ORIGINAL ARTICLE

Thiosemicarbazones and their antimycobacterial effects

Thiosemikarbazony a jejich antimykobakteriální účinky

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Summary

Antimycobacterial effects of thiosemicarbazones were discovered in the late 1940s. The best known representative of these compounds is thioacetazone that has been used in the therapy of tuberculosis since the turn of the 1940s and 1950s. At present, it is used only rarely since it exhibits severe side effects. This paper deals with the antimycobacterial effects of thiosemicarbazones and *N*,*N*-dimethylthiosemicarbazones derived from 5-alkyl-2-acetylpyrazines. Some of these compounds displayed high inhibition of the growth of *Mycobacterium tuberculosis* $H_{37}Rv$, but were excluded from the *in vivo* studies due to their cytotoxic effects. Nonetheless, they can be used as model compounds for studying the mechanisms of antimycobacterial action of thiosemicarbazones.

Keywords: tuberculosis • thiosemicarbazones of acetylpyrazines • antimycobacterial effects

Souhrn

Antimykobakteriální účinky thiosemikarbazonů byly objeveny ve druhé polovině čtyřicátých let 20. století. Nejznámějším zástupcem těchto sloučenin je thioacetazon používaný v terapii tuberkulózy od přelomu čtyřicátých a padesátých let. Pro závažné vedlejší účinky se dnes používá jen málokdy. Tato práce pojednává o antimykobakteriálních účincích thiosemikarbazonů a *N*,*N*-dimethylthiosemikarbazonů odvozených od 5-alkyl-2-acetylpyrazinů. Některé z těchto sloučenin významně inhibovaly růst *Mycobacterium tuberculosis* $H_{37}Rv$, ale pro svoji toxicitu nepostoupily do *in vivo* studií. Nicméně mohou být využity jako modelové sloučeniny pro studium mechanismů antimykobakteriálních účinků thiosemikarbazonů.

Klíčová slova: tuberkulóza • thiosemikarbazony acetylpyrazinů • antimykobakteriální účinky

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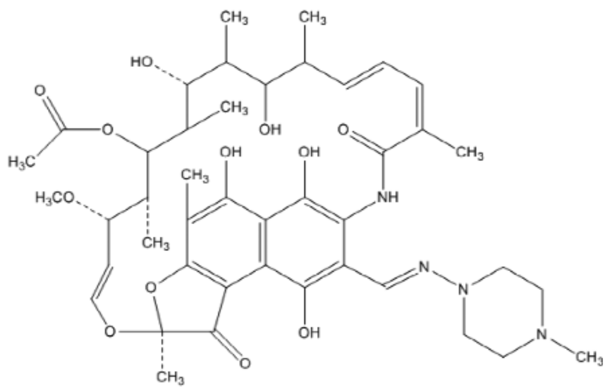
Introduction

Tuberculosis is a very old disease. It is thought that the progenitor of the *Mycobacterium tuberculosis* complex arouse from a soil bacterium and that the human bacillus may have been derived from the bovine form following domestication of cattle approx. 10 000 years ago[1–3]. Over the past 100 years, tuberculosis (TB) has probably killed 100 million people[4]. According to WHO Global Tuberculosis Report 2012, there were an estimated 8.7 million new cases of tuberculosis and 1.4 million died from TB in 2011, despite the availability of treatment that will cure most cases of TB[5]. Increased incidence of infections caused by the *M. tuberculosis* and *M. avium* complex in HIV-infected individuals[6, 7] as well as evolution of multidrug-resistant tuberculosis (MDR-TB)



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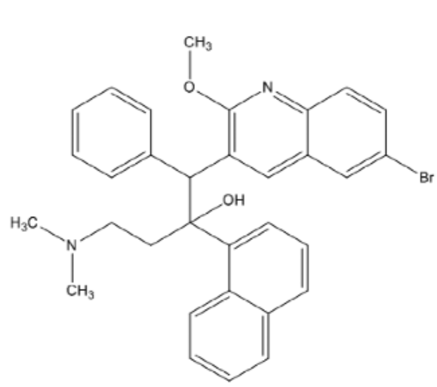

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*Fig. 1. Structures of rifampicin **(1)** and bedaquiline **(2)***

