Charles University

Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

Derivatives of Pyrazinecarboxylic Acid as Potential Antimycobacterial Active Drugs

Doctoral Thesis (A Commentary on Published Articles)

MSc. Ghada Bouz

Supervisor: **Prof. PharmDr. Martin Doležal, Ph.D.**

Advisor: **Assoc. Prof. PharmDr. Jan Zitko, Ph.D.**

Hradec Králové, 2020

Holding a PhD degree has always been a dream of mine, and I -thereby- dedicate my doctoral thesis to my beloved parents, you are the reason behind the person I am today, to my Sarah and Luis, you are my backbone, to the love of my life, you make me complete, and to my late beloved grandfather, you are always with me.

"I declare that this thesis is my original author's work. Literature and other resources used were in-text cited and referenced accordingly. The work has not been submitted elsewhere to obtain the same or another title."

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MSc. Ghada Bouz

Abstract

Charles University, Faculty of Pharmacy in Hradec Králové.

Department of	Department of Pharmaceutical Chemistry and Pharmaceutical Analysis
Candidate	Ghada Bouz
Supervisor	Prof. PharmDr. Martin Doležal, Ph.D.
Title of Doctoral Thesis	Derivatives of Pyrazinecarboxylic Acid as Potential Antimycobacterial Active Drugs

This doctoral thesis is focused on the design, synthesis, and *in vitro* antimicrobial evaluation of potentially active compounds structurally derived from the first line antitubercular, pyrazinamide. The introduction briefly highlights the serious issue of tuberculosis in the present time, along with the reasons behind the failure to eradicate this ancient infection. The lack of a proper animal model that can replicate tuberculosis in its latent and active form was discussed more in detail, where zebrafish (*Danio rerio*) was suggested as a novel animal model suitable for multi-research purposes connected to tuberculosis.

A total of 112 compounds were synthesized and published as part of this doctoral work. They are divided into four main structural types, namely 3-(phenylcarbamoyl)pyrazine-2-carboxylic acids, 3-aminopyrazine-2-carboxamides, ureidopyrazines, and *N*-(pyrazin-2-yl)benzenesulfonamides. All prepared compounds were *in vitro* screened against five mycobacterial strains (with the main focus on *Mycobacterium tuberculosis* H37Rv), eight bacterial strains, and eight fungal stems, along with *in vitro* cytotoxicity evaluation on HepG2 liver cancer cells. The synthetic routes and structure-activity-relationships based on obtained biological results are discussed, emphasising the structures of the most active compounds of each series. Ongoing synthesis with preliminary results is also mentioned in brief to inspire future efforts. The most promising compound among all for further optimization was propyl 5-(3-phenylureido)pyrazine-2-carboxylate (MIC_{Mtb} = 1.56 µg/mL, 5.2 µM) belonging to the ureidopyrazine series.

Abstrakt

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové.

Katedra	Katedra farmaceutické chemie a farmaceutické analýzy
Kandidát	Ghada Bouz
Školitel	Prof. PharmDr. Martin Doležal, Ph.D.
Název disertační práce	Deriváty pyrazinkarboxylové kyseliny jako potenciální antimykobakteriální léčiva

Tato disertační práce je zaměřena na návrh, syntézu a *in vitro* antimikrobiální hodnocení potenciálně účinných látek strukturně odvozených od pyrazinamidu, antituberkulotika první linie. Úvod krátce upozorňuje na závažnost problému tuberkulózy a na důvody, které dosud znemožňují eradikaci této nemoci. Mezi tyto důvody patří absence zvířecího modelu, který by umožnil studovat tuberkulózu ve stádiu latentní i aktivní infekce. V této souvislosti jsme popsali rybí model dánia pruhovaného (*Danio rerio*), který byl navržen jako nový zvířecí model vhodný pro výzkum tuberkulózy.

Celkem bylo v rámci řešení této disertační práce připraveno a publikováno 112 sloučenin. Sloučeniny jsou rozděleny do čtyř hlavních strukturních typů, jmenovitě 3-(fenylkarbamoyl)pyrazin-2-karboxylové kyseliny, 3-aminopyrazin-2-karboxamidy, ureidopyraziny a *N*-(pyrazin-2-yl)benzensulfonamidy. Všechny připravené sloučeniny byly testovány na *in vitro* aktivitu vůči pěti mykobakteriálním kmenům (s důrazem na *Mycobacterium tuberculosis* H37Rv), osmi bakteriálním kmenům a osmi kmenům hub. Dále byla hodnocena cytotoxicita *in vitro* na HepG2 buněčné linii karcinomu jater. Byly popsány syntetické postupy a diskutovány vztahy struktura-účinek založené na získaných biologických výsledcích, s důrazem na struktury nejaktivnějších sloučenin každé série. V závěru práce byl stručně popsán v současnosti probíhající výzkum na dalších sériích sloučenin a jeho předběžné výsledky. Celkově se nejslibnější sloučeninou pro další vývoj jeví propyl 5-(3-fenylureido)pyrazin-2-karboxylát (MIC$_{Mtb}$ = 1.56 µg/mL, 5.2 µM) patřící do série ureidopyrazinů.

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1. List of Abbreviations

°C	Degree centigrade
aq	Aqueous
BCG	Bacille Calmette-Guerin
CDI	1,1'-Carbonyldiimidazol
DHPS	Dihydropteroate synthase
DMSO	Dimethyl sulfoxide
DprE1	Decaprenylphosphoryl-β-D-ribose oxidase
FAS I	Fatty acid synthase I
h	Hour
His	Histidine
HMDPdiP	6-hydroxymethyl-7,8-dihydropterin diphosphate
IC	Inhibitory concentration
MIC	Minimum inhibitory concentration
min	Minute
MMP-8	Matrix metalloproteinase-8
Mtb	Mycobacterium tuberculosis
MW	Microwave
NAD	Nicotinamide adenine dinucleotide
PDIM	Phthiocerol dimycocerosate
POA	Pyrazinoic acid
PZA	Pyrazinamide
QAPRTase	Quinolinic acid phosphoribosyl transferase
RpsA	Ribosomal protein S1
RT	Room temperature
SI	Selectivity index
SMILES	Simplified molecular-input line-entry system
sol	Solution
TB	Tuberculosis
W	Watt
WHO	World Health Organization

