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Design, synthesis and anti-mycobacterial evaluation of some new *N*-phenylpyrazine-2-carboxamides

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N-Phenylpyrazine-2-carboxamides (anilides of pyrazinoic acids with simple substituents in various positions) were previously shown to possess significant biological activities in vitro, markedly anti-mycobacterial and photosynthesis-inhibiting activity. Based on structure-activity relationships (SAR) extracted from previously published series, 25 new anilides of non-substituted pyrazinoic acid (POA), 5-CH_3-POA, 6-Cl-POA, 5-*tert*-butyl-POA and 5-*tert*-butyl-6-Cl-POA were designed and synthesised. The phenyl part was substituted with simple hydrophobic substituents chosen from methyl and halogens. 5-*tert*-Butyl-*N*-(5-fluoro-2-methylphenyl)pyrazine-2-carboxamide (**9**), *N*-(3-chloro-4-methylphenyl)-5-methylpyrazine-2-carboxamide (**12**), 6-chloro-*N*-(3-chloro-4-methylphenyl)pyrazine-2-carboxamide (**13**) and 6-chloro-*N*-(5-iodo-2-methylphenyl)pyrazine-2-carboxamide (**18**) possessed whole cell anti-mycobacterial activity in vitro against *Mycobacterium tuberculosis* H37Rv with minimum inhibitory concentration (MIC) of around 10 μM. Importantly, no cytotoxicity in the HepG2 model was detected in vitro at the concentrations tested and the estimated IC_{50} values were in hundreds of μM, indicating promising selectivity. *N*-(3-Chloro-4-methylphenyl)pyrazine-2-carboxamide (**11**) and *N*-(4-chloro-2-iodophenyl)pyrazine-2-carboxamide (**21**) exerted significant activity against *Mycobacterium kansasii* with MIC 12.6 μM and 8.7 μM, respectively. No activity was detected against *Mycobacterium avium*. SARs were in accordance with those observed for the derivatives previously published.

Keywords: anilide, anti-mycobacterial activity, cytotoxicity in vitro, lipophilicity, pyrazinoic acid

Introduction

Tuberculosis (TB) is one of the most lethal and frequent infectious diseases worldwide. According to the World Health Organisation (2014), there were 9 million new cases of TB and 1.5 million deaths associated with TB (including 0.36 million deaths of HIV-positives) in 2013. The alarming increase in drug-resistant TB strains, namely multi-drug-resistant (MDR) and extensively drug-resistant (XDR), as well as the increasing number of patients co-infected with HIV (1.1 million, which represents approximately 13 % of new TB cases in 2013) constitutes a serious problem and emphasises the need for novel anti-tubercular agents. Accordingly, the search for new anti-tuberculosis drugs is an important research topic (Nemeček et al., 2013; Krátký et al., 2015).

Pyrazinamide (PZA), a first-line anti-tubercular agent, is a model compound used as a starting point for the design of *N*-phenylpyrazine-2-carboxamides presented in this paper. Although PZA has been used in clinical practice since the 1950s, its complex mechanism of action is not yet fully understood. The non-

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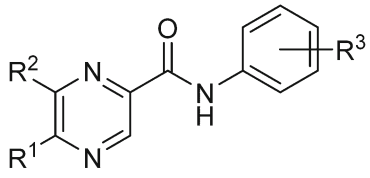
specific mechanism based on the accumulation of the pyrazinoic acid (POA; active metabolite) in the mycobacterial cell and the acidification of the cytoplasm is generally recognised. However, recent studies have shown that POA also has a specific intracellular target. Most importantly, Shi et al. (2011) showed that POA prevents the binding of tmRNA to the ribosomal protein S1 (RpsA), leading to the inhibition of trans-translation, the vital process of rescuing stalled ribosomes. Recently, Yang et al. (2015) determined the structure of POA-RpsA co-crystallised complex and, on the basis of the interactions observed, proposed possible favourable modifications of POA in respect of anti-mycobacterial activity. This clearly demonstrates that PZA, POA and their derivatives are still in focus.

