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Research Article

# Synthesis and Antimicrobial Evaluation of 6-Alkylamino-*N*-phenylpyrazine-2-carboxamides

**Barbora Servusova-Vanaskova[1,*], Pavla Paterova[1,2], Vladimir Garaj[3], Jana Mandikova[1], Jiri Kunes[1], Lieve Naesens[4], Petr Jílek[1], Martin Dolezal[1] and Jan Zitko[1,*]**

[1]*Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, Hradec Králové 500 05, Czech Republic*
[2]*Department of Clinical Microbiology, University Hospital Hradec Králové, Sokolská 581, Hradec Králové 500 05, Czech Republic*
[3]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University, Odbojárov 10, Bratislava 832 32, Slovakia*
[4]*Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, Leuven 3000, Belgium*
**Corresponding authors: Barbora Servusova, barbora.servusova@faf.cuni.cz; Jan Zitko, jan.zitko@faf.cuni.cz*

**This work presents synthesis and antimicrobial evaluation of nineteen 6-alkylamino-*N*–phenylpyrazine-2-carboxamides. Antimycobacterial activity was determined against *Mycobacterium tuberculosis* H37Rv, *M. kansasii* and two strains of *M. avium.* Generally, the antimycobacterial activity increased with prolongation of simple alkyl chain and culminated in compounds with heptylamino substitution (3e, 4e) with MIC = 5–10 μM against *M. tuberculosis* H37Rv. On the contrary, derivatives with modified alkyl chain (containing *e.g.* terminal methoxy or hydroxy group) as well as phenylalkylamino derivatives were mainly inactive. The most active compounds (with hexyl to octylamino substitution) were evaluated for their *in vitro* activity against drug-resistant strains of *M. tuberculosis* and possessed activity comparable to that of the reference drug isoniazid. None of the tested compounds were active against *M. avium.* Some derivatives exhibited activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (best MIC = 7.8 μM), while Gram-negative strains as well as tested fungal strains were completely unsusceptible. Active compounds were tested for in vitro toxicity on various cell lines and in most cases were non-toxic up to 100 μM.**

**Key words:** antibacterial evaluation, antimycobacterial evaluation, cytotoxicity, multidrug-resistant strains, pyrazinamide derivatives

**Abbreviations:** CRFK, Crandell feline kidney cells; FAS I, fatty acid synthase I; HEL, Human embryonic lung fibroblasts; HeLa, Human cervix epithelial cells; HepG2, Human hepatocellular carcinoma cells; INH, isoniazid; MDR, multidrug-resistant; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; *Mtb*, *Mycobacterium tuberculosis*; Nam, nicotinamide; PncA, pyrazinamidase/nicotinamidase; POA, pyrazinoic acid; PZA, pyrazinamide; RpsA, ribosomal protein S1; SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; TB, tuberculosis; Vero, African green monkey kidney cells; XDR, extensively drug-resistant.

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Tuberculosis (TB) is one of the most lethal and frequent infection diseases worldwide. In 2013, an estimated 9.0 million people fell ill with TB (64% were newly diagnosed cases) and 1.5 million died from TB (1). Resistant TB forms, namely multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, as well as the increasing number of patients co-infected with HIV (1,2) (1.1 million of all TB cases in 2013) (1) constitute a serious problem and emphasize the need for novel antitubercular drugs. Pyrazinamide (PZA) is one of the most important first-line drugs used in TB therapy (3). Apart from sterilizing activity (synergistic effect with rifampicin) (4), PZA has multiple mechanisms of action. It acts directly as an active compound or is a prodrug. As a prodrug, PZA is metabolized via pyrazinamidase (EC 3.5.1.19) to pyrazinoic acid (POA) (5,6). Recently, the specific targets for PZA and/or POA were recognized (7–12). Both PZA and POA inhibit mycobacterial fatty acid synthase I (FAS I) (13), an enzyme that participates in the synthesis of mycolic acids which are essential components of the mycobacterial cell wall (7–11). In 2011, ribosomal protein S1 (RpsA), a vital protein involved in protein translation and the ribosome-sparing process of trans-translation, was identified as a specific target for POA (12). During the last decade, variety of substituted *N*-phenylpyrazine-2-carboxamides was designed, prepared and intensively screened for antimycobacterial activity (14–18). As a complementary test, most of these compounds were also screened for *in vitro* antibacterial and antifungal activity. A substantial number of tested *N*-phenylpyrazine-2-carboxamides selectively inhibited the growth of Mycobacterium tuberculosis H37Rv. With some exceptions, no significant antibacterial and antifungal activity was observed. Based on the results of



