 Cite this: *Med. Chem. Commun.*,
2018, 9, 685

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

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Hybrid compounds based on a combination of the first-line antitubercular pyrazinamide (PZA) and a 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* by Microplate Alamar Blue Assay at neutral pH. Active compounds were tested for *in vitro* cytotoxicity in the human hepatocellular carcinoma cell line (HepG2). The most active 6-chloro-*N*-[4-(4-fluorophenyl)thiazol-2-yl]pyrazine-2-carboxamide (9b) also had the broadest spectrum of activity and inhibited *M. tuberculosis*, *M. kansasii*, and *M. avium* with MIC = 0.78 $\mu\text{g mL}^{-1}$ (2.3 μM) and a selectivity index related to HepG2 cells of SI > 20. Structure–activity relationships within the series are discussed. Based on its 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.

 Received 30th January 2018,
Accepted 21st February 2018

DOI: 10.1039/c8md00056e

rsc.li/medchemcomm

Introduction

According to the WHO Global Tuberculosis Report 2017, an 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 as the leading cause of death among all infectious diseases, followed by HIV/AIDS. New estimates account for 23% of the 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 for developing active TB. Globally in 2016, 477 000 new HIV-positive TB cases were reported (46% of the estimated incidents). Almost three-quarters of these cases were in the African Region.¹ The Global Plan to Stop Tuberculosis (2006–2015)³ aimed at reducing the prevalence of and deaths due to TB by 50% compared with the 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

Contrary to the positive trends in global epidemiology of TB, widespread 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 drawback with 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 a 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 the *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 a minimum inhibitory concentration (MIC) of 1.56–6.25 $\mu\text{g mL}^{-1}$.⁷ Enzyme inhibition assays

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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/c8md00056e

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

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, which is a part of the fatty acid synthase II complex (FAS II). In mycobacteria, FabH constitutes a crucial link between FAS I and FAS II systems.⁸

For the antimycobacterial activity, the 4-(pyridin-2-yl)thiazol-2-amine scaffold, as represented by the general structure **2**, was discovered by a phenotypic whole cell high-throughput screening campaign run by the 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 an amino group substituted mostly by acyls derived from simple substituted benzoic acids and showed that such *N*-benzoyl derivatives were superior in activity compared to derivatives with an unsubstituted amino group or an 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. The best compounds inhibited the growth of *Mtb* H37Rv with MIC in the low micromolar range. Unfortunately, all promising compounds exerted significant *in vitro* cytotoxicity on CHO cells with IC₅₀ at low micromolar levels. The superiority of *N*-benzoyl derivatives of the 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 a higher selectivity index (MIC/EC₅₀). The best compounds exerted MIC at submicromolar levels and cytotoxicity (Vero cells), with EC₅₀ 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 and the superiority of *N*-benzoyl

derivatives over *N*-phenyl derivatives, and revealed a 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 acids. 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 from 1.6–6.3 μM .¹¹ From this observation, we were inspired to design similar compounds incorporating the pyrazinoyl moiety. The mechanism of the antimycobacterial activity of compounds of the general structure **3** was neither proposed by Mjambilli nor Meissner.

Another comprehensive SAR study of aminothiazole derivatives similar to structures **2** and **3** was published by Kesicki *et al.*,¹² who altered the substitution at both C-2 and C-4 of the central aminothiazole core. Consistent with previous findings, the 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 linkers) and aryl substituents (amino linker), but *N*-acyl substituted compounds were indicated to have lower cytotoxicity (Vero cells) and therefore more favourable selectivity index values.

All the 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. In contrast, the research group of Pieroni *et al.*¹³ published a series of antimycobacterial aminothiazole derivatives (Fig. 1, **4**) having a 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 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 ($IC_{50} > 128 \mu M$ in Vero cells). The activity of derivatives **4** could have been lowered by a 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 the general structure **4** with $R^1 = R^2 = 4\text{-F}$ having MIC = $6.25 \mu M$ 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 also be 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

The first-line antitubercular agent pyrazinamide (PZA; pyrazine-2-carboxamide) exerts synergistic effects with rifampicin and is active even against a 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 a concentration of $6.25 \mu g mL^{-1}$.²⁸ Anilides of POA (Fig. 2) with simple substituents on the pyrazine ring (R^3 is H, methyl, *tert*-butyl, chloro or a combination thereof) and simple substituents on the benzene ring (R^4 is short alkyl, OH, halogen, nitro or a combination thereof) showed *in vitro* growth inhibiting activity against *Mtb* H37Rv, the best compounds with MIC at micromolar levels (2–20 μM). Their SAR have been reviewed elsewhere.^{29–31}

