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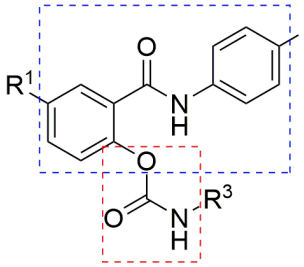

salicylanilide motif
(antimycobacterial)
R^1=H, Cl
R^2=H, F, Cl, Br, CF_3

carbamate moiety
(improving properties)
R^3=cyclohexyl, Ph, Bn,
phenethyl, adamantan-1-yl

Potent antimycobacterial activity
M. tuberculosis $H_{37}Rv$: MIC from ≤0.5 μM
MDR- and XDR-TB strains: MIC from 0.5 μM
nontuberculous mycobacteria: MIC from ≤0.79 μM
Mild **inhibition of mycobacterial isocitrate lyase**
Mostly **non-cytotoxic compounds**

Phenolic *N*-Monosubstituted Carbamates: Antitubercular and Toxicity Evaluation of Multi-targeting Compounds

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Abstract

The research of novel antimycobacterial drugs represents a cutting-edge topic. Thirty phenolic *N*-monosubstituted carbamates, derivatives of salicylanilides and 4-chlorophenol, were investigated against *Mycobacterium tuberculosis* $H_{37}Ra$, $H_{37}Rv$ including multidrug- and extensively drug-resistant strains, *Mycobacterium avium*, *Mycobacterium kansasii*, *Mycobacterium aurum* and *Mycobacterium smegmatis* as representatives of nontuberculous mycobacteria (NTM) and for their cytotoxic and cytostatic properties in HepG2 cells. Since salicylanilides are multi-targeting compounds, we determined also inhibition of mycobacterial isocitrate lyase, an enzyme involved in the maintenance of persistent tuberculous infection. The minimum inhibitory concentrations were from ≤0.5 μM for both drug-susceptible and resistant *M. tuberculosis* and from ≤0.79 μM for NTM with no cross-resistance to established drugs. The presence of halogenated salicylanilide scaffold results into an improved activity. We have verified that isocitrate lyase is not a key target, presented carbamates showed only moderate inhibitory activity (up to 18% at a concentration of 10 μM). Most of the compounds showed no cytotoxicity for HepG2 cells and some of them were without cytostatic activity. Cytotoxicity-based selectivity indexes of several carbamates for *M. tuberculosis*, including resistant strains, were higher than 125, thus favouring some derivatives as promising features for future development.

Keywords

antimycobacterial activity; carbamate; cytotoxicity; multi-targeting; *Mycobacterium tuberculosis*; salicylanilide

1. Introduction

The development of novel antimicrobial agents represents an essential public health topic. Tuberculosis (TB), a severe infectious contagious disease caused by *Mycobacterium tuberculosis* (*Mtb.*) complex is one of the leading causes of morbidity and mortality worldwide. According to the World Health Organization, TB remains a global emergency. In 2017, an estimated 10.3 million people fell ill with TB, and approximately 1.3 million HIV-negative people died from this disease. HIV and TB co-infection as well as development of drug-resistant TB forms have brought additional problems. There were an estimated 558,000 new cases of rifampicin (RIF)-resistant and multidrug-resistant tuberculosis (MDR-TB). MDR-TB consists of *in vitro* resistance to both isoniazid (INH) and rifampicin, the two most effective first-line oral drugs. XDR-TB was defined as an MDR-TB plus resistance to at least one drug in both essential classes of medicines used in an MDR-TB regimen: fluoroquinolones and second-line injectable drugs (amikacin, capreomycin, and kanamycin).[1] Their treatment is more challenging with limited therapeutic possibilities.

Ubiquitous nontuberculous (also called atypical) mycobacteria (NTM) are recognised more frequently as causative agents of serious opportunistic human infections in both immunocompetent and immunocompromised patients. They affect various tissues including respiratory tract, skin, joints, bones and soft tissues. Their incidence is expected to continue to increase at least up to 2050. There are multiple reasons for this fact, including heightened awareness, better diagnostics, an increased number of vulnerable hosts and increasing medical tourism. Their very long treatment requires administration of multiple drugs in combination, but there is a high level of both intrinsic and acquired resistance. That is why the current treatment regimens have only a limited efficacy and the outcome is poor. Unfortunately, many NTM cases are misdiagnosed as TB, however the treatment of NTM caused diseases is not like that of TB.[2,3,4]

To achieve a global control against epidemics of resistant mycobacterial strains, there is an urgent need for development of novel drugs. Ideally, they should avoid any cross-resistance to current therapeutic options.

Carbamate bond has been highlighted in medicinal chemistry as both drugs and prodrugs. This structure motif has been incorporated in many molecules of various indication classes in clinical practice, illustratively antiviral, antiparasitic or central nervous system targeting drugs. Generally, carbamate functional group provides sufficient chemical stability and capability to increase permeability across biological membranes.[5] Carbamates have been involved in prodrug design of phenolic compounds frequently. They are more stable towards enzymatic hydrolysis than corresponding esters of carboxylic acids.[5,6]

Diverse carbamates have been proposed and evaluated as potential antimycobacterial agents active against not only *Mtb.*[7,8,9,10,11,12] but also NTM like highly chemoresistant *Mycobacterium abscessus*[8], *Mycobacterium avium* and *Mycobacterium kansasii*[7,10]; for some of them truly promising results were reported. They have been able to inhibit also MDR- and XDR-TB strains.[7,9]

Carbamates obtained from salicylanilides (2-hydroxy-*N*-phenylbenzamides) have shown, e.g., significant antimycobacterial properties[7,9,10], activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus*[10,13], yeasts and fungi[10,13], or inhibition of acetylcholinesterase and butyrylcholinesterase.[14,15] Importantly, they provided a better *in vitro* antitubercular activity concomitantly with a reduced cytotoxicity and improved physicochemical properties (e.g., low lipophilicity required for passive diffusion *via* cellular membranes), i.e., the main disadvantages of their parent salicylanilide compounds.[16]

Salicylanilide-based molecules have been reported as inhibitors of many bacterial and/or mycobacterial enzymes targeting various metabolic pathways and physiological functions: D-Ala-D-Ala ligase[17], transglycosylase[18,19], isocitrate lyase and methionine aminopeptidase[20], L-alanine dehydrogenase, lysine ε-aminotransferase, chorismate mutase, pantothenate synthetase[21] or they are able to disrupt proton gradient as uncouplers.[22] However, there is no one "leading" target identified as crucial for anti-TB activity and, likely, their antimycobacterial properties are a result of interaction with multiple cellular proteins and structures. This "multi-targeting" nature may provide a benefit of more difficult development of an acquired resistance.

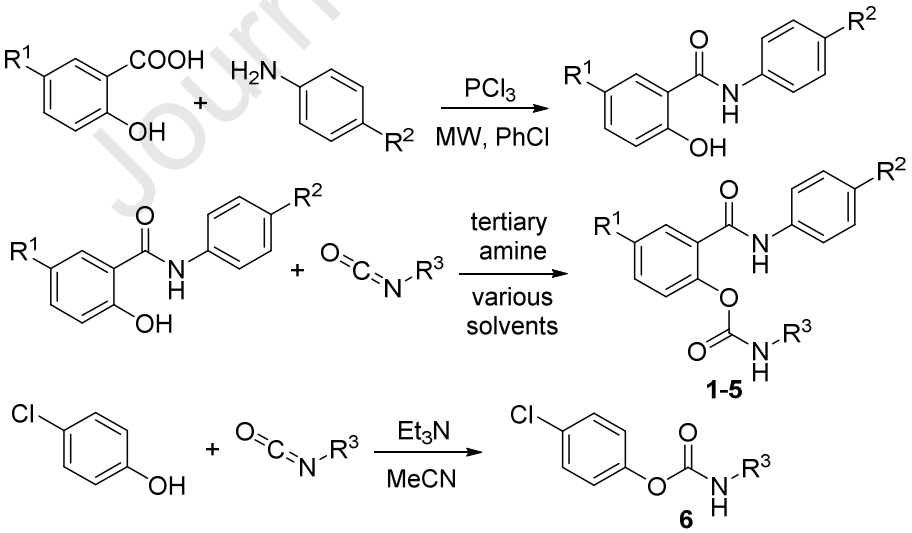
Keeping in mind especially a breakthrough study of Férriz *et al*.[7] reporting salicylanilide *N*-*n*-alkyl carbamates highly active against MDR-TB strains with minimum inhibitory concentrations (MIC) from 0.5 μM, we completed synthetically the series of our phenolic *N*-cycloalkyl/phenyl/phenylalkyl carbamates[13,15] and investigated them against various mycobacterial strains and HepG2 cell line. We have identified salicylanilide derivatives as inhibitors of isocitrate lyase; that is why we also evaluated their *in vitro* potency to inhibit this mycobacterial enzyme involved in the maintenance of latency.[10,20,23,24,25]