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aforementioned studies, two anilides (**1** and **2**) derived from 6-chloropyrazine-2-carboxylic acid were prepared and screened for antimycobacterial, antibacterial and antifungal activity. Unfortunately, no significant activity was observed. Following recently published studies, in which the positive influence of long alkyl chain (C6–C8) on antimycobacterial activity was observed (19–21), our attention was refocused on alkylamino derivatives of PZA. 6-Chloro-*N*-phenylpyrazine-2-carboxamide (**1**) and 6-chloro-*N*-(2–chlorophenyl)pyrazine-2-carboxamide (**2**) served as initial leads for compounds mentioned in this study. The anilide part of the compound was preserved, while 6-chlorine was substituted by alkylamines in the range from propylamine to octylamine to yield final compounds (**3a**–**f**, **4a**–**f**). Methylamine and ethylamine were omitted due to negligible activity of methylamino and ethylamino pyrazine derivatives in previously published series (19–21). Based on the first results of antimycobacterial screening and to study the influence of a simple alkyl chain on activity, series of phenylalkylamino, hydroxyalkylamino and methoxyalkylamino derivatives were prepared and evaluated (marked as **3g**–**m**). MycPermCheck (22) is an online tool used for the prediction of permeability of small molecules (MW < 500 Daltons) through Mycobacterium tuberculosis cell wall. The prediction is based on a logistic regression model of selected physico-chemical molecular descriptors of compounds which were active in whole-cell *in vitro* assays (and are therefore considered to be permeable). MycPermCheck was used to predict the permeability of studied compounds and to compare obtained data with whole-cell *in vitro* antimycobacterial activity. Most of the authors agree that PZA acts as a prodrug that needs to be converted by the mycobacterial enzyme pyrazinamidase/nicotinamidase PncA (EC 3.5.1.19) (6,12). However, PZA and some of its simple derivatives, for example 5-chloropyrazinamide, were shown to act in non-hydrolysed carboxamide form as competitive inhibitors of mycobacterial fatty acid synthase I (10,11,23). A molecular docking study was therefore performed to predict whether the active compounds of the presented series could be substrates of PncA to be converted into their hydrolysed acidic form.

## Methods and Materials

All chemicals were purchased from Sigma-Aldrich (Höhenkirchen, Germany). All organic solvents used for the synthesis were of analytical grade. Detailed synthetic procedures and analytical data of presented compounds are available in the Supporting Information. Biological methods were described in full details in previous publications (18,21) and can be found in the Supporting Information as well.

