ARTICLE

Benzaldehyde thiosemicarbazone derivatives against replicating and nonreplicating *Mycobacterium tuberculosis*

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Abstract

In this article, we report a series of benzaldehyde thiosemicarbazone derivatives possessing high activity toward actively replicating *Mycobacterium tuberculosis* strain with minimum inhibitory concentration (MIC) values in the range from 0.14 to 2.2 μM. Among them, two compounds—2-(4-phenethoxybenzylidene)hydrazine-1-carbothioamide (**13**) and 2-(3-isopropoxybenzylidene)hydrazine-1-carbothioamide (**20**) also demonstrate submicromolar antimycobacterial activity against *M. tuberculosis* under hypoxia with MIC values of 0.68 and 0.74 μM, respectively. The activity of compounds **13** and **20** toward five investigated isoniazid-, rifampicin-, and fluoroquinolone-resistant *M. tuberculosis* isolates is similar to commercially available antituberculosis drugs. The compounds **13** and **20** possess good ADME properties and have low cytotoxicity toward human liver cells (HepG2). Therefore, 2-(4-phenethoxybenzylidene)hydrazine-1-carbothioamide (**13**) and 2-(3-isopropoxybenzylidene)hydrazine-1-carbothioamide (**20**) are valuable candidates for further preclinical studies.

Introduction

Tuberculosis (TB) is one of the most serious human diseases and public problems throughout the world. The treatment of TB is impaired by the long duration of therapy, which leads to a high risk of drug resistance development. Resistance to the first-line antituberculosis antibiotics such as rifampicin and isoniazid is called multidrug resistant (MDR) tuberculosis. The resistance to fluoroquinolones, which are key second-line anti-tuberculosis drugs, increases the chances of getting extremely drug resistant (XDR) and totally drug resistant (TDR) tuberculosis. Therefore, there is an urgent need for new drugs with improved anti-tuberculosis efficiency. Unfortunately, the development of novel antituberculosis agents has been very slow. In 2012, the novel antibiotic bedaquiline was approved by the U.S.

Food and Drug Administration (FDA) for MDR tuberculosis treatment. This antibiotic was the first new medicine to fight tuberculosis in more than 40 years. Recently, the cases of bedaquiline resistance also were reported [1–5]. This fact highlights the need to keep pursuing the research on novel antituberculosis drugs able to kill resistant isolates of *Mycobacterium tuberculosis*.

Concerning antituberculosis drug discovery, the phenotypic high-throughput screening has demonstrated to be a more efficient method to produce lead candidates in comparison to the target-based approaches. For example, bedaquiline was discovered using whole cell phenotypic assay.

Using phenotypic screening assay, we have identified benzaldehyde thiosemicarbazone derivatives with antituberculosis activity. Thiosemicarbazone derivatives have been already reported in the literature as antibacterial, antifungal, and anti-tubercular agents [6–9].

The molecular mechanism of action for thiosemicarbazone derivatives is still not clearly understood. There are some indications concerning the mechanism of action for antituberculosis drug from this chemical class—thiacetazone. It has been shown that thiacetazone is a prodrug that requires activation by the mycobacterial monooxygenase EthA. The activated thiacetazone affects mycolic acid synthesis, probably by inhibiting certain

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cyclopropane mycolic acid synthases [10, 11]. Moreover, it is known that thiosemicarbazone group chelates metal cations strongly and the antitubercular action of thiacetazone is enhanced by copper [12]. Therefore, antituberculosis action of thiosemicarbazone derivatives may be due to complex action.

Taking into account the biological potential of thiosemicarbazone derivatives, we have investigated a series of benzaldehyde thiosemicarbazones for their activity toward pathogenic *M. tuberculosis* strain H37Rv and isoniazid, rifampicin, and fluoroquinolone resistant isolates.

Materials and methods

Chemical synthesis

Thiosemicarbazide (1 mmol) was dissolved in 20 ml of ethanol under heating and appropriate aldehyde (1 mmol) was added. The resulting mixture was heated to solid formation. Solid products were filtered, washed with ethanol (2 × 5 ml), and dried. The compounds have been already patented by us [13].