2. Introduction

Annually, the World Health Organization (WHO) releases the cause-of-death statistics to help authorities orient their public health actions. The top 10 causes of death list included -put in order- ischemic heart disease, stroke, chronic obstructive pulmonary disease, lower respiratory infections, Alzheimer disease and other dementias, respiratory system cancers, diabetes mellitus, road injuries, dehydration from diarrheal diseases, and tuberculosis (TB).[1] Despite the fact that TB has fallen from the 6th global cause of death in 2000 to the 10th in 2016, it is still the number one killer among infectious diseases.[2] The available Bacille Calmette-Guerin (BCG) vaccine prevents only the disseminated forms of the infection in children, yet fails to protect against adult TB infection.[3] The lengthy standard drug-sensitive TB treatment [initial phase for a period of two months with a combination of four different first-line antituberculars (Figure 1), namely isoniazid, rifampicin, pyrazinamide (PZA), and ethambutol, followed by continuation phase of minimum four months with isoniazid and rifampicin] with sub-optimal antimycobacterial activity and serious adverse effects, along with the inaccessibility to health care in developing countries, led to poor adherence to anti-TB regimen and subsequently high rate of treatment failure and emergence of antimicrobial resistance.[2] Furthermore, the increased numbers of immunocompromised people and the global mobilization movement have put developed countries at high risk of both drug-sensitive and drug-resistant TB.[4] Treatment for drug-resistant cases is even more challenging and requires longer periods with the use of second line agents.[4] Second line agents include levofloxacin, amikacin, ethionamide, and *p*-aminosalicylic acid, refer to Figure 1. These facts drove research efforts worldwide to find new approaches to prevent or treat this lethal infection. The ultimate goals of developing new antituberculars are shortening and simplifying treatment regimen, improving safety profile, overcoming resistance, and treating the infection in its latent stage.[5]
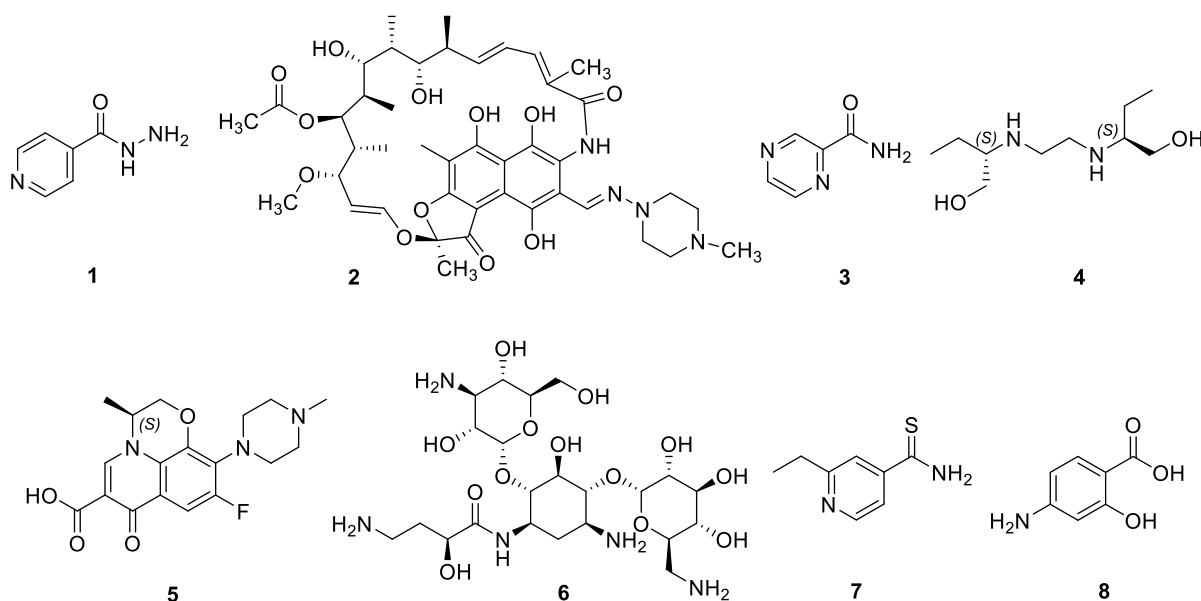
Figure 1: The chemical structures of first line antituberculars [(1) isoniazid; (2) rifampicin; (3) PZA; (4) ethambutol] and the mentioned second line antituberculars [(5) levofloxacin; (6) amikacin; (7) ethionamide; (8) *p*-aminosalicylic acid].

However, beside pharmaceutical industry apathy, advancement in developing new vaccines and treatments against TB is hindered by the lack of a proper animal model that could replicate the human TB infection in both the latent and active phases.[5,6] One attractive animal model in TB field is the tropical fish, *Danio rerio*, commonly known as zebrafish. The absence of lungs is not the only interesting fact when it comes to choosing zebrafish as a model for such pulmonary infection; the histological and pathological similarities between zebrafish *Mycobacterium marinum* infection and human *Mycobacterium tuberculosis* (*Mtb*) infection, along with their small size, low cost, transparency of their larvae, and the ease of manipulation added further advantages to the use of zebrafish model.[7,8] Nowadays, *Mycobacterium marinum* infected zebrafish model is used in evaluating new vaccines against tuberculosis, high throughput small molecule screening (see Figure 2), repurposing established drugs as antituberculars, and assessing hepatotoxicity of novel anti-TB agents.[9-12] More information on the use of zebrafish in TB research can be found in the review "The zebrafish model of tuberculosis - no lungs needed" in Section 7 (Publication **P1**).
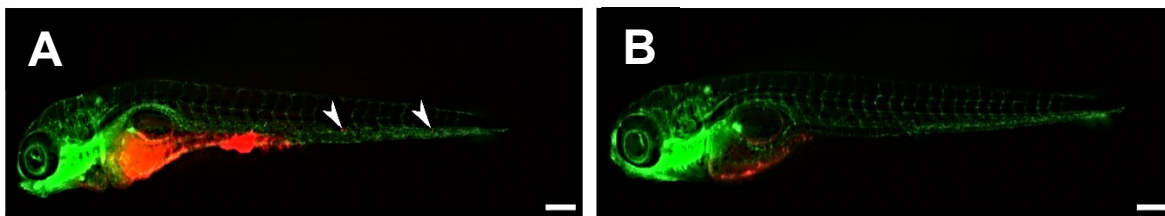
Figure 2: *Mycobacterium marinum* infected zebrafish model use in drug screening: effect of treatment with combination of isoniazid and rifampicin on bacterial growth (in red) in 6-day-old, fli1-egfp zebrafish larvae with gfp-labelled vasculature. A: fluorescent image of untreated larva; B: fluorescent image of larva after treatment. Adapted from [13] under CC BY license.

Proudly, our research group is part of this movement toward new antitubercular development. Our research focus is mainly on preparing new potentially active compounds structurally derived from the first line antitubercular, PZA. Among all anti-TB drugs, PZA has the highest capability to shorten treatment duration due to its sterilizing effect on semi-dormant *Mtb* bacilli.[14] It was discovered during *in vivo* phenotypic screening of nicotinamide analogues.[15] Interestingly, PZA exerts modest *in vitro* antimycobacterial activity.[15] PZA itself is hydrolysed by mycobacterial amidase PncA or host enzymes to the free pyrazinoic acid (POA).[16] It should be noted that mutations in *pncA* gene is the major pathway of resistance against PZA.[16] The mechanism of antimycobacterial activity of PZA/POA is complex involving multiple targets vital for mycobacterial survival, which in return widens the window for derivatization approach. Table 1 summarizes the most up-to-date findings regarding PZA/POA mode of action.

