel derivatives of nitro-substituted salicylic acids: Synthesis, antimicrobial activity and cytotoxicity

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Abstract
Inspired by the high antituberculous activity of novel nitro-substituted derivatives and based on promising predicted ADMET properties we have synthesized a series of 33 salicylanilides containing nitro-group in their salicylic part and evaluated them for their *in vitro* antimycobacterial, antimicrobial and antifungal activities. The presence of nitro-group in position 4 of the salicylic acid was found to be beneficial and the resulting molecules exhibited minimum inhibitory concentrations (MICs) ranging from 2 to 32 μM against *Mycobacterium tuberculosis*. The best activity was found for 2-hydroxy-4-nitro-*N*-[4-(trifluoromethyl)phenyl]benzamide (MIC = 2 μM). 4-Nitrosalicylanilides were also found to be active against all *Staphylococcus* species tested while for MRSA strain 2-hydroxy-4-nitro-*N*-[4-(trifluoromethyl)phenyl]benzamide's MIC was 0.98 μM. None of the nitrosalicylanilides was active against *Enterococcus* sp. J 14365/08 and no considerable activity was found against Gram-negative bacteria or fungi. The hepatotoxicity of all nitrosalicylanilides was found to be in the range of their MICs for HepG2 cells.

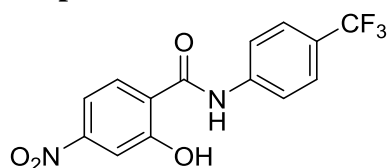**Keywords**
Salicylanilides
Nitro group
In silico ADMET prediction
In vitro antimycobacterial activity
In vitro antimicrobial activity
Cytotoxicity

Graphical abstract

M. tuberculosis MIC: 2 μM
nontuberculous mycobacteria: MICs 2-32 μM
Staphylococcus species (including MRSA): MICs from 0.98 μM

1. Introduction

According to the World Health Organization, tuberculosis (TB) is second only to Human Immunodeficiency Virus (HIV) as the greatest killer worldwide due to a single infectious agent.[1] The numbers reported at the Global Tuberculosis Report for 2014 are highlighting the severity of the disease. During 2013, 9 million people fell ill with TB and 1.5 million people died. Whilst TB is treatable and curable, standard anti-TB drugs, like isoniazid (INH) and rifampicin (RMP), have been used for decades, and resistance to the medicines is widespread. Clinical strains that are resistant to a single anti-TB drug have been documented in every country surveyed giving rise to multidrug-resistant tuberculosis (MDR-TB), extensively drug-resistant tuberculosis (XDR-TB) and the recently reported totally drug-resistant tuberculosis (TDR-TB).[2-4] In addition, the standard treatment for adult respiratory TB is a regimen of drugs developed many years ago and is connected with several adverse effects like hepatotoxicity, skin reactions, gastrointestinal and neurological disorders.[5] Among them hepatotoxicity is the most serious one [6], directing the current research towards the need to enrich the pipeline of available drugs against TB, preferably with candidates offering lower cytotoxicity and with new mode of action in comparison to existing drugs.

On the contrary, various opportunistic human infections are caused by nontuberculous mycobacteria (NTM) and, according to the predictions, they will continue to increase their incidence at least up to year 2050 mainly due to an increasing elderly population.[7] The treatment of NTM caused infections is complicated and involves multiple medications due to the high levels of natural and acquired antibiotic resistance. Furthermore, their current treatment share limited efficacy [8], and therefore there is a need for new therapeutic strategies to be developed. Moreover, severe hospital-acquired infections can be caused due to notable drug-resistance complications that been reported for Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae* and *Enterococci*.[9]

Salicylanilides (2-hydroxy-*N*-phenylbenzamides) have been the subject of several medicinal chemistry studies due to their significant *in vitro* antimicrobial and antifungal activities.[10-12] A number of salicylanilide derivatives have been developed so far with proven activity against *Mycobacterium tuberculosis* (Mtb) and INH-resistant strains.[13-15] However, the precise molecular mode of action of salicylanilides is not clear. It has been previously reported that a free phenolic hydroxyl on the salicylic acid moiety is required for activity and suggested that they might function as proton shuttles that kill bacterial cells by destroying the cellular proton gradient.[16] On the other hand, salicylanilides and their ester derivatives have been reported to act as moderate inhibitors of mycobacterial and human methionine aminopeptidases and may target the function of mycobacterial isocitrate lyase which could enhance its efficacy due to its particular relevance to mycobacterial latency.[17] These findings have suggested that salicylanilides affect multiple targets to exert their antimicrobial properties. Furthermore, salicylanilide esters, carbamates and additional derivatives resulting from their conjugation with existing antimicrobial agents, have been suggested to work as pro-drugs that are hydrolysed in order to express their activity.[18]

Although salicylanilides and their derivatives (esters, carbamates etc.) are very potent against Mtb, they often share a non-preferable cytotoxic profile.[16, 19] This drawback has limited their utility as potential therapeutics so far. Recent work has focused on the discovery of salicylanilide derivatives with decreased cytotoxicity and, under this concept, novel 2-

(phenylcarbamoyl)phenyl 4-substituted benzoates were synthesized and found to be active against several mycobacteria (0.125-8 μM) while they exhibited no cytotoxicity at concentrations of up to 50 μM against a hepatocyte cell line (HepG2).[20] The esterification products of 5-halogenated salicylanilides with 4-substituted benzoic acids were found to be, not only more potent inhibitors of Mtb growth but also to share a preferable cytotoxic profile in contrast with the parent salicylanilides. The increased lipophilicity of the afforded derivatives limited their solubility in aqueous media and hence their drugability.

Our ongoing efforts explore the influence of different substitutions on the parent structure of salicylanilides, in terms of their activity and cytotoxicity. The introduction of several substituents is under investigation. In general, it has been previously reported that an electron withdrawing group is favored in the aniline part while the influence of the substituents from the acyl moiety is more complex.[21] The best substituents of the parent salicylanilide structure reported so far is –Br or –Cl atoms in position 5 of the salicylic acid part while a –CF$_3$ group is favoured in position 4 of the aniline part. More specifically, 5-bromo-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide and 5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide are inhibiting the growth of Mtb at the concentrations of 1 and 2 μM respectively.

Current research in the field of novel antituberculotics on their way to the clinic has revealed that many nitro-substituted compounds owe their antimycobacterial activity to the presence of a nitro group in their scaffold.[22, 23] For example, the nitro-reduction of nitroimidazole OPC-67683 (also known as delamanid) [24] and PA-824 [25] by F$_{420}$-deazaflavin-dependent nitroreductase (Ddn) releases nitric oxide, which is thought to inhibit cytochrome oxidase and other targets. In addition, benzothiazinones (BTZ), i.e., BTZ-043 [26] and BTZ-derived inhibitors, i.e., CT139 [27] may interact through their nitro-group with a cysteine residue of decaprenylphosphoryl-β-D-ribose 2'-epimerase (DprE1), either covalently or not, thus blocking the first step in the epimerization reaction of decaprenyl-phosphoryl-D-ribose (DPR) to decaprenyl-phosphoryl- D-arabinose (DPA) the arabinan donor for arabinogalactan. In a similar manner dinitrobenzamides, i.e., DNB1 [28] are forming a covalent bond with Cys387 within the active site of DprE1. More recently, novel dinitrobenzyl-bearing benzazole and tetrazole derivatives exhibited high and selective antimycobacterial activity.[29, 30]

Herein we describe the influence of the introduction of a nitro-group at the salicylic part of salicylanilides to their antimycobacterial, antimicrobial and antifungal activities. To the best of our knowledge, a structure activity relationship (SAR) concerning the preferable nitro-substitution of the salicylic part of salicylanilides has not been reported in the literature to date, even if some nitro-substituted salicylanilides have been previously presented.[16, 21, 31-34]

2. Results and Discussion

2.1. ADMET properties prediction

The novel salicylanilides were screened *in silico*, prior to their synthesis, for their ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties. The software of choice was ADMET Predictor (Simulation Plus, Lancaster, CA), one of the leading computer software for advanced predictive modelling of ADMET properties.[35] The program uses molecular description values as inputs to independent mathematical models (generally,

nonlinear machine learning technics) in order to generate estimates for each of the ADMET properties.[36] ADMET Predictor not only rapidly estimates a number of vital ADMET properties from molecular structures, but it is able to validate a series of "ADMET Risk" or "Tox Risk" parameters. They are parameterized to include thresholds for a wide range of calculated and predicted properties that represent potential obstacles to a compound being successfully developed as a drug.

For the majority of the compounds, the analyses indicated no potential risk of either ADME/absorption problems related with physico-chemical properties or any stability risk related to fast hepatic metabolism. Furthermore, no potential interaction related to inhibition of drug biotransformation enzymes was indicated. Most importantly, the tested compounds were not found to be potentially related with toxicity or carcinogenicity as indicated by parameters of acute toxicity and carcinogenicity in rats expressed as TD_{50} and LD_{50} values, respectively, mutagenic chromosomal aberrations, or maximum recommended therapeutic dose (MRTD).

More specifically, 17 out of 33 compounds showed a preferable ADMET Risk (score 0-2). 5 out of 33 compounds presented an acceptable ADMET Risk (score 3), limited by potential high lipophilicity and absorption related risk. 11 out of 33 compounds showed a non-preferable ADMET Risk profile (score 4-5). On the other hand, 28 out of 33 compounds showed no potential risk of toxicity (score 0-2) and only 5 out of 33 compounds showed an acceptable risk of toxicity (score 3). There was no direct correlation between the position of the nitro group and the potential risk of toxicity. Furthermore, the substituent on the aniline part is more likely affecting the potential ADMET properties, as halogens in position 3 is preferred while trifluoromethyl group and nitro group are not preferable. The ADMET Risk, as well as the Tox Risk scores of the screened compounds are summarized in **Table S1**. With the encouraging prediction results the synthesis of the molecules was decided.

2.2. Chemistry

The synthesis of nitro-containing salicylanilides (**1-33**) is described in **Scheme 1**. The final compounds were products of the conjugation of nitro-salicylic acids with selected anilines in the presence of PCl_3 under microwave irradiation. All compounds were isolated with good to moderate yields.
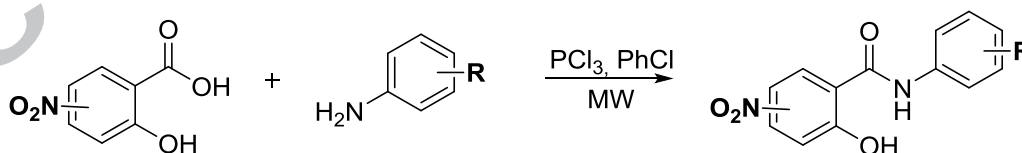
Scheme 1: Synthesis of nitro-substituted salicylanilides.