Antibacterial activity under aerobic conditions

The antimicrobial effect of compounds against *M. tuberculosis* H37Rv grown under aerobic conditions was assessed by determining the minimum inhibitory concentration (MIC). The assay is based on the measurement of growth in liquid medium of a fluorescent reporter strain H37Rv expressing dsRED under the control of constitutively expressed *rpsA* promoter, where the readout is either optical density (OD) or fluorescence [14–17]. The use of two readouts minimizes the problems caused by compound precipitation or autofluorescence. A linear relationship between OD and fluorescence readout has been established justifying the use of fluorescence as a measure of bacterial growth. MICs generated from the OD are reported in the "Results and discussion" section.

The antimicrobial activity of compounds against five resistant isolates of *M. tuberculosis* was assessed only by OD readout.

The MIC of compound was determined by measuring bacterial growth after 5 days in the presence of test compounds. Compounds were prepared as 10-point two-fold serial dilutions in DMSO (Fisher) and diluted into 7H9-TwOADC medium (4.7 g L^{-1} 7H9 base broth (VWR), 0.05% w/v Tween 80 (Fisher), 10% v/v OADC Supplement (VWR)) in 96-well plates with a final DMSO concentration of 2%. The highest concentration of compound was 200 μM. Each plate included assay controls for background (medium/DMSO only, no bacterial cells), zero growth

(100 μM rifampicin (Sigma-Aldrich)), and maximum growth (DMSO only), as well as a rifampicin dose response curve. Plates were inoculated with *M. tuberculosis* and incubated for 5 days: growth was measured by OD$_{590}$ and fluorescence (Ex 560/Em 590) using a BioTek™ Synergy 4 plate reader. Growth was calculated separately for OD$_{590}$ and RFU. To calculate the MIC, the 10-point dose response curve was plotted as % growth and fitted to the Gompertz model using GraphPad Prism 5. The MIC was defined as the minimum concentration at which growth was completely inhibited and was calculated from the inflection point of the fitted curve to the lower asymptote (zero growth).

Antibacterial activity under hypoxic conditions

The antimicrobial activity of compounds under hypoxic conditions was assessed using the low oxygen recovery assay (LORA) [18]. Test compounds were prepared as 20-point two-fold serial dilutions in DMSO and diluted into DTA medium (6.5 g L^{-1} Dubos broth base (Fisher), 10% (v/v) Dubos medium albumin, 0.05% w/v Tween 80) in 96-well plates with a final DMSO concentration of 2%. Control compounds were prepared as 20-point two-fold serial dilutions in DMSO and diluted into DTA medium in 96-well plates with a final DMSO concentration of 2%. The highest concentration of compound was 200 μM.

M. tuberculosis constitutively expressing the *luxABCDE* operon was inoculated into DTA medium in gas-impermeable glass tubes and incubated for 18 days to generate hypoxic conditions (Wayne model of hypoxia). At this point, bacteria are in a non-replicating state (NRP stage 2) induced by oxygen depletion.

Oxygen-deprived bacteria were inoculated into compound assay plates and incubated under anaerobic conditions for 10 days followed by incubation under aerobic conditions (outgrowth) for 28 h. Growth was measured by luminescence.

Intracellular activity assay

The intracellular activity of compounds was measured using a macrophage cell line infected with *M. tuberculosis* [19]. The activity of compounds against intracellular bacteria was determined by measuring viability in infected THP-1 human monocytic cell line (ATCC) after 3 days in the presence of test compounds. Compounds were prepared as 10-point three-fold serial dilutions in DMSO (Fisher). The highest concentration of compound tested was 50 μM where compounds were soluble in DMSO at 10 mM.

