CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Doctoral Thesis

2011 Martin Krátký

Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Inorganic and Organic Chemistry

Design and Synthesis of New Compounds Active Especially against Multidrug-Resistant Mycobacterial Strains

Ph.D. thesis

Author: Martin Krátký
Study Program: Organic Chemistry
Specialization: Bioorganic Chemistry
Supervisor: Prof. Dr. Jarmila Vinšová, Ph.D.

Hradec Králové

December 2011

Universita Karlova v Praze
Farmaceutická fakulta v Hradci Králové
Katedra anorganické a organické chemie

Design a syntéza nových sloučenin působících zejména vůči multilékově resistentním kmenům mykobakterií

Disertační práce

Autor: Martin Krátký
Studijní program: Organická chemie
Studijní obor: Bioorganická chemie
Školitel: Prof. RNDr. Jarmila Vinšová, CSc.

Hradec Králové

Prosinec 2011

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně pod vedením prof. RNDr. Jarmily Vinšové, CSc. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

I declare that this work is my original authorial work which I developed independently under the supervision of Professor Dr. Jarmila Vinšová, Ph.D. All literature and other sources which I used during processing of this thesis are listed in the list of references and cited properly in the thesis.

Hradec Králové, ...

Abstract

Charles University in Prague, Faculty of Pharmacy in Hradec Králové
Department of Inorganic and Organic Chemistry
Candidate Martin Krátký
Supervisor Prof. Dr. Jarmila Vinšová, Ph.D.
Title of Doctoral Thesis Design and Synthesis of New Compounds Active Especially against Multidrug-Resistant Mycobacterial Strains

This work is focused mainly on the field of searching of new potential antimicrobial agents, particularly against multidrug-resistant *Mycobacterium tuberculosis* strains, based on the modification of the salicylanilide (2-hydroxy-(*N*-phenyl)benzamide) group. The second research topic is a study of the rearrangement in the series of salicylanilide amino acids esters to form 2-hydroxy-*N*-[2-oxo-2-(phenylamino)-alkan-2-yl]benzamides called "diamides".

At the beginning, the thesis summarizes some basic facts about tuberculosis, a very important and serious bacterial infectious disease caused by *Mycobacterium tuberculosis* complex, about its treatment and related troubles and limitations. Proper attention is given for the problematic of drug-resistant tuberculosis, especially multidrug-resistant and extensively drug-resistant forms, their epidemiology and therapy.

The development of new drugs against multidrug-resistant tuberculosis is largely discussed, including requirements for them, recent advances and present status of some perspective molecules in clinical and preclinical stages of the investigation and the specific targeting on mycobacteria, a promising contemporary approach.

Salicylanilides have revealed a wide spectrum of pharmacological activities including antiviral, antifungal and antibacterial ones and their mechanism of the action seems to be complex and multiple; new targets have been found. The thesis describes some facts about biological properties of salicylanilides and their ester prodrugs, as well as about prodrug formation, which may bring some advantages, e.g. a higher activity, better passing through mycobacterial cell wall or a decreased toxicity.

In the experimental part, the rearrangement of the salicylanilide amino acids esters after liberation of amino group to form 2-hydroxy-*N*-[2-oxo-2-(phenylamino)-alkan-2-yl]benzamides ("diamides") was investigated to propose a new mechanism of the rearrangement *via* five-membered imidazoline ring.

The thesis reports the facts about salicylanilide modification based on the esterification *via* *N,N´*-dicyclohexylcarbodiimide as an activating agent or by direct acylation of salicylanilide salts. The synthesis, physico-chemical properties, spectral characteristics and mainly antimycobacterial, antifungal and antibacterial activities are presented and discussed. Five series of salicylanilide esters were designed, synthesized and evaluated – esters with *N*-acetyl-L-phenylalanine, benzoic acid, 4-(trifluoromethyl)benzoic acid, pyrazine-2-carboxylic acid and benzenesulfonic acid – and the series of sulfonamide derivatives containing 5-chlorosalicylamide and 5-chlorosalicylaldehyde scaffolds. Many of newly described derivatives exhibited excellent *in vitro* activity towards mycobacteria (minimum inhibitory concentrations ≥ 0.25 μmol/L, for drug-resistant strains even ≥ 0.125 μmol/L), Gram-positive bacteria and moulds (≥ 0.49 μmol/L). The structure-activity relationships are also discussed.

Moreover, some synthesized compounds were investigated as potential inhibitors of two essential mycobacterial enzymes, isocitrate lyase and methionine aminopeptidase. The results which revealed moderate inhibition of both these enzymes are reported.

Keywords

Antimicrobial agents; biological activity; multidrug-resistant tuberculosis; rearrangement; salicylanilides; salicylanilide esters; targeting.

Abstrakt

Universita Karlova v Praze, Farmaceutická fakulta v Hradci Králové

Katedra anorganické a organické chemie

Kandidát	Martin Krátký
Školitel	prof. RNDr. Jarmila Vinšová, CSc.
Název disertační práce	Design a syntéza nových sloučenin působících zejména vůči multilékově resistentním kmenům mykobakterií

Tato práce je zaměřena na hledání nových potenciálních antimikrobních látek, působících zejména vůči multilékově resistentním kmenům *Mycobacterium tuberculosis*, a to na bázi modifikací salicylanilidů (2-hydroxy-*N*-(fenyl)benzamidů). Druhým tématem je studie přesmyku popsaného v sériích salicylanilidových esterů s aminokyselinami, který poskytuje 2-hydroxy-*N*-(2-oxo-2-(fenylamino)-alkan-2-yl)benzamidy, zkráceně nazývané "diamidy".

Disertační práce na začátku shrnuje některé základní poznatky o tuberkulóze, jež představuje závažné infekční onemocnění způsobené bakteriemi komplexu *Mycobacterium tuberculosis*. Zabývá se její léčbou a s ní souvisejícími problémy. Pozornost je věnována především problematice lékově resistentní tuberkulózy, zvláště multilékově a extrémně resistentním formám, jejich epidemiologii a léčbě.

Je diskutován vývoj potenciálních léčiv vůči multilékově resistentní tuberkulóze, zejména požadavky na nová léčiva, poslední pokroky a současný stav některých perspektivních molekul podstupujících preklinické a klinické hodnocení, stejně jako moderní a nadějná problematika enzymového cílení mykobakterií.

Salicylanilidy prokázaly řadu zajímavých farmakologických účinků včetně antivirové, antimykotické a antibakteriální aktivity; pravděpodobně působí komplexně více mechanismy a bylo popsáno několik cílových míst jejich zásahu. Tato práce shrnuje základní poznatky o biologické aktivitě salicylanilidů a jejich esterů jakožto potenciálních proléčiv, věnuje se i významu přípravy proléčiv, která mohou být výhodná např. vyšší účinností, lepším průchodem skrze buněčnou stěnu mykobakterií či sníženou toxicitou.

V experimentální části práce shrnuje zkoumání mechanismu přesmyku aminokyselinových esterů salicylanilidů po uvolnění amino skupiny za vzniku 2-hydroxy-*N*-(2-oxo-2-(fenylamino)-alkan-2-yl)benzamidů ("diamidů"). Byl navržen nový mechanismus přesmyku probíhající přes pětičlenný imidazolinový cyklus jako meziprodukt.

Práce se zabývá esterifikací salicylanilidů pomocí aktivačního činidla *N,N´*-dicyklohexylkarbodiimidu či přímou acylací salicylanilidových solí, věnuje se syntéze esterů, jejich fyzikálně-chemickým a spektrálním vlastnostem a zejména jejich antimykobakteriální, antifungální a antibakteriální aktivitě. Bylo připraveno a zhodnoceno pět sérií salicylanilidových esterů – s *N*-acetyl-L-fenylalaninem, benzoáty, 4-(trifluormethyl)benzoáty, pyrazinoáty a benzensulfonáty – a série sulfonamidových derivátů-amidů s kyselinou 5-chlorsalicylovou a Schiffových bazí odvozených od 5-chlorsalicylaldehydu. Řada nově připravených derivátů vykázala vynikající *in vitro* účinnost vůči mykobakteriím (minimální inhibiční koncentrace ≥ 0.25 μmol/L), vůči lékově resistentním dokonce od 0.125 μmol/L, grampozitivním bakteriím a vláknitým houbám (≥ 0.49 μmol/L). Je diskutována problematika struktury a biologické aktivity.
Některé sloučeniny byly testovány jako potenciální inhibitory dvou esenciálních mykobakteriálních enzymů, isocitrát lyasy a methionin aminopeptidasy. Výsledky ukazují středně silnou inhibici těchto enzymů.

Klíčová slova

Antimikrobiální látky; biologická aktivita; multilékově resistentní tuberkulóza; přesmyk; salicylanilidy; salicylanilidové estery; cílení.

Acknowledgements

Firstly, I would like to thank heartily to my supervisor and mentor, Prof. Jarmila Vinšová, for her valuable advice, constant guidance and strong support during all my study. I am grateful to her for the help with the development of this thesis and she represents an unbeatable image of the scientific worker.

This thesis would not have been possible unless the constant support of my parents and especially my girlfriend Jaroslava Urbanová and my daughter Kateřina; I thank them for patience and tolerance, to Slávka additionally for checking of English grammar in this thesis.

My thanks are also headed to all people who measured physical characteristics of synthesized compounds at the Faculty of Pharmacy, i.e. Assoc. Prof. J. Kuneš, Mrs. D. Cardová, Mrs. I. Vencovská, Mrs. H. Jílková-Mikešová, as well as to people determining the biological activities of synthesized compounds – Mrs. I. Dufková, Dr. J. Stolaříková, Dr. V. Ulmann and their co-workers, Dr. E. Novotná, Prof. J. O. Liu.

I would like to thank to all my friends and colleagues in our laboratory and research group, especially to Dr. Eva Vavříková and Ing. Ján Kozic, for fruitful collaboration and cheerful moments and events.

The work on this thesis was financially supported by GAUK 27610/2010, the Research Project MSM 0021620822, IGA NS 10367-3 and SVV 2011-263-001.

Last, but not at least, it is honour for me to thank to The Supreme Truth and Affection as my life-guide.