2.3. Antimycobacterial activity

The nitro-containing salicylanilides (**1-33**) were evaluated to determine their *in vitro* antimycobacterial activity against *Mycobacterium tuberculosis* 331/88 (i.e., $H_{37}R_V$), and the following nontuberculous mycobacteria: *Mycobacterium avium* 330/88 and *Mycobacterium kansasii* 235/80 and 6509/96 (a clinical isolated strain). INH, the first-line anti-TB drug, was chosen for the comparison performed in this study. **Table 1** reports their minimum inhibitory concentrations (MICs) as well as their calculated log*P* values.

Table 1. Antimycobacterial activity of nitrosalicylanilides **1-33**

	R	R^1	C logP	Mtb. 331/88 (H$_{37}$Rv)		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
1	3-NO$_2$	3-I	3.44	62.5	62.5	500	500	62.5	125	125	125	125	125
2	3-NO$_2$	3-Br	3.03	62.5	62.5	250	250	62.5	125	125	125	125	125
3	3-NO$_2$	3-Cl	2.76	62.5	62.5	500	500	62.5	125	250	125	125	125
4	3-NO$_2$	3-F	2.28	62.5	62.5	500	500	62.5	125	250	125	250	250
5	3-NO$_2$	3,4-diCl	3.38	16	32	250	250	32	62.5	62.5	62.5	62.5	62.5
6	3-NO$_2$	3-CF$_3$	3.12	32	32	500	500	32	62.5	62.5	125	250	250
7	3-NO$_2$	4-CF$_3$	3.12	32	32	250	250	62.5	125	125	62.5	62.5	125
8	3-NO$_2$	3-CF$_3$, 4-NO$_2$	3.08	16	16	250	500	32	62.5	125	32	62.5	62.5
9	3-NO$_2$	3-NO$_2$	2.1	62.5	62.5	>500	>500	62.5	250	250	62.5	250	250
10	3-NO$_2$	4-NO$_2$	2.1	32	32	125	125	32	62.5	125	62.5	125	125
11	3-NO$_2$	3,5-diCF$_3$	4.1	**8**	**8**	125	125	16	32	32	32	32	32
12	4-NO$_2$	3-I	3.44	16	16	32	62.5	8	16	16	16	16	16
13	4-NO$_2$	3-Br	3.03	16	16	32	62.5	8	16	16	16	16	16
14	4-NO$_2$	3-Cl	2.76	8	16	32	62.5	8	16	16	16	16	16
15	4-NO$_2$	3-F	2.28	16	16	62.5	125	16	16	32	16	32	32
16	4-NO$_2$	3,4-diCl	3.38	**4**	**8**	32	62.5	4	8	8	8	16	16
17	4-NO$_2$	3-CF$_3$	3.12	**8**	**8**	62.5	62.5	8	16	16	8	16	16
18	4-NO$_2$	4-CF$_3$	3.12	**2**	**4**	32	32	**2**	**4**	**8**	**4**	**8**	**8**
19	4-NO$_2$	3-CF$_3$, 4-NO$_2$	3.08	16	16	62.5	62.5	16	32	32	16	32	32
20	4-NO$_2$	3-NO$_2$	2.1	32	32	125	125	32	32	62.5	32	32	32
21	4-NO$_2$	4-NO$_2$	2.1	8	16	62.5	62.5	8	16	32	32	32	32
22	4-NO$_2$	3,5-diCF$_3$	4.1	**4**	**8**	32	32	8	16	16	16	16	16
23	5-NO$_2$	3-I	3.44	16	16	125	125	16	32	62.5	32	62.5	62.5
24	5-NO$_2$	3-Br	3.03	16	16	125	250	32	62.5	62.5	32	62.5	62.5
25	5-NO$_2$	3-Cl	2.76	16	16	125	125	16	32	62.5	32	62.5	62.5
26	5-NO$_2$	3-F	2.28	16	32	125	125	16	62.5	62.5	32	62.5	62.5
27	5-NO$_2$	3,4-diCl	3.38	8	16	125	125	16	32	32	32	32	32
28	5-NO$_2$	3-CF$_3$	3.12	8	16	125	125	16	32	62.5	32	62.5	62.5
29	5-NO$_2$	4-CF$_3$	3.12	**8**	**8**	62.5	62.5	8	16	32	16	32	32
30	5-NO$_2$	3-CF$_3$, 4-NO$_2$	3.08	32	32	125	125	32	62.5	125	62.5	62.5	125
31	5-NO$_2$	3-NO$_2$	2.1	62.5	62.5	125	125	16	62.5	125	62.5	125	125
32	5-NO$_2$	4-NO$_2$	2.1	62.5	125	250	250	16	62.5	125	62.5	125	125
33	5-NO$_2$	3,5-diCF$_3$	4.1	16	16	32	32	16	32	32	16	32	32

INH	-	0.5	1	>250	>250	>250	>250	>250	4	8	8

INH = isoniazid

According to the nitro-substitution of the salicylic part, the tested compounds could be divided into three groups: Group 1 includes 3-NO_2 derivatives (**1-11**); Group 2 includes 4-NO_2 derivatives (**12-22**); and Group 3 includes 5-NO_2 derivatives (**23-33**). It was observed that the most preferable position of nitro-group is position 4 of the salicylic part, followed by the nitro-substitution in position 5. Nitro-substitution in position 3 of the salicylic part was the least preferable.

Some nitrosalicylanilides exhibited good to moderate activity against Mtb. For the 4-nitro substituted salicylanilides, the activity ranged between 2-32 μM. The best activity was found for 2-hydroxy-4-nitro-*N*-[4-(trifluoromethyl)phenyl]benzamide (**18**) (MIC = 2 μM). The favorable substitutions in the aniline part, regardless of the position of the nitro-group at the salicylic part, were the mono- or bis-trifluoromethyl- substitution. This is probably due to the increased lipophilicity (higher calculated log*P* value) of the molecule when bearing the trifluoromethyl moiety and is in accordance with previous results regarding the preferable substitution in the aniline part.[21] Furthermore, it is noteworthy that the presence of a nitro group in the aniline part of the molecule resulted less active molecules as a result of their decreased lipophilicity (lower calculated log*P* values).

Nitro-substituted salicylanilides did not show any considerable active against *M. avium* 330/88 (MIC ≥ 32 μM). On the other hand, all tested molecules were more active than INH against *M. kansasii* 235/80, while 2-hydroxy-4-nitro-*N*-[4-(trifluoromethyl)phenyl]benzamide (**18**) was the most active (MIC = 8 μM after 21 days). The same derivative (**18**) was similarly active with INH against *M. kansasii* 6509/96 (MIC = 8 μM after 21 days).

2.4. Antibacterial and antifungal activity

Our efforts to further investigate the biological activity of the nitrosalicylanilides, the derivatives that bear the nitro-group in positions 4 and 5 of the salicylic part involved testing against eight bacterial and eight fungal strains. More specifically, the selected molecules were tested against *Staphylococcus aureus* CCM 4516/08, methicillin-resistant *Staphylococcus aureus* H 5996/08 (MRSA), *Staphylococcus epidermis* H 6966/08, *Enterococcus* sp. J 14365/08, *Escherichia coli* CCM 4517, *Klebsiella pneumoniae* D 11750/08, extended spectrum β-lactamases producing *Klebsiella pneumoniae* J 14368/08, and *Pseudomonas aeruginosa* CCM 1961. Furthermore, the selected nitrosalicylanilides were tested against four *Candida* strains (*Candida albicans* ATCC 44859, *Candida tropicalis* 156, *Candida krusei* E28, and *Candida glabrata* 20/I), *Trichosporon asahii* 1188 and three filamentous fungi (*Aspergillus fumigatus* 231, *Absidia corymbifera* 272, and *Trichophyton mentagrophytes* 445). Benzylpenicillin (PNC) and fluconazole (FLU) were used for comparison during antibacterial and antifungal activity studies respectively.

Similar to their antimycobacterial activity, 4-nitro substituted salicylanilides were found to be more active against all different *Staphylococcus* species than their 5-nitro substituted analogues (**Table 2**). The range of MIC values were between 1-62.5 μM and 3.9-500 μM, respectively. The 3,5-bis(trifluoromethyl) moiety was the best substitution for the aniline part for all the different *Staphylococcus* species tested. The most active compound against all different *Staphylococcus* species was *N*-[3,5-bis(trifluoromethyl)phenyl]-2-hydroxy-4-nitrobenzamide (**22**), which exhibited MIC of 1.95 μM for *S. aureus* CCM 4516/08 and *S. epidermis*, while for MRSA strain the compound's MIC was 0.98 μM. Once again, the presence of a nitro-group in the aniline part of the molecule was found to drastically decrease

the activity. On the other hand, no considerable activity was found against *Enterococcus* sp. J 14365/08 (MIC $\geq$ 62.5 μM). In comparison with PNC, none of the nitrosalicylanilides was more active against *Staphylococcus aureus* CCM 4516/08 while 4-nitrosalicylanilides were always more active against MRSA and *Staphylococcus epidermis* H 6966/08.