The design of the title compounds of our study aimed to take the best from previously published series and incorporate the PZA fragment into a 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-OCH₃ as in **4**, and modifications. Substituents of the pyrazine core were inspired by previously published derivatives of 5- or 6-chloropyrazinoic 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 another 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** and **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 the theoretical yield after simple recrystallization from hot water and EtOH, consequently. The identity of 4-phenylthiazol-2-amines was confirmed by the melting points and ¹H NMR spectra in comparison with data published in the literature. Similarly, we prepared 4-(pyridin-2-yl)thiazol-2-amine (**6f**) by condensation of 2-acetopyridine with thiourea in the presence of I₂.

The corresponding pyrazinoic acid (POA, 5-Cl-POA, or 6-Cl-POA) was converted to its acyl chloride by thionyl chloride (SOCl₂) with a 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 the 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.

The final products were isolated as off-white, yellow or orange solids. Products were characterized using their melting point, ¹H, ¹³C NMR, and IR spectra and elemental analysis. The 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}, attributed to the amidic carbonyl C=O. In the ¹H 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, and 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. In contrast, in series derived from chloropyrazinoic acid (**8** and **9**), compounds with $R^1 =$ H (**8a**

Fig. 2 Anilides of POA with antimycobacterial activity.

Scheme 1 Synthesis of final compounds.

and 9a), methoxy (8d and 9d) or dimethoxy (8e and 9e) suffered from low solubility. Pyridinyl containing final compounds (7f, 8f and 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 the 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 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 $\mu\text{g mL}^{-1}$. Regarding the activity against *Mtb* H37Rv and antimycobacterial activity in general, the most advantageous variants 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). In contrast, compounds derived from non-halogenated POA (series 7) were completely inactive, with the exception of the 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 the 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

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	<i>Mtb</i> $\mu\text{g mL}^{-1}$	<i>M. kans.</i> $\mu\text{g mL}^{-1}$	<i>M. avium</i> $\mu\text{g mL}^{-1}$	<i>M. smegm.</i> $\mu\text{g mL}^{-1}$	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	0.620
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 – MycPermCheck³⁵ – predicted the probability of permeation into mycobacterial cells; a value of 1.0 equals 100%; n.a. – not available; DMSO soluble – estimation of solubility in DMSO at RT at a concentration intended for the ¹H NMR experiment (15 mg in 600 μL of DMSO). In hot – soluble in hot DMSO (55–80 °C).

mycobacterial FabH, the suggested target (see section 'On the possible mechanism of action' and the ESI†).

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 the 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 $R^1 = 4\text{-F}$ (compounds **8b** and **9b**), followed by $R^1 = 4\text{-OCH}_3$ (**8d** and **9d**). This is in agreement with the most successful substitutions in formerly published series 3 ($R = 3\text{-halogen}$) and 4 ($R = 4\text{-OCH}_3$). Compounds with non-substituted phenyl ($R^1 = \text{H}$, **7a**, **8a** and **9a**) or 4-chlorophenyl ($R^1 = 4\text{-Cl}$, **7c**, **8c** and **9c**) were inactive in the tested concentrations. It should be noted that the assay was complicated by the low solubility of some compounds in the DMSO/broth testing system. Therefore, the term of inactivity is not equal among compounds with different solubilities. Consult Table 1 for the maximum concentrations of the test 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 with the activity against *Mtb* H37Rv. The only exception was compound **9b**, which preserved the same level of activity ($\text{MIC} = 0.78 \mu\text{g mL}^{-1}$) against *Mtb*, *M. kansasii* and *M. avium*.

The activity against *M. smegmatis* was low and sporadic. Interestingly, the most successful substituent at C-4 of the phenyl ring was Cl or methoxy, in contrast to other strains, where a rather small fluorine substituent was needed.

