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PII: S0960-894X(17)31044-2
DOI: https://doi.org/10.1016/j.bmcl.2017.10.050
Reference: BMCL 25379

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 30 August 2017
Revised Date: 17 October 2017
Accepted Date: 20 October 2017

Please cite this article as: Krátký, M., Bősze, S., Baranyai, Z., Stolař íková, J., Vinšová, J., Synthesis and biological evolution of hydrazones derived from 4-(trifluoromethyl)benzohydrazide, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: https://doi.org/10.1016/j.bmcl.2017.10.050

Synthesis and biological evolution of hydrazones derived from 4-(trifluoromethyl)benzohydrazide

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Abstract

Reflecting the known biological activity of isoniazid-based hydrazones, seventeen hydrazones of 4-(trifluoromethyl)benzohydrazide as their bioisosters were synthesized from various benzaldehydes and aliphatic ketones. The compounds were screened for their *in vitro* activity against *Mycobacterium tuberculosis*, nontuberculous mycobacteria (*M. avium*, *M. kansasii*), bacterial and fungal strains. The most antimicrobial potent derivatives were also investigated for their cytostatic and cytotoxic properties against three cell lines. Camphor-based molecule, 4-(trifluoromethyl)-*N'*-(1,7,7-trimethylbicyclo[2.2.1]heptan-2-ylidene)benzohydrazide, exhibited the highest and selective inhibition of *M. tuberculosis* with the minimum inhibitory concentration (MIC) of 4 μM, while *N'*-(4-chlorobenzylidene)-4-(trifluoromethyl)benzohydrazide was found to be superior against *M. kansasii* (MIC = 16 μM). *N'*-(5-Chloro-2-hydroxybenzylidene)-4-(trifluoromethyl)benzohydrazide showed the lowest MIC values for gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* as well as against two fungal strains of *Candida glabrata* and *Trichophyton mentagrophytes* within the range of ≤0.49-3.9 μM. The convenient substitution of benzylidene moiety at the position 4 or the presence of 5-chloro-2-hydroxybenzylidene scaffold concomitantly with a sufficient lipophilicity are essential for the noticeable antimicrobial activity. This 5-chlorosalicylidene derivative avoided any cytotoxicity on two mammalian cell cultures (HepG2, BMM*Φ*) up to the concentration of 100 μM, but it affected the growth of MonoMac6 cells.

Keywords

Antibacterial activity; antifungal activity; cytostasis; cytotoxicity; hydrazone; *Mycobacterium tuberculosis*; nontuberculous mycobacteria; 4-(trifluoromethyl)benzohydrazide[*]

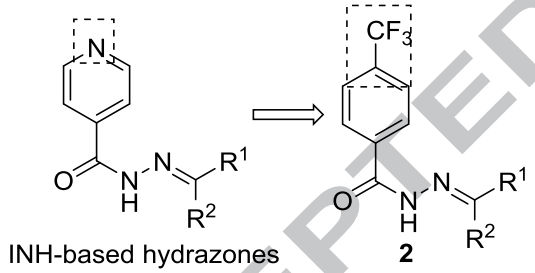
[*]Abbreviations: BAC: bacitracin; BMM*Φ*: murine bone marrow culture-derived macrophages; FLU: fluconazole; INH: isoniazid; MRSA: methicillin-resistant *Staphylococcus aureus*; *Mtb.*: *Mycobacterium tuberculosis*; NTM: non-tuberculous (atypical) mycobacteria.

Development of novel antimicrobial agents represents an up-to-date research topic to achieve a global control of drug-resistant microbial strains. Modification of compounds with a known activity to improve their properties belongs to the powerful trends in medicinal chemistry.