Table 2. Antibacterial activity of nitrosalicylanilides **12-33** (Gram-positive bacteria)

	R^1	R^2	MIC/IC$_{95}$ (μmol/L)							
			SA		MRSA		SE		EF	
			24h	48h	24h	48h	24h	48h	24h	48h
12	4-NO$_2$	3-I	3.9	3.9	3.9	3.9	15.62	15.62	>500	>500
13	4-NO$_2$	3-Br	3.9	3.9	3.9	3.9	3.9	3.9	500	500
14	4-NO$_2$	3-Cl	7.81	15.62	7.81	15.62	7.81	15.62	500	>500
15	4-NO$_2$	3-F	15.62	31.25	15.62	31.25	15.62	31.25	500	>500
16	4-NO$_2$	3,4-diCl	3.9	3.9	1.95	1.95	1.95	1.95	125	500
17	4-NO$_2$	3-CF$_3$	3.9	3.9	1.95	1.95	1.95	7.81	250	500
18	4-NO$_2$	4-CF$_3$	7.81	7.81	1.95	1.95	1.95	7.81	250	250
19	4-NO$_2$	3-CF$_3$, 4-NO$_2$	7.81	7.81	3.9	3.9	7.81	7.81	250	500
20	4-NO$_2$	3-NO$_2$	62.5	62.5	125	125	31.25	62.5	>250	>250
21	4-NO$_2$	4-NO$_2$	62.5	62.5	31.25	31.25	62.5	62.5	500	>500
22	4-NO$_2$	3,5-diCF$_3$	1.95	1.95	0.98	0.98	1.95	1.95	62.5	125
23	5-NO$_2$	3-I	31.25	31.25	31.25	31.25	31.25	31.25	250	>500
24	5-NO$_2$	3-Br	31.25	62.5	31.25	62.5	31.25	250	500	>500
25	5-NO$_2$	3-Cl	31.25	62.5	62.5	62.5	62.5	62.5	500	>500
26	5-NO$_2$	3-F	125	125	125	125	125	250	>500	>500
27	5-NO$_2$	3,4-diCl	31.25	31.25	31.25	31.25	62.5	62.5	125	125
28	5-NO$_2$	3-CF$_3$	31.25	31.25	31.25	31.25	31.25	31.25	>500	>500
29	5-NO$_2$	4-CF$_3$	31.25	31.25	31.25	31.25	15.62	15.62	>500	>500
30	5-NO$_2$	3-CF$_3$, 4-NO$_2$	62.5	62.5	31.25	31.25	125	125	>500	>500
31	5-NO$_2$	3-NO$_2$	500	500	500	500	500	500	>500	>500
32	5-NO$_2$	4-NO$_2$	250	250	250	250	500	500	>500	>500
33	5-NO$_2$	3,5-diCF$_3$	3.9	3.9	7.81	7.81	15.62	15.62	500	500
PNC			0.98	0.98	62.5	125	250	250	7.81	15.62

SA: *Staphylococcus aureus* CCM 4516/08, MRSA: Methicillin-resistant *Staphylococcus aureus* H 5996/08, SE: *Staphylococcus epidermidis* H 6966/08, EF: *Enterococcus sp.* J 14365/08. PNC: benzylpenicillin.
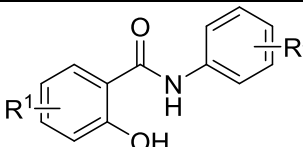
None of the tested compounds was found to be active against any of the tested Gram-negative bacteria (**Table S2**). In addition, nitrosalicylanilides were not found to possess any considerable antifungal activity (**Table S3**).

2.5. Cytotoxicity evaluation

12 of the synthesized nitrosalicylanilides were evaluated for their cytotoxic properties using an MTT assay in the HepG2 cell model. HepG2 cells represent the most likely target

tissue of antituberculotics-related toxicity, which often complicates the treatment of TB.[37] The tested compounds were chosen according to their antimycobacterial activity and the position of the nitro-group in the salicylic part. More specifically, 4 compounds from each different nitro-substitution of the salicylic part were selected for cytotoxicity experiments. The substitution for the aniline part was: 3,4-diCl, 3-CF$_3$, 4-CF$_3$ and 3,5-diCF$_3$. The cytotoxicity results as well as the selectivity indexes (SI) for the tested compounds are summarized in **Table 3**.

Table 3. Cytotoxicity of selected nitrosalicylanilides

	R^1	R^2	IC$_{50}$ (μM) for HepG2 cells	Selectivity index (SI) for Mtb
5	3-NO$_2$	3,4-diCl	5.9	0.184-0.369
6	3-NO$_2$	3-CF$_3$	8.9	0.278
7	3-NO$_2$	4-CF$_3$	8.0	0.250
11	3-NO$_2$	3,5-diCF$_3$	7.4	0.925
16	4-NO$_2$	3,4-diCl	1.4	0.175-0.350
17	4-NO$_2$	3-CF$_3$	2.8	0.350
18	4-NO$_2$	4-CF$_3$	1.2	0.300-0.600
22	4-NO$_2$	3,5-diCF$_3$	4.4	0.550-1.100
27	5-NO$_2$	3,4-diCl	8.9	0.556-1.113
28	5-NO$_2$	3-CF$_3$	7.4	0.463-0.925
29	5-NO$_2$	4-CF$_3$	3.5	0.438
33	5-NO$_2$	3,5-diCF$_3$	13.9	0.869

SI = IC$_{50}$/MIC$_{100}$

The IC$_{50}$ values for the tested compounds were in the range of 1.2 to 13.9 μM. According to the position of the nitro group, 4-nitro substituted salicylanilides showed consistently the lowest IC$_{50}$ values (1.2-4.4 μM), while the 3- and 5-nitro- substituted analogues showed higher IC$_{50}$ values (3.5-13.9 μM). The highest rate of cytotoxicity was found for 2-hydroxy-4-nitro-*N*-[4-(trifluoromethyl)phenyl]benzamide (**18**). On the other hand, the highest IC$_{50}$ was found for *N*-[3,5-bis(trifluoromethyl)phenyl]-2-hydroxy-5-nitrobenzamide (**33**). There is no direct correlation between the substitution at the aniline part and cytotoxicity. SI values for the tested compounds was always ≤ 1.113 indicating that antimycobacterial activity trends with cytotoxicity.

3. Conclusions

In order to investigate the influence of the introduction of a nitro group at the salicylic part of salicylanilides on their antimycobacterial, antimicrobial and antifungal activities, a series of novel salicylanilides, containing nitro group in their salicylic part, were designed and synthesized. All compounds were screened *in silico* with ADMET Predictor prior to their synthesis, in order to obtain an estimated prediction about their potential ADME properties and toxicity risk. The prediction revealed no potential risk of either ADME/absorption problems related with physico-chemical properties or any stability risk related to fast hepatic metabolism for the majority of the screened compounds. In addition the nitrosalicylanilides showed minimum to acceptable risk of toxicity as indicated from their Tox Risk score.

The synthesized compounds were evaluated for their antimycobacterial activity against Mtb and found to possess activity in the low micromolar range, comparable with the 5-halogenated salicylanilides which are the most active salicylanilides presented to date. The most preferable position of nitro-group was found to be position 4 of the salicylic part, followed by the nitro substitution in position 5. Nitro substitution in position 3 of the salicylic part was the least preferable. Although nitro-substituted salicylanilides did not show any considerable activity against *M. avium*, they were more active than INH against *M. kansasii* 235/80. 2-hydroxy-4-nitro-*N*-[4-(trifluoromethyl)phenyl]benzamide (**18**) was found to possess similar activity with INH against *M. kansasii* 6509/96. Furthermore, 4-nitro substituted salicylanilides were found to be more active against all *Staphylococcus* species tested than their 5-nitro substituted analogues while none of the nitrosalicylanilides presented considerable activity against *Enterococcus* sp. J 14365/08. In addition, the synthesized compounds were found to be inactive against all tested Gram-negative bacteria and fungi. Nitro substituted salicylanilides were found to share a non-preferable cytotoxic profile as their IC_{50} values against HepG2 cells were found to be in the range of their MIC values.

The presence of a free phenolic hydroxyl at the salicylanilide scaffold has been suggested to be responsible for the cytotoxicity of these molecules.[16] Several derivatives of salicylanilides have been proved to retain their activity while possessing a less cytotoxic profile.[20, 38] Under this concept, 4-nitrosalicylanilides could be potential candidates for further development by preparing their 4-(substituted)benzoates. This method was previously reported by our group for 5-halogenated salicylanilides affording the more active salicylanilide (4-substituted)benzoates with a preferable cytotoxic profile than the parent salicylanilides.[20] The only limitation observed for the (4-substituted)benzoates of 5-halogenated salicylanilides was their poor solubility in aqueous media. It is expected that the presence of a nitro group at the salicylic part, instead of a halogen, will have a positive impact at the solubility of 4-(substituted)benzoates of 4-nitrosalicylanilides. The suggestion concerning the solubility is in agreement with the comparison of the predicted calculated log*P* values between 4-(substituted)benzoates of 4-nitrosalicylanilides and 4-(substituted)benzoates of 5-halogenated salicylanilides (**Table S4**).

4. Experimental part

4.1. Chemistry

4.1.1. General methods

All of the reagents and solvents were purchased from Sigma-Aldrich (Darmstadt, Germany) or Penta Chemicals (Prague, Czech Republic) and used as received. The reactions and the purity of the products were monitored by thin-layer chromatography using a mixture of hexane to ethyl acetate of 4:1 or 2:1 as eluent. The plates were coated with 0.2-mm Merck 60 F254 silica gel and were visualized by UV irradiation (254 nm). The melting points were determined on a Büchi Melting Point B-540 apparatus using open capillaries, and the reported values are uncorrected.

Elemental analysis (C, H and N) was performed on an automatic microanalyser CHNS-O CE instrument (FISONS EA 1110, Milano, Italy). Infrared spectra (ATR) were recorded on a FT-IR spectrometer (Nicolet 6700 FT-IR) in the range of 400-4000 cm^{-1}. The NMR spectra were measured in DMSO at ambient temperature using a Varian V NMR S500 instrument (500 MHz for ^{1}H and 125 MHz for ^{13}C, Varian Comp., Palo Alto, CA, USA) or a Varian

Mercury-Vxbb 300 (300 MHz for ^{1}H and 75.5 MHz for ^{13}C, Varian, Inc., Palo Alto, CA, USA). The chemical shifts, δ, are given in ppm with respect to tetramethylsilane, which was used as an internal standard. The coupling constants (J) are reported in Hz.

The calculated log$P$ values (Clog$P$), which are the logarithms of the partition coefficients for octan-1-ol/water, were determined using the CS ChemOffice Ultra program (version 12.0, Cambridge-Soft, Cambridge, MA, USA).

4.1.2. Synthesis

All salicylanilides (**1-33**) were synthesized *via* a previously described method [39] with yields of 60-85%. This microwave assisted syntheses were performed in a microwave reactor (530 W, 600 rpm, MicroSYNTH Milestone) for 30-60 min to reflux. All compounds were purified by recrystallization from ethanol or acetone or mixture of ethanol/acetone.

4.1.2.1. 2-Hydroxy-*N*-(3-iodophenyl)-3-nitrobenzamide (1). Yellow solid; yield 74%; mp 188.5-189.5 ^{o}C. IR (ATR): 3376, 3083, 1676 (CO). ^{1}H NMR (500 MHz, DMSO): δ 10.73 (1H, s, NH), 8.17 (1H, s, H2'), 8.16 (1H, dd, J = 8.0 Hz, J = 1.0 Hz, H4), 8.13 (1H, dd, J = 8.0 Hz, J = 1.0 Hz, H6), 7.70 (1H, d, J = 8.5 Hz, H4'), 7.53 (1H, d, J = 7.5 Hz, H6'), 7.20 (1H, t, J = 8.0 Hz, H5'), 7.15 (1H, t, J = 8.0 Hz, H5). ^{13}C NMR (125 MHz, DMSO): δ 166.35, 152.94, 138.38, 138.23, 134.08, 133.30, 130.97, 129.37, 128.75, 122.11, 120.43, 118.73, 94.59. Anal. Calcd for C$_{13}$H$_9$IN$_2$O$_4$ (384.13): C, 40.65; H, 2.36; N, 7.29. Found: C, 40.60; H, 2.38; N, 7.28.