Table 1: Molecular targets of PZA/POA in *Mtb*.

| Target	Comments
Cellular and intracellular membranes	It was thought that POA acts like a proton ionophore causing the collapse of membrane potential and intracellular acidification.[17] However, this old theory was later disputed as PZA has to be present at concentrations 10 times higher than its minimum inhibitory concentration (MIC) to exert such structure-nonspecific action.[18]
Ribosomal protein S1 (RpsA)	RpsA is involved in trans-translation process needed for mycobacterial survival by degrading toxic protein accumulation in stressed mycobacteria.[19] It was thought that POA inhibits the trans-translation via binding to RpsA.[20] However, this mechanism was later disputed as it was found that a single strand of RNA is the one that binds to RpsA and not POA, and that the overexpression of *rpsA* gene was not associated with resistance against PZA.[20]
Fatty acid synthase I (FAS I)	FAS I is responsible for mycolic acid synthesis.[21] However, the IC_{50} of POA for FAS I is orders of magnitude greater than that of 5-Cl-PZA despite having similar MIC values.[22] Such finding suggests that the inhibition of FAS I is a downstream consequence of PZA treatment rather than a target.[22]
Quinolinic acid phosphoribosyl transferase (QAPRTase)	It is proposed that PZA inhibits the catalytic activity of QAPRTase, which is involved in the biosynthesis of nicotinamide adenine dinucleotide (NAD).[23]
Aspartate decarboxylase (PanD)	PanD encoded by *panD* gene is involved in the biosynthesis of pantothenate (B5) and thus the essential acyl carrier coenzyme A (CoA).[24] Treatment with POA was found to cause degradation in PanD.[24] However, *Mtb* strains lacking *panD* gene are still susceptible to PZA, suggesting that aspartate decarboxylase is not the only molecular target for PZA.[25]
Gps1	Gps1 is involved in DNA polymerization and phosphorylation.[26] *Mtb* strains with *gps1* mutations had higher MIC values against PZA.[26]
ATP-dependent ATPase ClpC1	ATP-dependent ATPase ClpC1 is encoded by a new gene *clpC1* and is responsible for protein degradation.[27] This target was discovered by identifying resistant strains not bearing mutations in *pncA*, *rpsA* or *panD* genes.[27] This ATPase is also the target of three new cyclic peptide antibiotics, namely cyclomarin A [28], lassomycin [29], and ecumicin [30].
Phthiocerol dimycocerosate (PDIM)	PDIM is a virulence factor encoded by the *in vitro* nonessential polyketide synthase genes *mas* and *ppsA-E*.[31] This interference of PZA/POA with PDIM may explain its excellent *in vivo* efficacy versus the poor *in vitro* activity.[31]

3. Commentary on Published Results

From the literature, structure-activity-relationships studies showed that the presence of pyrazine heterocycle and a carboxamide at C2 are essential for the antitubercular activity of PZA.[5] Therefore, our design and synthesis was mainly based on pyrazine-2-carboxamide. Synthesized compounds were evaluated *in vitro* for their antimycobacterial activities against five different mycobacterial strains (*Mtb* H37Rv, *Mycobacterium kansasii*, *Mycobacterium avium*, *Mycobacterium smegmatis*, and *Mycobacterium aurum*). As complementary testing, compounds were also screened against four Gram-positive (*Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*) and four Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*) bacterial strains and eight fungal stems (*Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Absidia corymbifera*, *Trichophyton interdigitale*) using microplate dilution assay with MICs determined by naked eye. All compounds were evaluated for *in vitro* cytotoxicity in HepG2 liver cancer cell line. A hepatocellular cancer model was selected since antitubercular drugs are known to carry the risk of hepatotoxicity, which is even augmented when agents are administered in combination.[32,33] Obtained results were presented by the inhibitory concentration that reduces the viability of cancer cell population to 50% (IC_{50}). For biological evaluation details, refer to corresponding publications and their supplementary materials in Section 7. Mainly, activities against *Mtb* H37Rv will be discussed in this commentary. If any other significant activities were found, then they shall be mentioned.

3.1. Expansion of previous work

In this section, the two series are originally designed by my colleague Lucia Semelková, Ph.D., yet I contributed to the synthesis, purifications, analytical data interpretation, and writing original drafts.

3.1.1. 3-(Phenylcarbamoyl)-pyrazine-2-carboxylic acids (Publication P2, Ref. No. 34)

In order to prepare a more lipophilic derivatives of pyrazinecarboxylic acid, we prepared a series of 3-(phenyl-carbamoyl)pyrazine-2-carboxylic acids and their methyl and propyl esters as prodrugs, refer to scheme 1. The most active compounds against *Mtb* H37Rv were the free acid 3-[(4-nitrophenyl)carbamoyl]pyrazine-2-carboxylic acid (R = 4-NO$_2$; MIC$_{Mtb}$ = 1.56 µg/mL, 5 µM; IC$_{50}$ > 750 µM, SI* > 150) and the ester propyl 3-{[4-(trifluoromethyl)phenyl]carbamoyl}pyrazine-2-carboxylate (R = 4-CF$_3$; MIC$_{Mtb}$ = 3.13 µg/mL, 9 µM; IC$_{50}$ > 525.4 µM, SI > 25.2) with no observed *in vitro* cytotoxicity in tested concentrations. No other significant antimicrobial activity was detected in this series. Based on obtained biological results it was concluded that esterification did not improve antitubercular activity with the mentioned propyl ester being an exception.
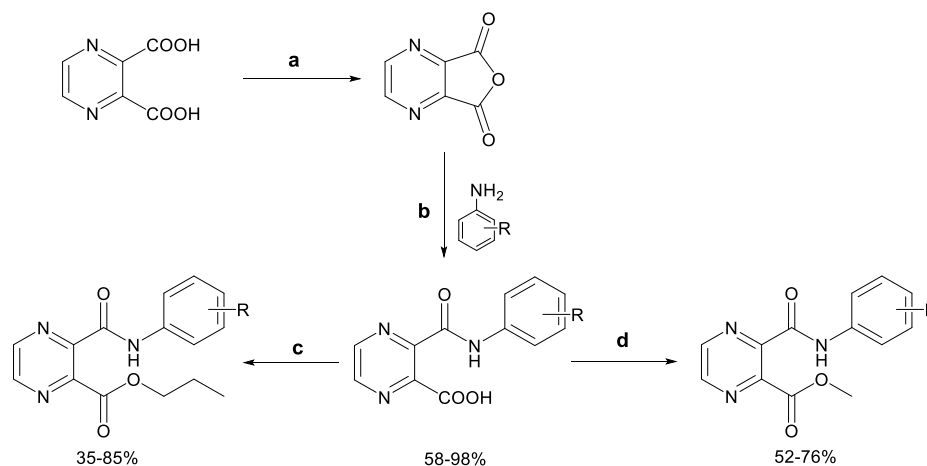
Scheme 1: Synthetic procedures of prepared series. *Reagents and Conditions:* (a) acetic anhydride, reflux, 1 h; (b) 1. tetrahydrofuran, RT, 1 h, 2. water, NaHCO$_3$ sol.; (c) propanol, H$_2$SO$_4$, microwave reactor (MW): 120 °C, 20 min, 50 W; (d) methanol, H$_2$SO$_4$, MW: 120 °C, 20 min, 50 W. Adapted from [34] under CC BY 4.0 license.

Due to the structural similarities between title compounds and the original quinoxaline inhibitor of mycobacterial decaprenylphosphoryl-β-D-ribose oxidase

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*SI = selectivity index; SI was calculated by dividing the toxicity parameter IC$_{50}$ (determined in HepG2 cells) by the MIC in µM against *Mtb* H37Rv. Values above 10 are favorable. Due to low solubility of some compounds, the IC$_{50}$ could not be accurately determined and the IC$_{50}$ value at highest tested concentration was used (values with the ">" sign).

