Charles University

Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

Synthesis, development and biological evaluation of new antimicrobial compounds

Ph.D. Dissertation
(Commentary on Published Articles)

Mgr. Vinod Sukanth Kumar Pallabothula

Supervisor: Assoc. Prof. PharmDr. Jan Zitko, Ph.D.

Hradec Králové, 2024

Declaration

"I declare that this thesis is my original work of authorship, which I have written independently (under the guidance of my supervisor Assoc. Prof. PharmDr. Jan Zitko, Ph.D.). All literature and other sources I have drawn upon in the preparation of this thesis are listed in the reference list and duly cited in the thesis. The thesis has not been used to obtain another or the same degree."

Hradec Králové, 2024 **Mgr. Vinod Sukanth Kumar Pallabothula**

Acknowledgment

First and foremost, I would like to express my deepest gratitude to my supervisor, doc. PharmDr. Jan Zitko, Ph.D. His invaluable support, encouragement, and expertise were influential throughout my Ph.D. journey. I am particularly thankful for his innovative ideas, feedback on scientific papers, grant proposals, and texts; being very patient during the challenging moments, and giving me complete freedom to explore my ideas. During my entire Ph.D. studies, he was like more or less "*Try it Vinod, don't worry about the funding, we can manage."* I am very thankful for his invaluable support through *in-silico* studies to back our hypotheses and discover new outcomes.

My Ph.D. experience was significantly enriched by the guidance of **Prof. PharmDr. Martin Doležal**, **Ph.D.**, the leader of the "Design and Development of New Antimicrobial Agents" research group. Prof. Doležal fostered a vibrant and supportive research environment that extended far beyond the lab. The regular "coffee meets", group trips, and social events encouraged a sense of friendship and made my time truly enjoyable. I will always cherish the memories and lessons learned during this time.

I would like to especially thank the following people for their scientific collaboration and/or technical assistance throughout my whole research:

- **PharmDr. Ondřej Janďourek, Ph.D.**; **RNDr. Klára Konečná, Ph.D., Ms. Dufková** and **MUDr. Pavla Paterová, Ph.D.** for their invaluable support with antimicrobial and antifungal studies.
- **doc. PharmDr. Jiří Kuneš, Ph.D.** for measuring several hundreds of NMR samples.
- **Mgr. Pavel Bárta, Ph.D.** and **RNDr. Novák Martin, Ph.D.** for invitro biological assays.
- **Dr. Matteo Mori, Ph.D.,** and **Dr. Enrico M. A. Fassi, Ph.D.** for their teaching assistance during my traineeship at the University of Milan, Milan, Italy.

A big thanks to my awesome lab partner and friend, **PharmDr. Martin Juhás, Ph.D.** He's put up with me for way too long, and I'm sure it's been a two-way street! I'm super grateful to Martin for sharing his knowledge and teaching me new stuff. He was a huge help with the computer stuff, too.

I would like to thank my favorite person in the Czech Republic, **MSc. Ghada Bouz, Ph.D.** a well-wisher and more like a big sister. She was a great help during my troubled times, supporting me emotionally and trying to cheer me up with a lot of social events, friendly meets, and so on. I'm grateful to **PharmDr. Marta Kučerová, Ph.D.**, **Dr. Jibin A. Abraham**, **Ph.D.**, **Manikaran S. Mahal**, and the rest of the Department members and friends for being part of my Ph.D. journey. Your advice and friendly company helped me feel supported and connected.

I would like to acknowledge the use of artificial intelligence (AI) tools such as Google's Gemini and OpenAI's ChatGPT to enhance language proficiency and Grammarly for language corrections in this dissertation.

Finally, I am eternally grateful to the Almighty for guiding me to this momentous achievement. My heartfelt thanks go to my parents for their unwavering love, support, sacrifices, and financial support. I couldn't have lived comfortably without my mom's secret 'research' fund. There are no words to describe how grateful I am to my parents to be at this stage.

Funding acknowledgement

- Faculty of Pharmacy in Hradec Kralove, Charles University, for the doctoral stipend.
- Charles University - Project SVV 260 666.
- Grant schemes of Charles University (reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935).
- Grant agency of Charles University (GAUK) – Project no. 349 721.
- Co-funded by Project National Institute of Virology and Bacteriology (Programme EXCELES, ID Project No. LX22NPO5103) - Funded by the European Union - Next Generation EU.

Abstract

Charles University, Faculty of Pharmacy in Hradec Králové

Department	Department of Pharmaceutical Chemistry and Pharmaceutical Analysis
Doctoral Study Program	Pharmaceutical Chemistry
Candidate	Mgr. Vinod Sukanth Kumar Pallabothula
Supervisor	Assoc. Prof. PharmDr. Jan Zitko, Ph.D.
Doctoral dissertation title	Synthesis, development and biological evaluation of new antimicrobial compounds

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains a substantial global health burden. Regardless of the availability of modern antibiotics, the incidence and mortality rates of TB continue to advance due to drug resistance, highlighting the need for innovative strategies to combat this persistent disease.

The introductory part of this doctoral dissertation briefly describes TB and current challenges in the treatment regimen and the need for novel antibacterials to combat drug resistance. Mycobacterial prolyl-tRNA synthetase (mtProRS) is an essential enzyme for protein synthesis and it was our main cellular target of interest to combat TB, hopefully without effecting human homologue hsProRS. Aspartate decarboxylase (PanD) was an auxiliary target for some specific final compounds due to structural similarity to previously reported inhibitors.

The design of antimycobacterial compounds and potential inhibitors of mtProRS in this research was based on confirmed inhibitors of human-ProRS (hsProRS) containing a pyrazine scaffold. The final compounds exhibited firm structure-activity relationships (SAR) with MIC values ranging from 1.95–31.25 µg/mL against Mtb with consistent activities against multidrug-resistant Mtb strains with low toxicity on HepG2 cells. Several pyrazine-containing cyclic derivatives were synthesized within the main synthetic framework, and tested for antimycobacterial properties. These compounds are prone to metabolize to their respective pyrazinoic acids and open the door to the prodrug approach for PanD inhibitors. This dissertation also contains commentary on my work in complementary publications (co-author) focused on antimicrobial research.

Abstrakt

Univerzita Karlova, Farmaceutická Fakulta v Hradec Králové

Katedra	Katedra farmaceutické chemie a farmaceutické analýzy
Doktorský studijní program	Farmaceutická chemie
Kandidát	Mgr. Vinod Sukanth Kumar Pallabothula
Školitel	Doc. PharmDr. Jan Zitko, Ph.D.
Název doktorské disertační práce	Syntéza, vývoj a biologické hodnocení nových antimikrobních sloučenin

Tuberkulóza (TB), způsobená patogenem *Mycobacterium tuberculosis* (Mtb), zůstává významnou globální zdravotní zátěží. Bez ohledu na dostupnost moderních antibiotik se incidence a úmrtnost na TB nadále zvyšuje v důsledku lékové rezistence, což zdůrazňuje potřebu inovativních strategií pro boj s tímto přetrvávajícím onemocněním.

Úvodní část této doktorské disertační práce stručně popisuje TB a aktuální výzvy v léčebném režimu a potřebu nových antibiotik pro boj s lékovou rezistencí. Mykobakteriální prolyl-tRNA syntetáza (mtProRS) je nezbytný enzym pro syntézu proteinů a byl naším hlavním buněčným cílem v boji proti TB, pokud možno bez ovlivnění lidského homologu hsProRS. Aspartát dekarboxyláza (PanD) byla doplňkovým cílem pro vybrané finální sloučeniny na základě jejich strukturní podobnosti s dříve popsanými inhibitory.

Návrh antimykobakteriálních sloučenin a potenciálních inhibitorů mtProRS v tomto výzkumu byl založen na potvrzených inhibitorech lidské ProRS (hsProRS) obsahujících pyrazinové jádro. Finální sloučeniny vykazovaly zřejmé vztahy mezi strukturou a aktivitou (SAR) s hodnotami MIC v rozmezí 1,95–31,25 μg/ml proti Mtb s konzistentními aktivitami proti multilékově-rezistentním kmenům Mtb s nízkou toxicitou na buňkách HepG2. V rámci hlavního syntetického rámce bylo syntetizováno několik cyklických derivátů obsahujících pyrazin; a tyto byly rovněž testovány na antimykobakteriální aktivitu. Tyto sloučeniny jsou náchylné k metabolizaci na jejich příslušné pyrazinové kyseliny a otevírají tak možnost jejich využití jako proléčiv inhibitorů PanD. Tato disertační práce také obsahuje komentáře k mé práci provedené v doplňkových publikacích (spoluautor), tematicky rovněž zaměřených na antimikrobiální výzkum.

Table of Contents

List of Abbreviations

Mtb	*Mycobacterium tuberculosis*
TB	Tuberculosis
LTBI	Latent Tuberculosis Infection
Mil	Million
PZA	Pyrazinamide
PZase	nicotinamidase/pyrazinamidase
INH	Isoniazid
AMR	Antimicrobial resistance
MDR-TB	Multidrug-Resistant TB
XDR-TB	Extensively Drug-Resistant TB
pre-XDR	pre-Extensively Drug-Resistant
POA	Pyrazinoic acid
aaRSs	Aminoacyl-tRNA synthetases
ProRS	Prolyl-tRNA Synthetase
HFG	Halofuginone
EPRS	glutamyl-prolyl-tRNA synthetase
PanD	Aspartate decarboxylase
CoA	Coenzyme A
ITC	Isothermal calorimetry
DCM	Dichloromethane
MeCN	Acetonitrile
Anhyd.	Anhydrous
SAR	Structure-Activity Relationship
hsProRS	Human prolyl-tRNA synthetase

MIC	Minimal inhibitory concentration
MW	Microwave irradiation
THF	Tetrahydrofuran
NOE	Nuclear Overhauser effect
HLM	Human liver microsomes
mtMetAP1	Mycobacterial methionine aminopeptidase 1
CDI	Carbonyldiimidazole
2-AMT	2-aminothiazole
2-AMO	2-aminooxazole
DMF	Dimethyl formamide
TSA	Thermal shift assay

1. Introduction

This doctoral dissertation is a collection of published works with commentary along with various contributions throughout the doctoral study program. In our research work, we mainly focused on the design, synthesis, and evaluation of novel antimicrobial agents. More specifically, this dissertation work involves the investigation of novel pyrazine-containing molecules to target *Mycobacterium tuberculosis* (Mtb), followed by *in silico* investigations to understand the mechanism of action.

1.1. Tuberculosis (TB)

Tuberculosis (TB) is one of the oldest and deadliest diseases ever known to humankind. It is mainly caused by *Mycobacterium tuberculosis* (Mtb).[1, 2] Mtb has infected approx. one-fourth of the entire world's population and this phenomenon is called Latent TB infection (LTBI). People with LTBI are at a 10% cumulative lifetime risk of developing the active disease.[3, 4] Every year, about 10 mil. (million) people fall ill with this deadly disease and at least 1.3 mil. people lost their lives in 2022.[4] The trend continues to rise from 2020 to 2022 with the rise in the number of people suffering due to TB from 10.0 mil in 2020 to 10.3 mil in 2021 and 10.6 mil in 2022.[4] This estimated trend from 2020 – 2022 was misinterpreted and was majorly impacted due to COVID-19 global pandemic.[4] It is estimated that approx. USD 15 billion is required to prevent, diagnose, and for the treatments by the '*Global Plan to end TB'* (2018 to 2022). Additionally, it is estimated that USD 2.0 billion is required annually to conduct TB research to combat this deadly disease. However, the funding falls far short of the global requirement by almost half the requirement.[5]

1.1.1. Current Treatment Regimen for LTBI & TB

The drug regimen for LTBI recommended by the *Center for Disease Control and Prevention* (www.cdc.gov) is given below.[6]

- Rifamycin-based regimens:

This includes including a) three months of weekly-once isoniazid along with rifapentine, b) four months of everyday rifampicin, c) three months of everyday isoniazid along with rifampin are the preferred recommendations due to their safety, effectiveness, and high treatment completion rates.[6]

- Isoniazid-based monotherapy regimen:

This Regimen may include either 6 or 9 months of daily isoniazid as an alternative treatment regimen; although this treatment is highly efficacious, it also has higher toxicity risk and lower treatment completion rates, which decreases the overall effectiveness.[6]

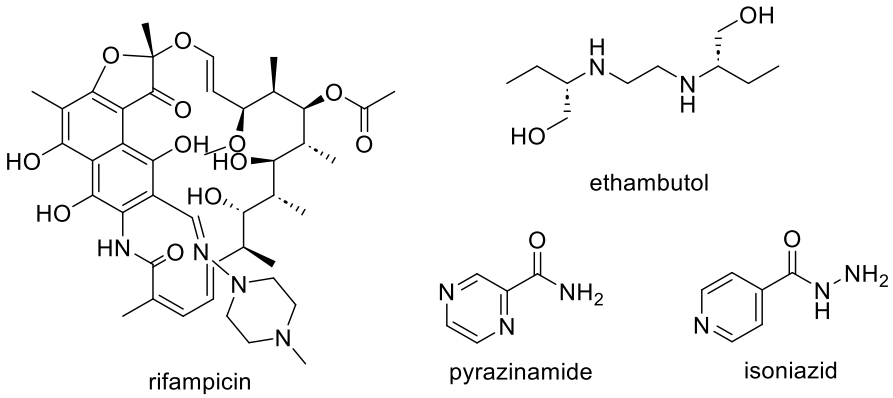


Figure 1. Four 1st line drugs to treat Tuberculosis[7]

The treatment for drug susceptible active TB involves combinations of the above-mentioned (see Figure **1**) four 1st line anti-tubercular drugs such as rifampicin, ethambutol, pyrazinamide, and isoniazid for a period of 6 months.[7-9] The long-term usage of the cocktail of these recommended drugs causes liver damage, nerve pain, vision issues, and skin rashes. It is quite challenging for the patient to use these many drugs for a long period and hence may lead to drug misuse. When the drugs are not properly used, may create an environment for Mtb bacteria to develop resistance mechanisms and it will be quite difficult to treat the disease in the future.[10]

- Rifampicin – It displays a modest early bactericidal activity, but it is one of the most significant sterilizing drugs available for TB treatment. It initially binds to the β-subunit of mycobacterial DNA-dependent RNA polymerase enzyme, followed by inhibition of gene transcription.[7, 11]

- Ethambutol – Ethambutol is generally used in combination therapy against multidrug-resistant forms of Mtb. The cell-wall synthesis is quite complex in Mtb compared to other regular bacteria. The membrane-embedded (Emb) proteins like EmbA, EmbB, and EmbC, which are responsible for cell-wall biosynthesis, are regarded as ethambutol targets and disrupt the cell-wall synthesis.[12, 13]

- Pyrazinamide – Pyrazinamide (PZA) is a special drug that exhibits a sterilizing effect on both drug-sensitive and MDR-TB.[14, 15] The mechanism of action is not yet fully understood and is the subject of debate. However, PZA is a prodrug that is converted into pyrazinoic acid (POA) in its active form by nicotinamidase/pyrazinamidase (PZase), followed by high accumulation of POA intracellularly and kills the bacteria by multiple mechanisms.[14, 16] Aspartate decarboxylase (PanD), is one of interesting target present only bacteria for POA derivatives (refer **section 1.2.3.** for more information).
- Isoniazid – Isoniazid (INH) is a prodrug that enters the cytoplasm of Mtb via passive diffusion and is then activated inside Mtb by an enzyme KatG. A reactive intermediate of INH inhibits the biosynthesis of mycolic acids, which are necessary for cell-wall formation.[17-19] INH kills only actively-multiplying mycobacteria; no killing occurs when mycobacteria are in the stationary phase or dividing under anaerobic conditions.[20]

1.1.2. Drug Resistance

The rise of antimicrobial resistance (AMR) has been almost inevitable and is one of the major challenges for TB treatment.[21] Since the beginning of the development of antimicrobials and their use, scientists have observed the nearly guaranteed emergence of resistance.[22] While antimicrobial resistance (AMR) is a natural process of occurrence in bacteria, the overuse, underuse, and misuse of antimicrobials in both humans and animals have significantly accelerated this phenomenon.[22-24]

Multidrug-Resistant Tuberculosis and Extensively Drug-Resistant Tuberculosis (XDR-TB)

Multidrug-resistant tuberculosis (MDR-TB)[25] is a serious form of TB where the mycobacteria is resistant to immediate 1st line drugs such as isoniazid and rifampicin. Worldwide, about 0.41 million people developed MDR-TB with an estimate of 3.3% among TB new cases and 17% among previously treated.[4] Studies show that previous TB treatment increases the risk of MDR-TB by 14 times. Extensively drug-resistant TB (XDR-TB)[25] is an even more severe form where MDR-TB strains become resistant to any fluoroquinolone plus at least one additional powerful drug (linezolid or bedaquiline). The World Health Organization (WHO) recognizes additional classifications of drug-resistant TB. Pre-

extensively drug-resistant (pre-XDR) TB is resistant to rifampicin and one of the fluoroquinolone antibacterials.[26]

Beyond XDR-TB, TB infections can become completely untreatable due to further resistance. This significantly increases the risk of death and creates major challenges in controlling the spread of the disease.[26] The lack of effective treatment for these highly resistant forms of TB leads to higher death rates and emphasizes the need for new strategies to combat TB transmission. In order to combat drug-resistant TB, the development of new treatment regimens and new drugs and exploring new targets are essential. Some of the drugs used to treat MDR-TB are given the given Table **1**.

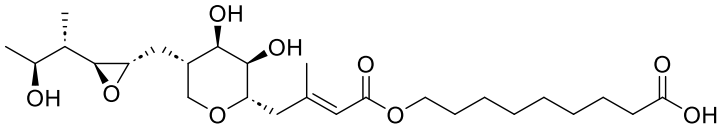
Table 1. Drugs used for MDR-TB treatment.