4.1.2.2. 2-Hydroxy-*N*-(3-bromophenyl)-3-nitrobenzamide (2). Yellow solid; yield 80%; mp 160 ^{o}C. IR (ATR): 3375, 3084, 1677 (CO). ^{1}H NMR (300 MHz, DMSO): δ 10.79 (1H, s, NH), 8.16 (1H, dd, J = 8.1 Hz, J = 1.5 Hz, H4), 8.13 (1H, dd, J = 7.2 Hz, J = 1.8 Hz, H6), 8.03 (1H, s, H2'), 7.67-7.65 (1H, m, H5'), 7.37-7.35 (2H, m, H4', H6'), 7.15 (1H, t, J = 8.1 Hz, H5). ^{13}C NMR (75 MHz, DMSO): δ 166.20, 152.64, 139.45, 138.03, 133.99, 130.80, 128.57, 127.20, 123.32, 122.20, 121.46, 119.76, 118.64. Anal. Calcd for C$_{13}$H$_9$BrN$_2$O$_4$ (337.13): C, 46.32; H, 2.69; N, 8.31. Found: C, 45.83; H, 2.90; N, 8.02.

4.1.2.3. 2-Hydroxy-*N*-(3-chlorophenyl)-3-nitrobenzamide (3). Yellow solid; yield 85%; mp 153-154 ^{o}C. IR (ATR): 3365, 3091, 1667 (CO). ^{1}H NMR (300 MHz, DMSO): δ 10.81 (1H, s, NH), 8.16 (1H, dd, J = 7.8 Hz, J = 1.2 Hz, H4), 8.13 (1H, dd, J = 8.4 Hz, J = 1.5 Hz, H6), 7.89 (1H, t, J = 1.8 Hz, H2'), 7.62 (1H, dd, J = 8.1 Hz, J = 0.9 Hz, H6'), 7.15 (1H, t, J = 7.8 Hz, H5'), 7.25 (1H, dd, J = 7.8 Hz, J = 1.5 Hz, H4'), 7.15 (1H, t, J = 8.1 Hz, H5). ^{13}C NMR (75 MHz, DMSO): δ 166.21, 152.63, 139.33, 138.02, 134.00, 133.06, 130.51, 128.56, 124.31, 122.27, 120.47, 119.37, 118.65. Anal. Calcd for C$_{13}$H$_9$ClN$_2$O$_4$ (292.68): C, 53.35; H, 3.10; N, 9.57. Found: C, 52.80; H, 3.51; N, 9.32.

4.1.2.4. 2-Hydroxy-*N*-(3-fluorophenyl)-3-nitrobenzamide (4). Yellow solid; yield 76%; mp 111-112 ^{o}C. IR (ATR): 3366, 3088, 1662 (CO). ^{1}H NMR (500 MHz, DMSO): δ 10.81 (1H, s, NH), 8.16 (1H, dd, J = 7.8 Hz, J = 1.4 Hz, H4), 8.13 (1H, dd, J = 8.3 Hz, J = 1.5 Hz, H6), 7.67 (1H, dt, J = 11.5 Hz, J = 2.1 Hz, H2'), 7.48 (1H, d, J = 8.3 Hz, H6'), 7.43 (1H, dt, J = 10.0 Hz, J = 7.4 Hz, H5') 7.15 (1H, t, J = 7.9 Hz, H5), 7.00 (1H, td, J = 8.5 Hz, J = 2.2 Hz, H4'). ^{13}C NMR (125 MHz, DMSO): δ 166.17, 162.00 (d, J = 242.2 Hz), 152.60, 139.57 (d, J = 11.1 Hz), 137.98, 134.00, 130.43 (d, J = 9.4 Hz), 128.51, 122.30, 118.64, 111.08 (d, J = 21.0 Hz), 107.77 (d, J = 26.3 Hz). Anal. Calcd for C$_{13}$H$_9$FN$_2$O$_4$ (276.22): C, 56.53; H, 3.28; N, 10.14. Found: C, 55.94; H, 3.67; N, 9.92.

4.1.2.5. 2-Hydroxy-*N*-(3,4-dichlorophenyl)-3-nitrobenzamide (5). Yellow solid; yield 73%; mp 153-154 ^{o}C. IR (ATR) 3365, 3107, 1673 (CO). ^{1}H NMR (300 MHz, DMSO): δ

10.88 (1H, s, NH), 8.13 (2H, d, J = 8.1 Hz, H4, H6), 8.07 (1H, s, H2'), 7.65 (2H, s, H5', H6'), 7.15 (1H, t, J = 8.1 Hz, H5). ^{13}C NMR (75 MHz, DMSO): δ 166.10, 152.42, 138.09, 138.01, 134.08, 131.03, 130.74, 128.56, 126.09, 122.55, 122.07, 120.83, 118.72. Anal. Calcd for $C_{13}H_8Cl_2N_2O_4$ (327.12): C, 47.73; H, 2.47; N, 8.56. Found: C, 47.02; H, 2.78; N, 8.09.

4.1.2.6. 2-Hydroxy-*N*-[3-(trifluoromethyl)phenyl]-3-nitrobenzamide (6). Yellow solid; yield 76%; mp 142-143.5 $^{\circ}$C. IR (ATR): 3364, 3089, 1665 (CO). ^{1}H NMR (500 MHz, DMSO): δ 10.94 (1H, s, NH), 8.17 (1H, dd, J = 7.8 Hz, J = 1.4 Hz, H4), 8.16 (1H, d, J = 1.5 Hz, H2'), 8.14 (1H, dd, J = 8.3 Hz, J = 1.5 Hz, H6), 7.95 (1H, d, J = 8.3 Hz, H4'), 7.64 (1H, t, J = 8.0 Hz, H5'), 7.52 (1H, d, J = 7.8 Hz, H6'), 7.16 (1H, t, J = 7.8 Hz, H5). ^{13}C NMR (125 MHz, DMSO): δ 160.30, 152.54, 138.72, 138.05, 134.02, 130.07, 129.48 (q, J = 31.8 Hz), 128.55, 124.51, 124.03 (q, J = 270.00 Hz), 122.35, 120.87 (q, J = 3.8 Hz), 118.66, 117.06 (q, J = 4.00 Hz). Anal. Calcd for $C_{14}H_9F_3N_2O_4$ (326.23): C, 51.54; H, 2.78; N, 8.59. Found: C, 51.03; H, 2.94; N, 8.22.

4.1.2.7. 2-Hydroxy-*N*-[4-(trifluoromethyl)phenyl]-3-nitrobenzamide (7). Yellow solid; yield 69%; mp 156-157 $^{\circ}$C. IR (ATR): 3362, 3093, 1678 (CO). ^{1}H NMR (500 MHz, DMSO): δ 10.96 (1H, s, NH), 8.16 (1H, dd, J = 7.8 Hz, J = 1.4 Hz, H4), 8.14 (1H, dd, J = 8.3 Hz, J = 1.5 Hz, H6), 7.94 (2H, d, J = 8.5 Hz, H3',H5'), 7.76 (2H, d, J = 8.5 Hz, H2',H4'), 7.16 (1H, t, J = 8.0 Hz, H5). ^{13}C NMR (125 MHz, DMSO): δ 166.22, 152.41, 141.63, 137.95, 134.21, 128.47, 126.05 (q, J = 3.8 Hz), 124.42 (q, J = 32.5 Hz), 124.27 (q, J = 270.0 Hz), 122.87, 120.74, 118.70. Anal. Calcd for $C_{14}H_9F_3N_2O_4$ (326.23): C, 51.54; H, 2.78; N, 8.59. Found: C, 51.10; H, 2.98; N, 8.37.

4.1.2.8. 2-Hydroxy-3-nitro-*N*-(4-nitro-3-[trifluoromethyl)phenyl]benzamide (8). Yellow solid; yield 78%; mp 161-162 $^{\circ}$C. IR (ATR): 3331, 3096, 1686 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.38 (1H, s, NH), 8.39 (1H, d, J = 2.0 Hz, H2'), 8.26 (1H, d, J = 8.9 Hz, H5'), 8.21 (1H, dd, J = 9.0 Hz, J = 2.1 Hz, H6'), 8.14 (1H, dd, J = 8.2 Hz, J = 1.7 Hz, H4), 8.08 (1H, dd, J = 7.7 Hz, J = 1.6 Hz, H6), 7.16 (1H, t, J = 7.9 Hz, H5). ^{13}C NMR (125 MHz, DMSO): δ 166.12, 151.80, 142.97, 142.05, 138.00, 134.51, 128.49, 127.63, 123.90, 123.67, 122.84 (q, J = 33.3 Hz), 122.02 (q, J = 271 Hz), 118.85, 118.54 (q, J = 6.0 Hz). Anal. Calcd for $C_{14}H_8F_3N_3O_6$ (371.23): C, 45.30; H, 2.17; N, 11.32. Found: C, 44.65; H, 2.61; N, 10.87.

4.1.2.9. 2-Hydroxy-3-nitro-*N*-(3-nitrophenyl)benzamide (9). Yellow solid; yield 66%; mp 204-205 $^{\circ}$C IR (ATR): 3350, 3112, 1670 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.06 (1H, s, NH), 8.71 (1H, t, J = 2.1 Hz, H2'), 8.16 (1H, dd, J = 5.7 Hz, J = 1.6 Hz, H6'), 8.14 (1H, dd, J = 6.2 Hz, J = 1.7 Hz, H4'), 8.08 (1H, dd, J = 8.2 Hz, J = 1.3 Hz, H4), 8.01 (1H, dd, J = 8.1 Hz, J = 2.3 Hz, H6), 7.68 (1H, t, J = 8.2 Hz, H5'), 7.16 (1H, t, J = 8.1 Hz, H5). ^{13}C NMR (125 MHz, DMSO): δ 166.32, 152.39, 147.90, 139.17, 138.02, 134.14, 130.26, 128.59, 126.72, 122.62, 118.97, 118.77, 114.93. Anal. Calcd for $C_{13}H_9N_3O_6$ (303.23): C, 51.49; H, 2.99; N, 13.96. Found: C, 50.68; H, 3.24; N, 13.47.