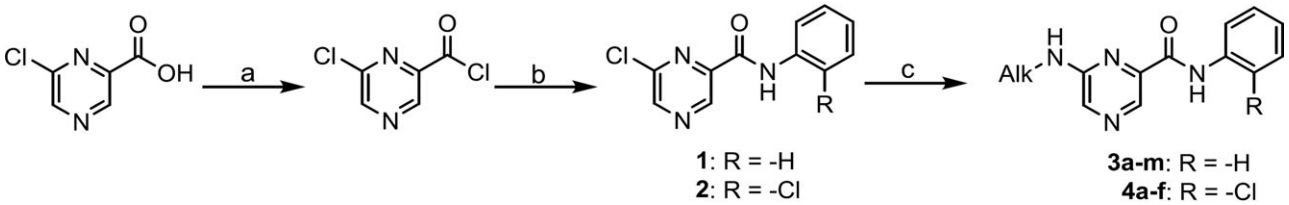
## Results and Discussion

### Chemistry

As shown in Scheme 1, 6-chloro-*N*-phenylpyrazine-2-carboxamide (**1**) and 6-chloro-*N*-(2-chlorophenyl)pyrazine-2-carboxamide (**2**) were prepared by convenient two-step synthesis using 6-chloropyrazine-2-carboxylic acid (24), which was treated with thionyl chloride to form 6-chloropyrazine-2-carbonyl chloride. Aniline or 2-chloroaniline was used for aminolysis of carbonyl chloride to form corresponding compounds **1** or **2** (purified by flash column chromatography). Final structures **3a**–**f** and **4a**–**f** were prepared by means of nucleophilic substitution of chlorine by non-aromatic amines (for the structures, please see Table 1). Analytical data of all prepared compounds were in accordance with proposed structures. IR spectrum of all compounds (**1**, **2**, **3a**–**m** and **4a**–**f**) had carbonyl (C=O) transmittance peak in the range of 1667–1685 $cm^{-1}$. $^{1}H$ NMR spectra exhibited amidic hydrogen (-CONH-) as a broad singlet (independently of the solvent) in the range of 10.45–9.57 ppm. Shift of hydrogen of amino group was significantly dependent on the solvent used for measurement. For compounds measured in DMSO-$d_6$ (**3b**–**m**), hydrogen of –NH– group was observed as triplet in the range of 7.56–7.44 ppm. For the compounds measured in $CDCl_3$ (**3a**, **4a**–**f**), broad singlet in the range of 5.13–4.98 ppm was observed. $^{13}C$ NMR spectra of all compounds exhibited carbonyl carbon (–CONH-) in the range of 162.82–159.51 ppm and carbons of simple aliphatic chain (**3a**–**f**, **4a**–**f**) in the range of 43.28–11.48 ppm.

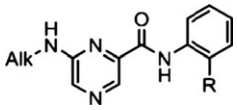
### In vitro antimycobacterial activity

All prepared compounds were evaluated for antimycobacterial activity against four mycobacterial strains. Results were expressed as minimal inhibitory concentration (MIC) in $\mu$g/mL or in $\mu$M with respect to the molecular weight. Table 1 provides an overview of the antimycobacterial activity against the strain *Mycobacterium tuberculosis* H37Rv (*Mtb*). Both tested strains of *Mycobacterium avium*




**Scheme 1:** Synthesis of starting compounds **1** and **2** and subsequent synthesis of final compounds **3a**–**m** and **4a**–**f**. Reagents and conditions: (a) $SOCl_2$, toluene, reflux, 1.5 h; (b) aniline/2-chloroaniline, acetone, TEA, RT, 2 h; (c) non-aromatic amines, TEA, EtOH, reflux, 8 h.

**Table 1:** Summary of prepared compounds, their properties and whole-cell *in vitro* antimycobacterial activity against *Mycobacterium tbc* H37Rv (*Mtb*)



| No. | R | Alk | log *P* | MIC | | Perm[a] |
|---|---|---|---|---|---|---|
| | | | | *Mtb* (μg/mL) | *Mtb* (μM) | |
| **3a** | H | *n*-$C_3H_7$ | 1.63 | 12.5 | 49 | 0.438 |
| **3b** | H | *n*-$C_4H_9$ | 2.04 | 6.25 | 23 | 0.541 |
| **3c** | H | *n*-$C_5H_{11}$ | 2.46 | 3.13 | 11 | 0.640 |
| **3d** | H | *n*-$C_6H_{13}$ | 2.88 | 1.56 | 5 | 0.727 |
| **3e** | H | *n*-$C_7H_{15}$ | 3.29 | 1.56 | 5 | 0.798 |
| **3f** | H | *n*-$C_8H_{17}$ | 3.71 | 3.13 | 10 | 0.853 |
| **3g** | H | -$(CH_2)_2$Ph | 2.81 | >100 | >313 | 0.998 |
| **3h** | H | -$(CH_2)_3$Ph | 3.23 | 100 | 301 | 0.999 |
| **3i** | H | -$(CH_2)_3$OH | 0.39 | 100 | 367 | 0.074 |
| **3j** | H | -$(CH_2)_4$OH | 0.84 | 100 | 349 | 0.095 |
| **3k** | H | -$(CH_2)_5$OH | 1.26 | 50 | 167 | 0.164 |
| **3l** | H | -$(CH_2)_2OCH_3$ | 0.65 | 100 | 367 | 0.089 |
| **3m** | H | -$(CH_2)_3OCH_3$ | 0.75 | 50 | 175 | 0.123 |
| **4a** | Cl | *n*-$C_3H_7$ | 2.18 | 25 | 86 | 0.634 |
| **4b** | Cl | *n*-$C_4H_9$ | 2.60 | 25 | 82 | 0.692 |
| **4c** | Cl | *n*-$C_5H_{11}$ | 3.02 | 6.25 | 20 | 0.773 |
| **4d** | Cl | *n*-$C_6H_{13}$ | 3.44 | 6.25 | 19 | 0.782 |
| **4e** | Cl | *n*-$C_7H_{15}$ | 3.85 | 3.13 | 9 | 0.884 |
| **4f** | Cl | *n*-$C_8H_{17}$ | 4.27 | 6.25 | 17 | 0.898 |
| **1** | H | – | 1.49 | 25 | 107 | 0.891 |
| **2** | Cl | – | 2.05 | 100 | 373 | 0.874 |
| **PZA**[b] | | | −1.31 | 12.5 | 102 | – |
| **INH**[c] | | | −0.64 | 0.2–1.56 | 2–11 | – |