For several years, *N*-phenylpyrazine-2-carboxamides (i.e. anilides of POA) have been the focus of the working group led by Doležal at the Faculty of Pharmacy in Hradec Králové (Hradec Králové, Czech Republic). More than one hundred anilides were synthesised and evaluated as potential anti-infective agents with anti-mycobacterial, antibacterial or antifungal activity (Dolezal et al., 2002, 2006, 2008, 2009, 2010). Many *N*-phenylpyrazine-2-carboxamides also proved to be effective photosynthesis inhibitors in the spinach chloroplast model, interrupting the photosynthetic electron transport (PET) in photosystem II (PS II) (Dolezal et al., 2002, 2006, 2008, 2010). For further information and SAR on the PET-inhibiting activity, (Dolezal & Kralova, 2011). Several other research groups have focused on potential anti-tuberculosis agents containing the carboxanilide pattern. Gonec et al. (2015) prepared and tested derivatives of naphthalene-2-carboxanilides (Kos et al., 2015a), and quinoline-2-carboxanilides (Kos et al., 2015b) as potential anti-tuberculosis agents. The working group led by Vinsova focused on anti-tuberculosis salicylanilides (Kratky & Vinsova, 2011). Recently, these researchers developed salicylanilides esterified with POA (compounds combining two active anti-tuberculosis fragments – the salicylanilide and POA). These compounds exhibited promising micromolar to sub-micromolar activity in vitro against multidrug-resistant mycobacterial strains (Kratky et al., 2014).

The most comprehensive review (Dolezal et al., 2012) published on the anti-mycobacterial activity of the *N*-phenylpyrazine-2-carboxamides in question contains the activity data on 103 derivatives of general formula depicted in Fig. 1. The anilides reported were derived from non-substituted POA, 6-Cl-POA, 5-*tert*-butyl-POA and 5-*tert*-butyl-6-Cl-POA. The phenyl ring was mono-, di- or tri-substituted with small substituents R^3 (Fig. 1) chosen from short alkyl (methyl, isopropyl), methoxy, halogen, CF_3 and hydroxy substituents. The anti-mycobacterial activity was measured in a high-throughput screening campaign run by the Tuberculosis Antimicrobial Acquisition and Co-



R^1; H, *tert*-butyl
R^2; H, Cl
R^3; H, OH, OCH_3, CH_3, isopropyl, CF_3, X

Fig. 1. General formula of *N*-phenylpyrazine-2-carboxamides; X – halogen.

ordinating Facility (TAACF), established by the National Institute of Allergy and Infectious Diseases (NIAID; Bethesda, MD, USA). In the primary screening against *Mycobacterium tuberculosis* H37Rv (ATCC 27294), the activity was expressed as a percentage of growth inhibition at 6.25 μg mL^{-1} in the BACTEC 12B medium using the microplate alamar blue assay (MABA) (Collins & Franzblau, 1997).

These comprehensive data were used to extract the basic structure-activity relationships (SAR) in respect of the calculated lipophilicity of the compounds and their substitution in the anilide part of the molecule (R^3; Fig. 1). Analysis showed that the most anti-mycobacterial activity-enhancing substituents R^3 were 3-CF_3, 4-CH_3, 4-$CH(CH_3)_2$, 3-F, and 3,5-CF_3 (Dolezal et al., 2012), that is, the electron-withdrawing substituent in the *meta* position and the electron-donating substituent in the *para* position. In the current study, the two counteracting substituents were combined in one molecule and the R^3 = 3-Cl-4-CH_3 pattern was proposed as the substitution of choice. This was also inspired by the fact that a previous publication showed *N*-(3-iodo-4-methylphenyl)pyrazine-2-carboxamide to be one of the most active anilides examined, inhibiting the growth of *M. tuberculosis* H37Rv by 95 % at 6.25 μg mL^{-1} (Dolezal et al., 2011). Other substituent patterns R^3 of the compounds in this article (Fig. 2) originated on the basis of the mutual exchange of various halogen atoms (chlorine for other halogens) and/or positional isomerism, to further confirm that the 3,4-disubstitution is superior to other combinations.

From previous experience with different series of pyrazine derivatives evaluated as potential anti-tuberculosis agents, it is known that activity usually grows with increasing lipophilicity to a certain point but a further increase in lipophilicity often leads to diminished or lost activity of such derivatives. For example, this pertained for various series of PZA derivatives with alkylamino substituents with increasing lengths of carbon chain, where the activity culminated in hexyl- or heptyl-amino derivatives (Servusova et al.,

