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Study Of Pyridine Derivatives As Potential Antimycobacterial Active Drugs.

Doctoral thesis (a commentary on published articles)

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Hradec Králové, 2024

I would like to express my gratitude to an important group of people:

To the most important ones - my family (*Mama*, *Babcia* i *Wujek*), who is always there for me. I cannot imagine completing PhD studies without them.

Ghada Bouz - A true friend in times of need and beyond. Statistically improved my likelihood of persisting and completing PhD studies (statistical significance $P \leq 0.001$). Always a supportive companion in both work and life.

Negar Maafi - An amazing person who prioritized my personal development through my stay in ČR. Made me meet new people (painful), get bruised while snowboarding (less painful), play squash, learn Czech language, and eat excellent food.

Małgorzata Morkis - Studying at MISMaP was one of my best choices, because I met you.

Priam-Amedeo Houngebedji - Thank you for handling what I prefer not to.

I am grateful that *Barbora Koutníková* chose to collaborate with our research group. I only wish that others were as excellent students.

I want to express my gratitude to *Klara Konečná*, *Pavla Paterová*, and *Pavel Bárta* for their contributions that enabled us to publish the work presented in this commentary. I deeply admire their ethics, dedication, and hard work.

Patric Gámez, Ana B. Caballero and *Ernesto Nicolas*. Thanks to them I could fully enjoy my stay abroad in Barcelona. They granted me a place in their research group and showed how passionate about science one can be.

Finally, I want to express my gratitude to *Professor Martin Doležal.* I am truly grateful for the opportunity granted to me, the mentorship and what I have learnt during my stay in Czech Republic.

There are many others to mention, yet this would end up as a book.

To my all et al.

Mischief Managed.

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

Daria Nawrot

……, Hradec Králové

Abstract

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Title of Doctoral Thesis *Study of pyridine derivatives as potential antimycobacterial active drugs.*

Besides the period when COVID-19 was the biggest threat to health systems efficiency worldwide, tuberculosis remains the most important cause of death by an infectious disease. This, coupled with a rise in antibacterial resistance, remains a substantial challenge for the new drug research pipeline.

The theoretical part of this commentary briefly highlights issues related to tuberculosis, specifically antimicrobial resistance. In the experimental part, the design, synthesis, and biological evaluation of novel compounds investigated during the doctoral program are discussed. In total, three publications are addressed.

The designed compounds were prepared and screened for their *in vitro* activity against selected mycobacterial strains (*M. avium*, *M. aurum*, *M. kansasii*, *M. smegmatis* were used alongside strains of main interest - *Mtb* H37Ra and *Mtb* H37Rv), eight fungal strains (*Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Absidia corymbifera*, and *Trichophyton interdigitale*) and eight bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus spp*., *Klebsiella pneumonia*, and *Acinetobacter baumannii*). The compounds were tested *in vitro* for their cytotoxicity against the HepG2 cancer cell line and where applicable on the *Galleria mellonella in vivo* model. Some selected compounds were tested for their *in vitro* antiproliferative activity against human epithelial kidney cancer cell line A498, human prostate cancer cell line PC-3 and human glioblastoma cell line U-87MG. The more advanced *in vivo* testing in a murine tuberculosis model was also performed where justified.

Unpublished work is briefly summarized at the end of this commentary.

Abstrakt

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Název disertační práce: *Studium derivátů pyridinu jako potenciálních antimykobakteriálních léčiv.*

Mimo období kdy tvořil COVID-19 největší hrozbu pro účinnost zdravotnických systémů na celosvětovém měřítku, zůstává tuberkuloza nejdůležitější příčinou úmrtí na infekční chorobu. Tato skutečnost, spolu se stoupající antibakteriální rezistencí, tvoří zásadní výzvu pro výzkum nových léčiv.

Teoretická část tohoto komentáře krátce zvýrazňuje problematiku tuberkulozy a konkrétněji antimikrobiální rezistence. V experimentální části je vedena diskuze o návrhu, syntéze a biologickém hodnocení nových sloučenin zkoumaných během doktorského studia.

Navržené sloučeniny byly připraveny a testovány na in vitro aktivitu proti vybraným mykobakteriálním kmenům (*M. Avium*, *M. aurum*, *M. kansasii*, *M. smegmatis* spolu s kmeny hlavního zájmu - Mtb H37Ra a H37Rv), osmi fungálním kmenům (*Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Absidia corymbifera*, a *Trichophyton interdigitale*) a osmi bakteriálním kmenům (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, Enterococcus spp., *Klebsiella pneumonia*, a *Acinetobacter baumannii*). Sloučeniny byly testovány in vitro na cytotoxicitu proti HepG2 rakovinným buněčním liniím a kde možné na Galleria mellonella In vivo model. Vybrané sloučeniny byly testovány na in vitro protiproliferační aktivitu proti lidským buněcím liniím rakoviny epitelu ledvin A498, lidským buněčním liniím rakoviny prostaty PC-3, a buněčným liniím lidkského glioblastomu U-87MG. Pokročilejší in vivo testování na myších modelech tuberkulozi bylo provedené kde vhodné.

Nepublikovaná práce je krátce shrnuta na konci tohoto komentáře.

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List of Abbreviations

TB	Tuberculosis
Mtb	*Mycobacterium Tuberculosis*
WHO	World Health Organization
BCG	Bacillus Calmette-Guérin
MDR	multidrug-resistant
XDR	extensively resistant
SA	*Staphylococcus aureus*
MIC	Minimum inhibitory concentration
CDI	1,1'-Carbonyldiimidazol
PAINS	Pan-assay interference compounds

Introduction

Tuberculosis – a worldwide concern

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). Aside from the period of the COVID-19 pandemic, TB stands out as the most significant cause of death among infectious diseases [1, 2]. Meanwhile, it ranks as the 13th in global causes of all death, following conditions like cancer or cardiovascular diseases [3]. What is noteworthy about TB is that approximately one-fourth of the global population is infected with the bacteria, but only a small percentage will develop an active form of the disease (5-10% of those carrying the infection) (see fig. 1 for estimated TB incidence) [4, 5]. Those more susceptible to the disease are usually malnourished, HIV-positive or those with other coexisting diseases, such as diabetes [6]. TB is preventable, and even after developing the disease, one can be successfully cured. However, on average, about 1.5 million people die annually due to TB[3]. This number was even higher during the COVID-19 pandemic due to a reduced number of diagnosed and treated patients [7, 8]. The prevention of TB is primarily based on infants' vaccination with attenuated *Mycobacterium bovis* vaccine (also known as BCG - Bacillus Calmette-Guérin, from the names of its inventors) [9]; Its first use in humans dates back to 1921, and since 1977, it has been listed as World Health Organization (WHO) Essential Medicine. However, the efficacy and the duration of the protective effect are not clearly known [10, 11]. The vaccine's effectiveness also varies with the geographical location [12-15]. Besides children, all age groups are rather equally susceptible to the disease [16]. BCG is the only vaccine administered against Mtb; several new vaccine candidates are now in clinical trials. An example is M72/AS01$_E$, a protein adjuvant vaccine, which successfully completed phase 2b trials in adults and participants with well-controlled HIV demonstrating a significant positive effect against TB [17-20].

In those with already developed infection, the treatment regimen shall be based on drug susceptibility testing and concordant treatment. In the first line treatment for drug-susceptible TB four drugs are commonly used: isoniazid, pyrazinamide, ethambutol, and rifampicin (fig. 2). When needed streptomycin may be added but due to high incidence of resistance, such a change is not often practiced [21].

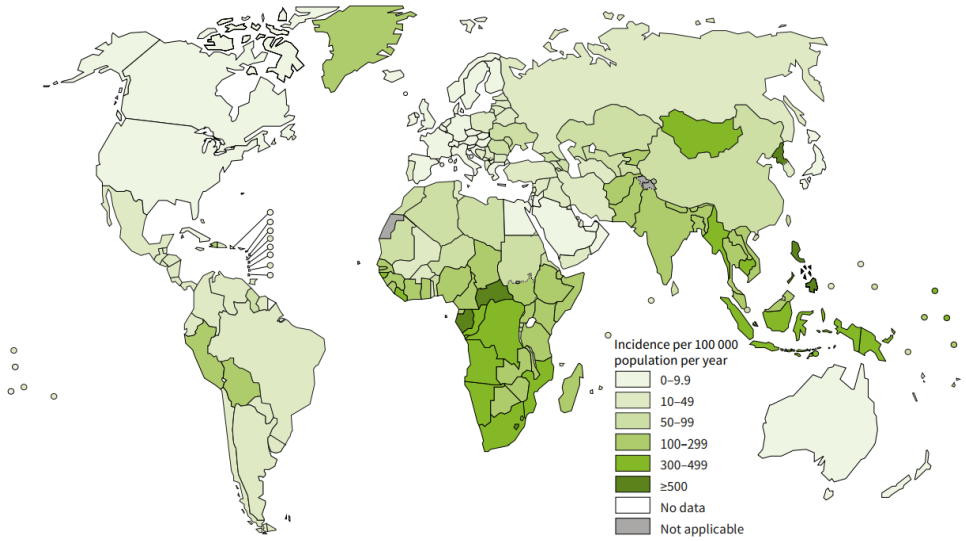


Fig. 1. WHO map: Estimated TB incidence rates in 2022 [5].

