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Novel salicylanilides from 4,5-dihalogenated salicylic acids: Synthesis, antimicrobial activity and cytotoxicity

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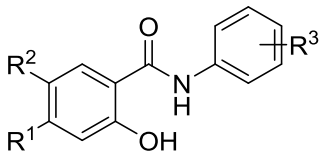
Abstract

Salicylanilides have proved their activity against tuberculosis (TB). One weak electron-withdrawing substituent is favored at the salicylic part, specially Cl or Br atoms at positions 4 or 5. On the other hand, the antimycobacterial activity of salicylanilides is negatively affected when a strong electron-withdrawing substituent ($-NO_2$) is present at the same positions. Herein we describe the synthesis and characterization of novel salicylanilides possessing two weak electron-withdrawing groups (halogen atoms) at their salicylic part and compare their antitubercular activity with their monohalogenated analogues. All dihalogenated derivatives proved to possess antitubercular activity at a very narrow micromolar range (MIC= 1-4 μM), similar with their most active monohalogenated analogues. More importantly, the most active final molecules were further screened against multidrug resistant strains and found to inhibit their growth at the range of 0.5-4 μM.

Keywords

Salicylanilides
Double halogenation
Tuberculosis
In vitro antimycobacterial activity
Cytotoxicity

Graphical abstract



R^1, R^2 = Br or Cl
R^3 = 4-Br, 3,4-diCl,
4-CF_3, 3,5-diCF_3

M. tuberculosis MIC: 1-4 μM
MDR strains MIC: 0.5-4 μM

1. Introduction

The World Health Organization (WHO) recently reported that around 9.6 million people were diagnosed with tuberculosis (TB) in 2014, having about 1.5 million of those died from the disease [1]. According to these data, TB joined Human Immunodeficiency Virus (HIV) on the top rank of the deadliest infectious diseases, while being responsible for one fourth of HIV-related deaths. Currently available drugs are not able to eliminate *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB, while residing in a dormant state after the initial infection. Apart from that, acquired immunodeficiency, treatments with immunosuppressive drugs or concomitant diseases may reactivate *Mtb* [2, 3]. Standard antimycobaterial agents, like isoniazid (INH) and rifampicin (RIF), have been used for decades in order to fight the disease. Their overuse or misuse resulted the appearance of multi-drug resistant (MDR-TB) strains [4-6], extensively drug-resistant TB (XDR-TB) strains [7] and totally drug-resistant TB (TDR-TB) [8]. The TB drug-development pipeline is focusing on new regiments to short and improve outcomes of TB treatment and it mainly includes new and repurposed antimicrobials as well as host directed drugs [9].

2-Hydroxy-*N*-phenylbenzamides, commonly known as salicylanilides, have been highly noticed due to their significant *in vitro* antimicrobial and antifungal activities [10-12]. Many analogues of salicylanilides have been developed so far with proven activity against *Mtb* and INH-resistant nontuberculous mycobacterial strains [13, 14]. In terms of their mode of action, there is no clear report up to date. The presence of a free phenolic hydroxyl on the salicylic acid moiety was proved as necessary, and the hypothesis that the molecules function as proton shuttles that kill bacterial cells by destroying the cellular proton gradient has been suggested [15]. On the other hand, salicylanilides and their ester derivatives have been reported to moderate inhibit the 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 [16]. Furthermore, they have been reported to inhibit the two-component regulatory systems [10] as well as multiple bacterial enzymes, like transglycosylase [17], sortase A [18] and D-alanine-D-alanine ligase [19]. Finally, salicylanilide esters, carbamates and additional derivatives resulted from their conjugation with existing antimicrobial agents, have been suggested to work as pro-drugs that are hydrolyzed in order to express their activity [20]. Thus, it can be concluded that salicylanilides affect multiple targets with more than one way in order to exert their antimicrobial and antimycobacterial properties.

Despite their high anti-tubercular activity, salicylanilides and their derivatives (esters, carbamates etc.) often share a non-preferable cytotoxic profile [15], which limits their

potential as therapeutics. Recent efforts of our group has been focused on a 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 cancer cell line (HepG2) [21]. The esterification products of 5-halosalicylanilides with 4-substituted benzoic acids 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. This way, increased cytotoxicity can be overcome while the activity of the final molecules remains intact.

The importance of different substituents at the salicylic or aniline part of salicylanilides (**Figure 1**) in order to express their antimycobacterial or antimicrobial activities has been previously highlighted. Up to date, extensive structure-antimycobacterial activity relationship (SAR) studies at the aniline part (**Figure 1, ring B**) have been performed and proved that activity is always favorably influenced by higher lipophilicity and electron-accepting substituents [15, 22, 23] while trifluoromethyl group at position 4 has been found to be the best substituent. On the other hand, the influence of the electronic parameters at the acyl part (**Figure 1, ring A**) cannot be evaluated in a simple way. To the best of our knowledge, the preferred substituents for ring A referred up to date is chlorine at position 4 or 5 and bromine at position 5. 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 1-2 μM respectively and they are the most active salicylanilides reported so far [22]. The antimycobacterial properties of salicylanilides possessing bromine at position 4 of the salicylic part have not been previously reported.

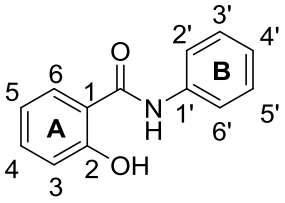


Figure 1. General structure of salicylanilides consisting of salicylic part (ring A) and aniline part (ring B)

During our ongoing efforts in order to explore the influence of different substitutions on the parent structure of salicylanilides, in terms of activity and cytotoxicity, the introduction of several substituents is under investigation. We have recently reported that the insertion of a strong electron-withdrawing group, like nitro-group, at the salicylic part of salicylanilides resulted active molecules with MICs at the range of 2-32 μM against *Mycobacterium tuberculosis*, while the nitro group was mostly favored at position 4 [24]. Despite the high activity of 4-nitrosalicylanilides, the presence of one halogen instead of nitro group was still more favored (lower MIC).

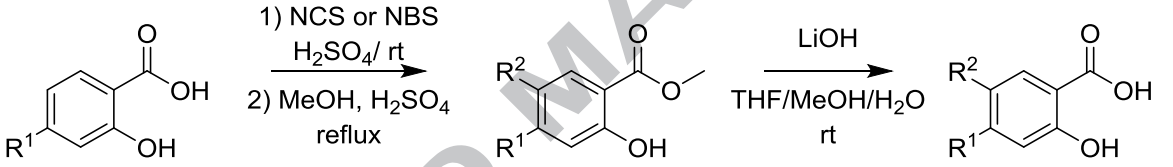
In our current approach, we examined how antimycobacterial activity is influenced when two weak electron-withdrawing groups (Cl and Br) are at positions 4 and 5 of the salicylic part instead of one strong electron-withdrawing during our previous attempt [24]. Furthermore, we compared their antimycobacterial activities and their cytotoxic profile against HepG2 cells with their monohalogenated analogues.