Content

List of the Abbreviations

24/48 h	24/48 hours of incubation
7/14/21 d	7/14/21 days of incubation
ADME	An acronym for absorption, distribution, metabolism and excretion
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
ATR	Attenuated total reflectance
BA	Benzoic acid
BCG	Bacillus Calmette-Guérin
BOC	*N*-*tert*-Butyloxycarbonyl
N-Cbz	*N*-Benzyloxycarbonyl
CF_3-BA	4-(Trifluoromethyl)benzoic acid
DCC	*N*,*N*′-Dicyclohexylcarbodiimide
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOTS	Directly observed treatment short-course
EMB	Ethambutol
ESBL	Extended-spectrum β-lactamase
ETH	Ethionamide
FAS	Fatty acid synthase
FLU	Fluconazole
HIV	Human immunodeficiency virus
IC	Inhibitory concentration
ICL	Isocitrate lyase
IFN-γ	Interferon-gamma
IL	Interleukin
INH	Isoniazid
IR	Infrared
log*P*	Logarithm of the octan-1-ol/water partition coefficient
M.	*Mycobacterium*
MDR-TB	Multidrug-resistant tuberculosis
MetAP	Methionine aminopeptidase

MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
OD	Optical density
PAS	*para*-Aminosalicylic acid (4-amino-2-hydroxybenzoic acid)
PCR	Polymerase chain reaction
PNC	Benzylpenicillin (G-penicillin)
POA	Pyrazinoic acid (pyrazine-2-carboxylic acid)
PZA	Pyrazinamide
PZase	Nicotinamidase/pyrazinamidase
RIF	Rifampicin
RNA	Ribonucleic acid
S.	*Staphylococcus*
SDS	Sodium dodecyl sulphate
STM	Streptomycin
TB	Tuberculosis
TDR-TB	Totally (or super extensively) drug-resistant tuberculosis
TNF-α	Tumour necrosis factor α
UV	Ultraviolet
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis
Z-	*N*-Benzyloxycarbonyl
δ	Chemical shift

1. Introduction

The coexistence of human beings and infectious diseases accompanies humankind from the beginnings. Novel interventions to reduce consequences of the infections were searched from that time, but the first significant success in the field of specific antimicrobial agents were obtained in the first half of the 20th century. Discovering and recognizing of the potent antibacterial action of sulfonamides, penicillin and streptomycin followed by other antimicrobial drugs having a broad range of the activity, was assumed very optimistic without justification that bacterial infections were controlled sufficiently and they would be eradicated. Unhappily, the inappropriate use of antibiotics and chemotherapeutics was very frequent. During next years, many bacterial species have developed a resistance to widely applied drugs, e.g. *Staphylococcus aureus*, enterococci or Gram-negative bacilli. At this moment, some authors predict a possible end of the "antibiotic epoch". There is an obvious effort for the properly management of the antimicrobial drugs in the industrial countries. The development of novel potential antimicrobial agents may help to reduce or even turn away the dark perspectives; it is one of the most emergent priorities of the new drugs development.

The situation for tuberculosis and the infections caused by atypical mycobacteria is quite similar. After introduction of effective antimycobacterial agent (streptomycin, *para*-aminosalicylic acid, isoniazid and cycloserine belonged to the first approved drugs), tuberculosis was not considered being a severe threat despite its relevance; it was strongly believed that tuberculosis would be totally eradicated in a short time. Regardless of this meaning, the resistance to antituberculous drugs developed and now it is spreading including the most serious forms of multidrug- and extensively drug-resistance. Furthermore, no sufficient or ideal drug has been invented; probably this aim is inaccessible. Therefore new agents to combat tuberculosis are required.

The synthesis and evaluation of new antimicrobial agents, especially antimycobacterial and antifungal ones, belong to the traditional research topics at the Department of Inorganic and Organic Chemistry, Faculty of Pharmacy in Hradec Králové, Charles University in Prague. This area has been studied here intensively for many years. There have been described many promising compounds with good or even excellent *in vitro* minimum inhibitory concentration towards *Mycobacterium tuberculosis*, atypical mycobacteria and fungi. Salicylanilides (2-hydroxy-*N*-(phenyl)benzamides), their esters and related compounds could be considered being such a potent group. Since prodrug design has

proved in many cases and for many purposes like a better bioavailability, a lower toxicity, an improved activity etc., the prodrug design may be useful also for salicylanilides. The study of structure-activity relationship may bring interesting and important findings prospectively useful in the further research.

2. The Aim of the Thesis

This thesis entitled “Design and Synthesis of New Compounds Active Especially against Multidrug-Resistant Mycobacterial Strains” follows the development efforts of the research group headed by Prof. RNDr. Jarmila Vinšová, CSc., and especially continues in the area outlined and initiated in the theses of Ing. Aleš Imramovský, Ph.D.[1], and Mgr. Juana Monreal Férriz, Ph.D.[2] It is focused on searching of new potential antimicrobial agents, especially against *Mycobacterium tuberculosis*, atypical mycobacteria, fungi and Gram-positive bacteria. This current topic is one component of the scientific interests at the Department of Inorganic and Organic Chemistry, Faculty of Pharmacy, Charles University in Prague.

The goals of the thesis have been postulated being:

- brief summarization of the problem of the tuberculosis (TB) treatment and related topics with the orientation to multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB),
- overview of recent trends in the development of novel potential agents towards MDR- and XDR-TB,
- review of some important aspects of salicylanilides and salicylanilide biological activity related to the topic of the thesis,
- study of salicylanilide esters reactivity leading to the unexpected formation of “diamides”,
- synthesis and evaluation of new salicylanilide derivatives, salicylanilide esters and related compounds as potential antimicrobial agents with respect to structure-activity relationships and comparison of the individual groups of derivatives.

3. Theoretical Part

3.1. Some Facts about Tuberculosis

3.1.1 Brief Characteristics of Tuberculosis

Tuberculosis (TB) has been responsible for millions of human deaths and currently this disease still represents a global threat. Pulmonary TB is known since the time of Hippocrates as phthisis. Robert Koch performed one of the greatest works on TB in 1882 when he isolated and cultured *M. tuberculosis* from crushed tubercles. His experimental work identified the bacterium as the etiological agent of tuberculosis. Later, the tuberculin skin test became the principal tool for infection diagnosis. In the same period, Koch developed staining methods for the identification of the mycobacteria; these techniques were improved by Paul Ehrlich whose method provided the basis for the development of the even presently used Ziehl-Nielsen staining[3].

In 1993 the WHO declared TB a global public health problem, being the only disease so far to warrant that designation. Although the treatment of TB has brought a considerable reduction in incidence to developed countries, historical data indicate that there have not been great effects on the global problem since the time of Koch. TB is the most fatal infectious disease, standing for 26 % of all preventable deaths and 7 % of all deaths (data from the year 1996)[3].

TB resumption has been attributed to several factors such as the increase in drug-resistance, default of the treatment, the human immunodeficiency virus (HIV) expansion, the increase of injectable drug users, the migration, the aging of the world population, the active transmission amongst environments of human accumulation (prisons, hospitals, homeless), the degradation of health care systems or especially social-economic changes and inequity impairing other impacts. On the other side, we can assign adequate health services, directly observed treatment short-course (DOTS), education, optimal nutrition level, human resources for TB control and degree of political participation of the population to the protective factors[3].

In human medicine the most significant group of mycobacteria are the members of the *Mycobacterium tuberculosis* complex (consisting of *M. tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium pinipendi* and *Mycobacterium canettii*[4]. Human TB is a contagious infectious disease mainly caused by *Mycobacterium tuberculosis*. It is a weak Gram-positive rod-shaped bacterium that has no

flagellum, does not form spores nor produces toxins and has no capsule. The microbe´s width and height vary from 0.3 to 0.6 and 1 to 4 μm, respectively. It is a macrophage intracellular pathogen that establishes its infection preferentially to the respiratory system where it is usually conditioned into a dormancy state as long as host´s immune system prevails[3]. The complete genome sequence of *M. tuberculosis* strain $H_{37}Rv$ was published in 1998. It consists of 4,411,529 base pairs, contains around 4,000 genes and has a very high proportion of guanine-cytosine pair[5]. This progress brought new opportunities for the targeted research.

The principal mean of transmission occurs by infective particles. Experiments with animals demonstrated that suspended particles containing 1 to 10 bacilli are enough to cause an infection. Upon infection, inhaled bacilli are phagocytised by alveolar macrophages and can either be eliminated or grow in the intracellular environment in localized lesions called tubercles. From two to six weeks past infection are usually followed by the establishment of cellular immunity and subsequent lymphocyte and activated macrophage infiltration into the lesion, which leads to the elimination of most portion of the bacilli and the end of the primary infection, commonly without symptom manifestation. A great proportion of the infected phagocytes and lung parenchymal cells are also killed, which produces a characteristic solid caseous necrosis where some bacilli may survive. When host immunity predominates, the lesion is contained, causing simply residual damage to the lungs. In the case the necrosis reaction expands breaking into a bronchus, a lung cavity can be formed, which may result in a massive bacterial dissemination into the air through coughing. However, more commonly, mycobacteria can coexist within its host as a quiescent or dormant form of infection, which establishes a bacterial reservoir[3].

Approximately 15 % of the patients with the active disease present extra-pulmonary TB, which is caused by granuloma evolution due to bacteria invading the blood vessel and disseminating the pathogens to various parts of the body. So called miliary TB frequently invades pleura, lymph nodes, liver, spleen, bones and joints, heart, brain, genital-urinary system, meninges, peritoneum and skin[3].

The pathogenesis produces typical TB symptoms such as weakness, fever, weight loss, night sweat, chest pain, respiratory insufficiency and excessive cough, whereas advanced pathology may also cause bleeding, which leads to haemoptysis. Many TB symptoms including tissue destruction are preferentially mediated by the host´s immune response instead of the bacterial virulence itself[3].

Latent tuberculosis infection is defined as an infection with *M. tuberculosis* manifested by a positive tuberculin skin test or an interferon-γ (INF-γ) release assay results simultaneously without evidence of active TB symptoms, progressive radiographic changes or microbiological evidence of replicating organisms (e.g. positive culture)[6]. The terms dormancy, persistence and latency have been used interchangeably generally to refer to the non multiplying state of mycobacteria, tolerance to antituberculotics or an infection without active disease. Dormancy is a physiological state of arrested growth, persistence consists in the phenotypic transient tolerance to the antibacterial effects of drugs[7]. Latent tuberculosis is established as a result of the interaction of immunity with *M. tuberculosis* infection, forcing it into a quiescent state in the infected tissue. Bacteria reduce their metabolism, as a consequence of the action of cellular immune response, and which can, to a certain point, contain, but not eradicate, the infection. Latent TB is not an infectious disease and, therefore, does not represent a public health threat. The bacterial intracellular survival depends on the phagosome acidification in infected macrophages thus preventing the phagosome-lysosome fusion. As soon as the immune system begins to debilitate from many causations, latent infection can be reactivated, leading to the development of active TB[3]. AIDS/HIV infection, post-transplantation state, silicosis, haemodialysis, carcinoma, TB infection before two years and later, TNF-α inhibitors and immunosuppressive treatment, diabetes mellitus etc. were referred being important risk factors of this reactivation[6]. For common population, the rate does not exceed 5 %, but at HIV-infected patients it is increased up to 50 %[8].