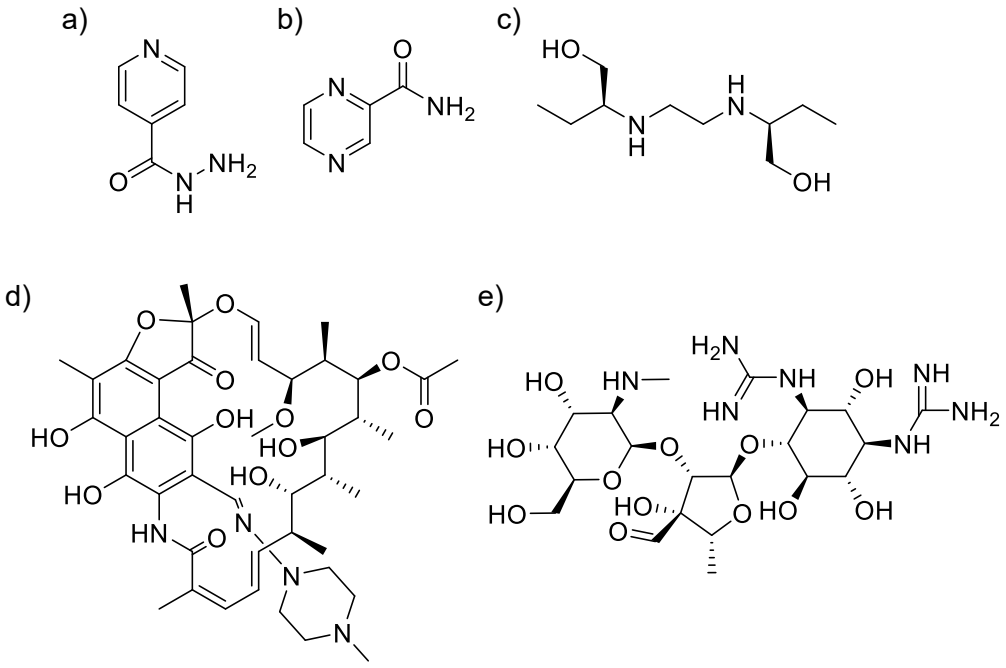


Fig. 2. First-line agents used in TB treatment. a) isoniazid; b) pyrazinamide; c) ethambutol; d) rifampicin; e) streptomycin.

Despite promising outcomes in TB eradication, such as a 19% decrease in TB-caused deaths between 2015 and 2022 [22], significant obstacles still impede faster progress. The recent COVID-19 pandemic has disrupted the progress made towards eliminating the disease and most likely contributed to the growth of antimicrobial resistance. Alongside this, the main challenges persist, including the increased incidence of drug-resistant tuberculosis [23] and a lack of proper funding to develop drugs acting on novel targets. Other issues that shall be addressed are ongoing global conflicts (the war in Ukraine [24], armed conflicts in African countries [25]) and food insecurity [26, 27].

The dedicated program ‘End TB strategy’, led and monitored by the World Health Organization (WHO), aims to reduce the incidence rate by 90%, and the death rate by 95%, and eliminate catastrophic costs caused by tuberculosis worldwide [28]. Globally we are far from achieving the desired numbers. Even in Europe, the continent with the lowest TB incidence [29], meeting the 2030 target seems unlikely unless a breakthrough occurs by 2025 [28]. Annually, the WHO releases the Global Tuberculosis Report, summarizing efforts, conclusions from previous years, and providing recommendations for the coming years. Based on that report, recent advances in the R&D pipeline for tuberculosis do not make one optimistic - even though there are new entities in clinical trials (both drugs and vaccines), most of them act by already-known mechanism. For example, sutezolid (currently in clinical trial phase 2 [30-34]) acts by the same mechanism as linezolid [35]. In addition to the lack of novel entities and novel targets, the problem also lies in already used drugs: antimicrobial resistance prolongs the treatment and lowers success rates [36]; the treatment for drug-resistant TB due to side effects may not be well tolerated [36]. Additionally, there is a lack of a proper formulation for a second-line regimen for paediatric use [37, 38], and the treatment cannot be afforded by all [39].

Antimicrobial resistance

Alongside tuberculosis, another problem needs to be tackled by modern scientists as soon as possible. It constitutes a major threat to public health and exceeds the field of medicine and pharmacy. Antimicrobial resistance is associated with a variety of bacteria, viruses, fungi, and parasites. Examples include methicillin-resistant *Staphylococcus aureus*, fluconazole-resistant *Candida albicans*, artemisinin-resistant malaria, or acyclovir-resistant *Herpes simplex* virus. While antimicrobial resistance is a natural outcome of evolution, poor patient adherence, lack of proper disease prevention and insufficient access to antibiotics contribute to a problem that cannot be ignored [40]. The lack of proper measures results in ‘superbugs’ - multi- and pan-resistant species, such as the so-called New Delhi bacteria (*K. pneumoniae* New Delhi metallo-ß-lactamase), which are increasingly found in hospitals [41-43]. In 2017, WHO published a list of antibiotic-resistant pathogens that shall be a priority in designing new antibiotics [44]. This list includes the ESKAPE panel of bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*.) which, alongside *mycobacterium*, falls under the interest of our research group. Alongside the apparent growth in antimicrobial resistance due to the factors mentioned earlier, the last years of the COVID pandemic, marked by mis- and over-use of antibiotics (for example ceftriaxone and azithromycin [45]) and disinfectants [46], may further exacerbate resistance. However, conclusive data remain limited [47], varying from country to country depending on the measures taken during the pandemic.

Antimicrobial resistance is also present in tuberculosis. Currently, the first-line treatment regimen and its mentioned alterations serve their purpose, but not universally. Tuberculosis exhibits various types of resistance, but usually, two or three major categories are distinguished: multidrug-resistant TB (MDR-TB), pre-extensively resistant TB (pre-XDR-TB), and extensively resistant TB (XDR-TB). In MDR-TB, bacteria do not respond to isoniazid and rifampicin treatment. In the case of pre-XDR, bacteria do not respond to rifampicin and may not react to isoniazid but also do not respond to one of the fluoroquinolones (moxifloxacin or levofloxacin). For both MDR and pre-XDR cases, the WHO Consolidated Guidelines on Tuberculosis 2022 recommend a 6-month initial choice regimen consisting of pretomanid, linezolid, bedaquiline and moxifloxacin (conditional recommendation, with low certainty of evidence, fig. 3). In the latter case, the infection is additionally not prone to the treatment containing Group A drugs (linezolid or bedaquiline) [48]. In this instance, WHO considers lobectomy or wedge resection surgery [49, 50], previously known and obsolete methods of limiting the spread of TB, as useful where sole chemotherapy fails.

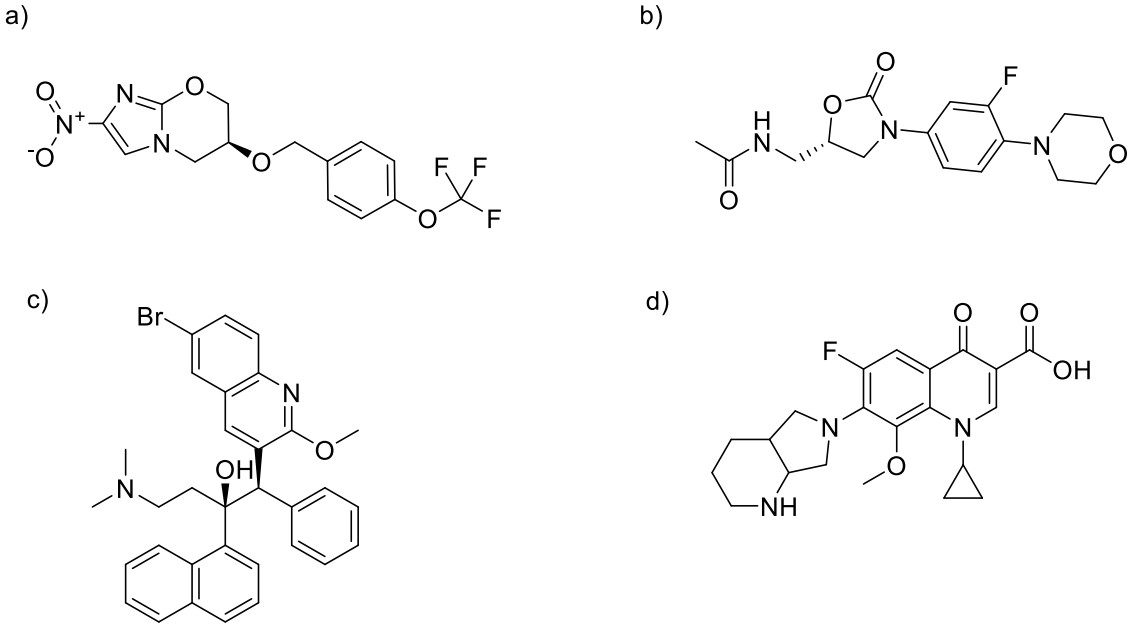


Fig. 3. MDR-TB treatment of choice regimen. a) pretomanid; b) linezolid; c) bedaquiline; d) moxifloxacin.