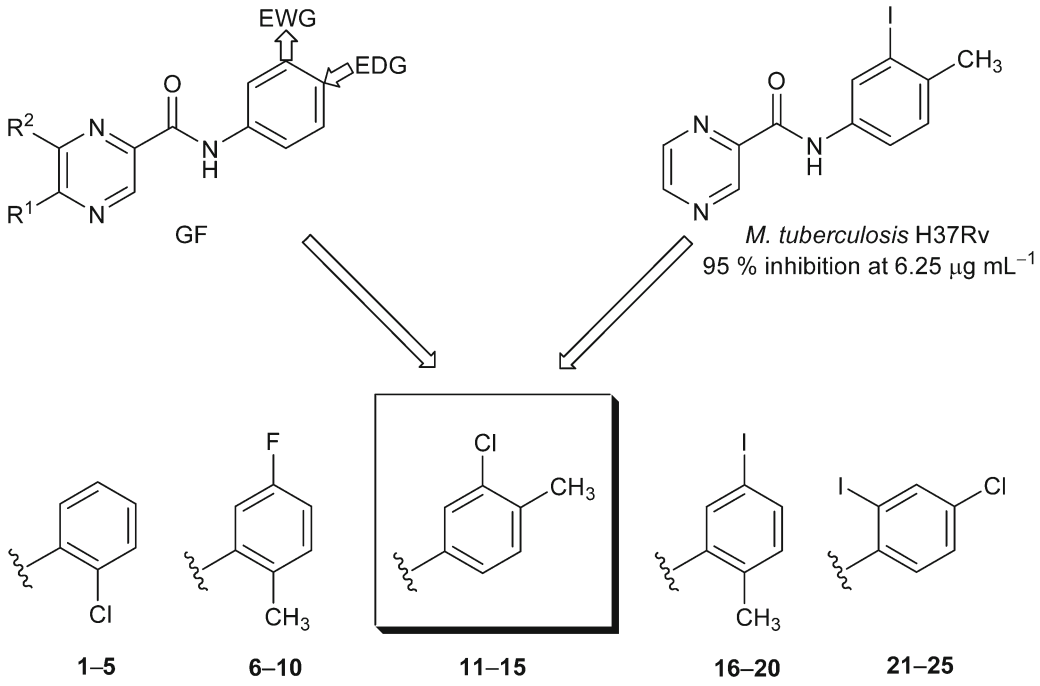


Fig. 2. Design of title compounds (**1**–**25**) based on previously determined SAR.

2014; Servusova-Vanaskova et al., 2015a, 2015b; Zitko et al., 2015). Increased lipophilicity of a compound is beneficial for permeation of the highly lipophilic mycobacterial envelope (Brennan, 2003). On the other hand, excessive lipophilicity is associated with insufficient water solubility and the ability of a compound to reach therapeutic concentrations is impaired. These two counteracting phenomena give rise to the optimal range of lipophilicity. The MycPermCheck model developed by Merget et al. (2013) is based on compounds with whole cell anti-mycobacterial activity in vitro found in the literature. The model establishes the optimal lipophilicity range for good permeation as QPlogPo/w = 2.779–4.479 where QPlogPo/w is the octanol/water partition coefficient predicted by the QikProp module (Schrödinger, USA) (Merget et al., 2013). The anilides designed in this study exhibited increased lipophilicity due to their R^3 substituent, hence they were used to probe the upper lipophilicity limit for the anti-mycobacterial activity in vitro.

In summary, 25 new anilides, which had not previously been described, were designed, synthesised and tested in vitro for anti-mycobacterial activity against *M. tuberculosis* H37Rv and non-tuberculous mycobacterial strains of *M. kansasii* and *M. avium.*

Experimental

All the organic solvents used for the synthesis were of analytical grade. Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Germany). The reactions were monitored using Merck Silica 60 F_{254} TLC plates (Merck, Germany). Compounds were purified using an automated chromatograph CombiFlash R_f (Teledyne Isco, USA) using columns filled with Kieselgel 60, 0.040–0.063 mm (Merck); gradient elution (hexane/ethyl acetate), detection wavelength of 260 nm, monitor wavelength of 280 nm. NMR analysis was performed on a Varian Mercury VX-BB 300 (Varian, USA) at 300 MHz for ^{1}H and 75 MHz for ^{13}C or on a Varian Mercury-Vx BB 500 (Varian) at 500 MHz for ^{1}H and 125 MHz for ^{13}C. The chemical shifts were recorded in δ and were indirectly referenced to tetramethylsilane (TMS). The IR spectra were measured in ATR mode using a Ge crystal-plate on a Nicolet Impact 400 (Nicolet, USA). Elementary analysis was performed on a CE Instruments EA-1110 CHN analyser (CE Instruments, UK). Melting points were determined on a Stuart SMP30 melting point apparatus (Bibby Scientific Limited, UK) and are uncorrected. Log$P$ (the logarithm of the partition coefficient for *n*-octanol/water) values were calculated using the CS ChemBioDraw Ultra program ver. 14.0 (CambridgeSoft, USA).

6-Chloropyrazine-2-carboxylic (Abe et al., 1969) (6-Cl-POA), 5-*tert*-butylpyrazine-2-carboxylic (Dolezal et al., 1999) (5-*tert*-Bu-POA) and 5-*tert*-butyl-6-chloropyrazine-2-carboxylic acid (Dolezal et al., 1999) (5-*tert*-Bu-6-Cl-POA) were synthesised following the known synthetic procedures described elsewhere (Dolezal et al., 1999; Servusova et al., 2014; Venturello & D'Aloisio, 1986). The analytical data of the prepared starting acids were fully in accordance with the data in the literature and ^{1}H NMR as well as the melting points are available in the Supplementary Data. POA and 5-methylpyrazine-2-

carboxylic acid (5-CH_3-POA) were purchased from Sigma–Aldrich and used as received.