The concept of (bio)isosters could be considered as one of the successful approaches for the rational drug design. It is based on the assumption that single atoms, groups or molecules that exhibit similar volume, shape, and/or physicochemical properties can produce broadly similar pharmacologic effects. However, the outcomes of isosteric replacement cannot be predicted absolutely, since conversion of the action was also observed occasionally. In the molecules of bioisosters, a different substituent or a group exchanges one moiety from the parent lead compound to impart similar biological effects. This change can modify both pharmacokinetics and pharmacodynamics to fine tune of them. Examples of broadly similar isosteric substitution comprise, e.g., replacement of hydrogen by fluorine, ester bond by amide, phenyl ring by thiophene, carboxylic acid by tetrazole etc., that are based on chemical and/or physical similarities.[1,2]

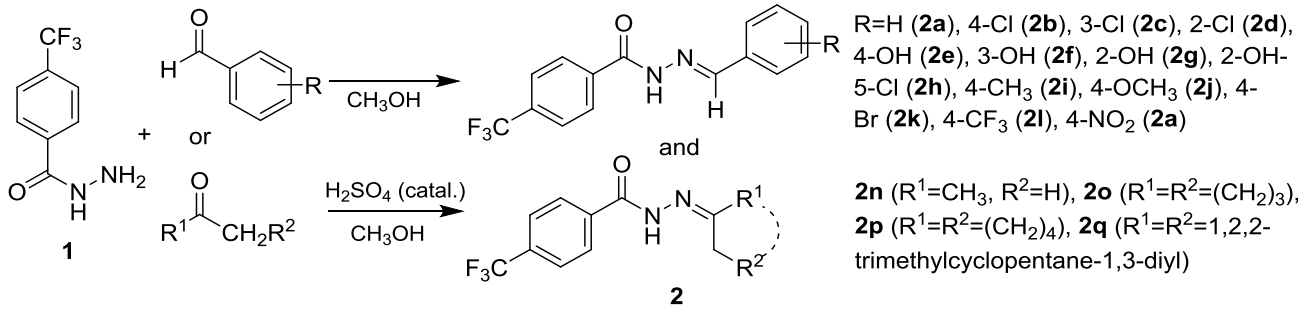
Hydrazones obtained from isoniazid (INH), a first-line antimycobacterial agent, have been reported as potent antimycobacterial,[3,4,5,6,7,8] antibacterial[5,7,9] and antifungal[5,7,9] agents. An analogue of INH where heterocyclic nitrogen is replaced by a carbon substituted with a strong electron-withdrawing group, 4-(trifluoromethyl)benzohydrazide **1** inhibits the growth of *Mycobacterium tuberculosis*[10] (*Mtb.*). Similarly, 4-(trifluoromethyl)benzohydrazide-derived INH isosters preserved antimycobacterial properties.[11,12] Illustratively, hydrazones of **1** with various aldehydes and ketones have exhibited antimycobacterial,[10,11] antibacterial,[13,14] antifungal,[14] antiparasitic[15] and cytotoxic[16,17] properties.

Based on here summarized facts, we designed and synthesized 4-(trifluoromethyl)benzohydrazide hydrazones **2**, bioisosters of INH hydrazones (Scheme 1), as potential antimicrobial agents.



Scheme 1. Bioisosteric design of 4-(trifluoromethyl)benzohydrazide hydrazones **2**

The synthesis of *N'*-alkyl/cycloalkyl/benzylidene-4-(trifluoromethyl)benzohydrazides **2** is depicted in Scheme 2. It involves the reaction of the hydrazide **1** (1.0 equivalent) with commercially available aldehydes and ketones (1.1 of eq.). If any ketone (acetone, cyclopentanone, cyclohexanone, camphor) was one of the reactants, a catalytic amount of concentrated sulphuric acid was added into the reaction mixture. The refluxing in boiling methanol for 2 hours provided targeted compounds **2** in 85-99% yields (aldehydes) and 68-87% for ketones (Scheme 2). The lowest yield was observed when camphor was used as a carbonyl compound.



R=H (**2a**), 4-Cl (**2b**), 3-Cl (**2c**), 2-Cl (**2d**), 4-OH (**2e**), 3-OH (**2f**), 2-OH (**2g**), 2-OH-5-Cl (**2h**), 4-CH_3 (**2i**), 4-OCH_3 (**2j**), 4-Br (**2k**), 4-CF_3 (**2l**), 4-NO_2 (**2a**)

2n (R^1=CH_3, R^2=H), **2o** (R^1=R^2=$(CH_2)_3$), **2p** (R^1=R^2=$(CH_2)_4$), **2q** (R^1=R^2=1,2,2-trimethylcyclopentane-1,3-diyl)

Scheme 2. Synthesis of *N´*-substituted 4-(trifluoromethyl)benzohydrazides **2**.