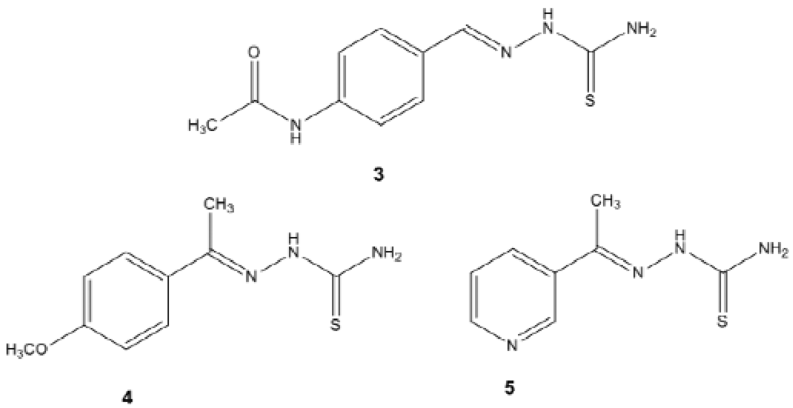


*Fig. 2. Structures of thioacetazone **(3)**, SRI-224 **(4)** and SRI-286 **(5)**v*

and extensively drug-resistant tuberculosis (XDR-TB) present a serious problem[8–11]. Moreover, the last first-line antimycobacterial agent – rifampicin **1** (RIFADIN®, Fig. 1) – was introduced more than 40 years ago[12]. In the end of 2012, bedaquiline **2** (SIRTURO®, Fig. 1) was approved by the FDA through an accelerated approval process only for patients who have multidrug-resistant TB, which can require up to 2 years of treatment. Phase III in MDR-TB patients are currently under way and may take 5 years. Only then the full potential of bedaquiline can be evaluated[13]. Thus, more-effective vaccines, diagnostic tools, drugs and therapeutic regimes are urgently needed[14–16].

Antimycobacterial effects of thiosemicarbazones were discovered in the 1940s[17] and resulted in a rapid introduction of thioacetazon (TAZ) **3** (CONTEBEN®, Fig. 2) into the therapy of tuberculosis[19, 20]. In spite of frequent side effects, TAZ may still be considered for the treatment of new cases if there is a lack of other antituberculous drugs and for the management of MDR-TB[20]. Several analogous thiosemicarbazones **4–5** (Fig. 2) have recently been studied[21–24].

The unsubstituted acetylpyrazine thiosemicarbazone **6a** (Fig. 3) has been known since 1952[25]. Almost 50 years later, its congeners modified in the thiosemicarbazide part of the molecule **6b–6s** and **7a–7g** (Fig. 3) were studied as potential antituberculous drugs. Based on *in vitro* results, compound **6h** was chosen for further testing on mice. Its potency was the same or even better than that of isoniazid and ethambutol. The best results were obtained with the dose of 5 mg/kg. The acute toxicity value (LD_{50}) after *per os* administration was 90 mg/kg, and only small organic changes were observed[26].

Within our studies aimed at finding new antimicrobial drugs, we have prepared and evaluated thiosemicarbazones **8a–8h** and *N*,*N*-dimethylthiosemicarbazones **9a–9h** alkylated on the pyrazine ring. The compounds exhibited iron chelating, antitumor and antifungal activities[27]. Thiosemicarbazones **8i** and **9i** derived from acetophenone were prepared for comparison (Fig 4).

The present paper deals with antimycobacterial effects of these compounds.

Table 1. 5-alkylacetylpyrazine thiosemicarbazones and their antimycobacterial effects