2. Results and Discussion

2.1. Chemistry

In this study, the synthesis of the selected 4- or 5-monohalogenated salicylanilides at ring A performed following direct conjugation of the 4- or 5-halogenated salicylic acid with the corresponding aniline in the presence of PCl_3 using chlorobenzene as solvent under microwave conditions [25]. The aniline part was modified with selected substituents that proved before to afford the most active analogues [15, 22]. More specifically, the aniline part modifications were 4-CF_3, 3,5-diCF_3, 3,4-diCl and 4-Br. The synthesis and mycobacterial activity for the most 4-chloro-, 5-bromo- and 5-chloro-salicylanilides have been previously reported by our group and others [15, 21-23, 26] while salicylanilides of 4-bromosalicylic acid are presented herein for the first time.

The synthesis of 4,5-dihalogenated salicylic acids from 4-halogenated salicylic acids performed in three steps (**Scheme 1**).



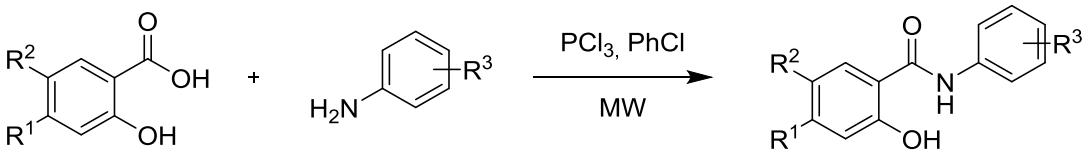
(I) R^1= Br
(II) R^2=Cl

(III) R^1= Br, R^2= Cl
(IV) R^1= Br, R^2= Br
(V) R^1= Cl, R^2= Cl
(VI) R^1= Cl, R^2= Br

(VII) R^1= Br, R^2= Cl
(VIII) R^1= Br, R^2= Br
(IX) R^1= Cl, R^2= Cl
(X) R^1= Cl, R^2= Br

Scheme 1. Synthesis of 4,5-dihalogehated salicylic acids

Initially, 4-bromo- or 4-chloro-salicylic acid reacted with *N*-bromosuccinimide (NBS) or *N*-chlorosuccinimide (NCS) in the presence of concentrated H_2SO_4. While repeated attempts to purify the reaction mixture were unsuccessful, the crude material was subsequently esterified with MeOH/H_2SO_4. The new crude material was purified by column chromatography and the isolated appropriate methyl ester was finally hydrolyzed, resulting the desired 4,5-dihalogenated salicylic acid. Conjugation of the isolated 4,5-dihalogenated salicylic acid with substituted aniline afforded the final molecules (**Scheme 2**).



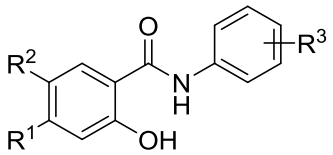
Scheme 2. Synthesis of final salicylanilides

2.2. Antimycobacterial activity

The synthesized salicylanilides (**1a-8d)** 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, as a first-line anti-TB drug, and *p*-aminosalicylic acid (PAS), a structurally related second-line drug, were chosen for the comparison performed in this study. **Table 1** reports the minimum inhibitory concentrations (MICs) of salicylanilides containing one halogen at positions 4 or 5 of their salicylic part and **Table 2** reports the MICs of salicylanilides containing two halogens at positions 4 and 5. Calculated Clog*P* values are reported as well.

The presence of one halogen at ring A of salicylanilides resulted molecules that are inhibiting the growth of *Mycobacterium tuberculosis* at 1-16 μM. In all cases, bromine derivatives are more active against their chlorine analogues with the same aniline substitution, regardless the position of the halogen. This is most likely because of the higher calculated Clog*P* values of the final salicylanilides when they possess a bromine over a chlorine atom. Among the tested, the best substitution for the aniline part was the trifluoromethyl group at position 4. The most active candidates of the series are 5-bromo-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**4c**), 4-bromo-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (**2b**) and 4-bromo-2-hydroxy-*N*-[(4-(trifluoromethyl)phenyl)]benzamide (**2c**) with MIC=1 μM after 14 days. These molecules are sharing a similar inhibition profile with INH, a first line anti-TB drug, while all monohalogenated analogues are inhibiting *Mtb* more effectively than PAS. The growth of *Mycobacterium avium* is less effectively inhibited by monohalogenated analogues, in comparison with *Mtb*, at 1-32 μM while 5-bromo-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**4c**) is the most active molecule with MIC=1μM after 21 days. 5-Chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**3c**) is the most active inhibitor of *Mycobacterium kansasii* (235/80) with MIC=1μM after 21 days while all other candidates of the monohalogenated series had an inhibition range of 2-16 μM for the specific strain. The growth of the clinical isolated strain of *Mycobacterium kansasii* (6509/96) was also inhibited at the range of 2-16 μM while 5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**3c**) was the most active compound.

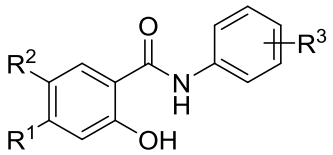
Table 1: Antimycobacterial activities of salicylanilides possessing one halogen at salicylic part



	R1	R2	R3	MIC (μM) *Mtb.* 331/88 ($H_{37}Rv$) 14 d	*Mtb.* 331/88 ($H_{37}Rv$) 21 d	*M. avium* 330/88 14 d	*M. avium* 330/88 21 d	*M. kansasii* 235/80 7 d	*M. kansasii* 235/80 14 d	*M. kansasii* 235/80 21 d	*M. kansasii* 6509/96 7 d	*M. kansasii* 6509/96 14 d	*M. kansasii* 6509/96 21 d	Clog*P*	IC_{50} (μM) HepG2	SI
1a	Cl	H	4-Br	4[a]	4[a]	16[a]	16[a]	4	4	4	4	8	8	3.84	3.24	0.81
1b	Cl	H	3,4-diCl	4[a]	4[a]	16[a]	16[a]	4	8	8	4	4	8	4.12	2.23	0.56
1c[b]	Cl	H	4-CF_3	4	4	8	8	2	4	4	2	2	4	3.93	2.64	0.66
1d	Cl	H	3,5-diCF_3	4	4	16	16	8	8	16	8	8	8	4.85	9,55	2.39
2a	Br	H	4-Br	2	4	16	16	4	8	8	8	8	8	4.11	7.59	1.90
2b	Br	H	3,4-diCl	1	2	16	16	4	4	4	4	4	8	4.40	17,67	8.84
2c	Br	H	4-CF_3	1	2	8	8	2	2	4	2	4	4	4.20	15,04	7.52
2d	Br	H	3,5-diCF_3	2	4	32	32	8	8	16	8	16	16	5.12	8,08	2.02
3a[b]	H	Cl	4-Br	8	16	8	8	4	4	4	4	8	8	3.84	3.14	0.20
3b[c]	H	Cl	3,4-diCl	4	8	16	16	4	4	4	8	8	8	4.12	1.99	0.25
3c	H	Cl	4-CF_3	2	2	8	8	1	1	1	2	2	2	3.93	1.89	0.95
3d	H	Cl	3,5-diCF_3	4	8	32	32	8	16	16	8	16	16	4.85	10,19	1.27
4a	H	Br	4-Br	4[d]	4[d]	16[d]	16[d]	4	8	8	8	8	8	4.11	23,60	5.9
4b	H	Br	3,4-diCl	4[d]	4[d]	8[d]	8[d]	8	8	8	2	2	4	4.40	31,47	7.87
4c	H	Br	4-CF_3	1[a]	1[a]	1[a]	1[a]	2	2	4	2	4	4	4.20	1.81	1.81
4d	H	Br	3,5-diCF_3	2	2	16	16	4	8	8	4	8	8	5.12	7,80	3.40
INH				0.5	1	>250	>250	>250	>250	>250	4	8	8			
PAS				62.5	62.5	32	125	125	1000	>1000	32	125	500			