Synthesis of N-phenylpyrazine-2-carboxamides (1–25)

The corresponding pyrazinoic acid (5.0 mmol) was dispersed in dry toluene (20 mL) and mixed with 1.5 eq. of thionyl chloride (0.55 mL, 7.5 mmol). The reaction mixture was heated to reflux for approximately 1 h. Next, the excess of thionyl chloride was removed by repeated evaporation with dry toluene under vacuum. The crude acyl chloride was dissolved in dry acetone (20 mL) and added drop-wise to a stirred solution of the corresponding aniline (5.0 mmol) with triethylamine (5.0 mmol) in dry acetone (30 mL). The reaction mixture was stirred at ambient temperature for up to 6 h. The completion of the reaction was monitored by TLC (eluent: hexane/ethyl acetate; φ_r = 2 : 1). The crude product adsorbed on silica gel by solvent evaporation was purified by flash chromatography (hexane/ethyl acetate gradient elution).

The analytical data of the prepared compounds were fully consistent with the proposed structures and are available in the Supplementary Data.

Anti-mycobacterial evaluation in vitro

Microdilution panel method (Servusova et al., 2012). The anti-mycobacterial evaluation was carried out by the Department of Clinical Microbiology, University Hospital and Faculty of Medicine in Hradec Králové, Charles University (Hradec Králové, Czech Republic). Four mycobacterial strains were used: *M. tuberculosis* H37Rv CNCTC My 331/88, *M. avium* CNCTC My 80/72, *M. avium* CNCTC My 152/73 and *M. kansasii* CNCTC My 235/80 (Czech National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic). The tested compounds were dissolved and serially diluted in dimethylsulphoxide (DMSO), mixed with Šula's semi-synthetic medium (Trios, Czech Republic) and placed in a microdilution panel. The tested species were added in the form of a suspension in an isotonic saline solution. The final concentrations of tested compunds were of 100 μg mL^{-1}, 50 μg mL^{-1}, 25 μg mL^{-1}, 12.5 μg mL^{-1}, 6.25 μg mL^{-1}, 3.125 μg mL^{-1} and 1.563 μg mL^{-1}), The final concentration of DMSO did not exceed 1 vol. %; this concentration of DMSO did not affect the growth of mycobacteria. The cultures were grown in Šula's semi-synthetic medium at pH 6.0 and 37 °C. The anti-mycobacterial activity was determined visually after 14 days (6 days for *M. kansasii* and *M. avium*) of incubation as the minimum inhibition concentration (MIC, in μg mL^{-1}), *i.e.* the lowest concentration of the tested substance that inhibited the growth of mycobacteria.

Cytotoxicity measurement

The human hepatocellular liver carcinoma cell line HepG2 (passage 40–41) purchased from Health Protection Agency Culture Collections (ECACC, UK) was routinely cultured in a minimum essentials eagle medium (MEM; Sigma–Aldrich) supplemented with 10 vol. % foetal bovine serum (PAA, Austria), 1 % L-glutamine solution (Sigma–Aldrich) and 1 vol. % non-essential amino acid solution (Sigma–Aldrich) in a humidified atmosphere containing 5 % of CO_2 at 37 °C. For sub-culturing, the cells were harvested after trypsin/EDTA (Sigma–Aldrich) treatment at 37 °C. The cells treated with the tested substances were used as the experimental groups and untreated HepG2 cells were used as the control groups. The cells were seeded in a density of 1×10^4 cells per well in a 96-well plate. The next day they were treated with the tested substances dissolved in DMSO (maximal incubation concentration of DMSO was 1 vol. %). The tested compounds were prepared at incubation concentrations of 1–7500 μM. The treatment was carried out in triplicates in a humidified atmosphere containing 5 % of CO_2 at 37 °C for 24 h. The controls representing 100 % cell viability, 0 % cell viability (cells treated with 10 vol. % DMSO), no-cell controls and vehiculum controls were incubated in triplicate simultaneously. After 24 h exposure, the reagent from the kit CellTiter 96® Aqueous one solution cell proliferation assay (Promega, USA) was added according to the manufacturer's recommendation. After 2 h incubation at 37 °C in a humidified, 5 % of CO_2 atmosphere, the absorbance was recorded at 490 nm. A standard toxicological parameter IC_{50} was calculated by non-linear regression analysis of the inhibitory curves using GraphPad Prism software version 6 (GraphPad Software, Inc., CA, USA).

