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Design, synthesis and antimycobacterial activity of hybrid molecules combining pyrazinamide with 4-phenylthiazol-2-amine scaffold

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Hybrid compounds based on a combination of first-line antitubercular pyrazinamide (PZA) and formerly identified antimycobacterial scaffold of 4-arylthiazol-2-amine were designed. Eighteen compounds were prepared, characterized and tested for in vitro growth inhibition activity against *M. tuberculosis* H37Rv, *M. kansasii*, *M. avium* and *M. smegmatis* in a Microplate Alamar Blue Assay at neutral pH. Active compounds were tested for *in vitro* cytotoxicity in human hepatocellular carcinoma cell line (HepG2). The most active 6-chloro-*N*-[4-(4-fluorophenyl)thiazol-2-yl]pyrazine-2-carboxamide (**9b**) had also the broadest spectrum of activity and inhibited *M. tuberculosis*, *M.* kansasii, and *M. avium* with MIC = 0.78 μg/mL (2.3 μM) and selectivity index related to HepG2 cells of SI>20. Structure-activity relationships within series are discussed. Based on structural similarity to known inhibitors and the results of a molecular docking study, we suggest mycobacterial beta-ketoacyl-(acyl-carrier-protein) synthase III (FabH) as a potential target.

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Electronic Supplementary Information (ESI) available: Contents - NMR spectra of representative final compounds, experimental procedure and full results of MycPermCheck calculation, and experimental procedure and full results of molecular docking to mycobacterial FabH. See DOI: 10.1039/x0xx00000x

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Introduction

According to the WHO Global Tuberculosis Report 2017, estimated 10.4 million people worldwide developed active tuberculosis (TB) in 2016.[1] In 2016, TB was the causative agent of 1.7 million deaths, including 0.4 million deaths in people with HIV/TB co-infection.[1] This ranks TB the leading cause of death from all infectious diseases, followed by HIV/AIDS. New estimates account for 23% of global population (1.7 billion people) being latently infected with TB.[2] Immunosuppression (caused by HIV as well as modern antiproliferative therapies and treatment of autoimmune diseases) is a significant risk factor of developing active TB. Globally in 2016, 477,000 new HIV-positive TB cases were reported (46% of the estimated incidence). Almost three-quarters of these cases were in the African Region.[1] The Global Plan to Stop Tuberculosis (2006-2015) [3] aimed for reducing the prevalence of and deaths due to TB by 50% compared with a baseline of 1990. Globally speaking, these goals were nearly met. In 2015, the prevalence rate was 42% lower and the mortality was 47% lower than in 1990.[4]

On contrary to the positive trends in global epidemiology of TB, the widespread of drug-resistant TB is threatening the TB control policy. In 2016, there were an estimated 490,000 new cases of multidrug-resistant TB (MDR-TB; resistant to rifampicin and isoniazid) and an additional 110,000 people with rifampicin-resistant TB (RR-TB).[1] The basic regimen for non-complicated, non-resistant TB is a cocktail of at least four first-line antitubercular agents (rifampicin, isoniazid, ethambutol and pyrazinamide) administered for six months. The shortest MDR-TB treatment regime recommended by WHO takes 6–9 months and includes the necessity of injection application.[5] Such prolonged and unpleasant administration is, of course, a draw-back with the respect to side effects and compliance. Therefore, there is an urgent need for the development of new TB drugs leading to shorter therapeutic regimens. Ideally, these drugs should have different mechanism of action to currently used antituberculars.

Various 4-(hetero)arylthiazol-2-amines were shown to possess interesting antibacterial, antimycobacterial or general anti-infective activity *in vitro*. Zhu et al.[6, 7] studied *in vitro* antibacterial activity of acylated 4-phenylthiazol-2-amine derivatives. The most active compound (Fig. 1, **1**) inhibited the growth of both Gram-negative (*Escherichia coli, Pseudomonas aeruginosa*) and Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) bacteria with minimum inhibitory concentration (MIC) of 1.56–6.25 μg/mL.[7] Enzyme inhibition assays suggested that inhibition of β-ketoacyl-(acyl-carrier-protein) synthase III (FabH or KAS III) of *E. coli* is the candidate mechanism of action of **1** and similar derivatives. FabH is an enzyme involved in the fatty acid biosynthesis, a part of Fatty Acid Synthase II complex (FAS II). In mycobacteria, FabH constitutes a crucial link between FAS I and FAS II systems.[8]

[INSERT FIG 1, DOUBLE COLUMN WIDTH]

For the antimycobacterial activity, the 4-(pyridin-2-yl)thiazol-2-amine scaffold as represented by general structure **2** was discovered by phenotypic whole cell high-throughput screening campaign run by Tuberculosis Antimicrobial Acquisition and Coordination Facility (TAACF),[9] This antitubercular aminothiazole scaffold was further investigated by several groups. Mjambilli et al.[10] prepared derivatives (**3**) with amino group substituted mostly by acyls derived from simply substituted benzoic acids, and showed that such *N*-benzoyl derivatives were superior in activity compared to derivatives with unsubstituted amino group or amino group substituted by phenyl. The most potent substituent R in the phenyl part was 4-Br, followed by 3-Br and 2-Br. Exchange of 2-pyridinyl for 3- or 4-pyridinyl led to a significant decrease of activity. Best compounds inhibited the growth of *Mtb* H37Rv with MIC in low micromolar range. Unfortunately, all promising compounds exerted significant *in vitro* cytotoxicity on CHO cells with IC_{50} at low micromolar levels. The superiority of *N*-benzoyl derivatives of general structure **3** over *N*-phenyl derivatives was confirmed in another study by Meissner et al.[11] In this study, the benzoyl core tolerated various substituents (both electron donating and withdrawing), but the meta-substituted derivatives as 3-Br or 3-Cl exerted higher selectivity index (MIC/EC_{50}). Best compounds exerted MIC at submicromolar level and cytotoxicity (Vero cells) with EC_{50} usually at units of μM, sparingly at tens of μM. Both SAR studies of Mjambilli et al.[10] and Meissner et al.[11] confirmed the necessity of the 4-(pyridin-2-yl)thiazole core, confirmed the superiority of *N*-benzoyl derivatives over *N*-phenyl derivatives, and pointed out similar substitution pattern on the benzoyl core (3-Cl, 3-Br). Meissner et al. also modified the *N*-acyl part to prepare derivatives of pyridine carboxylic acid. Picolinoyl (R = pyridin-2-yl), nicotinoyl (R = pyridin-3-yl), and isonicotinoyl (R = pyridin-4-yl) derivatives were less potent than benzoyl derivatives, but still possessed a reasonable activity with MIC ranging 1.6–6.3 μM.[11] From this observation, we were inspired to design similar compounds incorporating the pyrazinoyl moiety. The mechanism of antimycobacterial activity of compounds of general structure **3** was proposed neither by Mjambilli nor by Meissner.

Another comprehensive SAR study of aminothiazole derivatives similar to structures **2** and **3** was published by Kesicki et al.,[12] who altered substitution at both C-2 and C-4 of the central aminothiazole core. Consistently with previous findings, authors found the pyridin-2-yl substituent at C-4 important for the antimycobacterial activity. The amino group at C-2 was more tolerant for various substitutions and active compounds recruited both from acyl substituents (resulting in amide linker) and aryl substituents (amino linker), but *N*-acyl substituted compounds were denoted with lower cytotoxicity (Vero cells) and therefore more favourable selectivity index values.

All aforementioned studies of Mjambilli et al.,[10] Meissner et al.[11] and Kesicki et al.[12] argued that the pyridin-2-yl group at position C-4 of the central thiazole ring is essential for antimycobacterial activity. On contrary, research group of Pieroni et al.[13] published a series of antimycobacterial aminothiazole derivatives (Fig. 1, **4**) having 4-phenyl substituent instead of 4-(pyridin-2-yl). Importantly, they showed that the 4-phenyl substituent could not be moved to position C-5 of the thiazole ring without losing the activity. The most active 4-phenyl based compound had MIC = 13 μM

against *Mtb* H37Rv. This is at least 10 times lower activity compared to pyridin-2-yl derivatives (**3**), but, on the other hand, no *in vitro* cytotoxicity was detected (IC_{50} >128 μM in Vero cells). The activity of derivatives **4** could have been lowered by the change to the linker (as they are *N*-phenyl derivatives, not *N*-acyl derivatives) rather than by the pyridinyl to phenyl exchange. Roy et al. published a derivative of general structure **4** with R^1 = R^2 = 4-F having MIC = 6.25 μM against *Mtb* H37Rv (cytotoxicity not determined).[14]

To recapitulate the SAR from the previously mentioned studies on 4-(hetero)arylthiazol-2-amines as antimycobacterial compounds: a) *N*-aroyl derivatives are significantly more active than *N*-phenyl derivatives[10-12] and usually also of lower cytotoxicity[12]; b) *N*-acyl can be also of a heterocyclic acid as exemplified by pyridine-2-, 3-, or 4-carboxylic acid[11]; c) 4-phenyl derivatives are less active but also less toxic than 4-(pyridin-2-yl) derivatives.[13]