2. Results and discussion

2.1 Chemistry

The synthetic overview is depicted in Scheme **1**. First, salicylanilides were obtained by treatment of salicylic or 5-chlorosalicylic acid with appropriate anilines in the presence of phosphorus trichloride under microwave irradiation.[26]

5-Chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide was converted to novel carbamates **5** by a reaction with commercially available isocyanates (1.1 of equivalents) in the presence of 1 eq. of triethylamine. Dry acetonitrile was employed as a solvent. The reaction time was 2 hours at rt, only the preparation of adamantan-1-yl isocyanate **4e** required 8 h under reflux conditions. Yields ranged from 70 to 94%, symmetrical 1,3-disubstituted ureas resulting from isocyanates[13] were observed as side products in the case of phenylalkyl isocyanates and especially phenyl isocyanate, but not for cyclohexyl isocyanate. Salicylanilide bearing CF_3 group was chosen since it and also its derivatives have shown the most potent anti-TB activity in our previous studies.[9,10,20,21,23,24,28] The synthesis and characterization of the carbamates **1**-**3**, **5** and **6** were reported previously by our group.[13,15] To evaluate an influence of halogenation on biological activity and also the contribution of 2-[*N*-(substituted phenyl)carbamoyl] moiety, we also included non-halogenated salicylanilide carbamates **5** and corresponding "simplified" 4-chlorophenyl carbamates **6**, respectively. The parent salicylanilides of here presented derivatives are identical to salicylanilides involved in our previous study dealing with *N*,*N*-disubstituted carbamates to allow the direct comparison of these both groups.[10]



Scheme 1. Synthesis of salicylanilides, salicylanilide carbamates **1**-**5** and 4-chlorophenyl carbamates **6** [R^1 = H, Cl; R^2 = H, Cl, Br, F, CF_3; R^3 = cyclohexyl **a**, phenyl **b**, benzyl **c**, phenethyl **d**, adamantan-1-yl **e**; MW: microwave irradiation (530 W, 600 rpm, 22 min); PhCl: chlorobenzene; MeCN: acetonitrile; Et_3N: triethylamine].

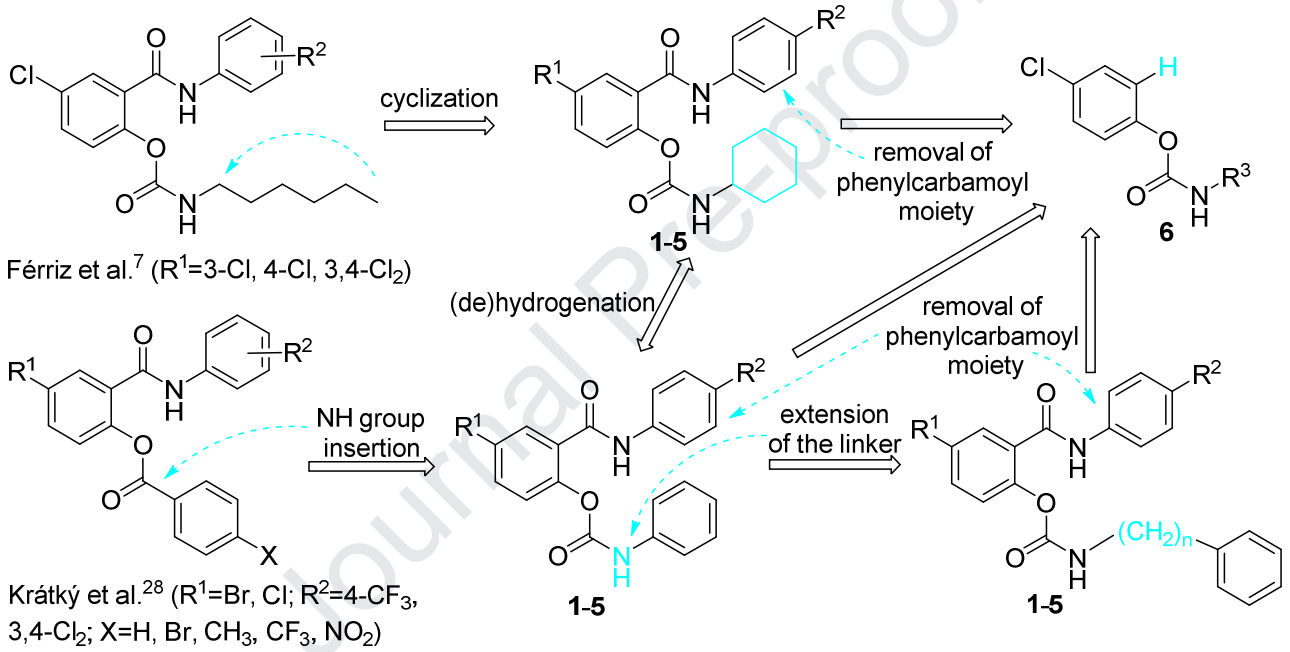
The design of the carbamic part of these molecules is outlined in Scheme **2**. Férriz *et al*.[7] identified salicylanilide *N*-hexylcarbamates as the most active against *Mtb*. Thus, we can obtain by an imaginary cyclization of this substituent *N*-cyclohexylcarbamates **a**. Their dehydrogenation provides phenylcarbamates **b**, which can also be homologues of highly antitubercular active

salicylanilide benzoates[27,28] derived *via* an incorporation of -NH- group between ester and phenyl moiety. Benzyl- and phenethylcarbamates **c** and **d** are higher homologues of phenylcarbamates obtained by an elongation of the linker connecting planar aromatic portion with a more hydrophilic carbamate functional group. Adamantane molecule (carbamates **e**) represents one of the frequent and proved scaffolds for anti-TB drugs used for a sufficient lipophilicity control.[29]

The new compounds were characterized by melting points, IR and NMR spectra; the purity was checked by the thin-layer chromatography and elemental analysis. In the IR spectra, sharp and strong bands appear at around 1710–1722 cm^{-1} (carbamic C=O) and 1659–1668 (amide I). Amide II bands were present at 1522–1536 cm^{-1}. Most of the derivatives displayed also two clearly visible N–H stretch bands (3261–3336 cm^{-1}).

In some NMR spectra, we observed the existence of two rotamers (*syn*/*anti*) due to a hindered rotation of C-N "pseudo-double" bond. For the carbamates, this phenomenon is well known. Secondary carbamates usually prefer *anti*-conformation.[5] The existence of two distinct rotamers was the most pronounced in the spectra of the phenethylcarbamate **5d**; the ratio of the dominant and minority conformer was approx. 100:18.

All of the derivatives investigated in this study are summarized in Table **1**.



Scheme 2. Design of antimycobacterial salicylanilide **1-5** and phenolic carbamates **6** (structural modifications-new fragments are highlighted using blue colour)

2.2 Antimycobacterial activity

Initially, salicylanilide and 4-chlorophenol-based carbamates **1-6** were investigated *in vitro* against mycobacteria actively growing in culture: *Mtb.* 331/88 (i.e., $H_{37}Rv$), three NTM strains – *Mycobacterium avium* 330/88, *M. kansasii* 235/80 and *M. kansasii* 6509/96 (a clinical isolate). 4-Chlorophenol, isoniazid (INH), the first-line anti-TB drug, and *p*-aminosalicylic acid (PAS) as a structurally similar compound were involved as the reference compounds for comparison.

Presented *N*-cycloalkyl/phenyl/phenylalkyl carbamates **1-6** (Table **1**) did not exhibit a uniform behaviour. This group should be divided into two: highly active halogenated salicylanilide derivatives **1-4** (MIC values of ≤0.5 to 62.5 µM) and, on the other hand, non-halogenated salicylanilide carbamates **5** together with slightly less potent 4-chlorophenyl carbamates **6** (MIC of 62.5 to 1000 µM).