4.1.2.10. 2-Hydroxy-3-nitro-*N*-(4-nitrophenyl)benzamide (10). Yellow solid; yield 60%; mp 221-222 $^{\circ}$C. IR (ATR): 3355, 3100, 1677 (CO). ^{1}H NMR (300 MHz, DMSO): δ 11.74 (1H, s, NH), 8.27 (2H, d, J = 9.1 Hz, H3',H5'), 8.09 (2H, d, J = 7.8 Hz, H4,H6), 7.97 (2H, d, J = 9.1 Hz, H2',H6'), 7.03 (1H, t, J = 7.9 Hz, H5). ^{13}C NMR (75 MHz, DMSO): δ 165.99, 153.86, 144.69, 142.73, 138.30, 134.68, 128.59, 124.93, 123.83, 120.09, 117.29. Anal. Calcd for $C_{13}H_9N_3O_6$ (303.23): C, 51.49; H, 2.99; N, 13.96. Found: C, 50.72; H, 3.37; N, 13.40.

4.1.2.11. *N*-[3,5-Bis(trifluoromethyl)phenyl]-2-hydroxy-3-nitrobenzamide (11). Yellow solid; yield 71%; mp 183-184 $^{\circ}$C. IR (ATR): 3355, 3109, 1671 (CO). ^{1}H NMR (300 MHz, DMSO): δ 11.21 (1H, s, NH), 8.41 (2H, s, H2',H6'), 8.14 (1H, dd, J = 8.0 Hz, J = 1.5 Hz, H4), 8.12 (1H, dd, J = 8.0 Hz, J = 1.5 Hz, H6), 7.17 (1H, t, J = 8.1 Hz, H5). ^{13}C NMR (75 MHz, DMSO): δ 166.28, 152.09, 140.09, 138.10, 134.20, 130.79 (q, J = 33.0 Hz), 128.60,

123.17 (q, J = 271.0 Hz), 122.95, 120.40, 118.81, 117.18 (q, J = 3.7 Hz). Anal. Calcd for $C_{15}H_8F_6N_2O_4$ (394.23): C, 45.70; H, 2.05; N, 7.11. Found: C, 45.08; H, 2.31; N, 6.89.

4.1.2.12. 2-Hydroxy-*N*-(3-iodophenyl)-4-nitrobenzamide (12). Yellow solid; yield 77%; mp 243-245 °C. IR (ATR): 3337, 3125, 1640 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.75 (1H, bs, OH), 10.49 (1H, s, NH), 8.23 (1H, s, H2'), 7.92 (1H, d, J = 9.2 Hz, H6), 7.78-7.74 (2H, m, H3,H5), 7.68 (1H, d, J = 8.1 Hz, H4'), 7.50 (1H, d, J = 9.2 Hz, H6'), 7.17 (1H, t, J = 8.0 Hz, H5'). ^{13}C NMR (125 MHz, DMSO): δ 164.01, 156.57, 149.49, 139.74, 132.66, 130.83, 130.81, 128.30, 127.10, 119.44, 113.66, 111.16, 94.55. Anal. Calcd for $C_{13}H_9IN_2O_4$ (384.13): C, 40.65; H, 2.36; N, 7.29. Found: C, 40.48; H, 2.59; N, 7.01.

4.1.2.13. *N*-(3-Bromophenyl)-2-hydroxy-4-nitrobenzamide (13). Yellow solid; yield 68%; mp 220-221.5 °C. IR (ATR): 3313, 3085, 1639 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.79 (1H, bs, OH), 10.49 (1H, s, NH), 8.08 (1H, s, H2'), 7.92 (1H, d, J = 9.2 Hz, H6), 7.78-7.74 (2H, m, H3,H5), 7.67-7.65 (1H, m, H6'), 7.35-7.30 (2H, m, H4',H5'). ^{13}C NMR (125 MHz, DMSO): δ 164.06, 156.56, 149.50, 139.93, 130.84, 130.77, 127.07, 126.72, 122.46, 121.54, 118.98, 113.65, 111.17. Anal. Calcd for $C_{13}H_9BrN_2O_4$ (337.13): C, 46.32; H, 2.69; N, 8.31. Found: C, 45.91; H, 3.01; N, 7.96.

4.1.2.14. *N*-(3-Chlorophenyl)-2-hydroxy-4-nitrobenzamide (14). Yellow solid; yield 71%; mp 227-228 °C. IR (ATR): 3329, 3080, 1637 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.80 (1H, bs, OH), 10.57 (1H, s, NH), 7.95-7.91 (2H, m, H6,H2'), 7.78-7.74 (2H, m, H3,H5), 7.61 (1H, dd, J = 8.2 Hz, J = 1.0 Hz, H4'), 7.39 (1H, t, J = 8.1 Hz, H5'), 7.19 (1H, dd, J = 7.8 Hz, J = 1.8 Hz, H6'). ^{13}C NMR (125 MHz, DMSO): δ 164.06, 156.56, 149.50, 139.80, 133.11, 130.85, 130.47, 127.06, 123.82, 119.62, 118.59, 113.65, 111.17. Anal. Calcd for $C_{13}H_9ClN_2O_4$ (292.68): C, 53.35; H, 3.10; N, 9.57. Found: C, 52.98; H, 3.41; N, 9.30.

4.1.2.15. *N*-(3-Fluorophenyl)-2-hydroxy-4-nitrobenzamide (15). Yellow solid; yield 75%; mp 238-239 °C. IR (ATR): 3393, 3110, 1684 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.80 (1H, bs, OH), 10.59 (1H, s, NH), 7,93 (1H, d, J = 8.2 Hz, H6), 7.78-7.74 (2H, m, H3,H5), 7.72 (1H, d, J = 11.6 Hz, H2'), 7.47 (1H, d, J = 8.3 Hz, H6'), 7.43-7.37 (1H, m, H5'), 7.19 (1H, td, J = 8.4 Hz, J = 2.3 Hz, H4'). ^{13}C NMR (125 MHz, DMSO): δ 164.02, 162.26 (d, J = 241.6 Hz), 156.52, 149.49, 140.07 (d, J = 11.0 Hz), 130.87, 130.44 (d, J = 9.5 Hz), 127.13, 115.91, 113.66, 111.16, 110.58 (d, J = 21.1 Hz), 106.93 (d, J = 26.3 Hz). Anal. Calcd for $C_{13}H_9FN_2O_4$ (276.22): C, 56.53; H, 3.28; N, 10.14. Found: C, 55.87; H, 3.65; N, 9.98.

4.1.2.16. *N*-(3,4-Dichlorophenyl)-2-hydroxy-4-nitrobenzamide (16). Yellow solid; yield 83%; mp 230-232 °C. IR (ATR): 3325, 3115, 1638 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.79 (1H, bs, OH), 10.67 (1H, s, NH), 8.12 (1H, d, J = 2.0 Hz, H2'), 7.91 (1H, d, J = 8.2 Hz, H6), 7.78-7.74 (2H, m, H3,H5), 7.66 (1H, dd, J = 8.8 Hz, J = 2.2 Hz, H6'), 7.61 (1H, d, J = 8.8 Hz, H5'). ^{13}C NMR (125 MHz, DMSO): δ 164.13, 156.53, 149.54, 138.51, 131.06, 130.90, 130.73, 127.10, 125.57, 121.31, 120.18, 113.64, 111.18. Anal. Calcd for $C_{13}H_8Cl_2N_2O_4$ (327.12): C, 47.73; H, 2.47; N, 8.56. Found: C, 47.24; H, 2.71; N, 8.29.

4.1.2.17. 2-Hydroxy-4-nitro-*N*-[3-(trifluoromethyl)phenyl]benzamide (17). Yellow solid; yield 65%; mp 215-216 °C. IR (ATR): 3316, 3085, 1639 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.80 (1H, bs, OH), 10.73 (1H, s, NH), 8.23 (1H, s, H2'), 7.96-7.91 (2H, m, H6,H4'), 7.78-7.74 (2H, m, H3,H5), 7.61 (1H, t, J = 8.0 Hz, H5'), 7.48 (1H, d, J = 7.7 Hz, H6'). ^{13}C NMR (125 MHz, DMSO): δ 164.50, 156.84, 149.73, 139.35, 131.03, 130.21, 129.71 (q, J = 31.6 Hz), 127.23, 124.26 (q, J = 271.3 Hz), 123.96, 120.61 (q, J = 3.7 Hz), 116.44 (q, J = 4.1 Hz), 113.80, 111.39. Anal. Calcd for $C_{13}H_9F_3N_2O_4$ (326.23): C, 51.54; H, 2.78; N, 8.59. Found: C, 51.11; H, 2.96; N, 8.20.

4.1.2.18. 2-Hydroxy-4-nitro-*N*-[4-(trifluoromethyl)phenyl]benzamide (18).[31] Yellow solid; yield 69%; mp 215-216 °C. IR (ATR): 3401, 3109, 1665 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.80 (1H, bs, OH), 10.74 (1H, s, NH), 8.23 (1H, s, H2'), 7.97-7.91 (3H, m, H6,H2', H6'), 7.78-7.75 (2H, m, H3,H5), 7.73 (2H, d, *J* = 8.7 Hz, H3',H5'). ^{13}C NMR (125 MHz, DMSO): δ 164.25, 156.49, 149.53, 141.99, 130.94, 127.29, 126.09 (q, *J* = 3.7 Hz), 124.31 (q, *J* = 270.0 Hz), 124.05 (q, *J* = 32.1 Hz), 120.03, 113.67, 111.16. Anal. Calcd for $C_{13}H_9F_3N_2O_4$ (326.23): C, 51.54; H, 2.78; N, 8.59. Found: C, 51.08; H, 2.99; N, 8.14.

4.1.2.19. 2-Hydroxy-4-nitro-*N*-[4-nitro-3-(trifluoromethyl)phenyl]benzamide (19). Yellow solid; yield 69%; mp 194-195 °C. IR (ATR): 3406, 3120, 1667 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.68 (1H, bs, OH), 11.16 (1H, s, NH), 8.42 (1H, d, *J* = 1.1 Hz, H2'), 8.24 (1H, d, *J* = 9.0 Hz, H5'), 8.20 (1H, dd, *J* = 9.0 Hz, *J* = 1.8 Hz, H6'), 7.90 (1H, d, *J* = 8.2 Hz, H6), 7.79-7.74 (2H, m, H3,H5). ^{13}C NMR (125 MHz, DMSO): δ 164.79, 156.73, 149.74, 143.17, 141.87, 131.04, 127.66, 127.10, 123.29, 122.94 (q, *J* = 33.0 Hz), 122.05 (q, *J* = 271.5 Hz), 118.16 (q, *J* = 6.0 Hz), 113.48, 111.26. Anal. Calcd for $C_{13}H_8F_3N_3O_6$ (371.23): C, 45.30; H, 2.17; N, 11.32. Found: C, 44.72; H, 2.63; N, 10.92.