First-line antitubercular agent pyrazinamide (PZA; pyrazine-2-carboxamide) exerts synergistic effects with rifampicin and is active even against dormant subpopulation of *M. tuberculosis* (*Mtb*).[15] The strengthened interest in PZA can be documented by recent studies, which revealed new specific mechanisms of action for PZA and/or its active metabolite pyrazinoic acid (POA). PZA, POA, or their simple structure derivatives were shown to act as inhibitors of mycobacterial Fatty Acid Synthase I (FAS I),[16-20] aspartate decarboxylase (PanD),[21-23] and quinolinic acid phosphoribosyltransferase (QAPRTase).[24] POA acts as an inhibitor of *trans*-translation, the process of rescuing of ribosomes stalled during translation.[25, 26] In general, the perception of PZA and its metabolite POA has changed from a non-specific cytosol acidifier to a multi-target inhibitor of specific mycobacterial enzymes and processes.[27]

Amides (Fig. 1, **5**) derived from substituted POA and aminothiazole exerted 15–65% inhibition of *Mtb* growth *in vitro* at the concentration of 6.25 μg/mL.[28] Anilides of POA (Fig. 2) with simple substituents on the pyrazine ring (R^3 is H, methyl, *tert*-butyl, chloro or combination thereof) and simple substituents on the benzene ring (R^4 is short alkyl, OH, halogen, nitro or combination thereof) possessed *in vitro* growth inhibiting activity against *Mtb* H37Rv, best compounds with MIC at micromolar level (2–20 μM). Their SAR have been reviewed elsewhere.[29-31]

[INSERT FIG 2, SINGLE COLUMN WIDTH]

The design of title compounds of our study was to take the best from previously published series and incorporate the PZA fragment to the verified anti(myco)bacterial scaffold based on 2-aminothiazole. Three series (Scheme 1, **7**–**9**) of hybrid compounds combining the PZA and 4-(hetero)arylthiazol-2-amine fragments were designed and synthesised. Substituents in the phenyl part were inspired by the most active aminothiazole derivatives presented above (see Fig. 1) - 4-halogen as in **1**, 4-OCH_3 as in **4**, and modifications. Substituents of the pyrazine core were motivated by previously published derivatives of 5- or 6-clhloropyrazinoic acid with *in vitro* antimycobacterial activity.[30, 32, 33] We chose the linker connecting the pyrazine and the aminothiazole to be carboxamidic, because *N*-acyl derivatives of 2-aminothiazole were more active than *N*–phenyl derivatives as discussed above. From other perspective, the final series **7**–**9** can be viewed as analogues of antimycobacterial anilides of POA, where the aniline part is exchanged for the substituted aminothiazole.

Results and discussion

Chemistry

4-Phenylthiazol-2-amines (**6a**, **6b**, **6d**, **6e**) were prepared by cyclic condensation of substituted acetophenone and thiourea in the presence of iodine (Scheme 1, a).[34] The yields varied between 58–76% of theoretical yield after simple recrystallization from hot water and EtOH, consequently. The identity of 4-phenylthiazol-2-amines was confirmed by melting points and ^{1}H NMR spectra in comparison to data published in literature. Similarly, we prepared 4-(pyridin-2-yl)thiazol-2-amine (**6f**) by condensation of 2-acetopyridine with thiourea in the presence of I_2.

Corresponding pyrazinoic acid (POA, 5-Cl-POA, or 6-Cl-POA) was converted to its acyl chloride by thionyl chloride ($SOCl_2$) with catalytic amount of *N,N*-dimethylformamide (DMF) (Scheme 1, b). The crude pyrazinoyl chloride was reacted with the corresponding 4-phenylthiazol-2-amine (**6a-e**) or 4-(pyridin-2-yl)thiazol-2-amine (**6f**) in the presence of triethylamine (TEA) as a base (Scheme 1, c). The aminolysis of the acyl chloride proceeded at RT and was complete typically in 1–2 hours as indicated by absence of the corresponding aminothiazole on TLC (silica, 30% EtOAc in hexane). Nevertheless, for organisational purposes, the reaction mixture was usually stirred overnight before workup.

[INSERT SCHEME 1, DOUBLE COLUMN WIDTH]

The final products were isolated as off-white, yellow or orange solids. Products were characterized with melting point, ^{1}H and ^{13}C NMR, IR spectra and elemental analysis. Results of the analyses were fully consistent with the proposed structures. In the IR spectra, the final compounds exerted a strong absorption at 1663–1698 cm^{-1} of the amidic carbonyl C=O. In ^{1}H NMR spectra, the signal of the H5′ aminothiazole hydrogen was a singlet at 7.58–7.92 ppm independent of the solvent; the signal of the amidic hydrogen appeared as a singlet at 12.02–12.84 ppm in DMSO-d_6 and 14.01–14.33 ppm in pyridine-d_5. However, this singlet in pyridine-d_5 was broad and of low intensity, therefore not recognizable in some compounds.

Some of the prepared compounds were hardly soluble or practically insoluble in DMSO at RT. From the series derived from non-substituted POA (series **7**), compounds with R^1 = halogen were troublesome. On contrary, in series derived from chloropyrazinoic acid (**8** and **9**), compounds with R^1 = H (**8a**, **9a**), methoxy (**8d**, **9d**) or dimethoxy (**8e**, **9e**) suffered from low solubility. Pyridinyl containing final compounds (**7f**, **8f**, **9f**) were of low solubility in all three series. The extent of solubility is roughly reported in Table 1 and can be judged from the interpretation of NMR spectra – compounds which were not soluble in DMSO at RT were measured at elevated temperatures, and those not soluble in hot DMSO were measured in pyridine. The limited solubility of final compounds

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can influence biological assays if compounds need to be dissolved in DMSO prior to testing.

Antimycobacterial activity

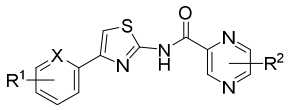
All of the final compounds were tested for *in vitro* whole cell growth inhibition activity against *Mtb* H37Rv, *M. kansasii*, *M. avium* and *M. smegmatis* by adjusted Microplate Alamar Blue Assay (MABA) based on binary dilution. See Table 1 for results expressed as minimum inhibitory concentrations (MIC) in μg/mL. Regarding the activity against *Mtb* H37Rv and antimycobacterial activity in general, the most advantageous variant of the *N*-acyl part were the derivatives of 6-Cl-POA (series **9**, three compounds with micromolar activity), followed by 5-Cl-POA (series **8,** two derivatives with MIC < 10 μM). On contrary, compounds derived from non-halogenated POA (series **7**) were completely inactive, with the exception of weakly active pyridin-2-yl derivative (**7f**). The superiority of 6-Cl-POA derivatives might be based on the structural similarity to derivatives presented by Meissner et al.,[11] as the most valuable derivatives of general structure **3** possessed halogen on C-3 of the phenyl ring, which corresponds to C-6 of the pyrazine ring in our compounds. This hypothesis was further confirmed by the results of molecular docking to mycobacterial FabH, the suggested target (see section 'On the possible mechanism of action' and Supplementary Information).

Regarding the substituent at C-4 of the aminothiazole ring, substituted phenyl produced significantly better derivatives than isosteric pyridin-2-yl. This is in sharp contrast with the series of Mjambilli et al. and Meissner et al., which preferred pyridin-2-yl substituent over phenyl as discussed in the introduction of this article. The problem of pyridin-2-yl based derivatives in our series might be (at the level of theory) in the insufficient permeation into mycobacteria (as explained in the following section). The most promising substituent on the phenyl ring was R^1 = 4-F (compounds **8b**, **9b**), followed by R^1 = 4-OCH_3 (**8d**, **9d**). This is in agreement with the most successful substitutions in formerly published series **3** (R = 3-halogen) and **4** (R = 4-OCH_3). Compounds with non-substituted phenyl (R^1 = H, **7a**, **8a**, **9a**) or 4-chlorophenyl (R^1= 4-Cl, **7c**, **8c**, **9c**) were inactive in the tested concentrations. It should be noted that the assay was complicated by low solubility of some compounds in the DMSO/broth testing system. Therefore, the term of inactivity is not equal among compounds with different solubility. Consult Table 1 for maximum concentrations of tested compounds achieved in individual assays.

The activity against non-tuberculous strains of *M. kansasii* and *M. avium* was at least 2–3 steps on the dilution scale lower in comparison to activity against *Mtb* H37Rv. The only exception was compound **9b**, which preserved the same level of activity (MIC = 0.78 μg/mL) against *Mtb*, *M. kansasii* and M. *avium*.

Activity against *M. smegmatis* was low and sporadic. Interestingly, the most successful substituents at C-4 of the phenyl ring were Cl or methoxy, on contrary to other strains, where rather small fluorine substituent was needed.