(DprE1),[35] we docked the most active free acid 3-[(4-nitrophenyl)carbamoyl]pyrazine-2-carboxylic acid to DprE1 in order to have an insight on its mechanism of action, refer to Figure 3. DprE1 is an essential enzyme for the synthesis of arabinogalactan, an important component of mycobacterial cell wall, and thus its inhibition results in cell death.[35] Docking results revealed that exchanging the -NH-CH$_2$- linker in the original ligand with -CONH- group in title compounds did not negatively alter the position of the scaffold in DprE1. However, the large lipophilic substituent in the original ligand, represented by the condensed benzene ring and trifluoro methyl group, is essential for filling the hydrophobic subpocket of the enzyme and positively contributes to antitubercular activity. Since such lipophilic fragment is missing in title compounds, we were inspired to prepare another series of compounds bearing this additional condensed benzene core (general structure bearing quinoxaline instead of pyrazine, Section 4.1.). For further information, refer to the paper entitled "Design, Synthesis, Antimycobacterial Evaluation, and *In Silico* Studies of 3-(Phenylcarbamoyl)-pyrazine-2-carboxylic Acids" in Section 7.
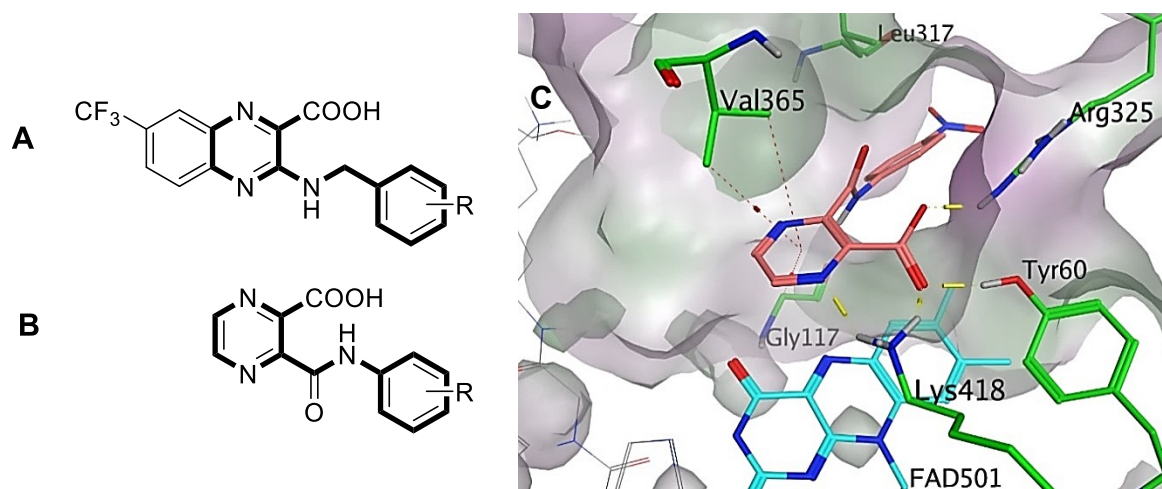

Figure 3: DprE1 as a possible target for the most active free acid, 3-[(4-nitrophenyl)carbamoyl]pyrazine-2-carboxylic acid. A: Basic scaffold of established DprE1 inhibitors; B: General structure of title compounds; C: Predicted binding mode of the most active acid against *Mtb* (in pink). Adapted from [34] under CC BY 4.0 license.

3.1.2. 3-Aminopyrazine-2-carboxamides (Publication P3, Ref. No. 39)

We prepared a series of 3-aminopyrazine-2-carboxamides with various substituents (R) on the carboxamidic nitrogen as the last block in our long-term investigation on the effect of different substituents at position 3 on the pyrazine ring (free amino group vs. chlorine atom [36] vs. *N*-alkyl/*N*-benzyl substituted amino group [36,37]) on antimicrobial activity, refer to Figure 4. We have previously also synthesized aminopyrazinamide derivatives with free amino groups at position C5 on the pyrazine ring, yet they had no significant antimycobacterial activity.[38] Prepared compounds were further subdivided based on the substituent (R) into benzyl derivatives, alkyl derivative, and phenyl derivatives, refer to Figure 4. Benzyl derivatives were prepared by using procedures (a) and (b) in Scheme 2, yet due to low yields, procedure (c) was followed to prepare the remaining alkyl and phenyl derivatives, which in return gave higher yields (some benzyl derivatives were resynthesized by the latter procedure to verify higher yields).
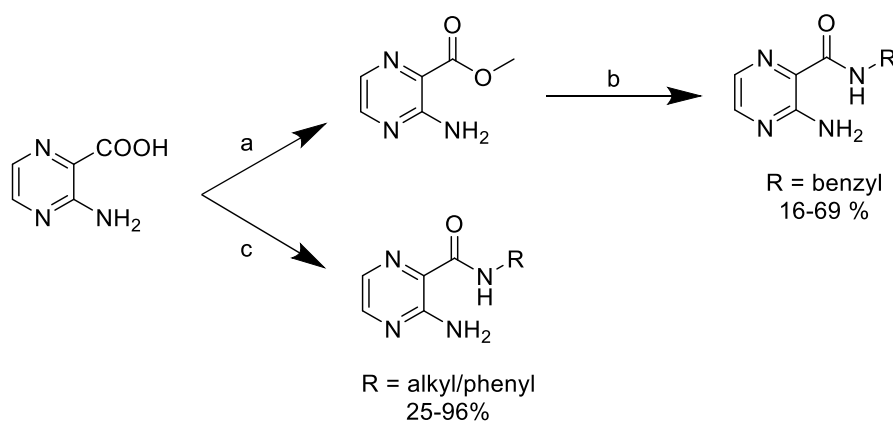
Scheme 2: Synthesis of *N*-substituted 3-aminopyrazine-2-carboxamides. (a) H_2SO_4, methanol, 48 h, RT; (b) substituted benzylamine, NH_4Cl, methanol, MW: 130 °C, 40 min, 90 W; (c) 1. CDI, DMSO; 2. benzylamine/alkylamine/aniline, MW: 120 °C, 30 min, 100 W. Adapted from [39] under CC BY 4.0 license.

The most active compound against *Mtb* was the phenyl derivative 3-amino-*N*-(2,4-dimethoxyphenyl)pyrazine-2-carboxamide (R = 2,4-diOCH$_3$; MIC$_{Mtb}$ 12.5 µg/mL, 46 µM; IC$_{50}$ > 250 µM, SI > 6). Generally, phenyl and alkyl derivatives were more active than the benzyl derivatives. Regarding benzyl derivatives, mono substitution at position C3 or C4 exerted low activity, while the disubstituted were