Results and discussion

Chemistry

Fig. 3 shows that the target derivatives (**1**–**25**) were prepared by a convenient two-step synthesis using corresponding starting pyrazinoic acid, which was treated with thionyl chloride to form carbonyl chloride. Various ring-substituted anilines were then used for the aminolysis of carbonyl chloride to form final compounds (**1**–**25**) (purified by flash column chromatography); see Table 1. The analytical data of all the prepared compounds were in accordance with the proposed structures and are available in the Supplementary Data. The yields of chromatographically pure products ranged from 17.8 % to 86.6 %. The IR spectrum of all the compounds had a carbonyl (C═O) transmittance peak in the range of 1670–1698 cm^{-1}. The ^1H NMR spectra exhibited amidic hydrogen (-CONH-) as a broad singlet (independently

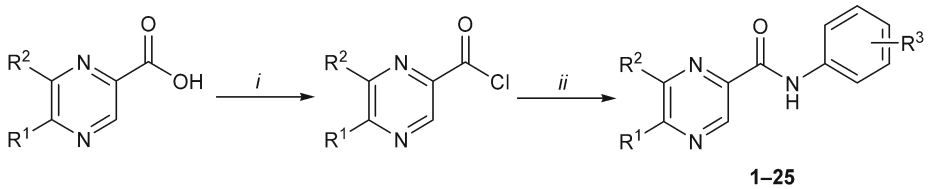


Fig. 3. Synthesis of final compounds (**1**–**25**); reagents and conditions: (*i*) $SOCl_2$, toluene, reflux, 1 h; (*ii*) substituted aniline, TEA, acetone, AT, up to 6 h.

Table 1. Summary of prepared compounds. Anti-mycobacterial activity against *M. tuberculosis* H37Rv and *M. kansasii* in vitro expressed as MIC

No.	Molecular mass	Substituent			MIC/(μg mL^{-1})		log*P*
		R^1	R^2	R^3	*M. tuberculosis*	*M. kansasii*	
1	233.66	H	H	2-Cl	50	50	1.15
2	247.68	CH_3	H	2-Cl	25	> 50	1.85
3	268.10	H	Cl	2-Cl	100	> 100	2.05
4	289.76	$C(CH_3)_3$	H	2-Cl	> 100	> 100	3.28
5	324.21	$C(CH_3)_3$	Cl	2-Cl	> 100	> 100	4.18
6	231.23	H	H	2-CH_3-5-F	25	25	1.23
7	245.26	CH_3	H	2-CH_3-5-F	n.a.	n.a.	1.94
8	265.67	H	Cl	2-CH_3-5-F	> 100	> 100	2.13
9	287.34	$C(CH_3)_3$	H	2-CH_3-5-F	3.13	> 100	3.36
10	321.87	$C(CH_3)_3$	Cl	2-CH_3-5-F	> 100	> 100	4.26
11	247.68	H	H	3-Cl-4-CH_3	12.5	3.13	1.63
12	261.71	CH_3	H	3-Cl-4-CH_3	3.13	12.5	2.34
13	282.12	H	Cl	3-Cl-4-CH_3	3.13	100	2.53
14	303.79	$C(CH_3)_3$	H	3-Cl-4-CH_3	> 100	> 100	3.76
15	338.23	$C(CH_3)_3$	Cl	3-Cl-4-CH_3	> 100	> 100	4.66
16	339.14	H	H	2-CH_3-5-I	> 100	> 100	2.43
17	353.16	CH_3	H	2-CH_3-5-I	> 100	> 100	3.14
18	373.58	H	Cl	2-CH_3-5-I	3.13	> 100	3.33
19	395.24	$C(CH_3)_3$	H	2-CH_3-5-I	> 100	> 100	4.56
20	429.69	$C(CH_3)_3$	Cl	2-CH_3-5-I	> 100	> 100	5.46
21	359.55	H	H	2-I-4-Cl	6.25	3.13	2.50
22	373.58	CH_3	H	2-I-4-Cl	> 50[a]	> 50[a]	3.21
23	393.99	H	Cl	2-I-4-Cl	> 100	25	3.41
24	415.66	$C(CH_3)_3$	H	2-I-4-Cl	> 50[a]	> 50[a]	4.63
25	450.10	$C(CH_3)_3$	Cl	2-I-4-Cl	> 50[a]	> 50[a]	5.53
INH	137.14	–	–	–	0.2–0.78	1.56–6.25	–0.64
PZA	123.12	–	–	–	12.5	> 100	–1.31

a) Measurement at 100 μg mL^{-1} not performed due to precipitation of tested compound in testing medium; INH – isoniazid, PZA – pyrazinamide, n.a. – data not available.

of the solvent) in the range of δ 10.96–9.31. The ^{13}C NMR spectra of all the compounds exhibited carbonyl carbon (-CONH-) in the range of δ 168.2–159.3.