INH: Isoniazid, PAS: *p*-aminosalicylic acid, [a]MICs are reported in Ref. [22], [b]MICs are reported in Ref [26], [c]MICs are reported in Ref. [21], [d]MICs are reported in Ref. [23]

Table 2: Antimycobacterial activities of salicylanilides possessing two halogens at salicylic part



				MIC (μM)										ClogP	IC_{50} (μM) HepG2	SI
	R^1	R^2	R^3	*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			
5a	Br	Cl	4-Br	2	2	32	32	4	8	8	4	8	8	4.67	9.78	4.89
5b	Br	Cl	3,4-diCl	2	4	125	125	8	8	8	8	8	8	4.95	11.07	2.76
5c	Br	Cl	4-CF_3	1	2	32	32	4	4	4	4	4	4	4.76	6.52	3.26
5d	Br	Cl	3,5-diCF_3	2	2	32	32	8	8	8	8	8	8	5.68	4.80	2.40
6a	Br	Br	4-Br	4	4	32	32	4	8	8	8	8	8	4.94	17.88	4.47
6b	Br	Br	3,4-diCl	2	4	62.5	62.5	8	8	8	8	8	8	5.22	7.96	1.99
6c	Br	Br	4-CF_3	2	2	32	62.5	4	4	4	4	4	4	5.03	6.25	3.13
6d	Br	Br	3,5-diCF_3	2	2	16	32	4	8	8	8	8	8	5.95	3.00	1.50
7a	Cl	Cl	4-Br	2	4	32	32	8	8	8	8	8	8	4.40	21.41	5.35
7b	Cl	Cl	3,4-diCl	2	2	62.5	62.5	4	8	8	8	8	8	4.68	12.42	6.21
7c	Cl	Cl	4-CF_3	2	2	62.5	62.5	4	8	8	4	8	8	4.49	9.87	4.94
7d	Cl	Cl	3,5-diCF_3	2	2	32	32	8	8	16	8	16	16	5.41	4.86	2.43
8a	Cl	Br	4-Br	2	4	32	32	4	8	8	8	8	8	4.67	10.83	2.71
8b	Cl	Br	3,4-diCl	2	4	125	125	8	8	8	8	8	8	4.95	7.65	1.91
8c	Cl	Br	4-CF_3	1	2	32	62.5	4	4	4	4	4	4	4.76	11.03	5.52
8d	Cl	Br	3,5-diCF_3	2	4	16	32	8	8	8	8	8	8	5.68	5.07	1.27
INH				0.5	1	>250	>250	>250	>250	>250	4	8	8			
PAS				62.5	62.5	32	125	125	1000	>1000	32	125	500			

INH: Isoniazid, PAS: *p*-aminosalicylic acid

Introduction of a second halogen at the ring A of salicylanilides afforded molecules that inhibit the growth of *Mtb* at a narrow micromolar range (MICs=1-4 μM). When these results compared with ring A nitro substituted salicylanilides [24], it is obvious that two weak electron-withdrawing groups are preferred at the salicylic part instead of one strong electron-withdrawing group. On the other hand, the dihalogenated products are similarly active with their most active monohalogenated analogues. Even if the presence of the second halogen affects the Clog*P* values of the final molecules, no direct correlation between activities and Clog*P* values was noticed. The best substitution for the aniline part is trifluoromethyl group at position 4, like for the monohalogenated analogues. The most active inhibitors of the series are 4-bromo-5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**5c**) and 5-bromo-4-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**8c**) with MIC=1 μM after 14 days. The growth of *Mycobacterium avium* is less effectively inhibited by dihalogenated analogues, in comparison with *Mtb* as well as in comparison with the monohalogenated analogues, at 16-62.5 μM. The most active compounds against *Mycobacterium avium* were *N*-[3,5-bis(trifluoromethyl)phenyl]-4,5-dibromo-2-hydroxybenzamide (**6d**) and *N*-[3,5-bis(trifluoromethyl)phenyl]-5-bromo-4-chloro-2-hydroxybenzamide (**8d**) with MIC=32 μM after 21 days, proving the superiority of 3,5-bis(trifluoromethyl) substitution on ring B. *Mycobacterium kansasii* (235/80) was inhibited in the range of 4-16 μM from ring A dihalogenated analogues, without important differences between the different substitutions. The growth of *Mycobacterium kansassi* (6509/96) was inhibited at the same range (4-16 μM) while the highest activity observed for 4,5-dibromo-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**6c**) and was 4 μM after 21 days.

The most active salicylanilides against *Mtb* among all monohaloganated and dihalogenated ring A salicylanilides were those possessing a trifluoromethyl group at position 4 of the aniline part. These molecules were selected for further screening against five MDR-TB strains with various resistance patterns (234/2005, 9449/2007, 242/2015, Praha 1 and Praha 4) and one XDR-TB strain (Praha 131). The results are summarized in **Table 3**.

Table 3: Antimycobacterial activities of selected salicylanilides against MDR-TB and XDR-TB strains.

Code	MIC [μM]											
	M. tuberculosis 234/2005		*M. tuberculosis* 9449/2007		*M. tuberculosis* 242/2015		*M. tuberculosis* Praha 1		*M. tuberculosis* Praha 4		*M. tuberculosis* Praha 131	
	14 d	21 d	14 d	14 d	21 d	14 d	14 d	21 d	14 d	21 d	14 d	21 d
1c	2	2	2	1	1	1	1	1	1	2	2	2
2c	1	2	1	1	1	1	1	1	1	2	1	1
3c	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
4c	2	4	2	1	1	2	1	1	2	4	2	4
5c	2	2	2	2	2	2	2	2	2	4	2	2
6c	2	2	2	1	1	2	1	1	2	2	2	2
7c	1	2	2	1	1	1	1	1	1	2	1	2
8c	2	2	2	2	2	2	2	2	2	2	2	2
INH	16	32	16	16	16	16	16	16	16	16	16	16

INH: Isoniazid

All tested salicylanilides exhibited excellent activities against MDR and XDR strains ranging between 0.5-4 μM which are similar or even lower than the MICs observed for drug-sensitive strains. The insertion of a second halogen at ring A doesn't seem to dramatically affect the antimycobacterial activity of the final molecules against these strains even if the ring A dihalogenated compounds are slightly less active than their monohalogenated analogues. The most active ring A monohalogenated derivative was 5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**3c**) with MIC=0.5 μM against all tested strains while the most active dihalogenated derivatives were 4,5-dibromo-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**6c**) and 4,5-dichloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**7c**) with MIC=1-2 μM against all resistant strains.

2.3. Cytotoxicity evaluation

Since hepatotoxic effects are a very common and serious problem in patients who are undergoing treatment for tuberculosis [27], all synthesized salicylanilides were evaluated for their *in vitro* cytotoxic properties using an MTT assay in the human liver model of HepG2 cell line. The cytotoxicity results as well as the selectivity indexes (SI= IC_{50}/MIC against *Mtb* in 21 days) for the tested compounds are summarized in **Table 1** and **Table 2** together with their antimycobacterial activities.