[a]Perm. = probability of permeation through mycobacterial cell wall calculated by MycPermCheck; a value of 1000 equals 100%.
[b]PZA pyrazinamide.
[c]INH isoniazid.

were completely resistant to prepared compounds (MIC >100 μg/mL). Except for compounds **3f** and **4d** (MIC = 6.25 μg/mL), no significant activity was observed against *Mycobacterium kansasii* (MIC > 100 μg/mL). Starting compounds 6-chloro-*N*-phenylpyrazine-2-carboxamide (**1**, MIC = 107 μM) and 6-chloro-*N*-(2-chlorophenyl)pyrazine-2-carboxamide (**2**, MIC = 373 μM) exhibited only poor antimycobacterial activity against *Mtb* and were also completely inactive against other tested mycobacterial strains. Substitution of chlorine by simple aliphatic alkylamine led to an appreciable increase in activity for an extended range of amines (from propylamine to octylamine, MIC = 5–86 μM). Compounds with non-substituted phenyl derived from **1** displayed slightly better activities than corresponding 2-chlorophenyl derivatives synthesized from **2**. Taking into account the molecular weight (MIC values converted to molar concentration in μM), most active compounds (**3d**–**f** and **4e**, MIC = 5–10 μM) exhibited similar activity as the therapeutically used standard isoniazid (**INH**, MIC = 2–11 μM), yet markedly higher activity than the other reference drug pyrazinamide (**PZA**, MIC = 102 μM) and both starting anilides **1** and **2** as well. Attempted structural changes in simple aliphatic chain led to decrease or complete loss of activity. The insertion of an aromatic nucleus into the aliphatic chain resulted in completely inactive compounds (**3g**, **3h**). The activity of derivatives substituted with aminoalcohols (**3i**–**k**) or methoxyalkylamines (**3l**, **3m**) was negligible (MIC = 167–367 μM).

The probability value calculated by MycPermCheck (22) serves to express the probability of a compound to penetrate through the mycobacterial cell wall. Compounds with a probability value over 0.82 are ranked as permeable, while compounds with probability value under 0.55 are predicted to be impermeable (22).

The ability to penetrate the mycobacterial cell wall is an important criterion for activity, but obviously it is not the only condition that must be met by a drug candidate. In this study, starting compounds **1** and **2** as well as **3g** and **3h** are hypothetically permeable (probability value over 0.82) but have no significant activity. Incorporation of a terminal hydroxyl or methoxy group into the aliphatic chain drastically reduced the predicted permeability and led to inactive compounds (**3i**–**m**). Prolongation of alkyl

chain increased the probability of permeation, for example in series **3** going from theoretically impermeable propylamino derivative **3a**, through pentylamino (**3c**) and heptylamino (**3e**) derivatives with moderate permeation, to octylamino derivative **3f** with good permeability. Generally, the MycPermCheck prediction tool supports our previously formulated hypothesis (21) that a long alkylamino chain is a structural factor that facilitates permeation of the mycobacterial cell wall. Among other determinants, permeability is closely associated with lipophilicity. We therefore compared the log *P* of the compounds with their antimycobacterial activity. A significant dependence between the length of the alkyl chain (lipophilicity) and antimycobacterial activity was observed for compounds with a simple aliphatic chain (**3a**–**f**, **4a**–**f**, likewise in previously published series) (19–21). The highest activity (MIC <10 $\mu$M) was observed for compounds with a log *P* value in the range of 2.5–4.0 (corresponding to compounds with hexyl- to octylamino substitution), while inactive compounds (MIC >100 $\mu$M) usually had log *P* value lower than 1.5 (see Graph 1**)**.