Anti-mycobacterial activity

Final compounds (**1**–**25**) were assayed in vitro for whole cell anti-mycobacterial activity against *M. tuberculosis* H37Rv, *M. kansasii*, and *M. avium* using a two-fold dilution microplate method. The results (Table 1) are expressed as MIC (μg mL^{-1}).

To determine the substituents in the pyrazine (R^1, R^2) and anilide (R^3) parts of the molecule with a positive effect on anti-mycobacterial activity within the newly prepared series of compounds (**1**–**25**), the individual compounds were scored with points according to the following rule: high activity, MIC = 3.13–6.25 μg mL^{-1}, 3 points; moderate activity, MIC = 12.5–25 μg mL^{-1}, 2 points; weak activity, MIC = 50–100 μg mL^{-1}, 1 point; no activity, MIC > 100 μg mL^{-1} (MIC > 50 μg mL^{-1} for compounds (**22**), (**24**) and (**25**)), 0 points. Table 2 presents the scores of the individual substituents for both *M. tuberculosis* and *M. kansasii* activity; the number in parentheses is the total number of active compounds (high, moderate or weak activity) with the respective substitution. Fig. 4 graphically summarises the level of activity against

Table 2. Activity score of various substitution patterns and their hydrophobic substituent constants π

	Anilide part (R^3)					Pyrazine part (R^1, R^2)				
	2-Cl	2-CH_3-5-F	3-Cl-4-CH_3	2-CH_3-5-I	2-I-4-Cl	H	5-CH_3	6-Cl	5-*tert*-Bu	5-*tert*-Bu-6-Cl
π	0.56	0.64	1.04	1.84	1.91	0	0.7	0.9	2.13	3.03
M. tuberculosis	4 (3)	5 (2)	8 (3)	3 (1)	3 (1)	8 (4)	5 (2)	7 (3)	3 (1)	0 (0)
M. kansasii	1 (1)	2 (1)	6 (3)	0 (0)	5 (2)	9 (4)	2 (1)	3 (2)	0 (0)	0 (0)

π – Hydrophobic substituent constant; calculated as log$P$(RX) – log$P$(RH), where RX is substituted derivative and RH is non-substituted derivative. See text for rules used for calculation of the score; numbers in parentheses represent the total number of active compounds with the respective substitution.

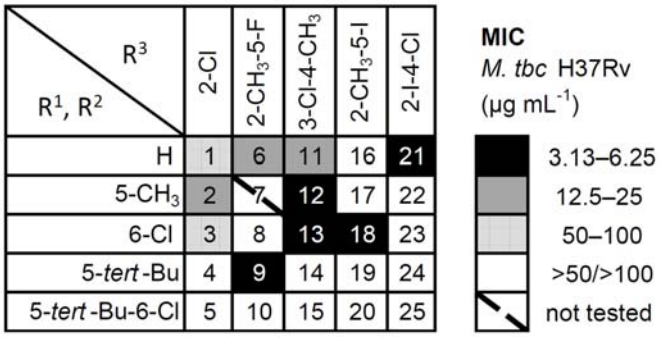


Fig. 4. Level of antimycobacterial activity against *M. tuberculosis* H37Rv according to combinations of substituents in pyrazine and phenyl part.

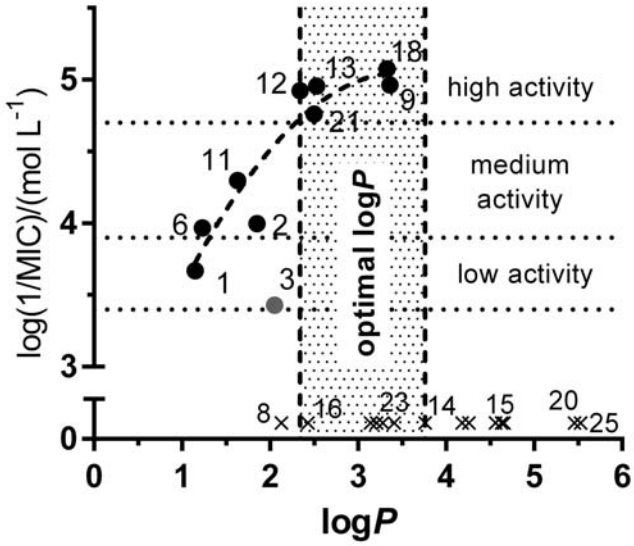


Fig. 5. Plot of anti-mycobacterial activity against *M. tuberculosis* H37Rv vs. calculated lipophilicity for compounds (**1**–**25**); ○ – active, × – not active.