The IC_{50} values for the salicylanilides bearing one halogen at ring A were in the range of 1.81 to 31.47 μM. The majority of the compounds of this group possess a non-preferable cytotoxic profile, since their IC_{50} values are in the range of their MICs, thus having SI values around 1. The non-preferable cytotoxic profile is expected for these molecules, since the presence of a free phenolic hydrogen is closely connected, not only with the activity of salicylanilides but also with their cytotoxicity [15]. Interestingly, two of the most active salicylanilides of 4-bromosalicylic acid, which are presented here for the first time, are having a comparatively high value of SI. More specifically, 4-bromo-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (**2b**) and 4-bromo-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**2c**) are having SI values of 8.84 and 7.52 respectively.

The insertion of a second halogen on the ring A afforded molecules characterized by a generally more preferable cytotoxic profile in comparison with their monohalogenated analogues, since SI values are in the range of 1.27-6.21. It is noteworthy, that the presence of two trifluoromethyl groups at the aniline part is always negatively affecting the cytotoxicity of the final salicylanilides (lower SI values). In terms of SI, the best compounds of the series are 4,5-dichloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (**7b**), 5-bromo-4-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**8c**) and *N*-(4-bromophenyl)-4,5-dichloro-2-hydroxybenzamide (**7a**) with SI=6.21, 5.52 and 5.35 respectively, while their activities against *Mtb* are 2, 2 and 4 μM after 21 days.

3. Conclusions

Herein, the effect on antimycobacterial activity of salicylanilides when two weak electron-withdrawing groups (bromine and chlorine atoms) are present at the positions 4 and 5 of their salicylic part has been examined. Initially, salicylanilides possessing bromine at position 4 of salicylanilides were synthesized in order to complete the series of 4 and 5 monohalogenated salicylanilides. To the best of our knowledge, 4-bromosalicylanilides, together with their antimycobacterial activities, are presented herein for the first time. 4,5-Dihalogenated salicylic acids synthesized from 4-halogenated salicylic acids and used for the synthesis of novel salicylanilides possessing two halogens at positions 4 and 5 of their salicylic part. All

new compounds were screened for their antimycobacterial activities against *Mtb* 331/88 (i.e., $H_{37}R_V$) and three strains of nontuberculous mycobacteria.

Introduction of a second halogen at ring A resulted salicylanilides sharing a high antimycobacterial activity, similar with the most active monohalogenated salicylic acid derivatives. The growth of *Mtb* is inhibited at a very narrow micromolar range (MICs=1-4 μM). Furthermore, as a continuation of our previous findings [24], the insertion of a second weak electron-withdrawing substituent at ring A is much preferable over the presence of one single strong electron-withdrawing substituent. The cytotoxicity of the monohalogenated salicylic acid derivatives, confirmed once more that the parent structure of salicylanilides is having a non-preferable cytotoxic profile, since IC_{50} values were close to the range of their MICs. Small exception noticed for two 4-bromosalicylic acid derivatives which exhibited SI>7 against *Mycobacterium tuberculosis*. The insertion of a second halogen, did not prove to dramatically affect the overall cytotoxic profile of the final salicylanilides, even if afforded molecules with generally more acceptable selectivity indexes (all values were >1). Finally, the synthesized compounds were proved to be very active against all multidrug-resistant and one extensively drug-resistant mycobacterial strains (MICs = 0.5-4 μM). Our results suggest that multiple halogen substitution has a positive effect to the overall antimycobacterial activity of the final molecules and that multiple substitution at ring A is a promising method for the discovery of novel molecules with increased activity over *Mtb*.

4. Experimental part

4.1. Chemistry

4.1.1. General methods

All 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 (TLC). 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 Bűchi 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 deuterated solvents at ambient temperature using a Varian V NMR S500 instrument (500 MHz for ^1H and 125 MHz for ^{13}C, Varian Comp., Palo Alto, CA, USA) or a Varian Mercury-Vxbb 300 (300 MHz for ^1H 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 Clog*P* values, which are the logarithms of the partition coefficients for octan-1-ol/water, were determined using the CS ChemOffice Ultra program (version 15.0, CambridgeSoft, Cambridge, MA, USA).

4.1.2. Synthesis

4.1.2.1. Synthesis of methyl esters of dihalogenated salicylic acids (**III-VI**), *general procedure A*:

4-Chloro or 4-bromosalicylic acid (1.0 eq) was dissolved in concentrated H_2SO_4 (10 mmol salicylic acid/30 mL H_2SO_4). *N*-Chlorosuccinimide or *N*-bromosuccinimide (1.3 eq) were slowly added in small portions while keeping the temperature between 0-5 ^oC during the addition. The reaction mixture was stirred at r.t. and, after complete consumption of starting material, water was carefully added. The formed precipitate was filtered and washed with cold water. The crude product was dissolved in MeOH and concentrated H_2SO_4 was added to the solution (1 mL H_2SO_4/10 mL MeOH). The reaction mixture was heated at boiling temperature for 8-16 hours. After cooling at r.t., AcOEt was added (equal amount with MeOH) followed by extensive washing with saturated solution of $NaHCO_3$. The organic phase was dried over anhydrous Na_2SO_4, filtrated and evaporated to dryness. The crude material was purified by column chromatography with hexane/AcOEt as an eluent in order to afford the pure product. Yields were 35-42% after the described two-step procedure, calculated from the initial amount of 4-monohalogenated salicylic acid.

4.1.2.2. Synthesis of 4,5-dihalogenated salicylic acids (**VII-X**), *general procedure B*:

The methyl ester of 4,5-dihalogenated salicylic acid (1 eq) was dissolved in a mixture of THF:MeOH:H_2O (4:1:1, 0.15 mmol acid/1 mL solution). LiOH was added (4 eq) to the solution and the reaction mixture stirred at r.t until complete consumption of starting material (1-2 hours). Solvents were removed under reduced pressure and the resulting solid dissolved in water. The solution was acidified with 2M HCl until pH=3 (precipitate formed) and washed with AcOEt (3 x equal volume). The combined organic phases were dried (Na_2SO_4), filtrated and evaporated to dryness to afford the desired 4,5-dihalogenated salicylic acid with yields of 94-97 %.

4.1.2.3. Synthesis of salicylanilides, *general procedure C*:

All salicylanilides (**1a-8d**) were synthesized via a previously described microwave assisted (MicroSYNTH Milestone) method [25]. Briefly, the appropriately substituted salicylic acid (1 eq) and the substituted aniline (1 eq) were suspended or dissolved in chlorobenzene (1mL/0.15 mmol of acid). Phosphorus trichloride (PCl_3) was added (0.5 eq) and the mixture was stirred (600 rpm) and radiated (530 W) for 30-60 min. The mixture was filtrated while hot and was let cool, initially at r.t. and overnight at 4 ^oC. The formed precipitate was filtered and recrystallized with ethanol or mixture of hexane/AcOEt in order to afford the final compounds with yields of 41-83%.