In silico estimation of mycobacterial cell wall permeability

We used the 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 a regression model of the physico-chemical properties (calculated descriptors) of permeable antimycobacterial compounds extracted from the literature. Compounds with a probability value above 0.60 are ranked as permeable while compounds with a 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 the ESI† for details). According to the prediction, the theoretical permeability is hampered in 4-OCH₃ and 3,4-(OCH₃)₂ derivatives, which are ranked as impermeable (**7d**, **7e**, **8e**, and **9e**) or with intermediate permeability (**8d** and **9d**). Analysing the descriptor values, the negative effect of the methoxy group dwells mainly in the increased number of H-bond accepting groups in the molecule. The theoretical permeability of pyridin-2-yl derivatives (**7f**, **8f** and **9f**) is decreased by the combined effect of lowered lipophilicity and higher than optimal number of H-bond acceptors. Derivatives

with non-substituted phenyl ($R^1 = \text{H}$) or phenyl with a simple halogen substituent ($R^1 = 4\text{-F}$, 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 substituents 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 the number of H-bond acceptors (acceptHB = 6.5, recommended range 3.75–6.00). See the ESI† for full results.

In vitro HepG2 cytotoxicity

All compounds with significant antimycobacterial activity ($\text{MIC} \leq 6.25 \mu\text{g mL}^{-1}$) were assessed for *in vitro* cytotoxicity in a 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). The limited solubility in the culture medium did not allow the determination of the exact IC₅₀ value for most of the compounds (in other words, IC₅₀ was significantly above the maximal achieved concentration, see Table 2). Significant toxicity was found for compound **8b** with IC₅₀ of approximately 25 μM and this value was confirmed in the prolonged exposure test (after 48 h). In contrast, the trends of inhibitory curves for most of the other test compounds predicted IC₅₀ of several hundreds of μM or higher. No cytotoxicity was observed for **9b** (with the best antimycobacterial activity and a broad activity spectrum), not even in the confirmatory test after 48 h of exposure.

On the possible mechanism of action

As stated in the Introduction, *N*-acyl-4-phenyl-2-aminothiazoles of the 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 on this enzyme (PDB: 3IL9) and showed that the predicted pIC₅₀ (based on the assessment of the inhibitor–enzyme interaction energy predicted by the scoring function) correlated with real pIC₅₀ values.⁷ In contrast, no molecular target in mycobacteria was suggested for derivatives of structures 3 and 4. The 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 of whether these derivatives could work by inhibiting the mycobacterial FabH (note that FabH is known to be structurally and functionally conserved among many bacterial organisms³⁶). We assessed this question by molecular docking, as reported in the ESI† (see for comprehensive results).

Indeed, the molecular docking study showed that the most active compounds of the general structures **1**, **3** and **9** occupied the same space near the catalytic triad of mycobacterial FabH and showed strong stabilizing interactions. Moreover, they were predicted to take the same binding mode.

Table 2 Cytotoxicity of selected compounds in HepG2 cells expressed as IC₅₀ in comparison with MIC against *Mtb* H37Rv

No.	<i>Mtb</i> MIC [μ M] ^a	HepG2 IC ₅₀ [μ M]	Tested concentrations [μ M]	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 [μ M] calculated as MIC [μ g mL⁻¹]/MW. ^b Measurement at higher concentrations not possible due to precipitation in the cell culture medium. ^c Values in parentheses represent hypothetical IC₅₀ values calculated from the trend of the inhibitory curve. ^d SI calculated based on hypothetical IC₅₀ values.

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

No.	IC ₅₀ (μ M) after 24 h	IC ₅₀ (μ M) after 48 h	Tested concentrations (μ M)
8b	22.43	18.05	1–500
9b	>50 ^a	>50 ^a	0.1–100

^a Measurement at higher concentrations not reproducible due to precipitation in the cell culture medium.

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 the 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.