Table 1. *In vitro* whole cell antimycobacterial activity expressed as MIC and predicted permeability (MycPermCheck) of prepared compounds in comparison with isoniazid (INH).



No.	R^1	X	R^2	MW	*Mtb* μg/mL	*M. kans.* μg/mL	*M. avium* μg/mL	*M. smegm.* μg/mL	DMSO soluble	MPC
7a	H	CH	H	282.32	>50	>50	n.a.	>250	yes	0.628
7b	4-F	CH	H	300.31	>12.5	>12.5	>12.5	>250	no	0.627
7c	4-Cl	CH	H	316.76	>12.5	>12.5	>12.5	62.5	no	0.614
7d	4-OCH_3	CH	H	312.35	>100	>100	>100	>250	yes	0.516
7e	3,4-$(OCH_3)_2$	CH	H	342.37	>50	>50	>50	>125	yes	0.435
7f	H	N	H	283.31	6.25	>50	>50	>125	in hot	0.539
8a	H	CH	5-Cl	316.76	>100	>100	>100	>500	in hot	0.663
8b	4-F	CH	5-Cl	334.75	3.13	12.5	>50	>500	yes	0.650
8c	4-Cl	CH	5-Cl	351.21	>50	>50	>50	>500	yes	0.662
8d	4-OCH_3	CH	5-Cl	346.79	1.56	>50	>50	>500	in hot	0.553
8e	3,4-$(OCH_3)_2$	CH	5-Cl	376.82	>12.5	>12.5	>12.5	>250	no	0.475
8f	H	N	5-Cl	317.75	>50	>50	>50	>125	in hot	0.576
9a	H	CH	6-Cl	316.76	>50	>50	n.a.	>500	in hot	0.634
9b	4-F	CH	6-Cl	334.75	0.78	0.78	0.78	>250	yes	0.62
9c	4-Cl	CH	6-Cl	351.21	>12.5	>12.5	>12.5	62.5	yes	0.633
9d	4-OCH_3	CH	6-Cl	346.79	1.56	12.5	>12.5	62.5	no	0.521
9e	3,4-$(OCH_3)_2$	CH	6-Cl	376.82	1.56	>12.5	>12.5	>125	no	0.443
9f	H	N	6-Cl	317.75	>50	>50	>50	>125	in hot	0.545
INH	-	-	-	137.14	0.1–0.2	12.5–25	6.25–12.5	7.8–15.6	yes	n.a.

MPC – MycPermCheck[35] – predicted probability of permeation into mycobacterial cell, a value of 1.0 equals 100%; n.a. – not available; DMSO soluble – estimation of solubility in DMSO at RT at concentration intended for ^{1}H NMR experiment (15 mg in 600 μL of DMSO). In hot – soluble in hot DMSO (55–80 °C).

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In silico estimation of mycobacterial cell wall permeability

We used MycPermCheck v1.1 (MPC)[35] online-tool to calculate the probability of permeation of individual final compounds into mycobacterium. The MycPermCheck prediction is based on regression model of the physico-chemical properties (calculated descriptors) of permeable antimycobacterial compounds extracted from literature. Compounds with probability value above 0.60 are ranked as permeable while compounds with probability value under 0.52 as impermeable. (N.B. These cut-off limits changed between MycPermCheck versions 1.0 and 1.1 – see the online reference in Supplementary Information for details). According to the prediction, the theoretical permeability is hampered in 4-OCH_3 and 3,4-$(OCH_3)_2$ derivatives, which are ranked as impermeable (**7d**, **7e**, **8e**, **9e**) or with intermediate permeability (**8d**, **9d**). Analysing the descriptor values, the negative effect of methoxy group dwells mainly in the increased number of *H*-bond accepting groups in the molecule. Theoretical permeability of pyridin-2-yl derivatives (**7f**, **8f**, **9f**) is decreased by combined effect of lowered lipophilicity and higher than optimal number of *H*-bond acceptors. Derivatives with non-substituted phenyl (R^1 = H) or phenyl with simple halogen substitution (R^1 = 4-F, 4-Cl) are predicted to permeate the mycobacterial cell wall.

Without overestimating the significance of such theoretical prediction, we can conclude that the most favourable substitutions of the *N*-(4-phenylthiazol-2-yl)pyrazine-2-carboxamide core (which is, in fact, compound **7a**) are substituents that do not lower the lipophilicity and at the same time do not add up to the number of *H*-bond acceptors. The core itself is already at the upper limit for number of *H*-bond acceptors (accptHB = 6.5, recommended range 3.75–6.00). See Supplementary Information for full results.

In vitro HepG2 cytotoxicity

All compounds with significant antimycobacterial activity (MIC ≤ 6.25 μg/mL) were assessed for *in vitro* cytotoxicity in human hepatocyte carcinoma (HepG2) model using a standard protocol (Table 2). Selected compounds were further evaluated for cytotoxicity after prolonged exposure of 48 hours (Table 3). Limited solubility in the culture medium did not allow the determination of exact IC_{50} value for most of the compounds (in other words, IC_{50} was significantly above the maximal achieved concentration, see Table 2). Significant toxicity was found for compound **8b** with IC_{50} at approximately 25 μM and this value was confirmed in the prolonged exposure test (after 48 h). On contrary, the trends of inhibitory curves for most of the other tested compounds predicted IC_{50} at several hundreds of μM or higher. No cytotoxicity was observed for **9b** (best antimycobacterial activity, broad activity spectrum), not even in the confirmatory test after 48 h of exposure.

Table 2. Cytotoxicity of selected compounds in HepG2 cells expresses as IC_{50} in comparison with MIC against *Mtb* H37Rv.

No.	*Mtb* MIC [μM][a]	HepG2 IC_{50} [μM]	Tested concentrations [μM]	SI (IC_{50}/MIC)
7f	22.1	>25[b]	0.1–100	>1.1
8b	9.4	24.79	1–1000	2.7
8d	4.5	>75b (834.9)[c]	1–500	>16.7 (186)[d]
9b	2.3	>50[b]	1–500	>21.5
9d	4.5	>25b (622.8)[c]	0.1–100	>5.6 (138)[d]
9e	4.1	>25[a]	0.1–100	>6.0

[a] MIC[μM] calculated as MIC[μg/mL] / MW. [b] Measurement at higher concentration not possible due to precipitation in cell culture medium. [c] Values in parentheses represent hypothetic IC_{50} value calculated from the trend of inhibitory curve. [d] SI calculated based on hypothetic IC_{50} value.

Table 3. Confirmatory test of cytotoxicity of compounds **8b** and **9b** in HepG2 cells

No.	IC_{50} (μM) after 24 h	IC_{50} (μM) after 48 h	Tested concentrations (μM)
8b	22.43	18.05	1-500
9b	>50[a]	>50[a]	0.1-100

[a] Measurement at higher concentration not reproducible due to precipitation in cell culture medium.

On the possible mechanism of action

As stated in the Introduction, *N*-acyl-4-phenyl-2-aminothiazoles of general structure **1** were confirmed by enzyme inhibition assay as inhibitors of FabH of *E. coli*.[7] The authors of the study performed molecular docking to this enzyme (pdb: 3IL9) and showed that the predicted pIC_{50} (based on the assessment of the inhibitor-enzyme interaction energy predicted by the scoring function) correlated with real pIC_{50} values.[7] On contrary, no molecular target in mycobacteria was suggested for derivatives of structure **3** and **4**. High structural similarity of confirmed *E. coli* FabH inhibitors of structure **1** to antimycobacterial derivatives **3**, **4**, and our compounds is a solid base for raising the question whether these derivatives could work by inhibiting the mycobacterial FabH. (Note that FabH is known to be structurally and functionally conserved among many bacterial organisms [36]). We assessed this question by molecular docking as reported in the Supplementary Information (see for comprehensive results).

Indeed, the molecular docking study showed that the most active compounds of general structure **1**, **3** and **9** occupied the same space near to the catalytic triad of mycobacterial FabH and showed strong stabilizing interactions. Moreover, they were predicted to take the same binding mode. This indicates that previously reported 4-(pyridin-2-yl)-*N*-benzoyl-thiazol-2-amines (**3**) as well as (at least some of) our title compounds might act through inhibition of mycobacterial FabH. Of course, this is only a presumption to be confirmed by future enzyme inhibition assays or studies on mutants overproducing FabH.

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Selectivity versus promiscuity

Some aminothiazole derivatives are considered to be potential PAINS (Pan-assay interference compounds),[37] potentially having non-specific activity against many protein targets. However, PAINS rules were designed to rule out compounds that might interfere with high-throughput screening assays, and especially in biochemical assays on isolated targets.[38] Our testing of biological activity, on contrary, was done by human, and growth/no growth was indicated not only by Alamar blue readout (theoretically, the reduction of the stain could be caused by chemical properties of tested compounds) but additionally by visual inspection of growing mycobacteria based on turbidity. Further, in our series we also observed the selectivity of activity. as exemplified by the most active **9b**, which inhibited the growth of *Mtb, M. kansasii* and *M.* avium, but was totally inactive against *M. smegmatis* and also did not significantly alter the growth of HepG2 cells in tested concentrations. Selectivity of aminothiazole derivatives towards mycobacteria was reported repeatedly.[12, 39] Also it should not be forgotten that aminothiazole fragment is present in clinically used drugs (17 FDA approved drugs with aminothiazole moiety were found in the Zinc15 database FDA subset[40] on 2018-01-12). For all those reasons, we believe that despite PAINS properties of some aminothiazole derivatives, aminothiazoles should not be generally disregarded in the drug design.