THP-1 cells were cultured in complete RPMI (RPMI-1640 (Fisher), 10% v/v FBS (Fisher), 2 mM GlutaMAX (Fisher),

1 mM sodium pyruvate (Fisher)) and differentiated into macrophage-like cells using 80 nM PMA (Sigma-Aldrich) overnight at 37 °C, 5% CO_2. THP-1 cells were infected with a luminescent strain of H37Rv (which constitutively expresses *luxABCDE*) at a multiplicity of infection of 1 and incubated overnight at 37 °C, 5% CO_2. Infected cells were recovered using Accutase/EDTA solution (Accutase, 5 mM EDTA), washed twice with PBS (Fisher) to remove extracellular bacteria and seeded into assay plates. Compound dilutions were added to a final DMSO concentration of 0.5%. Assay plates were incubated for 72 h at 37 °C, 5% CO_2. Each run included isoniazid (Sigma-Aldrich) as a control. Relative luminescent units (RLU) were measured using a Biotek Synergy 2 plate reader. The dose response curve was fitted using the Levenberg–Marquardt algorithm. The IC_{50} and IC_{90} were defined as the compound concentrations that produced 50% and 90% inhibition of growth, respectively.

Plasma protein binding

Plasma protein binding for compounds **13** and **20** was determined by equilibrium dialysis [20]. Compounds were added to human plasma (Bioreclamation Inc.) at a fixed concentration of 5 μM. The mixture was dialyzed in a RED device (Rapid Equilibrium Dialysis, Pierce) against PBS and incubated on an orbital shaker for 4 h at 37 °C. Aliquots from plasma and PBS sides were collected; an equal volume of PBS was added to the plasma sample, and an equal volume of plasma was added to the PBS sample. Three volumes of methanol (containing the internal binding standard propranolol (Sigma)) was added to precipitate the proteins and release the compound. Samples were centrifuged, the supernatant was recovered and analyzed by LC-MS/MS. Each experiment included warfarin (Sigma) as a high-binding control.

Caco-2 cell permeability

The permeability of test compounds was assessed using a Caco-2 cell monolayer [21–25]. Caco-2 cells (ATCC) were trypsinized, resuspended in medium, and dispensed into a Millipore 96-well Caco-2 plate. The cells were allowed to grow and differentiate for 3 weeks, with feeding at 2-day intervals. For apical to basolateral (A→B) permeability, the compound was added to the apical (A) side and amount of permeation was determined on the basolateral (B) side; for basolateral to apical (B→A) permeability, the compound was added to the B side and the amount of permeation was determined on the A side. Each experiment included the control compounds atenolol (MP Biomedicals) (low permeability), propranolol (Sigma) (high permeability), and talinolol (TRC) (P-gp efflux control).

Quality control

The A-side contained 100 μM Lucifer yellow (Sigma) in transport buffer (1.98 g L^{-1} glucose (Teknova) in 10 mM HEPES (Sigma), 1× Hank's Balanced Salt Solution (Lonza) pH 6.5), and the B-side contained transport buffer (1.98 g L^{-1} glucose (Teknova) in 10 mM HEPES (Sigma), 1× Hank's Balanced Salt Solution (Lonza) pH 7.4). Caco-2 cells were incubated with these buffers for either 1 or 2 h, and the receiver side buffer was removed for analysis by LC-MS/MS. Aliquots of the cell buffers were analyzed by fluorescence to determine the transport of the impermeable dye Lucifer yellow to verify the Caco-2 cell monolayers were properly formed. Any deviations from control values were reported. Data are expressed as permeability, $P_{app} = (dQ/dt)/(C_0/A)$, in which dQ/dt is the rate of permeation, C_0 is the initial concentration of compound, and A is the area of monolayer.

HepG2 cytotoxicity

The cytotoxicity of compounds towards eukaryotic cells was determined using the human liver cells (HepG2) [26–29]. The cytotoxicity of compounds was determined by measuring HepG2 (ATCC) cell viability growth after 3 days in the presence of test compounds. Compounds were prepared as 10-point three-fold serial dilutions in DMSO. The highest concentration of compound tested was 100 μM where compounds were soluble in DMSO at 10 mM. HepG2 cells were cultured in complete DMEM (DMEM medium (Invitrogen), 1× penicillin–streptomycin solution (Fisher), 2 mM Corning Glutogro supplement (Fisher), 1 mM sodium pyruvate (Fisher), 10% v/v Fetal Bovine Serum (Fisher)), inoculated into 384-well assay plates containing compounds and incubated for 24 h at 37 °C, 5% CO_2. Compounds were added and cells were cultured for a further 72 h. The final DMSO concentration was 1%. Cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) and measuring RLU. The dose response curve was fitted using the Levenberg–Marquardt algorithm. The IC_{50} was defined as the compound concentration that produced 50% decrease in viable cells. Each run included staurosporine (Santa Cruz Biotechnology) as a control.