3.1.1.1 Incidence of the Tuberculosis

The number of people who fell ill with TB dropped to 8.8 million in 2010; the number has been falling since 2005, but very slowly. The estimated global incidence rate mildly fell to 128 cases per 100,000 people in 2010. About 1.4 million people died from TB in this year[9]. The epidemiologic situation in the Czech Republic is quite favourable when compared with other countries. There were reported 680 cases of TB of all localisations (6.5/100,000 inhabitants) in 2010. Of that number 59 cases were extra-pulmonary localised tuberculosis. The trend shows a slight but significant decreasing of this illness. The portion of immigrants takes 17.2 % of all notified TB. Most of them were from Ukraine, Vietnam and Mongolia, i.e. countries with high TB burden. Only 28 patients died for tuberculosis (0.3/100 000 inhabitants) in the Czech Republic in 2010[10]. The trends of changes of the

total number of tuberculosis incidence and incidence related to 100 000 inhabitants brings Figure 1. The similarity of both curves is obvious.

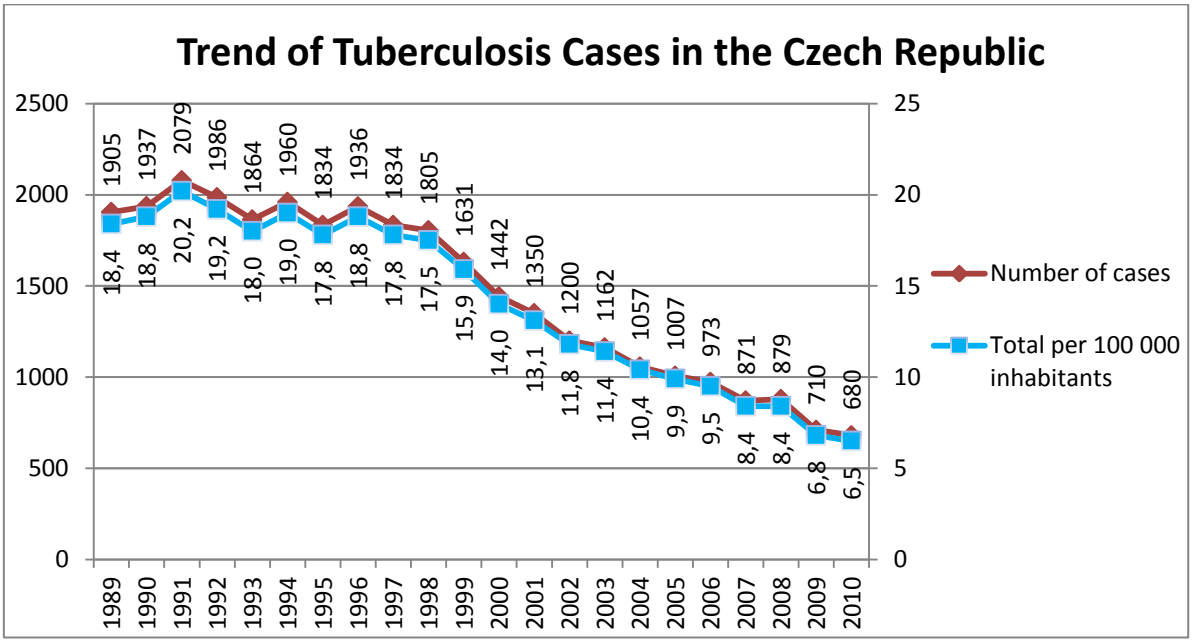


Figure 1. Trend of number of notified TB cases (1989 – 2010) in the Czech Republic (data was taken from lit.[10])

Rapid expansion of a standardised approach to tuberculosis diagnosis and treatment cured more than 36 million people between years 1995 and 2008, averting up to 6 million deaths. However, regardless of great effort of many organizations and individuals, TB remains still a huge global public health concern, especially in the developing countries and for the poorest people. TB is a leading cause of death of people in the most economically productive age-groups, concurrently it poses appreciable economical encumbrance[11].

3.1.2 Treatment and Prevention of Tuberculosis

The aims of the tuberculosis treatment in the accordance with WHO are[12]:

- to cure the patient and restore quality of life and productivity,
- to prevent death from active TB or its late effects,
- to prevent relapse of TB,
- to reduce transmission of TB to others, and
- to prevent the development and transmission of drug resistance.

The history of attempts how to heal tuberculosis is motley. From ancient period and for centuries, milk has been practically considered the cure for tuberculosis. Other curious methods were bloodletting, thoracic poultices, drinking wine, surgical methods, worship

of the god Asclepius etc. Medical plants and honey were popular, too. During the 16th and 17th centuries, sulphur, arsenic, mercury and every type of plant from the New World such as quinine, cocoa and tobacco were all applied. In 19th century, novel various medications such as tannin, iodine, tar, creosote and alcohol continued to be used ineffectively. Interestingly, malaria was considered being an antagonist disease to TB[13].

In 1854, H. Brehmer proposed the idea that tuberculosis was indeed a curable disease. The introduction of the sanatorium cure provided the first big step to the treatment due to healthier climate, fresh air and good nutrition. These and improving social and sanitary conditions strengthen the defence forces of the human body against infection. Several methods of direct tuberculosis treatment were discovered, e.g. artificial pneumothorax, radiation and chemotherapy. With the introduction of artificial pneumothorax and surgical methods to reduce the lung volume, active therapy for tuberculosis began. The discovery of Roentgen´s rays enabled to monitor the progress and severity of a malady[14]. In 1920, A. Calmette and C. Guérin found a bovine species of mycobacterium with a rare virulence that could possibly be developed into active immunity against tuberculosis. The BCG (Bacillus Calmette-Guérin) vaccine was a great hope and progress but because of its widespread use, actually became an obstacle for the modern chemotherapy[13]. The first really highly efficacious therapy lied in the discovering of streptomycin in 1943 by S. A. Waksman and in its administration to patients one year later[14] and then followed by *para*-aminosalicylic acid in 1944 and isoniazid[13].

Now, the therapeutic approaches and regimens differ from various situations. Patients, those who have no history of prior TB treatment or who received less than one month anti-TB drugs, are presumed to have drug-susceptible TB. However, local epidemiologic situation (a high prevalence of isoniazid-resistance in new patients) and individual characteristics (active TB after known contact with a patient infected by documented drug-resistant TB) should be respected. New patients with pulmonary TB and some kinds of extra-pulmonary forms should receive next 6 months regimen consisted of two month taking of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) and then four month of INH and RIF. WHO uses next abbreviation: H, R, Z and E, respectively, but there are preferred abbreviations derived from international nonproprietary names of these drugs in this thesis. Replacement of RIF by EMB in continuing phase is associated with more relapses and deaths and should be avoided. In populations with known or suspected high INH-resistance levels, new patients may receive INH, RIF and EMB as therapy in the continuation phase[12].

It is assumed that susceptible mycobacteria will be eradicated from sputum rapidly, usually within starting 2 months and a continuation phase is required to prevent relapses[15].

The systematic review found little evidence of differences in failure or relapse rates with daily or three times per week regimens. However, patients receiving three times weekly dosing throughout therapy had higher rates of acquired drug-resistance than patients who received drugs daily throughout treatment. Therefore the daily administration is an optimal choice, but other possibilities are eventually possible for patients without HIV infection. There is an insufficient evidence of the efficacy of twice weekly dosing[12].

Table 1 presents recommended doses of the first-line antimycobacterial agents for adult patients.

Table 1. Recommended dosage of first-line antituberculous drugs (according to lit.[12])

Recommended doses of first-line antituberculotics for adults				
Drug	Daily administration		Administration three times per week	
	Standard dose (mg/kg)	Maximal dose (mg)	Standard dose (mg/kg)	Daily maximal dose (mg)
Isoniazid (INH)	5	300	10	900
Rifampicin (RIF)	10	600	10	600
Pyrazinamide (PZA)	25	-	35	-
Ethambutol (EMB)	15	-	30	-
Streptomycin (STM)	15	-	15	1 000

The mechanisms of the action of a range of antimycobacterial agents are briefly summarized e.g. in our review[16]. A concise review was published in 2008[17].

It has been speculated that at least four significant mycobacterial subpopulations exist and for each one different drug(s) are efficacious[18]:

- active growing mycobacteria killed by INH,
- bacteria with sporadic metabolic bursts could be killed by RIF,
- those with low metabolic activity due to acidic and hypoxia environment susceptible to PZA,
- dormant bacilli that are not killed by any current agents.

In the standard treatment protocol, INH reduces the mycobacterial load by around 95 % over the first two days of treatment and thus prevents threat of TB transmission. INH displays equal efficiency as a "classic" four-combination. Despite of this excellent early bactericidal activity, INH is no more effective than other drugs after this period; however, it is important for reducing of the emergence of drug-resistance to other drugs. In the next stage, RIF represents the most crucial drug against sporadically active *M. tuberculosis* to

reduce relapse rate. PZA represents a pivotal component of the killing of a subset of bacteria at specific conditions unaffected by other drugs. Its inclusion reduces the therapy course up to a half. Therefore it is obvious that during the therapy, particular drugs play various roles at subsequent periods[18].

Although this first-line therapy is very effective (greater than 90 %), it could fail to cure TB infection for several reasons even including initially fully drug-sensitive strains; the effectiveness of present treatment regimen ranges within 55 – 85 %. Firstly, the treatment takes minimally six month, which is too long. The length of the therapy with multidrug treatment results in a poor adherence, a lack of compliance, and also contributes to the emergence of drug-resistant forms. Reducing the duration of the treatment from six to two months or less could result in the significant increase of the adherence and cost reduction. Secondly, these anti-TB drugs have toxic effects including hepatotoxicity, neurotoxicity and drug-drug interaction[19,20]. Therefore new options are needed to shorten the duration of drug administration (see chapter 3.2.2.1).

In 2011, WHO proclaimed and published Global Tuberculosis Control 2011 plan with a lot of goals for DOTS, drug-resistant TB, TB/HIV co-infection and laboratory strengthening. There exists an objective need for the searching of new technologies – the document exactly says that the progress in TB care and control is constrained by old technologies. During the past decade, efforts to develop new diagnostics, drugs and vaccines for TB have been intensified with partial success. For example, the first-line antituberculotics are around fifty years and more old and new drugs are required[21].