Pyridine and its place in antitubercular research

Since the establishment of our research group ‘Design and Development of New Antimicrobial Agents’ led by Prof. Martin Doležal, we have taken an interest in developing drugs based on pyrazinamide, a well-known antitubercular agent. WHO in its operational handbook on tuberculosis describes pyrazinamide as a routinely present agent in the treatment regimen, provided no contraindications are present (mainly hepatitis) [51]. Another reason why pyrazinamide is a good candidate for further structural modification -besides its ubiquity in the treatment regimen- is its synergic effect when combined with bedaquiline (also clarithromycin or rifampicin) [52-56].

As mentioned earlier, isoniazid is a well-known example of pyridine-based antitubercular. It belongs to the first-line treatment and acts as a bactericidal agent on growing mycobacterium by interrupting mycobacterial cell wall synthesis. Isoniazid (prodrug) is converted by the catalase-peroxidase enzyme (KatG) to a radical form, which later forms an adduct with nicotinamide adenine dinucleotide (NAD) and inhibits NADH-dependant enoyl-acyl carrier protein reductase InhA (fig. 4).

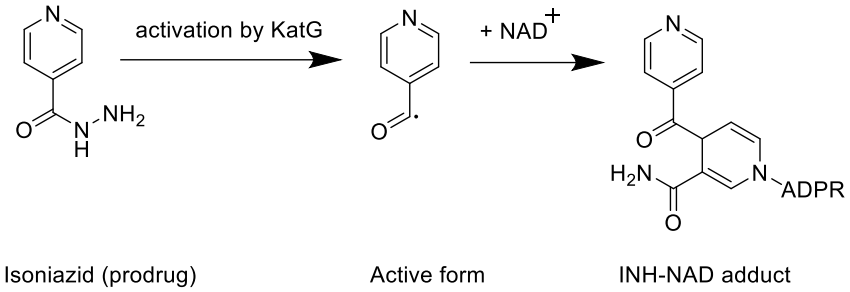


Fig. 4. Isoniazid mechanism of action. ADPR – adenosine diphosphate ribose.

Eight per cent of all TB cases worldwide exhibit resistance to isoniazid, with this number varying between 5 and 11 % across regions [57]. This resistance results in prolonged treatment and a reduced success rate of standard treatment [58, 59]. The side effects of isoniazid may manifest as acute or chronic toxicity, typically seen as peripheral neuropathy or hepatotoxicity [60-63]. Several modifications of the isoniazid structure have been proposed, with the main ones being iproniazid (fig. 5), and ethionamide (fig. 5). Iproniazid was initially designed as an antituberculotic but was later found to act as a non-selective MAO inhibitor [64], leading to its discontinued use. Ethionamide is still in use in MDR-TB; like isoniazid, it inhibits InhA, and its activation is mediated by EthA monooxygenase. Ethionamide's side effects include gastrointestinal disturbance, hepatotoxicity, and hypothyroidism [65].

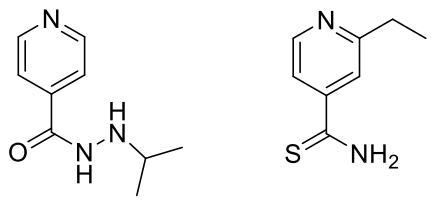


Fig. 5. Iproniazid (left) and ethionamide (right) chemical structures.

Importance of bioisosterism in drug development

Bioisosterism, a long-known and widely used concept in medicinal chemistry, refers to structural modifications employed to alter properties of lead compounds in order to enhance its desired properties. These modifications help to address various challenges encountered during structure optimization, including undesired biological activity, short half-life, low solubility, toxicity, or lack of selectivity [66-69]. Importantly, those parameters are often the

main reasons why new drug candidates fail to advance to the clinical trial stage, rather than a lack of biological activity. Bioisosteres are expected to retain the biological activity of the 'parent' compound while, ideally, other parameters such as efficacy and safety are improved.

The application of bioisosterism is diverse, and this commentary also presents published work that has employed this approach. In the publications discussed, isosterism is represented by functional group inversion and classic isosteric replacement. A well-known example illustrating group inversion in drug design is the comparison between lidocaine and procainamide, where the -CONH- group is switched to -NHCO- (fig. 6). Additionally, procainamide and procaine may serve as an example of classic isosteric replacement, as the structural difference between them lies in the presence of ester oxygen or amidic nitrogen (fig. 6).

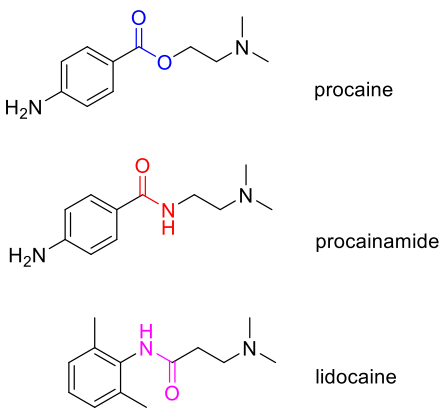


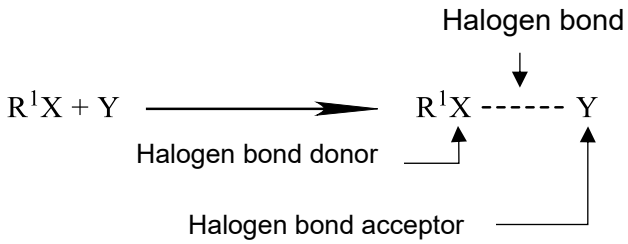
Fig. 6. Example of bioisosterism and retroisomerism on procaine, procainamide, and lidocaine.

Isosteric replacements may be implemented in both chain structures and cycles. Heterocycles, privileged scaffold in drug design, are present in around 85-90% of emerging drugs [70, 71], have wide range of applications and provide a scaffold that can readily undergo structural modifications.

Since we can modify the heterocycle structure, we may also modify its substitution. Although it may not neatly align with the definition of bioisosterism (according to the author's perspective), halogen insertion, particularly chlorine, may enhance the overall biological performance of the structure. Examples of successful halogen implementation may be both HIV reverse transcriptase inhibitor [72] leading to improved activity, or the insertion of a chlorine atom to the pyrazinamide [73, 74], which resulted in shifting the mechanism of action towards mycobacterial FAS I. Introducing a chlorine atom to the structure improves the lipophilic profile, a crucial factor in antituberculars development, considering the challenging permeability of the thick mycobacterial cell wall. Another rationale for introducing chlorine is the potential formation of a halogen bond with targeted structure. A halogen bond is a type of

noncovalent interaction between the electrophilic region of halogen, acting as a Lewis acid, and any electron-donating moiety (fig. 7a). It must be noted that not only halogens are able to interact in this manner; analogously to 'halogen bond' interactions such as 'chalcogen bond' [75], 'aerogen bond' [76] are also possible.

a)



b)

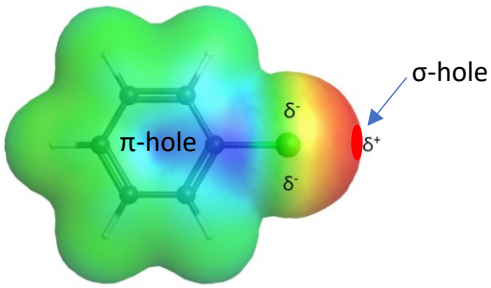


Fig. 7. a) Halogen bond scheme. X – halogen bond donor (Cl, Br, I). Y – halogen bond acceptor (aromatic compounds with conjugated π electrons, multiple bonds carbonyl compounds, free radicals, anions); b) An example of a chemical structure with potential binding sides for Lewis bases. δ – surface electrostatic potential.