In general, *Mtb.* is more susceptible than NTM. It was inhibited by the carbamates **1-4** with MIC up to 16 µM with trifluoromethyl derivatives (**4a**, **4b**, **4d** and **4e**) superiority (≤1 µM). This activity is comparable to INH. Non-halogenated salicylanilide carbamates **5** led to a single MIC of 125 µM, while 4-chlorophenyl derivatives inhibited this strain at 62.5-500 µM with **6e** superiority. MIC for

atypical mycobacteria of the first group of derivatives ranged from 2 to 62.5 µM, for the second one of 125-1000 µM. The highest activity was found for four carbamates again (**4a**, **4b**, **4d**, **4e**; 2-8 µM), followed by brominated derivatives **2**. The activities against *M. avium* and two strains of *M. kansasii* were comparable (±one dilution). Usually, salicylanilide derivatives have supressed *M. kansasii* stronger (e.g.,[28]) than *M. avium*. All of the salicylanilide carbamates **1**-**5** and some of the carbamates **6** exhibited lower MIC than INH against INH-resistant strains of *M. avium* and *M. kansasii* 235/80. A major part of phenolic derivatives (**1a**-**2e**, **3b**, **3d**-**4e**) showed an activity comparable to INH against the clinical isolate of *M. kansasii* (6509/96). All halogenated compounds **1**-**4** exhibited a significantly better activity against *Mtb.* than PAS (up to >125 times), whereas other investigated derivatives were usually comparable (**5**, adamantane-based molecule **6e**) to this second-line anti-TB drug. The carbamates **1**-**4** showed also identical (**3c**) or the remaining ones even substantially lower MIC values for *M. avium*, especially after 3 weeks of incubation. Illustratively, the derivative **4e** more than 31 times more potent than PAS. Additionally, both strains of *M. kansasii* were dramatically more susceptible to halosalicylanilide carbamates **1**-**4** than to PAS (up to >250 times). With one exception, also non-halogenated salicylic **5** and 4-chlorophenyl carbamates **6** produced a better growth inhibition of *M. kansasii* 235/80 after 2 and 3 weeks of incubation; the activities against *M. kansasii* 6509/96 are comparable mutually (i.e., equal or ±one dilution). Surprisingly, the modification of 4-chlorophenol with mild intrinsic antimycobacterial properties (MIC ≥125 µM) to form more lipophilic carbamates **6** did not result in any significant improvement in activity.

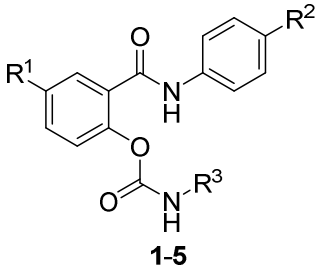
Focusing on structure-activity relationships, following issues were identified:

1) The presence of the salicylanilide scaffold is essential for the low MIC values. The removal of 2-[*N*-(substituted phenyl)carbamoyl] fragment to provide less lipophilic carbamates of 4-chlorophenol (derivatives **6**) led to significantly lower antimycobacterial properties (up to two orders of magnitude).

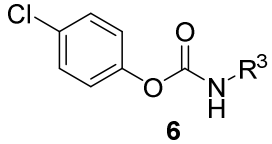
2) The halogenation of the salicylanilide core and corresponding increased lipophilicity are required for the outstanding activity (compounds **1**-**4** vs. **5**) with CF_3 group superiority (**4**) for both *Mtb.* H_{37}Rv and NTM. Among particular halogens, fluorine atom is the best one for *Mtb.* but not NTM, where brominated derivatives led to the most potent growth inhibition.

3) Carbamates **1**, **2** and **5** obtained from 5-chloro-*N*-(4-chlorophenyl)-2-hydroxybenzamide, *N*-(4-bromophenyl)-5-chloro-2-hydroxybenzamide and unsubstituted salicylanilide, respectively, exhibited equal MIC values (±one dilution) despite carbamic *N*-substituent. On the other hand, among fluorine derivatives **3** and **4**, *N*-benzyl compounds **c** showed a decreased activity when compared to other carbamates. *N*-Adamantan-1-yl group (**6e**) represents the most convenient moiety for 4-chlorophenyl carbamates.

Table 1. Antimycobacterial activity of the carbamates **1**-**6**



1-5



6

	R^1	R^2	R^3	MIC [µM]										log*P*
				Mtb. 331/88		*M. avium*		*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	
1a	Cl	Cl	Cyclohexyl	16	16	16	16	16	32	32	8	8	8	5.0
1b	Cl	Cl	Phenyl	16	16	16	16	16	32	32	8	16	16	5.11
1c	Cl	Cl	Benzyl	16	16	**8**	16	**8**	**8**	16	**4**	8	8	5.18
1d	Cl	Cl	Phenethyl	8	16	**8**	**8**	**8**	**8**	16	**4**	8	8	5.46
1e	Cl	Cl	Adamantan-1-yl	16	16	16	16	**8**	16	16	8	16	16	5.28

2a	Cl	Br	Cyclohexyl	8	16	**8**	16	**4**	**8**	**8**	**4**	8	8	5.27
2b	Cl	Br	Phenyl	8	16	**8**	16	**4**	**8**	**8**	**4**	8	8	5.38
2c	Cl	Br	Benzyl	16	16	**8**	16	**4**	**8**	**8**	**4**	8	8	5.45
2d	Cl	Br	Phenethyl	8	8	**8**	**8**	**4**	**8**	**8**	**4**	8	8	5.73
2e	Cl	Br	Adamantan-1-yl	8	8	**8**	**8**	**4**	**8**	**8**	**4**	8	8	5.55
3a	Cl	F	Cyclohexyl	4	8	32	32	16	32	32	16	16	32	4.60
3b	Cl	F	Phenyl	4	8	32	32	**8**	16	32	8	16	16	4.71
3c	Cl	F	Benzyl	8	16	62.5	62.5	16	32	32	16	32	32	4.78
3d	Cl	F	Phenethyl	4	4	32	32	**8**	16	16	8	16	16	5.06
3e	Cl	F	Adamantan-1-yl	4	4	32	32	**8**	**8**	16	8	8	16	4.88
4a	Cl	CF_3	Cyclohexyl	**1**	**1**	**8**	**8**	**4**	**4**	**8**	**2**	**4**	**4**	5.36
4b	Cl	CF_3	Phenyl	**≤0.5**	**1**	**8**	**8**	**4**	**4**	**4**	**2**	**4**	**4**	5.48
4c	Cl	CF_3	Benzyl	2	4	32	32	**8**	16	16	8	16	16	5.54
4d	Cl	CF_3	Phenethyl	**≤0.5**	**1**	**8**	**8**	**4**	**4**	**8**	**2**	**4**	**4**	5.82
4e	Cl	CF_3	Adamantan-1-yl	**≤0.5**	**1**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	5.64
5a	H	H	Cyclohexyl	125	125	125	125	125	250	250	125	250	250	3.88
5b	H	H	Phenyl	125	125	125	125	125	250	250	125	250	250	4.0
5c	H	H	Benzyl	125	125	125	125	125	250	250	125	250	250	4.07
5d	H	H	Phenethyl	125	125	125	125	125	250	250	125	250	250	4.35
5e	H	H	Adamantan-1-yl	125	125	125	125*	125	125*	125*	125	250	250	4.16
6a	-	-	Cyclohexyl	500	500	500	1000	250	500	1000	125	250	250	3.63
6b	-	-	Phenyl	250	500	500	1000	125	250	500	125	250	500	3.75
6c	-	-	Benzyl	125	250	500	500	125	250	500	125	250	500	3.82
6d	-	-	Phenethyl	250	250	250	500	125	250	500	125	250	500	4.10
6e	-	-	Adamantan-1-yl	62.5	125	125	250	125	125*	125*	125	125*	125*	3.91
4-Cl-phenol				125	250	>250	>250	250	>250	>250	125	250	>250	2.20
INH				0.5	1	>250	>250	>250	>250	>250	4	8	8	-0.60
PAS				62.5	62.5	32	125	125	1000	>1000	32	125	500	0.88

INH: isoniazid; PAS: *p*-aminosalicylic acid; * at this concentration, the growth of the strain was observed; at duplex concentration, a precipitate and/or a turbidity was present, therefore it was not possible to determine the exact MIC value.

Based on these promising data, we decided to extend the commonly used panel of mycobacteria to confirm an excellent wide-spectrum antimycobacterial activity against actively growing mycobacteria. We involved *Mycobacterium tuberculosis* H_{37}Ra ITM-M006710 and two NTM: *Mycobacterium aurum* DSM 43999 and *Mycobacterium smegmatis* DSM 43465. Avirulent strain of *Mtb*. is commonly used in screening showing comparable results to virulent strain with benefit shown in lower risk of infection. Other two strains are counted between fast growing mycobacteria and their benefit is in generation time that is much shorter than *Mtb*. Three first-line oral anti-TB drugs, INH, rifampicin (RIF) and ethambutol (EMB) were used as the standards. The results are summarized in Table **2**.