4.1.2.20. 2-Hydroxy-4-nitro-*N*-(3-nitrophenyl)benzamide (20). Yellow solid; yield 63%; mp 221-222 °C. IR (ATR): 3400, 3091, 1655 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.77 (1H, bs, OH), 10.85 (1H, s, NH), 8.77 (1H, s, H2'), 8.04 (1H, dd, *J* = 7.8 Hz, *J* = 1.3 Hz, H4'), 7.98 (1H, dd, *J* = 8.2 Hz, *J* = 2.2 Hz, H6'), 7.93 (1H, d, *J* = 9.3 Hz, H6), 7.80-7.73 (2H, m, H3,H5), 7.65 (1H, t, *J* = 8.2 Hz, H5'). ^{13}C NMR (125 MHz, DMSO): δ 164.42, 156.58, 149.58, 147.95, 139.55, 130.91, 130.22, 127.09, 126.13, 118.60, 114.23, 113.67, 111.20. Anal. Calcd for $C_{13}H_9N_3O_6$ (303.23): C, 51.49; H, 2.99; N, 13.86. Found: C, 50.94; H, 3.24; N, 13.52.

4.1.2.21. 2-Hydroxy-4-nitro-*N*-(4-nitrophenyl)benzamide (21). Yellow solid; yield 61%; mp 253-255 °C. IR (ATR): 3392, 3090, 1655 (CO). ^{1}H NMR (500 MHz, DMSO): δ 10.90 (1H, s, NH), 8.30-8.21 (2H, m, H3',H5'), 8.02-7.96 (2H, m, H2',H6'), 7.94 (1H, d, *J* = 8.4 Hz, H6), 7.80-7.73 (2H, m, H3,H5). ^{13}C NMR (125 MHz, DMSO): δ 164.23, 156.47, 149.60, 144.30, 142.75, 130.79, 126.86, 124.60, 119.74, 113.38, 111.11. Anal. Calcd for $C_{13}H_9N_3O_6$ (303.23): C, 51.49; H, 2.99; N, 13.86. Found: C, 50.98; H, 3.18; N, 13.61.

4.1.2.22. *N*-[3,5-Bis(trifluoromethyl)phenyl]-2-hydroxy-4-nitrobenzamide (22). Yellow solid; yield 74%; mp 253-255 °C. IR (ATR): 3340, 3116, 1640 (CO). ^{1}H NMR (300 MHz, DMSO): δ 11.72 (1H, bs, OH), 10.98 (1H, s, NH), 8.43 (2H, s, H2',H6'), 7.92 (1H, d, *J* = 8.0 Hz, H6), 7.83 (1H, s, H4'), 7.80-7.73 (2H, m, H3,H5). ^{13}C NMR (75 MHz, DMSO): δ 164.69, 156.48, 149.69, 140.36, 130.97, 130.81 (q, *J* = 32.3 Hz), 126.98, 123.20 (q, *J* = 271.5 Hz), 119.91 (q, *J* = 3.75 Hz), 116.90 (q, *J* = 3.8 Hz), 113.71, 111.19. Anal. Calcd for $C_{13}H_8F_6N_2O_4$ (394.23): C, 45.70; H, 2.05; N, 7.11. Found: C, 45.24; H, 2.39; N, 8.89.

4.1.2.23. 2-Hydroxy-*N*-(3-iodophenyl)-5-nitrobenzamide (23). White solid; yield 80%; mp 239-240 °C. IR (ATR): 3332, 3129, 1637 (CO). ^{1}H NMR (500 MHz, DMSO): δ 10.58 (1H, s, NH), 8.72 (1H, d, *J* = 2.8 Hz, H6), 8.28 (1H, dd, *J* = 9.1 Hz, *J* = 2.8 Hz, H4), 8.20 (1H, s, H2'), 7.69 (1H, d, *J* = 8.2 Hz, H6'), 7.51 (1H, d, *J* = 7.8 Hz, H4'), 7.18 (1H, t, *J* = 10.0 Hz, H5'), 7.16 (1H, d, *J* = 10.0 Hz, H3). ^{13}C NMR (125 MHz, DMSO): δ 164.15, 163.07, 139.45, 139.33, 132.85, 130.79, 128.76, 128.40, 125.78, 119.88, 119.72, 117.96, 94.49. Anal. Calcd for $C_{13}H_9IN_2O_4$ (384.13): C, 40.65; H, 2.36; N, 7.29. Found: C, 40.11; H, 2.71; N, 6.94.

4.1.2.24. *N*-(3-Bromophenyl)-2-hydroxy-5-nitrobenzamide (24). White solid; yield 76%; mp 231-232 °C. IR (ATR): 3333, 3077, 1617 (CO). ^{1}H NMR (300 MHz, DMSO): δ 10.64 (1H, s, NH), 8.70 (1H, d, *J* = 2.9 Hz, H6), 8.28 (1H, dd, *J* = 9.2 Hz, *J* = 2.9 Hz, H4), 8.06 (1H, s, H2'), 7.65 (1H, m, H5'), 7.35 (2H, m, H4',H6'), 7.16 (1H, d, *J* = 9.2 Hz, H3). ^{13}C NMR (75 MHz, DMSO): δ 164.16, 162.98, 139.70, 139.36, 130.78, 128.42, 126.93, 125.85,

122.91, 121.52, 119.89, 119.40, 117.95. Anal. Calcd for $C_{13}H_9BrN_2O_4$ (337.13): C, 46.32; H, 2.69; N, 8.31. Found: C, 45.91; H, 2.97; N, 8.04.

4.1.2.25. N-(3-Chlorophenyl)-2-hydroxy-5-nitrobenzamide (25).[32] White solid; yield 76%; mp 227.5-228.5 °C. IR (ATR): 3331, 3085, 1630 (CO). ^{1}H NMR (500 MHz, DMSO): δ 10.64 (1H, s, NH), 8.71 (1H, d, J = 2.9 Hz, H6), 8.28 (1H, dd, J = 9.0 Hz, J = 2.9 Hz, H4), 7.92 (1H, s, H2'), 7.62 (1H, m, H6'), 7.40 (1H, t, J = 8.1 Hz, H5'), 7.20 (1H, d, J = 8.2 Hz, H4'), 7.16 (1H, d, J = 9.1 Hz, H3). ^{13}C NMR (125 MHz, DMSO): δ 164.14, 162.95, 139.54, 139.36, 133.08, 130.45, 128.39, 125.84, 124.01, 120.07, 119.86, 119.01, 117.94. Anal. Calcd for $C_{13}H_9ClN_2O_4$ (392.68): C, 53.35; H, 3.10; N, 9.57. Found: C, 52.89; H, 3.52; N, 9.10.

4.1.2.26. N-(3-Fluorophenyl)-2-hydroxy-5-nitrobenzamide (26).[32, 33] White solid; yield 73%; mp 218-219 °C. IR (ATR): 3333, 3085, 1612 (CO). ^{1}H NMR (500 MHz, DMSO): δ 10.66 (1H, s, NH), 8.70 (1H, d, J = 2.8 Hz, H6), 8.28 (1H, dd, J = 9.1 Hz, J = 2.8 Hz, H4), 7.71 (1H, d, J = 11.5 Hz, H2'), 7.47 (1H, d, J = 8.2 Hz, H6'), 7.41 (1H, dd, J = 15.0 Hz, J = 8.0 Hz, H5'), 7.16 (1H, d, J = 9.1 Hz, H3), 6.98 (1H, td, J = 8.5 Hz, J = 2.4 Hz, H4'). ^{13}C NMR (125 MHz, DMSO): δ 164.07, 162.88, 162.07 (d, J = 240.0 Hz), 139.81 (d, J = 11.0 Hz), 139.38, 130.41 (d, J = 9.4 Hz), 128.36, 125.86, 119.97, 117.92, 116.32 (d, J = 2.7 Hz), 110.78 (d, J = 21.1 Hz), 107.37 (d, J = 26.2 Hz). Anal. Calcd for $C_{13}H_9FN_2O_4$ (276.22): C, 56.53; H, 3.28; N, 10.14. Found: C, 56.29; H, 3.61; N, 9.85.

4.1.2.27. N-(3,4-Dichlorophenyl)-2-hydroxy-5-nitrobenzamide (27).[32, 33] White solid; yield 84%; mp 273-274 °C. IR (ATR): 3391, 3097, 1655 (CO). ^{1}H NMR (300 MHz, DMSO): δ 10.70 (1H, s, NH), 8.67 (1H, d, J = 2.8 Hz, H6), 8.27 (1H, dd, J = 9.2 Hz, J = 2.9 Hz, H4), 8.09 (1H, d, J = 2.1 Hz, H2'), 7.67 (1H, dd, J = 8.8 Hz, J = 2.2 Hz, H6'), 7.61 (1H, d, J = 8.8 Hz, H5'), 7.15 (1H, d, J = 9.0 Hz, H3). ^{13}C NMR (75 MHz, DMSO): δ 164.13, 162.87, 139.34, 138.26, 131.04, 130.68, 128.46, 125.91, 125.80, 121.75, 120.56, 119.92, 117.94. Anal. Calcd for $C_{13}H_8Cl_2N_2O_4$ (327.12): C, 47.73; H, 2.47; N, 8.56. Found: C, 47.21; H, 2.78; N, 8.12.

4.1.2.28. 2-Hydroxy-5-nitro-N-[3-(trifluoromethyl)phenyl]benzamide (28).[33] White solid; yield 71%; mp 181-183 °C. IR (ATR): 3342, 3199, 1632 (CO). ^{1}H NMR (500 MHz, DMSO): δ 10.79 (1H, s, NH), 8.73 (1H, d, J = 2.8 Hz, H6), 8.29 (1H, dd, J = 9.1 Hz, J = 2.9 Hz, H4), 8.21 (1H, s, H2'), 7.94 (1H, d, J = 8.1 Hz, H4'), 7.62 (1H, t, J = 8.0 Hz, H5'), 7.50 (1H, d, J = 7.7 Hz, H6'), 7.17 (1H, d, J = 9.0 Hz, H3). ^{13}C NMR (125 MHz, DMSO): δ 164.40, 163.03, 139.34, 138.90, 130.00, 129.49 (q, J = 31.6 Hz), 128.42, 125.83, 124.06 (q, J = 271.0 Hz), 124.21, 120.61 (q, J = 3.7 Hz), 119.82, 117.96, 116.73 (q, J = 4.1 Hz). Anal. Calcd for $C_{14}H_9F_3N_2O_4$ (326.23): C, 51.54; H, 2.78; N, 8.59. Found: C, 51.08; H, 3.01; N, 8.10.