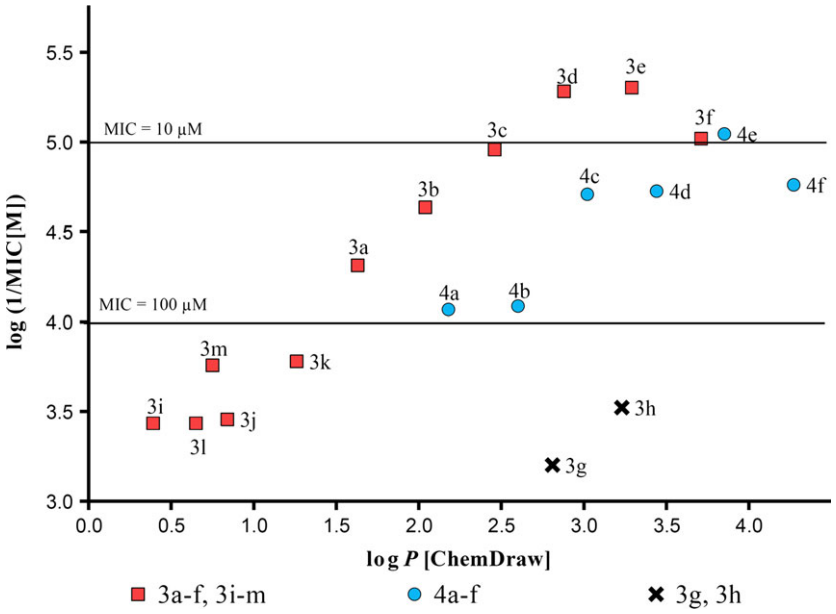
The negligible activity of hydroxyalkylamino derivatives (**3i**–**k**) and methoxylalkylamino derivatives (**3l**, **3m**) demonstrates that modification of long alkyl chain with polar groups is not desirable. As seen in Graph 1, the activity of phenylalkylamino derivatives (**3g**, **3h**) was trifling compared to simple alkylamino derivatives. This decrease of activity could be caused by steric effect of phenyl core. Selected active compounds were also evaluated for their activity against seven drug-resistant strains of *Mycobacterium tuberculosis* with different resistance patterns (clinical isolates; Table 2). All tested compounds exhibited moderate activity, which was in general lower than the activity of INH. However, compound **3f** showed promising activity against all tested strains with MIC values comparable to those of INH.

### *In vitro* antibacterial and antifungal activity

To complete the biological study, all prepared compounds including starting compounds were screened for activity against eight bacterial and eight fungal strains. Compounds **3b**–**e** and **4d** exhibited activity against Gram-positive strains *Staphylococcus aureus*, methicillin-resistant *S. aureus* and *S. epidermidis* (Table 3), while Gram-negative strains were completely resistant to these compounds. The other prepared compounds (including starting compounds **1** and **2**) did not affect the growth of Gram-positive strains. Compound **3b** showed only moderate antibacterial activity (125–250 $\mu$M). More importantly, compounds **3c**–**e** exhibited relatively potent antibacterial activity (MIC = 7.81–62.5 $\mu$M) against methicillin-resistant *S. aureus*. Also activity against *S. epidermidis* was in a similar range (MIC = 7.81–31.3 $\mu$M) for compounds **3c**–**e** and **4d**. These activities were fully comparable or even superior to the activity of reference compounds. Gram-negative strains as well as all fungal strains were completely resistant against all prepared compounds (including the starting compounds **1** and **2**) even at the highest concentrations used in the assay (MIC > 500 $\mu$M).

### *Cytotoxicity*

*In vitro* cytotoxicity (25–27) assays were performed for compounds with heptylamino (**3e**, **4e**) and octylamino substitution (**3f**, **4f**). The results were expressed as the compound concentration causing minimal changes in cell morphology (MCC) or as 50% cytotoxic concentration ($CC_{50}$, compound reducing cell viability by 50%, as




**Graph 1:** Dependence of antimycobacterial activity on lipophilicity log *P*.

**Table 2:** Antimycobacterial activity of selected derivatives against MDR-TB and XDR-TB strains, minimal inhibitory concentrations in $\mu$M