Results and discussion

In order to find novel antituberculosis compounds, we have performed phenotypic screening of the library containing about 1000 compounds from different chemical classes against *M. tuberculosis* H37Rv strain. As a result, a series of benzaldehyde thiosemicarbazone derivatives possessing high activity toward actively replicating *M. tuberculosis*

strains has been identified. As shown in Table 1, a number of investigated benzaldehyde thiosemicarbazones demonstrate moderate to excellent anti-tubercular activities under aerobic conditions.
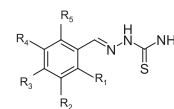
We have analyzed the impact of substituents on the antimycobacterial activity of benzaldehyde thiosemicarbazone derivatives. It was revealed that the substitution of hydrogen atom (compound **1**) in the position R_1 with fluorine atom (compound **25**) or pentoxy group (compound **27**) leads to the decrease of antimycobacterial activity (corresponding MIC values are 3.2, 6.3, and 17 μM).

It was identified that the antimycobacterial activity of benzaldehyde thiosemicarbazone derivatives depends on the structure of R_3 substituents. The presence of pentoxy group as R_3 substituent (compound **2**, MIC = 0.14 μM) is more favored than propoxy (compound **3**, MIC = 0.22 μM) or ethoxy group (compound **4**, MIC = 0.54 μM). Therefore, the increase in the inhibition of *M. tuberculosis* growth is attributed to the increase in alkyl chain length. The substitution of alkyl chain with benzyloxy group (compound **5**), 4-chloro-benzyloxy (compound **6**), or 4-bromo-benzyloxy (compound **7**) leads to decrease in antimycobacterial activity (corresponding MIC values are 0.78, 2.2, and 2.9 μM). From the obtained results, the order of potency for the substituent R_3 in a series of compounds with R_2 = methoxy group, could be proposed as following: pentoxy group > propoxy group > ethoxy group > benzyloxy > 4-chloro-benzyloxy > 4-bromo-benzyloxy. It has been observed that for a series of compounds **13–18** with R_2 = hydrogen atom the order of potency for R_3 substituent is following: 2-phenylethoxy > dimethylamine > methyl >> bromine > hydroxyl group > nitro group (corresponding MIC values are 0.18, 0.32, 0.48, 0.86, 1.5, and 3.2 μM).

The insertion of additional substituent R_2 (methoxy group) in the structure of R_3-substituted benzaldehyde thiosemicarbazone derivatives leads to the decrease in antimycobacterial activity. To compare this effect, one can refer to compound pairs such as **15**, **6**; **18**, **8** (corresponding MIC values are 0.43, 2.2; 1.5, 2.8 μM). The substitution of methoxy group (compound **9**) with ethoxy group (compound **10**) does not have a significant influence on the potency of investigated compounds (MIC = 19 μM).

As shown in Table 1, the substitution of hydrogen atom (compound **1**) at the position R_4 with isopropoxy group (compound **20**) results in appreciable rise of antimycobacterial activity (MIC equal to 3.2 and 0.71 μM, respectively). The introduction of bromine atom (compound **9**) instead of hydrogen (compound **8**) at the position R_4 leads to significant decrease in antimycobacterial activity (corresponding MIC values are 19 and 2.8 μM).