completely inactive (MIC$_{Mtb}$ > 100 µg/mL). For alkyl derivatives, we observed that the antimicrobial activity against *Mtb* and *Mycobacterium kansasii* increased with increasing the number of carbons in the alkyl side chain, which is consistent with our previous findings.[38] Antibacterial activity was observed for phenyl and alkyl derivatives, but not for benzyl derivatives. Marginal antifungal activity was detected for the three structural types, mainly against *Trichophyton interdigitale* and *Candida albicans*. We compared obtained results with that of previously prepared, structurally related compounds from our group and summarized structure-activity relationships in Figure 4. For further information, refer to the paper entitled "Derivatives of 3-Aminopyrazine-2-carboxamides: Synthesis, Antimicrobial Evaluation, and *in Vitro* Cytotoxicity" in Section 7.
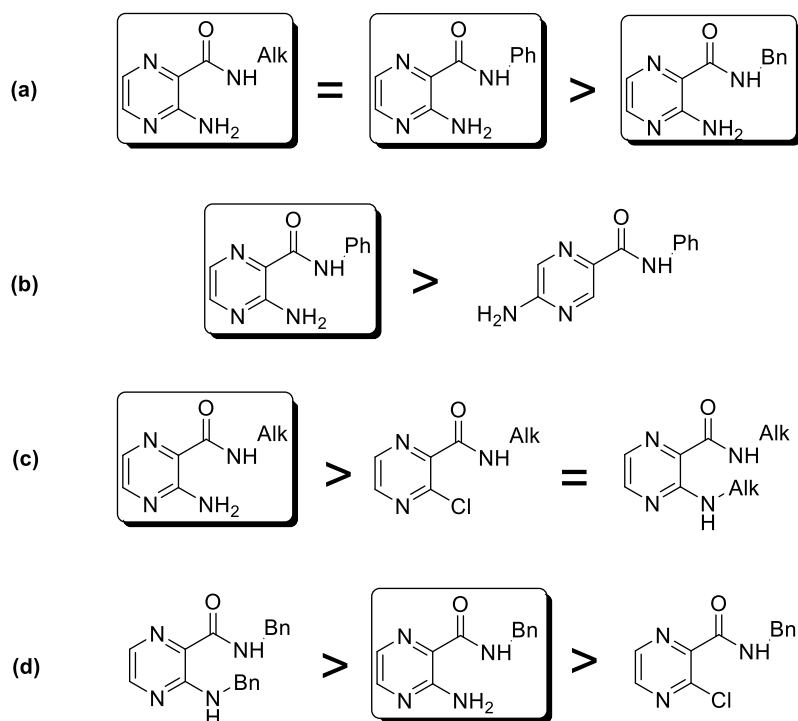
Figure 4: A comparison between antimycobacterial activity against *Mtb* (a) between the three structural subtypes of the current series (b) between phenyl derivatives belonging to this series and their positional isomers [38] (c) among different alkyl derivatives [40] (d) among different benzyl derivatives [41]. Structures in rounded rectangles belong to the current series. Alk = alkyl; Ph = phenyl; Bn = benzyl; R = alkyl, phenyl, benzyl. Adapted from [39] under CC BY 4.0 license.

3.2. New series

All compounds in this section are my original design.

3.2.1. Ureidopyrazine derivatives (Publication P4, Ref. No. 44)

In general, compounds containing urea moiety exert wide range of pharmacological activities, such as anticancer, hypoglycaemic, anticonvulsant, and antimicrobial activities.[42] Therefore, we introduced urea moiety to pyrazine core by reacting aryl isocyanates with propyl 5-aminopyrazine-2-carboxylate (Scheme 3, I) or by reacting aryl/alkyl isocyanates with aminopyrazine (Scheme 3, II and III respectively) in pressurized vials using a CEM Discover microwave reactor with a focused field, refer to Scheme 3. The two compounds, propyl 5-(3-phenylureido)pyrazine-2-carboxylate (MIC_{Mtb} = 1.56 µg/mL, 5.2 µM; IC_{50} > 25 µM, SI > 5) and propyl 5-(3-(4-methoxyphenyl)ureido)pyrazine-2-carboxylate (MIC_{Mtb} = 6.25 µg/mL, 19 µM; IC_{50} > 50 µM, SI > 2.6), had potent antimycobacterial activity against *Mtb* H37Rv with no significant *in vitro* cytotoxicity on HepG2cell line. From the obtained biological results, we concluded that the ester moiety is important for activity probably by increasing the compound's lipophilicity. In general, increasing the lipophilicity of antituberculars has positive influence on antimycobacterial activity, presumably due to enhanced penetration through the lipophilic mycobacterial cell wall.[43] When the most active compound, propyl 5-(3-phenylureido)pyrazine-2-carboxylate, was hydrolysed to its free acid form, it lost its antimycobacterial activity. Among non-ester compounds, we found that aryl substituted ureidopyrazines had higher antitubercular activity against *Mtb* than the alkyl derivatives. Regarding alkyl derivatives, 1-octyl-3-(pyrazin-2-yl)urea (MIC_{Mtb} = 25 µg/mL, 99.9 µM; IC_{50} =1000 µM, SI = 10), was the most active compound, in agreement with our previous finding that the optimal alkyl chain length for antitubercular activity is 6–8 carbons.[29] No other significant antimicrobial activity was detected. For further information, refer to the paper entitled "Ureidopyrazine Derivatives: Synthesis and Biological Evaluation as Anti-Infectives and Abiotic Elicitors" in Section 7.
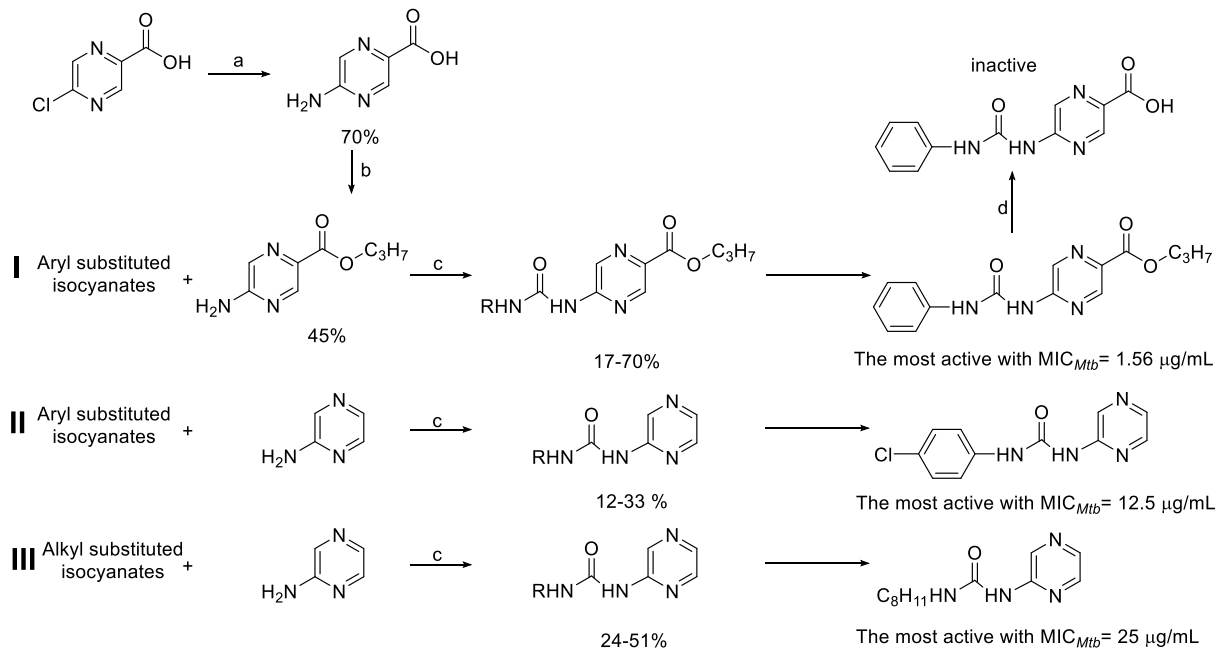
Scheme 3: Synthetic procedures leading to ureidopyrazines and depiction of most active compounds. *Reagents and Conditions*: (a) NH_3 (25% aq.sol), MW:100 °C, 30 min, 80 W; (b) esterification with propanol, H_2SO_4, MW: 100 °C, 1 h, 80 W; (c) hexane, MW:120 °C, 1 h, 80 W; (d) base catalysed hydrolysis with anhydrous K_2CO_3 in methanol, 85 °C under reflux, 6 h. Adapted from [44] under CC BY 4.0 license.