3.1.2.1 Treatment of Latent Tuberculosis

Treatment of latent TB infection is a key component of tuberculosis control programmes. Firstly, INH was found to be effective in preventing disease as well and it had constituted the only regimen for latent TB therapy for many years, given efficacy of 90 % if completed properly. However, its standard duration reaches nine month of daily administration and from more reasons this treatment fails sometimes[6]. The bactericidal activity of INH is virtually complete after two days of treatment and then reverts to low levels, since it seems very slow in killing persisting bacilli. Another hypothesis is that INH has a slow sterilizing action because some of the persisting bacilli move temporarily into a rapidly growing phase when they can be killed[22]. Six or twelve month INH therapies, daily or twice weekly were evaluated with wobble rate of attainments; some regimens are not convenient for all threatened individuals. Due to the problems with INH, over the past years, alternative

regimens using different drugs were assayed successfully. Main advantages could be shorter, safer and yet equally effective therapy[6]. For a drug to kill persistent/latent form of mycobacteria is necessary to target some aspect of cell metabolism that remains vital[15].

Monotherapy may be realized sufficiently by three or four month of daily RIF, whereas EMB appears to have no sterilizing activity at conventional and even at high doses. Combination regimens may be by three or four month administration of both INH and RIF (the continuing bactericidal activity seems to be due almost entirely to the RIF component and it is doubtful whether INH plays an important role either in killing persisters or in preventing the emergence of resistance), INH and rifapentine, albeit here are still some important questions. Combined two-month therapy of RIF and PZA exhibited an excellent efficacy, but shares a higher risk of adverse events[6,22].

Nevertheless, no treatment has been demonstrated to be efficacious for the treatment of latent TB caused by INH- nor both INH- and RIF- resistant *M. tuberculosis*. There is recommended to use four months daily rifampicin. Advices for the MDR-TB treatment are based on expert opinion and Table 2 summarizes them. The duration for all MDR latent TB treatment takes 6 – 12 months. The potency of fluoroquinolones to eliminate latent mycobacteria decreases in a row moxifloxacin = gatifloxacin > levofloxacin >> ofloxacin > ciprofloxacin[6].

Table 2. Recommended (non evidence-based) treatment of latent MDR tuberculosis infection (data was taken from[6])

Drug resistance pattern	Recommended regimen
INH + RIF	fluoroquinolone monotherapy or PZA + EMB or fluoroquinolone + PZA or fluoroquinolone + EMB
INH + RIF + EMB	fluoroquinolone monotherapy or fluoroquinolone + PZA
INH + RIF + PZA	fluoroquinolone monotherapy or fluoroquinolone + EMB
INH + RIF + EMB + PZA	fluoroquinolone monotherapy or fluoroquinolone + ETH
INH + RIF + EMB + PZA + ethionamide	fluoroquinolone monotherapy or fluoroquinolone + cycloserine
INH + RIF + EMB + PZA + fluoroquinolones	cycloserine + PAS or ETH + PAS or ETH + cycloserine

3.1.2.2 Prevention of the Tuberculosis

Beside the treatment of both open and latent form of tuberculosis, i.e. intervention on infected patients, there is a question of prevention of mycobacterial infections. The combination of a new highly effective pre-exposure vaccine with a more effective preventive therapy would potentially have a dramatic effect on incidence. A highly effective post-exposure vaccine may have the same effect as a preventive treatment. Unfortunately, these methods are not yet available[11].

BCG vaccine is currently the most widely used only available vaccine against TB. It has been administered up to three billion people without serious complications. Although it displays some advantages (easy administration, it can confer immunity for a long period, a very efficient adjuvant for immunity induction, a low cost), recent molecular analysis have revealed that genetic modifications formed substrains along time and a complete avirulence acquisition is also suspected for the vaccine. Another problem is that there can be a gradual loss of T-memory cell population during in the course of time. Regrettably, BCG vaccination has had the limited impact on the global burden of TB. Although vaccine has been effective to prevent severe and fatal forms of TB in young children (e.g. meningeal form), it does not protect against pulmonary TB in adults sufficiently. Since the efficacy varies from zero to 80 %, the new improved vaccine is searched constantly[3,22].

A second approach consists of thorough prevention leading to the reduction of such factors, which increase the risk of progression from infection to disease; HIV, the malnutrition, smoking, diabetes mellitus and alcohol misuse are individual risk factors that can double or triple the risk of development of active tuberculosis. Indoor air pollution is an additional possible causal factor. A wide range of disorders (e.g. silicosis, malignant diseases and chronic systemic illnesses) and immunosuppressive treatment are established risk factors for TB; some other common situations were proposed, but very little research has been done to test these hypotheses (chronic helminth infections, depression, mental illness, pregnancy and the postpartum, outdoor air pollution)[11].

3.1.3 Nontuberculous Mycobacteria

Except *Mycobacterium tuberculosis* complex there exist nontuberculous mycobacteria comprised of many species responsible for a wide range of clinical syndromes. They encompass all mycobacterial species other than *M. tuberculosis* complex and *Mycobacterium leprae* and, traditionally, they are classified into four groups or otherwise

into slowly and rapidly growing mycobacteria. Nontuberculous mycobacteria are not obligate pathogens and are not considered being contagious. Clinically important species include *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium gordonae*, *Mycobacterium scrofulaceum*, *Mycobacterium xenopi*, *Mycobacterium avium-intracellulare*, *Mycobacterium ulcerans*, *Mycobacterium celatum*, *Mycobacterium fortuitum*, *Mycobacterium chelonae*, *Mycobacterium abscessus* and others. Nontuberculous mycobacteria are generally free-living organisms ubiquitous in the environment. Important reservoirs include water, soil, animals and dairy products. The true prevalence of infection caused by these pathogens is unknown. Nonetheless, there is an evidence of a recent increase described in the developed world in the face of a falling incidence of TB. The spectrum of clinical infections caused by nontuberculous mycobacteria varies widely. However, it can cause a broad spectrum of infections – pulmonary and respiratory infections, lymphadenitis, soft tissue and skeletal infections, gastrointestinal disorders, disseminated diseases (especially in patients with late HIV infection or other forms of severe immunosuppression) and iatrogenic infections[4,23].

Treatment of these infections is difficult, still not entirely clarified and requires long courses of multidrug therapy, sometimes with adjunctive surgical intervention. For most species, macrolide-based (azithromycin, clarithromycin) regimens are an effective option, although a lot of adverse effects, treatment failure and resistance may develop. Other drugs referred being useful in the therapy include INH, rifamycines, EMB, PZA, some quinolones (moxifloxacin, ciprofloxacin), β-lactams (cefoxitin, imipenem), aminoglycosides (streptomycin, amikacin, tobramycin), sulfonamides and cotrimoxazole, tetracyclines (doxycycline, tigecycline, minocycline), linezolid, telithromycin or clofazimine. However, the development of new antibiotics may offer more successful and safe treatment options[4,23].

3.1.4 Drug-Resistant Tuberculosis

Resistance of any kind to antimicrobial drugs brings a lot of possible difficulties. Drug-resistant tuberculosis is not a recent phenomenon. *M. tuberculosis* strains that were resistant to streptomycin, the first really effective anti-TB drug, appeared soon after its introduction into treatment of TB in 1944. The currently prevailing scenarios of drug-resistant tuberculosis are particularly alarming and they evoke a serious challenge for the effective control of the tuberculosis[24]. TB is only one of more illnesses caused by resistant

strains that have become major public health problem, e.g. gonorrhoea, malaria, methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant enterococci infections[25].

There are various possibilities and approaches how to classify and sort the drug-resistance. Drug-resistant tuberculosis can be classified into three categories when considering the origin of the resistance during therapy – primary, acquired (secondary) and mixed (initial):

- primary resistance is defined as an infection of a naive (i.e. previously untreated) patient with an already resistant tuberculosis strain,
- acquired resistance develops in patients originally infected with a drug-susceptible strain that during the treatment becomes resistant to multiple medications. Main reasons of this and then secondary therapeutic failure are inadequate, inappropriate or irregular treatment, non-adherence to treatment protocols and prescribed therapy or non-compliance,
- mixed resistance lies between primary and acquired resistance. It develops in people who claim to be treated first, but their previous drug history cannot be found out or confirmed. In reality this category consists of true primary resistance due to original infection with a drug-resistant strain and an unknown amount of undisclosed acquired resistance from previous therapy[25,26].

Inappropriate chemotherapy is defined as the use of a single drug, inadequate drug combinations, short treatment period and low absorption of the administered drugs[3].

Drug-resistance of mycobacteria should be divided into two main groups – intrinsic and acquired types:

- intrinsic one has been attributed mainly to the unusual structure of cell wall containing mycolic acid with a low permeability for many compounds including antimicrobial agents, efficient efflux mechanisms, lacking of porins or the presence of β-lactamases. Intrinsic resistance limits the number of drugs available for the treatment[27],
- acquired resistance is caused mainly by spontaneous chromosomal mutations resulting in the selection of resistant strains during inadequate therapy, unlike the situation in other bacterial species. For most of the main antituberculotics, spontaneous mutation occurs at a rate of $10^{-6} – 10^{-9}$ mutations per cell division (for INH, STM and EMB $1 \text{ in } 10^6$ and for RIF $1 \text{ in } 10^8$); when a combination of antimycobacterial drugs is applied the likelihood of a presence of a mutant having

two resistance mutations is < 10^{-14} up to 10^{-18}, three of 10^{-27} etc. Naturally occurring two-drug resistance is very uncommon, therapy with two (or more) drugs prevents the emergence of progressive resistance. Moreover, this rate can be reduced due to human intervention. No single pleiotropic mutation producing MDR-TB phenotype has been found, but a possible complex association between classical mutations associated with resistance to one drug could be related to initial steps in the resistance to other agents[25,26,27].

Classical theory postulated the mechanism of drug-resistance emergence based on the selection of naturally pre-existing resistant mutants in the mycobacterial population by "drug pressure". If INH is prescribed for cavitary pulmonary TB alone, it will kill all of the susceptible cells, including those resistant to another drugs, but INH-resistant mutants will survive. These will multiply their population. The probability of this event is enhanced by the duration of monotherapy; at 25 % of the people receiving INH alone for two weeks, 60 % after six months and 80 % among those receiving it for two years. When rifampicin alone is then added, by the same mechanism a MDR strain is selected; therefore an addition of only single drug to failing therapy is a wrong step[26]. Unfortunately, the combination therapy of patients infected by susceptible strains does not consistently prevent the drug-resistance, especially if patients take medications irregularly. There were proposed four basic mechanisms to explain how drug-resistance emerges as a result of poor compliance – differential bactericidal effects during initial killing, monotherapy during sterilization of specific populations, differential subinhibitory drug concentrations during regrowth and differential bacterio-pausal effects during regrowth. From other point of view, several cycles of killing caused by drugs and regrowth (when drug-taking stops) are required. In each of these cycles, there is a selection favouring the resistant mutants relative to the susceptible bacterial population. Regrowth to the size of the original population may occur and result in an increasing proportion of resistant bacilli[26,28].