A more in-depth explanation for why such interaction is possible lies in the presence of electron anisotropy in the halogen atom participating in covalent bonding (R-X type). This is manifested as a low electron density region present along the extension of the RX bond (fig.7b). This electron-deficient region of the halogen atom is then available to interact with a Lewis base creating a halogen bond. In 2007 Peter Politzer et al. referred to this electron-deficient region as 'σ-hole' (fig. 7b) and rationalized in their publication [77] how two centres of strongly negative agents can interact with each other. As mentioned earlier, σ-hole, an effect of electron anisotropy, is directed along the extension of the R-X bond. However, what if the anisotropy is present perpendicularly to the plane of the structure? This concept is known now as 'π-hole' (fig. 7b). Halogen bond is a directional interaction and while the angle for σ-hole interaction revolves around 180º (160º-180º, depending on the conditions), for π-hole is much lower and deviating around 90º (65.9º-108º, depending on the conditions) [78]. The strength of the bond for halogens increases from fluorine to iodine, in agreement with their decreasing electronegativity, increasing size, and ability to be polarized. While fluorine is the least likely to

form such bonding, it is not impossible — some examples and more thorough explanations are described in the review by Wang et al. [78]. The halogen bond is a long-known interaction; however, the phenomena behind it were proven experimentally only recently (σ-hole in 2020 and π-hole in 2023 by B. Mallada et al. [79, 80]). Although it may not be the most crucial among other possible interactions, its role in drug development shall not be underestimated. Its presence may influence structure stabilization [81], and improve affinity [82] as well as biological efficacy [83, 84]. There are many possible moieties serving as a target to form a halogen bond; Wilcken et al. [83] present examples where moieties in amino acids such as carboxylate group, nitrogen and carbonyl oxygen are described as possible targets.

Biological evaluation of obtained compounds

The biological testing in this commentary was not conducted by the author; however, it remains the most crucial aspect and the primary objective of drug discovery. The publications presented in this commentary include a variety of biological tests, with the primary emphasis on *in vitro* evaluation conducted on different mycobacterial strains – specifically, *Mtb* H37Rv and *Mtb* H37Ra, which are the strains of main interest. Both strains are commonly used to assess the antitubercular potency of novel entities. Notably, Mtb H37Ra, serving as an avirulent surrogate model for *Mtb* H37Rv, is less pathogenic. According to the literature [ref] its minimum inhibitory concentration (MIC) can be compared to those obtained during testing on Mtb H37Rv.

Additional testing was also conducted on two nontuberculous strains: *M. avium*, *M. kansasii*, and two strains of mycolicibacteria: *M. smegmatis* and *M. aurum*. *M. avium* and *M. kansasii*, are slow-growing types of mycobacteria and are causative agents of mycobacterioses[85]. In contrast, *M. smegmatis* and *M. aurum* belong to fast-growing strains that can cause opportunistic infections in immunodeficient patients [ref]. For more advanced testing, where applicable, MDR strains were utilised (Table 1).

Table 1. Resistance panel of used MDR-*Mtb* strains.

Mtb IZAK	Resistant to isoniazid, rifampicin, streptomycin
Mtb MATI	Resistant to isoniazid, rifampicin, pyrazinamide, streptomycin

Biological testing included screening against a panel of bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus spp*., *Klebsiella pneumonia*, and *Acinetobacter baumannii*) and fungi (*Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Lichtheimia corymbifera*, and *Trichophyton interdigitale*).

All compounds underwent testing for cytotoxicity. Basic testing included cytotoxicity on the HepG2 carcinoma cell line, while additional testing was performed on the human epithelial kidney cancer cell line A498, human prostate cancer cell line PC-3 human glioblastoma cell line U-87MG.

Additional *in vivo* testing, where applicable, was conducted on *Galleria Mellonella* wax moth to assess cytotoxicity and on a murine model of tuberculosis to estimate the antitubercular properties of the prepared compounds.

Commentary on Published Results

In this commentary, I present a selection of publications where I was the first or one of the authors and where such conditions are fulfilled: structures were based on pyridine derivatives, allowing the investigation of the effects of isosteric replacement from pyrazine to pyridine core, and exploring their antimycobacterial activity.

In this commentary, I describe the rationale behind the design of discussed structures, their synthesis, evaluation of biological activity, and interpretation of obtained results.

N-pyridinylbenzamides

(Publication P1, Ref No.[86])

The title series serves as a continuation of a previous publication (Zitko et al. [87]) and an exploration into the effects of isosteric change (N to C) in the main core of pyrazine on antimicrobial activity. To design the title structures, we retained previously used linker type(-NHCO-, fig. 8b.), investigated in a prior publication [87]. This approach was based on the discovery that such a linker may provide lower hepatotoxicity, as tested on the HepG2 cells, compared to a typical amidic linker (-CONH-, fig. 8a) [87]. Hepatotoxicity is a significant concern in newer antitubercular regimes, given the known fact that antitubercular treatment is usually hepatotoxic and lengthy.

In total, we synthesized 44 unique compounds representing two structural types: either *N*-pyridin-2-yl (lack of N-4 in respect to pyrazine) or *N*-pyridin-3-yl structural type (lack of N-1 in respect to pyrazine) (fig. 8 c and d). Compounds were synthesized through a simple reaction between an amine (2-amino- or 3-amino-pyridine) and the corresponding benzoyl chloride in the presence of a base (pyridine) to avoid diacylation, based on observations in publication[88]. In terms of the antimycobacterial activity, four compounds exhibited the best activity in the series with a MIC of 7.81 μg/ml against the strain of main interest: *Mtb* H37Ra (R^1 = 5-Cl, R^2 = 3-CF3; R^1 = 6-Cl, R^2 = 3-CF3; R^1 = 6-Cl, R^2 = 4-tBu; R^1 = 6-Cl, R^2 = 4-Et), out of which two compounds (R^1 = 5-Cl, R^2 = 3-CF3; R^1 = 6-Cl, R^2 = 3-CF3;) exhibited activity against other

tested strains (which are mentioned in the introduction). We concluded that a small alkyl chain or lipophilic moiety are the most beneficial substituents at the benzoyl core for antimycobacterial activity, potentially linked to the better permeability of the mycobacterial cell wall. No additional activity against tested bacterial strains or fungi stems was detected. Our research confirmed that the title series compounds, as previously, showed a low risk of hepatotoxicity. Regarding the isosteric changes, based on our findings we demonstrated that N-1 nitrogen (*N*-pyridin-2-yl structural type) was vastly more important for the antimycobacterial activity than N-4 nitrogen.

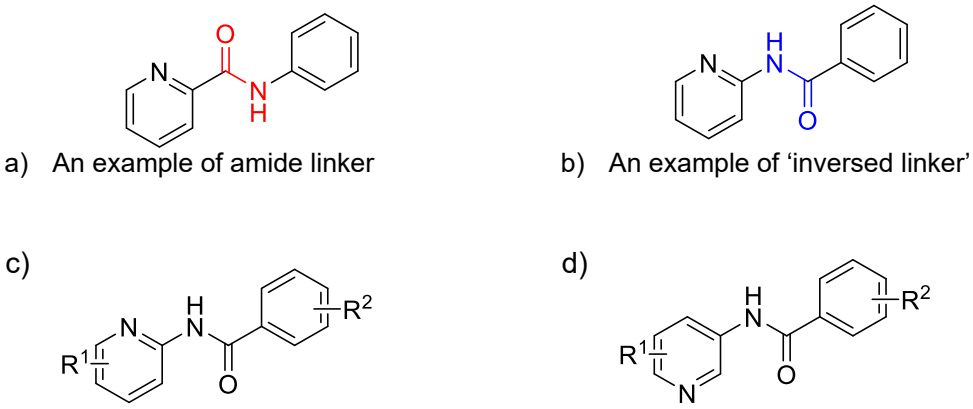


Fig. 8. Structural types a & b: linker design, c & d: title series structures presented in P1.

For further insight, please refer to publication P1: "*N*-pyridinylbenzamides: an isosteric approach towards new antimycobacterial compounds" which can be found in section 7.

2-Aminooxazoles

(Publication P2, Ref No [89])

This series of compounds was not personally designed by me. However, as one of the authors of this publication, I contributed to the synthesis (specifically compounds with phenyl-2-aminooxazole), purification, analysis of the data and the writing of the publication.

The title series explores the influence of isosteric exchange (S to O) in the thiazole moiety on physicochemical properties and possible biological activity against selected mycobacterial strains.