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, especially with biochemical assays on isolated targets.³⁸ Our testing of biological activity, in contrast, was conducted by humans, and growth/no growth was indicated not only by the Alamar blue readout (theoretically, the reduction of the stain could be caused by the 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 the tested concentrations. The selectivity of aminothiazole derivatives towards mycobacteria was reported repeatedly.^{12,39} Also, it should not be forgotten that the aminothiazole fragment is present in clinically used drugs (17 FDA approved drugs with the aminothiazole moiety were found in the Zinc15 database FDA subset⁴⁰ on 2018-01-12). For all these reasons, we believe that despite the 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 F₂₅₄ TLC plates (Merck, Darmstadt, Germany). Flash chromatography of the final compounds was run on a CombiFlash Rf 200 automated chromatograph (Teledyne Isco, Lincoln, NE, USA) using columns filled with Kieselgel 60 (0.040–0.063 mm; Merck, Darmstadt, Germany) and a detection wavelength of 260 nm. NMR spectra were recorded on Varian VNMR S500 (Varian, Palo Alto, CA, USA) at 500 MHz for ¹H and 125 MHz for ¹³C. The spectra were recorded in DMSO-*d*₆ or pyridine-*d*₅ at ambient or elevated temperature, as indicated by 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 the ATR-Ge method. Elemental analysis was performed using a vario MICRO cube elemental analyzer (Elementar Analysensysteme, Hanau, Germany). All values obtained from elemental analyses are given as percentages. Melting points were determined in open capillaries using 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 the purification steps. Molecular docking was performed using 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 procedure³⁴ 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 an air-cooled condenser at 100 °C with stirring. Upon heating, the mixture liquefied. After 2 hours, the mixture was cooled and it solidified again. A saturated aqueous solution of sodium thiosulfate (Na₂S₂O₃) was added (with concomitant mechanical disruption of the solid matrix) in a sufficient amount to reduce the remaining iodine (until the 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 and filtered, and the hot filtrate was adjusted to pH = 9 with aqueous Na₂CO₃. 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 a round-bottom flask (250 mL) with a magnetic stirrer. While stirring, 1.0 mL of thionyl chloride (SOCl₂) was added followed by a catalytic amount (1–2 drops) of *N,N*-dimethylformamide (DMF). The flask was placed in an oil bath under an air-cooled condenser and heated at 110 °C for 1 hour with vigorous stirring (stirring is needed to prevent the formation of deposits of the solid starting acid, which tend to be charcoaled by aggressive SOCl₂). 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 SOCl₂, 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 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 an additional 2 hours (minimum) under TLC control (silica, 30% EtOAc in hexane). After completion, the reac-

tion 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:** Pyrazinoyl chloride is violet-black in colour and in contrast to chlorides of 5-Cl-POA or 6-Cl-POA it tends to sublime in the PhMe evaporation step; therefore, we advise 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). ¹H NMR (500 MHz, CDCl₃) 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, NH₂). ¹³C NMR (125 MHz, CDCl₃) 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 solid. Yield: 58%. mp 105–108 °C (lit.³⁴ 108–110 °C). ¹H 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, NH₂), 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). ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.74–7.67 (m, 2H, H-Ph), 6.99 (bs, 2H, NH₂), 6.93–6.89 (m, 2H, H-Ph), 6.81 (s, 1H, H-thiazole), 3.76 (s, 3H, OCH₃).

4-(3,4-Dimethoxyphenyl)thiazol-2-amine (6e). Off-white solid. Yield: 71%. mp 182–185 °C (lit.⁴⁴ 185–189 °C). ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.37–7.31 (m, 2H, H-Ph), 7.06 (bs, 2H, NH₂), 6.93 (d, *J* = 8.3 Hz, 1H, H-Ph), 6.87 (s, 1H, H-thiazole), 3.78 (s, 3H, OCH₃), 3.76 (s, 3H, OCH₃).

4-(Pyridin-2-yl)thiazol-2-amine (6f). Pink solid. Yield: 51%. mp 168–170 °C (lit.¹⁰ 166–168 °C). ¹H 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, NH₂). ¹³C 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 notations in ¹H NMR spectra, positions without a prime denote pyrazine hydrogen atoms (*e.g.* H3), those with a single prime denote thiazole hydrogen atoms (*e.g.* H5'), and those with a double prime denote phenyl or pyridinyl hydrogen atoms (*e.g.* H3'').