| | | | MIC ($\mu$M) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Mtb.* 234/2005 | | *Mtb.* 9449/2007 | | *Mtb.* 7357/1998 | | *Mtb.* 8666/2010 | | *Mtb.* Praha 1 | | *Mtb.* Praha 4 | | *Mtb.* Praha131 | |
| No. | R | Alk | 14d | 21d | 14d | 21d | 14d | 21d | 14d | 21d | 14d | 21d | 14d | 21d | 14d | 21d |
| **3d** | -H | -*n*-$C_6H_{13}$ | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| **3e** | -H | -*n*-$C_7H_{15}$ | 32 | 32 | 16 | 32 | 16 | 32 | 16 | 32 | 32 | 32 | 16 | 32 | 16 | 32 |
| **3f** | -H | -*n*-$C_8H_{17}$ | 16 | 16 | 16 | 32 | 16 | 32 | 16 | 16 | 16 | 16 | 16 | 32 | 16 | 16 |
| **4d** | -Cl | -*n*-$C_6H_{13}$ | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| **4e** | -Cl | -*n*-$C_7H_{15}$ | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| **4f** | -Cl | -*n*-$C_8H_{17}$ | 32 | 62.5 | 32 | 62.5 | 32 | 62.5 | 62.5 | 62.5 | 62.5 | 62.5 | 32 | 62.5 | 32 | 62.5 |
| **INH** | | | 16 | 16 | 16 | 16 | 16 | 32 | 16 | 32 | 16 | 16 | 16 | 16 | 16 | 16 |

MDR-TB strains: **234/2005** and **7357/1998** both resistant to INH, RIF, rifabutine, streptomycin, ethambutol and ofloxacin; **Praha 1** resistant to INH, RIF, rifabutine, streptomycin, ethambutol and clofazimine; **8666/2010** resistant to INH, RIF, rifabutine; **9449/2006** and **Praha 4** both resistant to INH, RIF, rifabutine, ethambutol and streptomycin. XDR-TB strain: **Praha 131** resistant to INH, RIF, rifabutin, streptomycin, ethambutol, ofloxacin, gentamicin and amikacin.

**Table 3:** Antibacterial activity of the most active derivatives, MIC values defined as 95% inhibition of bacterial growth read after 24 and 48 h

| | MIC ($\mu$M) | | | | | |
|---|---|---|---|---|---|---|
| | SA | | MRSA | | SE | |
| No. | 24 h | 48 h | 24 h | 48 h | 24 h | 48 h |
| **3b** | 125 | 125 | 250 | 250 | 250 | 250 |
| **3c** | 31.3 | 125 | 31.3 | 31.3 | 15.6 | 31.3 |
| **3d** | 250 | >500 | 7.81 | 62.5 | 7.81 | 31.3 |
| **3e** | 250 | >500 | 7.81 | 15.6 | 7.81 | 31.3 |
| **4d** | >500 | >500 | >500 | >500 | 31.3 | 31.3 |
| **Neomycin** | 1.95 | 3.9 | 3.9 | 7.81 | 15.6 | 15.6 |
| **Bacitracin** | 7.81 | 7.81 | 7.81 | 31.3 | 15.6 | 31.3 |
| **Penicillin G** | 0.49 | 0.98 | 62.5 | 125 | 125 | 250 |

SA, *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*; SE, *S. epidermidis*.

**Table 4:** Cytotoxic effect of selected compounds on different cell lines expressed as $CC_{50}$[a], MCC[a] or $IC_{50}$ in $\mu$M

| No. | CRFK[b] $CC_{50}$ $\mu$M | HEL[c] MCC $\mu$M | HeLa[d] MCC $\mu$M | Vero[e] MCC $\mu$M | HepG2[f] $IC_{50}$ $\mu$M |
|---|---|---|---|---|---|
| **3e** | >100 | >100 | >100 | >100 | ND |
| **3f** | >100 | >100 | >100 | >100 | 30.7 |
| **4e** | >100 | >100 | >100 | >100 | ND |
| **4f** | >100 | >100 | >100 | >100 | ND |

ND, not done.
[a]Cytotoxicity is expressed as the minimum cytotoxic concentration (MCC; compound concentration producing minimal changes in cell morphology, as estimated by microscopy), or the 50% cytotoxic concentration ($CC_{50}$; estimated by the MTS cell viability assay). Value preceded by the sign '>' means that at the indicated concentration, no significant cytotoxicity was observed.
[b]Crandell Rees feline kidney cells.
[c]Human embryonic lung fibroblasts.
[d]Human cervix epithelial cells.
[e]African green monkey kidney cells.
[f]Human hepatocellular liver carcinoma cell line.

assessed by a colorimetric formazan assay). As shown in Table 4, all tested compounds were not cytotoxic at a concentration of 100 $\mu$M.