Drugs[27] used to treat MDR-TB	**Mechanism of action**	**Effect**
Bedaquiline[28-30]	inhibits ATP synthase's proton pump	bactericidal
Nitroimidazoles: *Delamanid*[31, 32] and *Pretomanid*[33, 34]	disrupts cell wall synthesis by inhibiting mycolic acid synthesis	Bactericidal and sterilizing
Oxazolidinones: *Linezolid*[35, 36] and *Tedizolid*[37, 38]	inhibit bacterial protein synthesis by interfering with translation	Bacteriostatic
Fluoroquinolones: *Levofloxacin, Moxifloxacin*[17, 39, 40]	inhibits Mtb DNA gyrase enzyme, eventually leads to blocking of DNA supercoiling	Bactericidal
Clofazimine[41-43]	not completely understood; suspected to target bacterial outer membrane[44]	Bactericidal
Aminoglycosides: *Streptomycin,*[45] *neomycin, paromomycin, gentamicin, and kanamycin, amikacin*[46-48]	disrupts protein synthesis by targeting mycobacterial ribosomes	Bactericidal
Ethionamide and **Prothionamide**[49-51]	prodrugs that need activation by monooxygenase *EthA,* inhibits biosynthesis of mycolic acids	Bacteriostatic
Cycloserine and **Terizidone**[27, 52, 53]	disrupts the cell-wall formation by targeting D-alanine racemase and D-alanine:D-alanine ligase	Bacteriostatic

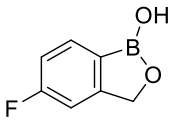
1.2. Theory and background for this research

1.2.1. Aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases (aaRSs), a family of 20 enzymes essential for protein synthesis,[54] have emerged as promising candidates for the development of next-generation antimicrobials and anti-cancer drugs.[55, 56] Their primary function is to covalently link the correct amino acid to its corresponding tRNA molecule, a critical step in protein biosynthesis.[54] Since bacteria rely on aaRSs for protein production as well, these enzymes present themselves as attractive targets for novel antimicrobials.[57] Mupirocin (antibacterial)[58] used to treat boils and impetigo[59] and Tavaborole (antifungal) used to treat onychomycosis, specifically targeting leucyl-tRNA synthetase,[60] serve as successful examples of aaRS-based antimicrobials.



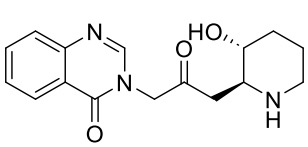
mupirocin



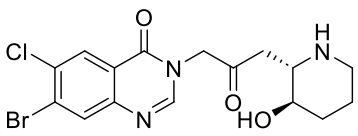
tavaborole

1.2.2. Prolyl-tRNA synthetase (ProRS)

Prolyl-tRNA synthetase (ProRS), a member of the aaRS family, is responsible for attaching the amino acid proline to its cognate tRNA to maintain the essential process of protein synthesis.[57] Inhibition of this enzyme leads to disruption of protein synthesis and subsequent death of bacteria. Blockade of protein synthesis is one of the major approaches for many MDR-TB antitubercular drugs (refer to Table **1**) and others, such as macrolides and tetracyclines.



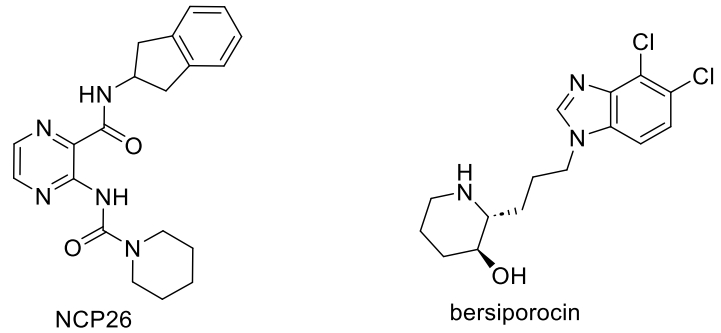
febrifugine



halofuginone

Febrifugine and its derivatives, including halofuginone (HFG), are established ProRS inhibitors known to bind to both the proline and tRNA binding sites.[61, 62] While HFG demonstrates promising activity against malaria, fibrosis, and cancer, it presents its own limitations.[62] Notably, HFG can lead to drug resistance in malaria parasites and exhibit reduced effectiveness in fibrosis treatment due to proline accumulation.[63] Researchers are actively seeking alternative ProRS inhibitors to address the limitations associated with HFG.[63] One approach involves developing non-competitive proline inhibitors or proline-

dependent inhibitors.[64] These strategies aim to prevent proline accumulation and potentially circumvent drug resistance issues.



ProRS inhibition holds promise for cancer therapy as well.[57] Studies have revealed upregulation of glutamyl-prolyl-tRNA synthetase (EPRS), which harbors ProRS activity, in certain cancers like multiple myeloma.[65] Inhibiting ProRS activity within EPRS has been identified as a potential therapeutic approach for such cancers. NCP26,[66] a newly synthesized inhibitor with a pyrazinamide scaffold, demonstrates effectiveness in cellular models and offers a promising candidate for EPRS-related cancer treatment.[66] Bersiporocin (DWN12088),[67] as an inhibitor of hsProRS, in Phase 2 clinical trials (ClinicalTrials.gov ID. NCT05389215) for the treatment of idiopathic pulmonary fibrosis.

In this current dissertation, two confirmed hsProRS binders and an antimycobacterial candidate (represented as **A**,[68, 69] **B**,[68] and **C**,[70] respectively, in Figure **2**) were considered as an anchor point to extend the horizon into antimycobacterial research.

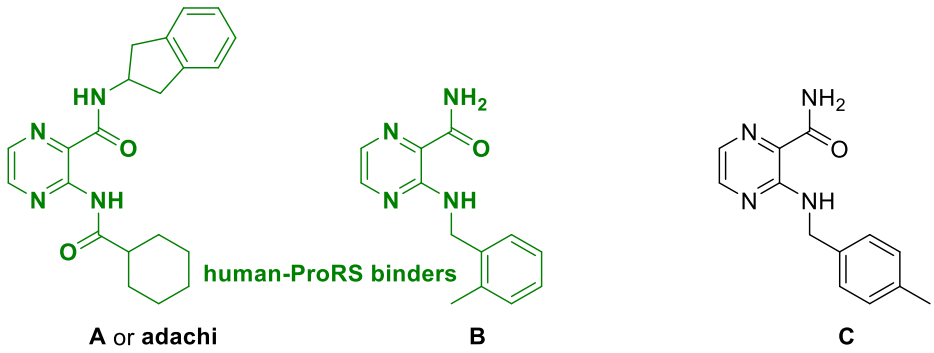


Figure 2. A, B - Pyrazinamide scaffold containing confirmed hsProRS binders; **C** – pyrazinamide containing anti-Mtb candidate.

The key differences between these compounds were clearly identified based on their target binding affinity, antimycobacterial property, and their cytotoxicity as follows.

- Candidate **A** described in Adachi et al. 2017,[69] is a confirmed hsProRS binder (PDB: 5VAD) with high structural similarity with NCP26 (EPRS inhibitor for cancer

treatment)[66], which was initially developed based on candidate **A**; cytotoxic on HepG2 cell-line IC_{50} = 19.6 µM and shows mediocre antimycobacterial activity Mtb H37Ra MIC = 62.5 µg/mL from our studies (results were published in **P1**; refer to page #56)[71]

- Candidate **B** described in Pang et al. 2021,[68, 70] is also a confirmed hsProRS binder (PDB: 7OSZ) with a lower binding affinity (evaluated by thermal-shift assay) compared to candidate **A**. This is due to the missing carbonyl oxygen and hence, missing an interaction with the protein. This compound does not exhibit any promising mycobacterial activity.
- Candidate **C** described in Jandourek et al. 2017,[68, 70] does not exhibit any binding confirmation with hsProRS yet, but shows promising antimycobacterial properties with MIC 1.56 µg/mL against H37Rv.

Hence, from the above structures and key differences, the following elements were considered for this dissertation research for potential ProRS inhibitors.

- The 3-amino pyrazinamide scaffold-based candidate should possess the additional carbonyl group in position 3, which was missing in candidate **B**.
- For potential hsProRS binding, 2' sub in the aromatic ring with short alkyl or X is favorable.
- For potential mtProRS binding, 4' sub in the aromatic ring with short alkyl or X are relevant as they tend to exhibit excellent antimycobacterial activity.

The complete research based on the above criteria along with additional substitutions and other structural modifications were designed and evaluated their SAR derived from antimycobacterial activity (refer to **P1** & **P2**; page #56).

1.2.3. Aspartate decarboxylase (PanD)

Mycobacteria possess a unique biosynthetic pathway for pantothenate, a precursor to the essential Coenzyme A (CoA). CoA is critical for various functions of the bacterial cell such as fatty acid metabolism, amino acid metabolism, and citric acid cycle.[72] A key enzyme in this pathway, aspartate decarboxylase (PanD), encoded by the *panD* gene, catalyzes the conversion of L-aspartate to β-alanine, a crucial step in pantothenic acid biosynthesis (see Figure **3**). PanD is a pyruvoyl-dependent enzyme, containing a covalently bonded pyruvoyl group for its catalytic activity.[73]

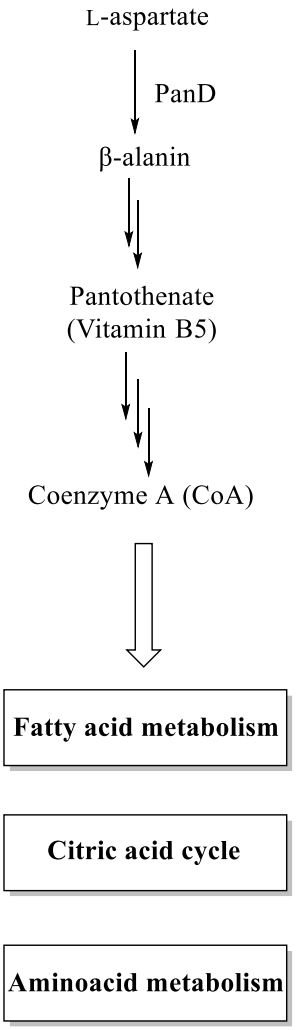
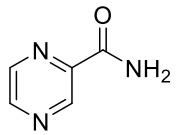


Figure 3. Pantothenate and CoA biosynthesis pathway in mycobacteria.

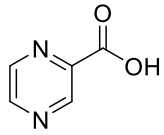
This distinction between humans, who acquire pantothenate from their diet, and bacteria, which rely solely on their internal biosynthesis pathway, presents a promising target for the development of novel antitubercular drugs. By inhibiting PanD, we could potentially disrupt CoA production within Mtb, crippling its essential metabolic processes and hindering its ability to survive and multiply. This targeted approach offers a potential strategy for developing effective antibacterials to combat the growing problem of AMR.[74]

One of the important drugs in the TB treatment regimen is pyrazinamide (PZA), a prodrug converted to produce pyrazinoic acid (POA) to its active form by pyrazinamidase (PZase).[14, 75, 76] PZA is a first-line antitubercular drug that acts explicitly on dormant non-growing Mtb and has limited activity against growing active Mtb with its unique sterilizing activity.[77-79] PZA shortens the TB treatment from 9–12 months to 6 months.[80-83] The PZA prodrug and its corresponding metabolite POA exhibit multiple modes of action.[16, 84] The recent studies[85]

strongly suggest a direct influence of POA's inhibitory effect on the catalytic activity of aspartate decarboxylase PanD in the biosynthesis of β-alanine. However, the inhibitory effect of POA is quite weak even at higher concentrations, but it has a very good binding affinity K_D of 6.1 μM ± 0.88 μM. In contrast, no binding activity was noticed for PZA during isothermal titration calorimetry (ITC) experiments.[85, 86] POA also triggers PanD degradation by the ClpC1-ClpP complex, thus reducing the production of β-alanine rather than inhibiting the PanD.[86] A similar study of POA on various PanD mutants indicates that minor protein alterations can significantly impact POA efficacy through various mechanisms, such as diminished drug binding, changes in protein assembly, and/or altered enzyme function.[87]



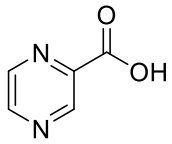
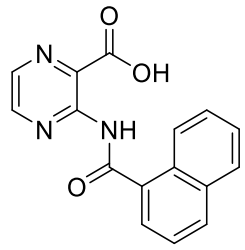
Pyrazinamide (prodrug)

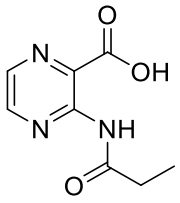


Pyrazinoic acid (POA)

However, derivatives of POA, containing additional modifications at pyrazine C3 have greatly influenced the production of β-alanine catalyzed by the Mtb PanD. POA analogue 1 (see Table **2**) containing bulky groups at pyrazine C3 exhibits a higher inhibitory effect of β-alanine production with 95.5% efficiency. POA exhibits only 4.8% efficiency even at 200 μM concentration (see Table. **2**).[87]

Table 2. Comparative study of POA and its representative derivatives.[87]

Compound	Mechanism of action	Inhibitory effect of β-alanine production at different concentrations.		K_D μM
		2.5 μM	200 μM	
 **POA**	• Major – triggers PanD degradation • Minor – PanD inhibition	-	4.8%	20 ± 0.2
 **POA analogue 1**	• Major – PanD inhibition	48.4%	95.5%	20 ± 0.5

 POA analogue 2	• Major – PanD inhibition (less active)	-	-	200 ± 0.9

Hence, POA and pyrazine pharmacophore scaffold could be a starting point for the PanD inhibitors. In this Ph.D. dissertation, a series of cyclic pyrazinooxazinone derivatives were synthesized as a pilot study to investigate their potential as prodrugs for pyrazinoic acids (POA) and their activities against *Mycobacterial spp*. (**P2**; refer to page #56)

1.2.4. Methionine aminopeptidase 1 (Brief Introduction)

Methionine aminopeptidase 1 (MetAP1), a binuclear metalloproteinase, plays a vital role in nutrient acquisition and protein turnover by removing the initiator methionine from the N-terminus of newly synthesized proteins (nascent polypeptides).[88, 89] This process, occurring either during translation (co-translationally) or after (post-translationally), allows for essential post-translational modifications. Interestingly, MetAP1 requires divalent metal ions like Co(II), Mn(II), Ni(II), and Fe(II) as cofactors for its enzymatic function, and its efficacy depends greatly on the metal cofactor.[89, 90] Insufficient activity of MetAP1 demonstrated fatal outcomes in bacteria in several knock-out experiments. Mycobacterial MetAP1 (mtMetAP1) is therefore a potential target for anti-TB drug candidates. Some of the mtMetAP1 inhibitors with anti-TB activity are given in Figure **4**.[91]

As I have not participated in the design and conceptualization of mtMetAP1 inhibitors, refer to **P3**; page #56 for detailed information on MetAP1.

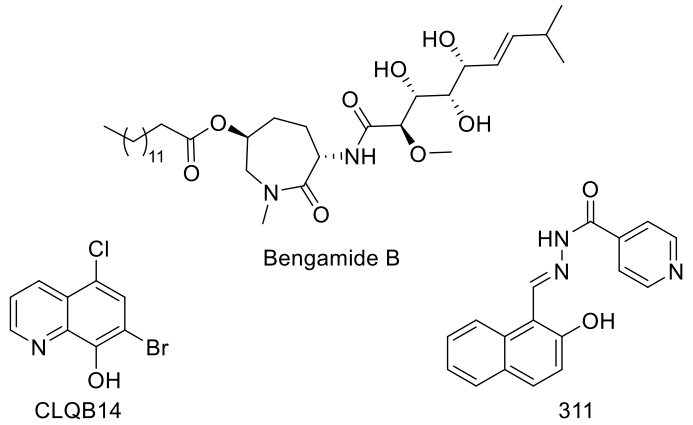


Figure 4. Examples of mtMetAP1 inhibitors with anti-TB activity.[92-94]

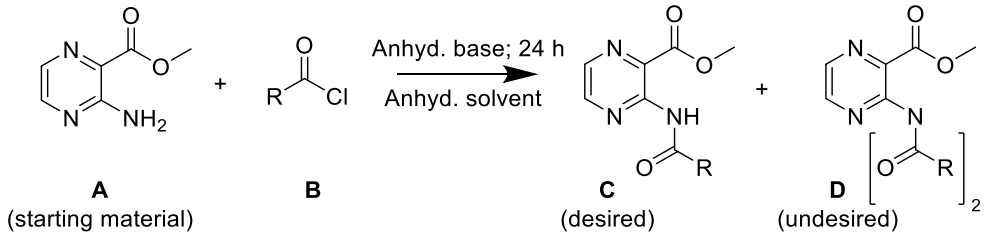
2. Methodology

2.1. Synthetic Methods

A commentary on synthetic optimizations performed (other than those mentioned in the commentary on published articles section) is provided in this chapter for comparability and readability.

2.1.1. Reactions with acyl chlorides (acylation step)

Acyl chlorides with methyl 3-aminopyrazine-2-carboxylate



Scheme 1. Acylation reaction with acyl chlorides.

Table 3. Optimizations made for acylation reaction.