4.1.3. Compounds' characterization

4.1.3.1. **Methyl 4-bromo-5-chloro-2-hydroxybenzoate (III)**

Prepared by general procedure A using 4-bromosalicylic acid and *N*-chlorosuccinimide. Purified by column chromatography, eluent hexane/AcOEt:20/1. White solid; yield 42% in two steps; mp 120.5-122.0 ^oC. IR (ATR): 3152, 1668, 1599, 1455, 1438, 1200, 1100, 788. ^1H NMR (500 MHz, $CDCl_3$): δ 10.66 (s, 1H), 7.89 (s, 1H), 7.31 (s, 1H), 3.96 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.2, 159.9, 130.3, 130.0, 124.7, 122.9, 112.6, 77.2, 52.8. Anal. Calcd for $C_8H_6BrClO_3$ (265.49): C, 36.19; H, 2.28. Found: C, 36.16; H, 2.26.

4.1.3.2. **Methyl 4,5-dibromo-2-hydroxybenzoate (IV):** Prepared by general procedure A using 4-bromosalicylic acid and *N*-bromosuccinimide. Purified by column chromatography, eluent Hexane/AcOEt:15/1. White solid; yield 38% in two steps; mp 107.0-108.0 ^{o}C. IR (ATR): 3152, 1669, 1597, 1451, 1439, 1193, 1100, 902, 787. ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.67 (s, 1H), 8,04 (s, 1H), 7.31 (s, 1H), 3.96 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.6, 161.0, 134.3, 132.8, 123.5, 114.3, 113.6, 53.4. Anal. Calcd for $C_8H_6Br_2O_3$ (309.94): C, 31.00; H, 1.95. Found: C, 30.97; H, 1.96.

4.1.3.3. **Methyl 4,5-dichloro-2-hydroxybenzoate (V):** Prepared by general procedure A using 4-chlorosalicylic acid and *N*-chlorosuccinimide. Purified by column chromatography, eluent hexane/AcOEt:50/1. White solid; yield 35% in two steps; mp 100.0-101.0 ^{o}C. IR (ATR): 3093, 1669, 1606, 1463, 1449, 1342, 1248, 1210, 1103, 935, 794. ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.69 (s, 1H), 7.90 (s, 2H), 7.12 (s, 2H), 3.96 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.5, 160.5, 140.0, 131.1, 123.2, 119.9, 112.5, 53.2. Anal. Calcd for $C_8H_6Cl_2O_3$ (221.03): C, 43.47; H, 2.74. Found: C, 43.44; H, 2.72.

4.1.3.4. **Methyl 5-bromo-4-chloro-2-hydroxybenzoate (VI):** Prepared by general procedure A using 4-chlorosalicylic acid and *N*-bromosuccinimide. Purified by column chromatography, eluent hexane/AcOEt (50:1). White solid; yield 38% in two steps; mp 93.5-94.5 ^{o}C. IR (ATR): 3097, 1672, 1601, 1493, 1439, 1323, 1242, 1207, 1104, 917, 788. ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.71 (s, 1H), 8.06 (s, 2H), 7.13 (s, 1H), 3.96 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.1, 160.9, 141.6, 134.2, 119.6, 112.7, 111.6, 52.9. Anal. Calcd for $C_8H_6BrClO_3$ (265.49): C, 36.19; H, 2.28. Found: C, 36.18; H, 2.27

4.1.3.5. **4-Bromo-5-chloro-2-hydroxybenzoic acid (VII):** Prepared by general procedure B from methyl 4-bromo-5-chloro-2-hydroxybenzoate. White solid; yield 96%; mp 222.5-223.5 ^{o}C. IR (ATR): 3051, 2851, 1657, 1599, 1461, 1439, 1313, 1237, 1199, 887, 863. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.59 (bs, 1H), 7.86 (s, 2H), 7.41 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.9, 159.5, 130.7, 128.1, 123.0, 122.4, 114.6. Anal. Calcd for $C_7H_4BrClO_3$ (251.46): C, 33.44; H, 1.60. Found: C, 33.42; H, 1.59

4.1.3.6. **4,5-Dibromo-2-hydroxybenzoic acid (VIII):** Prepared by general procedure B from methyl 4,5-dibromo-2-hydroxybenzoate. White solid; yield 94%; mp 226.0-228.0 ^{o}C. IR (ATR): 2922, 2851, 1659, 1596, 1460, 1439, 1311, 1236, 1198, 882, 863. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.08 (bs, 1H), 7.98 (s, 1H), 7.41 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.8, 160.0, 134.0, 130.4, 122.4, 115.0, 112.5. Anal. Calcd for $C_7H_4Br_2O_3$ (295.91): C, 28.41; H, 1.36. Found: C, 28.40; H, 1.35

4.1.3.7. **4,5-Dichloro-2-hydroxybenzoic acid (IX):** Prepared by general procedure B from methyl 4,5-dichloro-2-hydroxybenzoate. White solid; yield 97%; mp 208.0-209.0 ^{o}C. [lit[28]: mp 210-211 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.8. **5-Bromo-4-chloro-2-hydroxybenzoic acid (X):** Prepared by general procedure B from methyl 5-bromo-4-chloro-2-hydroxybenzoate. White solid; yield 95%; mp

213.0-214.5 ^{o}C. [lit[28]: mp 208-213 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.9. ***N*-(4-Bromophenyl)-4-chloro-2-hydroxybenzamide (1a):** Prepared by general procedure C. White solid; yield 73%; mp 220.0-221.0 ^{o}C. [lit[22]: mp 220.0-222.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.10. **4-Chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (1b):** Prepared by general procedure C. White solid; yield 77%; mp 222.0-223.0 ^{o}C. [lit[22]: mp 221.0-223.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.11. **4-Chloro-2-hydroxy-*N*-(4-(trifluoromethyl)phenyl)benzamide (1c):** Prepared by general procedure C. White solid; yield 71%; mp 212.0-213.5 ^{o}C. [lit[22]: mp 213.0-216.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.12. ***N*-(3,5-Bis(trifluoromethyl)phenyl)-4-chloro-2-hydroxybenzamide (1d):** Prepared by general procedure C. White solid; yield 82%; mp 203.0-204.5 ^{o}C. [lit[29]: mp 204.0-205.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.13. **4-Bromo-*N*-(4-bromophenyl)-2-hydroxybenzamide (2a):** Prepared by general procedure C. White solid; yield 63%; mp 229.0-230.0 ^{o}C. IR (ATR): 3322, 3087, 1602, 1590, 1550, 1448, 1225, 1076, 827, 810. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.89 (brs, 1H), 10.42 (s, 1H), 7.82 (d, J = 8.5 Hz, 1H), 7.69 (d, J = 9.0 Hz, 2H), 7.55 (d, J = 8.5 Hz, 2H), 7.19 (d, J = 2.0 Hz, 1H), 7.16 (dd, J = 8.3, 2.0 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 165.4, 158.5, 137.6, 131.6, 131.0, 126.1, 122.6, 122.1, 119.6, 118.0, 115.9. Anal. Calcd for $C_{13}H_9Br_2NO_2$ (371.03): C, 42.08; H, 2.44; N, 3.78. Found: C, 42.11; H, 2.45; N, 3.76.