4.1.2.29. 2-Hydroxy-5-nitro-N-[4-(trifluoromethyl)phenyl]benzamide (29).[31] White solid; yield 76%; mp 255-256 °C. IR (ATR): 3391, 3097, 1632 (CO). ^{1}H NMR (300 MHz, DMSO): δ 10.79 (1H, s, NH), 8.69 (1H, d, J = 2.9 Hz, H6), 8.28 (1H, dd, J = 9.2 Hz, J = 2.9 Hz, H4), 7.94 (2H, d, J = 8.6 Hz, H3',H5'), 7.73 (2H, d, J = 8.7 Hz, H2',H6'), 7.17 (1H, d, J = 9.2 Hz, H3). ^{13}C NMR (75 MHz, DMSO): δ 164.21, 162.78, 141.80, 141.79, 139.38, 128.40, 126.07 (q, J = 3.75 Hz), 124.12 (q, J = 31.5 Hz), 122.51, 120.41, 120.26, 117.91. Anal. Calcd for $C_{14}H_9F_3N_2O_4$ (326.23): C, 51.54; H, 2.78; N, 8.59. Found: C, 51.12; H, 3.12; N, 8.03.

4.1.2.30. 2-Hydroxy-5-nitro-N-[4-nitro-3-(trifluoromethyl)phenyl]benzamide (30). White solid; yield 62%; mp 255-256 °C. IR (ATR): 3358, 3094, 1665 (CO). ^{1}H NMR (500 MHz, DMSO): δ 11.15 (1H, s, NH), 8.63 (1H, d, J = 2.9 Hz, H6), 8.42 (1H, s, H2'), 8.59 (1H, dd, J = 9.1 Hz, J = 2.9 Hz, H4), 8.24 (1H, d, J = 9.1 Hz, H5'), 8.22 (1H, dd, J = 9.0 Hz, J = 1.9 Hz, H6'), 7.17 (1H, d, J = 9.2 Hz, H3). ^{13}C NMR (125 MHz, DMSO): δ 164.70, 162.76, 143.15, 142.14, 139.45, 128.74, 127.72, 126.27, 123.77, 122.73 (q, J = 31.3 Hz), 122.26 (q, J

= 273Hz), 120.63, 118.66 (q, J = 6.25 Hz), 118.05. Anal. Calcd for $C_{14}H_9F_3N_2O_4$ (371.23): C, 45.30; H, 2.17; N, 11.32. Found: C, 44.88; H, 2.46; N, 11.01.

4.1.2.31. 2-Hydroxy-5-nitro-*N*-(3-nitrophenyl)benzamide (31).[32, 34] White solid; yield 66%; mp 235-236 °C. IR (ATR): 3342, 3091, 1626 (CO). ^{1}H NMR (500 MHz, DMSO): δ 10.89 (1H, s, NH), 8.74 (1H, t, J = 1.9 Hz, H2'), 8.70 (1H, d, J = 2.8 Hz, H6), 8.28 (1H, dd, J = 9.1 Hz, J = 2.8 Hz, H4), 8.06 (1H, d, J = 8.1 Hz, H4'), 7.99 (1H, dd, J = 8.1 Hz, J = 2.0 Hz, H6'), 7.66 (1H, d, J = 8.2 Hz, H5'), 7.17 (1H, d, J = 9.1 Hz, H3). ^{13}C NMR (125 MHz, DMSO): δ 164.44, 162.95, 147.91, 139.32, 139.31, 130.17, 128.49, 126.50, 125.90, 119.87, 118.73, 117.95, 114.63. Anal. Calcd for $C_{13}H_9N_3O_6$ (303.23): C, 51.49; H, 2.99; N, 13.86. Found: C, 51.12; H, 3.14; N, 13.55.

4.1.2.32. 2-Hydroxy-5-nitro-*N*-(4-nitrophenyl)benzamide (32). White solid; yield 67%; mp 208-210 °C. IR (ATR): 3375, 3088, 1660 (CO). ^{1}H NMR (300 MHz, DMSO): δ 11.04 (1H, s, NH), 8.63 (1H, d, J = 2.7 Hz, H6), 8.27 (3H, m, H4,H3',H5'), 7.98 (2H, d, J = 9.1 Hz, H2',H6'), 7.16 (1H, d, J = 9.1 Hz, H3). ^{13}C NMR (75 MHz, DMSO): δ 164.23, 156.48, 149.61, 144.31, 142.76, 130.79, 126.86, 124.60, 119.74, 113.38, 111.12. Anal. Calcd for $C_{13}H_9N_3O_6$ (303.23): C, 51.49; H, 2.99; N, 13.86. Found: C, 51.21; H, 3.21; N, 13.47.

4.1.2.33. *N*-[3,5-Bis(trifluoromethyl)phenyl]-2-hydroxy-5-nitrobenzamide (33).[16] White solid; yield 69%; mp 223-224 °C. IR (ATR): 3387, 3097, 1645 (CO). ^{1}H NMR (300 MHz, DMSO): δ 11.02 (1H, s, NH), 8.69 (1H, d, J = 2.9 Hz, H6), 8.42 (2H, s, H2',H6'), 8.28 (1H, dd, J = 9.1 Hz, J = 2.9 Hz, H4), 7.82 (1H, s, H4'), 7.16 (1H, d, J = 9.1 Hz, H3). ^{13}C NMR (75 MHz, DMSO): δ 164.64, 162.90, 140.15, 139.29, 130.73 (q, J = 32.9 Hz), 128.58, 125.97, 125.90, 123.19 (q, J = 272.8 Hz), 120.32, 120.29, 119.78, 117.98, 117.96, 116.96 (q, J = 3.7 Hz). Anal. Calcd for $C_{15}H_8F_6N_2O_4$ (394.23): C, 45.70; H, 2.05; N, 7.11. Found: C, 45.24; H, 2.57; N, 6.87.

4.2. Biology

4.2.1 In vitro antimycobacterial evaluation

The *in vitro* antimycobacterial activity of the synthesized compounds was determined against *Mycobacterium tuberculosis* My 331/88 (H$_{37}$R$_V$), *M. avium* My 330/80, *M. kansasii* 235/80 and *M. kansasii* 6509/96. All strains were obtained from the Czech National Collection of Type Cultures (CNCTC, Brno, Czech Republic) except of *M. kansasii* 6509/96, which was clinically isolated. Basic suspensions of the mycobacterial strains were prepared according to a 1.0 McFarland standard. From the basic suspension, subsequent dilutions of each strain were made: *M. tuberculosis* 10^{-3}, *M. avium* 10^{-5} and *M. kansasii* 10^{-4}. The antimycobacterial activity of the compounds was determined in a Šula's semisynthetic medium (SEVAC, Prague, Czech Republic) via the micromethod for the determination of the minimum inhibitory concentration (MIC) at 37 °C after 14 and 21 days and after 7, 14 and 21 days for *M. kansasii*.[40] The tested compounds were added to the medium as DMSO solutions while INH was used as a standard in a sterile water solution. The concentrations of the tested compounds were used as following: 500, 250, 125, 62.5, 32, 16, 8, 4, 2, 1 and 0.5 μM. The same concentrations within the range from 0.5 to 250 μM were used for INH.

4.2.2 In vitro antibacterial evaluation

The *in vitro* antibacterial activity was assayed against eight Gram-positive and Gram-negative strains: *Staphylococcus aureus* CCM 4516/08, methicillin-resistant *Staphylococcus aureus* H 5996/08 (MRSA), *Staphylococcus epidermis* H 6966/08, *Enterococcus aureus* H

6966/08, *Enterococcus* sp. J 14365/08, *Escherichia coli* CCM 4517, *Klebsiella pneumoniae* D 11750/08, ESBL-positive *Klebsiella pneumoniae* J 14368/08, and *Pseudomonas aeruginosa* CCM 1961.

The microdilution broth method modified according to standard M07-A07 in Mueller-Hinton broth (HiMedia Laboratories, Mumbai, India) adjusted to pH 7.4 ($\pm$0.2) was used. The tested compounds were dissolved in DMSO to the final concentrations ranging from 500 to 0.49 µmol/L. Benzylpenicillin (penicillin G) was used as a comparative drug. Bacterial inoculum in sterile water was prepared to match 0.5 McFarland scale (1.5 ± 10^8 CFU/mL). The minimum inhibitory concentrations were assayed as 95% (IC_{95}) or higher reduction of growth when compared to the control. The determination of results was performed visually and spectrophotometrically (at 540 nm). The values of MICs were determined after 24 and 48 h of incubation in the darkness at 35 ^{o}C ($\pm$0.1) in a humid atmosphere.

4.2.3 In vitro antifungal evaluation

The antifungal properties were evaluated in vitro against four *Candida* strains (*Candida albicans* ATCC 44859, *Candida tropicalis* 156, *Candida krusei* E28, and *Candida glabrata* 20/I), *Trichosporon asahii* 1188 and three filamentous fungi (*Aspergillus fumigatus* 231, *Absidia corymbifera* 272, and *Trichophyton mentagrophytes* 445). The microdilution broth method was used according to the CLSI M27-A3 and M38-A2 guidelines in RPMI 1640 with glutamine (KlinLab, Prague, Czech Republic) buffered to pH 7.0 with 0.165 mol of 3-morpholino-propane-1-sulphonic acid (Sigma-Aldrich, Darmstadt, Germany). DMSO served as a diluent for all of the compounds. In yeast, the final size of the inoculum was $5 \times 10^3 \pm 0.2$ CFU/mL. In the case of the moulds *Aspergillus*, *Trichophyton* and *Absidia corymbifera*, the spores were harvested after cultivation of a given fungal strain grown on Sabouraud agar from 3 to 10 days. The final size of the inoculum was $0.5-5 \times 10^4$ CFU/mL. The sizes of the fungal inocula were checked using a Bürker chamber. Fluconazole was used as a reference drug. The MIC values for yeasts and filamentous fungi were assayed as a reduction of growth of at least 80% (IC_{80}) or of at least 50% (IC_{50}) compared with the control, respectively. The results were analyzed visually and spectrophotometrically (at 540 nm). The MIC values were determined after 24 and 48 h of incubation in the dark at 35 ^{o}C ($\pm$0.1) in a humid atmosphere, but for *T. mentagrophytes*, the final MIC values were determined after 72 and 120 h of incubation.

4.2.4 Cytotoxicity

Twelve nitrosalicylanilides, selected depending on the position of their nitro-substitution and their antimycobacterial activity, were tested for cytotoxicity in the human hepatocellular liver carcinoma cell line HepG2 (passages 33-37; ECACC, Salisbury, UK) using a standard colorimetric method measuring a tetrazolium salt reduction (CellTiter(R) 96 AQueous One Solution Assay, Promega G3580, Madison, WI, USA).