Experimental

General

All chemicals and solvents (unless stated otherwise) were purchased from Sigma-Aldrich (Schnelldorf, Germany). 6-Chloropyrazine-2-carboxylic acid (6-Cl-POA) was available from our previous study.[41] 5-Cl-POA (Fluorochem, Derbyshire, UK) and POA (Sigma-Aldrich) were obtained commercially and used without any purification.

The reaction process and the purity of final compounds were checked using Merck Silica 60 F_{254} TLC plates (Merck, Darmstadt, Germany). Flash chromatography of the final compounds was run on automated chromatograph CombiFlash Rf 200 (Teledyne Isco, Lincoln, NE, USA) using columns filled with Kieselgel 60, 0.040–0.063 mm (Merck, Darmstadt, Germany), detection wavelength 260 nm. NMR spectra were recorded on Varian VNMR S500 (Varian, Palo Alto, CA, USA) at 500 MHz for ^{1}H and 125 MHz for ^{13}C. The spectra were recorded in DMSO-d_6 or pyridine-d_5 at ambient or elevated temperature as indicated along the interpretation. The chemical shifts as δ values in ppm are indirectly referenced to tetramethylsilane (TMS) *via* the solvent signal. IR spectra were recorded on Nicolet Impact 400 (Nicolet, Madison, WI, USA) using ATR-Ge method. Elemental analysis was performed on vario MICRO cube Element Analyzer (Elementar Analysensysteme, Hanau, Germany). All values regarding elemental analyses are given as percentages. Melting points were determined in open capillary on Stuart SMP30 melting point apparatus (Bibby Scientific Limited, Staffordshire, UK) and are uncorrected. Yields are given as percentages and refer to the amount of chromatographically pure product after all purification steps. Molecular docking was performed in Molecular Operating Environment (MOE), v2016.0802 (Chemical Computing Group Inc., Montreal, QC, Canada).

Chemistry

Synthesis of 4-substituted 2-aminothiazoles

4-Substituted aminothiazoles (**6a**, **6b**, **6d-f**) were prepared by a published procedure [34] with modifications. Thiourea (40 mmol, 2 molar equiv.) was mixed with iodine beads (20 mmol, 1 molar equiv.) in a mortar and the mixture was homogenised with a pestle. The solids were scratched off into a 250 mL round-bottom flask with a magnetic stirring bar and charged with the corresponding acetophenone or 2-acetopyridine (20 mmol, 1 molar equiv.). The flask was heated in an oil bath under air-cooled condenser at 100 °C with stirring. Upon heating, the mixture liquefied. After 2 hours, the mixture was cooled and it solidified again. Saturated aqueous solution of sodium thiosulfate ($Na_2S_2O_3$) was added (with concomitant mechanical disruption of the solid matrix) in sufficient amount to reduce the remaining iodine (until brown colour disappeared). The product as a suspension was filtered off and carefully washed with approx. 20 mL of diethyl ether (caution: product partially soluble) to remove the residues of unreacted acetophenone (or 2-acetopyridine). The crude product was dissolved in hot water, filtered, and the hot filtrate was adjusted to pH = 9 by aqueous Na_2CO_3. After cooling, the precipitated product was collected and recrystallized from hot EtOH if needed.

Representative procedure for compounds 8a–f

5-Chloropyrazine-2-carboxylic acid (5-Cl-POA, 300 mg, 1.89 mmol) was dispersed in 40 mL of dry toluene (PhMe) in round-bottom flask (250 mL) with a magnetic stirrer. While stirring, 1.0 mL of thionyl chloride ($SOCl_2$) was added followed by catalytic amount (1–2 drops) of *N,N*- dimethylformamide (DMF). The flask was placed into an oil bath under an air-cooled condenser and heated at 110 °C for 1 hour with vigorous stirring. (The stirring is needed to prevent the formation of deposits of the solid staring acid, which tend to be charcoaled by aggressive $SOCl_2$). In the reaction, the dispersed acid is consumed to form the soluble acyl chloride and the reaction mixture turns red to brown. When all of the starting acid was consumed, the solvents were evaporated under reduced pressure to obtain the crude 5-chloropyrazinoyl chloride as a dark red-brown liquid residue. To remove the traces of $SOCl_2$, 15 mL of dry PhMe was added to the residue and evaporated under reduced pressure repeatedly, usually 2–3 times.

The crude 5-chloropyrazinoyl chloride was dissolved in 15 mL of anhydrous acetone (solution A). 1.7 mmol (0.9 molar equiv. related to the starting 5-Cl-POA) of the corresponding 4-phenylthiazol-2-amine or 4-(pyridin-2-yl)thiazol-2-amine was dissolved in anhydrous acetone (20 mL) with 383 mg (3.8 mmol, 2 molar equiv.) of triethylamine (TEA) as an indifferent

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organic base (solution B). The final condensation was performed by drop-wise addition of solution A into stirred solution B at RT. The product usually started to form after several minutes as a precipitate. The reaction mixture was stirred for additional 2 hours (minimum) under TLC control (silica, 30% EtOAc in hexane). After completion, the reaction mixture was adsorbed on silica/sea sand (by evaporation of solvents under reduced pressure) and subjected to flash-chromatography (silica, gradient elution EtOAc in hexane 0–30%).

Compounds **9a–f** were prepared analogously from 6-chloropyrazinoic acid (6-Cl-POA).

Compounds **7a–f** were prepared analogously from pyrazine-2-carboxylic acid (POA). **Note**: The pyrazinoyl chloride is violet-black in colour and on contrary to chlorides of 5-Cl-POA or 6-Cl-POA it tends to sublime in the step of PhMe evaporation, therefore we advise for careful evaporation just at the pressure needed to distil PhMe.

Analytical data

4-Phenylthiazol-2-amine (**6a**). Off-white solid. Yield: 76%. mp 145–147 °C (lit.[42] 148–149 °C). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.81–7.76 (m, 2H, H-Ph), 7.43–7.36 (m, 2H, H-Ph), 7.33–7.28 (m, 1H, H-Ph), 6.72 (s, 1H, H-thiazole), 5.28 (bs, 2H, NH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.40, 151.18, 134.58, 128.56, 127.70, 125.95, 102.70, 102.69.

4-(4-Fluorophenyl)thiazol-2-amine (**6b**). Yellow crystalline. Yield: 58%. mp 105–108 °C (lit.[34] 108–110 °C). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.85–7.77 (m, 2H, H-Ph), 7.22–7.13 (m, 2H, H-Ph), 7.05 (bs, 2H, NH_2), 6.97 (s, 1H, H-thiazole).

4-(4-Chlorophenyl)thiazol-2-amine (**6c**). Purchased from Sigma-Aldrich and used without any purification.

4-(4-Methoxyphenyl)thiazol-2-amine (**6d**). Off-white solid. Yield: 63%. mp 200–202 °C (lit.[43] 204–207 °C). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.74–7.67 (m, 2H, H-Ph), 6.99 (bs, 2H, NH_2), 6.93–6.89 (m, 2H, H-Ph), 6.81 (s, 1H, H-thiazole), 3.76 (s, 3H, OCH_3).

4-(3,4-Dimethoxyphenyl)thiazol-2-amine (**6e**). Off-white solid. Yield: 71%. mp 182–185 °C (lit.[44] 185–189 °C). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.37–7.31 (m, 2H, H-Ph), 7.06 (bs, 2H, NH_2), 6.93 (d, *J* = 8.3 Hz, 1H, H-Ph), 6.87 (s, 1H, H-thiazole), 3.78 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3).

4-(Pyridin-2-yl)thiazol-2-amine (**6f**). Pink solid. Yield: 51%. mp 168–170 °C (lit.[10] 166–168 °C). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.54–8.49 (m, 1H, H-pyridine), 7.83–7.76 (m, 2H, H-pyridine), 7.25–7.21 (m, 2H, H-pyridine, H-thiazole), 7.09 (bs, 2H, NH_2). ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.62, 152.62, 150.30, 149.39, 137.14, 122.38, 120.25, 105.52.

In the following notation of ^{1}H NMR spectra, positions without a prime denote pyrazine hydrogens (e.g. H3), single prime denotes thiazole hydrogens (e.g. H5´), and double prime denotes phenyl or pyridinyl hydrogens (e.g. H3´´).