M. tuberculosis H37Rv according to combinations of substituents R^1, R^2 and R^3.

On reviewing the activity against *M. tuberculosis* H37Rv as depicted in Table 2 and Fig. 4, it is obvious that the most beneficial substitution on the phenyl is R^3 = 3-Cl-4-CH_3, which produced two compounds with high activity (**12**, **13**) and one with moderate activity (**11**), scoring 8 points. This substitution pattern is fully compliant with the general formula GF (Fig. 2), bearing the electron-withdrawing *meta* halogen and electron-donating methyl in the *para* position. Interestingly, this substitution pattern also performed best against *M. kansasii*. The activity of compounds with R^3 = 2-CH_3-5-F (**6**–**10**) and, in particular, R^3 = 2-CH_3-5-I (**16**–**20**) is probably reduced by the *ortho* methyl group, the negative effect of which on anti-mycobacterial activity was identified in the previously published review (Dolezal et al., 2012). In the present case, the negative effect of *ortho* methyl often counteracts the positive effect of *meta* withdrawing halogens. Similarly, *ortho*-halogen substitution was also disadvantageous, e.g. R^3 = 2-Cl in compounds (**1**–**5**) and R^3 = 2-I-4-CH_3 in (**22**–**25**). Moreover, these compounds lack the *meta*-withdrawing substituent, apparently important for the anti-mycobacterial activity. From the activities published in the review (Dolezal et al., 2012), it appears that the phenyl part does not tolerate *ortho* substituents (e.g. 2,6-Cl_2, 2-CH_3; 2-X-5-OH, where X is halogen). This may be due to steric hindrance with the structure of the potential target, although this cannot currently be confirmed.

Considering the pyrazine part of the molecule, the best scores were achieved by non-substituted pyrazine, followed by 6-Cl and 5-CH_3 (it should be noted that 5-CH_3 substitution might have been hampered due to missing activity data for compound (**7**)). The anilides derived from 5-*tert*-butyl-6-chloropyrazine-2-carboxylic acid were completely inactive. This observation is in contrast with the SAR extracted from the review (Dolezal et al., 2012), where the series derived from 5-*tert*-butyl-6-chloropyrazine-2-carboxylic acid possessed significant activities. However, these compounds often bore the hydrophilic substituent (R^3 = 2-OH, 3-OH, or 4-OH) counteracting the lipophilic substitution of the pyrazine part, or the substituent with a small increase in lipophilicity such as R^3 = 3-F (π_{Ph} = + 0.16). The combination of highly lipophilic phenyl substitution patterns used in this study with a highly lipophilic substitution of the pyrazine ring (R^1 = *tert*-Bu, R^2 = Cl) is, therefore, disadvantageous.

Fig. 5 presents the plot of activity (log(1/MIC) in μM) against *M. tuberculosis* H37Rv vs. calculated

Table 3. Cytotoxicity of tested substances in HepG2 cells

No.	$IC_{50}/\mu M$	MIC of *M. tuberculosis*/μM	$SI = IC_{50}/MIC$
9	$> 50^a$ $(573.3)^b$	10.9	> 4.59 $(52.6)^c$
11	$> 250^a$	50.5	> 4.95
12	$> 100^a$ $(343.5)^b$	12.0	> 8.36 $(28.7)^c$
13	$> 250^a$ $(820.7)^b$	11.1	> 22.54 $(74.0)^c$
18	$> 50^a$ $(653.8)^b$	8.4	> 5.97 $(78.0)^c$
21	$> 100^a$	17.4	> 5.75

a) Measurement at higher concentration not reproducible (precipitation of compound in cell culture medium); *b*) number in parentheses represents hypothetic IC_{50} value calculated from the trend of inhibitory curve; *c*) number in parentheses represents SI calculated based on hypothetic IC_{50} value.

lipophilicity log*P*. Among the active compounds, their activity increased with lipophilicity. The compounds with high activity (MIC = 3.13–6.25 μg mL^{-1}) possessed log*P* in the range from 2.34 to 3.36. This can be regarded as the optimal lipophilicity in the series under discussion. The compounds with $\log P \geq 3.76$ were inactive. This limit value can be deduced from compounds (**11**–**15**) with the successful substitution pattern R^3 = 3-Cl-4-CH_3, where compounds (**11**–**13**) are active and compounds (**14**) ($\log P = 3.76$) and (**15**) ($\log P = 4.76$) are completely inactive. However, it is obvious that lipophilicity is only a secondary determinant of anti-mycobacterial activity, probably facilitating the penetration through the lipophilic mycobacterial cell wall. Compounds (**4**), (**16**), (**17**), and (**22**) lie within the optimal lipophilicity range but still are inactive. This can be explained by the negative influence of substituent R^3, for example, substituent (2-CH_3, 2-Cl, or 2-I) in the *ortho* position as discussed above or non-compliance with the *meta*-EWG rule. This negative steric effect is obvious for 2-Cl and 2-I substitution; the effect of 2-CH_3 is unclear, as compound (**18**) (R^1 = H, R^2 = Cl, R^3 = 2-CH_3-5-I) is highly active, but other compounds with the same phenyl substitution (**16**), (**17**), (**19**) and (**20**) are inactive.