4.1.3.14. **4-Bromo-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (2b):** Prepared by general procedure C. Light purple solid; yield 54%; mp 234.5-235.5 ^{o}C. IR (ATR): 3304, 3161, 1631, 1597, 1586, 1442, 1223, 1136, 899, 819. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.75 (brs, 1H), 10.50 (s, 1H), 8.10 (d, J = 2.5 Hz), 7.77 (d, J = 8.5 Hz, 1H), 7.66 (dd, J = 8.5, 2.5 Hz, 1H), 7.61 (d, J = 9.0 Hz, 1H), 7.20 (d, J = 2.0 Hz, 1H), 7.18 (dd, J = 8.3, 2.0 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 165.4, 158.1, 138.4, 131.1, 130.9. 130.6, 126.1, 125.5, 122.2, 121.7, 120.5, 119.6, 118.2. Anal. Calcd for $C_{13}H_8BrCl_2NO_2$ (371.03): C, 42.25; H, 2.23; N, 3.88. Found: C, 42.28; H, 2.22; N, 3.89.

4.1.3.15. **4-Bromo-2-hydroxy-*N*-(4-(trifluoromethyl)phenyl)benzamide (2c):** Prepared by general procedure C. White solid; yield 44%; mp 224.0-225.5 ^{o}C. IR (ATR): 3314, 2934, 1609, 1591, 1572, 1421, 1227, 1107, 1070, 914, 840. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.79 (brs, 1H), 10.58 (s, 1H), 7.94 (d, J = 8.5 Hz, 2H), 7.81 (d, J = 8.5 Hz, 1H), 7.73 (d, J = 8.5 Hz, 2H), 7.21 (d, J = 2.0 Hz, 1H), 7.17 (dd, J = 8.5, 2.0 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 165.7, 158.3, 142.1, 131.4, 126.2 (q, J = 3.63 Hz), 126.3,

124.5 (q, *J* = 264.3 Hz), 124.1 (q, *J* = 44.4 Hz), 122.4, 120.6, 119.8, 118.6. Anal. Calcd for $C_{14}H_9BrF_3NO_2$ (360.13): C, 46.69; H, 2.52; N, 3.89. Found: C, 46.70; H, 2.50; N, 3.86.

4.1.3.16. ***N*-(3,5-Bis(trifluoromethyl)phenyl)-4-bromo-2-hydroxybenzamide (2d):** Prepared by general procedure C. White solid; yield 47%; mp 218.0-219.0 ^{o}C. IR (ATR): 3316, 3169, 1642, 1381, 1275, 1184, 1130, 906, 693. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.62 (brs, 1H), 10.79 (s, 1H), 8.43 (s, 2H), 7.81 (s, 1H), 7.76 (d, *J* = 8.1 Hz, 1H), 7.21 (d, *J* = 1,5 Hz, 1H), 7.17 (dd, *J* = 8.4 Hz, 1,8 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 65.9, 158.1, 140.3, 131.2, 130.7 (q, *J* = 33.0 Hz), 126.7, 123.1 (q, *J* = 294.3 Hz), 122.2, 120.3 (q, *J* = 3.8 Hz), 119.6, 118.3, 116.8 (q, *J* = 3.8 Hz). Anal. Calcd for $C_{15}H_8BrF_6NO_2$ (428.12): C, 42.08; H, 1.88; N, 3.27. Found: C, 42.04; H, 2.00; N, 3.85.

4.1.3.17. ***N*-(4-Bromophenyl)-5-chloro-2-hydroxybenzamide (3a):** Prepared by general procedure C. White solid; yield 56%; mp 240.0-241.5 ^{o}C. [lit[23]: mp 240-241 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.18. **5-Chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (3b):** Prepared by general procedure C. Light purple solid; yield 59%; mp 246.0-248.0 ^{o}C. [lit[23]: mp 248.0-250.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.19. **5-Chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (3c):** Prepared by general procedure C. White solid; yield 54%; mp 218.0-219.0 ^{o}C. [lit[22]: mp 217.0-218.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.20. ***N*-[3,5-Bis(trifluoromethyl)phenyl]-5-chloro-2-hydroxybenzamide (3d):** Prepared by general procedure C. White solid; yield 62%; mp 169.0-170.0 ^{o}C. [lit[15]: mp 172.0-173.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.21. **5-Bromo-*N*-(4-bromophenyl)-2-hydroxybenzamide (4a):** Prepared by general procedure C. White solid; yield 67%; mp 240.0-241.5 ^{o}C. [lit[23]: mp 240.0-241.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.22. **5-Bromo-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (4b):** Prepared by general procedure C. White solid; yield 63%; mp 235.0-236.0 ^{o}C. [lit[23]: mp 234.0-236.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.23. **5-Bromo-2-hydroxy-*N*-[4-(trifluoromethyl)]benzamide (4c):** Prepared by general procedure C. White solid; yield 61%; mp 202.0-203.0 ^{o}C. [lit[22]: mp 202.0-203.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.24. ***N*-[3,5-Bis(trifluoromethyl)phenyl]-5-bromo-2-hydroxybenzamide (4d):** Prepared by general procedure C. White solid; yield 83%; mp 201.0-202.0 ^{o}C. [lit[15]: mp 194.0-195.0 ^{o}C]. All spectroscopic characterization was in accordance with previously reported values.

4.1.3.25. **4-Bromo-*N*-(4-bromophenyl)-5-chloro-2-hydroxybenzamide (5a):** Prepared by general procedure C. White solid; yield 45%; mp 262.0-263.0 ^{o}C. IR (ATR): 3322, 3086, 1605, 1549, 1488, 1402, 1237, 1078, 820. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.90 (brs, 1H), 10.44 (s, 1H), 8.03 (s, 1H), 7.68 (d, J = 8.5 Hz, 2H), 7.55 (d, J = 8.5 Hz, 2H), 7.37 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.1, 156.5, 137.4, 131.6, 130.1, 125.5, 123.0, 122.5, 121.8, 120.2, 116.0. Anal. Calcd for $C_{13}H_8Br_2ClNO_2$ (405.47): C, 38.51; H, 1.99; N, 3.45. Found: C, 38.50; H, 2.01; N, 3.44.

4.1.3.26. **4-Bromo-5-chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (5b):** Prepared by general procedure C. Light purple solid; yield 47%; mp 264.0-265.5 ^{o}C. IR (ATR): 3321, 3089, 1626, 1599, 1541, 1476, 1402, 1237, 1135, 858, 818, 671. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.79 (brs, 1H), 10.52 (s, 1H), 8.08 (d, J = 2.0 Hz, 1H), 7.98 (s, 1H), 7.65 (dd, J = 9.0, 2.0 Hz, 1H), 7.61 (d, J = 9.0 Hz, 1H), 7.36 (s, 1H, H3). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.3, 155.4, 138.4, 131.2, 130.8, 130.3, 125.9, 125.7, 123.3, 122.0, 121.9, 120.7, 120.5. Anal. Calcd for $C_{13}H_7BrCl_3NO_2$ (395.46): C, 39.48; H, 1.78; N, 3.54. Found: C, 39.44; H, 1.81; N, 3.41.