***N*-(4-Phenylthiazol-2-yl)pyrazine-2-carboxamide (7a).** Off-white solid. Yield: 31%. mp 193.8–194.4 °C. ¹H 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''). ¹³C 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). 1H 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. 1H 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. 1H 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, OCH₃). ^{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. 1H 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, OCH₃), 3.78 (s, 3H, OCH₃). ^{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. 1H 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'), 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. 1H 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. 1H 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. 1H 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. 1H 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, OCH₃). ^{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. 1H 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, OCH₃), 3.79 (s, 3H, OCH₃). ¹³C 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 C₁₆H₁₃ClN₄O₃S (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. ¹H 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, H_{6''}), 8.02 (d, 1H, *J* = 7.8 Hz, H_{3''}), 7.91–7.84 (m, 2H, H_{5'}, H_{4''}), 7.34–7.28 (m, 1H, H_{5''}). ¹³C 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 C₁₃H₈ClN₅OS (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. ¹H 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, H_{2''}, H_{6''}), 7.73 (s, 1H, H_{5'}), 7.44 (t, 2H, *J* = 7.6 Hz, H_{3''}, H_{5''}), 7.36–7.31 (m, 1H, H_{4''}). ¹³C 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 C₁₄H₉ClN₄OS (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. ¹H 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, H_{2''}, H_{6''}), 7.85 (s, 1H, H_{5'}), 7.32–7.21 (m, 2H, H_{3''}, H_{5''}). ¹³C 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 C₁₄H₈ClFN₄OS (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. ¹H 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', H_{2''}, H_{6''}), 7.84 (s, 1H, H_{5'}), 7.53–7.48 (m, 2H, AA', BB', H_{3''}, H_{5''}). ¹³C 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 C₁₄H₈Cl₂N₄OS (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. ¹H 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', H_{2''}, H_{6''}), 7.60

(s, 1H, H_{5'}), 7.21–7.15 (m, 2H, AA', BB', H_{3''}, H_{5''}), 3.79 (s, 3H, OCH₃). ¹³C 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, 709. Anal. calcd. for C₁₅H₁₁ClN₄O₂S (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. ¹H 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, H_{2''}), 7.76 (dd, 1H, *J* = 8.3 Hz, *J* = 1.7 Hz, H_{6''}), 7.59 (s, 1H, H_{5'}), 7.05 (d, 1H, *J* = 8.3 Hz, H_{5''}), 3.82 (s, 3H, OCH₃), 3.78 (s, 3H, OCH₃). ¹³C 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 C₁₆H₁₃ClN₄O₃S (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. ¹H 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, H_{6''}), 8.02 (d, 1H, *J* = 7.8 Hz, H_{3''}), 7.95 (s, 1H, H_{5'}), 7.89 (t, 1H, *J* = 7.8 Hz, H_{4''}), 7.36–7.30 (m, 1H, H_{5''}). ¹³C 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 C₁₃H₈ClN₅OS (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. Test strains *M. tuberculosis* H37Rv CNCTC My 331/88 (ATCC 27294), *M. kansasii* Hauduroy CNCTC My 235/80 (ATCC 12478), and *M. avium* ssp. *avium* Chester CNCTC My 80/72 (ATCC 15769) were obtained from the Czech National Collection of Type Cultures (CNCTC), National Institute of Public Health, Prague, Czech Republic. For culturing and testing, we used Middlebrook 7H9 broth of declared pH = 6.6 (Sigma-Aldrich, Steinheim, Germany) enriched with 0.4% (v/v) glycerol (Sigma-Aldrich) and 10% (v/v) OADC supplement (oleic acid, albumin, dextrose, catalase; HiMedia, Mumbai, India). 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) in 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. The final concentrations of the test compounds in the wells were 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 μg mL⁻¹. Isoniazid (INH) was used as a positive control

(inhibition of growth). The 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 inhibitory concentration (MIC, µg mL⁻¹) was determined as the lowest concentration that prevented the blue to pink colour change, as seen by the 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 the microdilution broth panel method using 96-well microtitration plates. The 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. The final density was adjusted to a value ranging from 0.5 to 1.0 according to the McFarland scale and diluted in a 1:20 ratio with the broth. The test compounds were dissolved in DMSO (Sigma-Aldrich) and broth was added to obtain a concentration of 2000 µg mL⁻¹. The 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⁻¹ 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⁻¹, 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⁻¹. The final concentration of DMSO did not exceed 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, the plates were sealed with a polyester adhesive film and incubated in the dark at 37 °C without agitation. The addition of a 0.01% solution of resazurin sodium salt followed after 48 hours of incubation. The stain was prepared by dissolving resazurin sodium salt (Sigma-Aldrich) in deionised water to obtain a 0.02% solution. Then a 10% aqueous solution of Tween 80 (Sigma-Aldrich) was prepared. Both liquids were mixed, making use of the same volumes and filtered through a syringe membrane filter. The microtitration panels were then incubated for a further 2.5 hours for the determination of activity. The antimycobacterial activity was expressed as the minimum inhibitory concentration (MIC) and the value was read based on the stain colour change (blue colour – no growth; pink colour – growth). The standards used for the activity determination were isoniazid (INH), rifampicin (RIF) and ciprofloxacin (CPX) (Sigma-Aldrich). The MIC values for the standards were in the range 7.81–15.625 µg mL⁻¹ for