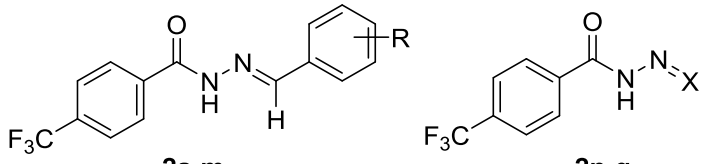
We chose substituents with different electronic effects as a substitution pattern for benzaldehyde ring: no substituent (R = H), those with electron-donating properties – methyl (+I effect), hydroxy and methoxy groups (-I and +M effects), as well as strong electron-withdrawing groups of NO_2 (-I and -M effects) and CF_3 (-I effect). Halogens (Cl, Br) as weak deactivating substituents with -I and +M electronic effects were involved too. Based on the known antimicrobial activity of 5-chloro-2-hydroxybenzylidene derivatives,[18,19] 5-chlorosalicylaldehyde was used. We also investigated various positional isomers (*ortho*, *meta*, *para*; R = Cl, OH) and the activity of hydrazones obtained from four ketones. In spite of different electronic effects, these derivatives differ also in lipophilic behaviour and steric parameters.

All of the compounds **2** were characterized by spectroscopic data involving ^{1}H, ^{13}C and IR spectra (see Supplementary information), and the purity was checked additionally by TLC and elemental analysis. In the ^{1}H NMR spectra, hydrazone (CONHN=) protons appeared as singlets at 12.31-11.83 ppm for benzylidene scaffold-based compounds **2a-m** concomitantly with azomethine singlets (N=CH) observed at 8.88-8.36 ppm. Ketone-derived hydrazones **2n-q** share CONH proton signals shifted to upfield within the range of 10.84-10.41 ppm. The ^{13}C NMR spectra contain C=O and C=N peaks at 162.43-161.83 ppm and 149.14-144.65 ppm, respectively. In the spectra of ketone-based hydrazones **2n-q**, the C=N singlet is shifted to 174.92-161.53 ppm.

The hydrazide **1** and all of the hydrazones **2a-q** were screened *in vitro* for their antimicrobial properties (see Supplementary information). The panel of pathogens involved *Mycobacterium tuberculosis* 331/88 (*i.e.*, $H_{37}Rv$), *Mycobacterium avium* 330/88 (resistant to INH, rifamycines, ethambutol and ofloxacin), *Mycobacterium kansasii* 235/80 and 6509/96 (a clinical isolate; Table 1); gram-positive bacteria: *Staphylococcus aureus* CCM 4516/08, methicillin-resistant *Staphylococcus aureus* H 5996/08 (MRSA), *Staphylococcus epidermidis* H 6966/08, *Enterococcus faecalis* J 14365/08; *Escherichia coli* CCM 4517, *Klebsiella pneumoniae* D 11750/08, extended spectrum beta-lactamase (ESBL)-positive *Klebsiella pneumoniae* J 14368/08, and *Pseudomonas aeruginosa* CCM 1961 (gram-negative strains),[20] and fungal species of *Candida albicans* ATCC 44859, *Candida tropicalis* 156, *Candida krusei* E28, *Candida glabrata* 20/I, *Trichosporon asahii* 1188, *Aspergillus fumigatus* 231, *Absidia corymbifera* 272, and *Trichophyton mentagrophytes* 445 (Table 2).[21] This panel of twenty microbial species covers a wide range of important human pathogens including those with an acquired resistance. It is a useful tool for an initial identification of potential antimicrobial activity of novel compounds. Bacitracin (BAC), fluconazole (FLU) and isoniazid were employed as the comparative drugs for antibacterial, antifungal and antimycobacterial activity, respectively.

The results from antimycobacterial evaluation expressed as minimum inhibitory concentrations (MICs) are overviewed in Table 1; only active molecules are involved.