Inhibition of *M. tuberculosis* $H_{37}Rv$						
Compd.	**X**	**R[1]**	**% inhibition at 6.25 μg/ml**	**MIC (μg/ml)**	**IC_{50} (μg/ml)**	**SI**
8a	N	H	ND[a]	ND[a]	ND[a]	ND[a]
8b	N	propyl	95	6.25	1.28	0.20
8c	N	isopropyl	88	ND[a]	ND[a]	ND[a]
8d	N	butyl	98	0.39	0.43	1.10
8e	N	isobutyl	96	6.25	0.63	0.10
8f	N	*tert*-butyl	95	6.25	0.42	0.07
8g	N	pentyl	95	3.13	0.36	0.12
8h	N	hexyl	93	3.13	0.10	0.03
8i	C	H	ND[a]	ND[a]	ND[a]	ND[a]
9a	N	H	55	ND[a]	ND[a]	ND[a]
9b	N	propyl	6	ND[a]	ND[a]	ND[a]
9c	N	isopropyl	24	ND[a]	ND[a]	ND[a]
9d	N	butyl	39	ND[a]	ND[a]	ND[a]
9e	N	isobutyl	47	ND[a]	ND[a]	ND[a]
9f	N	*tert*-butyl	51	ND[a]	ND[a]	ND[a]
9g	N	pentyl	100	1.56	0.20	0.13
9h	N	hexyl	100	3.13	0.17	0.05
9i	C	H	24	ND[a]	ND[a]	ND[a]
isoniazid	–	–	ND[a]	0.025–0.05	< 1000	< 40 000
rifampicin	–	–	98	0.015–0.125	< 100	< 800

[a]ND = not determined

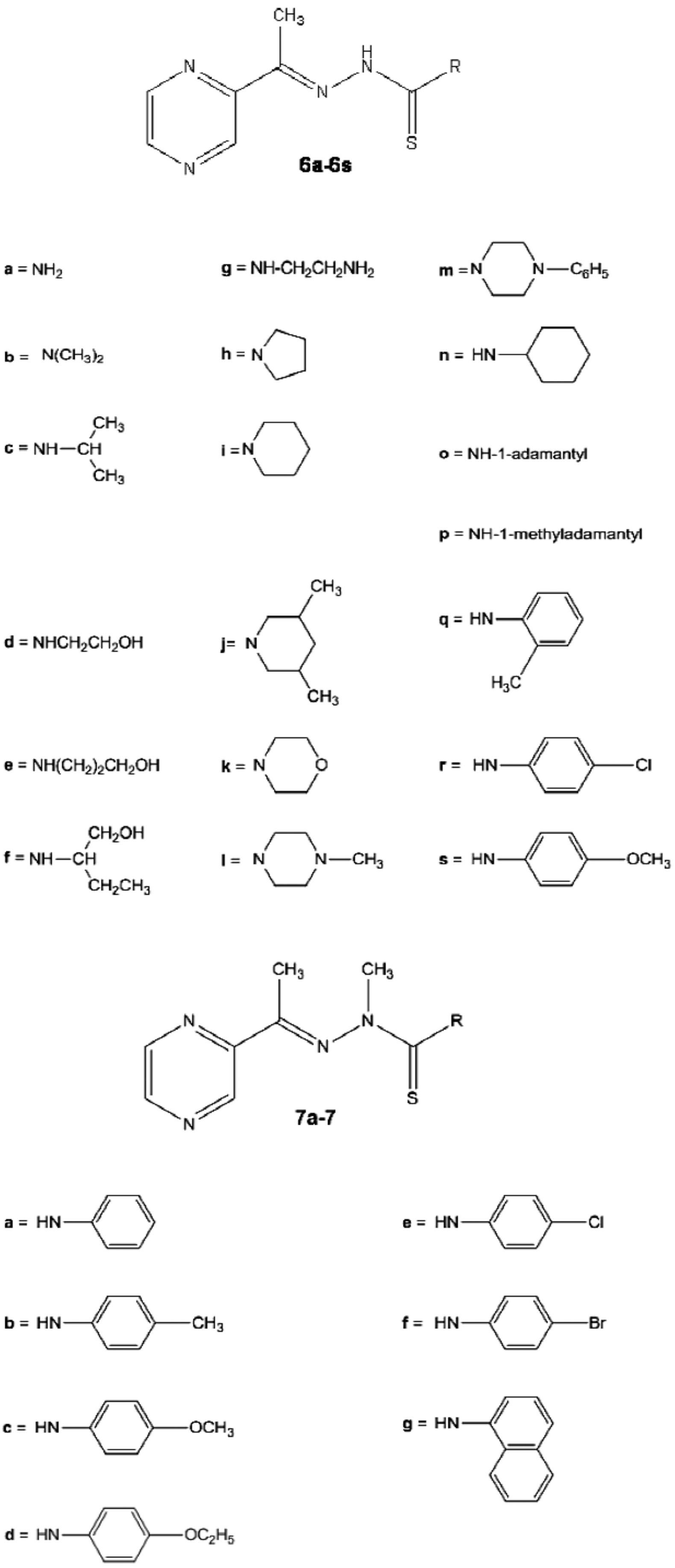
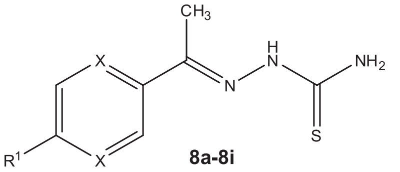
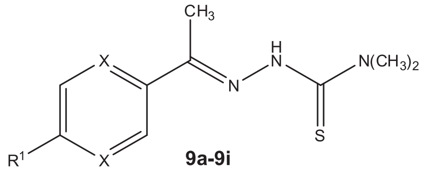