The mechanisms of resistance to particular drugs including the role of involved genes are surveyed in many concise reviews, e.g. lit.[24,25,27,29] and in brief in our book[16].

As mentioned before, it is important to intervene the risk factors of the acquired resistance development. Next factors have been described[12,29]:

- previous treatment for TB is an important risk factor for inducing the drug-resistance, particularly MDR-TB (prevalence of primary MDR is 1.4 % and 13.0 % in previously treated patients) due to inadequate treatment; it consists of inappropriate chemotherapy regimens, drug supply, unsatisfactory patients or

clinicians compliance, lack of any supervision under the treatment and absence of infection control measures in hospitals;

- a history of using the anti-TB drugs of poor or unknown quality,
- who remain sputum smear-positive at month 2 or 3 of treatment,
- whose prior course of therapy included rifampicin throughout,
- exposing in institutions with an outbreak or a high prevalence of MDR (such as certain prisons or mines),
- immigration (3- to 10- fold higher risk chance) due to lack of health care services and inadequate socio-economic conditions,
- age – the group of 35 – 64 years has a significantly higher proportion of MDR-TB,
- sex – MDR-TB is more predominant in male, but there is no influence of sex on the association between MDR-TB. It has been hypothesized that women are more compliant with the treatment and therefore less likely to receive inadequate treatment. Contrary to MDR-TB, female gender has been found as a significant risk factor in XDR-TB patients;
- HIV infection – there is no clear association between HIV and MDR-TB, however, it has been found that HIV infection favours the transmission of MDR strains (these connections exist in some settings),
- co-morbidity with malabsorption or rapid-transit diarrhoea,
- alcoholism bringing a poor adherence to the treatment,
- smoking,
- diabetes mellitus where exists a significant association with MDR-TB,
- socio-economic factors like drug abuse, poverty and homelessness.

WHO document Anti-Tuberculosis Drug Resistance in the World from 2008 presented data of the prevalence of any drug-resistance among new cases of TB. The values ranged from 0 % up to 56.3 % (Azerbaijan). The spread of any resistance among previously treated patients with tuberculosis is much higher up to 85.9 % in Uzbekistan[30].

Drug-resistance may be classified generally into five groups. Monoresistant TB strain is resistant *in vitro* towards only one antimycobacterial agent, polyresistant express insusceptibility to more drugs with both concomitant isoniazid and rifampicin resistance being an exception[31]. Multidrug-resistance, extremely drug-resistance and more recent totally drug-resistance are defined in detail in the chapter 3.1.5.

Data from the Czech Republic (recapitulating the year 2010) reveal that resistance to RIF was detected in 5 %, to INH in 4.5 %, to STM in 3.1 % and 1.7 % to EMB; these values are higher than those presented in the preceding year. Monoresistance was described in 5.0 %, multidrug-resistance in 1.9 % similarly as in the preceding year and polyresistance in 1.4 %[10].

3.1.5 Multidrug-Resistant, Extensively Drug-Resistant and Totally Drug-Resistant Tuberculosis

3.1.5.1 Characteristics of Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis

Multidrug-resistant tuberculosis (MDR-TB) is defined as the infection that is resistant to multiple antituberculosis drugs, at least to isoniazid and rifampicin (rifampin is a widely used synonymous term), the most effective first-line oral agents used to treat mycobacterial infections[16,25]. The first evidence of multidrug-resistant strain dated back in 1970 after RIF introduction into therapeutic regimen[31].

With the increasing rate of tuberculosis insensitivity to used drugs, the extensively drug-resistance (XDR) was defined by WHO. The first specification from March 2006 postulated that XDR is such a mycobacterial strain which is resistant to INH and RIF and additionally at least to three second-line anti-TB drugs groups of the total of six (fluoroquinolones, aminoglycosides, polypeptides, thioamides, cycloserine and *p*-aminosalicylic acid). According to newer, now recommended and widely used definition from October 2006, XDR-TB consists in MDR (i.e. INH and RIF insusceptibility) in combination with both resistance to any fluoroquinolone and at least one second-line injectable drug (kanamycin, amikacin, capreomycin)[31,32].

However, this definition is not accepted uniformly without doubts. Caminero[32] gives evidence that this definition enables the possibility of the susceptibility to fluoroquinolones and aminoglycosides and/or polypeptides in some XDR-TB patients and their use in the treatment, which improves success rates > 80 % as very effective bactericidal agents. The possibility of success in XDR-TB patients using an injectable and all of the second-line drugs could be very close to in patients with only MDR-TB. Although it was shown that the current definition of XDR-TB predicts a poorer clinical outcome than MDR-TB, this definition may still be inappropriate. Furthermore, it permits the possibility of susceptibility to EMB and/or PZA. Their embracing into regimen improves clinical

outcomes significantly; the combination of PZA, ETH and cycloserine reached more than 90% efficiency. Therefore Caminero proposes another, stricter definition: XDR strains should comprise the resistance to all first-line drugs and to fluoroquinolones and all of the injectables (not just to one). These patients have a possible treatment success rate of < 50% and clearly stand apart from MDR-TB patients[32]. This is concordant with the assertion that XDR-TB is more expensive and difficult to treat than MDR-TB and outcomes for patients are much worse[30].

3.1.5.2 Epidemiology of MDR-TB and XDR-TB

In 2010, there was an estimation of 650,000 cases of MDR-TB (see Figure 2) and in 2008 it was estimated about 150,000 MDR-TB deaths annually[9]. Globally, it was estimated that 3.3 % of all new TB cases was MDR-TB in 2009[33]. The median prevalence of MDR-TB in new TB cases was 1.6% and in previously treated TB cases 11.7%[28]. XDR-TB has been reported from 69 countries (by the end of the year 2010) and there are estimated 25,000 cases of XDR-TB emerging annually. The XDR-TB cases were reported also in Central Europe from Austria, Czech Republic, Germany, Poland and Slovenia[33].

Prevalence of MDR among new TB cases ranged from 0 % (Andorra, Cuba, Luxembourg, Malta, Slovenia, Aragon, Spain and Uruguay) to 22.3 % in Azerbaijan (see Figure 3). When concentrated on the prevalence among previously treated cases, MDR-TB was reported up to 62.5 % ratio in Lebanon[30].

The trends in resistance have not displayed uniformity around the world. Some countries (the USA, Hong Kong) reported a faster reduction in MDR-TB compared with all TB cases, Russia, Peru and South Korea have increasing trends in the prevalence and estimated incidence of MDR-TB. In most high-resource countries with low a prevalence of TB (the United Kingdom, France and Germany), trends in MDR-TB were stable with a low number and proportion of drug-resistant forms[28].

There is a relatively favourable situation in the Czech Republic – MDR-TB comprises about 2 % of all TB patients, i.e. circa twenty people per year. In concordance with world trends, about one third of this number is comprised of foreigners from countries with high resistant tuberculosis load[31].

WHO declared that twenty seven countries are threatened by a high burden of MDR-TB and XDR-TB; they represent approximately over 85 % of the world's number of incidence of these both drug-resistant forms of tuberculosis. Armenia, Azerbaijan, Belarus, Bulgaria,

Estonia, Kazakhstan, Latvia, Lithuania, Republic of Moldova, Russian Federation and Ukraine are European and nearby countries are included in this list[33].

MDR and XDR-TB are the major threats in the controlling of TB around the world[34].

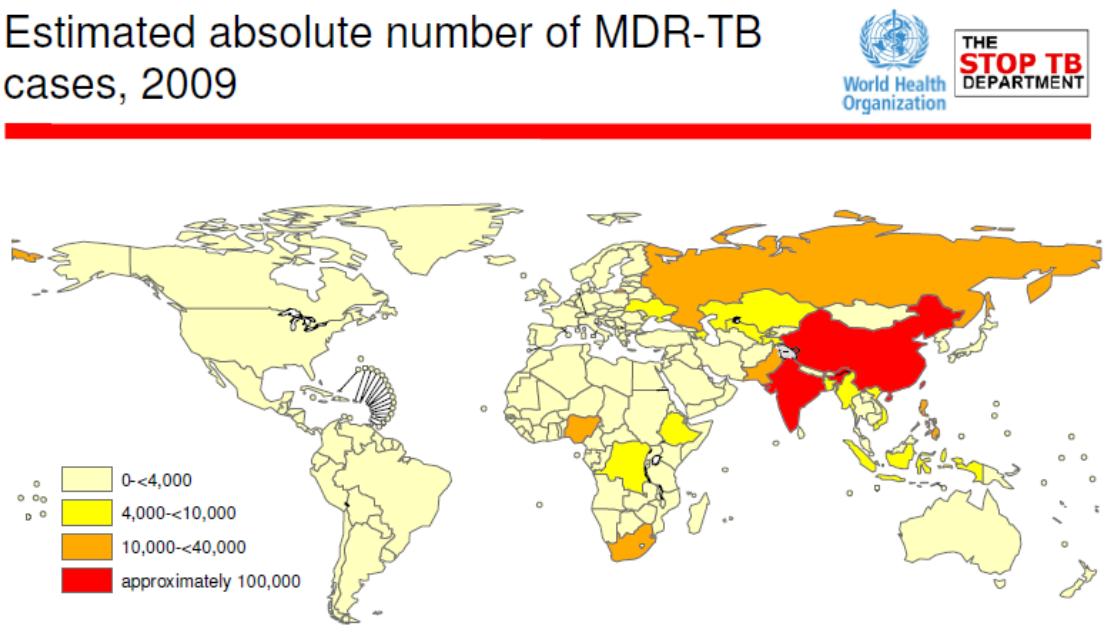


Figure 2. An estimation of total multidrug-resistant tuberculosis cases in 2009 (this picture was taken from http://www.who.int/tb/challenges/mdr/drs_maps_feb2011.pdf, accessed 28. 10. 2011)