A	**B** (eq.)	**Solvent**	**Base**	**Temp.** (°C)	**C** (% yield)	**D** (% yield)
1 m.mol	1.5	DCM	Pyridine (1.6 eq)	25 (ambient)	<40	<10
1 m.mol	1.5	Chloroform	Pyridine (1.6 eq)	25 (ambient)	<30	<10
1 m.mol	1.5	Acetone	Pyridine (1.6 eq)	25 (ambient)	<40	<10
1 m.mol	1.5	DCM	Triethylamine (1.6 eq)	25 (ambient)	<30	<30
1 m.mol	2.2	DCM	Pyridine (1.6 eq)	25 (ambient)	<40	<10
1 m.mol	**2.2**	**MeCN**	**Pyridine (2.5 eq)**	**70**	**<5**	**<90**

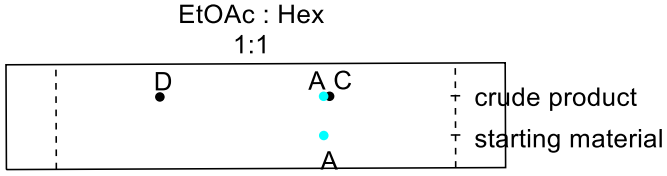
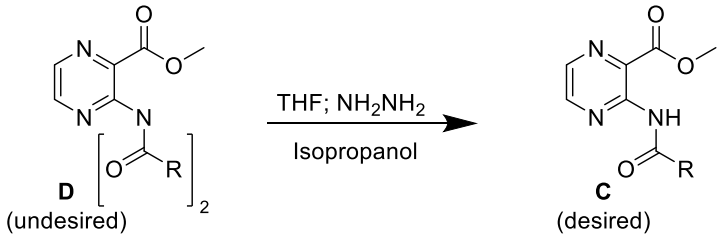


Figure 5. Representative TLC for the above acylation reaction. A – starting compound, C – desired monoacylated product, D – unwanted diacylated side-product

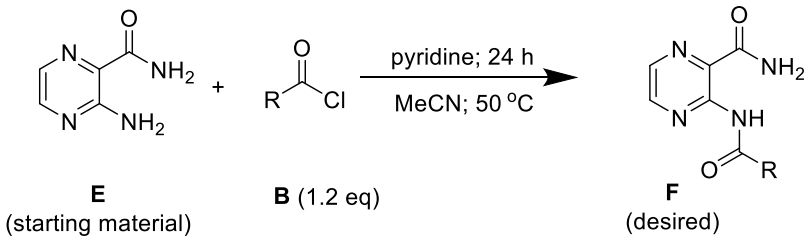
From the above acylation reaction optimizations (see Table **3)**, in most cases, it is very clear that reactivity during acylation was very low, with no positive progress of reaction after 24 h. Due to this, there was large quantities of unreacted started material in the final crude product, which made the chromatographic purifications very difficult (small R_F difference between **A** & **C**; see Figure **5**) with multiple attempts on flash chromatography.

However, during the last case with MeCN as solvent at 70 °C, the starting material **A** was totally consumed and favored high yields of undesired diacylated product **D** with easier purifications. Although the obtained product **D** was undesired, it was reduced to monoacylated product **C** using 1.0 eq. of hydrazine as mentioned below in Scheme **2,** with yields >85%.



Scheme 2. Reduction of diacylated side-product to monoacylated product using hydrazine.

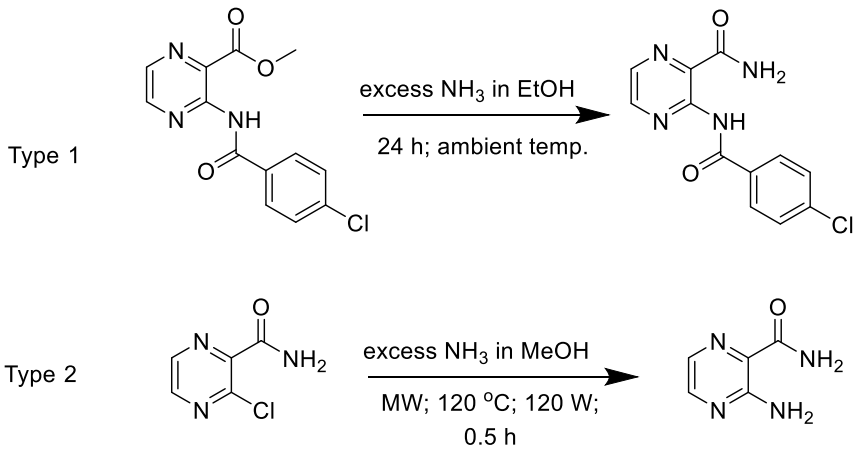
Acyl chlorides with 3-aminopyrazine-2-carboxamide



Scheme 3. Acylation of 3-aminopyrazine-2-carboxamide

In a similar acylation reaction with **E** (3-aminopyrazine-2-carboxamide) from the above scheme **3** using MeCN at 50 °C, the reaction favored only monoacylation with total consumption of starting material **E** with final yields >80%. This higher selectively of monoacylation with 3-aminopyrazine-2-carboxamide is likely due to reduced steric hindrance from the unsubstituted amide moiety. The amide at C2 pyrazine in **E** is less bulky compared to the ester moiety in 3-aminopyrazine-2-carboxylic acid methyl ester (**A**) mentioned above.

2.1.2. Reactions with ammonia in ethanol/methanol



Scheme 4. Reactions performed using excess ammonia in EtOH

In type 1, ammonolysis reaction (Scheme **4**), the ester moiety was directly converted to 1° amide using excess ammonia in EtOH at room temperature (ambient) for 24 h. However, in type 2 (Scheme **4**), to replace Cl with amine, higher temperature was required by using microwave reactor (MW) in pressured vials. After the reaction was completed, the excess ammonia and solvent were evaporated under *vacuo,* and the product yield was 100% with no further purifications.

2.2. *In vitro* antimicrobial evaluation

2.2.1. Antimycobacterial evaluation

Final compounds were evaluated for *in vitro* antimycobacterial activity against five different mycobacterial strains (avirulent strain - *Mtb* H37Ra, *M kansasii*, *M avium*, *M smegmatis*, and *M aurum*). The study was conducted by PharmDr. Ondřej Janďourek, Ph.D. at the Department of Biological and Medical Sciences of our faculty.

Most active and selected compounds were tested further against *Mtb* H37Rv (virulent strain) and multidrug-resistant isolates such as *Mtb* IZAK, *Mtb* MATI, and *Mtb* SORO. The study was conducted by MUDr. Pavla Paterová, Ph.D. at Department of Clinical Microbiology, University Hospital Hradec Králové.

Table 4. Activity profiles used in this dissertation for reference.

S.No	MIC conc. (µg/mL) range	Activity profile
1	≥1.95 to ≥7.8	High activity
2	≥15.625 to ≥31.25	Medium/moderate activity
3	≥62.5	Low/minimal activity

The studies were conducted using modified Microplate Alamar Blue Assay (MABA) and the results of antimycobacterial activity were measured as MIC in μg/mL. The arbitrarily set activity limits used in the text descriptions in this thesis are reported in Table **4**. Antimycobacterial evaluation

As a complementary study, compounds were also tested against four Gram-positive bacterial strains (*Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*) and four Gram-negative bacterial strains (*Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*), followed by eight fungal stems (*Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Aspergillus fumigatus*, *Aspergillus flavu*s, *Lichtheimia corymbifera*, *Trichophyton interdigitale*). None of the synthesized compounds displayed any promising antibacterial and antifungal properties, so I won't be discussing their results in this dissertation. The study was conducted by RNDr. Klára Konečná, Ph.D., and co-workers at the Department of Biological and Medical Sciences of our faculty.

2.3. *In vitro* cytotoxicity study

Most first-line antituberculosis drugs are hepatotoxic, and therefore new antitubercular drug candidates should not possess significant hepatotoxicity. The cytotoxicity of the synthesized compounds was measured using HepG2 cell-line. These are hepatocellular carcinoma cells, which are a commonly used model of substance toxicity in antimycobacterial research. The study was conducted by PharmDr. Pavel Bárta, Ph.D. at the Department of Pharmacology and Toxicology of our faculty.

2.4. Stability assessment and *in vitro* human liver microsomes (HLM) metabolism study

The chemical stability of lactone **29** (**P2**; refer to page #56) was studied under various conditions, such as mixtures of solvents using HPLC-HRMS. The compound was further evaluated for metabolites upon incubation with human liver microsomes (HLM). The study was conducted by RNDr. Martin Novák, Ph.D. at Biomedical Research Centre, University Hospital Hradec Králové.

2.5. *In silico* investigation

The homology model of mycobacterial Prolyl-tRNA synthetase was generated by AlphaFold using 3D coordinates of mtProRS (downloaded from the AlphaFold database; UniProt ID: P9WFT9) and overlaid with experimental coordinates of ProRS from *Enterococcus*

faecalis (PDB id: 2J3L). *In silico* predictions such as molecular docking and molecular dynamics were performed in MOE (Molecular Operating Environment, Chemical Computing Group, Ontario, Canada). The study was conducted by doc. PharmDr. Jan Ziko, Ph.D., PharmDr. Martin Juhás, Ph.D. and Mgr. Marek Kerda at the Department of Pharmaceutical Chemistry and Pharmaceutical Analysis of our faculty.

3. Aims and Objectives

This doctoral dissertation investigates the development of novel antimicrobial agents to combat the growing threat of antimicrobial resistance (AMR). The project employs a comprehensive approach, encompassing the design, synthesis, and in vitro biological evaluation of 3-amidopyrazine derivatives targeting mtProRS. By identifying promising candidates with potent activity, this research aims to contribute significantly to the pipeline of future antimicrobials against AMR targeting mtProRS. For this, we've identified and exploited key differences between human and mycobacterial Prolyl-tRNA synthetases using their respective ligands for this research.

Specific objectives:

- Design novel chemical entities derived from pyrazine scaffold as potential antimycobacterial compounds targeting mtProRS with minimal or no interaction with hsProRS, using computer-aided drug design.
- Synthesize the designed compounds and optimize the chemical reaction steps for better efficiency.
- Evaluate all the synthesized compounds using various *in vitro* biological assays to identify their bio-significance (performed by designated professionals under mutual collaboration).
- Identify the Structure Activity Relationship in correlation with bioactivity followed by *in silico* investigation of the mechanism of action.

Apart from the above-mentioned, I will participate in various other projects through grants and collaborations in search of novel antimicrobials.

4. Commentary on Published Articles

4.1. Generation 1: Substituted 3-amidopyrazine-2-carboxamide derivatives as potential antimycobacterial agents

(**P1**; Refer to page #56)[71]

Design and Rationale

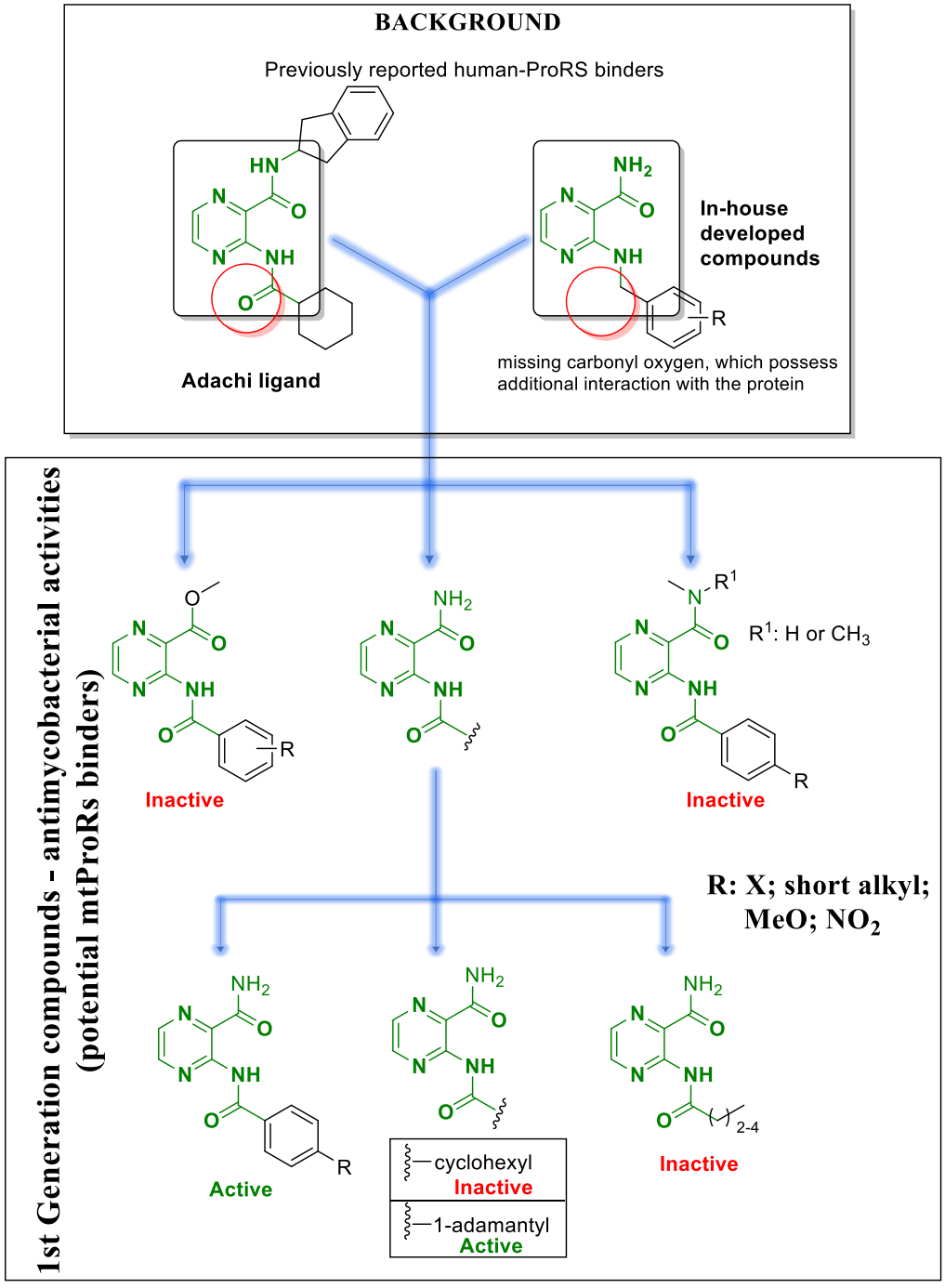


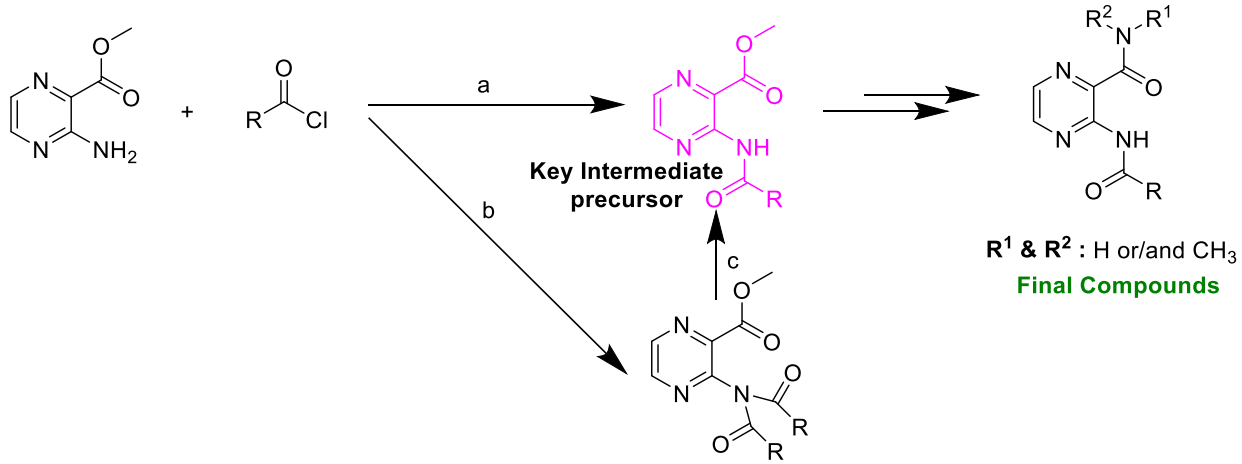
Figure 6. Design and development of potential ProRS inhibitors exhibiting antimycobacterial activity.

The current design of 3-amidopyrazine-2-carboxamides as potential ProRS inhibitors exhibiting antimycobacterial activity starts from the rationale of two different molecules containing 3-aminopyrazinamide scaffold (refer to Figure **6**; Background).

- Adachi ligand (reported by Adachi. R., et al. 2016)[69] is a confirmed novel hsProRS inhibitor. From our studies, the compound exhibits low activity against Mtb H37Ra with MIC 62.5 µg/mL and is cytotoxic on the HepG2 cell-line with IC_{50} 19.6 µM.
- 3-aminopyrazinamide derivatives (In-house developed compounds)[68, 70] with simpler structure compared to Adachi ligand. The simplified in-house ligands bind to hsProRS, although they have a weaker affinity than the Adachi ligand. This weaker binding could be attributed to the absence of the carbonyl oxygen. However, the 4' sub on the benzene ring favors Mtb inhibition but no hsProRS binding, whereas the 2' sub on the benzene ring favors hsProRS binding with no Mtb growth inhibition.[68, 70]

In this paper, we synthesized 3-amidopyrazine-2-carboxamides (Generation 1) as anti-mycobacterials, supposedly targeting mtProRS, by the inclusion of carbonyl oxygen for additional interaction with the protein. For an in-depth understanding of the structure-activity relationships (SAR), several other structural derivatives were synthesized by implying modifications at both C2 and C3 substitutions of pyrazine scaffold.

Results and Discussion



Scheme 5. Synthesis of key intermediate precursor

Conditions a) anhyd. DCM; anhyd. pyridine; 1.5 eq. acylchloride; ambient temp.; 24 h.
b) anhyd.MeCN; anhyd. pyridine; 24 h; 2.2 eq. acylchloride; 70 °C 24 h.
c) hydrazine, THF, isopropanol; 1 h.