4.1.3.27. **4-Bromo-5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (5c):** Prepared by general procedure C. White solid; yield 43%; mp 236.0-238.0 ^{o}C. IR (ATR): 3320, 3087, 1609, 1414, 1324, 1232, 1117, 1070, 841, 708, 673. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.81 (brs, 1H), 10.62 (s, 1H), 8.01 (s, 1H), 7.92 (d, J = 8.5 Hz, 2H), 7.73 (d, J = 9.0 Hz, 2H), 7.37 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.2, 156.2, 141.8, 130.2, 126.1 (q, J = 3.5 Hz), 125.5, 124.0 (q, J = 270.0 Hz), 124.3 (q, J = 32.0 Hz), 123.1, 121.8, 121.8, 120.7, 120.4. Anal. Calcd for $C_{14}H_8BrClF_3NO_2$ (394.57): C, 42.62; H, 2.04; N, 3.55. Found: C, 42.59; H, 2.06; N, 3.51.

4.1.3.28. ***N*-[3,5-Bis(trifluoromethyl)phenyl]-4-bromo-5-chloro-2-hydroxybenzamide (5d):** Prepared by general procedure C. White solid; yield 46%; mp 208.5-209.5 ^{o}C. IR (ATR): 3322, 3297, 1639, 1379, 1275, 1188, 1177, 1127, 896, 702. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.67 (brs, 1H), 10.82 (s, 1H), 8.41 (s, 2H), 7.99 (s, 1H), 7.82 (s, 1H), 7.38 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.6, 156.2, 140.1, 133.4, 130.7 (q, J = 32.5 Hz), 130.2, 125.8, 123.3 (q, J = 234.5 Hz), 123.1, 121.8, 120.3, 120.2 (q, J = 3.8 Hz), 119.9, 116.9 (q, J = 3.3 Hz). Anal. Calcd for $C_{15}H_7BrClF_6NO_2$ (462.57): C, 38.95; H, 1.53; N, 3.03. Found: C, 38.92; H, 1.55; N, 3.00.

4.1.3.29. **4,5-Dibromo-*N*-(4-bromophenyl)-2-hydroxybenzamide (6a):** Prepared by general procedure C. White solid; yield 53%; mp 262.0-263.0 ^{o}C. IR (ATR): 3323, 3086, 1604, 1548, 1488, 1402, 1237, 820. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.91 (brs, 1H), 10.44 (s, 1H), 8.14 (s, 1H), 7.68 (d, J = 8.6 Hz, 2H), 7.55 (d, J = 8.6 Hz, 2H), 7.37 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.0, 157.0, 137.4, 133.3, 131.6, 127.9, 122.5, 121.9, 120.4,

116.0, 112.6. Anal. Calcd for $C_{13}H_8Br_3NO2$ (405.47): C, 34.70; H, 1.79; N, 3.11. Found: C, 34.65; H, 1.82; N, 3.09.

4.1.3.30. **4,5-Dibromo-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (6b):** Prepared by general procedure C. White solid; yield 52%; mp 263.0-264.0 °C. IR (ATR): 3324, 3087, 1597, 1540, 1477, 1398, 1236, 858, 818, 680. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.80 (brs, 1H), 10.53 (s, 1H), 8.10 (s, 1H), 8.08 (d, *J* = 2.1 Hz, 1H), 7.65 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.62 (d, *J* = 8.8 Hz, 1H), 7.37 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.1, 156.8, 138.2, 133.3, 131.0, 130.6, 128.0, 125.7, 121.8, 121.7, 120.6, 120.5, 112.6. Anal. Calcd for $C_{13}H_7Br_2Cl_2NO_2$ (439.91): C, 35.49; H, 1.60; N, 3.18. Found: C, 35.48; H, 1.60; N, 3.17.

4.1.3.31. **4,5-Dibromo-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (6c):** Prepared by general procedure C. White solid; yield 48%; mp 240.5-241.5 °C. IR (ATR): 3319, 3084, 1631, 1589, 1414, 1323, 1116, 1069, 841, 702. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.83 (brs, 1H), 10.62 (s, 1H), 8.12 (s, 1H), 7.93 (d, *J* = 8.5 Hz, 2H), 7.74 (d, *J* = 8.5 Hz, 2H), 7.38 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.2, 156.7, 141.8, 127.9, 126.0 (q, *J* = 3.7 Hz), 124.3 (q, *J* = 268.8 Hz), 124.1 (q, *J* = 31.9 Hz), 121.8, 120.9, 120.4. Anal. Calcd for $C_{14}H_8Br_2F_3NO_2$ (439.02): C, 38.30; H, 1.84; N, 3.19. Found: C, 38.26; H, 1.86; N, 3.18.

4.1.3.32. ***N*-[3,5-Bis(trifluoromethyl)phenyl]-4,5-dibromo-2-hydroxybenzamide (6d):** Prepared by general procedure C. White solid; yield 53%; mp 206.0-207.0 °C. IR (ATR): 3224, 1642, 1379, 1275, 1173, 1127, 896, 701. ^{1}H NMR (500 MHz, DMSO-d_6): δ 16.42 (s, 0H), 15.59 (s, 1H), 13.18 (s, 2H), 12.87 (s, 1H), 12.59 (s, 1H), 12.14 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.6, 156.8, 140.1, 133.4, 130.7 (q, *J* = 32.9 Hz), 128.2, 123.2 (q, *J* = 272.8 Hz), 121.9, 120.6, 120.3 (d, *J* = 3.8 Hz), 117.1 – 116.9 (d, *J* = 3.8 Hz), 112.6. Anal. Calcd for $C_{15}H_7Br_2F_6NO_2$ (50.02): C, 35.53; H, 1.39; N, 2.76. Found: C, 35.50; H, 1.41; N, 2.75.

4.1.3.33. ***N*-(4-Bromophenyl)-4,5-dichloro-2-hydroxybenzamide (7a):** Prepared by general procedure C. White solid; yield 59%; mp 266.0-267.0 °C. IR (ATR): 3327, 3091, 1606, 1548, 1489, 1404, 1241, 1099, 820. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.96 (brs, 1H), 10.45 (s, 1H), 8.06 (s, 1H), 7.68 (d, *J* = 9.0 Hz, 2H), 7.55 (d, *J* = 8.5 Hz, 2H), 7.23 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.0, 156.7, 137.4, 134.9, 131.6, 130.4, 122.5, 121.0, 119.6, 118.7, 116.1. Anal. Calcd for $C_{13}H_8BrCl_2NO_2$ (361.02): C, 43.25; H, 2.23; N, 3.88. Found: C, 43.22; H, 2.23; N, 3.86.

4.1.3.34. **4,5-Dichloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (7b):** Prepared by general procedure C. White solid; yield 67%; mp 262.0-263.5 °C. IR (ATR): 3321, 3089, 1658, 1625, 1413, 1324, 1233, 1121, 1105, 842, 710, 682. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.83 (brs, 1H), 10.53 (s, 1H), 8.08 (d, *J* = 2 Hz, 1H), 8.01 (s, 1H), 7.62 (dd, *J* = 9.0, 2.0 Hz, 1H), 7.61 (d, *J* = 9.0 Hz, 1H), 7.23 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.1, 156.5, 138.2, 135.0, 131.0, 130.7, 130.5, 125.7, 121.7, 121.0, 120.5, 119.8, 118.6. Anal. Calcd for $C_{13}H_7Cl_4NO_2$ (351.01): C, 44.48; H, 2.01; N, 3.99. Found: C, 44.41; H, 2.04; N, 3.95.