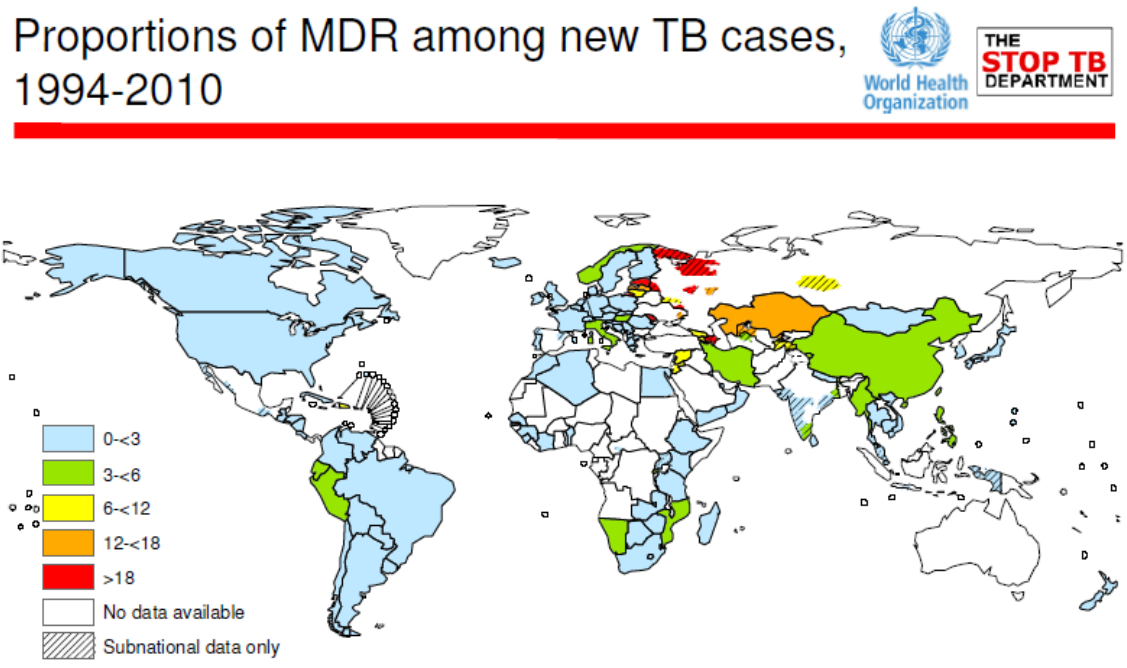


Figure 3. Proportion of multidrug resistance among new cases of tuberculosis (this picture was taken from http://www.who.int/tb/challenges/mdr/drs_maps_feb2011.pdf, accessed 28. 10. 2011)

3.1.5.3 Treatment of MDR and XDR-TB

Drugs are classified for the purpose of treatment of drug-resistant (MDR-TB) forms of the tuberculosis to five groups (see Table 3) chosen with a stepwise selection process based on the efficacy, safety, experience of use and cost.

Table 3. The classification of the drugs used for the treatment of drug-resistant tuberculosis (data was taken and compiled from lit.[12,16,35,36])

The groups of drugs used for the treatment of drug-resistant tuberculosis	
Group/drug	Recommendation/drug
1. First-line oral antituberculosis agents	**(use all possible drugs)**
isoniazid[1]	ethambutol
rifampicin[1]	pyrazinamide
rifabutin[2]	
2. Injectable agents	**(use one because of similar targets)**
capreomycin	streptomycin[3]
kanamycin	(viomycin)
amikacin	(enviomycin)
3. Fluoroquinolones[4]	**(use one because of the same target)**
ofloxacin	moxifloxacin
levofloxacin	(gatifloxacin)
4. Second-line oral antituberculosis agents	**(use all possible drugs if necessary)**
ethionamide[5]	cycloserine
prothionamide[5]	terizidone[6]
p-aminosalicylic acid	
5. Agents with unclear role in the treatment of MDR-TB	**(use all necessary drugs to complete four-combination of antituberculotics)**
clofazimine	clarithromycin[8]
amoxicillin/clavulanate	high-dosed isoniazid
linezolid	thioacetazone
imipenem[7]/cilastatin	

drugs in brackets may be considered belonging into particular groups
[1]: not useful in the treatment of MDR-TB,
[2]: may share cross-resistance to rifampicin, as well as other rifamycines,
[3]: high rate of resistance, it should be classified into first-line drugs, but with parenteral administration,
[4]: ciprofloxacin and other prior-generation of quinolones have not been recommended already,
[5]: fully interchangeable,
[6]: a derivative of cycloserine with assumed equal efficacy,
[7]: meropenem shares the core structure and the mechanism of the action,
[8]: also other macrolides of higher generation and new macrolide derivatives have showed *in vitro* antitubercular potency

In the therapy of MDR-TB, WHO recommends administration of a fluoroquinolone, better from later-generations (levofloxacin, moxifloxacin, gatifloxacin and sparfloxacin; ciprofloxacin should not be used) and a thioamide drug (ethionamide or prothionamide). Four second-line antituberculosis drugs likely to be effective, as well as PZA, should be included in the intensive phase. In sum, regimens should contain at least PZA, one fluoroquinolone, a parenteral agent (kanamycin or amikacin, capreomycin, where is no

superiority to any other; the use of STM is not recommended), ETH (or prothionamide) and either cycloserine or PAS (if cycloserine or thioamide cannot be used). When the individual effect of amoxicillin/clavulanate, clofazimine, macrolides (azithromycin, clarithromycin and roxithromycin) and thioacetazone was analysed, no significant association with the cure could be discerned. EMB was associated with a marginal but statistically significant reduction in likelihood of the cure among patients not previously treated for MDR-TB; therefore it is not yet recommended as a basal part of standard regimen and it was replaced by one more oral second-line agent[37].

Caminero and co-workers in their excellent review[35] suggest that the recommended regimen should combine at least four drugs to which the *M. tuberculosis* isolate is likely to be susceptible, although more than four might be necessary. The intensive phase of treatment (with the injectable drug) should take for at least 6 months (or 4 months after culture conversion) and the continuation phase should last until 18 months. Surgery approach may be also used under specific conditions.

Among the oral first-line drugs high-dosed INH (10 – 15 mg/kg; according to[12] 16 – 20 mg/kg), PZA and EMB are thought as an adjunct for the treatment of MDR- and XDR-TB for the entire treatment duration, but they should never be included among the main four drugs used to treat patients with resistant TB due to possible previous mismanagement with one or more therapeutic cycles and the low reliability of the susceptibility tests to EMB and PZA. Interestingly, the resistance to low but not high doses of INH with the resistance to ethionamide is common in up to 15 % of patients with INH-resistant tuberculosis, in whom high doses of isoniazid might, despite of *in vitro* resistance, be clinically useful to overcome the problem of the cross-resistance to ETH. It seems to be a promising approach, but further studies are needed. About 15 – 20 % of RIF-resistant mycobacterial strains are likely to be susceptible to rifabutin, but due to more reasons rifamycins are not recommended in the treatment of drug-resistant TB. Many MDR- and XDR-TB retain susceptible to PZA and EMB[35].

Fluoroquinolones have been widely used for drug-resistant TB treatment since the late 1980s, while ciprofloxacin revealed this potency in 1984[15]. The resistance to fluoroquinolones increases the risk of treatment failure and death in patients with MDR- or XDR-TB; because of these drugs deliver better clinical outcomes than the drugs in the other groups. The cross-resistance between fluoroquinolones is not always complete – about half of the ofloxacin-resistant isolates could be susceptible to moxifloxacin and high-dosed levofloxacin. High-dose levofloxacin (1 g per day) is the optimal choice, closely

followed by gatifloxacin and moxifloxacin; moxifloxacin seems to have the best sterilising activity. One of them should be routinely included in the treatment regimens of MDR-TB, but not XDR-TB. Ciprofloxacin is somewhat less effective than others and is not recommended nowadays[35].

The injectable drugs should be used in the following order – capreomycin, kanamycin and amikacin. Cyclic peptide capreomycin has a different structure and mechanism of the action from the aminoglycosides. As with the fluoroquinolones, there is no reason to use more than one injectable in the treatment. All these drugs display similar efficacy and adverse effects profile. In most cases, an injectable drug is one of the four key drugs, even for patients with XDR-TB. Streptomycin should never be used to treat these resistant forms of tuberculosis, even if susceptibility testing indicates a susceptible isolate, because the clinical reliability is not very high and the resistance is very common, especially in patients with INH-resistance. Amikacin should always be avoided as a first choice because of the possible cross-resistance and an increased risk of serious adverse events. Recent studies have revealed that isolates with acquired resistance to STM are usually susceptible to kanamycin, amikacin and capreomycin; capreomycin-resistant isolates to kanamycin and amikacin; most isolates bearing acquired resistance to amikacin are resistant to both kanamycin and capreomycin. The kanamycin-resistant strains show different levels of cross-resistance[35]. However, WHO recommends kanamycin or amikacin as drugs of choice and capreomycin is suggested in the case of STM, kanamycin and amikacin resistance[12].

The second-line drugs are advised to be applied in the ranking of thioamides, cycloserine (and terizidone) and then PAS. It is useful to combine drugs from these three chemically and functionally different groups. Thioamides expressed being more bactericidal, cheaper and less toxic than remaining others. They are used as anchors in regimens for patients with MDR- or XDR-TB, as well as cycloserine. PAS was recently reintroduced into therapy in connection with the expanding spread of drug-resistant mycobacterial infections. However, PAS has a very low effectiveness and a poor tolerability; nevertheless it plays an important role in the treatment of many MDR- and XDR-TB patients. In sum, the second-line drugs are substantially less effective than three previously reported groups of antimycobacterial agents[35].

Drugs in the fifth group (i.e. heterogeneous molecules that have showed a low effectiveness, a high toxicity or with no sufficient clinical evidence) should be used as minor or adjuvant drugs; each one should be counted as only half of one of the four

basic drugs needed to treat MDR- and XDR-TB. The following order has been advised according to their efficacy, adverse events and costs – clofazimine, amoxicillin/clavulanate, linezolid, carbapenems (imipenem or meropenem), thioacetazone and clarithromycin[35].

Amoxicillin/clavulanate or carbapenems may overcome the action of mycobacterial β-lactamases by two different ways – either by inhibition of these enzymes or by using an antibiotic, which is not a substrate for it; mycobacterial strains are sensitive *in vitro* and these drugs have been successfully used to treat some patients with MDR-TB, but this approach has met much scepticism. The possibility of using linezolid as a basal drug of choice in the management of MDR- and XDR-TB (even outside the group five) is severely hampered by its high cost and a high toxicity in the long term. Two main disadvantages of thioacetazone are only a bacteriostatic action and high toxicity, especially in HIV-infected patients. Clarithromycin is not very effective against *M. tuberculosis*, in contrast to atypical mycobacteria, and more information about its role in the therapy of drug-resistant TB is lacking[16,35].