This study describes the synthesis and biological evaluation of **56 compounds** followed by *in silico* investigation. We investigated their antimycobacterial, antifungal, and antibacterial

activities, followed by cytotoxicity assessment and SAR analysis. The synthesis of a key intermediate, an ester derivative, significantly impacted the overall efficiency of our approach (ref. Scheme **5**). Initially, the acylation reaction (step a) was carried out using 1.5 eq. of substituted acyl chloride, with solvent dichloromethane (DCM) at room temperature. This approach resulted in low yields of the desired monoacylated product (<40%) due to poor reactivity. Additionally, purifying the monoacylated product proved challenging due to the presence of unreacted starting material (low R_f difference) on chromatography. Due to this limitation, the solvent was changed from DCM to MeCN and reaction temp. to 70 ℃ with 2.2 eq. of acyl chloride to increase the reactivity. This allowed us to produce high yields of undesired diacylated product (>90%) with complete consumption of the starting material. The diacylated product was converted to the monoacylated product using a simple reduction reaction using hydrazine (refer to **P1** for complete synthesis). The double-step reaction (steps b & c) saved a lot of time and materials compared to the single-step (step a) reaction mentioned earlier using DCM, where the product yields were low and multiple chromatographic separations were needed and sometimes multiple repetitions of the same reaction were required to isolate a sufficient quantity of the product. For optimizations, refer to **section 2.1.1.** in this dissertation.

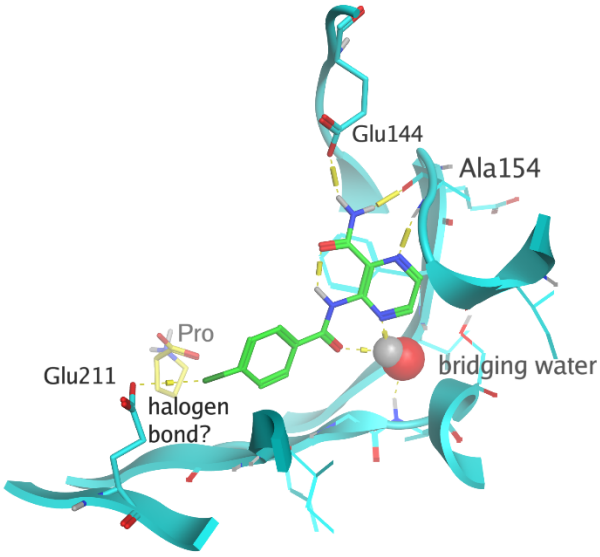


Figure 7. Representative binding mode of compound **15** as optimized by molecular dynamics (taken from **P1**)[71]

To explain the SAR, a large sample of data was collected by synthesizing **56 compounds** in total (referred to as Generation 1) with modifications at C2 and C3 of pyrazine (See Figure **6**; design and rationale) and evaluated for antimycobacterial activity, with additional

antibacterial and antifungal evaluation. The most potent antimycobacterial derivatives possessed halogen substituents at the 4' position of the benzine ring, with 4-Br sub. being the most active (MIC = 1.95 µg/mL) against Mtb H37Ra. Interestingly, high activity was not solely linked to halogen electron-withdrawing groups but also influenced by the bulkiness of the substituted moiety. Alkyl substitutions such as 4-Me and 4-*t*Bu (MIC = 7.81 µg/mL; and MIC = 15.625 µg/mL, respectively) are more potent compared to 4-F substitution (MIC = 31.25 µg/mL) against Mtb H37Ra. No antimycobacterial activity was noticed in compounds with 2' or 3' substitutions, as they are in concordance with the knowledge of the design part of this study and hence these may not be suitable candidates for mtProRs inhibition. To summarize, the enhanced activity of larger halogens (chlorine and bromine) could potentially involve halogen bond formation with Glu211 of the receptor (Figure **7**). The potential halogen bond was further studied by *in silico* investigation, using molecular dynamics on one of the most active compounds docked into the homology model of mycobacterial ProRS.

The compounds containing ester moiety at C2 pyrazine were completely inactive confirmed NH_2 hydrogen bond donor in the C2 carboxamide moiety plays a crucial role in the interaction with the supposed cellular target. Upon further investigation into C2 pyrazine substitution, we prepared and evaluated *N*-methylcarboxamide and *N*,*N*-dimethylcarboxamide derivatives. The *N*,*N*-dimethyl derivatives were inactive as expected due to their inability to form the crucial H-bond to the Ala154 backbone (see Figure **7**). In case of *N*-monomethyl derivatives, despite their structural similarity, exhibit low or no activity. This cannot be attributed to a mismatched binding mode, as one carboxamidic hydrogen remains compatible with the mtProRS target. For complete SAR and in silico results, refer to P1. Furthermore, all the final compounds were assessed for cytotoxic evaluation and the most promising compounds were tested against two MDR clinical isolates of Mtb (Mtb IZAK and Mtb MATI). The most active compounds showed consistent activities against MDR Mtb strains with MIC = 6.25–12.5 µg/mL.

4.2. Generation 2: 3-urediopyrazinamide derivatives as a Hit-Expansion series of Generation 1

(**P2**; refer to page #56)[95]

Design and Rationale

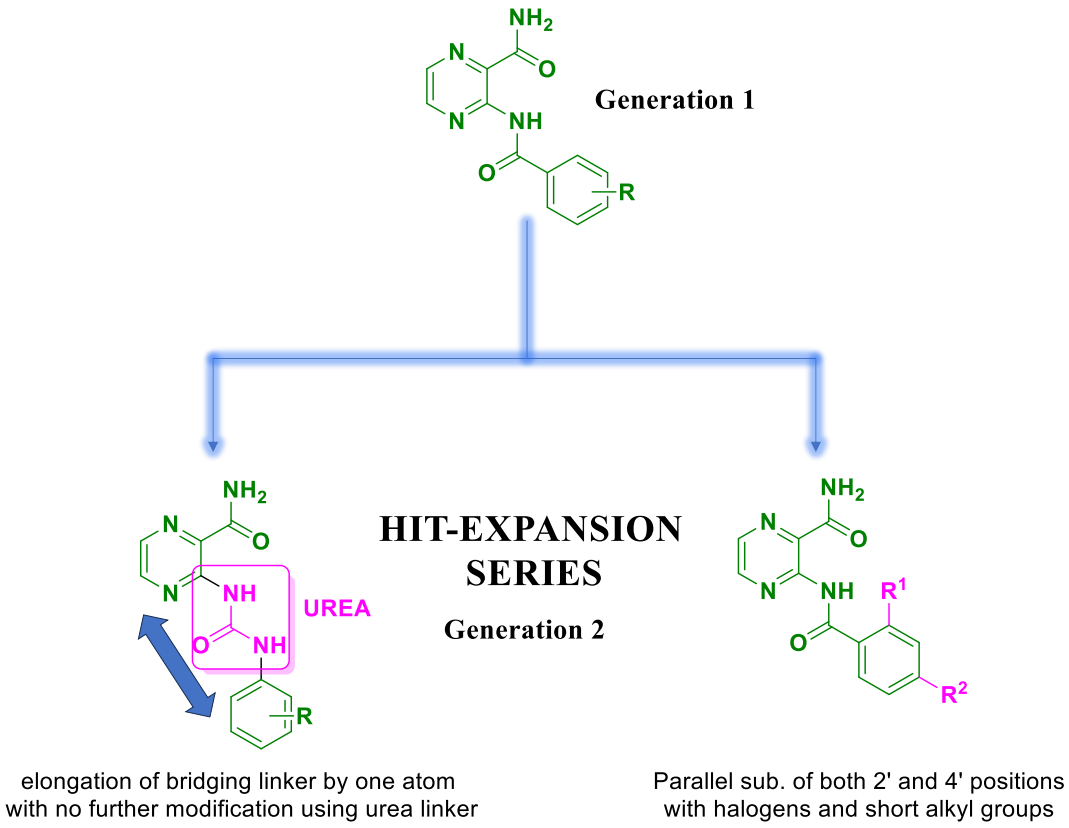


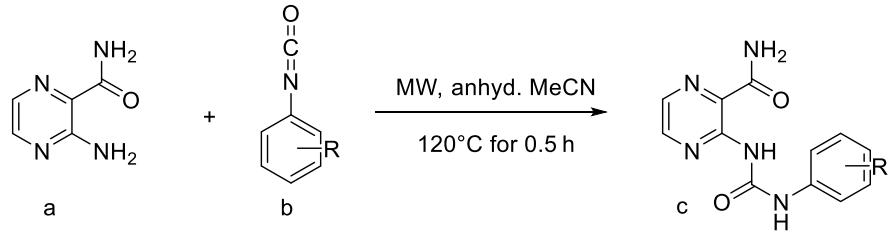
Figure 8. Design of Generation 2 hit expansion series

The current research article is a hit expansion of the 3-amidopyrazine-2-carboxamides (Generation 1) that contain mainly two individual series (see Figure **8**), and with an additional spin-off series which will be discussed in **section 4.3**.

- Ureidopyrazine-2-carboxamides were derived by replacing the amidic linker with urea linker while causing the elongation of the linker bridge.
- 2,4-disub. benzamidopyrazine-2-carboxamides are the combination of both 4' substituted compounds from Generation 1 (antimycobacterial activity)[71] and 2' substituted compounds from 3-aminopyrazine-2-carboxamide derivatives exhibiting hsProRS binding property.[68]

Results and Discussion

This study describes the synthesis and evaluation of **29 compounds** for their antimycobacterial activity (series 1, 2 & 3). The urea derivatives (series 1) were synthesized in three steps using microwave irradiation (MW) in pressurized vials, starting from 3-chloropyrazine-2-carbonitrile (detailed synthesis in **P2**). However, certain reaction optimizations performed at the last step of the synthesis (Scheme **6**) are described below (see Table **5**), while temperature and reaction time are kept constant (120 ˚C and 0.5 h, respectively).



Scheme 6. Synthesis of final compounds

Table 5. Reaction optimizations for final step reaction from Scheme 2 (where R = H)

a (eq.)	b (eq.)	solvent	c (% of theor. yield)
1	1	Hexane	No reactivity
1	1	Hexane/THF (1:1)	<5
1	1	THF	<5
1	>3	Neat (no solvent)	Product + carbonisations
1	**3**	**MeCN**	**~20–30**

Microwave irradiation, temperature 120 ˚C, reaction time 0.5 h for all entries.

The final compounds exhibited poor solubility in common organic solvents like DCM, hexane, and acetone. This low solubility facilitated easier purification by washing the crude product with these solvents to remove soluble impurities, followed by recrystallization.

However, such poor solubility limited the measurement of biological activities at higher concentrations. Additionally, compounds containing chlorine or bromine at the 2' position of the benzene ring could not be evaluated for antimycobacterial activity due to immediate precipitation in the (water-based) growth media.

In contrast to Generation 1 (**P1**), amidic derivatives (series 2 from the publication[95]) were readily synthesized in a single step by reacting 3-aminopyrazine-2-carboxamide with various 2,4-disubstituted benzoyl chlorides at the temperature of 50 ℃. This one-step reaction achieved selective monoacylation, unlike the acylation step in a previous study (**P1**), which

required harsher conditions with dual reaction steps (starting with diacylation followed by reduction reaction using hydrazine to achieve a monoacylated product). Refer to **section 2.1.1.** for synthetic optimizations and acylation reaction comparisons.

All the final compounds were evaluated against various *Mycobacteria spp.*; none of the urea derivatives (series 1 in **P2**; refer to page #56) exhibited any antimycobacterial activity. A minor change in the linker (amidic to urea linker) resulted in no activity. Upon further investigations into the 3D structure of urea **1** (**P2**; series 1; compound **1**), the urea derivative can exist in four main structural conformers (*trans–trans*, *cis–trans*, *trans–cis*, or *cis-cis*).

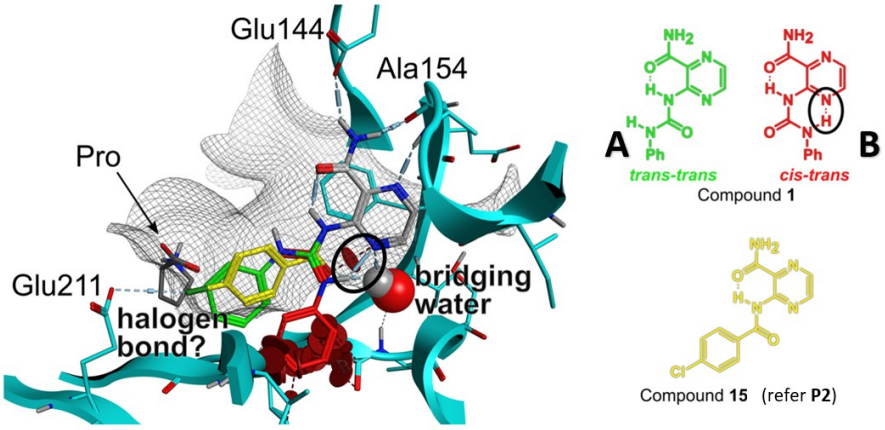


Figure 9. Overlay of urea **1** in *cis–trans* (**B**; red carbons) and *trans–trans* (**A**; green carbons) conformation with probable binding mode of compound **15** from published work (**P1**)[71] (yellow carbons) in the active site of mtProRS. The visualized surface corresponds to the van der Waals interaction surface of the binding site. Red cylinders around the phenyl ring represent a steric clash of *cis–trans* conformer with the protein. The black ellipse highlights the discussed intramolecular H-bond. (figure taken and modified)[95]

Based on DFT electronic structure calculations, the *cis–trans* conformer seems to be more prevalent due to additional stabilization by an intramolecular hydrogen bond (IMHB) with pyrazine nitrogen (see Figure **9**; **B**). This conformation arrangement was further confirmed by nuclear overhauser effect (NOE) NMR experiments. Since the *trans–trans* conformer (see Figure **9**; **A**) would be most suitable to adopt the desired binding mode of the amidic derivatives from Generation 1 (**P1**; compound **15**; refer to Figure **9**) the obtained *cis–trans* conformer was not compatible with the shape of the active site of mtProRS.

In contrast, the 2',4'-disubstituted derivatives (series 2) exhibited interesting and promising results, since the 4' sub. with halogen or bulky group along with 2-H on benzene ring exhibited excellent antimycobacterial activity in Generation 1. The current series 2 obeyed the same characteristics with bulky or halogen moiety at 4' position and only 2-F on the

benzene ring was tolerated as the size of F is as almost as small as H. The compounds **21** and **22** with 4-Cl and 4-Br containing 2-F exhibited decent activities on all strains of *Mycobacteria spp.*; however, activity was diminished against Mtb H37Ra compared to Generation 1 compounds. Compounds with bulkier groups instead of F at 2' position lost the activity against all strains of *Mycobacteria spp.* The most promising compounds (**21** and **22**) retained activities against MDR Mtb strains (Mtb IZAK, Mtb SORO, and Mtb MATI) with MIC 6.25–25 µg/mL.

4.3. Cyclic derivatives: spin-off series

(**P1** & **P2**; refer to page #56)[71, 95]

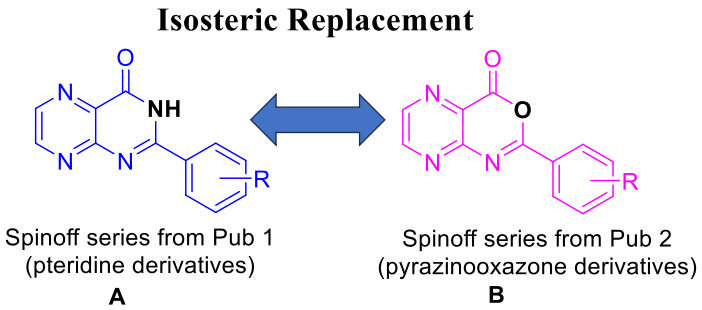


Figure 10. Cyclic derivatives – Spin-off series in **P1** & **P2**; refer to page #56

Pteridine derivatives (**A**) and pyrazinooxazinone derivatives (**B**) are two spin-off series (Figure **10**). A total of **10 compounds** were synthesized and evaluated for antimycobacterial properties in **P1** & **P2** together as a part of ongoing research and further explorations. The synthesis of these cyclic derivatives was performed as mentioned in the literature.[96] Pteridine derivatives were synthesized by cyclization of amide derivatives of Generation 1 using 0.5M KOH solution in water (**P1**). Pyrazinooxazinones were synthesized by the cyclization of ester derivatives of Generation 1 using 1,2-dibromo tetrachloroethane and triphenylphosphine with triethylamine as a base.

The pteridine derivatives exhibited very moderate to minimal activities against Mtb H37Ra (**P1**). From these obtained results, we have expanded these cyclic derivatives by isosteric replacement of 'N' to 'O' (see Figure **10**) and synthesized pyrazinooxazinones (**P2**) and investigated them for their biological properties. Unlike the pteridine derivatives, pyrazinooxazinones containing the lactone ring were less stable (lactone ring cleavage) and prone to biological metabolism.[97]

The stability and metabolic studies were conducted on pyrazinooxazinones, and the results were quite interesting. The resulting metabolites are 3-substituted pyrazinoic acids (POA), which are well-known PanD inhibitors. However, the prodrug approach (pyrazinooxazinones being prodrugs of POA-based PanD inhibitors) may seem slightly problematic due to their sensitivity towards methanol (ester formation) and buffer (hydrolysis to produce free carboxylic acid) used during biological assay. These changes were measured on HPLC-HRMS and confirmed with reference standards (synthesized separately). However, upon continuation into the HLM metabolism assay, additional hydroxylation was observed (see Figure **11**); refer to **P2**; page #56 for details of the study.