In addition, for compound **3f**, an *in vitro* hepatotoxicity assay was carried out in human HepG2 hepatoma cells. The decrease in viability of the HepG2 cells was measured using a colorimetric assay based on the reduction of tetrazolium (28,29) and results expressed as $IC_{50}$ (concentration causing 50% inhibition of cell proliferation). Based on these HepG2 model, 6–octylamino-*N*-phenylpyrazine-2-carboxamide (**3f**) appears to have moderate cytotoxicity ($IC_{50}$ = 30.7 $\mu$M).

## Docking

We performed a molecular docking study to estimate whether the active compounds of the presented series can be substrates of PncA and could be hydrolysed to their acidic form. Initially, we tried to apply a simple docking procedure described in our previous publication (21), in which the probability of conversion was determined simply based on the orientation of the carboxamido group of studied PZA derivatives at the active site of enzyme PncA (pdb: 3PL1). In that study, the carboxamide moiety of docked 5-alkylamino and 6-alkylaminopyrazine-2-carboxamides was directed significantly away from the catalytic site of the enzyme, preventing the catalytic reaction. Using this described procedure, we docked compounds **3a**–**f** but found that the results were unclear. Because of sterically demanding substituents at both ends of the molecule (i.e. alkylamino and phenyl substituent), some conformations of the docked compounds had their carboxamide moiety relatively close and quite well oriented towards the

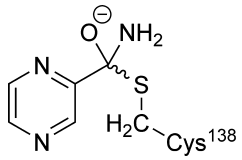



**Figure 1:** General structure of the proposed substrate–enzyme tetrahedral intermediate.

catalytic triad of the enzyme, which is compatible with the possibility for hydrolytic cleavage of the compounds. However, docking of small molecules into the active site of an enzyme by itself is not sufficient to distinguish between enzyme inhibitors and substrates (30). Enzymatic hydrolysis of a carboxamide by PncA proceeds through the formation of the acyl–enzyme covalent complex, originating from the nucleophilic attack on the carboxamide carbon by the reactive thiol group of Cys138 (31). We hypothesize that this acyl–enzyme complex is preceded by a tetrahedral intermediate (Figure 1), analogous to the catalytic mechanism described for the related nicotinamidase from *Acinetobacter baumannii* (32).

Formation of the tetrahedral intermediate and its stabilization by interaction with active site residues are crucial for substrate conversion. We thus used a covalent docking protocol embedded in Schrödinger Suite to simulate the formation of the ligand–enzyme tetrahedral intermediates. The intermediates were simulated for compounds **3a** (short alkyl chain, moderate activity) and **3e** (long alkyl chain, high activity), and compared with intermediates constructed from known substrates of PncA, that is nicotinamide (Nam) and pyrazinamide (PZA). The covalent docking protocol produced highly stabilized tetrahedral ligand–protein intermediates for both known substrates Nam and PZA. These Nam and PZA intermediates shared the same interaction pattern. Namely, their carbonyl oxygen (oxoanion) forms *H*-bonds to the backbone NH groups of Cys138 and Ala134. The ligand's $-NH_2$ group forms an *H*-bond interaction to the side chain carboxyl of Asp8. The ring nitrogen in *meta* position to the carboxamide forms an *H*-bond to water 220, which itself is coordinated to a $Fe^{2+}$ ion. Furthermore, the heteroaromatic nucleus is stabilized by $\pi$–$\pi$ stacking interaction (T-shaped) with the planar ring of Trp68. Figure 2 represents Nam covalently bound to PncA and the interactions that stabilize its tetrahedral intermediate. The PZA-derived intermediate showed the same interactions, and the superposition of PZA and Nam intermediates is presented in Figure 3. To sum up, the covalent docking protocol that we used was able to predict ligand–enzyme tetrahedral intermediates with all important stabilizing interactions described in a published Petrella *et al.* (31). model of acyl–enzyme complex for PZA and PncA. In this model, water 202 is positioned under the carbonyl carbon to prepare for final hydrolysis of the acyl–enzyme complex. Therefore, we first tried to perform the covalent docking with HOH202