Table 2. Activity of the carbamates **1-6** against additional mycobacterial strains

	R^1	R^2	R^3	MIC [µg/mL] (µM)		
				Mtb. H_{37}Ra	*M. aurum*	*M. smegmatis*
				48 h	72 h	120 h
1a	Cl	Cl	Cyclohexyl	3.125	1.56	1.56
1b	Cl	Cl	Phenyl	1.56	1.56	1.56
1c	Cl	Cl	Benzyl	1.56	1.56	1.56
1d	Cl	Cl	Phenethyl	1.56	1.56	1.56
1e	Cl	Cl	Adamantan-1-yl	3.125	3.91	3.91
2a	Cl	Br	Cyclohexyl	1.56	1.56	1.56
2b	Cl	Br	Phenyl	1.56	1.56	1.56
2c	Cl	Br	Benzyl	3.91	1.98	1.98
2d	Cl	Br	Phenethyl	0.78 (1.65)	1.56	3.125
2e	Cl	Br	Adamantan-1-yl	1.56	1.56	3.125
3a	Cl	F	Cyclohexyl	3.125	1.56	3.91
3b	Cl	F	Phenyl	3.125	1.98	1.98
3c	Cl	F	Benzyl	3.125	3.125	3.125
3d	Cl	F	Phenethyl	3.125	3.125	3.125

3e	Cl	F	Adamantan-1-yl	0.99 (2.24)	1.98	1.98
4a	Cl	CF_3	Cyclohexyl	≤0.39 (≤0.88)	≤0.39 (≤0.88)	≤0.39 (≤0.88)
4b	Cl	CF_3	Phenyl	≤0.39 (≤0.90)	≤0.39 (≤0.90)	≤0.39 (≤0.90)
4c	Cl	CF_3	Benzyl	≤0.39 (≤0.87)	≤0.39 (≤0.87)	0.78 (1.74)
4d	Cl	CF_3	Phenethyl	0.39-0.78 (0.84-1.69)	≤0.39 (≤0.84)	≤0.39 (≤0.84)
4e	Cl	CF_3	Adamantan-1-yl	0.99 (2.00)	≤0.39 (≤0.79)	1.98 (4.00)
5a	H	H	Cyclohexyl	15.625	15.625	15.625
5b	H	H	Phenyl	31.25	31.25	31.25
5c	H	H	Benzyl	15.625	31.25	31.25
5d	H	H	Phenethyl	15.625	31.25	31.25
5e	H	H	Adamantan-1-yl	250	≥250	250
6a	-	-	Cyclohexyl	500	≥500	≥500
6b	-	-	Phenyl	7.81	15.625	15.625
6c	-	-	Benzyl	31.25	125	125
6d	-	-	Phenethyl	31.25	125	62.5
6e	-	-	Adamantan-1-yl	62.5	≥125	≥125
INH				0.125-0.25 (0.91-1.82)	3.91	15.625
RIF				0.0039-0.0078 (0.005-0.009)	0.39 (0.47)	12.5-25
EMB				0.5 (1.80)	0.25 (0.90)	0.5 (1.80)

INH: isoniazid; RIF: rifampicin; EMB: ethambutol. For the most active compounds (MIC lower than 1 μg/mL) we calculated also MIC in μM for direct comparison with values reported in Table 1.

Obviously, the carbamates **1**-**5** were also able to inhibit more mycobacterial strains effectively: *Mtb.* H_{37}Ra, *M. smegmatis* and *M. aurum*. We confirmed the excellence of the compounds substituted by CF_3 group (derivatives **4**) as well as significantly higher MIC values of non-halogenated salicylanilide carbamates **5** and 4-chlorophenyl carbamates **6**.

The most active derivatives are fully comparable or superior to clinically used drugs INH and EMB at low micromolar or even submicromolar concentrations. In contrast to INH and RIF, there were no differences between MIC values of the carbamates **1**-**6** against *Mtb.* and NTM strains predominantly. Thus, we confirmed the broad-spectrum antimycobacterial action of the investigated carbamates. Interestingly, generally fewer active compounds **5**-**6** inhibited the additional mycobacterial strains in this follow-up assay. Other important finding is that the activity our compounds does not depend on the medium used for the determination of MIC; in this assay, Middlebrook 7H9 medium was used. Comparison of results between virulent and avirulent strain also showed that values of MIC are comparable and reliable. The advantage of using *Mtb*. H_{37}Ra is also connected with a shorter time of screening (results are read after 120 hours compared to 14 or 21 days).

2.2.1 Activity against drug-resistant TB strains

The carbamates with the highest *in vitro* efficacy against drug-susceptible *Mtb.* H_{37}Rv (i.e., **4a**, **4b**, **4d**, and **4e** with MIC of ≤1 μM, all being the derivatives of 5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide) were evaluated also against MDR- and XDR-TB additionally. They exhibited also an interesting activity against six multidrug-resistant *Mtb.* strains and one XDR-TB strain within the range of 0.5-2 μM, independently of the resistance patterns (Table **3**). *N*-Phenethylcarbamate **4d** showed only a negligibly lower *in vitro* efficacy. Importantly, these values are comparable or identical to those obtained for *Mtb.* H_{37}Rv indicating that there is no cross-resistance with currently used drugs for the treatment of both drug-susceptible and resistant TB (INH, RIF, rifabutin, ethambutol, streptomycin, fluoroquinolones represented by ofloxacin, clofazimine, and aminoglycosides). Regarding these facts, the salicylanilide carbamates seem to be promising wide-spectrum anti-TB and antimycobacterial agents against drug-susceptible, drug-resistant and NTM mycobacterial strains.

Table 3. MIC of salicylanilide carbamates **4** for drug-resistant *Mtb.* strains

	MIC [μM]													
	Mtb. 7357/1998		*Mtb.* 9449/2006		*Mtb.* 8666/2010		*Mtb.* 234/2005		*Mtb.* Praha 1		*Mtb.* Praha 4		*Mtb.* Praha 131	
	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d
4a	**0.5**	**1**	**0.5**	**1**	**0.5**	**1**	**0.5**	**1**	**0.5**	**1**	**0.5**	**1**	**0.5**	**1**
4b	**0.5**	**1**	**1**	**1**	**0.5**	**1**	**1**	**1**	**0.5**	**1**	**0.5**	**1**	**1**	**1**
4d	**1**	2	2	2	2	2	2	2	**1**	**1**	2	2	2	2
4e	**0.5**	**1**	**1**	**1**	**0.5**	**1**	**1**	**1**	**0.5**	**1**	**0.5**	**1**	**1**	**1**

MIC values equal or lower than 1 μM are given in bold.

MDR-TB strains: 7357/1998 resistant to INH, RIF, rifabutin, streptomycin, ethambutol and ofloxacin; 9449/2006 resistant to INH, RIF, rifabutin and streptomycin; 234/2005 resistant to INH, RIF, rifabutin, streptomycin and ethambutol; 8666/2010 resistant to INH, RIF, rifabutin, streptomycin, ethambutol, ofloxacin and clofazimine; Praha 1 resistant to INH, RIF, rifabutin, streptomycin, ethambutol and clofazimine; Praha 4 resistant to INH, RIF, rifabutin, streptomycin, ethambutol, ofloxacin and clofazimine. XDR-TB strain: Praha 131 resistant to INH, RIF, rifabutin, streptomycin, ethambutol, ofloxacin, gentamicin and amikacin.

Comparing to previous salicylanilide carbamates, the most active derivatives **1**-**4** are comparable to salicylanilide *N*-*n*-alkylcarbamates and chloroalkycarbamates[7,9], and significantly superior to *N*,*N*-disubstituted carbamates.[10]

2.3 Evaluation of isocitrate lyase inhibition

Since salicylanilide derivatives are multi-targeting agents, we investigated the carbamates against one of the targets described earlier. Previous works have reported salicylanilides[10,20], their esters with various carboxylic acids[20,23,24], benzenesulfonic acid[20], *N*,*N*-disubstituted (thio)carbamates[10] and benzamides[25] as *in vitro* inhibitors of mycobacterial isocitrate lyase (ICL1), but to the best of our knowledge, inhibition activity of phenolic *N*-monosubstituted carbamates has not been reported to date.