4.1.3.35. **4,5-Dichloro-2-hydroxy-*N*-(4-(trifluoromethyl)phenyl)benzamide (7c):** Prepared by general procedure C. White solid; yield 58%; mp 238.0-240.0 ^{o}C. IR (ATR): 3318, 2922, 1613, 1414, 1324, 1232, 1120, 1071, 842, 710. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.88 (brs, 1H), 10.63 (s, 1H), 8.04 (s, 1H), 7.93 (d, J = 8.5 Hz,2H), 7.73 (d, J = 8.5 Hz, 2H), 7.24 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 154.2, 146.5, 131.7, 124.9, 120.6, 116.0 (q, J = 3.6 Hz), 114.14 (q, J = 32.1 Hz), 114.06 (q, J = 277.2 Hz), 111.1, 110.4, 110.1, 108.6. Anal. Calcd for $C_{14}H_8Cl_2F_3NO_2$ (350.12): C, 48.03; H, 2.30; N, 4.00. Found: C, 47.85; H, 2.36; N, 3.81.

4.1.3.36. ***N*-[3,5-Bis(trifluoromethyl)phenyl]-4,5-dichloro-2-hydroxybenzamide (7d):** Prepared by general procedure C. White solid; yield 57%; mp 216.0-217.0 ^{o}C. IR (ATR): 3334, 3161, 1641, 1379, 1276, 1190, 1180, 1128, 895, 703. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.72 (brs, 1H), 10.82 (s, 1H), 8.42 (s, 2H), 8.02 (s, 1H), 7.83 (s, 1H), 7.24 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.7, 156.7, 140.3, 135.4, 130.9 (q, J = 32.5Hz), 130.5, 123.3 (q, J = 271.6 Hz), 121.2, 120.4 (q, J = 3.5 Hz), 119.9, 118.9, 117.1 (q, J = 3.5 Hz). Anal. Calcd for $C_{15}H_7Cl_2F_6NO_2$ (418.12): C, 43.09; H, 1.69; N, 3.35. Found: C, 32.98; H, 1.72; N, 3.31.

4.1.3.37. **5-Bromo-*N*-(4-bromophenyl)-4-chloro-2-hydroxybenzamide (8a):** Prepared by general procedure C. White solid; yield 41%; mp 256.0-258.0 ^{o}C. IR (ATR): 3326, 2930, 1605, 1548, 1488, 1402, 1240, 1098, 820. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.98 (brs, 1H), 10.44 (s, 1H), 8.18 (s, 1H), 7.67 (d, J = 8.5 Hz, 2H), 7.55 (d, J = 9.0 Hz, 2H), 7.23 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.0, 157.4, 137.4, 136.9, 133.5, 131.6, 122.5, 119.8, 118.6, 116.1, 110.2. Anal. Calcd for $C_{13}H_8Br_2ClNO_2$ (405.47): C, 38.51; H, 1.99; N, 3.45. Found: C, 38.46; H, 2.03; N, 3.41.

4.1.3.38. **5-Bromo-4-chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (8b):** Prepared by general procedure C. White solid; yield 47%; mp 261.0-263.0 ^{o}C. IR (ATR): 3326, 3090, 1625, 1601, 1541, 1476, 1401, 1238, 1136, 859, 818, 670. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.85 (brs, 1H), 10.53 (s, 1H), 8.13 (s, 1H), 8.07 (s, 1H), 7.65 (d, J = 8.5 Hz, 1H), 7.61 (d, J = 8.5 Hz, 1H), 7.22 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.0, 157.1, 138.2, 137.0, 133.6, 131.0, 130.6, 125.7, 121.7, 120.5, 120.0, 118.6, 110.2. Anal. Calcd for $C_{13}H_7BrCl_3NO_2$ (395.46): C, 39.48; H, 1.78; N, 3.54. Found: C, 39.43; H, 1.80; N, 3.51.

4.1.3.39. **5-Bromo-4-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (8c):** Prepared by general procedure C. White solid; yield 43%; mp 243.5-244.5 ^{o}C. IR (ATR): 3320, 3083, 1633, 1608, 1413, 1324, 1238, 1111, 1069, 840, 712, 658. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.89 (brs, 1H), 10.62 (s, 1H), 8.16 (s, 1H), 7.92 (d, J = 8.5 Hz, 2H), 7.73 (d, J = 9.0 Hz, 2H), 7.23 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.1, 157.1, 141.7, 136.9, 133.7, 124.2 (q, J = 262.5 Hz), 124.3 (q, J = 31.3 Hz), 126.0 (q, J = 3.63 Hz), 120.4, 120.3, 118.6, 110.2. Anal. Calcd for $C_{14}H_8BrClF_3NO_2$ (394.57): C, 42.62; H, 2.04; N, 3.55. Found: C, 42.57; H, 2.08; N, 3.50.

4.1.3.40. ***N*-[3,5-Bis(trifluoromethyl)phenyl]-5-bromo-4-chloro-2-hydroxybenzamide (8d):** Prepared by general procedure C. White solid; yield 49%; mp

228.5-229.5 °C. IR (ATR): 3312, 3092, 1637, 1379, 1275, 1186, 1133, 902, 704. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.70 (s, 1H), 10.83 (s, 1H), 8.41 (s, 2H), 8.13 (s, 1H), 7.83 (s, 1H), 7.24 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.5, 157.1, 140.1, 137.2, 133.7, 130.7 (q, J = 32.8 Hz), 123.2 (q, J = 272.8 Hz), 120.3 (q, J = 3.4 Hz), 120.2, 118.7, 117.0 (q, J = 3.4 Hz) 110.2. Anal. Calcd for $C_{15}H_7BrClF_6NO_2$ (462.57): C, 38.95; H, 1.53; N, 3.03. Found: C, 38.89; H, 1.57; N, 2.98.

4.2. Biology

4.2.1. *In vitro* antimycobacterial evaluation

The *in vitro* antimycobacterial activity of the synthesized compounds was determined against *Mtb* My 331/88 ($H_{37}R_V$; dilution of strain 10^{-3}), *M. avium* My 330/80 (dilution of strain 10^{-5}), *M. kansasii* 235/80 (dilution of strain 10^{-4}) and *M. kansasii* 6509/96 (dilution of strain 10^{-4}). A selected set of compounds was further tested against five MDR-TB strains (dilution of all MDR-TB strains was 10^{-3} while 234/2005 is resistant to INH, RIF, rifabutin, streptomycin, ethambutol and ofloxacin; 9449/2007 is resistant to INH, RIF, rifabutin, and streptomycin; 242/2015 is resistant to INH, RIF, rifabutin, streptomycin and ethambutol; Praha 1 is resistant to INH, (RIF), rifabutin and streptomycin and Praha 4 is resistant to INH, RIF, rifabutin, streptomycin, ethambutol, ofloxacin and clofazimine) and one XDR-TB strain (Praha 131 which is resistant to INH, RIF, rifabutin, streptomycin, ethambutol, ofloxacin, gentamicin and amikacin). 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. 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* [30]. 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. INH and PAS were chosen as reference drugs.

4.2.2. Cytotoxicity

All salicylanilides were tested for cytotoxicity against 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 °C. For subculturing, the cells were harvested after trypsin/EDTA (Sigma-Aldrich) treatment at 37 °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 x 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 ^{o}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 ^{o}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).

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