3.2.2. Substituted *N*-(pyrazin-2-yl)benzenesulfonamides (Publication P5, Ref. No. 47)

As an attempt to study the influence of isosteric replacement of the retro-amide moiety in the previously published series of *N*-(pyrazin-2-yl)benzamides (referred to as retro-amides; Figure 5b [45]) by the sulfonamide moiety, we prepared a series of *N*-(pyrazin-2-yl)benzenesulfonamides (Figure 5c) by reacting different sulfonyl chlorides with pyrazin-2-amine or 6-chloropyrazin-2-amine, refer to Scheme 4. Title compounds (Figure 5c) were also compared to original amides from the series pyrazinecarboxamides (Figure 5a).[46]
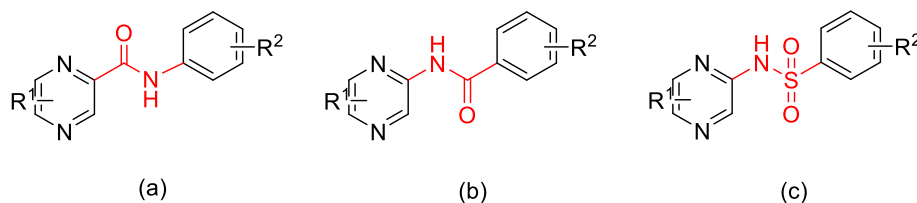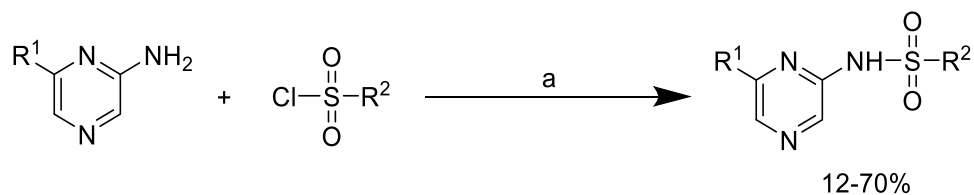

Figure 5: Design rationale: general structure of (a) pyrazinecarboxamides = amides; (b) *N*-pyrazinylbenzamides = retro-amides; and (c) title compounds = sulfonamides. Adapted from [47] under CC BY 4.0 license.

Scheme 4: General synthetic reaction. R^1 : H/Cl; R^2 : refer to corresponding publication **P5**. *Reagents and Conditions*: (a) pyridine and acetone, RT, overnight. Adapted from [47] under CC BY 4.0 license.

Although initially designed as antituberculars, title sulfonamides did not show significant antimicrobial activity against mycobacterial, bacterial, or fungal strains. Exceptions include two compounds, namely 4-amino-*N*-(pyrazin-2-yl)benzenesulfonamide (MIC_{Mtb} = 6.25 µg/mL, 25 µM; IC_{50} = 775.6 µM, SI = 31) and 4-amino-*N*-(6-chloropyrazin-2-yl)benzenesulfonamide (MIC_{Mtb} = 6.25 µg/mL, 22 µM; IC_{50} > 500 µM, SI > 23), both of which possess a free amino group in the *para* position of the benzene ring, satisfying the general requirement for antibacterial sulfonamides. Therefore, in order to verify this hypothesis, pteridine conjugates of the latter two compounds were docked to mycobacterial dihydropteroate synthase (DHPS), refer to Figure 5. Docking results showed good binding scores (-7.9 to -8.3) and that the substitution on the pyrazine ring with chlorine atom had no effect on the predicted binding mode, which justifies the similar antimycobacterial activity of the non-chlorinated and chlorinated compounds.
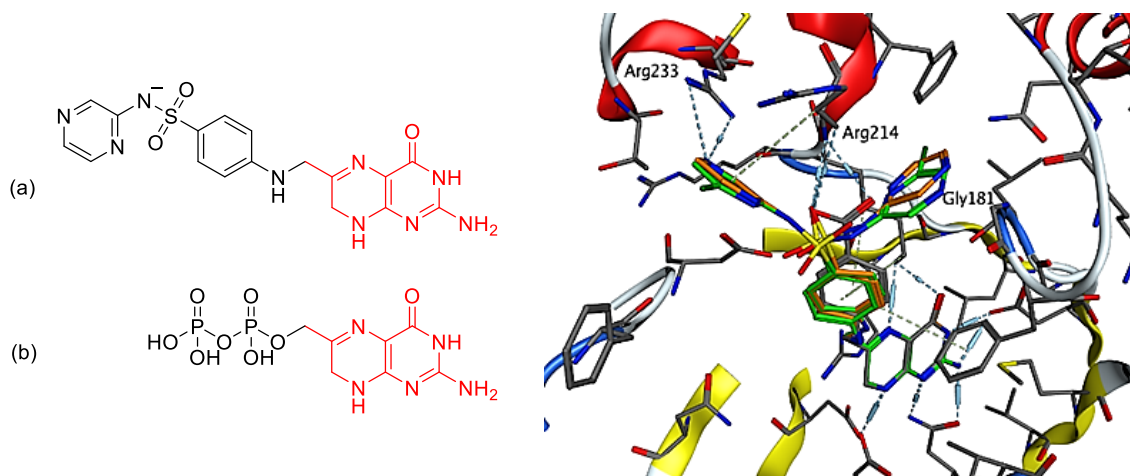

Figure 5: Structures of (a) the hypothetic product of compound 4-amino-*N*-(pyrazin-2-yl)benzenesulfonamide and (b) 6-hydroxymethyl-7,8-dihydropterin diphosphate (HMDPdiP) used for the docking experiments. Fragment

of HMDPdiP in the condensed product is in the rectangle. Product of mentioned compound (orange) and product of the chlorinated analogue (green) main poses from the docking experiments. The pose of 7,8-dihydropteroate (grey) corresponds to the experimentally determined position seen in bacterial DHPS. Adapted from [47] under CC BY 4.0 license.

In general, the substitution of retro-amide linker with the sulfonamide did not improve antimycobacterial activity against *Mtb*. We lastly performed target fishing, where we identified Matrix metalloproteinase-8 (MMP-8) as a potential target for our title compounds that is worth future exploration, refer to Figure 6. For further information, refer to the paper entitled "Substituted *N*-(Pyrazin-2-yl)benzenesulfonamides; Synthesis, Anti-Infective Evaluation, Cytotoxicity, and *In Silico* Studies" in Section 7.