ICL1, one of two enzymes involved in glyoxylate cycle, a metabolic pathway replenishing tricarboxylic acid cycle intermediates, and methylcitrate cycle represents an attractive target for combating especially persistent intracellular mycobacterial subpopulation during chronic infection. However, inhibition of ICL has no significant observable effect against actively growing mycobacteria[25] (here reported in Tables **1**-**3**). It was presumed that the ICL inhibitors may result in the shortening of TB therapy. Especially dual activity against both actively growing and non-replicating mycobacteria should provide an extra benefit.[25,30] Moreover, this enzyme has been proposed to be linked to endogenous resistance or a higher tolerance to anti-TB drugs.[31]

ICL inhibition assay was selected to extend the potential application to latent mycobacteria as an adjunctive target illustrating the multi-targeting nature of the carbamates **1**-**6**. We determined their activity at a single concentration of 10 μM using glyoxylate phenyl hydrazone formation assay (Table **4**). INH and PAS were employed as negative controls; 3-nitropropionic acid, a known substrate-analogue inhibitor[25], served as a positive control. We also intended to determine IC_{50} for the most potent inhibitors (i.e., inhibition rate comparable to 3-nitropropionic acid: 25%). However, all of the derivatives produced only a mild inhibition of the enzyme within the range of 5-18% with **2e** and **3a** superiority, even though the differences are rather marginal.

The highest activity was observed for the brominated and monofluorinated derivatives **2** and **3**, which are superior to dichlorocarbamates **1**. In contrast to whole-cell antimycobacterial activity, the presence of CF_3 group (**4a**-**4e**) does not offer any significant advantage. The halogenation improved the inhibition potency substantially (**5** vs. **1** and especially **2**-**4** vs. **5**). Comparing salicylanilide molecules **1**-**5** and 4-chlorophenyl carbamates **6**, the (4-halogenophenyl)carbamoyl fragment is not required for an effective ICL inhibition. Due to insignificant distinctions, it is also unfeasible to identify the most convenient carbamic *N*-substituent.

In accordance to some previous reports regarding salicylanilide carboxylates, benzenesulfonates esters and *N*,*N*-disubstituted (thio)carbamates[10,20,23,24], here presented phenolic carbamates were

found as mild ICL1 inhibitors comparable with the majority of them and, importantly, superior to parent salicylanilides with free phenolic group.

As expected[25,30], we did not identify a clear relationship of *in vitro* MIC against actively growing mycobacteria (low micromolar or submicromolar values; Tables **1**, **2** and **3**) and ICL inhibition (only partial inhibition at 10 µM; Table **4**). Moreover, there should not be a direct correlation between strong anti-TB properties under our experimental conditions and ICL suppression, since both tests are focused on different stages of mycobacterial life cycle. Since ICL is indispensable only in non-replicating mycobacteria, its inhibition has shown partial potential against persistent or latent mycobacteria during a long-term use in the TB therapy, but not in short-term assay determining suppression of actively growing extracellular bacteria used in this study. Using in combination with other drugs, it may help to increase susceptibility to them. Thus, the consideration of ICL as a key target of the carbamates **1**-**6** is inappropriate and it is in accordance with the known assumptions. Their mechanism of action against actively growing mycobacteria remains to be elucidated in depth and it is not related to described mild ICL inhibition. Analogous phenomenon was described for methyl 4-(4-methoxyphenyl)-4-oxobut-2-enoate. This ester inhibited ICL1 (IC_{50} = 30.9 µM) and showed an activity in model of intracellularly persistent mycobacteria, but it was also active against various mycobacterial strains in log-phase in culture (MIC_{90} 0.25-1 µg/mL). The explanation of this excellent activity cannot be attributed to moderate ICL inhibition suggesting likely an additional unknown actual target remaining to be confirmed.[32].

Giving together, the here reported ICL inhibition of the salicylanilide carbamates brings probably only a certain minor benefit concerning nonreplicating mycobacteria and latent TB. Actual and key anti-TB target of the carbamates **1**-**6** remains to be confirmed.

Table 4. Inhibition of isocitrate lyase

Code	R^1	R^2	R^3	Inhibition rate [%] at 10 µM
1a	Cl	Cl	Cyclohexyl	13 ± 1.56
1b	Cl	Cl	Phenyl	10 ± 1.56
1c	Cl	Cl	Benzyl	11 ± 1.58
1d	Cl	Cl	Phenethyl	10 ± 1.21
1e	Cl	Cl	Adamantan-1-yl	11 ± 1.58
2a	Cl	Br	Cyclohexyl	16 ± 3.70
2b	Cl	Br	Phenyl	17 ± 1.42
2c	Cl	Br	Benzyl	17 ± 2.94
2d	Cl	Br	Phenethyl	17 ± 1.15
2e	Cl	Br	Adamantan-1-yl	**18 ± 2.61**
3a	Cl	F	Cyclohexyl	**18 ± 0.75**
3b	Cl	F	Phenyl	16 ± 4.54
3c	Cl	F	Benzyl	15 ± 2.71
3d	Cl	F	Phenethyl	15 ± 2.49
3e	Cl	F	Adamantan-1-yl	14 ± 3.93
4a	Cl	CF_3	Cyclohexyl	14 ± 3.66
4b	Cl	CF_3	Phenyl	13 ± 2.47
4c	Cl	CF_3	Benzyl	14 ± 3.00
4d	Cl	CF_3	Phenethyl	11 ± 4.72
4e	Cl	CF_3	Adamantan-1-yl	15 ± 1.33
5a	H	H	Cyclohexyl	8 ± 2.77
5b	H	H	Phenyl	5 ± 1.56
5c	H	H	Benzyl	8 ± 1.35
5d	H	H	Phenethyl	6 ± 1.99
5e	H	H	Adamantan-1-yl	9 ± 1.58
6a	-	-	Cyclohexyl	16 ± 2.27
6b	-	-	Phenyl	14 ± 0.83
6c	-	-	Benzyl	14 ± 2.38
6d	-	-	Phenethyl	12 ± 1.10
6e	-	-	Adamantan-1-yl	13 ± 2.88

INH	0
PAS	0
3-nitropropionic acid	25 ± 4.1

INH: isoniazid; PAS: *p*-aminosalicylic acid.

2.4. Cytotoxic and cytostatic properties evaluation

All of the carbamates **1-6** underwent determination of their *in vitro* cytotoxic and cytostatic action on hepatocellular carcinoma cells (HepG2) using MTT assay. The HepG2 cell line serves as an *in vitro* model for the hepatotoxicity for early drug screen. Liver tissue represents the most likely target for the chronic toxicity of antitubercular drugs, which often complicates the six months long therapy of TB.[33] That is why we have chosen this cell line for selectivity indexes (SI) determination. The same approach was used for our previously reported salicylanilide derivatives[10,20,23,24,28] and allows a direct comparison of different series.

In general, the cytotoxicity of salicylanilide-based molecules has been reported as an unwanted effect of these potential antimicrobial agents.[16] The evaluation of cytotoxicity allows calculating the selectivity indexes, which are defined as a ratio of IC_{50} and MIC (cytotoxicity on HepG2 cells). Analogously to the therapeutic index, SI values higher than 10 suggest an acceptable toxicity. There were solubility problems with several derivatives preventing determination of IC_{50} values at higher concentrations (especially **3b**). Hence, the highest non-cytotoxic and concentrations with no precipitation observed are reported.

The evaluated derivatives can be divided into several groups regarding their toxicities (Table **5**). First, the carbamates that are neither cytotoxic nor cytostatic at the concentration of 125 µM or higher (all 4-chlorophenyl carbamates **6** and two adamantyl compounds **3e** and **5e**). Second, the largest group is comprised of the derivatives with no cytotoxicity but with cytostatic properties (**1a**-**1c**, **2a**-**2d**, **3b**, **3c**, **4a**, **4c**-**4e**, **5c** and **5d**). It means that they do not kill the cell directly, although they arrest cell growth, multiplication and proliferation. This cytostatic action together with no cytotoxicity was reported for salicylanilide 4-substituted benzoates.[28] The remaining molecules exhibited both cytotoxic and cytostatic action for HepG2 cells, although at variable concentrations (**1d**, **1e**, **2e**, **3a**, **3d**, **4b**, **5a** and **5b**).

The most cytotoxic compound was the adamantyl derivative **2e** (IC_{50} = 4.20 µM), on the other hand, the less toxic carbamates shared IC_{50} higher than 250 µM (**1a**-**1c**, **5e**). The majority of halogenated salicylanilide carbamates caused a significant cytostasis in HepG2 cells (i.e., IC_{50} ≤10 µM; **1b**-**3b**, **3d**, **4a**-**4e**), three of them with IC_{50} values of ≤1 µM (**1b**, **4a**, **4d**). The presence of halogenated salicylanilide scaffold is translated mostly into enhanced cytostatic properties but not cytotoxicity. Focusing on *N*-carbamic substitution, there is no clear structure-activity relationship. From an alternative point of view, especially 2-[(4-bromophenyl)carbamoyl]-4-chlorophenyl adamantan-1-ylcarbamate **2e** may be considered as a potent cytotoxic and cytostatic agent with a concomitant antibacterial, antifungal[13] and antimycobacterial properties.