In the treatment of MDR-TB patients, an intensive phase of at least eight months' duration is recommended, whereas the total treatment duration should be minimally twenty months. The WHO´s guidelines recommend continuing therapy for a minimum of 18 months after culture conversion. Extension of therapy to 24 months may be indicated in some cases. This new advice duration is two months longer than the minimum previously recommended (2008)[12,37].

Despite many studies, the treatment of MDR-TB is not still clarified and generally accepted, there remain some controversies and unclear questions[38].

3.1.5.4 Totally Drug-Resistant Tuberculosis

The situation has been recently becoming more serious with the development and notice of new forms of drug-resistant *Mycobacterium tuberculosis*; it is called totally drug-resistant (TDR) or super extensively drug-resistant tuberculosis. TDR-TB is defined as MDR strains that are resistant to all second-line drug classes (i.e. aminoglycosides, polypeptides, fluoroquinolones, thioamides, serine analogues and salicylic acid derivatives). This term was used firstly in the study from Iran published in 2009[39].

Authors reported that eight of 146 MDR-TB strains were extensively drug-resistant (5.4 %) and fifteen TDR isolates (10.3 %) were identified. In such cases, the smear and cultures remained positive after 18 months of medium treatment with second-line drugs

(ETH, PAS, cycloserine, ofloxacin, amikacin and ciprofloxacin). Additionally, the switch to amoxicillin-clavulanate or clarithromycin along with a high dose of INH (15 mg/kg) regimen was applied without any improvement for TDR cases. Twelve TDR strains were resistant to all first- and second-line drugs tested[39].

Aggressive treatment with second-line drugs has yielded a range of positive outcomes for XDR-TB cases, whereas the evolution and incidence of TDR-TB would return us to the preantibiotic era[40].

Although the term of TDR-TB is rather new, the reality of existence of such strains is older. Data from Russia demonstrate the existence of one case of totally drug-resistance as early as 1997, with three additional cases in the following year. It was described the resistance of some clinical isolates to up to ten drugs[40].

There were described microscopic morphological characteristics of TDR strains. Their cells contain an extraordinarily thick cell wall (up to 26 nm) resembling stationary or dormant phase bacilli. This cell wall type may effectively block the penetration of drugs[41].

Importantly, these facts about occurrence and bad treatment response of TDR-TB are alarming and bring both a significant deadly threat of virtually untreatable infection and urgently new challenges for the treatment and therefore for the development of new antimycobacterial drugs without cross-resistance with clinical used medicines.

3.1.6 Human Immunodeficiency Virus and Tuberculosis Co-Infection

Another problem connected with the spread of the tuberculosis is its coincidence with human immunodeficiency virus infection. The dual pandemics of tuberculosis and HIV called syndemics causes considerable morbidity and mortality; both diseases act synergistically to magnify their burdens[42]. This is an additional reason for the searching of new antimycobacterial agents. An ideal drug candidate should combine antimycobacterial and antiretroviral activity. Such a molecule may bypass some problems of the combined HIV-TB therapy, e.g. it tackles the consequences arising due to immune reconstitution and also minimizing of the pill burden could increase the patient compliance[43,44].

According to WHO data, TB is the leading cause of death among people infected with HIV – it causes almost one in four deaths. In 2010, 350,000 people died of HIV-associated TB. There were an estimated 1.1 million HIV positive new TB cases globally. Most of these patients live in sub-Saharan Africa. At least one-third of the 34 million people living with HIV worldwide are infected with TB. One way leading to the reduction of the burden

of TB among people living with HIV is isoniazid preventive therapy[45]. The two-fold higher fatality rate of TB in HIV-infected patients is caused due to more factors – e.g. a rapid progression of TB inflicted by immune failure, delayed diagnosis and treatment or higher occurrence of drug-resistant strains[42].

There was a widespread opinion that the tuberculosis had virtually been conquered by the early 1980s. This conviction was changed in the early 1990s, among others with the occurrence of miniepidemics of HIV-associated opportunistic TB in New York City. Since the early 1980s, infection with HIV, likewise increasing in prevalence, has emerged as the most important predisposing factor for developing overt tuberculosis in people co-infected with *M. tuberculosis*. The HIV infection enhances multiple the annual risk of developing active tuberculosis up to 8 %. The risk depends on the degree of immunosuppression. In addition, HIV bearers can be primarily infected or reinfected by the tubercle bacillus. It was demonstrated that HIV positive patients with tuberculosis are at a higher risk of dying[46]. On the other side, TB hastens HIV progression to AIDS by accelerating viral replication specifically and/or by inflammation[42,44].

Tuberculosis in HIV-infected people may have unusual clinical features and the diagnosis could be more difficult, e.g. due to a higher proportion of the extra-pulmonary manifestations, as well as the treatment is more complicated. Also adverse effects of the antituberculotics are more frequent and there is a large number of potential problems connected with concomitant drug combinations for dual infection by *M. tuberculosis* and HIV-1: high pill burdens, shared drug toxicities (especially nausea and gastrointestinal tract disturbance, peripheral neuropathy, cutaneous reactions, renal and liver toxicity), drug and drug-disease interactions, immune reconstitution inflammatory syndrome, co-morbid diseases, drug resistance and the treatment of latent TB infection. Certain drug combinations are contraindicated[44,46]. Unfortunately, in patients with both TB and HIV infections, especially those with low CD4 cell counts, *M. tuberculosis* develops most likely resistance to RIF during treatment, namely during the continuation phase[15]. A review reporting the molecular immunological mechanisms of HIV-TB co-infection was published[47].

On the other side, HIV *per se* is not a predisposing factor for the development of the multidrug resistance[46], however, WHO report[30] mentions a significant association between HIV and MDR-TB in some countries.

Growing HIV prevalence also increases the spread of non-tuberculous (atypical) mycobacterial infections, which are concerning the lungs, the lymphatic system, the skin or

the bones tissues or they are sometimes disseminated. These infections are more and more frequent among patients with HIV/AIDS. *Mycobacterium avium-intracellulare*, *M. xenopi*, *M. kansasii*, *M. fortuitum* and *M. chelonae* cause opportunistic infections, although these species are essentially saprophytic; they remain a serious challenge in the management of the immunocompromised host, including those infected with HIV[3,48,49].

3.2 Development of New Therapeutic Intervention, Especially against Multidrug-Resistant Strains

There exists an objective and indisputable need for the searching of new drugs towards mycobacterial infections. Some reasons are outlined in the chapter 3.1 – epidemiology, health and socio-economic seriousness of drug-sensitive tuberculosis, themes of latent TB and atypical mycobacteria infections therapy, as well as increasing emergence of drug-resistant forms of tuberculosis, especially MDR-, XDR- and TDR-TB and also coincidence of TB with HIV and AIDS.

Tuberculosis drug research has largely been declined from the 1960s due to introduction of RIF until the end of the 20th century. On the other side, last ten years is characterized by intensive searching for new antituberculosis agents[50]. The current research involves testing of new or transformed drugs, combinations of different drugs to shorten the therapy, a development of novel slow-release drug delivery systems that could reduce the frequency and an amount of drugs necessary during the treatment and the research of new molecular targets[16].

The searching for new novel molecules includes the chemical modification of existing drugs, the identification of drug targets, structure-based drug design and new types of structures. Besides chemotherapy immunotherapeutic approaches such as DNA vaccines, cytokines used in the combination with chemotherapy offer a promising prospect for the treatment of TB[16].

The treatment of MDR tuberculosis is quite challenging. There are four main goals of the development:

1) new agents to shorten and simplify therapy duration from current 6 – 9 months to two months or less. Compounds with sterilizing activity are requested,
2) development of new drugs to treat MDR-TB with a novel mechanism of the action to improve the efficacy and tolerability of the treatment without cross-resistance,
3) new molecules to improve the simultaneous treatment of TB and HIV among people living with HIV,
4) development of new drugs to improve the treatment of a latent TB infection[16,21].

Ideally, a new highly effective drug will achieve all goals. The acceptable product characteristics for new compounds are oral administration and favourable ADME properties, low likelihood of spontaneous mutation rate, *in vivo* activity towards both drug-sensitive and drug-resistant strains, administration once daily or less, safety with tolerable

side effects and toxicity, good clinical efficacy at six or less month administration, no serious drug-drug (especially with antiretroviral agents) and drug-comorbid disease interactions, minimal or no interaction with cytochrome P450. The relatively low cost is also imperative, especially with the respect to developing countries[22,36,50]. For MDR and XDR-TB life-rescue treatment may be these demands slightly more lenient[50].

The searching of perspective antituberculous agents consists of three main categories – investigation of novel drugs (TMC207, SQ109, LL3858), re-evaluation of present first-line TB drugs to optimize their efficacy (rifamycines) and already licensed drugs for other indications and "next-generation" compounds from established chemical classes being repurposed for TB. The engineering of existing scaffolds has brought many new candidates. During this process, chemical modifications are introduced into the core structure that may lead to improved antimycobacterial efficacy, safety, tolerability or superior pharmacokinetic properties. The progress was described in the fluoroquinolone group (nalidixic acid → ciprofloxacin → moxifloxacin and gatifloxacin), oxazolidinones (linezolid → PNU-100480 and AZD5847), nitroimidazoles (metronidazole → PA-824 and OPC-67683) or 1,2-ethylenediamines (EMB → SQ109)[8,51].

Until now, progress in the development of new antimycobacterial agents has been impeded by two main factors – the belief that there was a little need for new agents (due to unrealistic prognoses of total TB elimination) and the high cost of development with no clear guarantees of the return of invested sources. Pharmaceutical companies have not been interested in TB because the disease does not appear to be a major problem in industrialized nations[22].

There are some imperfections in the research and development pipeline that are impeding the discovery of new antituberculosis strategies[22]:

- basic research – targets and compounds identified through recent basic research are not being fully exploited,
- discovery – private companies are not willing to dedicate the screening resources or medicinal chemists to optimizing new compounds,
- preclinical development – companies do not have an interest in preclinical TB studies and the public sector has limited resources,
- process development/chemistry – development of appropriate manufacturing processes is inhibited by the lack of compounds available for scale-up, as well as the

unwillingness of pharmaceutical companies to dedicate process chemistry resources to TB chemotherapeutics,

- clinical trials – Phase III trials require an additional coordination, regulatory support and funding; however, they are irrelevant without promising novel compounds from preclinical studies,
- technology transfer – little commercialization activity is taking place because of the lack of novel compounds, pessimistic view of pharmaceutical companies of the TB market and concerns about toxicity associated with long-term use.

Their overcoming could help with the searching for novel therapeutic interventions.

Since monotherapy with a new agent would probably lead to the resistance emergency, the new drug should be added to an adequate regimen. However, this rational decision shields the individual contribution of this drug. It is difficult to distinguish the effect of single drug and its interactions with others[15].