INH, 0.78–1.56 µg mL⁻¹ for RIF and 0.098–0.195 µg mL⁻¹ 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 Eagle's minimum essential medium (MEM; Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (PAA, Austria), 1% (v/v) L-glutamine solution (Sigma-Aldrich) and 1% (v/v) non-essential amino acid solution (Sigma-Aldrich) in a humidified atmosphere containing 5% CO₂ 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 test substances were used as experimental groups whereas untreated HepG2 cells served as control groups.

HepG2 cells were seeded in a density of 1 × 10⁴ cells per well on a 96-well plate. On the next day (24 h after seeding), the cells were treated with test substances dissolved in DMSO at different concentrations ranging from 0.1 to 1000 µM (depending on the solubility, see Table 2). The maximal incubation concentration of DMSO in a well did not exceed 1% (v/v). The treatment was carried out in triplicate in a humidified atmosphere containing 5% CO₂ at 37 °C. The controls representing 100% cell viability (untreated cells) and 0% cell viability (cells treated with 10% DMSO), no-cell controls and vehiculum controls were incubated in triplicate simultaneously. After 24 h exposure to the test 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 the humidified, 5% CO₂ containing atmosphere, the absorbance was recorded at 490 nm. Inhibitory curves were constructed for each compound, plotting the incubation concentration *vs.* the percentage of absorbance relative to the untreated control. The standard toxicological parameter IC₅₀ 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 a density of 1 × 10⁴ cells per well on a 96-well plate. On the next day (24 h after seeding), they were treated with test substances dissolved in DMSO (maximal incubation concentration of DMSO was 1% v/v). The test substances were prepared according to their solubility in DMSO at incubation concentrations of 0.1–500 µM (see Table 3). The treatment was carried out in a humidified atmosphere containing 5% CO₂ at 37 °C in triplicate for 24 h and 48 h. The controls representing 100% cell viability and 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 triplicate simultaneously. After 24 h exposure, the reagent from the CellTox™ Green Cytotoxicity Assay (Promega, Madison, WI, USA) kit was prepared and added according to the recommendation of the manufacturer. After 15 min incubation at room

temperature, the fluorescence was measured at 485 nm_{Ex}/520 nm_{Em}. The measurement was repeated after 24 h to get results for the 48 h exposure period. Inhibitory curves were constructed for each compound, plotting the incubation concentration *vs.* the percentage of fluorescence relative to the untreated control. The standard toxicological parameter IC₅₀ 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 the first-line anti-tubercular pyrazinamide (PZA) and a formerly identified anti-mycobacterial scaffold of 4-arylthiazol-2-amine. The prepared compounds were fully characterized and tested for *in vitro* growth inhibition activity against *Mtb* H37Rv and several non-tuberculous mycobacterial strains using Microplate Alamar Blue Assay. We have identified several compounds with micromolar activity. The most active compound **9b** also had the broadest spectrum of activity and inhibited the growth of *Mtb*, *M. kansasii*, and *M. avium* with MIC = 0.78 µg mL⁻¹ (2.3 µM) and selectivity index SI > 20. Most of the test compounds were non-toxic to the HepG2 cell line. The molecular docking study suggested that our compounds could share the mechanism of action with very structurally similar derivatives of 2-aminothiazoles, that is, they act as inhibitors of mycobacterial FabH. (Mycobacterial FabH is considered a promising target in the 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 the general structure **3** were found to be rather metabolically unstable in mouse, rat and human liver microsomes, with half-lives 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-lives of aminothiazole derivatives **3** were strongly dependent on the substitution on the benzene ring in the acyl part of the molecule,^{10,11} so there is a fair chance that the exchange of the benzoyl for pyrazinoyl (as is present in the 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 6-Cl-pyrazine, can substitute the phenyl moiety of pattern compounds of the general structure **3**. Whether the introduction of a 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 anti-mycobacterial 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.

Conflicts of interest

The authors declare no competing interests.

Acknowledgements

This work was supported by the Czech Science Foundation (project no. 17-27514Y). The computations of QikProp descriptors (Schrödinger) were performed at the Toxicological and Antidoping Center (TAC) of the Faculty of Pharmacy UK in Bratislava.

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