In term of SI, ten derivatives provided a sufficient selectivity for *Mtb.* (**1a**-**1c**, **3d**-**4e**), even up to >125 with **4a**, **4d** and **4e** superiority. The adamantyl carbamate **4e** was found as the most selective one for NTM (SI >31.3), followed by **1c**, **4a** and **4d** (SI >15.6), whereas dichlorocarbamates **1a** and **1b** were selective certainly only for *M. avium*. Moreover, these most selective derivatives were comparably selective or even better than INH and PAS.

Table 5. Cytotoxic and cytostatic action of phenolic carbamates

Code	R^1	R^2	R^3	IC_{50} [µM] cytotoxicity	IC_{50} [µM] cytostasis	SI for *Mtb.*	SI for NTM
1a	Cl	Cl	Cyclohexyl	>250	33.60±8.66	**>15.6**	>7.8 (*Mk*), **>15.6** (*Ma*)
1b	Cl	Cl	Phenyl	>250	0.90±0.00	**>15.6**	>7.8 (*Mk*), **>15.6** (*Ma*)
1c	Cl	Cl	Benzyl	>250	6.40±1.56	**>15.6**	**>15.6**
1d	Cl	Cl	Phenethyl	15.00	5.10±3.39	1.9	>0.9
1e	Cl	Cl	Adamantan-1-yl	24.00	8.15±1.06	1.5	1.5-3
2a	Cl	Br	Cyclohexyl	>50	9.40±0.71	>6.3	>3.1

2b	Cl	Br	Phenyl	>50	5.45±0.07	>6.3	>3.1
2c	Cl	Br	Benzyl	>50	1.65±0.64	>3.1	>3.1
2d	Cl	Br	Phenethyl	>50	2.20±1.27	>6.3	>6.3
2e	Cl	Br	Adamantan-1-yl	4.20±0.99	4.35±0.07	0.5	0.5-1
3a	Cl	F	Cyclohexyl	21.20±4.67	8.60±1.84	2.7-5.3	0.7-1.3
3b	Cl	F	Phenyl	>20	3.25±1.34	>2.5	0.6-2.5
3c	Cl	F	Benzyl	>125	34.15±12.37	>7.8	>2
3d	Cl	F	Phenethyl	41.50±4.95	7.60±0.85	**10.4**	1.3-5.2
3e	Cl	F	Adamantan-1-yl	>125	>125	**31.3**	>3.9
4a	Cl	CF_3	Cyclohexyl	>125	0.75±0.35	**>125**	**>15.6**
4b	Cl	CF_3	Phenyl	9.80±3.54	2.45±1.77	9.8-**19.6**	1.2-9.8
4c	Cl	CF_3	Benzyl	>125	3.00±0.85	**>31.3**	>3.9
4d	Cl	CF_3	Phenethyl	>125	1.00±0.42	**>125**	**>15.6**
4e	Cl	CF_3	Adamantan-1-yl	>125	4.85±2.47	**>125**	**>31.3**
5a	H	H	Cyclohexyl	49.00±8.91	51.50	0.4	0.2-0.4
5b	H	H	Phenyl	57.65±9.40	26.80	0.5	0.2-0.5
5c	H	H	Benzyl	>125	54.30	>1	>0.5
5d	H	H	Phenethyl	>125	55.60	>1	>0.5
5e	H	H	Adamantan-1-yl	>250	233.00±24.04	>2	>0.5
6a	-	-	Cyclohexyl	>125	>125	>0.3	>0.1
6b	-	-	Phenyl	>125	>125	>0.3	>0.1
6c	-	-	Benzyl	>125	>125	>0.5	>0.3
6d	-	-	Phenethyl	>125	>250	>0.5	>0.3
6e	-	-	Adamantan-1-yl	>125	>250	>1	>0.5
INH	-	-	-	>250	ND	**>250**	**≥31.3** (*Mk* 6509/96)
PAS	-	-	-	2,240	ND	**35.8**	<2.2-**70**

INH: isoniazid; PAS: *p*-aminosalicylic acid. SI = IC50 for cytotoxicity/MIC. SI values higher than 10 are given in bold. *Mk = M. kansasii, Ma = M. avium.*

3. Conclusions

In this work, we completed the series of salicylanilide *N*-cycloalkyl/phenyl/phenylalkyl carbamates and together with analogous 4-chlorophenyl carbamates, they were screened for their biological activity. We assessed them against various mycobacterial strains (*Mtb.* including MDR- and XDR-TB, broad-spectrum of NTM), for their cytotoxicity and cytostatic properties in liver cells model and also inhibition of isocitrate lyase, one of the mycobacterial enzymes responsible for the maintenance of persistent TB infection.

Minimum inhibitory concentrations for both tuberculous and nontuberculous mycobacteria were in the micromolar concentration range and some of them were even submicromolar including activity against drug-resistant *Mtb.* strains. Importantly, the carbamic acid derivatives do not share any cross-resistance to clinically used anti-TB drugs. Their mechanism of action is multiple affecting many cellular targets. Moreover, all the compounds inhibited isocitrate lyase mildly and the majority of the carbamates were not cytotoxic, several derivatives also non-cytostatic. In sum, some here presented carbamates may represent attractive antimycobacterial molecules for future investigation, especially with respect to drug-resistant *Mtb*. and nontuberculous isoniazid-resistant species, thus confirming carbamates as an auspicious scaffold in medicinal chemistry for combating mycobacteria. Key mycobacterial target of the carbamates remains to be elucidated.

4. Material and Methods

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 they were used as received. The reactions and the purity of the products were monitored by the thin layer-chromatography using a mixture with a ratio of toluene to ethyl acetate 4:1 (v/v) as the eluent. Plates were coated with 0.2 mm Merck 60 F254 silica gel (Merck Millipore, Darmstadt, Germany) and were visualised by UV irradiation (254 nm).

The melting points were determined on a Büchi Melting Point B-540 apparatus (BÜCHI, Flawil, Switzerland) using open capillaries, and the reported values are uncorrected. Elemental analysis (C, H, 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 (ThermoFisher Scientific, Waltham, MA, USA) in the range of 650-4000 cm^{-1}. The NMR spectra were measured in DMSO-d_6 or THF-d_8 at ambient temperature using a Varian V NMR S500 instrument (500 MHz for ^1H and 126 MHz for ^{13}C; Varian Comp. Palo Alto, CA, USA). The chemical shifts, δ, are given in ppm with respect to tetramethylsilane 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 18.0 (Cambridge-Soft, Cambridge, MA, USA).

4.1.2 Synthesis
Salicylanilides were prepared *via* a previously reported microwave-assisted method.[26] The synthesis of the dihalogenated carbamates **1**-**4**[13] and 4-chlorophenyl carbamates **6**[15] was also already published by our group.
The carbamates **5** were synthesised according to the ref.[13] 5-Chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (1 mmol, 315.7 mg) was suspended under vigorous stirring in dry acetonitrile (MeCN; 8 mL), and then triethylamine (1 equivalent, 139.4 μL) was added in one portion. The mixture was stirred to allow complete dissolution of the salicylanilide. Then, appropriate isocyanate (1.1 of equivalents) was added when divided into two portions, second portion after 15 minutes, and the mixture was stirred at room temperature for additional 2 h (cyclohexyl, phenyl, benzyl and phenethyl isocyanates) or it was refluxed for 8 h (adamantan-1-yl isocyanate). After this time, the resulting precipitate was collected by filtration, washed with a small volume of cold methanol and dried. The products were recrystallized from ethyl acetate.