N-(4-Phenylthiazol-2-yl)pyrazine-2-carboxamide (**7a**). Off-white solid. Yield: 31%. mp 193.8–194.4 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.49 (bs, 1H, NH), 9.32 (s, 1H, H3), 8.94 (d, 1H, *J*=2.5 Hz, H6), 8.85–8.83 (m, 1H, H5), 7.95 (d, 2H, *J*=7.7 Hz, H2´´, H6´´), 7.76 (s, 1H, H5´), 7.44 (t, 2H, *J*=7.7 Hz, H3´´, H5´´), 7.36–7.30 (m, 1H, H4´´). ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.5, 157.4, 149.5, 148.4, 144.5, 144.0, 144.0, 134.3, 128.9, 128.1, 126.0, 109.3. IR (ATR-Ge, cm^{-1}): 3109, 3063, 1680 (C=O, CONH), 1570, 1317, 1022, 900, 714. Anal. calcd. for $C_{14}H_{10}N_4OS$ (MW 282.32): C, 59.56; H, 3.57; N, 19.85. Found: C, 59.41; H, 3.51; N, 19.71.

N-(4-(4-Fluorophenyl)thiazol-2-yl)pyrazine-2-carboxamide (**7b**), CAS 362482-53-9. Yellow solid. Yield: 36%. mp 220.4–221.0 °C (no mp reported in lit. so far). ^{1}H NMR (500 MHz, Pyridine-d_5) δ 14.01 (bs, 1H, NH), 9.68 (s, 1H, H3), 8.83 (d, 1H, *J*=2.4 Hz, H6), 8.68–8.64 (m, 1H. H5), 8.15–8.08 (m, 2H, H2´´, H6´´), 7.58 (s, 1H, H5´), 7.26–7.20 (m, 2H, H3´´, H5´´). ^{13}C NMR (125 MHz, Pyridine-d_5) δ 162.9 (d, *J*=246.1 Hz), 163.1, 158.9, 148.4, 145.4, 144.4, 143.6, 131.9, 131.8, 128.4 (d, *J*=7.5 Hz), 115.9 (d, *J*=22.0 Hz), 108.8. IR (ATR-Ge, cm^{-1}): 3160, 3071, 1673 (C=O, CONH), 1551, 1486, 1303, 1217, 1020, 903, 833, 734, 697. Anal. calcd. for $C_{14}H_9FN_4OS$ (MW 300.31): C, 55.99; H, 3.02; N, 18.66. Found: C, 56.19; H, 3.05; N, 18.87.

N-(4-(4-Chlorophenyl)thiazol-2-yl)pyrazine-2-carboxamide (**7c**). Pale yellow fluffy solid. Yield: 91%. mp 240.5–241.7 °C. ^{1}H NMR (500 MHz, Pyridine-d_5) δ 14.08 (bs, 1H, NH), 9.62 (s, 1H, H3), 8.33 (s, 1H, H6), 8.66 (s, 1H, H5), 8.12–8.01 (m, 2H, AA´, BB´, H2´´, H6´´), 7.63 (s, 1H, H5´), 7.51–7.43 (m, 2H, AA´, BB´, H3´´, H5´´). ^{13}C NMR (125 MHz, Pyridine-d_5) δ 163.1, 158.8, 148.3, 145.3, 144.3, 143.5, 135.0, 134.0, 133.5, 129.1, 127.9, 109.6. IR (ATR-Ge, cm^{-1}): 3103, 3051, 1673 (C=O, CONH), 1546, 1298, 1019, 902, 827, 735, 703. Anal. calcd. for $C_{14}H_9ClN_4OS$ (MW 316.76): C, 53.09; H, 2.86; N, 17.69. Found: C, 52.78; H, 2.88; N, 17.41.

N-(4-(4-Methoxyphenyl)thiazol-2-yl)pyrazine-2-carboxamide (**7d**). Yellow solid. Yield: 25%. mp 190–191.6 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.45 (bs, 1H, NH), 9.31 (s, 1H, H3), 8.94 (d, *J*=2.0 Hz, 1H, H6), 8.83 (bs, 1H, H5), 7.90–7.85 (m, AA´, BB´, 2H, H2´´, H6´´), 7.60 (s, 1H, H5´), 7.02–6.97 (m, AA´, BB´, 2H, H3´´, H5´´), 3.78 (s, 3H, OCH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.4, 159.3, 157.2, 149.4, 148.4, 144.5, 144.0, 127.4, 127.1, 114.3, 107.3, 55.3. IR (ATR-Ge, cm^{-1}): 2927, 2840, 1668 (C=O, CONH), 1550, 1296, 1247, 1171, 1020, 900, 832, 742, 702. Anal. calcd. for $C_{15}H_{12}N_4O_2S$ (MW 312.35): C, 57.68; H, 3.87; N, 17.94. Found: C, 57.97; H, 3.9; N, 17.58.

N-(4-(3,4-Dimethoxyphenyl)thiazol-2-yl)pyrazine-2-carboxamide (**7e**). Yellow solid. Yield: 25%. mp 200.5–201.7 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.44 (bs, 1H, NH), 9.31 (s, 1H, H3), 8.94 (d, *J*=2.0 Hz, 1H, H6), 8.83 (bs, 1H, H5), 7.65 (s, 1H, H5´), 7.52 (s, 1H, H2´´), 7.50 (d, 1H, *J*=8.3 Hz, H6´´), 7.00 (d, 1H, *J*=8.3 Hz, H5´´), 3.83 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.4, 157.1, 149.6, 149.0, 149.0, 148.4, 144.5, 144.0, 144.0, 127.3, 118.5, 112.1, 109.8, 107.6, 55.7. IR (ATR-Ge, cm^{-1}): 3117, 2959, 2835, 1663 (C=O, CONH), 1536, 1499, 1270, 1159, 1019, 906, 807, 760, 708. Anal. calcd. for $C_{16}H_{14}N_4O_3S$ (MW 342.37): C, 56.13; H, 4.12; N, 16.36. Found: C, 55.68; H, 4; N, 16.13.

N-(4-(Pyridin-2-yl)thiazol-2-yl)pyrazine-2-carboxamide (**7f**). Pale yellow solid. Fluffy. Yield: 60%. mp 244.6–245.7 °C. ^{1}H NMR (500 MHz, DMSO-d_6, 80 °C) δ 12.02 (bs, 1H, NH), 9.32 (s, 1H, H3), 8.98 (s, 1H, H6), 8.84–8.80 (m, 1H, H5), 8.61 (d, 1H, *J*=3.9 Hz, H6´´), 8.02 (d, 1H, *J*=8.0 Hz, H3´´), 7.92 (s, 1H, H5´),

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7.87 (t, 1H, *J*=8.0 Hz, H4´´), 7.35–7.28 (m, 1H, H5´´). ^{13}C NMR (125 MHz, Pyridine-d_5) δ 163.1, 158.9, 153.3, 151.0, 150.0, 148.4, 145.4, 144.4, 143.5, 137.2, 123.0, 120.9, 113.1. IR (ATR-Ge, cm^{-1}): 3153, 3073, 3007, 1677 (C=O, CONH), 1551, 1295, 1020, 903, 743, 706. Anal. calcd. for $C_{13}H_9N_5OS$ (MW 283.31): C, 55.11; H, 3.2; N, 24.72. Found: C, 54.78; H, 3.16; N, 24.46.

5-Chloro-*N*-(4-phenylthiazol-2-yl)pyrazine-2-carboxamide (**8a**). Pale yellow solid. Yield: 24%. mp 187.3–189.4 °C. ^{1}H NMR (500 MHz, DMSO-d_6, 55 °C) δ 12.37 (bs, 1H, NH), 9.14 (s, 1H, H3), 8.94 (s, 1H, H6), 7.94 (d, 2H, *J*=7.4 Hz, H2´´, H6´´), 7.72 (s, 1H, H5´), 7.43 (t, 2H, *J*=7.4 Hz, H3´´. H5´´), 7.36–7.30 (m, 1H, H4´´). ^{13}C NMR (125 MHz, Pyridine-d_5) δ 162.3, 158.7, 152.6, 150.8, 145.1, 143.6, 142.9, 135.7, 129.2, 128.3, 126.6, 109.2. IR (ATR-Ge, cm^{-1}): 3358, 3348, 2926, 1688 (C=O, CONH), 1537, 1129, 1023, 898, 741, 692. Anal. calcd. for $C_{14}H_9ClN_4OS$ (MW 316.76): C, 53.09; H, 2.86; N, 17.69. Found: C, 53.24; H, 2.95; N, 17.17.