Table 1. Antimycobacterial activity of hydrazones **2**



2a-m **2n-q**

Code	**R** or **X**	MIC [μM]										Clog*P*
		Mtb. 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96			
		14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d	
2b	4-Cl	**16**	**16**	>125	>125	**16**	**16**	**16**	**16**	**16**	**16**	4.84

2c	3-Cl	>125	>125	>125	>125	125	>125	>125	>125	>125	>125	4.84
2e	4-OH	125	125	250	500	62.5	125	125	250	500	500	3.9
2f	3-OH	250	250	250	250	62.5	125	250	250	500	500	3.9
2g	2-OH	125	250	**125**	250	250	250	250	250	250	250	3.9
2h	2-OH-5-Cl	62.5	62.5	**62.5**	**125**	62.5	125	125	**62.5**	125	125	4.45
2m	4-NO_2	125	>250	>250	>250	**32**	62.5	62.5	250	>250	>250	3.49
2n	propan-2-ylidene	>125	>125	>125	>125	125	>125	>125	>125	>125	>125	2.71
2o	cyclopentylidene	500	500	>125	>125	500	>1000	>1000	500	1000	1000	3.52
2p	cyclohexylidene	250	500	>125	>125	500	>1000	>1000	500	1000	1000	3.93
2q	1,7,7-trimethyl-bicyclo[2.2.1]heptan-2-ylidene	**4**	**4**	>250	>250	>250	>250	>250	>250	>250	>250	5.42
	BH (1)	250	500	>500	>500	>250	>250	>250	>250	>250	>250	1.61
	INH	0.5	1	>250	>250	>250	>250	>250	8	8	8	-

INH: isoniazid; BH: 4-(trifluoromethyl)benzohydrazide **1**; *Mtb.*: *Mycobacterium tuberculosis*. The lipophilicity (Clog*P*) was computed using the program CS ChemOffice Ultra version 16.0.
One or two of the best MIC value(s) for each strain are shown in bold.

Eleven hydrazones (**2b-c**, **2e-h**, **2m-q**) exhibited an antimycobacterial activity, remaining derivatives (**2a**, **2d**, **2i-l**) were inactive. Hydrazide **1** itself showed only a mild activity against *Mtb.* (250/500 μM). *M. avium* inhibited only by phenolic derivatives was the most resistant strain (MICs ≥ 62.5 μM). The lowest MIC values for *Mtb.* were produced by terpenic hydrazone obtained from camphor **2q** (4 μM concomitantly with an evident selectivity for this strain) followed by 4-chlorobenzylidene derivative **2a**. This molecule was also found to be the most active against both strains of *M. kansasii* (16 μM). *N'*-(4-Nitrobenzylidene)-4-(trifluoromethyl)benzohydrazide **2m** inhibited significantly only the growth of the collection strain of *M. kansasii* 235/80 with MIC values starting from 32 μM.

In sum, for the antimycobacterial activity it is essential the presence of one chlorine atom at the position of 4 (**2b**) since its structural isomers are virtually (**2c**) or totally inactive (**2d**). The phenolic group represents another favourable substituent preferably at the position 4 again (**2e**), but in this case isomeric hydroxybenzaldehydes provided derivatives (**2e-g**) with a similar biological response. The halogenation of salicylaldehyde improves antimycobacterial properties up to four times (**2g** vs. **2h**). Generally, an increased lipophilicity is translated into an enhanced antimycobacterial action (**2q**, **2b** vs. **2e** and **2m**, **2g** vs. **2h**). On the other hand, the most lipophilic benzaldehyde-based hydrazones (i.e., 4-Br **2k** and 4-CF_3 **2l** with C log*P* >5) did not display any growth inhibition. Although essential, the lipophilicity is not the only factor determining this property and similarly, the occupation of the 4-position does not ensure bioactivity automatically.

The chemical modification of the parent hydrazide **1** by carbonyl compounds led mainly to more potent antimycobacterial agents. Four derivatives (**2e-h**) exceeded the *in vitro* activity of INH for *M. avium* and *M. kansasii* 235/80, additional four hydrazones (**2b-c**, **2m-n**) produced lower MIC value(s) against the last strain demonstrably. *N'*-(4-Chlorobenzylidene)-4-(trifluoromethyl)benzohydrazide **2b** was comparable to INH against the clinical isolate of *M. kansasii* 6509/96 (± one dilution). Our study did not confirm previous finding about the activity of camphor-based hydrazones against NTM.[22]

The results of antibacterial and antifungal evaluation are reported in Table 2. Unfortunately, two compounds (**2k**, **2q**) are not sufficiently soluble in the media for both antibacterial and antifungal testing (Mueller-Hinton broth, RPMI-1640), thus making the MIC values determination unreliable.