The design of this series was primarily based on publications by Elisa Azzali et al. [90] (2-aminooxazole scaffold, fig. 9a) and Jan Zitko et al. [91] (4-phenylthiazol-2-amine scaffold, fig. 9b). Azzali and her colleagues in their publication [90] pondered whether the 2-aminothiazole scaffold, more often represented in the scientific literature, could be exchanged for the 2-aminooxazole motif. This change aimed to improve the physicochemical properties of sulphur-

containing structures which pose challenges in drug development (sulphur compounds usually exhibit poor metabolic stability, produce toxic intermediates, and belong to PAINS – pan-assay interference compounds). Their research demonstrated that such an exchange may be advantageous and did not negatively impact either metabolism or solubility. Moreover, based on incubation with glutathione the authors confirmed that the scaffold (2-aminooxazole) does not function as PAINS.

Meanwhile, Jan Zitko and colleagues presented a series [91] combining two scaffolds: pyrazine carboxylic acid, a known antitubercular agent, and 4-(hetero)arylthiazol-2-amine. The best compound of this series, namely 6-Chloro-*N*-(4-(4-fluorophenyl)thiazol-2-yl)pyrazine-2-carboxamide, exhibited a broad spectrum of activity and inhibited the growth of Mtb H37Rv at 0.78 μg/ml. However, the author highlighted a problem raised also by Azzali (metabolic stability). Moreover, the solubility of the compounds made some biological assays difficult to perform (cytotoxicity could not be properly assessed due to low solubility).

Based on these findings, the title series of publication P2 was designed. The title series utilized a combination of two scaffolds, one of them being derivatives of pyrazinoic acid combined with 2-aminooxazole or 2-aminothiazole (fig. 9a, subtype I in P2) or combined with 4-phenyloxazole-2-amine or 4-phenylthiazol-2-amine (fig. 9b, subtype II in P2). The scheme below presents the synthesis of title compounds. 4-Phenyl-2-aminothiazole and 4-phenyl-2-aminooxazole scaffolds were prepared by reacting 2-bromoacetophenone with thiourea in ethanol to obtain 4-phenyl-2-aminothiazole (fig. 10a); or with urea in acetonitrile to obtain 4-phenyl-2-aminooxazole (fig. 10b). All final compounds, except subtype I 2-aminothiazole scaffold-based structures, were prepared by acylation with acyl chloride (activation with thionyl chloride). Subtype I 2-aminothiazole compounds were prepared via coupling with 1,1'-carbonyldiimidazol (CDI). Later, the compounds underwent biological screening against mycobacterium strains, fungi and chosen bacteria. Additionally, compounds were tested for their cytotoxicity on the HepG2 cancer cell line.

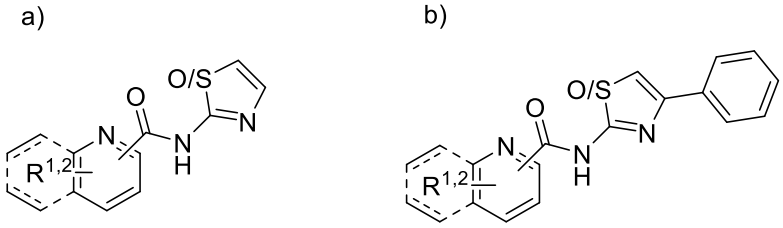


Fig. 9. Title series chemical structures. a) subtype I, 2-Aminooxazole/2-Aminothiazole structural type; c) subtype II, 4-Phenyl-2-aminooxazole/ 4-Phenylaminothiazole structural type.

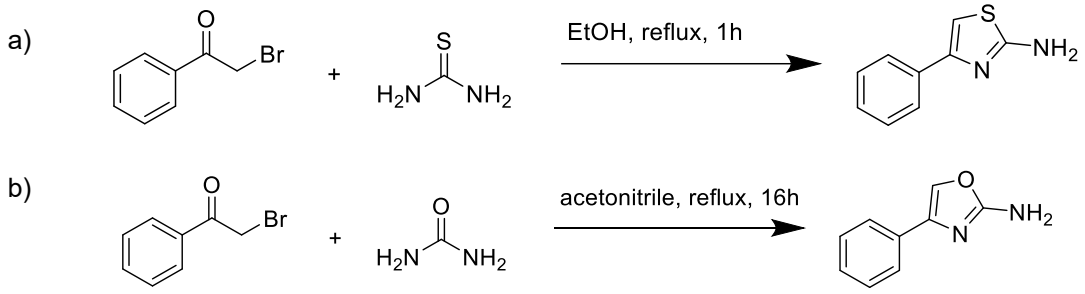


Fig. 10. Synthesis scheme of subseries II scaffold. a) 4-Phenyl-2-aminothiazole; b) 4-Phenyl-2-aminooxazole.

Based on these results, we identified compounds with promising antimycobacterial activity, with the lowest $MIC_{MtbH37Ra}$ in the title series being <3.91 µg/ml (compound **7b**). In terms of this commentary subtype I had a lower count of the most active compounds (MICs ≤7.81 µg/ml) than subtype II (3 compounds vs 6 compounds). It is worth noting that, when compared to pyrazine compounds, the pyridine-based compounds exhibited slightly better biological activity, although the sample to compare was too small to draw broader conclusions. Once again, for sulphur-based compounds, the estimation of cytotoxicity was not always possible due to solubility issues in the testing medium, while no such issues occurred in oxazole-based compounds. Regarding the differences between oxazole and thiazole compounds, in subtype I, the antimycobacterial activity difference was noticeable, with an advantage observed when the compound was oxazole-based. In subtype II, a little difference was noticeable, but what can be said is that compounds with oxazole usually retained the activity of their thiazole-based counterparts.

For further insight, please refer to publication P2: Improving Antimicrobial Activity and Physico-Chemical Properties by Isosteric Replacement of 2-Aminothiazole with 2-Aminooxazole.

Pyridine carboxamides

(Publication P3, Ref No [92])

The title series is based on compounds that are used as first-line and second-line agents in current tuberculosis treatment. We attempted to utilize pyrazine, isoniazid, 4-aminosalicylic acid and 4-aminobenzoic acid in hybrid compounds to investigate if such modifications can be beneficial for antimycobacterial activity. We divided the synthesised compounds into two groups based on their structure (fig. 11 a and b). All compounds were synthesised by coupling different pyridine carboxylic acid derivatives with corresponding amino components (2-aminopyrazine or 6-chloropyrazine or 4-aminosalicylic acid or 4-aminobenzoic acid). The coupling was performed as previously, either with CDI or oxalyl chloride (Fig.12).

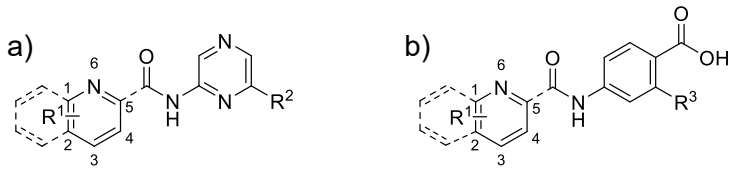


Fig. 11 General structures of final compounds. a) series I b) series II. R^2 = H or Cl, R^3 = H or OH.

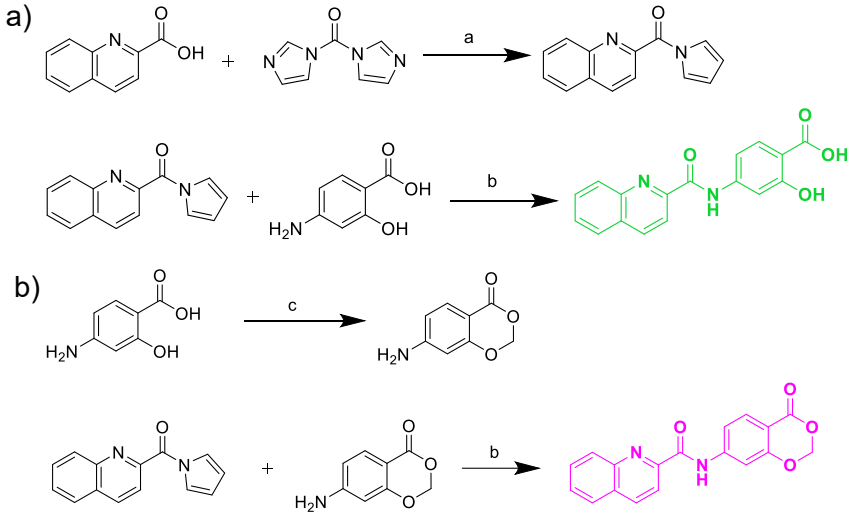


Fig. 12 Schematic synthesis of compound: a) 10c and b) its **prodrug**. Reagents and conditions: (a): DCM, heating, 30 min; (b): DCM, RT, overnight; (c): K_3PO_4 *3 H_2O, DCM, DMF, 10h, reflux.