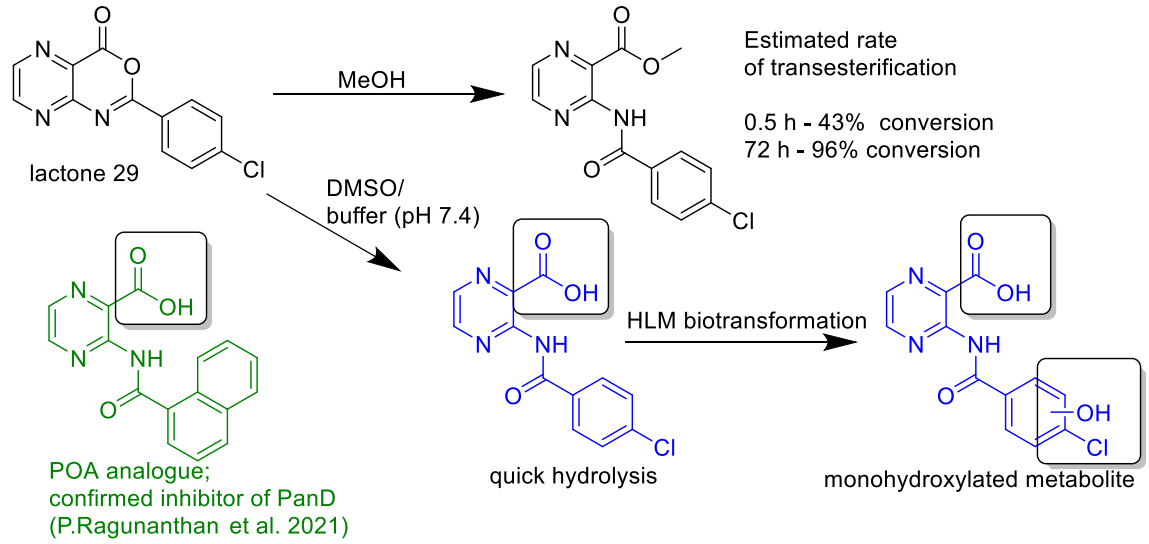


Figure 11. Overview of solvent stability and HLM metabolization of lactone 29 (modified figure; taken from **P2**)[95].

4.4. *N*-(thiazol-2-yl)pyrazine-2-carboxamides as inhibitors of mycobacterial methionine aminopeptidase 1

(**P3**; refer to page #56)[91]

The project was designed by my colleague PharmDr. Martin Juhás, Ph.D. As a coauthor in this publication,[91] I have participated in the synthesis and purification of compounds and analytical data interpretation.

Design and Rationale

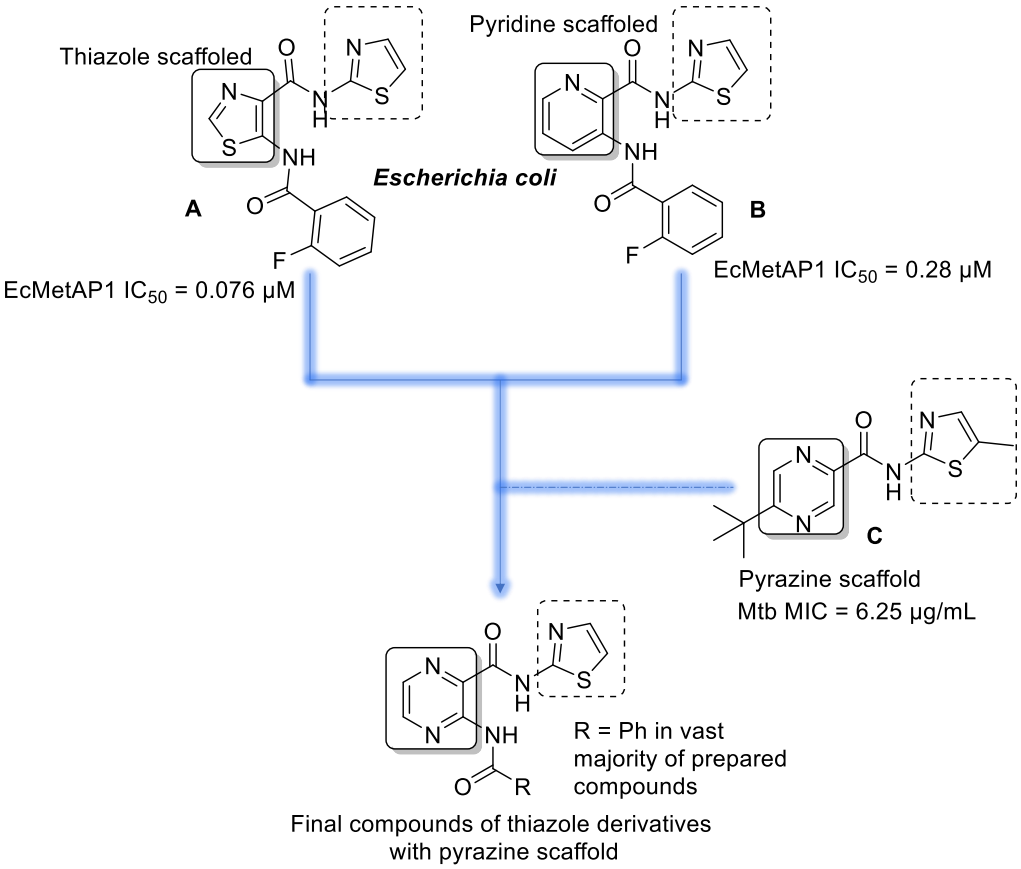


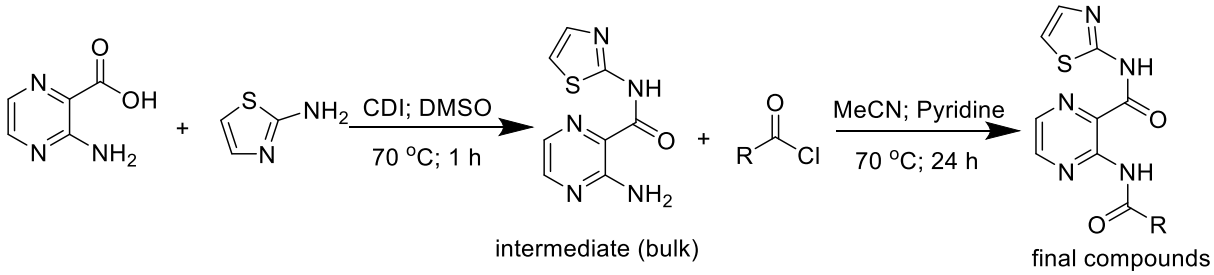
Figure 12. Design of Mycobacterial methionine aminopeptidase 1 (mtMetAP1) inhibitors

This study introduces pyrazine-based compounds as replacements for previously studied derivatives of thiazole-4-carboxamide (**A**)[98] and pyridine-2-carboxamide (**B**)[99] from the above Figure **12**. Both thiazole-4-carboxamide and pyridine-2-carboxamide derived compounds exhibited high potency against *E. coli* MetAP1 and also exhibited high selectivity to bacterial isoforms compared to that of human MetAP2 isoforms, a crucial feature for antimicrobial drugs.[98, 99] Pyrazine scaffold was used to replace thiazole/pyridine in final compounds (see Figure **12**) since a similar pyrazine derivative with peripheral thiazole

substitution (**C**) exhibited good antimycobacterial activity (Mtb H37Rv MIC = 6.25 µg/mL see Figure **12**).[100]

Results and Discussion

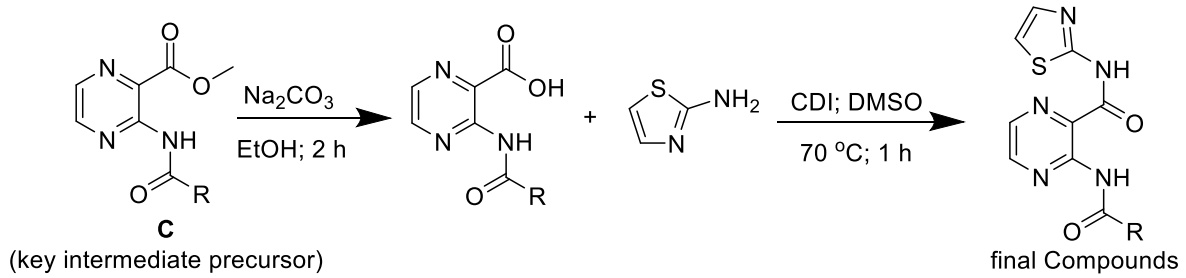
This article describes the design and synthesis of **20 compounds** containing pyrazine scaffold, alongside a reference pyridine-containing molecule (**B**) in Figure **12** for comparison, followed by enzymatic studies. Two synthetic methods were employed to produce the final compounds.



Scheme 7. Synthesis of final compounds (1st approach)

The first approach (see Scheme **7**) involved a bulk synthesis of a common intermediate. This intermediate was prepared by coupling 3-amino-2-pyrazinoic acid with 2-aminothiazole using CDI (1,1'-carbonyldiimidazole). The intermediate was then used to produce several final compounds through acylation with various substituted benzoyl chlorides. The acylation method was similar to that mentioned in **section 2.1.1**.

While this approach was straightforward, it had some limitations, and due to the low reactivity during CDI coupling, it resulted in lower yields (9–33%) and required extensive purification efforts.



Scheme 8. Synthesis of final compounds (2nd approach)

The second approach (see Scheme **8**) was more work-intensive due to its multiple steps:

- The strategy was to first perform the acylation of the 3-amino group of the methyl ester to yield various substituted methyl 3-benzamido-pyrazine-2-carboxylates **C** (key intermediate precursor, refer to **section 2.1.1.**)

- Hydrolysis of the key intermediate precursor (Scheme **8**) using sodium carbonate to produce substituted 3-benzamidopyrazine-2-carboxylic acids.
- Finally, these acids were coupled with 2-aminothiazole to obtain the final products.

Despite the additional steps, this approach provided higher yields (23–71%) with better reaction efficiency and easier purifications.

All the title compounds were evaluated for antimicrobial activity and enzyme inhibition assay on mtMetAP1a followed by *in silico* molecular docking to mtMetAP1c ('a' and 'c' are the isoforms of mtMetAP1). MetAP1 is a metalloproteinase and requires divalent metal ions like Co(II), Mn(II), Ni(II), and Fe(II) as cofactors. Its enzymatic activity differs and greatly depends on the metal cofactor.[89, 90] With our compounds, the inhibition of the enzyme was best seen in the presence of Ni^{2+}, followed by Fe^{2+}. From SAR studies, it was clearly notable that compounds with 2' substitution on the benzene ring, such as 2-Br and 2-Cl showed the highest activity, with IC_{50} inhibition concentration on the isolated enzyme below 1 μM. We have observed a clear correlation between the water solubility of the compounds and enzyme inhibition. Nevertheless, when the compounds were tested for antimycobacterial activity (whole-cell growth inhibition), very limited/poor activities were observed. Although enzymatic inhibition was clearly noticeable, we believe our compounds exhibit poor penetration through the cell wall and therefore may not be able to target the specific protein in the whole-cell format to obtain desired activity. For complete enzymatic inhibition results and antimycobacterial activities, refer to **P3**.

4.5. Aminooxazoles – How isosteric replacement of 'S' to 'O' produces significant changes in biological activities.

(**P4**; refer to page #56)[101]

The presented work in this section was originally designed and executed by my colleague PharmDr. Martin Juhás, Ph.D. As a coauthor in this publication,[101] I have participated in synthesizing and purifying compounds, interpreting analytical data, and editing the manuscript.

Design and Rationale

The design of this series was inspired by the works of Zitko et al. 2018 (see Figure **13**),[102] where they produced hybrid derivatives from the combination of two bioactive scaffolds of pyrazinamide and 4-aryl-2-aminothiazole. Although excellent overall antimycobacterial

activities were achieved, some compounds were of low solubility in DMSO and some were insoluble. Due to this, some of the key biological evaluations were not performed or non-reproducible due to solubility limitations. However, in this series of final compounds (see Figure **13**), several modifications were made on both sides of the thiazole scaffold with various substitutions.[102]

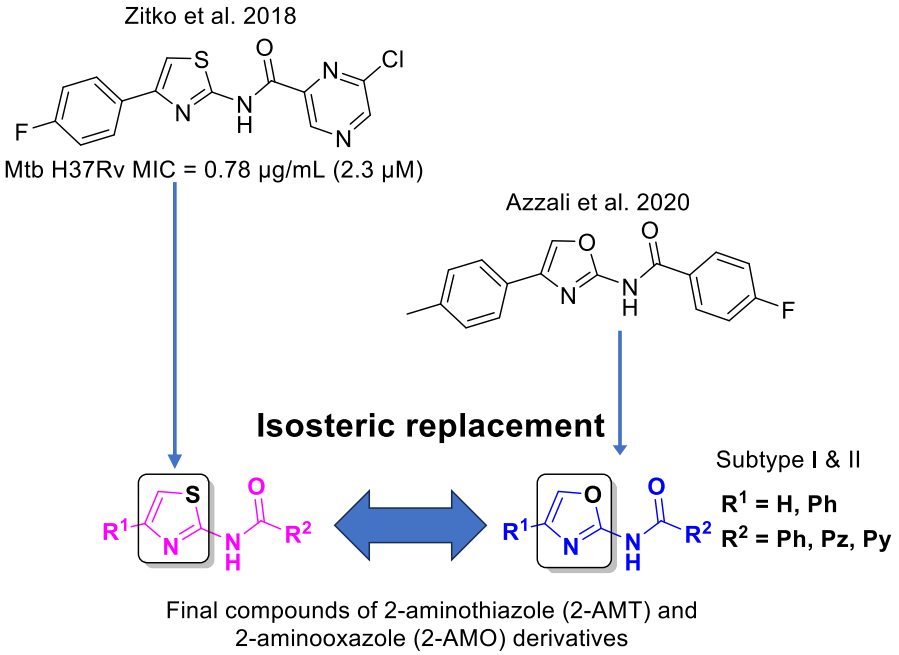
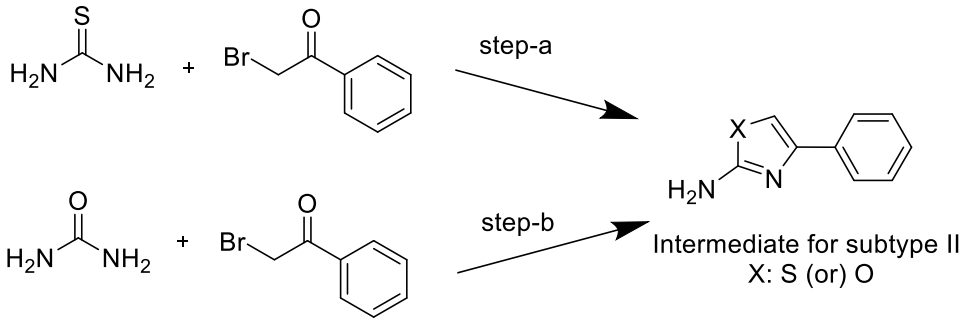


Figure 13. Design and rationale of 2-AMT and 2-AMO derivatives from **P4**

Along with 2-aminothiazole derivatives (2-AMT), an additional series of 2-aminooxazoles (2-AMO) derivatives was designed, synthesized for improved physicochemical properties using isosteric approach by replacement of 'S' with 'O' and evaluated for biological activities. This isosteric replacement approach was inspired by Assali et al. 2020,[103] where significant changes in the physicochemical properties like solubility and lipophilicity were improved while retaining the bioactivities.

Results and Discussion

This article describes the synthesis and biological evaluation of 2-aminothiazole (2-AMT) derivatives and 2-aminooxazole (2-AMO) derivatives (**34 compounds**), changes in the physicochemical properties due to isosteric replacement of 'S' with 'O' and their respective biological activity. Subtype I containing final compounds of 2-AMO and 2-AMT with R^1 = H and subtype II containing final molecules with R^1 = Phenyl group, while R^2 = sub. pyrazine; sub. pyridine and quinazoline moieties in final compounds.



Scheme 9. Synthesis of reaction intermediate for subtype II

Reaction step	Thiourea/Urea (eq.)	Solvent	Temperature	Duration
Step-a	1.1 eq. (thiourea)	EtOH	Reflux (80 ˚C)	~ 1 h
Step-b	10 eq. (urea)	DMF	120 ˚C	2 h
Step-b	10 eq. (urea)	MeCN	Reflux (85 ˚C)	16 h

Step a: Synthesis of intermediate 4-phenyl-2-aminothiazole using thiourea proceeds with higher reactivity and efficiency, leading to better yields and easier purification.

Step b: Synthesis of intermediate 4-phenyl-2-aminooxazole using urea is less reactive, requiring higher amounts of urea to achieve the desired conversion. While DMF favors quick and higher reactivity due to its higher boiling point compared to MeCN, but removal of DMF involves a multi-step extraction (> 6 times) process using ethyl acetate and water.