Table 1 Structures and antimycobacterial activity of benzaldehyde thiosemicarbazone derivatives toward H37Rv strain under aerobic conditions

Compound	R_1	R_2	R_3	R_4	R_5	MIC, μM
1	H	H	H	H	H	3.2
2	H	methoxy	pentoxy	H	H	0.14
3	H	methoxy	propoxy	H	H	0.22
4	H	methoxy	ethoxy	H	H	0.54
5	H	methoxy	benzyloxy	H	H	0.78
6	H	methoxy	4-chloro-benzyloxy	H	H	2.2
7	H	methoxy	4-bromo-benzyloxy	H	H	2.9
8	H	methoxy	OH	H	H	2.8
9	H	methoxy	OH	Br	H	19
10	H	ethoxy	OH	Br	H	19
11	H	methoxy	OH	NO_2	H	>200
12	OH	methoxy	H	NO_2	H	51
13	H	H	2-phenylethoxy	H	H	0.18
14	H	H	dimethylamine	H	H	0.32
15	H	H	4-chloro-benzyloxy	H	H	0.43
16	H	H	CH_3	H	H	0.48
17	H	H	Br	H	H	0.86
18	H	H	OH	H	H	1.5
19	H	H	NO_2	H	H	3.2
20	H	H	H	isopropoxy	H	0.71
21	H	H	benzyloxy	methoxy	H	0.83
22	H	H	4-methyl-benzyloxy	ethoxy	H	3.2
23	H	H	H	methoxy	OH	4.6
24	H	H	H	methoxy	methoxy	16
25	F	H	H	H	H	6.3
26	F	H	H	H	Cl	9
27	pentoxy	H	H	H	H	17
28	OH	Br	OH	Br	H	36
29	Cl	Cl	H	H	Cl	>200
30	H	NO_2	OH	ethoxy	H	200

It has been found that R_5 also impacts compound's antimycobacterial activity. The substitution of hydrogen atom (compound **25**) with chlorine atom (compound **26**) results in the increase of antimycobacterial activity (corresponding MIC values are 6.3 and 9 μM).

Therefore, structure–activity relationships (SAR) of benzaldehyde thiosemicarbazone derivatives with respect to their antitubercular activity revealed that the antimycobacterial activity of the studied compounds depends on the structure of all substituents. The results of SAR studies can be used for further chemical optimization of benzaldehyde thiosemicarbazone derivatives.

The most active 16 compounds identified in the primary screening were also evaluated against *M. tuberculosis* H37Rv under hypoxic conditions (Table 2). This method is used for screening of antimicrobial agents against non-replicating persistence *M. tuberculosis*. The compounds **13** and **20** which possess submicromolar activity have been taken for further biological investigations.

The intracellular activity of compounds **13** and **20** was measured using a macrophage cell line infected with *M. tuberculosis*. This is important because *M. tuberculosis* can survive inside macrophages, which contributes to the treatment failure and disease relapse [30–32]. Accordingly to the results presented in Table 3, MIC, IC_{50}, and IC_{90} values of compounds **13** and **20** are lower than for isoniazid. Also, it should be noted that intracellular activities of these compounds are significantly lower than the activities in axenic media.

The compounds **13** and **20** were tested for antibacterial activity against five resistant isolates of *M. tuberculosis*

Table 2 Antituberculosis activity for benzaldehyde thiosemicarbazone derivatives toward H37Rv strain under hypoxic conditions

Compound	MIC, μM
2	1.2
3	20
4	15
5	71
6	>200
7	92
8	>200
13	0.68
14	>200
15	8.9
16	58
17	>200
18	>200
19	>200
20	0.74
21	82

Table 3 Intracellular activity of compounds **13** and **20** toward THP-1 human monocytic cell line infected with *M. tuberculosis*

Compound	IC_{50}, μM	IC_{90}, μM	MIC, μM
13	<0.098	<0.098	<0.098
20	<0.098	<0.098	<0.098
Isoniazid	0.315	0.38	1.46

Table 4 Minimum inhibitory concentration (MIC, μM) of compounds **13** and **20** toward resistant isolates of *M. tuberculosis*

Compound	INH-R1[a]	INH-R2[b]	RIF-R1[c]	RIF-R2[d]	FQ-R1[e]
13	0.21	0.11	0.079	0.25	0.25
20	0.38	0.18	0.39	0.61	0.64
Rifampicin	0.020	0.011	3.0	>50	0.015
Isoniazid	>200	>200	0.36	0.71	0.80
Levofloxacin	1.4	2.3	1.3	1.6	26

[a]INH-R1—isoniazid resistant strain derived from H37Rv and is a *katG* mutant (Y155* = truncation)