4-Chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl cyclohexylcarbamate (**4a**)
White solid; yield 94%; mp 215-217 °C. IR (ATR): 3261 (N-H), 2930, 2857, 1710 (C=O carbamate), 1659 (amide I), 1602, 1552, 1529 (amide II), 1479, 1410, 1395, 1318, 1270, 1222, 1178, 1166, 1116, 1106, 1065, 1017, 883, 836, 743, 728, 699, 666 cm^{-1}. ^1H NMR (500 MHz, DMSO): δ 10.63 (1H, bs, amide NH), 7.95-7.87 (3H, m, carbamate NH, H2´, H6´), 7.77-7.66 (3H, m, H3, H3´, H5´), 7.58 (1H, dd, *J* = 8.7 Hz, *J* = 2.7 Hz, H5), 7.25 (1H, d, *J* = 8.7 Hz, H6), 3.24-3.14 (1H, m, CH), 1.74-1.45 (5H, m, cyclohexyl), 1.28-0.97 (5H, m, cyclohexyl). ^{13}C NMR (126 MHz, DMSO): δ 165.28, 152.87, 147.49, 142.81, 131.84, 131.21, 129.07, 128.81, 126.16 (q, *J* = 3.8 Hz), 125.34, 124.04 (q, *J* = 271.7 Hz), 123.86 (q, *J* = 32.8 Hz), 120.53, 119.75, 50.06, 32.42, 24.84, 24.61. Anal. Calcd. for $C_{21}H_{20}ClF_3N_2O_3$ (440.84): C, 57.21; H, 4.57; N, 6.35. Found: C, 57.13; H, 4.80; N, 6.27.

4-Chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl phenylcarbamate (**4b**)
White solid; yield 70%; mp 202.5-205 °C. IR (ATR): 3313 (N-H), 3281 (N-H), 1722 (C=O carbamate), 1663 (amide I), 1602, 1536 (amide II), 1511, 1478, 1446, 1405, 1330, 1264, 1224, 1207, 1184, 1160, 1116, 1102, 1066, 1015, 840, 833, 763, 745, 708, 691, 664 cm^{-1}. ^1H NMR (500 MHz, DMSO): δ 10.82 (1H, bs, amide NH), 10.28 (1H, bs, carbamate NH), 7.89 (2H, d, *J* = 8.5 Hz, H2´, H6´), 7.80 (1H, d, *J* = 2.6 Hz, H3), 7.72-7.63 (3H, m, H5, H3´, H5´), 7.47-7.39 (3H, m, H6, H2´´, H6´´), 7.27 (2H, t, *J* = 7.9 Hz, H3´´, H5´´), 7.01 (1H, t, *J* = 7.3 Hz, H4´´). ^{13}C NMR (126 MHz, DMSO): δ 165.28, 163.61, 151.16, 146.99, 139.91, 133.24, 131.49, 129.74, 129.02, 128.84, 128.77, 126.20 (q, *J* = 3.7 Hz), 124.02 (d, *J* = 32.0 Hz), 124.10 (q, *J* = 271.4 Hz), 121.99, 120.60, 118.37. Anal. Calcd. for $C_{21}H_{14}ClF_3N_2O_3$ (434.80): C, 58.01; H, 3.25; N, 6.44. Found: C, 58.12; H, 3.14; N, 6.39.

4-Chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl benzylcarbamate (**4c**)

White solid; yield 72%; mp 188.5-190 °C. IR (ATR): 3321 (N-H), 3032, 2926, 2874, 1713 (C=O carbamate), 1665 (amide I), 1626, 1610, 1573, 1541, 1525 (amide II), 1474, 1454, 1338, 1327, 1257, 1219, 1115, 1070, 1020, 837, 752, 730, 697, 659 cm^{-1}. ^{1}H NMR (500 MHz, DMSO): δ 10.74 (1H, bs, amide NH), 8.37 (1H, t, *J* = 6.2 Hz, carbamate NH), 7.92 (2H, d, *J* = 8.5 Hz, H2´, H6´), 7.76-7.69 (3H, m, H3, H3´, H5´), 7.61 (1H, dd, *J* = 8.7 Hz, *J* = 2.7 Hz, H5), 7.31 (1H, d, *J* = 8.7 Hz, H6), 7.25-7.15 (5H, m, H2´´, H3´´, H4´´, H5´´, H6´´), 4.21 (2H, d, *J* = 6.2 Hz, N-CH_2). ^{13}C NMR (126 MHz, DMSO): δ 163.67, 154.15, 147.37, 142.77, 139.14, 131.91, 131.28, 129.34, 128.59, 128.28, 126.95, 126.88, 126.14 (q, *J* = 3.9 Hz), 125.49, 124.56 (q, *J* = 271.2 Hz), 123.89 (q, *J* = 32.0 Hz), 119.86, 44.04. Anal. Calcd. for $C_{22}H_{16}ClF_3N_2O_3$ (448.82): C, 58.87; H, 3.59; N, 6.24. Found: C, 58.95; H, 3.47; N, 6.30.

4-Chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl phenethylcarbamate (**4d**)
White solid; yield 90%; mp 160.5-162 °C. IR (ATR): 3336 (N-H), 3280 (N-H), 2929, 1717 (C=O carbamate), 1662 (amide I), 1603, 1573, 1537, 1524 (amide II), 1513, 1476, 1409, 1327, 1265, 1215, 1165, 1123, 1067, 1021, 926, 836, 748, 738, 700, 662 cm^{-1}. ^{1}H NMR (500 MHz, DMSO): δ 10.70 (1H, bs, amide NH), 7.96-7.90 (3H, m, NH carbamate, H2´, H6´), 7.73-7.68 (3H, m, H3, H3´, H5´), 7.59 (1H, dd, *J* = 8.7 Hz, *J* = 2.7 Hz, H5), 7.27-7.10 (6H, m, H6, H2´´, H3´´, H4´´, H5´´, H6´´), 3.20 (2H, dt, *J* = 7.9 Hz, *J* = 6.2 Hz, N-CH_2), 2.66 (2H, t, *J* = 7.4 Hz, CH_2-Ph). ^{13}C NMR (126 MHz, DMSO): δ 163.65, 153.66, 147.30, 142.77, 139.26, 131.80, 131.15, 129.17, 128.77, 128.51, 128.40, 126.21, 126.15 (q, *J* = 3.6 Hz), 125.42, 124.53 (q, *J* = 271.2 Hz), 123.91 (q, *J* = 32.0 Hz), 119.83, 42.23, 35.23. Anal. Calcd. for $C_{23}H_{18}ClF_3N_2O_3$ (462.85): C, 59.68; H, 3.92; N, 5.68. Found: C, 59.90; H, 3.87; N, 5.77.

4-Chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl adamantan-1-ylcarbamate (**4e**)
White solid; yield 71%; mp 242-244.5 °C (decomp.). IR (ATR): 3303 (N-H), 2911, 2853, 1717 (C=O carbamate), 1668 (amide I), 1603, 1540, 1522 (amide II), 1508, 1475, 1457, 1381, 1356, 1343, 1319, 1214, 1188, 1163, 1107, 1065, 1017, 923, 854, 837, 750, 702, 665 cm^{-1}. ^{1}H NMR (500 MHz, THF): δ 10.40 (1H, bs, NH), 7.99 (1H, d, *J* = 2.7 Hz, H3), 7.94 (2H, d, *J* = 8.5 Hz, H2´, H6´), 7.69-7.65 (3H, m, NH carbamate, H2´, H6´), 7.40 (1H, dd, *J* = 8.7 Hz, *J* = 2.6 Hz, H5), 6.95 (1H, d, *J* = 8.9 Hz, H6), 1.91-1.89 (3H, m, CH), 1.74 (6H, s, C-CH_2), 1.70-1.63 (6H, m, CH-C$\underline{H_2}$). ^{13}C NMR (126 MHz, DMSO): δ 165.26, 152.56, 147.05, 142.64, 131.81, 131.15, 129.01, 128.83, 126.23 (q, *J* = 3.8 Hz), 125.37, 124.48 (q, *J* = 271.4 Hz), 123.92 (q, *J* = 32.4 Hz), 120.49, 119.64, 50.43, 41.00, 36.05, 28.92. Anal. Calcd. for $C_{25}H_{24}ClF_3N_2O_3$ (492.92): C, 60.92; H, 4.91; N, 5.68. Found: C, 61.01; H, 4.98; N, 5.33.

4.2 Biological activity

4.2.1 Antimycobacterial activity evaluation

4.2.1.1 Activity against *M. tuberculosis* $H_{37}Rv$, *M. avium* and *M. kansasii*

The *in vitro* antimycobacterial activity of the carbamates **1**-**6** against *Mycobacterium tuberculosis* 331/88 ($H_{37}Rv$) and three strains of nontuberculous mycobacteria (*Mycobacterium avium* 330/88 [resistant to isoniazid, rifamycines, ofloxacin, and ethambutol], *Mycobacterium kansasii* 235/80 from the Czech National Collection of Type Cultures, and a clinical isolate of *M. kansasii* 6509/96) was evaluated using a previously reported method.[23] The micromethod for the determination of MIC was used involving the Šula's semisynthetic medium (SEVAC, Prague, Czech Republic). The investigated compounds were added to the medium as solutions in DMSO; the final volume contained 1.0 % DMSO (v/v). The following concentrations were used: 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, 1 and 0.5 μM. The MIC were determined after incubation at 37 °C for 14 and 21 days, for *M. kansasii* additionally for 7 days. MIC (in μM) was the lowest concentration at which the complete inhibition of mycobacterial growth occurred. Two oral antitubercular drugs, isoniazid (INH) and *p*-aminosalicylic acid (PAS), were involved as reference drugs.