Synthesis of final compounds involved a single-step acylation of commercially available 2-aminooxazole or 2-aminothiazole for subtype I, or phenyl-substituted intermediates prepared in Scheme **9** for subtype II. The acylation was achieved by reaction with commercially available substituted benzoyl chloride or substituted acyl chlorides prepared from their respective heteroaromatic carboxylic acids using thionyl chloride (refer to **P4** for complete synthesis). In total, 34 final compounds were synthesized and evaluated for biological activities against various mycobacterial, fungal, and other bacterial species, followed by *in vitro* cytotoxicity evaluations. Since the design of 2-AMO derivatives as isosteres of 2-AMT derivatives was motivated by our attempt to improve physicochemical properties, the hypothesis was tested by measuring the water solubility (method based on water-induced DMSO precipitation of sample stock solutions) and lipophilicity (we determined the log k'_w

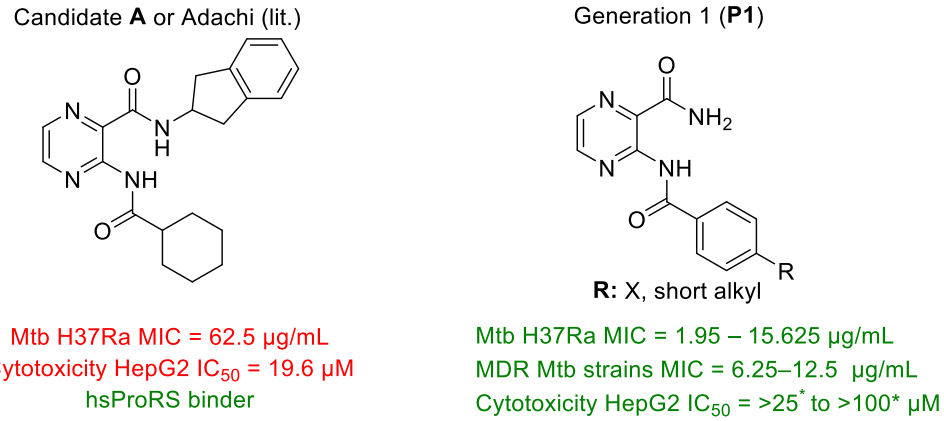
of all derivatives and compared these values between 2-AMT and 2-AMO derivatives) of final compounds. The study yielded promising outcomes as follows.

- 10 out of 34 final compounds are promising against Mtb with MICs ≤ 7.81 μg/mL.
- When a 2-AMT derivative is active against mycobacteria, their respective 2-AMO derived isostere is also active.
- Two additional 2-AMO derivatives (6b and 7b in publication **P4**) containing 2-Cl-pyridine and 2-Cl-6-Me pyridine moieties were active, while their counter 2-AMT derivatives were inactive.
- Increased solubilities were clearly observed in 2-AMO derivatives, while the 2-AMT derivatives were poorly soluble.
- Upon cytotoxic evaluation of all the compounds on the HepG2 cell line, most of the final compounds exhibited no toxicity when measured up to their highest achieved concentration.

From this study, we can clearly identify that the isosteric replacement of 'S' with 'O' favors certain 2-AMO derivatives with bioactivity due to improved physicochemical properties. There was not enough evidence to conclude that isolated isosteric exchange can turn an inactive 2-AMT derivative into an active 2-AMO drug candidate.

5. Summary

My doctoral research focused on the design and synthesis of novel pyrazine-based compounds with potential antimycobacterial activity targeting mycobacterial Prolyl-tRNA synthetase (mtProRS). A total of 56 compounds (3-benzamidopyrazine-2-carboxamide and similar derivatives) were synthesized and evaluated (**P1**), leading to the development and synthesis of an additional 29 compounds (3-ureidopyrazine-2-carboxamide, disub. 3-benzamidopyrazine-2-carboxamide, and similar derivatives) (**P2**). All synthesized compounds underwent antimycobacterial evaluation, structure-activity relationship analysis, and computational studies to predict their binding mode within the target protein using mtProRS homology model. Among the synthesized compounds, substituted 3-ureidopyrazine-2-carboxamide derivatives (series 1, **P2**) exhibited the lowest antimycobacterial activity. *In silico* conformational analysis and subsequent NOE NMR experiments indicated that these compounds adopt conformation incompatible with the binding cavity, likely contributing to their inactivity. Most promising compounds from both **P1** and **P2** (Generation 1 & 2; refer to Figure **14** and Figure **15**) retained activities against MDR Mtb strains with MIC = 6.25–25 µg/mL.



* IC_{50} cannot measured due to precipitation at higher concentration

Figure 14. Most active and promising compounds from **P1**[71] in comparison with Adachi ligand (Candidate **A**)[68, 69, 71]

In a secondary investigation, cyclic derivatives, including pteridines (**P1**) and pyrazinooxazinones (**P2**), were assessed for their antimycobacterial properties. Notably, pyrazinooxazinones showed potential for further development as prodrugs of PanD inhibitors targeting Mtb. The most promising compounds from **P2** are given below in Figure **15**.

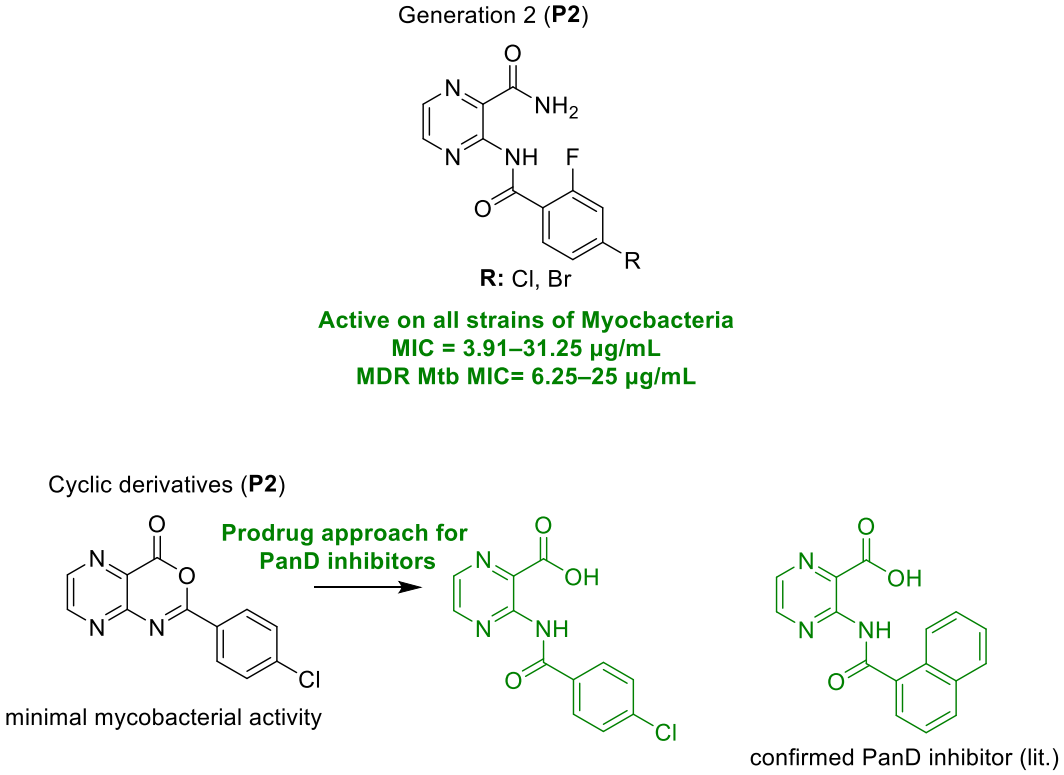


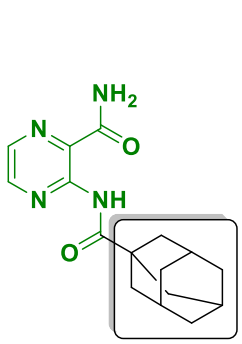
Figure 15. Most active and promising compounds from **P2**[95] and PanD inhibitor[87]

Additionally, as a contribution to antimicrobial research during my doctoral studies, I have participated in the synthesis of thiazole-containing pyrazine derivatives as potential inhibitors of mycobacterial methionine aminopeptidase 1 (mtMetAP1) (**P3**). While these compounds demonstrated promising enzymatic inhibitory activity, they did not exhibit sufficient activity in whole-cell *in vitro* antimycobacterial evaluation. I have also participated in synthesizing novel 2-AMT and 2-AMO derivatives (**P4**) as potential antimicrobials. The initial hypothesis to improve physicochemical properties by isosteric exchange of thiazole to oxazole was confirmed. 2-AMO derivatives preserved the antimycobacterial property and exhibited improved physicochemical properties such as water solubility compared to 2-AMT derivatives.

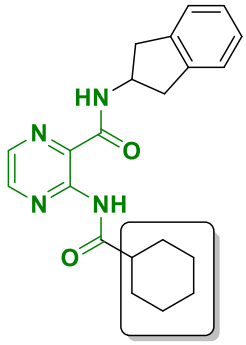
6. Ongoing research and prospects

In collaboration with Dr. Luping Pang, Ph.D. (Zhengzhou University, Zhengzhou, China), the most promising compounds are being evaluated for their target binding and enzymatic studies. For this, they initially produced human prolyl-tRNA synthetase (hsProRS) and mycobacterial prolyl-tRNA synthetase (mtProRS) and conducted thermal shift assay (TSA) experiments with/without the presence of proline to observe the effect of proline on binding affinity.

The preliminary data (TSA) indicate that selected compounds from Generation 1 & 2 are proline-dependant hsProRS binders. The results are consistent with the earlier published results[68] of similar derivatives. Compounds with 4' substitution with the most promising activities against Mtb do not exhibit binding to hsProRS (a promise for selectivity to mtProRS over hsProRS). When TSA was done with mtProRS, the results had minor discrepancies; hence, re-evaluations are being made. Nevertheless, the results of the derivative with adamantyl substitution (Generation 1 (28) or adam-NH3, Figure 15) were promising with binding to both hsProRS and mtProRS. This may be due to the close structural similarity with candidate **A** (Adachi ligand) containing cyclohexyl. (The study is in progress; will be published upon acquiring all the data)



Generation 1 (**28**) or adam-NH3 (lab code)
Mtb H37Ra MIC = 31.25 µg/mL
Cytotoxicity HepG2 IC_{50} = 366.6 µM



Candidate **A** or adachi
Mtb H37Ra MIC = 62.5 µg/mL
Cytotoxicity HepG2 IC_{50} = 19.6 µM
hsProRS binder

Figure 15. Structural comparison of Generation 1 (**28** in article)[71] with confirmed hsProRS inhibitor (Adachi)[68, 71]

In collaboration with Prof. Andrzej Joachimiak, Ph.D. (Argonne National Laboratory, Argonne, USA), a few selected compounds from Generation 1 & 2 were being used to make mtProRS co-crystals and will be evaluated for ligand-protein binding confirmation through X-ray crystallography. (Study is in progress; it will be published upon acquiring all the data)

7. Publication Contributions

P1 **PALLABOTHULA, V. S. K.**; KERDA, M.; JUHÁS, M.; JANĎOUREK, O.; KONEČNÁ, K.; BÁRTA, P.; PATEROVÁ, P.; ZITKO, J. Adenosine-Mimicking Derivatives of 3-Aminopyrazine-2-Carboxamide: Towards Inhibitors of Prolyl-tRNA Synthetase with Antimycobacterial Activity. *Biomolecules*, **2022**, vol. 12, article no. 1561.

IF_{2022} = 5.5 (Q2), doi: 10.3390/biom12111561

Author's share: *Methodology, Investigation, Analytical Interpretation, Writing and Editing the manuscript.*

P2 **PALLABOTHULA, V. S. K.**; ABDALRAHMAN, N. T.; MORI, M.; FEKRI, A. H.; JANĎOUREK, O.; KONEČNÁ, K.; PATEROVÁ, P.; NOVÁK, M.; DUDÁŠOVÁ-HATOKOVÁ, P.; ŠTĚRBOVÁ-KOVAŘÍKOVÁ, P.; CASTELLANO, C.; MENEGHETTI, F.; VILLA, S.; KUNEŠ, J.; JUHÁS, M.; & ZITKO, J. A hit expansion of 3-benzamidopyrazine-2-carboxamide: Toward inhibitors of prolyl-tRNA synthetase with antimycobacterial activity. *Archiv der Pharmazie,* **2024**, vol. 357, article no. e2400171.

IF_{2023} = 4.3 (Q2), doi: doi.org/10.1002/ardp.202400171

Author's share: *Methodology, Investigation, Analytical Interpretation, Writing and Editing the manuscript.*

P3 JUHÁS, M.; **PALLABOTHULA, V. S. K.**; GRABRIJAN, K.; ŠIMOVIČOVÁ, M.; JANĎOUREK, O.; KONEČNÁ, K.; BÁRTA, P.; PATEROVÁ, P.; GOBEC, S.; SOSIČ, I.; ZITKO, J. Design, synthesis and biological evaluation of substituted 3-amino-N-(thiazol-2-yl)pyrazine-2-carboxamides as inhibitors of mycobacterial methionine aminopeptidase 1. *Bioorganic Chemistry*, **2022**, vol. 118, article no. 105489.

IF_{2022} = 5.1 (Q2Q1), doi: 10.1016/j.bioorg.2021.105489

Author's share: *Investigation, Synthesis, and Analytical Interpretation.*

P4 JUHÁS, M.; BACHTIKOVÁ, A.; NAWROT, D.E.; HATOKOVÁ, P.; **PALLABOTHULA, V. S. K.**; DIEPOLTOVÁ, A.; JANĎOUREK, O.; BÁRTA, P.; KONEČNÁ, K.; PATEROVÁ, P.; SESTÁK, V.; ZITKO, J. Improving Antimicrobial Activity and Physico-Chemical Properties by Isosteric Replacement of 2-Aminothiazole with 2-Aminooxazole. *Pharmaceuticals*, **2022**, vol. 15, article no. 580.

IF_{2022} = 4.6 (Q2), doi: doi.org/10.3390/ph15050580

Author's share: *Synthesis, Analytical Interpretation and writing of designated parts of manuscript.*

8. Contributions at Scientific Conferences

8.1. Oral Presentations

- **Pallabothula, V. S. K.**, Zitko, J. *Synthesis of novel 2,3-disubstituted pyrazines as potential antimicrobials.* 10th Postgraduate and 8th Postdoc Conference, Hradec Králové, Czechia, 22–23 January, 2020.
- **Pallabothula, V. S. K.**, Zitko, J. *Design, synthesis and biological evaluation of 3-aminopyrazine-2-carboxamide derivatives as potential antimicrobials.* 11th Postgraduate and Postdoc Conference, Hradec Králové, Czechia, 27–28 January 2021.
- **Pallabothula, V. S. K.**, Zitko, J., Kerda, M. *Design, synthesis, SAR and in silico studies of 3-aminopyrazine-2-carboxamides as antimicrobials.* 49th Conference Synthesis and Analysis of Drugs 2021, Hradec Králové, Czechia, 16–19 September 2021.
- **Pallabothula, V. S. K.**, Kerda, M., Zitko, J. *Design, synthesis, and SAR of 3-amidopyrazine-2-carboxamides as antimicrobials.* 12th Postgraduate and Postdoc Conference, Hradec Králové, Czechia, 01–02 February 2022.
- **Pallabothula, V. S. K.**, Zitko, J., Mori, M., Meneghetti, F., Villa, S. *Synthesis of Furan derivatives, Microscale Thermophoresis, and Crystallographic studies.* 13th Postgraduate and Postdoc Conference, Hradec Králové, Czechia, 01–02 February 2023.
- **Pallabothula, V. S. K.**, Zitko, J. *Design, synthesis and biological evaluation of pyrazinamide derivatives and their structure active relationship.* 14th Postgraduate and Postdoc Conference, Hradec Králové, Czechia, 30–31 January 2024.

8.2. Poster presentations

- **Pallabothula, V. S. K.**, Zitko, J. *Novel 3-substituted pyrazine-2-carboxamides as potential antimycobacterial agents.* EFMC-ISMC & EFMC-YMCS Virtual Poster Session, 09 September 2020.
- **Pallabothula, V. S. K.**, Abdalrahman, N. T., Zitko, J. *Design, synthesis, and evaluation of 3-amido and 3-uredo substituted pyrazine-2-carboxamides as anti-mycobacterials targeting prolyl-tRNA synthetases.* 12th Joint Meeting on Medicinal Chemistry 2022, Bratislava (Virtual), Slovakia, 23–26 November 2022.

- **Pallabothula, V. S. K.**, Zitko, J. *Repurposing human proly-tRNA synthetase inhibitors to antimycobacterial agents with minimal cytotoxicity.* XII Paul Ehrlich PhD Network in Medicinal Chemistry, Thessaloniki, Greece, 16–18 July 2023.

9. Internships/Summer Schools

- **Internship:** Traineeship on Organic Synthesis, Microscale Thermophoresis and Single-Crystal Crystallographic Studies at Dept. of Pharmaceutical Sciences, University of Milan, Italy, September 2022 to December 2022. (Supervisor: Prof. Stefania Villa, Ph.D.)
- **Vienna Summer School 2021:** Drug Design and Workshop (Virtual), University of Vienna, Austria, 13–16 September 2021.
- **Prace Autumn School 2021:** Fundamentals of Biomolecular Simulations and Virtual Drug development, National Center for Supercomputing Applications, Sofia, Bulgaria, 20–24 September 2021

10. Grant contributions

- Grant agency of Charles University (GA UK) – Project No. 349721 - *Design, synthesis and biological evaluation of new antimicrobial compounds derived from inhibitors of aminoacyl-tRNA synthetases.* 01 January 2021 to 31 December 2023 (**Principal Investigator**).
- Grant schemes at UK - reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935 - *Design, synthesis and biological evaluation of 2-aminooxazoles as antibacterial compounds.* 01 April 2021 to 31 March 2023 (**Co-investigator**).

11. Thesis consultant for Master students

- Design, synthesis, and evaluation of heterocyclic compounds with potential antimicrobial activity IV. (https://dspace.cuni.cz/handle/20.500.11956/185475) (Author: PharmDr. Amirhossein Fekir; Supervisor: doc. PharmDr. Jan Zitko, Ph.D.)
- Design, synthesis, and evaluation of heterocyclic compounds with potential antimicrobial activity VI. (https://dspace.cuni.cz/handle/20.500.11956/185476) (Author: RNDr. Nechirwan Abdalrahman; Supervisor: doc. PharmDr. Jan Zitko, Ph.D.)