Fig. 3. Acetylpyrazine thiosemicarbazones modified in the thiosemicarbazide moiety

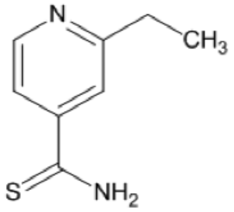


8a-8i



9a-9i

Fig. 4. Structures of the studied compounds



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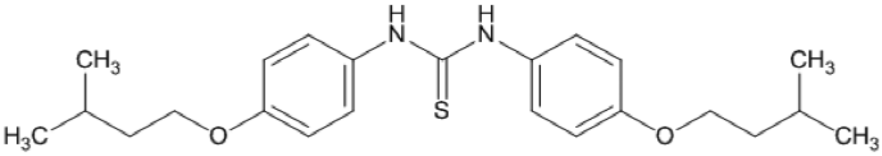

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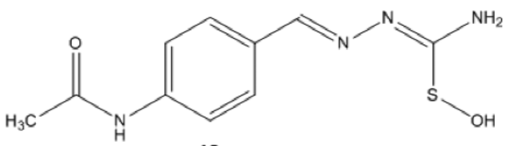
*Fig. 5. Structures of ethioamide(**10**) and thiocarlide (**11**)*



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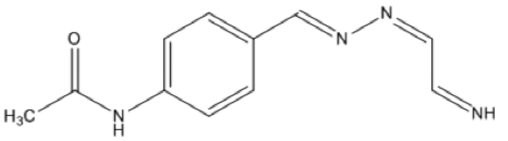

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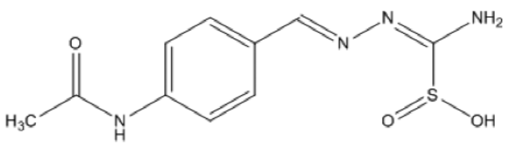

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*Fig. 6. Structures of TAZ-sulfenic acid (**12**), TAZ-carbodiimide (**13**) and TAZ-sulfinic acid (**14**)*

Experimental part

Primary screening of all compounds was conducted at 6.25 μg/ml against *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294) in the BACTEC 12B medium using the Microplate Alamar Blue Assay (MABA)[28]. Compounds exhibiting fluorescence were tested in the BACTEC 460-radiometric system[28]. Compounds demonstrating at least 90% inhibition in the primary screen were re-tested at lower concentrations against *M. tuberculosis* $H_{37}Rv$ to determine the actual minimum inhibitory concentration (MIC) in the MABA. The MIC is defined as the lowest concentration effecting a reduction in fluorescence of 90% relative to controls.

The compounds that exhibited promising antimycobacterial activity were tested for cytotoxicity (IC_{50}) in VERO cells at concentrations less than or equal to 10 times the MIC for *M. tuberculosis* $H_{37}Rv$. After 72-h exposure, viability was assessed on the basis of cellular conversion of 1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) into a formazan product using the Promega CellTiter 96 Non-radioactive Cell Proliferation Assay.

The selectivity index (SI) was then calculated as the ratio of the measured IC_{50} in VERO cells to the MIC described above. Generally, requirements for moving compound into *in vivo* testing include: MIC ≤ 6.25 g.ml^{-1} and an SI ≥ 10.