Out of all the prepared compounds (a total of 40), we concluded that those based on 4-aminosalicylic acid (series c in P3) were the most promising for further investigation. Compound **10c** underwent thorough *in vitro* and *in vivo* investigation, emerging as a potent drug candidate with a significant advantage over currently used 4-aminosalicylic acid. A major drawback of 4-aminosalicylic acid is its short half-life (11 min), and the investigated compound showed substantial improvement in this regard (630 min). Additionally, we successfully prepared a lactone prodrug of compound **10c** (fig. 12 b). While the prodrug retained the MIC of the parent drug, its solubility limited broader investigation. Exploring the impact of the loss of the hydroxyl group (change within R^3, Scheme 11b) on antimycobacterial activity revealed low to no activity when the hydroxyl group was not present. Despite all compounds being tested on bacterial strains and fungi stems, they exhibited selective activity towards mycobacterial strains. Cytotoxicity screening yielded a promising conclusion: compound **10c** was non-toxic when tested *in vitro* on the HepG2 cells as well as when tested *in vivo* on *Galleria mellonella* larvae. Subsequently, the compound was formulated as a sodium salt (10cNa) and subjected to *in vivo* antimycobacterial screening using the tuberculosis murine model. The results demonstrated a statistically significant decrease of CFUs in the spleens of mice compared to the untreated control group. However, there was insufficient evidence for an improvement in the lungs of treated mice (fig. 13). To investigate if our compound works on *Mtb* by the same

mechanism as 4-aminosalicylic acid, **10c** was tested *in vitro* on *Mtb* H37Ra in the presence of varying concentrations of methionine in the culture media. In conclusion, the compound does not target the dihydrofolate pathway, but it affects the synthesis of the mycolic acids and lipids (as 4-aminosalicylic acid does) and interferes with the metabolism of methionine. However, the specific target remains unknown. Additional *in silico* simulations suggested that the possible target may be methionyl-tRNA synthase.

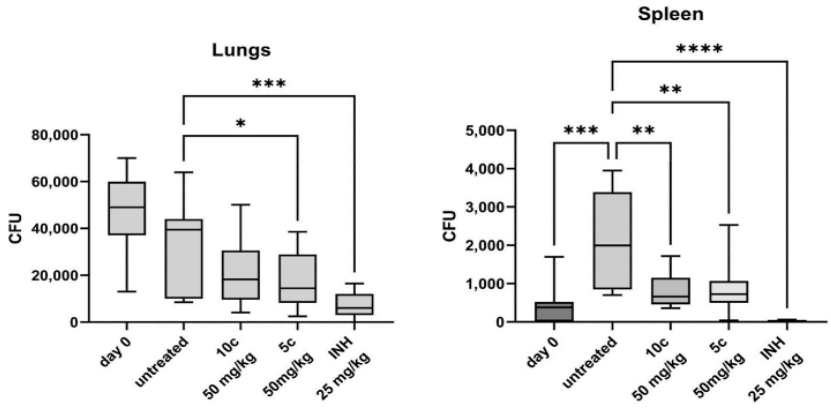


Fig. 13. Efficacy of compound 10cNa *in vivo* (murine model of TB). * - P ≤ 0.05; ** - P ≤ 0.01; *** - P ≤ 0.001; **** - P ≤ 0.0001.

For further insight, please refer to publication P3: Antimycobacterial pyridine carboxamides: From design to *in vivo* activity.

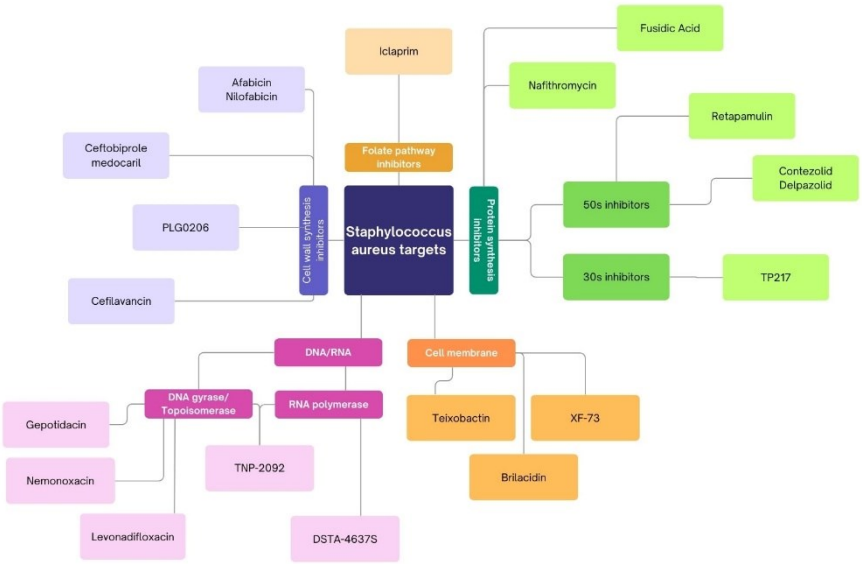
Ongoing research & future perspectives

Here, I briefly discuss some unpublished or unfinished work. The methods used to determine the biological activity of presented compounds are the same as in already published papers (P1, P2, P3).

Review on antistaphylococcal agents in clinical trials

This review was published in the *European Journal of Medicinal Chemistry* [93]. In this review, we attempted to delve deeper into clinical trials focused on interventional studies against a variety of *Staphylococcus aureus*-caused bacteraemia. Sadly, none of the agents currently in the clinical trials contain either pyrazine or pyridine moiety. Based on our analysis, what can be concluded, and is even more concerning, is that the development of new antibacterial agents is slow, not allowing for, statistically, one antibacterial drug to be approved by the FDA annually. In this work, the authors described 19 novel entities, (categorised by their mechanism of action) that were in clinical trials till June 2023 (fig. 14a), summarized the current status of eight monoclonal antibodies targeting *SA* (fig. 14b) and briefly mentioned agents that were approved to treat *Staphylococcus aureus* bacteraemia in last 10 years.

a)



b)

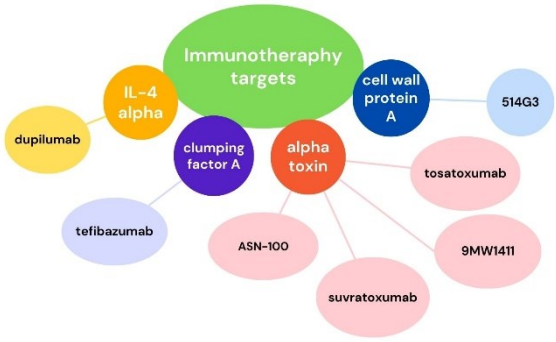


Fig. 14. Agents in clinical trials described in the publication. a) chemical agents b) biological agents.

Pyrazine-2-Carbohydrazides & *N*-benzylidene derivatives

In this series, we are exploring the possible antimicrobial activity of prepared intermediates and final compounds (fig. 15). Out of all prepared intermediates, those with the best MIC against tested strains were selected for further modifications. One of the modifications is to yield a silver complex that could have improved antimicrobial activity (such modification will be performed by Biljana Glisić group from the Faculty of Science, University of Kragujevac). Further, selected intermediates were modified to yield final compounds. Final compounds undergo screening for their possible mechanism of action and chelating properties. The schematic synthesis of intermediates and final compounds is presented below (fig. 15). Preliminary results were presented in an oral presentation during the 11th Postgraduate and Postdoc Conference FAF UK HK, 2021.

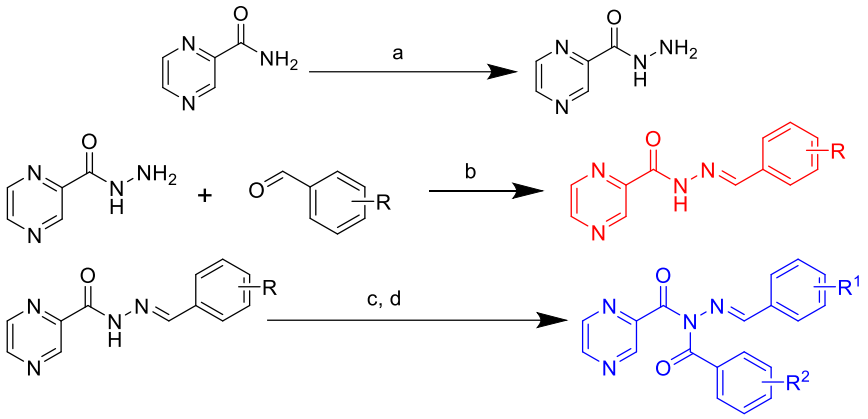


Fig. 15. Preparation of intermediates (in red) and final compounds (in blue). Reagents and conditions: (a): 60% NH_2NH_2 in H_2O, THF, 24h, reflux; (b): MeOH, 1h, reflux; (c): DCM, NaH, 24h, reflux; (d): benzoyl chloride derivative, 24h, reflux.