(**a**)　　　　　　　　　　　　　　(**b**)

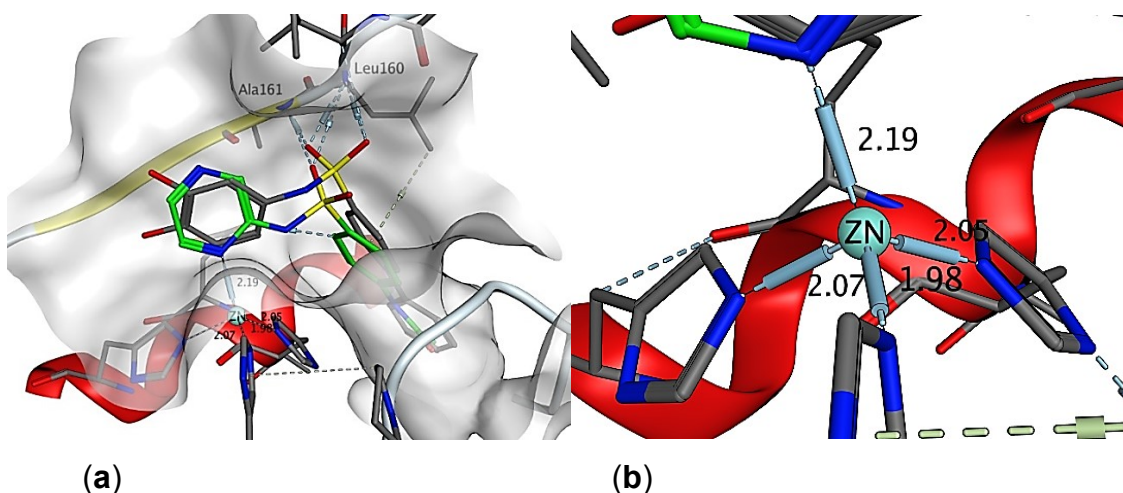**Figure 6:** Target fishing: (a) Compound *N*-(4-(*N*-(pyrazin-2-yl)sulfamoyl)phenyl)acetamide (green carbons) docked to MMP-8 (PDB ID: 5h8x) in comparison to the co-crystalized pose of the original catechol ligand (grey carbons). (b) Detail presenting interactions between pyrazine nitrogen N1′ and Zn2+ ion coordinated by His residues. (Measurements are in Å). Adapted from [47] under CC BY 4.0 license.

4. Ongoing Research and Future Plans

4.1. Derivatives of quinoxaline-2-carboxylic acid

As mentioned earlier in Section 3.1.1., the structure of the original inhibitor of DprE1 inspired us to prepare a number of compounds with quinoxaline core, refer to Scheme 5 for synthetic schemes and general structures.[48] Besides, quinoxaline-2-carboxylate 1,4-dioxide [49] and sugar conjugates of quinoxaline [50] are documented in the literature to have potent antimycobacterial activity. This series has three aims: to investigate the effect of different substituents (R) in each structural group on biological activity, to observe the influence of increasing the length of the linker between the amide and the benzene core on biological activity, and to compare title quinoxaline bearing compounds to previous structurally related pyrazine containing compounds. Preliminary results suggest that bulky substituents and extending the length of the linker have positive influence on antitubercular activity against *Mtb*.
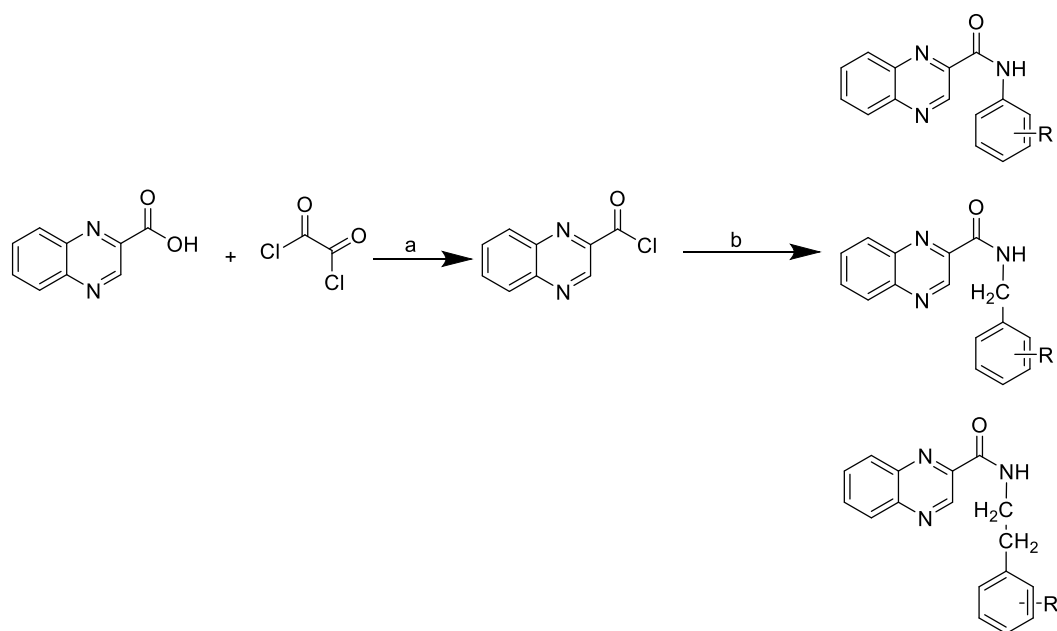
Scheme 5: Synthetic procedures. Reagents and Conditions: (a): DCM, DMF, RT, 30 minutes; (b) DCM, pyridine, corresponding amine, in ice bath, overnight.

4.2. Hybrid compounds combining pyrazinamide and *p*-aminosalicylic acid

Since TB is a classic example of a disease treated by multiple target therapy, we decided to apply hybridization approach of multiple target therapy and combine the first line PZA with the second line agent *p*-aminosalicylic acid to obtain a new chemical entity. Hybrid molecules may bring the advantage of having improved selectivity profile, new mode of action when compared to the original compounds, reduced unwanted side effects, improved pharmacokinetic profile, reduced manufacturing cost, and -of course- better adherence to therapy.[51,52] Final compounds were obtained by reacting different pyrazinecarboxylic acids with *p*-aminosalicylic acid according to Scheme 6. Then the *p*-aminosalicylic acid fragment of the most active compound was modified in order to optimize activity. Some compounds showed potent antitubercular activity against *Mtb* H37Rv with MICs below 1.56 µg/mL. Further structural modifications are attempted, and results of biological evaluation are still pending. Preliminary results were presented as oral communications at the 2018 meeting of Paul Ehrlich MedChem Euro-PhD Network in Porto and at the 6th EFMC Young Medicinal Chemist Symposium in Athens, 2019.

R: H, Cl, NH_2, CH_3; R^1: H, OH

50-90%
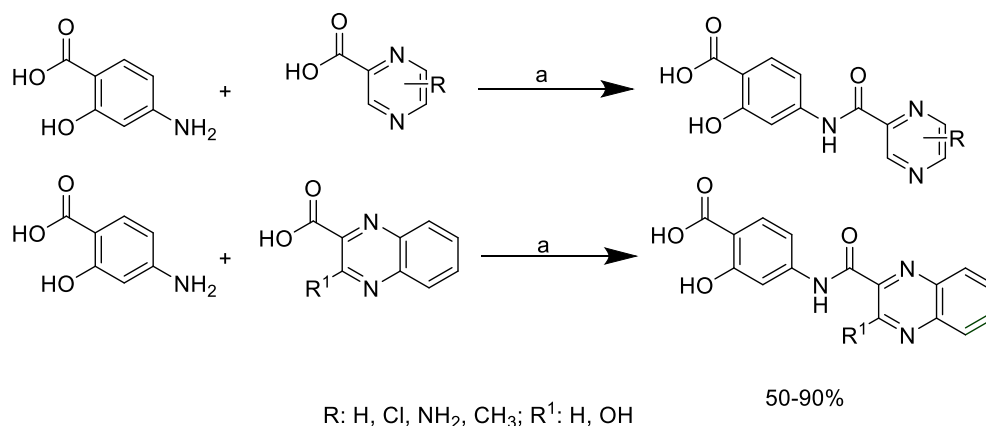
Scheme 6: Synthetic procedures. *Reagents and Conditions:* (a) DMSO, CDI, RT.