The majority of the hydrazones **2** as well as the parent hydrazide **1** avoided any growth inhibition of eight bacterial strains investigated. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* showed a complete resistance, *E. coli* was the only one gram-negative species susceptible to **2** (salicylaldehyde-based hydrazones **2g-h**, 250-500 μM). The MIC values of 5-chloro-2-

hydroxybenzylidene derivative **2h** against four gram-positive strains (≤3.9 μM) qualify this molecule for further investigation. Importantly, the MIC values of **2h** were uniformly clearly superior to BAC, an established antibiotic drug used for the topical treatment of gram-positive infection. The hydrazone **2b** obtained from 4-chlorobenzaldehyde exhibited MICs starting from 31.25 μM that was comparable to BAC in the case of *Staphylococcus epidermidis*. Interestingly, there is no cross-resistance to methicillin as could be observed from the comparison of identical MIC values against a drug-susceptible *Staphylococcus aureus* and a methicillin-resistant (MRSA) strain. The antibacterial activity of remaining compounds reported in the Table 2 (**2e**, **2g**, and **2n**) is rather detrimental (≥125 μM). The structure-activity relationships are similar to those obtained for the action against mycobacteria: the presence of chlorine atom (**2b**) or hydroxyl group (**2e** vs. **2g**) at the position 4 of the benzylidene moiety as well as chlorination of salicylic ring (**2g** vs. **2h**) modulate antibacterial properties positively.

Three positive hits from antibacterial screening, **2b**, **2g**, and **2h**, produced also an inhibition of four fungal strains: *Candida tropicalis*, *Candida krusei*, *Candida glabrata* and a filamentous fungus, *Trichophyton mentagrophytes*. Four species showed a complete resistance to all of the hydrazones **2** (*Candida albicans*, *Trichosporon asahii*, *Aspergillus fumigatus*, *Absidia corymbifera*). *N'*-(4-Chlorobenzylidene)-4-(trifluoromethyl)benzohydrazide **2b** exhibited a mild antifungal activity with MICs within the range of 62.5-125 μM. Salicylidene derivatives **2g**-**h** were able to affect *C. glabrata* and *T. mentagrophytes* at the concentration of ≤0.49-15.62 μM with the superiority of the halogenated counterpart **2h** again (up to 16 times). Almost all of these values are comparatively lower than those obtained for fluconazole, a clinically used antimycotic drug. Especially the inhibition of *C. glabrata* at low micromolar or submicromolar concentrations is worth. This pathogen is one of the most common causative agents of invasive candidoses with a high mortality rate of 40-50 % concomitantly with an increasing incidence and drug-resistance.[23]

4-(Trifluoromethyl)benzohydrazide **1** and its hydrazones **2** were also screened for their *in vitro* cytotoxic and cytostatic properties on two human cell cultures: hepatocellular carcinoma cells (HepG2)[24] and on monocytic cell line MonoMac6[25] using MTT-assay. The *in vitro* cytotoxic activity of a representative compound set was also determined on murine bone marrow culture-derived macrophages (BMM*Φ*)[26,27] using MTT assay.

Importantly, none of the derivatives **1**-**2** exhibited cytotoxicity (*i.e.*, direct killing of cells) for HepG2 cells up to the concentration of 100 μM and majority of them were non-cytostatic. The most antimicrobial active hydrazones (**2b**, **2g**-**h**, **2q**) were subjected to additional tests on MonoMac6 and BMM*Φ* cells (Table 3). The HepG2 cells represent an *in vitro* model for the hepatotoxicity, the MonoMac6 cells and BMM*Φ* are models of the macrophages.[28] The selectivity indexes (SI) were defined as a ratio of the MIC and IC_{50} value for cytotoxic action on HepG2 cells. Its values higher than 10 indicate an acceptable toxicity (based on the analogy of the therapeutic index).