The most active carbamates **4** (**4a**, **4b**, **4d**, **4e**) were evaluated against seven drug-resistant TB strains (dilution of the strains was 10^{-3}) with different resistance patterns. All of the strains are

resistant to INH, RIF, rifabutin, and streptomycin, and resistance to other drugs was observed in some cases: strain 7357/1998 was resistant additionally to ethambutol and ofloxacin, strain 234/2005 to ethambutol, strain 8666/2010 resistant to ethambutol, ofloxacin and clofazimine, strain Praha 1 exhibited an additional resistance to ethambutol and clofazimine; Praha 4 to ethambutol, ofloxacin and clofazimine (all MDR-TB strains); and Praha 131 was resistant to INH, rifamycines, streptomycin, ethambutol, ofloxacin, gentamicin and amikacin (i.e., XDR-TB strain). The following concentrations were used for drug-resistant strains: 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.064, and 0.032 μM.

4.2.1.2 Activity against *M. tuberculosis* H_{37}Ra, *M. aurum* and *M. smegmatis*

Antimycobacterial assay was performed with fast growing *Mycobacterium smegmatis* DSM 43465 (ATCC 607), *Mycobacterium aurum* DSM 43999 (ATCC 23366) from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and with avirulent strain of *Mycobacterium tuberculosis* H_{37}Ra ITM-M006710 (ATCC 9431) from Belgian Co-ordinated Collections of Micro-organisms. The technique used for activity determination was microdilution broth panel method using 96-well microtitration plates. Culturing medium was Middlebrook 7H9 broth (Sigma-Aldrich, Steinheim, Germany) enriched with 0.4% of glycerol (Sigma-Aldrich, Steinheim, Germany) and 10% of Middlebrook OADC growth supplement (Himedia, Mumbai, India).

Mycobacterial strains were cultured on Middlebrook 7H9 agar and suspensions were prepared in Middlebrook 7H9 broth. Final density was adjusted to value 1.0 according to McFarland scale and diluted in ratio 1:20 (for fast growing mycobacteria) or 1:10 (for *M. tuberculosis*) with broth.

Tested compounds were dissolved in DMSO (Sigma-Aldrich, Steinheim, Germany) then MB broth was added to obtain concentration 2000 μg/mL. Standards used for activity determination were isoniazid (INH), rifampicin (RIF) and ethambutol (EMB) (Sigma-Aldrich, Steinheim, Germany). Final concentrations were reached by binary dilution and addition of mycobacterial suspension and were set at 500, 250, 125, 62.5, 31.25, 15.625, 7.81 and 3.91 μg/mL or 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 μg/mL according to final MIC. Isoniazid was diluted in range 500-3.91 μg/mL for screening against fast growing mycobacteria and in range 1-0.0078 μg/mL for screening against *M. tuberculosis*. Rifampicin final concentrations ranged from 50 to 0.39 μg/mL for fast growing mycobacteria and from 1 to 0.0039 μg/mL for *M. tuberculosis*. Ethambutol was used for screening antimycobacterial activity with the final concentrations 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156 μg/mL. The final concentration of DMSO did not exceeded 2.5% (v/v) and did not affect the growth of *M. smegmatis*, *M. aurum* nor *M. tuberculosis*. Positive (broth, DMSO, bacteria) and negative (broth, DMSO) controls were included.

Plates were sealed with polyester adhesive film and incubated in dark at 37 °C without agitation. The addition of 0.01% solution of resazurin sodium salt followed after 48 hours of incubation for *M. smegmatis*, 72 hours of incubation for *M. aurum* and after 120 hours of incubation for *M. tuberculosis*, respectively. Stain was prepared by dissolving resazurin sodium salt (Sigma-Aldrich, Steinheim, Germany) in deionised water to get 0.02% solution. Then 10% aqueous solution of Tween 80 (Sigma-Aldrich, Steinheim, Germany) was prepared. Both liquids were mixed up making use of the same volumes and filtered through syringe membrane filter. Microtitration panels were then incubated for further 2.5 hours for determination of activity against *M. smegmatis*, 4 hours for *M. aurum* and 24 hours for *M. tuberculosis*, respectively.

Antimycobacterial activity was expressed as minimal inhibition concentration (MIC) and the value was read on the basis of stain colour change (blue colour – active compound; pink colour – not active compound). MIC values for standards were 15.625 μg/mL for INH, 12.5-25 μg/mL for RIF and 0.5 μg/mL for EMB against *M. smegmatis*, and 3.91 μg/mL for INH, 0.39 μg/mL for RIF and 0.25 μg/mL for EMB against *M. aurum*, and 0.125-0.25 μg/mL for INH, 0.0039-0.0078 μg/mL for RIF and 0.5 μg/mL for EMB against *M. tuberculosis*, respectively. All experiments were conducted in duplicate.

4.2.2 Isocitrate lyase inhibition determination

The isocitrate lyase (ICL1) inhibition activity was assayed according to Dixon and Kornberg procedure (glyoxylate phenyl hydrazone formation) using a single concentration of the carbamates **1**-**6** of 10 μM.[23] Results are expressed as inhibition rates [%] and the data represent an average of triplicate experiments ± standard deviation (SD). Isoniazid and *p*-aminosalicylic acid were employed as negative controls (inhibition of 0%), while 3-nitropropionic acid served as a positive control.

4.2.3 Cytostatic and cytotoxic activity determination

HepG2, human hepatocellular carcinoma cells (ATCC HB-8065) were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 160 μg/mL gentamicin at 37 °C, 5% CO_2 in water-saturated atmosphere and grown to confluence and were plated into 96-well plate with initial cell number of 5.0-10.0 × 10^3 per well. After 24 h incubation at 37 °C, cells were treated with the compounds in 100 μL final volume containing 2.0 % DMSO (v/v). Cells were incubated with the compounds at 0.0032-250 μM concentration range for overnight. Control cells were treated with serum free medium (RPMI-1640) only or with DMSO (c = 1.0 v/v %) at 37 °C for overnight. After incubation, the cells were washed twice with serum free medium (RPMI-1640). To determine the *in vitro* cytostatic effect, cells were cultured for a further 72 h in medium containing serum. To measure the *in vitro* cytotoxicity of the compounds, MTT-assay was carried out immediately after the washing step following the overnight treatment.

The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The solution of MTT (45 μL, 2 mg/mL) was added to each well, which was reduced to insoluble violet formazan dye crystals within the living cells. After 3.5 h of incubation at 37 °C the cells were centrifuged for 5 min (2000 rpm) and supernatant was removed. The obtained formazan crystals were dissolved in 100 μL of DMSO and the optical density (OD) of the samples was measured at λ = 540 and 620 nm, respectively, employing ELISA Reader instrument (iEMS Reader, Labsystems, Finland). OD_{620} values were subtracted from OD_{540} values and the percent of cytostasis or cytotoxicity was calculated using equation "Cytostatic effect/Cytotoxicity (%) = [1 − ($OD_{treated}$/$OD_{control}$)] × 100" (where $OD_{treated}$ and $OD_{control}$ correspond to the optical densities of the treated and the control cells, respectively). For each compound, at least two independent experiments were carried out with four parallel measurements.

The 50% inhibitory concentration (IC_{50}) values were determined from the dose-response curves. The curves were defined using MicrocalTM Origin1 (version 7.6) software: cytostasis (%) or cytotoxicity (%) was plotted as a function of concentration, fitted to a sigmoidal curve and, based on this curve, the half maximal inhibitory concentration (IC_{50}) value was determined representing the concentration of a compound required for 50% inhibition *in vitro*.

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Conflict of Interest

The authors declare no conflict of interest.

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Highlights

- Salicylanilide *N*-monosubstituted carbamates are highly active against mycobacteria.
- MIC against multidrug-resistant mycobacteria are from ≤0.5 μM.
- Salicylanilide carbamates are mild inhibitors of mycobacterial isocitrate lyase.
- The majority of carbamates are non-cytotoxic and selective for mycobacteria.