4.3. Miscellaneous

A large library of compounds was prepared by reacting the carboxylic acids of 6-membered heterocycles bearing one nitrogen [such as nicotinic acid (refer to Figure 7), isonicotinic acid, picolinic acid, substituted pyridine carboxylic acids, and quinolinecarboxylic acid] with aminopyrazine, 6-aminopyrazine, propyl ester of aminopyrazine, *p*-aminosalicylic acid, and *p*-aminobenzoic acid, separately, forming an amide bond. Some compounds showed potent antitubercular activity against *Mtb* H37Rv with MICs below 1.56 µg/mL. Preliminary results were presented as poster at the 2019 EFMC-ACSMEDI MedChem Frontiers in Krakow and at the 6th EFMC Young Medicinal Chemist Symposium in Athens, 2019.
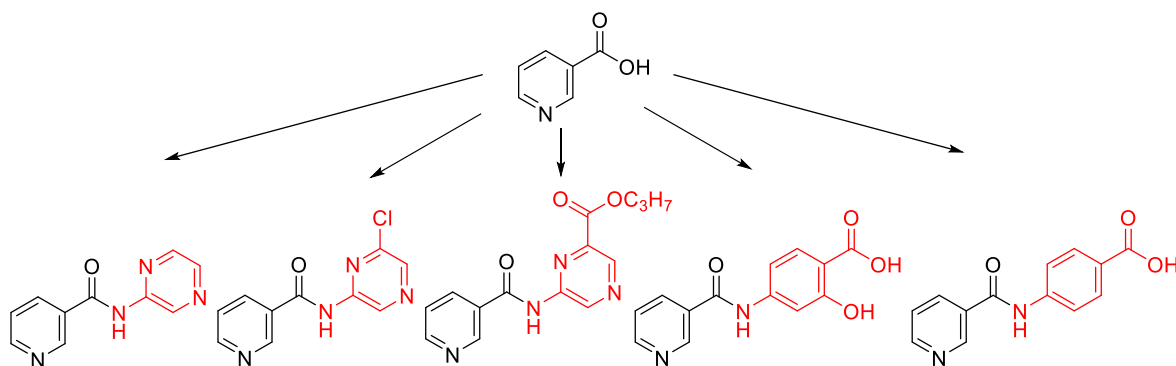

Figure 7: Obtained products when starting from nicotinic acid.

5. Conclusions and Recommendations

The urgent need for new agents to treat tuberculosis has brought efforts worldwide to investigate different compounds. The main drug discovery approaches in this field include repurposing established drugs, structural modifications of drugs with known antitubercular activity, and developing new structures against known or novel mycobacterial targets. The continuous discoveries and research on pyrazinamide (PZA) target identification enable chemists to design and synthesize various PZA-based compounds with different pharmacophores.

To conclude my work during my doctoral studies, I have prepared a number of compounds derived from the chemical structure of PZA. The attempted final compounds can be mainly divided into pyrazincarboxamides [3-(phenylcarbamoyl)-pyrazine-2-carboxylic acids (**P2**) and 3-aminopyrazine-2-carboxamides (**P3**)], ureidopyrazines (**P4**), *N*-(pyrazin-2-yl)benzenesulfonamides (**P5**), quinoxalinecarboxamides (section 4.1.), and hybrid compounds combining pyrazine/pyridine with *p*-aminosalicylic acid (sections 4.2. and 4.3.). From obtained biological evaluation against *Mycobacterium tuberculosis* (*Mtb*) H37Rv, it can be concluded that compounds bearing urea moiety exert high antimycobacterial activity, quinoxaline core may improve such activity by forming additional interactions with DprE1, the sulfonamidic bond does not improve antimycobacterial activity compared to amidic or retro-amidic bond, and having *p*-aminosalicylic acid fragment in the structure positively influence activity. The most active compounds from each published series are presented in Figure 8.
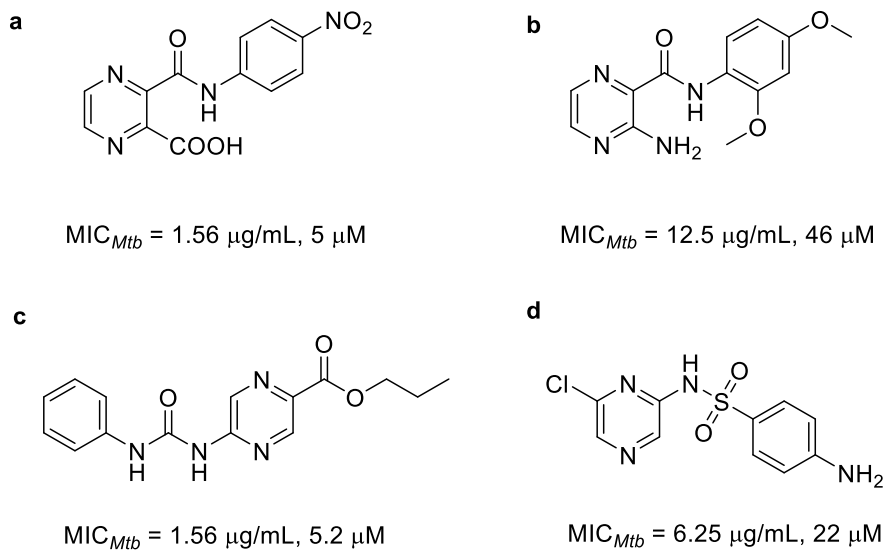
a

MIC$_{Mtb}$ = 1.56 μg/mL, 5 μM

b

MIC$_{Mtb}$ = 12.5 μg/mL, 46 μM

c

MIC$_{Mtb}$ = 1.56 μg/mL, 5.2 μM

d

MIC$_{Mtb}$ = 6.25 μg/mL, 22 μM

Figure 8: The chemical structures of the most active compound among (a) 3-(phenylcarbamoyl)-pyrazine-2-carboxylic acids (**P2**); (b) 3-aminopyrazine-2-carboxamides (**P3**); (c) ureidopyrazines (**P4**); and (d) *N*-(pyrazin-2-yl)benzenesulfonamides (**P5**).

As for future research, more focus should be directed toward synthesis mentioned in Section 3.3. as some prepared compounds exerted potent antimycobacterial activity. This ongoing work shall be part of my post-doc efforts and my colleagues' doctoral theses. I also suggest modifying the most promising compounds from this thesis by adding important functional groups essential for the interaction with target amino acids inspired from Table 1. Molecular docking studies of attempted structures to selected PZA target(s) is advised to be performed prior to synthesis in order to - along with biological testing results - influence the design of final compounds. A complete list of published compounds belonging to this thesis, including their SMILES and activities against *Mtb* H37Rv, is available as an attached CSV file for future use.

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7. Publications

7.1. Review Article

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7.2. Experimental Articles

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