The natural pharmacophore-based hydrazone **2q** exhibited neither cytotoxicity nor cytostasis for all three cell lines at a single concentration of 100 μM, thus targeting *Mtb.* selectively (SI>25) without any undesired effects on mammalian cells. 4-Chlorobenzylidene derivative **2b** did not affected both HepG2 and MonoMac6 at 100 μM, but it was cytotoxic on the BMM*Φ* cells (IC_{50} = 7.05 μM). These cells can be also considered as a sensitive macrophage model from normal primary cell culture, therefore a suitable choice to estimate selectivity of promising compounds.[26,27,28,29] Regarding hepatotoxicity, it is the most promising agent against NTM (SI>6.25 μM). In contrast to these two compounds, salicylaldehyde derivatives **2g-h** share a strong cytostatic behaviour (IC_{50} within the range of 1.70-13.55 μM), *i.e.*, they arrest cell growth, multiplication and proliferation. Simultaneously, they were cytotoxic on MonoMac6 model (IC_{50} values for **2g** and **2h** of 2.28 and 5.8 μM, respectively), but not for HepG2 and BMM*Φ* (IC_{50} >100 μM). Interestingly, the

concomitant cytostatic action together with no cytotoxicity was described for salicylanilide derivatives.[30]

Table 2. Antibacterial and antifungal activity of hydrazone derivatives **2**

Code	MIC/IC_{95}/IC_{50} [μM]																	
	SA		MRSA		SE		EF		EC		CT		CK		CG		TM	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	72 h	120 h
2b	62.5	62.5	62.5	62.5	31.25	62.5	62.5	>125	>125	>125	**125**	**125**	**62.5**	**62.5**	>125	>125	125	125
2e	250	250	250	250	125	125	>250	>250	>250	>250	>125	>125	>125	>125	>125	>125	>125	>125
2g	500	>500	500	>500	250	500	250	250	500	500	>125	>125	>125	>125	3.9	15.62	7.81	15.62
2h	**1.98**	**1.98**	**1.98**	**1.98**	**3.9**	**3.9**	**1.98**	**3.9**	**250**	**250**	>125	>125	>125	>125	**≤0.49**	**0.98**	**1.98**	**3.9**
2n	>500	>500	>500	>500	500	>500	250	500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
BH (1)	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
BAC	7.81	15.62	15.62	15.62	15.62	31.25	15.62	62.5	>500	>500	-	-	-	-	-	-	-	-
FLU	-	-	-	-	-	-	-	-	-	-	>500	>500	125	250	31.25	500	7.81	125

BAC: bacitracin; BH: 4-(trifluoromethyl)benzohydrazide **1**; FLU: fluconazole. Bacteria: SA: *Staphylococcus aureus* CCM 4516/08; MRSA: methicillin-resistant *Staphylococcus aureus* H 5996/08; SE: *Staphylococcus epidermidis* H 6966/08; EF: *Enterococcus* sp. J 14365/08; *Escherichia coli* CCM 4517. Fungi: CT: *Candida tropicalis*; CK: *Candida krusei* E28; CG: *Candida glabrata* 20/I; TM: *Trichophyton mentagrophytes* 445.

One or two of the best MIC value(s) for each strain are shown in bold.

In contrast to antimicrobial activity, the halogenation of salicylidene core decreases both cytotoxicity and cytostasis (**2g** vs. **2h**) thus being beneficial in term of the selectivity. *N'*-(5-Chloro-2-hydroxybenzylidene)-4-(trifluoromethyl)benzohydrazide **2h** was identified as the most potent and selective molecule against gram-positive cocci and several fungal strains (SI on HepG2 of >50.5 and >25.64, respectively).

Table 3. Cytostatic and cytotoxic properties of the most potent hydrazones **2**

Code	Cytotoxicity (HepG2) IC_{50} [μM]	Cytostasis (HepG2) IC_{50} [μM]	Cytotoxicity (MonoMac6) IC_{50} [μM]	Cytostasis (MonoMac6) IC_{50} [μM]	Cytotoxicity (BMMΦ) IC_{50} [μM]	SI for myco-bacteria	SI for *S. aureus*	SI for fungi[b]
2b	>100	>100	>100	>100	7.05±0.15	>6.25	>1.6	NA
2g	>100	6.20±1.0	2.28±0.32	1.70±0.20	>100	NA	NA	>6.40
2h	>100	13.55±0.75	5.8±0.10	10.3±2.40	>100	>1.6	**>50.5**	**>25.64**
2q	>100	>100	>100	>100	>100	**>25**[a]	NA	NA

SI = IC_{50} (cytotoxicity for HepG2)/MIC. *a* = only for *Mtb*. b = for *C. glabrata* and *T. mentagrophytes*. NA = not applicable.