The cells were routinely cultured in Minimum Essentials Eagle Medium (Sigma-Aldrich, Darmstadt, Germany) supplemented with 10% fetal bovine serum (PAA, Biotech, Prague, Czech Republic), 1% of L-glutamine solution (Sigma-Aldrich), and non-essential amino acids solution (Sigma-Aldrich) in a humidified atmosphere containing 5% CO_2 at 37 ^{o}C. For subculturing, the cells were harvested after trypsin/EDTA (Sigma-Aldrich) treatment at 37 ^{o}C. The cells treated with the tested substances were used as experimental groups. Untreated HepG2 cells were used as control groups.

The cells were seeded in density 1×10^4 cells per well in a 96-well plate with microscopic control. Next day, the cells were treated with each of the tested substances. All tested

substances were prepared at concentrations 0.1-100 μM and tested in triplicates. The following types of controls were included: determination of 100% viability and 0% viability (the cells treated by 10% DMSO), no cell control and vehiculum controls. These checking samples were prepared in triplicates.

The treated cells were incubated together with controls at 37 $^{\circ}$C for 24 h in 5% CO_2 atmosphere. After this time reagent from the kit CellTiter 96 AQueous One Solution Cell Proliferation Assay was added. The CellTiter 96 assay is based on the reduction of tetrazolium salt MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] to water-soluble formazan dye by metabolically active cells. The reduction of the reagent is attributed 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 tested plate was incubated for 2 h at 37 $^{\circ}$C and 5% CO_2 and after this time, the absorbance was recorded at 490 nm using a 96-well plate reader (TECAN, Infinite M200, Grödig, Austria).

The results were expressed as inhibitory concentration which is necessary to inhibit cell viability to 50% from the maximal (control) viability (IC_{50}). A standard toxicological parameter IC_{50} was calculated in each of the tested substances using GraphPad Prism software (version 6; GraphPad Software Inc. San Diego, CA, USA).

References

1. Global Tuberculosis Report 2014, World Health Organization
http://www.who.int/tb/publications/global_report/en/
2. Acosta, C.D.; Dadu, A.; Ramsay, A.; Dara, M. *Public health action*, **2014**, *4*, S3-s12.
3. Engstrom, A. *Infectious diseases (London, England)*, **2015**, 1-17.
4. Parida, S.K.; Axelsson-Robertson, R.; Rao, M.V.; Singh, N.; Master, I.; Lutckii, A.; Keshavjee, S.; Andersson, J.; Zumla, A.; Maeurer, M. *Journal of internal medicine*, **2015**, *277*, 388-405.
5. Tostmann, A.; Boeree, M.J.; Aarnoutse, R.E.; de Lange, W.C.; van der Ven, A.J.; Dekhuijzen, R. *J Gastroenterol Hepatol*, **2008**, *23*, 192-202.
6. Frieden, T.R.; Sterling, T.R.; Munsiff, S.S.; Watt, C.J.; Dye, C. *Lancet*, **2003**, *362*, 887-899.
7. Mirsaeidi, M.; Farshidpour, M.; Ebrahimi, G.; Aliberti, S.; Falkinham, J.O., 3rd *Eur J Intern Med*, **2014**, *25*, 356-363.
8. van Ingen, J.; Boeree, M.J.; van Soolingen, D.; Mouton, J.W. *Drug Resist Updat*, **2012**, *15*, 149-161.
9. Livermore, D.M. *Int J Antimicrob Agents*, **2012**, *39*, 283-294.
10. Macielag, M.J.; Demers, J.P.; Fraga-Spano, S.A.; Hlasta, D.J.; Johnson, S.G.; Kanojia, R.M.; Russell, R.K.; Sui, Z.; Weidner-Wells, M.A.; Werblood, H.; Foleno, B.D.;

Goldschmidt, R.M.; Loeloff, M.J.; Webb, G.C.; Barrett, J.F. *J Med Chem*, **1998**, *41*, 2939-2945.

11. De La Fuente, R.; Sonawane, N.D.; Arumainayagam, D.; Verkman, A.S. *Br J Pharmacol*, **2006**, *149*, 551-559.

12. Imramovský, A.; Vinšová, J.; Férriz, J.M.; Buchta, V.; Jampílek, J. *Bioorg Med Chem Lett*, **2009**, *19*, 348-351.

13. Vinšová, J.; Kozic, J.; Krátký, M.; Stolaříková, J.; Mandíková, J.; Trejtnar, F.; Buchta, V. *Bioorg Med Chem*, **2014**, *22*, 728-737.

14. Krátký, M.; Vinšová, J.; Novotná, E.; Stolaříková, J. *Eur J Pharm Sci*, **2014**, *53*, 1-9.

15. Pauk, K.; Zadražilová, I.; Imramovský, A.; Vinšová, J.; Pokorná, M.; Masaříková, M.; Čížek, A.; Jampílek, J. *Bioorg Med Chem*, **2013**, *21*, 6574-6581.

16. Lee, I.Y.; Gruber, T.D.; Samuels, A.; Yun, M.; Nam, B.; Kang, M.; Crowley, K.; Winterroth, B.; Boshoff, H.I.; Barry, C.E., 3rd *Bioorg Med Chem*, **2013**, *21*, 114-126.

17. Krátký, M.; Vinšová, J.; Novotná, E.; Mandíková, J.; Wsól, V.; Trejtnar, F.; Ulmann, V.; Stolaříková, J.; Fernandes, S.; Bhat, S.; Liu, J.O. *Tuberculosis (Edinb)*, **2012**, *92*, 434-439.

18. Krátký, M.; Vinšová, J. *Curr Pharm Des*, **2011**, *17*, 3494-3505.

19. Kratky, M.; Vinsova, J.; Stolarikova, J. *Molecules*, **2012**, *17*, 12812-12820.

20. Krátký, M.; Bősze, S.; Baranyai, Z.; Szabó, I.; Stolaříková, J.; Paraskevopoulos, G.; Vinšová, J. *Bioorg Med Chem*, **2015**, *23*, 868-875.

21. Waisser, K.; Bureš, O.; Holý, P.; Kuneš, J.; Oswald, R.; Jirásková, L.; Pour, M.; Klimešová, V.; Kubicová, L.; Kaustová, J. *Archiv der Pharmazie*, **2003**, *336*, 53-71.

22. Zumla, A.; Nahid, P.; Cole, S.T. *Nature reviews Drug discovery*, **2013**, *12*, 388-404.

23. Poce, G.; Cocozza, M.; Consalvi, S.; Biava, M. *Eur J Med Chem*, **2014**, *86*, 335-351.

24. Matsumoto, M.; Hashizume, H.; Tomishige, T.; Kawasaki, M.; Tsubouchi, H.; Sasaki, H.; Shimokawa, Y.; Komatsu, M. *PLoS Med*, **2006**, *3*, e466.

25. Lenaerts, A.J.; Gruppo, V.; Marietta, K.S.; Johnson, C.M.; Driscoll, D.K.; Tompkins, N.M.; Rose, J.D.; Reynolds, R.C.; Orme, I.M. *Antimicrob Agents Chemother*, **2005**, *49*, 2294-2301.

26. Trefzer, C.; Skovierova, H.; Buroni, S.; Bobovska, A.; Nenci, S.; Molteni, E.; Pojer, F.; Pasca, M.R.; Makarov, V.; Cole, S.T.; Riccardi, G.; Mikusova, K.; Johnsson, K. *J Am Chem Soc*, **2012**, *134*, 912-915.

27. Batt, S.M.; Jabeen, T.; Bhowruth, V.; Quill, L.; Lund, P.A.; Eggeling, L.; Alderwick, L.J.; Futterer, K.; Besra, G.S. *Proc Natl Acad Sci U S A*, **2012**, *109*, 11354-11359.

28. Christophe, T.; Jackson, M.; Jeon, H.K.; Fenistein, D.; Contreras-Dominguez, M.; Kim, J.; Genovesio, A.; Carralot, J.P.; Ewann, F.; Kim, E.H.; Lee, S.Y.; Kang, S.; Seo, M.J.; Park, E.J.; Skovierova, H.; Pham, H.; Riccardi, G.; Nam, J.Y.; Marsollier, L.; Kempf, M.; Joly-Guillou, M.L.; Oh, T.; Shin, W.K.; No, Z.; Nehrbass, U.; Brosch, R.; Cole, S.T.; Brodin, P. *PLoS Pathog*, **2009**, *5*, e1000645.

29. Karabanovich, G.; Roh, J.; Smutný, T.; Němeček, J.; Vicherek, P.; Stolaříková, J.; Vejsová, M.; Dufková, I.; Vávrová, K.; Pávek, P.; Klimešová, V.; Hrabálek, A. *Eur J Med Chem*, **2014**, *82*, 324-340.

30. Karabanovich, G.; Roh, J.; Soukup, O.; Pávková, I.; Pasdiorová, M.; Tambor, V.; Stolaříková, J.; Vejsová, M.; Vávrová, K.; Klimešová, V. *MedChemComm*, **2015**, *6*, 174-181.

31. Fukai, R.; Zheng, X.; Motoshima, K.; Tai, A.; Yazama, F.; Kakuta, H. *ChemMedChem*, **2011**, *6*, 550-560.

32. Waisser, K.; Hladůvková, J.; Kuneš, J.; Kubicová, L.; Klimesova, V.; Karajannis, P.; Kaustová, J. *Chem Pap*, **2001**, *55*, 121-129.

33. Zuo, M.; Zheng, Y.W.; Lu, S.M.; Li, Y.; Zhang, S.Q. *Bioorg Med Chem*, **2012**, *20*, 4405-4412.

34. Jadvah, G.V.; Nerlekar, P.G. *J Univ Bombay*, **1951**, *20*, 93.

35. Diaza, R.G.; Manganelli, S.; Esposito, A.; Roncaglioni, A.; Manganaro, A.; Benfenati, E. *SAR QSAR Environ Res*, **2015**, *26*, 1-27.

36. Ruiz, P.; Begluitti, G.; Tincher, T.; Wheeler, J.; Mumtaz, M. *Molecules*, **2012**, *17*, 8982-9001.

37. Senousy, B.E.; Belal, S.I.; Draganov, P.V. *Nat Rev Gastroenterol Hepatol*, **2010**, *7*, 543-556.

38. Kratky, M.; Volkova, M.; Novotna, E.; Trejtnar, F.; Stolarikova, J.; Vinsova, J. *Bioorg Med Chem*, **2014**, *22*, 4073-4082.

39. Krátký, M.; Vinšová, J.; Buchta, V.; Horvati, K.; Bősze, S.; Stolařiková, J. *Eur J Med Chem*, **2010**, *45*, 6106-6113.

40. Kaustová, J. *Klin Microbiol Inf Lék*, **1997**, *3*, 115-124.