The most active compounds achieved MIC against *M. tuberculosis* H37Rv (or *M. kansasii*) at the level of 3.13 μg mL^{-1} or 6.25 μg mL^{-1}, which are values comparable with the values of previously reported anilides (of comparable MW) with MIC = 2–8 μg mL^{-1} (Dolezal et al., 2009) or MIC = 3.13–6.25 μg mL^{-1} (Dolezal et al., 2011b). In recently published data on anilides of 5-Cl-POA (Zitko et al., 2013), the phenyl part tolerated a wider range of substituents than the anilides of non-substituted POA, 5-CH_3-POA, 6-Cl-POA, 5-*tert*-Bu-POA and 5-*tert*-Bu-6-Cl-POA currently described or in the review published previously. In the light of anti-mycobacterial activity, 5-Cl-POA derivatives appear to be special structures, as demonstrated by the ability of 5-chloropyrazine-2-carboxamide to inhibit the mycobacterial fatty acid synthase I (Ngo et al., 2007; Sayahi et al., 2012)

None of compounds (**1**–**25**) exhibited any activity against any of the two tested strains of *M. avium*.

Cytotoxicity

Drug-induced hepatotoxicity is an unfortunate common side-effect of many of the first line anti-tuberculosis agents (PZA, isoniazid, rifampicin) (Tostmann et al., 2008a). In view of the multidrug mixture approach used for the treatment of tuberculosis, it is highly probable that a newly developed anti-tuberculosis compound would be used in combination with clinically established agents. Due to possible toxicity synergism, the potential hepatotoxicity of new compounds developed as potential anti-tuberculosis agents should be assessed carefully. The human liver carcinoma cell line (HepG2) is used as a model for hepatotoxicity studies in vitro (Singh et al., 2011; Tostmann et al., 2008b).

A hepatotoxicity assay of human HepG2 hepatoma cells was performed in vitro for selected compounds: see Table 3. The decrease in viability of the HepG2 cells was measured using a colorimetric assay (Kratky et al., 2012; Owen, 1993) based on the reduction of tetrazolium dye and the results were expressed as IC_{50}. The limited solubility in the culture medium of all the compounds tested did not permit direct determination of the exact IC_{50} value, but no toxic effect on HepG2 cells was observed up to the highest concentration achieved during the measurement. The IC_{50} values of compounds (**9**), (**12**–**13**), and (**18**) (Table 3, values in parentheses) were calculated according to the trend of the inhibitory curves by GraphPad Prism software (version 6, non-linear regression analysis). The inhibitory curves for compounds (**11**) and (**21**) did not make it possible to calculate the exact IC_{50} value. Nevertheless, the IC_{50} values of all the compounds studied were hundreds of μM, which represents a significantly lower cytotoxicity than the previously published anilides of 5-chloropyrazine-2-carboxilic acid (IC_{50} mostly in units or tens of μM) (Zitko et al., 2013).

The estimated selectivity index (SI) defined as IC_{50}/MIC both in molar concentrations was calculated for activity against *M. tuberculosis* H37Rv. SI values over 10 are regarded as safe for a drug candidate, granting sufficient difference between efficient and cytotoxic concentrations.

Conclusions

Twenty five new, previously undescribed anilides of substituted pyrazinoic acids were synthesised by known methods. The anti-mycobacterial activity of the final compounds against *M. tuberculosis* H37Rv and non-tuberculous mycobacterial strains of *M. kansasii* and *M. avium* was evaluated in vitro. The compounds so prepared supplement the large family of anilides prepared by Doležal and co-workers. It was shown that similar structure-activity relationships pertain concerning the phenyl substitution pattern. Three new anilides (**12**, **13**, **18**) of substituted pyrazine-2-carboxylic acid exhibited whole cell antimycobacterial activity in vitro against *M. tbc* H37Rv with MIC around 10 μM. Importantly, no cytotoxicity in the HepG2 model was detected in vitro for the tested concentrations and the estimated IC_{50} values were in hundreds of μM, indicating promising selectivity. Compounds (**11**) and (**21**) exhibited significant activity against *M. kansasii* with MIC = 3.13 $\mu g\ mL^{-1}$ (12.6 μM and 8.7 μM, respectively). No activity was detected against *M. avium*.

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Supplementary Data

The supplementary data associated with this article can be found in the online version of this paper.

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