[b]INH-R2—isoniazid resistant strain ATCC35822 which has a complete deletion of the *katG* gene

[c]RIF-R1—rifampicin resistant strain derived from H37Rv and is an *rpoB* mutant (S522L)

[d]RIF-R2—rifampicin resistant strain ATCC35828 with a single G nucleotide absent at position 288 in the *pncA* gene

[e]FQ-R1—fluoroquinolone-resistant strain derived from H37Rv which is a *gyrB* mutant (D94N)

under aerobic conditions. Strains tested were two isoniazid resistant strains (INH-R1 and INH-R2), two rifampicin resistant strains (RIF-R1 and RIF-R2), and a fluoroquinolone resistant strain (FQ-R1). The assay was based on the measurement of growth in liquid medium of each strain where the readout is OD. INH-R1 was derived from H37Rv and is a *katG* mutant (Y155* = truncation). INH-R2 is strain ATCC35822 which has a complete deletion of the *katG* gene. RIF-R1 was derived from H37Rv and is an *rpoB* mutant (S522L). RIF-R2 is strain ATCC35828 with a single G nucleotide absent at position 288 in the *pncA* gene. FQ-R1 is a fluoroquinolone-resistant strain derived from H37Rv which is a *gyrB* mutant (D94N). Accordingly to the results of investigation, compounds **13** and **20** are highly potent toward all tested resistant isolates of *M. tuberculosis* (Table 4).

Plasma protein binding for compounds **13** and **20** was determined by equilibrium dialysis. As it can be seen from Table 5, compounds **13** and **20** are highly bound to plasma protein.

The permeability of test compounds was assessed using a Caco-2 cell monolayer. Compound permeability was measured in both directions. The experiment included the control compounds atenolol (low permeability), propranolol

Table 5 Plasma protein binding for compounds **13** and **20**, %

Compound	Mean plasma fraction unbound, %	Mean plasma fraction bound, %	Recovery, %
13	0.8	99.2	87.6
20	13.2	86.8	96
Propranolol	24.4	75.6	98.4
Warfarin	0.62	99.4	106

Table 6 Caco-2 permeability for compounds **13** and **20**

Compound	Assay duration (h)	Mean A→B P_{app}[a] (10^{-6} cm/s)	Mean B→A P_{app}[a] (10^{-6} cm/s)	Efflux ratio[b]
13	1	6.8	9.7	1.4
20	1	37.5	24.7	0.66
13	2	8.2	3.5	0.43
20	2	28.2	14.3	0.51
Atenolol	1	0.036	0.31	8.7
Atenolol	2	0.31	0.42	1.4
Propranolol	1	17.3	43.3	2.5
Propranolol	2	25.4	21.1	0.83
Talinolol	1	0.017	5.04	298
Talinolol	2	0.072	6.3	87.6

[a]P_{app} is the apparent permeability rate coefficient = $(dQ/dt)/(C_0A)$

[b]Efflux ratio (Re) is P_{app} (B→A)/P_{app} (A→B)

An Re > 2 indicates a potential substrate for P-glycoprotein or other active transporters

(high permeability), and talinolol (P-gp efflux control). As it can be seen from Table 6, compounds **13** and **20** have good permeability across Caco-2 cell monolayer in both directions.

The cytotoxicity of compounds towards eukaryotic cells was determined using the human liver cells (HepG2). IC_{50} values for compounds **13** and **20** were 41 and >100 μM, respectively, while staurosporine has IC_{50} value of 0.045 μM.

In conclusion, we have identified two compounds—2-(4-phenethoxybenzylidene)hydrazine-1-carbothioamide (**13**) and 2-(3-isopropoxybenzylidene)hydrazine-1-carbothioamide (**20**) possessing high antibacterial activity toward *M. tuberculosis* H37Rv under aerobic and hypoxic conditions. The compounds are active against isoniazid-, rifampicin-, and fluoroquinolone-resistant strains and also have good ADME properties and low cytotoxicity toward human liver cells (HepG2). These obtained data indicate that the compounds **13** and **20** can be valuable candidates for further preclinical studies.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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