In conclusion, seventeen hydrazones were synthesized by the treatment of 4-(trifluoromethyl)benzohydrazide with corresponding substituted benzaldehydes or ketones. These novel compounds were characterised and they underwent biological evaluation in order to determine their antibacterial, antifungal, antimycobacterial, cytotoxic and cytostatic action. Four molecules exhibited an interesting and potentially promising activity profile as potential antimicrobial agents especially against *Mycobacterium tuberculosis*, staphylococci including one MRSA strain, *Candida glabrata* and *Trichophyton mentagrophytes* with an acceptable toxicity and thus selectivity for eukaryotic cells. They represent auspicious hits for further structure optimization.

Acknowledgments

This work was supported by the Czech Science Foundation [grant number 17-27514Y]; and the Hungarian Research Fund [grant number OTKA K-104275].

We would like to thank Ida Dufková for the excellent performance of antibiotic and antimycotic susceptibility tests, the staff of the Department of Organic and Bioorganic Chemistry, Faculty of Pharmacy in Hradec Králové for the technical assistance and J. Urbanová, M.A., for improving the grammar of the manuscript. The authors also thank Dr. Rita Szabó for participating in the isolation and maintenance of murine bone marrow culture-derived cells.

Conflicts of interest: none.

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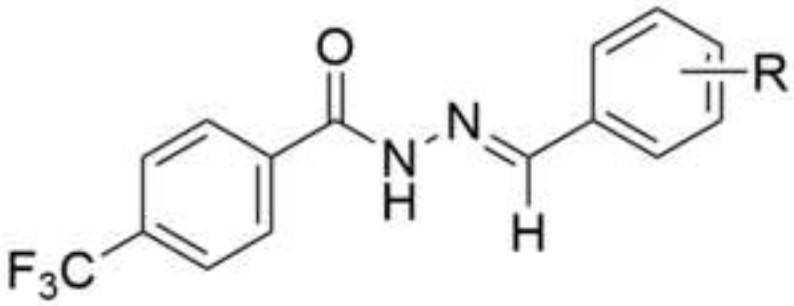
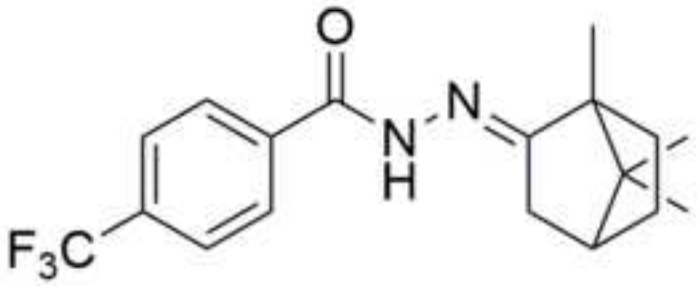


R = 4-Cl, 2-OH, 2-OH-5-Cl

MICs against *Mycobacterium tuberculosis* and nontuberculous mycobacteria from 16 μM
MICs for *Staphylococcus aureus* (including MRSA strain) from 1.98 μM
antifungal activity from 0.49 μM
no cytotoxicity for HepG2 (IC_{50} >100 μM)



selective against *M. tuberculosis*: MIC of 4 μM

Highlights

- Hydrazones of 4-(trifluoromethyl)benzohydrazide, an isoniazid isoster, were obtained.
- These hydrazones were investigated as potential antimicrobial agents.
- Hydrazone of camphor is selective inhibitor of *Mycobacterium tuberculosis* (MIC=4 μM).
- A high activity against gram-positive bacteria including MRSA strain (MIC ≥1.98 μM).
- Antifungal activity from ≤0.49 μM and no cytotoxicity for HepG2 cells.