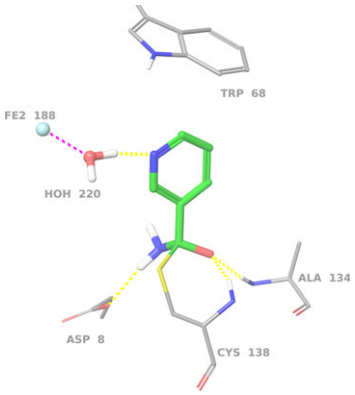



**Figure 2:** Stabilization of Nam-PncA tetrahedral intermediate.

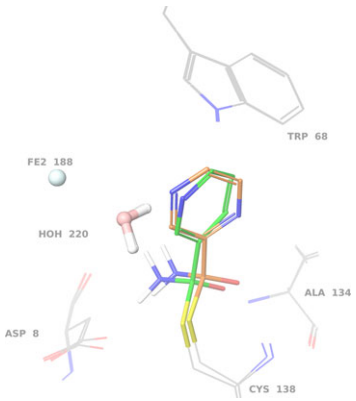



**Figure 3:** Superposition of PZA-PncA (orange carbons) and Nam-PncA (green carbons) intermediates.

(together with $Fe^{2+}$ co-ordinated HOH220 and HOH221), but we did not obtain tetrahedral intermediates with the desired orientation. Omitting the hydrolysing water 202 from the protein before covalent docking led to the intermediates described above, in which the space formerly occupied by HOH202 is reserved for the $-NH_2$ group of PZA or Nam. This can be interpreted as temporarily displacement of water 202 which returns to its hydrolysing position when the tetrahedral intermediate proceeds to the planar acyl–enzyme complex (i.e. the C–N bond breaks and the $-NH_2$ group is leaving).

Following the same procedure, we produced the tetrahedral intermediates for compounds **3a** and **3e**. These intermediates were not stabilized by any of the interactions describe above. Figure 4 shows the superposition of the intermediate form **3e** and Nam. The differences in the spatial arrangement and orientation of individual groups attached to the tetrahedral carbon explain the inability of the **3e** intermediate to form stabilizing interactions, either between its oxoanion and Cys138 and Ala134, or its –NH to the side chain carboxyl of Asp8. Due to the lack of stabilization of their tetrahedral intermediates, we conclude that compounds **3a** and **3e** are unlikely to be substrates for mycobacterial pyrazinamidase PncA.

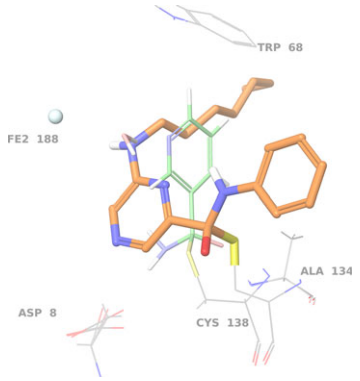



**Figure 4:** Superposition of PZA-PncA (orange carbons) and Nam-PncA (green carbons) intermediates.

## Conclusions

The main aim of this project was to increase the antimycobacterial activity of two newly prepared anilides (**1** and **2**) with negligible activity against *Mycobacterium tuberculosis* H37Rv. Two series of alkylamino derivatives (**3a**–**f**, **4a**–**f**) were designed, synthesized and screened for biological activities. All compounds with simple aliphatic chain exerted higher antimycobacterial activity than the corresponding starting compound **1** or **2**. Generally, the antimycobacterial activity increased with lipophilicity (corresponds to the length of alkyl chain) and culminated in compounds with heptylamino substitution (**3e**, **4e**). The most active compounds (with hexyl to octylamino substitution, marked from **d** to **f**) were also screened for activity against seven drug-resistant strains of *Mycobacterium tuberculosis*. All these compounds showed activity comparable to that of the reference drug isoniazid (INH). Except for compounds **3f** and **4d** (MIC = 6.25 $\mu$g/mL, *M. kansasii*, comparable with INH), no activity was observed against tested strains of *Mycobacterium kansasii* and *M. avium*. To study the influence of a simple aliphatic chain on antimycobacterial activity, part of the chain was replaced with phenyl ring (**3g**, **3h**), hydroxyl group (**3i**–**k**) or methoxy group (**3l** and **3m**). These substitutions led to significant decrease or complete loss of activity. The results of cytotoxicity assays suggest that the tested compounds are non-toxic at the highest concentration tested (100 $\mu$M). Compound **3f** displayed moderate cytotoxicity ($IC_{50}$ = 30.7 $\mu$M) in human hepatoma cells. A covalent docking procedure was used to predict potential hydrolysis of compounds **3a** and **3e** by PncA enzyme, to yield the corresponding carboxylic acid forms. On the basis of the lack of stabilization of the tetrahedral intermediates, we conclude that compounds **3a** and **3e** probably do not undergo hydrolysis by PncA and, thus, appear to act in their non-hydrolysed form.

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## Conflict of Interest

The authors declare no conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Experimental Section.