Derivatives of 2-phenyl-*N*-(pyridin-2-yl)acetamides and 2-phenyl-*N*-(pyrazin-2-yl)acetamides

Due to an extensive amount of the experimental material was divided into two series.

Part A

A series of unpublished work brings us back to publication P1 and previous publications of 'Design and Development of New Antimicrobial Agents' research group members [86, 87, 94]. The series presented here is a continuation of the structural design in P1 (fig. 16). In this series, we aim to investigate the influence of an elongated linker (an additional -CH_2- group) on the antimycobacterial and antiproliferative activities of such compounds, as well as the connection between the position of the substituent on phenyl and pyridine cores and their biological activity.

Title compounds were synthesised and are analysed for their antibacterial or antiproliferative activity, using the same methods as in publications presented in this commentary. Compounds were prepared by coupling a pyridine carboxylic acid derivative with the corresponding phenylacetic acid derivative, either by CDI or by activating the phenylacetic acid derivative with oxalyl chloride or by direct reaction with phenylacetic acid chloride if possible.

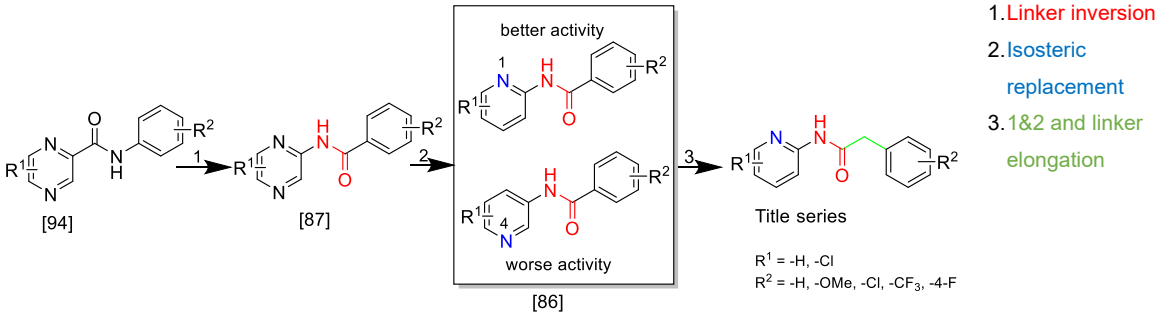


Fig. 16. Design rationale for the series of 2-phenyl-*N*-(pyridin-2-yl)acetamides [86, 87, 94].

Primary results showed that the hypothesis that elongated linker may be beneficial to the antimycobacterial activity was not resolved. We identified the position and type of the substituent R^1 and R^2 being the most beneficial to the antimycobacterial activity.

An interesting example involved two compounds that differed only by the position of chlorine on the pyridine core. Such a change altered the biological activity against the complementary strains. The antimycobacterial activity against the strain of main interest, *Mtb* H37Ra, was retained, the same was proved on *Mtb* H37Rv. However, one of those compounds did not show any efficacy against additional mycobacterial strains and did not affect the growth of bacteria or fungi that it was tested on. This compound had also twice lower IC_{50} value on the HepG2 cell line.

To test the antiproliferative activity, we selected three compounds based on their lack of antibacterial activity and low IC_{50} on the HepG2 cancer cell line. These compounds were tested on three cancer cell lines, namely the human epithelial kidney cancer cell line A498, human prostate cancer cell line PC-3 and human glioblastoma cell line U-87MG.

Preliminary results were presented as a poster at the Joint Meeting on Medicinal Chemistry Prague, Czech Republic 2019 and EFMC-ACSMEDI MedChem Frontiers 2019, the Joint Symposium on Medicinal Chemistry Frontiers, Kraków, Poland 2019.

Part B

In the second part, the main core of pyridine was exchanged once again for pyrazine, and the elongated linker was retained (fig. 17). Preliminary results showed that all tested compounds lacked activity against *Mtb* H37Ra. The activity was only observed against the strain of less interest, namely, *M. kansasii* and it was modest. Similarly, no biological activity was observed against the panel of bacteria and fungi stems frequently used in our work. Testing on the HepG2 cell line is yet to be performed.

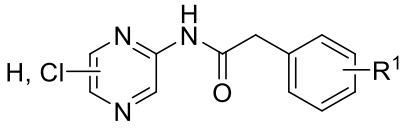


Fig. 17. Title series structure. R^1 = -H, -Cl, OMe, -CF_3, -4-F, -Me

Others

A few compounds, or rather motifs, based on *in silico* design (by Martin Juhás) were prepared as a part of the START project. I contributed to the synthesis of those compounds and their purification (fig. 18). Preliminary results were presented as a poster at EFMC-ISMC in Nice, France 2022.

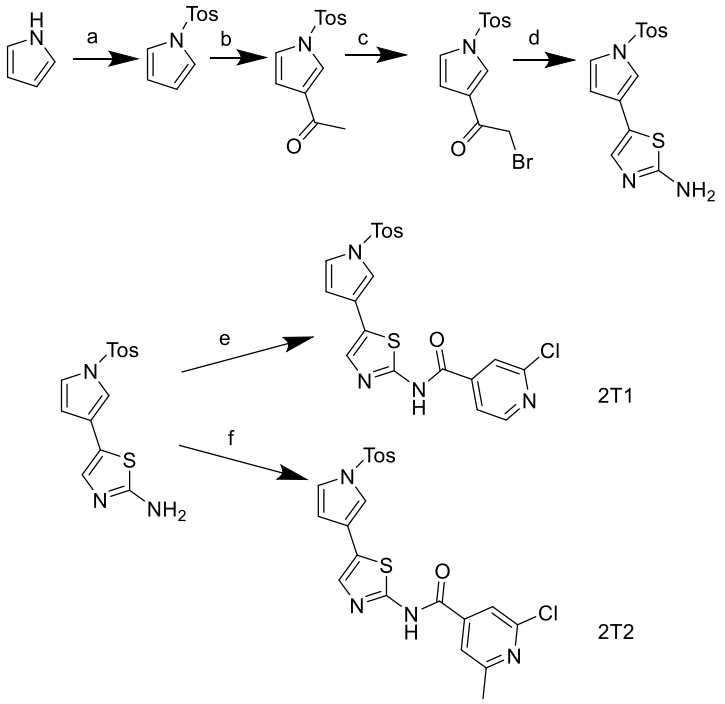


Fig. 18. Synthesis scheme for compounds 2T1 and 2T2. Reagents and conditions: (a): NaOH, DCM, 4-methylbenzenesulfonyl chloride, RT, overnight; (b): $AlCl_3$, DCM, acetic anhydride, 2h; (c): $CuBr_2$, 40ºC, 1h; (d): thiourea, EtOH, 2h, reflux; (e): 2-chloroisonicotinic chloride, DCM, overnight; (f): 2-chloro-6-methylisonicotinoyl chloride, DCM, overnight. Tos – toluene sulfonyl group.

Conclusions

To conclude my work as a PhD student, the primary aim was to develop potential drug candidates targeting tuberculosis, along with other clinically important bacteria or fungi. Where possible, final compounds were assessed for hepatotoxicity *in vitro*, cytotoxicity *in vivo* and biological activity *in vivo* in murine model of tuberculosis. Additionally, when justified, possible anticancer activity was undertaken.