The results of antimycobacterial and cytotoxicy assays are summarized in Table 1.

Results and discussion

Thiosemicarbazones **8a–8h** exhibited high inhibition of *M. tuberculosis*. The lowest MIC (0.39 μg/ml) was observed with (2*E*)-2-[1-(5-butylpyrazin-2-yl)ethylidene] hydrazinecarbo thioamide **8d.** *N,N*-dimethylthiosemicarbazones were less potent except for **9g** and **9h** which displayed potency similar to that of thiosemicarbazone **8d.** It indicates that the character of the alkyl is important. In both series longer non-branched alkyls seem to be preferable for the antimycobacterial activity. This is in agreement with a recent study performed with ring substituted benzaldehyde thiosemicarbazones[29]. Regarding lipophilicity, *N,N*-αdimethylation results in approx. a twofold increase of log *K* values determined experimentally by means of HPLC[27]. All compounds with MIC ≤ 3.13 μg/ml have log *K* in the range 1.02–1.72 for thiosemicarbazone series and 2.20–2.62 for *N,N*--dimethylthiosemicarbazones.

Due to high cytotoxicity (low SI) none of the studied compounds was chosen for *in vivo* testing. Nonetheless, they can serve as tools to study mechanisms of action of thiosemicarbazones.

It is known that TAZ is a prodrug. *In vivo* it undergoes oxidative activation by flavin monooxygenase EthA, the enzyme that bioactivates also ethionamide **10** (TRECATOR®) and tiocarlide **11** (ISOXYL®) (Fig. 5)[30, 31].

TAZ is also activated by human monooxidases FMO, FMO2.1 and FMO3 which may decrease the availability of the prodrug to the mycobacteria. The resulting metabolites – sulfenic acid **12** and carbodiimide **13,** but not sulfinic acid **14** (Fig. 6) – react with glutathione (GSH) and may contribute the cytotoxicity of TAZ[30–32].

Mycobacteria do not produce GSH but make mycothiol (MSH) which is essential for the growth of *M. tuberculosis*. MSH, like GSH, protects the cell against oxidative damage and electrophilic toxins. Metabolites of TZA that react with GSH should react with MSH in a similar manner thus lowering MSH concentration within the mycobacterial cell and sensitizing it to oxidative damage[30].

It has also be found that TAZ inhibits cyclopropanation of cell wall mycolic acids in mycobacteria by inhibition of the enzymes belonging to CMAS (Cyclopropanating Mycolic Acids Synthases) family[33, 34]. A computational analysis showed that TAZ fits well at the active site of cyclopropane mycolic acid synthases CmaA1and CmaA2. Moreover, TAZ metabolites – TAZ-carbodiimide **13** and TAZ-sulfinic acid **14** (Fig. 5) – may also bind at the active site by NH-π interactions analogous to TAZ. If these hypotheses are validated experimentally, new knowledge about the TAZ binding site will be obtained[35].

Other enzymes of CMAS family are also inhibited by TAZ even at extremely low doses with an exception of methoxy mycolic acid synthase MmaA4. Mutations in the *mmaA4* gene make the mycobacteria resistant to TAZ. According to Alahari et al., activation of TZA by EthA itself is not sufficient, and TAZ must be further activated by MmaA4 to get the active form capable to induce growth arrest of mycobacteria[34]. However, this hypothesis is not supported by Grzegorzewicz et al.[36].

Many other enzymes, especially those belonging to the fatty acid synthase (FAS) system, have recently been studied as new drug targets[37–39]. *M. tuberculosis* possesses not only FAS-II monofunctional enzymes (specific for prokaryotes and organelles), but also mega-enzyme FAS-I (a multifunctional enzyme found mainly in eukaryotes). These two systems are engaged in the synthesis of normal chain-length fatty acids together with specific long-chain mycolic acids[40]. Moreover, *M. tuberculosis* encodes more that 60 adenylating enzymes (AE) that are essential for virulence. Development of potent and selective AE inhibitors represents another strategy in the fight against pathogenic mycobacteria[41–43]. Let's hope that all these efforts will finally lead to more potent and safe antituberculous agents.

Acknowledgment

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