12. List of references

[1] SALEEM, A.; AZHER, M. The next Pandemic-Tuberculosis: The oldest disease of mankind rising one more time. *Br. J. Med. Pract.,* **2013**, *6*, 21-46.

[2] KIM, P. S.; SWAMINATHAN, S. Ending TB: the world's oldest pandemic. *J. Int. AIDS Soc.,* **2021**, *24*, e25698.

[3] NATARAJAN, A.; BEENA, P. M.; DEVNIKAR, A. V.; MALI, S. A systemic review on tuberculosis. *Indian J. Tuberc.,* **2020**, *67*, 295-311.

[4] *Global Tuberculosis Report 2023*, World Health Organisation, **2023**.

[5] *Global Tuberculosis Report 2021*, World Health Organisation, **2021**.

[6] STERLING TR, N. G., ZENNER D, ET AL. Guidelines for the treatment of latent tuberculosis infection: Recommendations from the National Tuberculosis Controllers Association and CDC, 2020. *MMWR. Recommendations and Reports,* **2020**, *69*, 1-11.

[7] ALSAYED, S. S. R.; GUNOSEWOYO, H., in *Int. J. Mol. Sci., Vol. 24*, **2023**.

[8] ZUMLA, A.; NAHID, P.; COLE, S. T. Advances in the development of new tuberculosis drugs and treatment regimens. *Nat. Rev. Drug Discovery,* **2013**, *12*, 388-404.

[9] GOOSSENS SANDER, N.; SAMPSON SAMANTHA, L.; VAN RIE, A. Mechanisms of Drug-Induced Tolerance in Mycobacterium tuberculosis. *Clin. Microbiol. Rev.,* **2020**, *34*, 10.1128/cmr.00141-00120.

[10] VAN DER WERF, M. J.; LANGENDAM, M. W.; HUITRIC, E.; MANISSERO, D. Multidrug resistance after inappropriate tuberculosis treatment: a meta-analysis. *Eur. Respir. J.,* **2012**, *39*, 1511-1519.

[11] WHITFIELD, M. G.; WARREN, R. M.; MATHYS, V.; SCOTT, L.; DE VOS, E.; STEVENS, W.; STREICHER, E. M.; GROENEN, G.; SIRGEL, F. A.; VAN RIE, A. The potential use of rifabutin for treatment of patients diagnosed with rifampicin-resistant tuberculosis. *J. Antimicrob. Chemother.,* **2018**, *73*, 2667-2674.

[12] ZHANG, L.; ZHAO, Y.; GAO, Y.; WU, L.; GAO, R.; ZHANG, Q.; WANG, Y.; WU, C.; WU, F.; GURCHA, S. S. Structures of cell wall arabinosyltransferases with the anti-tuberculosis drug ethambutol. *Science,* **2020**, *368*, 1211-1219.

[13] ZHU, C.; LIU, Y.; HU, L.; YANG, M.; HE, Z.-G. Molecular mechanism of the synergistic activity of ethambutol and isoniazid against Mycobacterium tuberculosis. *J. Biol. Chem.,* **2018**, *293*, 16741-16750.

[14] ZHANG, Y.; SHI, W.; ZHANG, W.; MITCHISON, D. Mechanisms of pyrazinamide action and resistance. *Microbiol. Spectrum,* **2014**, *2*, 10-1128.

[15] NJIRE, M.; TAN, Y.; MUGWERU, J.; WANG, C.; GUO, J.; YEW, W.; TAN, S.; ZHANG, T. Pyrazinamide resistance in Mycobacterium tuberculosis: Review and update. *Adv. Med. Sci.,* **2016**, *61*, 63-71.

[16] LAMONT, E. A.; DILLON, N. A.; BAUGHN, A. D. The bewildering antitubercular action of pyrazinamide. *Microbiol. Mol. Biol. Rev.,* **2020**, *84*, 10.1128/mmbr. 00070-00019.

[17] PELOQUIN, C. A.; DAVIES, G. R. The treatment of tuberculosis. *Clin. Pharm. Therap.,* **2021**, *110*, 1455-1466.

[18] MITCHISON, D. A. Role of individual drugs in the chemotherapy of tuberculosis. *Int. J. Tuberc. Lung Dis.,* **2000**, *4*, 796-806.

[19] FRIEDMAN, L. N.; DEDICOAT, M.; DAVIES, P. D. O., *Clinical Tuberculosis*, CRC Press, **2020**.

[20] UNISSA, A. N.; SUBBIAN, S.; HANNA, L. E.; SELVAKUMAR, N. Overview on mechanisms of isoniazid action and resistance in Mycobacterium tuberculosis. *Infect., Genet. Evol.,* **2016**, *45*, 474-492.
[21] MIGLIORI, G. B.; TIBERI, S. WHO drug-resistant TB guidelines 2022: what is new. *Int. J. Tuberc. Lung Dis.,* **2022**, *26*, 590-591.
[22] DHINGRA, S.; RAHMAN, N. A. A.; PEILE, E.; RAHMAN, M.; SARTELLI, M.; HASSALI, M. A.; ISLAM, T.; ISLAM, S.; HAQUE, M. Microbial resistance movements: an overview of global public health threats posed by antimicrobial resistance, and how best to counter. *Front. Public Health,* **2020**, *8*, 535668.
[23] FRANCO, B. E.; ALTAGRACIA MARTÍNEZ, M.; SÁNCHEZ RODRÍGUEZ, M. A.; WERTHEIMER, A. I. The determinants of the antibiotic resistance process. *Infect. Drug Resist.,* **2009**, 1-11.
[24] COATES, A. R. M.; HU, Y. Novel approaches to developing new antibiotics for bacterial infections. *Br. J. Pharmacol.,* **2007**, *152*, 1147-1154.
[25] JANG, J. G.; CHUNG, J. H. Diagnosis and treatment of multidrug-resistant tuberculosis. *Yeungnam Univ. J. Med.,* **2020**, *37*, 277-285.
[26] CHOWDHURY, K.; AHMAD, R.; SINHA, S.; DUTTA, S.; HAQUE, M. Multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) among children: Where we stand now. *Cureus,* **2023**, *15*, e35154.
[27] ESPINOSA-PEREIRO, J.; SÁNCHEZ-MONTALVÁ, A.; AZNAR, M. L.; ESPIAU, M. MDR Tuberculosis Treatment. *Medicina,* **2022**, *58*, 188.
[28] SARATHY, J. P.; GRUBER, G.; DICK, T. Re-Understanding the Mechanisms of Action of the Anti-Mycobacterial Drug Bedaquiline. *Antibiotics,* **2019**, *8*, 261.
[29] WORLEY, M. V.; ESTRADA, S. J. Bedaquiline: A Novel Antitubercular Agent for the Treatment of Multidrug-Resistant Tuberculosis. *Pharmacotherapy,* **2014**, *34*, 1187-1197.
[30] DESHKAR, A. T.; SHIRURE, P. A. Bedaquiline: A Novel Diarylquinoline for Multidrug-Resistant Pulmonary Tuberculosis. *Cureus,* **2022**, *14*, e28519.
[31] KHOSHNOOD, S.; TAKI, E.; SADEGHIFARD, N.; KAVIAR, V. H.; HADDADI, M. H.; FARSHADZADEH, Z.; KOUHSARI, E.; GOUDARZI, M.; HEIDARY, M. Mechanism of Action, Resistance, Synergism, and Clinical Implications of Delamanid Against Multidrug-Resistant Mycobacterium tuberculosis. *Front. microbiol.,* **2021**, *12*.
[32] NGUYEN, T. V. A.; ANTHONY, R. M.; CAO, T. T. H.; BAÑULS, A.-L.; NGUYEN, V. A. T.; VU, D. H.; NGUYEN, N. V.; ALFFENAAR, J.-W. C. Delamanid Resistance: Update and Clinical Management. *Clin. Infect. Dis.,* **2020**, *71*, 3252-3259.
[33] ANAND, P.; AKHTER, Y. A review on enzyme complexes of electron transport chain from Mycobacterium tuberculosis as promising drug targets. *Int. J. Biol. Macromol.,* **2022**, *212*, 474-494.
[34] MUDDE, S. E.; UPTON, A. M.; LENAERTS, A.; BAX, H. I.; DE STEENWINKEL, J. E. M. Delamanid or pretomanid? A Solomonic judgement! *J. Antimicrob. Chemother.,* **2022**, *77*, 880-902.
[35] TANG, M.; ZHAO, D.; LIU, S.; ZHANG, X.; YAO, Z.; CHEN, H.; ZHOU, C.; ZHOU, T.; XU, C. The Properties of Linezolid, Rifampicin, and Vancomycin, as Well as the Mechanism of Action of Pentamidine, Determine Their Synergy against Gram-Negative Bacteria. *Int. J. Mol. Sci.,* **2023**, *24*, 13812.
[36] YUAN, Y.; LI, J.; CHEN, Y.; CAI, Q.; XU, Y.; LIN, L.; LANG, Y.; GUO, S.; ZHANG, R.; CAI, X. Mechanism underlying linezolid-induced peripheral neuropathy in multidrug-resistant tuberculosis. *Front. pharmacol.,* **2022**, *13*.

[37] RUIZ, P.; CAUSSE, M.; VAQUERO, M.; CASAL, M. *In Vitro* Activity of Tedizolid against *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.,* **2019**, *63*, 10.1128/aac.01939-01918.
[38] RUTH, M. M.; KOEKEN, V. A. C. M.; PENNINGS, L. J.; SVENSSON, E. M.; WERTHEIM, H. F. L.; HOEFSLOOT, W.; VAN INGEN, J. Is there a role for tedizolid in the treatment of non-tuberculous mycobacterial disease? *J. Antimicrob. Chemother.,* **2019**, *75*, 609-617.
[39] PAIS, J. P.; POLICARPO, M.; PIRES, D.; FRANCISCO, A. P.; MADUREIRA, A. M.; TESTA, B.; ANES, E.; CONSTANTINO, L. Fluoroquinolone Derivatives in the Treatment of Mycobacterium tuberculosis Infection. *Pharmaceuticals,* **2022**, *15*, 1213.
[40] BAHUGUNA, A.; RAWAT, D. S. An overview of new antitubercular drugs, drug candidates, and their targets. *Med. Res. Rev.,* **2020**, *40*, 263-292.
[41] STADLER, J. A. M.; MAARTENS, G.; MEINTJES, G.; WASSERMAN, S. Clofazimine for the treatment of tuberculosis. *Front. pharmacol.,* **2023**, *14*.
[42] NUGRAHA, R. V.; YUNIVITA, V.; SANTOSO, P.; AARNOUTSE, R. E.; RUSLAMI, R. Clofazimine as a Treatment for Multidrug-Resistant Tuberculosis: A Review. *Sci. Pharm.,* **2021**, *89*, 19.
[43] KIM, S.; LOUIE, A.; DRUSANO, G. L.; ALMOSLEM, M.; KIM, S.; MYRICK, J.; NOLE, J.; DUNCANSON, B.; PELOQUIN, C. A.; SCANGA, C. A.; YAMADA, W.; NEELY, M.; SCHMIDT, S. Evaluating the effect of clofazimine against Mycobacterium tuberculosis given alone or in combination with pretomanid, bedaquiline or linezolid. *Int. J. Antimicrob. Agents,* **2022**, *59*, 106509.
[44] XU, J.; KOVAL, A.; KATANAEV, V. L. Clofazimine: A journey of a drug. *Biomed. Pharmacother.,* **2023**, *167*, 115539.
[45] SINGH, S. P.; QURESHI, A.; HASSAN, W. Mechanisms of action by antimicrobial agents: A review. *McGill Journal of Medicine,* **2021**, *19*.
[46] KUMAR, N.; SHARMA, S.; KAUSHAL, P. S. Protein synthesis in Mycobacterium tuberculosis as a potential target for therapeutic interventions. *Mol. Aspects Med.,* **2021**, *81*, 101002.
[47] PANG, A. H.; GREEN, K. D.; TSODIKOV, O. V.; GARNEAU-TSODIKOVA, S., in *Methods in Enzymology, Vol. 690* (Ed.: M. Lloyd), Academic Press, **2023**, pp. 369-396.
[48] RAAIJMAKERS, J.; SCHILDKRAUT, J. A.; HOEFSLOOT, W.; VAN INGEN, J. The role of amikacin in the treatment of nontuberculous mycobacterial disease. *Expert Opin. Pharmacother.,* **2021**, *22*, 1961-1974.
[49] KIM, C. G.; JOSE, J.; HAY, M. P.; CHOI, P. Novel Prodrug Strategies for the Treatment of Tuberculosis. *Chem. Asian J.*, *n/a*, e202400944.
[50] USHARANI, B.; VENKATESWARI, R.; SUGANTHI, P.; VIDYA RAJ CUPPUSAMY, K.; AAINA, M.; MUTHURAJ, M., in *Antibiotic Resistance - New Insights* (Eds.: M. Dr. Ghulam, K. S. Prof. Shailendra), IntechOpen, Rijeka, **2022**, p. Ch. 1.
[51] OH, S.; TRIFONOV, L.; YADAV, V. D.; BARRY, C. E.; BOSHOFF, H. I. Tuberculosis Drug Discovery: A Decade of Hit Assessment for Defined Targets. *Front. cell. infect. microbiol.,* **2021**, *11*.
[52] VAN DER WALT MARTHA, L.; SHEAN, K.; BECKER, P.; KEDDY KAREN, H.; LANCASTER, J. Treatment Outcomes and Adverse Drug Effects of Ethambutol, Cycloserine, and Terizidone for the Treatment of Multidrug-Resistant Tuberculosis in South Africa. *Antimicrob. Agents Chemother.,* **2020**, *65*, 10.1128/aac.00744-00720.

[53] FENG, Z.; BARLETTA RAÚL, G. Roles of Mycobacterium smegmatisd-Alanine:d-Alanine Ligase and d-Alanine Racemase in the Mechanisms of Action of and Resistance to the Peptidoglycan Inhibitor d-Cycloserine. *Antimicrob. Agents Chemother.,* **2003**, *47*, 283-291.
[54] GOMEZ, M. A. R.; IBBA, M. Aminoacyl-tRNA synthetases. *RNA,* **2020**, *26*, 910-936.
[55] PANG, L.; WEEKS, S. D.; VAN AERSCHOT, A. Aminoacyl-tRNA Synthetases as Valuable Targets for Antimicrobial Drug Discovery. *Int. J. Mol. Sci.,* **2021**, *22*, 1750.
[56] ZHOU, Z.; SUN, B.; NIE, A.; YU, D.; BIAN, M. Roles of aminoacyl-tRNA synthetases in cancer. *Front. cell dev. biol.,* **2020**, *8*, 599765.
[57] BOUZ, G.; ZITKO, J. Inhibitors of aminoacyl-tRNA synthetases as antimycobacterial compounds: an up-to-date review. *Bioorg. Chem.,* **2021**, *110*, 104806.
[58] GALINDO, E.; HEBERT, A. A. A comparative review of current topical antibiotics for impetigo. *Expert Opin. Drug Saf.,* **2021**, *20*, 677-683.
[59] GANGWAR, A.; KUMAR, P.; SINGH, R.; KUSH, P. Recent Advances in Mupirocin Delivery Strategies for the Treatment of Bacterial Skin and Soft Tissue Infection. *Future Pharmacol.,* **2021**, *1*, 80-103.
[60] HE, Z.; HUANG, D.-C.; GUO, D.; DENG, F.; SHA, Q.; ZHANG, M.-Z.; ZHANG, W.-H.; GU, Y.-C. Synthesis, fungicidal activity and molecular docking studies of tavaborole derivatives. *Adv. Agrochem,* **2023**, *2*, 185-195.
[61] XIE, S. C.; GRIFFIN, M. D.; WINZELER, E. A.; RIBAS DE POUPLANA, L.; TILLEY, L. Targeting aminoacyl tRNA synthetases for antimalarial drug development. *Annu. Rev. Microbiol.,* **2023**, *77*, 111-129.
[62] CHENG, B.; CAI, Z.; LUO, Z.; LUO, S.; LUO, Z.; CHENG, Y.; YU, Y.; GUO, J.; JU, Y.; GU, Q. Structure-guided Design of Halofuginone Derivatives as ATP-aided inhibitors against bacterial Prolyl-tRNA Synthetase. *J. Med. Chem.,* **2022**, *65*, 15840-15855.
[63] TYE, M. A.; PAYNE, N. C.; JOHANSSON, C.; SINGH, K.; SANTOS, S. A.; FAGBAMI, L.; PANT, A.; SYLVESTER, K.; LUTH, M. R.; MARQUES, S. Elucidating the path to Plasmodium prolyl-tRNA synthetase inhibitors that overcome halofuginone resistance. *Nature Communications,* **2022**, *13*, 4976.
[64] HWANG, J.; JIANG, A.; FIKRIG, E. A potent prolyl tRNA synthetase inhibitor antagonizes Chikungunya and Dengue viruses. *Antiviral Res.,* **2019**, *161*, 163-168.
[65] WU, J.; SUBBAIAH, K. C. V.; XIE, L. H.; JIANG, F.; KHOR, E.-S.; MICKELSEN, D.; MYERS, J. R.; TANG, W. H. W.; YAO, P. Glutamyl-prolyl-tRNA synthetase regulates proline-rich pro-fibrotic protein synthesis during cardiac fibrosis. *Circ. Res.,* **2020**, *127*, 827-846.
[66] KURATA, K.; JAMES-BOTT, A.; TYE, M. A.; YAMAMOTO, L.; SAMUR, M. K.; TAI, Y.-T.; DUNFORD, J.; JOHANSSON, C.; SENBABAOGLU, F.; PHILPOTT, M. Prolyl-tRNA synthetase as a novel therapeutic target in multiple myeloma. *Blood Cancer J.,* **2023**, *13*, 12.
[67] SHIN, W.; PARK, M. Y.; KIM, J.; KIM, J.; NAM, J. H.; CHOI, J.; YANG, A. Y.; YOO, H.; LEE, Y. S.; KIM, A. No drug–drug interactions between selective prolyl-tRNA synthetase inhibitor, bersiporocin, and pirfenidone or nintedanib in healthy participants. *Clin. Transl. Sci.,* **2024**, *17*, e13701.
[68] PANG, L.; WEEKS, S. D.; JUHÁS, M.; STRELKOV, S. V.; ZITKO, J.; VAN AERSCHOT, A. Towards Novel 3-Aminopyrazinamide-Based Prolyl-tRNA Synthetase Inhibitors: In Silico Modelling, Thermal Shift Assay and Structural Studies. *Int. J. Mol. Sci.,* **2021**, *22*, 7793.