5-Chloro-*N*-(4-(4-fluorophenyl)thiazol-2-yl)pyrazine-2-carboxamide (**8b**). Pale yellow solid. Yield: 16%. mp 197.4–198 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.63 (bs, 1H, NH), 9.13 (d, *J*=1.0 Hz, 1H, H3), 8.96 (d, *J*=1.0 Hz, 1H, H6), 8.01–7.93 (m, 2H, H2´´, H6´´), 7.74 (s, 1H, H5´), 7.29–7.23 (m, 2H, H3´´, H5´´). ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.0 (d, *J*=245.1 Hz), 161.7, 157.5, 151.6, 148.5, 144.5, 143.7, 142.7, 130.9, 128.0 (d, *J*=8.5 Hz), 115.8 (d, *J*=21.0 Hz), 109.2. IR (ATR-Ge, cm^{-1}): 3371, 3362, 3101, 1682 (C=O, CONH), 1541, 1132, 1024, 898, 846, 756, 706. Anal. calcd. for $C_{14}H_8ClFN_4OS$ (MW 334.75): C, 50.23; H, 2.41; N, 16.74. Found: C, 50.09; H, 2.39; N, 16.66.

5-Chloro-*N*-(4-(4-chlorophenyl)thiazol-2-yl)pyrazine-2-carboxamide (**8c**). Pale yellow solid. Yield: 33%. mp 219–220.1 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.70 (bs, 1H, NH), 9.14 (s, 1H, H3), 8.97 (s, 1H, H6), 8.01–7.90 (m, 2H, AA´, BB´, H2´´, H6´´), 7.83 (s, 1H, H5´), 7.54–7.44 (m, 2H, AA´, BB´, H3´´, H5´´). ^{13}C NMR (75 MHz, DMSO-d_6) δ 162.1, 157.9, 151.9, 148.6, 144.8, 144.1, 143.0, 133.4, 132.9, 129.2, 128.0, 110.4. IR (ATR-Ge, cm^{-1}): 3373, 3353, 3093, 1678 (C=O, CONH), 1540, 1132, 1017, 897, 840, 751, 703. Anal. calcd. for $C_{14}H_8Cl_2N_4OS$ (MW 351.21): C, 47.88; H, 2.3; N, 15.95. Found: C, 48.17; H, 2.25; N, 15.98.

5-Chloro-*N*-(4-(4-methoxyphenyl)thiazol-2-yl)pyrazine-2-carboxamide (**8d**). Pale yellow solid. Yield: 32%. mp 221.4–223.2 °C. ^{1}H NMR (500 MHz, DMSO-d_6, 65 °C) δ 12.22 (bs, 1H, NH), 9.13 (s, 1H, H3), 8.92 (s, 1H, H6), 7.89–7.81 (m, AA´, BB´, 2H, H2´´, H6´´), 7.53 (s, 1H, H5´), 7.02–6.95 (m, AA´, BB´, 2H, H3´´, H5´´), 3.80 (s, 3H, OCH_3). ^{13}C NMR (125 MHz, Pyridine-d_5) δ 162.2, 160.1, 158.6, 152.5, 150.7, 145.1, 143.6, 142.9, 128.2, 127.9, 114.6, 107.3, 55.3. IR (ATR-Ge, cm^{-1}): 3395, 3369, 3102, 1685 (C=O, CONH), 1541, 1253, 1130, 1020, 899, 840, 758, 703. Anal. calcd. for $C_{15}H_{11}ClN_4O_2S$ (MW 346.79): C, 51.95; H, 3.2; N, 16.16. Found: C, 51.58; H, 3.22; N, 15.94.

5-Chloro-*N*-(4-(3,4-dimethoxyphenyl)thiazol-2-yl)pyrazine-2-carboxamide (**8e**). Yellow solid. Yield: 27%. mp 210.7–212.4 °C. ^{1}H NMR (500 MHz, Pyridine-d_5) δ 14.33 (bs, 1H, NH), 9.37 (d, 1H, *J*=1.2 Hz, H3), 8.84 (d, 1H, *J*=1.2 Hz, H6), 7.82 (d, 1H, *J*=1.8 Hz, H2´´), 7.78 (dd, 1H, *J*=8.6 Hz, *J*=1.8 Hz, H6´´), 7.58 (s, 1H, H5´), 7.06 (d, 1H, *J*=8.6 Hz, H5´´), 3.84 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3). ^{13}C NMR (125 MHz, Pyridine-d_5) δ 162.2, 158.5, 152.5, 150.8, 145.0, 143.5, 142.9, 128.5, 119.1, 112.5, 110.7, 107.5, 55.8, 55.8. IR (ATR-Ge, cm^{-1}): 3380, 3095, 1693 (C=O, CONH), 1535, 1499, 1266, 1132, 1019, 953, 907, 873, 816, 740, 704. Anal. calcd. for $C_{16}H_{13}ClN_4O_3S$ (MW 376.82): C, 51; H, 3.48; N, 14.87. Found: C, 50.94; H, 3.59; N, 14.44.

5-Chloro-*N*-(4-(pyridin-2-yl)thiazol-2-yl)pyrazine-2-carboxamide (**8f**). Off-white solid. Yield: 66%. mp 244.3–245.3 °C. ^{1}H NMR (500 MHz, DMSO-d_6, 55 °C) δ 12.34 (bs, 1H, NH), 9.16 (s, 1H, H3), 8.92 (s, 1H, H6), 8.60 (d, 1H, *J*=4.4 Hz, H6´´), 8.02 (d, 1H, *J*=7.8 Hz, H3´´), 7.91–7.84 (m, 2H, H5´, H4´´), 7.34–7.28 (m, 1H, H5´´). ^{13}C NMR (125 MHz, Pyridine-d_5) δ 162.4, 158.9, 153.3, 152.6, 151.1, 150.0, 145.1, 143.6, 142.9, 137.2, 123.0, 120.9, 113.2. IR (ATR-Ge, cm^{-1}): 3356, 3113, 1689 (C=O, CONH), 1548, 1128, 1021, 922, 898, 803, 745, 703. Anal. calcd. for $C_{13}H_8ClN_5OS$ (MW 317.75): C, 49.14; H, 2.54; N, 22.04. Found: C, 49.47; H, 2.44; N, 21.95.

6-Chloro-*N*-(4-phenylthiazol-2-yl)pyrazine-2-carboxamide (**9a**). Yellow solid. Yield: 43%. mp 206.8–213.5 °C. ^{1}H NMR (500 MHz, DMSO-d_6, 55 °C) δ 12.51 (bs, 1H, NH), 9.26 (s, 1H, H3), 9.05 (s, 1H, H5), 7.95 (d, 2H, *J*=7.6 Hz, H2´´, H6´´), 7.73 (s, 1H, H5´), 7.44 (t, 2H, *J*=7.6 Hz, H3´´. H5´´), 7.36–7.31 (m, 1H, H4´´). ^{13}C NMR (125 MHz, Pyridine-d_5) δ 162.0, 158.7, 150.8, 148.4, 148.1, 144.3, 143.3, 135.3, 129.2, 128.3, 126.6, 109.3. IR (ATR-Ge, cm^{-1}): 3113, 3060, 1682 (C=O, CONH), 1550, 1296, 1170, 1010, 938, 904, 802, 765, 716. Anal. calcd. for $C_{14}H_9ClN_4OS$ (MW 316.76): C, 53.09; H, 2.86; N, 17.69. Found: C, 52.71; H, 2.84; N, 17.48.

6-Chloro-*N*-(4-(4-fluorophenyl)thiazol-2-yl)pyrazine-2-carboxamide (**9b**). Yellow-orange solid. Yield: 68%. mp 222.9–224.8 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.74 (bs, 1H, NH), 9.26 (s, 1H, H3), 9.07 (s, 1H, H5), 8.04–7.92 (m, 2H, H2´´, H6´´), 7.85 (s, 1H, H5´), 7.32–7.21 (m, 2H, H3´´, H5´´). ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.1 (d, *J*=245.1 Hz), 161.4, 157.5, 148.5, 148.2, 147.4, 143.8, 142.7, 130.9, 128.0 (d, *J*=7.5 Hz), 115.8 (d, *J*=22.0 Hz), 109.2. IR (ATR-Ge, cm^{-1}): 3119, 3060, 2925, 1682 (C=O, CONH), 1553, 1486, 1301, 1171, 1011, 937, 906, 834, 797, 739, 706. Anal. calcd. for $C_{14}H_8ClFN_4OS$ (MW 334.75): C, 50.23; H, 2.41; N, 16.74. Found: C, 50.48; H, 2.45; N, 16.7.

6-Chloro-*N*-(4-(4-chlorophenyl)thiazol-2-yl)pyrazine-2-carboxamide (**9c**). Yellow solid. Yield: 27%. mp 228.3–230.2 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.81 (bs, 1H, NH), 9.27 (s, 1H, H3), 9.08 (s, 1H, H5), 8.00–7.94 (m, 2H, AA´, BB´, H2´´, H6´´), 7.84 (s, 1H, H5´), 7.53–7.48 (m, 2H, AA´, BB´, H3´´, H5´´). ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.5, 157.6, 148.3, 148.2, 147.4, 143.8, 142.8, 133.1, 132.6, 129.0, 127.7, 110.2. IR (ATR-Ge, cm^{-1}): 3059, 1681 (C=O, CONH), 1547, 1298, 1091, 1011, 937, 904, 831, 734, 710. Anal. calcd. for $C_{14}H_8Cl_2N_4OS$ (MW 351.21): C, 47.88; H, 2.3; N, 15.95. Found: C, 47.89; H, 2.25; N, 15.85.