Contributing to the research on antitubercular agents I synthesized a number of compounds predominantly based on pyridine heterocycle. All publications in this commentary involved an isosteric exchange from pyrazine to pyridine. The presented work demonstrates that these subtle isosteric changes had interesting outcomes on biological activity and physicochemical properties (fig. 19). In the publication P1 improved activity was observed in 8 out of 10 comparable pairs of compounds (in two other pairs the activity was retained), with the most significant change in MIC from >500 μg/ml to 7.81 μg/ml (compound **2n** vs **24,** fig. 19a), achieved by removing of one nitrogen in the structure. This trend was sustained in publication P2, where the change from pyrazine to pyridine proved to be beneficial for biological activity. The main hypothesis regarding the benefit of isosteric exchange from thiazole to oxazole for the investigated structures' physicochemical was also confirmed (fig. 19 b). Isosteric changes (fig 19 c) in P3 included: lack of hydroxyl group in the salicylic acid moiety, exchange of the pyrazine core to pyridine, and insertion of a chlorine atom into pyridine core. These modifications led to the conclusion that the lack of hydroxyl worsened the compound's biological efficacy, supporting literature findings. The pyrazine-to-pyridine exchange improved biological activity and slightly influenced the mechanism of action. All presented publications and unpublished results (2-phenyl-*N*-(pyridin-2-yl)acetamides) suggest that isosteric exchange may be a viable strategy for chemical structure modification, potentially influencing biological activity against targeted pathogens. Regarding the importance of halogenation of the structure's biological efficacy, the effect was not consistently estimated as satisfactory. In publication P1 and unpublished 2-phenyl-*N*-(pyridin-2-yl)acetamides the effect was strong, suggesting that substitution with chlorine on the pyridine ring positively influenced biological activity, with position 6 being favoured. However, in publication P2 and P3, there are little to no evidence that such structural modification (a chlorine atom insertion) was beneficial to the biological activity.

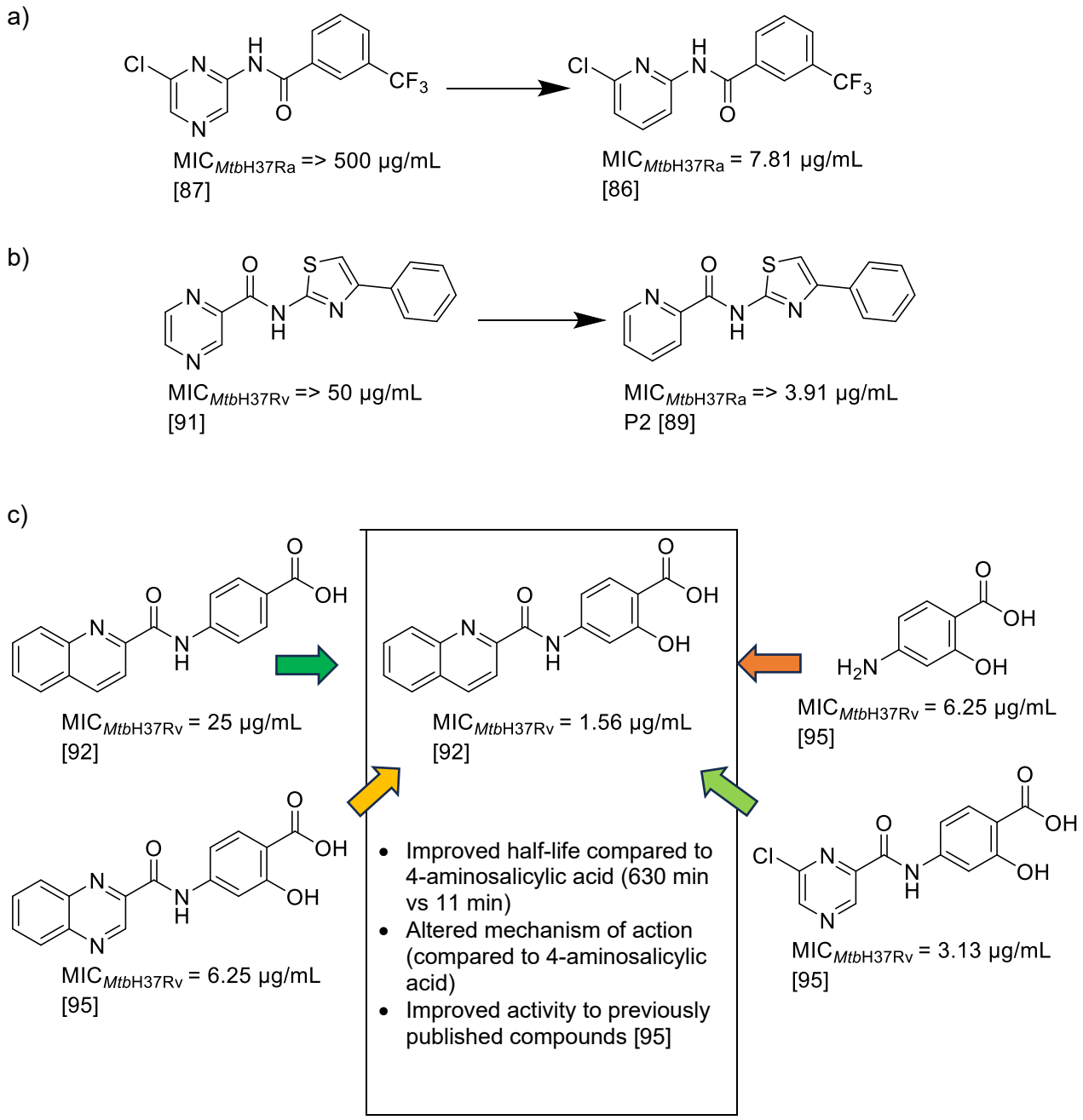


Fig. 19. Final conclusion on structural modifications leading to the best MICs from publications presented in the commentary.
a) Publication P1; b) Publication P2; c) Publication P3. [86, 87, 89, 91, 92, 95]

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Publications

Experimental Articles

P1 Nawrot, D., Suchánková, E., Janďourek, O., Konečná, K.; Bárta, P.; Doležal, M. *N-pyridinylbenzamides: an isosteric approach towards new antimycobacterial compounds.* Chemical Biology & Drug Design, 2021. **97**(3): p. 686-700.

P2 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, *Improving Antimicrobial Activity and Physico-Chemical Properties by Isosteric Replacement of 2-Aminothiazole with 2-Aminooxazole.* Pharmaceuticals, 2022. **15**(5): p. 580.

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Supplementary materials

Other publications

Nawrot D.; Kolenic M.; Kunes J.; Kostelansky F.; Miletin M.; Novakova V.; Zimcik P. *Transalkylation of alkyl aryl sulfides with alkylating agents*. Tetrahedron, 2018. **74**(5): p. 594-599.

Nawrot, D.; Ambrożkiewicz-Mosler, W.; Doležal, M.; Bouz, G. *Antistaphylococcal discovery pipeline; where are we now?*. European Journal of Medicinal Chemistry, 2023. **266**: p. 106077.

List of attended conferences and posters

Oral presentation: Dye-functionalized peptide synthesis and their interactions with beta-amyloid peptide, 13th Postgraduate and Postdoc Conference FAF UK HK, 1-2 February 2023. Section Bioorganic and Pharmaceutical Chemistry.

Poster presentation: De novo design and biological evaluation of 2-aminooxazoles as inhibitors of bacterial ß-ketoacyl-acyl carrier protein synthase III, EFMC-ISMC 4-8 September 2022, Nice, France

Oral presentation: 4-aminosalicylic acid hybrid compounds containing as potential antituberculotics 12th Postgraduate and Postdoc Conference FAF UK HK, 1-2 February 2022. Section Bioorganic and Pharmaceutical Chemistry.

Oral presentation: Hybrid compounds containing 4-aminosalicylic acid as potential antituberculotics, 49th Conference Synthesis and Analysis of Drugs 2021, Hradec Králové, Czech Republic

Oral presentation: Pyrazine-2-carbohydrazide derivatives as potential antituberculars, 11th Postgraduate and Postdoc Conference FAF UK HK, 27-28 January 2021. Section Bioorganic and Pharmaceutical Chemistry.

Poster presentation: Design, synthesis, and biological evaluation of positional derivatives of a series of *N*-pyridinylcarboxamides as potential antituberculotics, EFMC-YMCS 2020. Virtual Event.

Oral presentation: Design, synthesis and biological evaluation of positional derivatives of a series of *N*-(pyridin-2-yl)carboxamides, 10th Postgraduate and Postdoc Conference FAF UK HK, 22-23 January 2020. Section Bioorganic and Pharmaceutical Chemistry.

Poster presentation: Design, synthesis and biological evaluation of positional derivatives of a series of *N*-(pyridin-2-yl)carboxamides 2019, EFMC Young Medicinal Chemist Symposium 5-6.09.2019, Athens, Greece.

Poster presentation: Design, Synthesis and Biological Evaluation of *N*-(pyridyl)-benzamides and *N*-(pyridyl)-2-phenylacetamides as Potential Antimycobacterial Agents, Joint Meeting on Medicinal Chemistry (JMMC), 27-30.06.2019, Prague, Czech Republic.

Poster presentation: Design, Synthesis and Biological Evaluation of N-(pyridyl)-benzamides and *N*-(pyridyl)-2-phenylacetamides as Potential Antimycobacterial Agents, EFMC-ACSMEDI MedChem Frontiers 2019, Joint Symposium on Medicinal Chemistry Frontiers,10-3.06.2019, Kraków, Poland.