[69] ADACHI, R.; OKADA, K.; SKENE, R.; OGAWA, K.; MIWA, M.; TSUCHINAGA, K.; OHKUBO, S.; HENTA, T.; KAWAMOTO, T. Discovery of a novel prolyl-tRNA synthetase inhibitor and elucidation of its binding mode to the ATP site in complex with l-proline. *Biochem. Biophys. Res. Commun.,* **2017**, *488*, 393-399.

[70] JANDOUREK, O.; TAUCHMAN, M.; PATEROVA, P.; KONECNA, K.; NAVRATILOVA, L.; KUBICEK, V.; HOLAS, O.; ZITKO, J.; DOLEZAL, M. Synthesis of Novel Pyrazinamide Derivatives Based on 3-Chloropyrazine-2-carboxamide and Their Antimicrobial Evaluation. *Molecules,* **2017**, *22*, 223.

[71] PALLABOTHULA, V. S. K.; KERDA, M.; JUHÁS, M.; JANĎOUREK, O.; KONEČNÁ, K.; BÁRTA, P.; PATEROVÁ, P.; ZITKO, J. Adenosine-Mimicking Derivatives of 3-Aminopyrazine-2-Carboxamide: Towards Inhibitors of Prolyl-tRNA Synthetase with Antimycobacterial Activity. *Biomolecules,* **2022**, *12*, 1561.

[72] LEE, B. I.; SUH, S. W. Crystal structure of the schiff base intermediate prior to decarboxylation in the catalytic cycle of aspartate α-decarboxylase. *J. Mol. Biol.,* **2004**, *340*, 1-7.

[73] HU, Z.-C.; TIAN, Y.-H.; YANG, J.-L.; ZHU, Y.-N.; ZHOU, H.-Y.; ZHENG, Y.-G.; LIU, Z.-Q. Research progress of l-aspartate-α-decarboxylase and its isoenzyme in the β-alanine synthesis. *World Journal of Microbiology and Biotechnology,* **2023**, *39*, 42.

[74] SAW, W.-G.; LEOW, C. Y.; HARIKISHORE, A.; SHIN, J.; COLE, M. S.; ARAGAW, W. W.; RAGUNATHAN, P.; HEGDE, P.; ALDRICH, C. C.; DICK, T. Structural and Mechanistic Insights into Mycobacterium abscessus Aspartate Decarboxylase PanD and a Pyrazinoic Acid-Derived Inhibitor. *ACS Infect. Dis.,* **2022**, *8*, 1324-1335.

[75] NJIRE, M.; WANG, N.; WANG, B.; TAN, Y.; CAI, X.; LIU, Y.; MUGWERU, J.; GUO, J.; HAMEED, H. A.; TAN, S. Pyrazinoic acid inhibits a bifunctional enzyme in Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.,* **2017**, *61*, 10.1128/aac. 00070-00017.

[76] ZIMHONY, O.; VILCHEZE, C.; ARAI, M.; WELCH, J. T.; JACOBS JR, W. R. Pyrazinoic acid and its n-propyl ester inhibit fatty acid synthase type I in replicating tubercle bacilli. *Antimicrob. Agents Chemother.,* **2007**, *51*, 752-754.

[77] ZHANG, Y.; MITCHISON, D. The curious characteristics of pyrazinamide: a review. *Int. J. Tuberc. Lung Dis.,* **2003**, *7*, 6-21.

[78] GOPAL, P.; GRÜBER, G.; DARTOIS, V.; DICK, T. Pharmacological and molecular mechanisms behind the sterilizing activity of pyrazinamide. *Trends Pharmacol. Sci.,* **2019**, *40*, 930-940.

[79] ALMEIDA, D. V.; TYAGI, S.; LI, S.-Y.; WALLENGREN, K.; PYM, A. S.; AMMERMAN, N. C.; BISHAI, W. R.; GROSSET, J. H. Revisiting anti-tuberculosis activity of pyrazinamide in mice. *J. Mycobac. Dis.,* **2014**, *4*, 145.

[80] SHI, W.; ZHANG, X.; JIANG, X.; YUAN, H.; LEE, J. S.; BARRY 3RD, C. E.; WANG, H.; ZHANG, W.; ZHANG, Y. Pyrazinamide inhibits trans-translation in Mycobacterium tuberculosis. *Science,* **2011**, *333*, 1630-1632.

[81] FOX, W.; ELLARD, G. A.; MITCHISON, D. A. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946–1986, with relevant subsequent publications. *Int. J. Tuberc. Lung Dis.,* **1999**, *3*, S231-S279.

[82] THORACIC, A. B. A controlled trial of six months chemotherapy in pulmonary tuberculosis. Second report: results during the 24 months after the end of chemotherapy. British Thoracic Association. *Am. Rev. Respir. Dis.,* **1982**, *126*, 460-462.

[83] THORACIC, A. B. Short-course chemotherapy in pulmonary tuberculosis. *Lancet,* **1975**, 119-124.
[84] ROSSINI, N. D. O.; DIAS, M. V. B. Mutations and insights into the molecular mechanisms of resistance of Mycobacterium tuberculosis to first-line. *Genet. Mol. Biol.,* **2023**, *46*.
[85] GOPAL, P.; NARTEY, W.; RAGUNATHAN, P.; SARATHY, J.; KAYA, F.; YEE, M.; SETZER, C.; MANIMEKALAI, M. S. S.; DARTOIS, V.; GRÜBER, G. Pyrazinoic acid inhibits mycobacterial coenzyme A biosynthesis by binding to aspartate decarboxylase PanD. *ACS Infect. Dis.,* **2017**, *3*, 807-819.
[86] GOPAL, P.; SARATHY, J. P.; YEE, M.; RAGUNATHAN, P.; SHIN, J.; BHUSHAN, S.; ZHU, J.; AKOPIAN, T.; KANDROR, O.; LIM, T. K. Pyrazinamide triggers degradation of its target aspartate decarboxylase. *Nature Communications,* **2020**, *11*, 1661.
[87] RAGUNATHAN, P.; COLE, M.; LATKA, C.; ARAGAW, W. W.; HEGDE, P.; SHIN, J.; SUBRAMANIAN MANIMEKALAI, M. S.; RISHIKESAN, S.; ALDRICH, C. C.; DICK, T.; GRÜBER, G. Mycobacterium tuberculosis PanD Structure–Function Analysis and Identification of a Potent Pyrazinoic Acid-Derived Enzyme Inhibitor. *ACS Chemical Biology,* **2021**, *16*, 1030-1039.
[88] ZHANG, M.; HE, S.; HAN, X.; CUI, J.; WANG, H.; HUO, X.; YAN, F.; FENG, L.; WANG, C.; MA, X. Discovery of Potential Antituberculosis Agents Targeted Methionine Aminopeptidase 1 of Mycobacterium tuberculosis by the Developed Fluorescent Probe. *Anal. Chem.,* **2023**, *95*, 16210-16215.
[89] ROSE, J. A.; LAHIRI, S. D.; MCKINNEY, D. C.; ALBERT, R.; MORNINGSTAR, M. L.; SHAPIRO, A. B.; FISHER, S. L.; FLEMING, P. R. Novel broad-spectrum inhibitors of bacterial methionine aminopeptidase. *Bioorg. Med. Chem. Lett.,* **2015**, *25*, 3301-3306.
[90] LU, J.-P.; YE, Q.-Z. Expression and characterization of Mycobacterium tuberculosis methionine aminopeptidase type 1a. *Bioorg. Med. Chem. Lett.,* **2010**, *20*, 2776-2779.
[91] JUHÁS, M.; PALLABOTHULA, V. S. K.; GRABRIJAN, K.; ŠIMOVIČOVÁ, M.; JANĎOUREK, O.; KONEČNÁ, K.; BÁRTA, P.; PATEROVÁ, P.; GOBEC, S.; SOSIČ, I.; ZITKO, J. Design, synthesis and biological evaluation of substituted 3-amino-N-(thiazol-2-yl)pyrazine-2-carboxamides as inhibitors of mycobacterial methionine aminopeptidase 1. *Bioorg. Chem.,* **2022**, *118*, 105489.
[92] OLALEYE, O.; RAGHUNAND, T. R.; BHAT, S.; CHONG, C.; GU, P.; ZHOU, J.; ZHANG, Y.; BISHAI, W. R.; LIU, J. O. Characterization of clioquinol and analogues as novel inhibitors of methionine aminopeptidases from Mycobacterium tuberculosis. *Tuberculosis,* **2011**, *91*, S61-S65.
[93] QUAN, D. H.; NAGALINGAM, G.; LUCK, I.; PROSCHOGO, N.; PILLALAMARRI, V.; ADDLAGATTA, A.; MARTINEZ, E.; SINTCHENKO, V.; RUTLEDGE, P. J.; TRICCAS, J. A. Bengamides display potent activity against drug-resistant Mycobacterium tuberculosis. *Sci. Rep.,* **2019**, *9*, 14396.
[94] JOHN, S. F.; ANIEMEKE, E.; HA, N. P.; CHONG, C. R.; GU, P.; ZHOU, J.; ZHANG, Y.; GRAVISS, E. A.; LIU, J. O.; OLALEYE, O. A. Characterization of 2-hydroxy-1-naphthaldehyde isonicotinoyl hydrazone as a novel inhibitor of methionine aminopeptidases from Mycobacterium tuberculosis. *Tuberculosis,* **2016**, *101*, S73-S77.
[95] PALLABOTHULA, V. S. K.; ABDALRAHMAN, N. T.; MORI, M.; FEKRI, A. H.; JANĎOUREK, O.; KONEČNÁ, K.; PATEROVÁ, P.; NOVÁK, M.; DUDÁŠOVÁ-HATOKOVÁ, P.; ŠTĚRBOVÁ-KOVAŘÍKOVÁ, P.; CASTELLANO, C.; MENEGHETTI, F.; VILLA, S.; KUNEŠ, J.; JUHÁS, M.; ZITKO, J. A hit expansion

of 3-benzamidopyrazine-2-carboxamide: Toward inhibitors of prolyl-tRNA synthetase with antimycobacterial activity. *Arch. Pharm.,* **2024**, *357*, e2400171.

[96] WAMHOFF, H.; KROTH, E. Dihalogentriphenylphosphorane in der Heterocyclensynthese, 29. Eine einfache Synthese von Pteridin-4-onen aus 3-Amino-2-pyrazincarbonsäuremethylester und Pyrazino [3, 1] oxazin-4-onen. *Synthesis,* **1994**, *1994*, 405-410.

[97] BOUZ, G.; ŠLECHTA, P.; JAND'OUREK, O.; KONEČNÁ, K.; PATEROVÁ, P.; BÁRTA, P.; NOVÁK, M.; KUČERA, R.; DAL, N.-J. K.; FENAROLI, F.; ZEMANOVÁ, J.; FORBAK, M.; KORDULÁKOVÁ, J.; PAVLIŠ, O.; KUBÍČKOVÁ, P.; DOLEŽAL, M.; ZITKO, J. Hybridization Approach Toward Novel Antituberculars: Design, Synthesis, and Biological Evaluation of Compounds Combining Pyrazinamide and 4-Aminosalicylic Acid. *ACS Infect. Dis.,* **2023**, *9*, 79-96.

[98] CUI, Y.-M.; HUANG, Q.-Q.; XU, J.; CHEN, L.-L.; LI, J.-Y.; YE, Q.-Z.; LI, J.; NAN, F.-J. Identification of potent type I MetAP inhibitors by simple bioisosteric replacement. Part 1: Synthesis and preliminary SAR studies of thiazole-4-carboxylic acid thiazol-2-ylamide derivatives. *Bioorg. Med. Chem. Lett.,* **2005**, *15*, 3732-3736.

[99] LUO, Q.-L.; LI, J.-Y.; LIU, Z.-Y.; CHEN, L.-L.; LI, J.; QIAN, Z.; SHEN, Q.; LI, Y.; LUSHINGTON, G. H.; YE, Q.-Z. Discovery and Structural Modification of Inhibitors of Methionine Aminopeptidases from Escherichia c oli and Saccharomyces c erevisiae. *J. Med. Chem.,* **2003**, *46*, 2631-2640.

[100] DOLEZAL, M.; PALEK, L.; VINSOVA, J.; BUCHTA, V.; JAMPILEK, J.; KRALOVA, K. Substituted pyrazinecarboxamides: Synthesis and biological evaluation. *Molecules,* **2006**, *11*, 242-256.

[101] JUHÁS, M.; BACHTÍKOVÁ, A.; NAWROT, D. E.; HATOKOVÁ, P.; PALLABOTHULA, V. S. K.; DIEPOLTOVÁ, A.; JANĎOUREK, O.; BÁRTA, P.; KONEČNÁ, K.; PATEROVÁ, P.; ŠESTÁK, V.; ZITKO, J. Improving Antimicrobial Activity and Physico-Chemical Properties by Isosteric Replacement of 2-Aminothiazole with 2-Aminooxazole. *Pharmaceuticals,* **2022**, *15*, 580.

[102] ZITKO, J.; JAND'OUREK, O.; PATEROVÁ, P.; NAVRÁTILOVÁ, L.; KUNEŠ, J.; VINŠOVÁ, J.; DOLEŽAL, M. Design, synthesis and antimycobacterial activity of hybrid molecules combining pyrazinamide with a 4-phenylthiazol-2-amine scaffold. *MedChemComm,* **2018**, *9*, 685-696.

[103] AZZALI, E.; GIRARDINI, M.; ANNUNZIATO, G.; PAVONE, M.; VACONDIO, F.; MORI, G.; PASCA, M. R.; COSTANTINO, G.; PIERONI, M. 2-aminooxazole as a novel privileged scaffold in antitubercular medicinal chemistry. *ACS Med. Chem. Lett.,* **2020**, *11*, 1435-1441.

13. List of Publications

The publications along with supplementary files used in this dissertation are only available in the printed version of the doctoral thesis; Pages S 1 to S 225.

P1 **PALLABOTHULA, V. S. K.**; KERDA, M.; JUHÁS, M.; JANĎOUREK, O.; KONEČNÁ, K.; BÁRTA, P.; PATEROVÁ, P.; ZITKO, J. Adenosine-Mimicking Derivatives of 3-Aminopyrazine-2-Carboxamide: Towards Inhibitors of Prolyl-tRNA Synthetase with Antimycobacterial Activity. *Biomolecules*, **2022**, vol. 12, article no. 1561. **(Pages S 1 to S 52)**

IF_{2022} = 5.5 (Q2), doi: 10.3390/biom12111561

P2 **PALLABOTHULA, V. S. K.**; ABDALRAHMAN, N. T.; MORI, M.; FEKRI, A. H.; JANĎOUREK, O.; KONEČNÁ, K.; PATEROVÁ, P.; NOVÁK, M.; DUDÁŠOVÁ-HATOKOVÁ, P.; ŠTĚRBOVÁ-KOVAŘÍKOVÁ, P.; CASTELLANO, C.; MENEGHETTI, F.; VILLA, S.; KUNEŠ, J.; JUHÁS, M.; & ZITKO, J. A hit expansion of 3-benzamidopyrazine-2-carboxamide: Toward inhibitors of prolyl-tRNA synthetase with antimycobacterial activity. *Archiv der Pharmazie,* **2024**, vol. 357, article no. e2400171. **(Pages S 53 to S 133)**

IF_{2023} = 4.3 (Q2), doi: doi.org/10.1002/ardp.202400171

P3 JUHÁS, M.; **PALLABOTHULA, V. S. K.**; GRABRIJAN, K.; ŠIMOVIČOVÁ, M.; JANĎOUREK, O.; KONEČNÁ, K.; BÁRTA, P.; PATEROVÁ, P.; GOBEC, S.; SOSIČ, I.; ZITKO, J. Design, synthesis and biological evaluation of substituted 3-amino-N-(thiazol-2-yl)pyrazine-2-carboxamides as inhibitors of mycobacterial methionine aminopeptidase 1. *Bioorg. Chem.*, **2022**, vol. 118, article no. 105489. **(Pages S 134 to S 183)**

IF_{2022} = 5.1 (Q2Q1), doi: 10.1016/j.bioorg.2021.105489

P4 JUHÁS, M.; BACHTIKOVÁ, A.; NAWROT, D.E.; HATOKOVÁ, P.; **PALLABOTHULA, V. S. K.**; DIEPOLTOVÁ, A.; JANĎOUREK, O.; BÁRTA, P.; KONEČNÁ, K.; PATEROVÁ, P.; SESTÁK, V.; ZITKO, J. Improving Antimicrobial Activity and Physico-Chemical Properties by Isosteric Replacement of 2-Aminothiazole with 2-Aminooxazole. *Pharmaceuticals*, **2022**, vol. 15, article no. 580. **(Pages S 184 to S 225)**

IF_{2022} = 4.6 (Q2), doi: doi.org/10.3390/ph15050580