6-Chloro-*N*-(4-(4-methoxyphenyl)thiazol-2-yl)pyrazine-2-carboxamide (**9d**). Yellow-orange solid. Yield: 37%. mp 237.1–238.4 °C. ^{1}H NMR (500 MHz, Pyridine-d_5) δ 9.61 (s, 1H, H3), 9.00 (s, 1H, H5), 8.23–8.19 (m, 2H, AA´, BB´, H2´´, H6´´), 7.60 (s, 1H, H5´), 7.21–7.15 (m, 2H, AA´, BB´, H3´´, H5´´), 3.79 (s, 3H, OCH_3). ^{13}C NMR (125 MHz, Pyridine-d_5) δ 161.9, 160.1, 158.6, 150.8, 148.4, 148.1, 144.4, 143.3, 128.2, 127.9, 114.6, 107.4, 55.3. IR (ATR-Ge, cm^{-1}): 3110, 3058, 1681 (C=O, CONH), 1547, 1488, 1294, 1248, 1174, 1032, 1009, 938, 905, 826, 787, 741,

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709. Anal. calcd. for $C_{15}H_{11}ClN_4O_2S$ (MW 346.79): C, 51.95; H, 3.2; N, 16.16. Found: C, 51.76; H, 3.21; N, 15.96.

6-Chloro-*N*-(4-(3,4-dimethoxyphenyl)thiazol-2-yl)pyrazine-2-carboxamide (**9e**). Yellow to gold solid. Yield: 24%. mp 249.2–251.2 °C. ^{1}H NMR (500 MHz, Pyridine-d_5) δ 9.58 (s, 1H, H3), 8.92 (s, 1H, H5), 7.80 (d, 1H, *J*=1.7 Hz, H2´´), 7.76 (dd, 1H, *J*=8.3 Hz, *J*=1.7 Hz, H6´´), 7.59 (s, 1H, H5´), 7.05 (d, 1H, *J*=8.3 Hz, H5´´), 3.82 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3). ^{13}C NMR (125 MHz, Pyridine-d_5) δ 162.0, 158.6, 150.2, 148.4, 148.1, 144.4, 143.4, 128.6, 119.2, 112.6, 110.8, 107.6, 55.9, 55.9. IR (ATR-Ge, cm^{-1}): 3353, 3097, 1698 (C=O, CONH), 1537, 1497, 1265, 1171, 1142, 1016, 959, 874, 764, 739, 707. Anal. calcd. for $C_{16}H_{13}ClN_4O_3S$ (MW 376.82): C, 51; H, 3.48; N, 14.87. Found: C, 50.72; H, 3.52; N, 14.6.

6-Chloro-*N*-(4-(pyridin-2-yl)thiazol-2-yl)pyrazine-2-carboxamide (**9f**). Yellow fluffy solid. Yield: 59%. mp 241–243.1 °C. ^{1}H NMR (500 MHz, DMSO-d_6, 55 °C) δ 12.56 (bs, 1H, NH), 9.27 (s, 1H, H3), 9.06 (s, 1H, H5), 8.61 (d, 1H, *J*=4.9 Hz, H6´´), 8.02 (d, 1H, *J*=7.8 Hz, H3´´), 7.95 (s, 1H, H5´), 7.89 (t, 1H, *J*=7.8 Hz, H4´´), 7.36–7.30 (m, 1H, H5´´). ^{13}C NMR (125 MHz, DMSO-d_6, 55 °C) δ 161.2, 157.4, 151.9, 149.6, 149.4, 147.8, 147.2, 143.6, 142.4, 137.1, 122.8, 120.1, 112.7. IR (ATR-Ge, cm^{-1}): 3087, 1683 (C=O, CONH), 1553, 1423, 1295, 1011, 939, 909, 801, 762, 708. Anal. calcd. for $C_{13}H_8ClN_5OS$ (MW 317.75): C, 49.14; H, 2.54; N, 22.04. Found: C, 49.27; H, 2.5; N, 21.94.

Biological methods

Evaluation of In Vitro Antimycobacterial Activity

Microdilution panel method. Tested strains *M. tuberculosis* H37Rv CNCTC My 331/88 (ATCC 27294), *M. kansasii* Hauduroy CNCTC My 235/80 (ATCC 12478), *M. avium* ssp. *avium* Chester CNCTC My 80/72 (ATCC 15769) were obtained from Czech National Collection of Type Cultures (CNCTC), National Institute of Public Health, Prague, Czech Republic. Middlebrook 7H9 broth (Sigma-Aldrich, Steinheim, Germany) enriched with 0.4% (v/v) of glycerol (Sigma-Aldrich) and 10% (v/v) of OADC supplement (oleic acid, albumin, dextrose, catalase; Himedia, Mumbai, India) of declared pH = 6.6. Tested compounds were dissolved and diluted in DMSO, mixed with broth (25 µL of DMSO solution in 4.475 mL of broth and placed (100 µL) into microplate wells. Mycobacterial inocula were suspended in isotonic saline solution and the density was adjusted to 0.5–1.0 McFarland. These suspensions were diluted by 10^{-1} and used to inoculate the testing wells, adding 100 µL of mycobacterial suspension per well. Final concentrations of the tested compounds in wells were 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 µg/mL. Isoniazid (INH) was used as a positive control (inhibition of growth). Negative control (mycobacteria growth control) consisted of broth plus DMSO. Plates were statically incubated in a dark, humid atmosphere at 37 °C. After five days of incubation, 30 µL of Alamar Blue working solution (1:1 mixture of 0.1% resazurin sodium salt (aq. sol.) and 10% Tween 80) was added per well. Results were then determined after 24 h of incubation and interpreted according to Franzblau *et al.*[45] The minimum inhibition concentration (MIC, µg/mL) was determined as the lowest concentration that prevented the blue to pink colour change as indicated by naked eye.

In vitro growth inhibition of Mycobacterium smegmatis

The assay was performed with fast growing *Mycobacterium smegmatis* CCM 4622 (ATCC 607) from the Czech Collection of Microorganisms (Brno, Czech Republic). The technique used for activity determination was microdilution broth panel method using 96-well microtitration plates. Culturing medium was Middlebrook 7H9 broth of declared pH = 6.6 (Sigma-Aldrich, Steinheim, Germany) enriched with 0.4% (v/v) of glycerol (Sigma-Aldrich) and 10% (v/v) 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 ranging from 0.5 to 1.0 according to McFarland scale and diluted in ratio 1:20 with broth. Tested compounds were dissolved in DMSO (Sigma-Aldrich) and broth was added to obtain concentration 2000 µg/mL. Final concentrations were reached by binary dilution with broth and addition of mycobacterial suspension and were set as 500, 250, 125, 62.5, 31.25, 15.625, 7.81, and 3.91 µg/mL except for standards rifampicin (RIF), where the final concentrations were 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195 and 0.098 µg/mL, and ciprofloxacin (CPF), where the final concentrations were 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156, and 0.0078 µg/mL. The final concentration of DMSO did not exceeded 2.5% (v/v) and did not affect the growth of *M. smegmatis*. Positive growth control (broth, DMSO, mycobacteria) and negative growth control (broth, DMSO) were included.

After inoculation, 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. Stain was prepared by dissolving resazurin sodium salt (Sigma-Aldrich) in deionised water to get 0.02% solution. Then 10% aqueous solution of Tween 80 (Sigma-Aldrich) 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. Antimycobacterial activity was expressed as minimum inhibition concentration (MIC) and the value was read based on stain colour change (blue colour – no growth; pink colour – growth). Standards used for activity determination were isoniazid (INH), rifampicin (RIF) and ciprofloxacin (CPX) (Sigma-Aldrich). MIC values for standards were in ranges 7.81-15.625 µg/mL for INH, 0.78-1.56 µg/mL for RIF and 0.098-0.195 µg/mL for CPX. All experiments were conducted in duplicate.

HepG2 Cytotoxicity Determination

The human liver hepatocellular carcinoma cell line HepG2 (passage 32-34) purchased from Health Protection Agency Culture Collections (ECACC, Salisbury, UK) was routinely cultured in Minimum Essential Eagle Medium (MEM; Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (PAA, Austria), 1% (v/v) L-glutamine solution (Sigma-Aldrich) and 1%

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(v/v) non-essential amino acid solution (Sigma-Aldrich) in a humidified atmosphere containing 5% CO_2 at 37 °C. For subculturing, the cells were harvested after trypsin/EDTA (Sigma-Aldrich) treatment at 37°C. To evaluate the cytotoxicity, the HepG2 cells treated with the tested substances were used as experimental groups whereas untreated HepG2 cells served as control groups.

HepG2 cells were seeded in a density of 1×10^4 cells per well on a 96-well plate. Next day (24 h after seeding) the cells were treated with tested substances dissolved in DMSO at different concentrations ranging from 0.1 to 1000 µM (depending on the solubility, see Table 2). Maximal incubation concentration of DMSO in a well did not exceed 1% (v/v). The treatment was carried out in triplicates in a humidified atmosphere containing 5% of CO_2 at 37 °C. The controls representing 100% cell viability (untreated cells), 0% cell viability (cells treated with 10% DMSO), no-cell controls and vehiculum controls were incubated in triplicates simultaneously. After 24 h exposure to tested compounds, CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) reagent was added to each well according to the manufacturer`s recommendations. After 2 h incubation at 37 °C in humidified, 5% of CO_2 containing atmosphere, the absorbance was recorded at 490 nm. Inhibitory curves were constructed for each compound plotting incubation concentrations *vs.* percentage of absorbance relative to untreated control. The standard toxicological parameter IC_{50} was calculated by nonlinear regression analysis of the inhibitory curves using GraphPad Prism software, version 6 (GraphPad Software, Inc., CA, USA).

Confirmatory test of HepG2 Cytotoxicity

The HepG2 cells (see previous section) were seeded in density 1×10^4 cells per well on a 96-well plate. Next day (24 h after seeding) they were treated with tested substances dissolved in DMSO (maximal incubation concentration of DMSO was 1% v/v). The tested substances were prepared according to their solubility in DMSO at incubation concentrations 0.1–500 µM (see Table 3). The treatment was carried out in a humidified atmosphere containing 5% CO_2 at 37 °C in triplicates for 24 h and 48 h. The controls representing 100% cell viability, 0% cell viability (the cells treated with 10% DMSO and the cells treated with Lysis Solution 1:25), no-cell controls and vehiculum controls were incubated in triplicates simultaneously. After 24 h exposure, the reagent from the kit CellTox™ Green Cytotoxicity Assay (Promega, Madison, WI, USA) was prepared and added according to the recommendation of the manufacturer. After 15 min incubation at room temperature, the fluorescence was measured at $485nm_{Ex}/520nm_{Em}$. The measurement was repeated after 24 h to get results for the exposure period 48 h. Inhibitory curves were constructed for each compound plotting incubation concentrations vs. percentage of fluorescence relative to untreated control. The standard toxicological parameter IC_{50} was calculated by nonlinear regression analysis of the inhibitory curves using GraphPad Prism software version 6 (GraphPad Software, Inc., CA, USA).

Conclusion

We have designed and synthesized three series of hybrid compounds based on a combination of first-line antitubercular pyrazinamide (PZA) and formerly identified antimycobacterial scaffold of 4-arylthiazol-2-amine. Prepared compounds were fully characterized and tested for *in vitro* growth inhibition activity against *Mtb* H37Rv and several non-tuberculous mycobacterial strains in a Microplate Alamar Blue Assay. We have identified several compounds with micromolar activity. The most active compound **9b** had also the broadest spectrum of activity and inhibited the growth of *Mtb*, *M. kansasii*, and *M. avium* with MIC = 0.78 µg/mL (2.3 µM) and selectivity index SI > 20. Most of the tested compounds were non-toxic on HepG2 cell line. Molecular docking study suggested that our compounds could share the mechanism of action with structurally very similar derivatives of 2-aminothiazoles, that is, act as inhibitors of mycobacterial FabH. (Myco)bacterial FabH is considered promising target in development of antimicrobial compounds.[36]

Our compounds, especially **9b** for its broad spectrum of antimycobacterial activity, can be considered promising leads for further development. However, there are risks to assess. Firstly, similar 2-aminothiazole derivatives of general structure **3** were found to be rather metabolically unstable in mouse, rat and human liver microsomes, with half-time ranging from several minutes to half an hour.[10, 11] The metabolic instability of the described compounds of course has possible implications for their development as potential drug candidates. The fast metabolism can render the drug candidate useless for systemic administration. However, there is still the option of local administration, in the case of TB probably *via* pulmonary inhalation. The half-time of aminothiazole derivatives **3** was strongly dependant on the substitution on the benzene ring in acyl part of the molecule,[10, 11] so there is a fair chance that the exchange of the benzoyl for pyrazinoyl (as present in compounds of our study) could afford more stable compounds. Nevertheless, the metabolic stability of the compounds of our study remains to be assessed.

The compounds of this study were designed as hybrids of PZA and the 4-arylthiazol-2-amine scaffold. Based on our results, the pyrazine core, especially the 6-Cl-pyrazine, can substitute the phenyl moiety of pattern compounds of general structure **3**. Whether the introduction of PZA scaffold introduces the ability to hit another target (apart from supposed FabH and possibly connected with the mechanism of action of PZA), is not clear.

Note: After our study was finished and the manuscript written, Pieroni et al. published another article on aminothiazole derivatives with potent and selective antimycobacterial activity *in vitro*.[39] They showed that the phenyl or pyridin-2-yl at C-4 of the aminothiazole core could be exchanged for isoxazol-5-yl with polar substituents in position 3. In this series, the amino group of the thiazole core was substituted with phenyl. We recommend this publication to readers interested in the topic.

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Conflicts of interest

The authors declare no competing interest.

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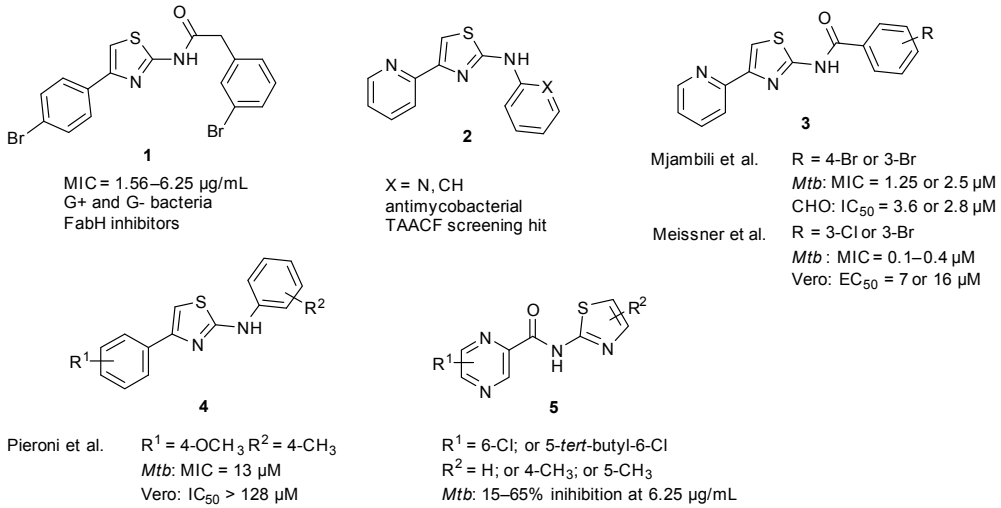

Fig. 1. Selected 2-aminothiazole derivatives with *in vitro* antibacterial or antimycobacterial activity.

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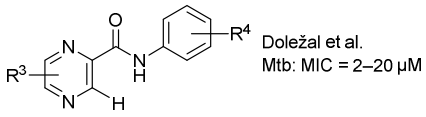
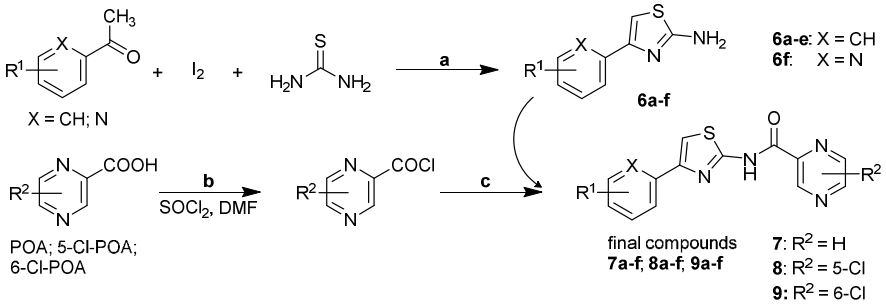


Fig. 2. Anilides of POA with antimycobacterial activity.

[SINGLE COLUMN]



Scheme 1. Synthesis of final compounds.

[DOUBLE COLUMN]

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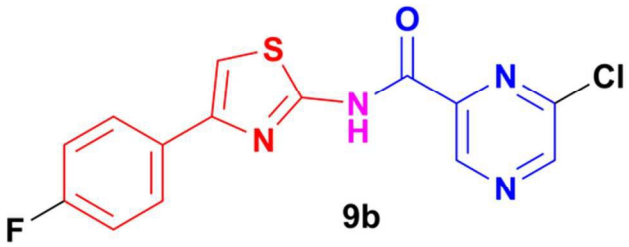

9b

pyrazinamide

4-aryl-2-aminothiazole

in vitro growth inhibition - MABA
M. tbc H37Rv, *M. kansasii*, *M. avium*
MIC = 2.3 μM

HepG2 IC_{50} > 50 μM; SI > 21

36x17mm (600 x 600 DPI)