The successful drug discovery process consists of next steps[22]:

- target selection – drug discovery programmes are aimed mostly at proteins essential to the bacterial function, especially targeting of the enzymes critical for the survival of latent mycobacterial population are welcomed,
- identification of the lead compounds based on high-throughput screening, known inhibitors or drugs, chemical intuition or *in silico* through the application of molecular docking algorithms,
- optimization of lead compounds,
- late discovery comprising formulation feasibility, pharmacokinetics (ADME) and pharmacodynamics, toxicology, safety pharmacology and intellectual property (patent) situation,
- preclinical development,
- process development/chemistry,
- clinical trials – Phase I, II and III,
- regulatory approval,
- technology transfer including postmarketing (Phase IV) studies.

The Global Alliance for TB Drug Development was established as a non-profit venture with the mission of accelerating discovery and/or development of new tuberculosis drugs[22].

The question if a new TB drug should produce broad-spectrum rather than narrow-spectrum agents is controversial. There are some arguments for both philosophies[22]:

- a carefully chosen broad-spectrum target allows the simultaneous development of therapeutics against multiple important pathogens, maximizing the efficient use of scarce resources,
- broad-spectrum agents stand a greater chance of development for diseases, such as TB, that might not financially inspire an independent programme,
- broad-spectrum agents are more likely to be produced in a large scale and therefore they have a higher probability of profitability,
- on the other hand, most broad-spectrum agents have a limited utility against *M. tuberculosis* because of its biochemical and physiological specificity,
- demonstrated efficacy of a broad-spectrum agent against tuberculosis might formally or informally restrict its use exclusively for TB,
- widespread use of broad-spectrum antibiotics in non-life-threatening human and agricultural applications might promote the development of drug resistance,
- broad-spectrum agents often disrupt normal symbiotic intestinal flora, thus increasing the potential for adverse side-effects.

WHO postulated inter alia next goals in the field of the novel antituberculosis drugs development until 2015[52]:

- a new four-month TB treatment regimen (including one new or repurposed drug approved by regulatory authorities for drug-sensitive TB) will be recommended by WHO and available for use,
- two new drugs will be approved for drug-sensitive TB,
- at least one new drug for the treatment of drug-resistant TB will be introduced into the market,
- a nine-month regimen for the treatment of drug-resistant TB (including at least one new drug) will be in a Phase III trial,
- a safer, higher-efficacy regimen will be available for treatment of latent TB infection,
- fixed-dose combinations for first-line drugs (including new drugs) will be available and in use.

In 2020, next progress should be achieved[52]:

- at least 1 – 3 month regimen (including one or more new or repurposed approved drugs) in clinical development,
- clinical trials for three new TB drug regimens for drug-susceptible TB (including one or more new or repurposed drugs) in progress,

- clinical trials for two new drug regimens for drug-resistant TB (including one new or repurposed drug) in progress,
- TB regimens compatible with antiretroviral therapy in late-stage clinical trials.

3.2.1 Searching for New Drugs against Multidrug-Resistant Tuberculosis

We reviewed the recent knowledge about development of new anti-MDR-TB drugs till the beginning of the year 2009 in our invited chapter Tuberculosis — The Development of New MDR-TB Drugs[16] in the book Drug-Resistant Tuberculosis: Causes, Diagnosis and Treatments[53], which was subsequently published due to offer from the publisher as separate publication Development of New MDR-Tuberculosis Drugs[54] with only minimal changes (**Appendix I**). This text was taken as a base of shorter review written in the Czech language and it was called "Advances in the Development of Antituberculotics Acting on Multidrug-Resistant Strains"[55] (**Appendix II**); for this purpose the original English text was translated and partially remade and simplified; the sourcing text is properly cited. There are presented some facts not included in these reviews or newer findings.

There are currently at least thirteen drugs in various stages of clinical evaluation for TB (see Table 4). Nevertheless, this progress is not still sufficient.

Table 4. Overview of clinical evaluated potential antituberculosis agents (freely according to ref.[18,51] and updated according to the website[56])

Drugs undergoing clinical trials as potential antituberculosis agents		
Drug	Target	Clinical trials phase
Novel drugs being developed for TB treatment		
TMC207	ATP synthase	II – III
SQ109	not exactly known, cell wall biosynthesis	II
LL3858 (Sudoterb)	unknown	II
Current first-line TB drugs being re-evaluated		
Rifampicin	RNA polymerase	II
Rifapentine	RNA polymerase	III
Repurposed drugs		
Gatifloxacin	DNA gyrase, DNA replication and transcription	III
Moxifloxacin	DNA gyrase, DNA replication and transcription	III
Linezolid	protein synthesis initiation complex	II
Metronidazole	unknown, probably cytochrome P450	II
Next generation repurposed drugs		
PNU100480	protein synthesis initiation complex	II
AZD5847	protein synthesis initiation complex	I
OPC-67683	multiple, cell wall biosynthesis	III
PA-824	multiple, mycolic acids and proteins syntheses	II

Here are characterized new facts about resurrected or repurposed known drugs or about new molecules affecting MDR-TB which are not involved in our review[16].

AstraZeneca's oxazolidinone AZD5847 ((*R*)-3-{4-[1-((*S*)-2,3-dihydroxypropanoyl)-1,2,3,6-tetrahydropyridin-4-yl]-3,5-difluorophenyl}-5-[(isoxazol-3-yloxy)methyl]oxazolidin-2-one; Figure 4) was originally intended as a broad-spectrum antibiotic agent; the antituberculosis activity was found later, similarly to linezolid. AZD5847 targets protein synthesis initiation complex. Two Phase I studies of AZD5847 have also been completed. It was evaluated for multiple dose safety, tolerability and pharmacokinetics when administered as an oral suspension over 14 days in healthy volunteers[36,51].

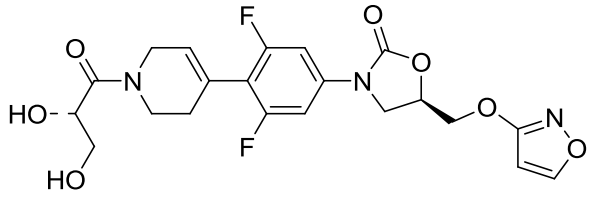


Figure 4. AZD5847

Another oxazolidinone, RBx 8700 ((*S*)-*N*-[(3-{3-fluoro-4-[4-(5-nitrothiophen-2-yl)piperazin-1-yl]phenyl}-2-oxooxazolidin-5-yl)methyl]acetamide; Figure 5) has demonstrated possessing potent and concentration-dependent activity *in vitro* against all slowly growing mycobacteria including MDR-TB strains as well as intracellular activity in infected macrophages. RBx 8700 exhibited MIC 0.25 – 1 μg/mL against MDR *M. tuberculosis*, whereas its MIC against single drug-resistant *M. tuberculosis* ranged from 0.032 to 0.5 μg/mL with lacking cytotoxicity[57,58].

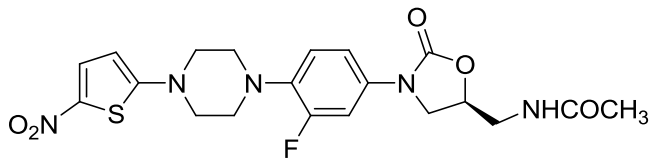


Figure 5. RBx 8700

For many years, the lack of activity of β-lactams against TB was thought to be due to their poor penetration into mycobacteria with β-lactamase-mediated resistance being only a minor factor[8]. Later, β-lactams have not been used widely because of the presence of highly active major chromosomally encoded β-lactamase BlaC hydrolyzing these agents; some of them have been evaluated with the result penicillin being inactive, while amoxicillin/clavulanate and ampicillin/sulbactam have exerted a partial effect. β-Lactams are only rarely used in the TB treatment[59,60]. However, the usage of agents, which are either poor substrate of this enzyme like carbapenems, or combination of β-lactams with

inhibitor of the enzyme, seems to have a potential benefit. Moreover, carbapenems target L,D-transpeptidases, until now believed to be non-sensitive[36].

Other potentially hopeful drugs with reported activity against XDR-TB are meropenem (Figure 6) with clavulanic acid (clavulanate; Figure 7). A co-administration of clavulanic acid, an irreversible β-lactamase inhibitor superior to both sulbactam and tazobactam, with a second-generation carbapenem meropenem was shown to have a potent activity (a sterilizing effect was observed within 14 days) *in vitro* under aerobic as well as anaerobic conditions mimicking the persistent state against both drug-susceptible and drug-resistant *M. tuberculosis*. This combination is favourable due to double sites how to overcome the hydrolyzing of β-lactame ring. Administration of any carbapenem with clavulanic acid reduced MIC of the carbapenem from 8 – 16 to 1 – 4 μg/mL or less. It inhibited the growth of XDR-TB at the same levels as drug-susceptible strains. *In vivo*, there is an evidence of the efficacy of these combinations, even for XDR-TB patients. Nevertheless, the necessity of parenteral application hampers partly the wide use; therefore an orally bioavailable penem is desired and it would have even greater potential value[36,59,60]. Interestingly, meropenem alone was described to act as an inhibitor of the BlaC; this activity might cause the enhanced activity of clavulanic acid against this enzyme when applied both drugs concomitantly[60].

The addition of clavulanic acid to various *M. tuberculosis* strains including resistant ones has shown to potentiate the effects of all classes of β-lactams. Clavulanic acid at the concentration of 2.5 μg/mL had only a modest effect on the MIC values of ampicillin and amoxicillin, but a significant effect on cephalothin, imipenem and meropenem[60].

New β-lactamase inhibitor NXL104 (sodium (2*R*,5*S*)-2-carbamoyl-7-oxo-1,6-diazabicyclo[3.2.1]octan-6-yl sulphate; Figure 8) may be of interest since it is a non-β-lactam β-lactamase inhibitor and is reported to be more potent than clavulanic acid or tazobactam[36].

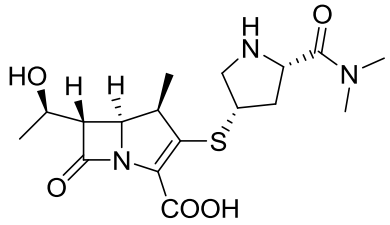


Figure 6. Meropenem

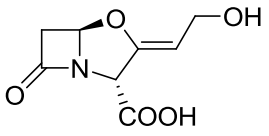


Figure 7. Clavulanic acid

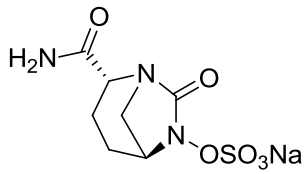


Figure 8. β-Lactamase inhibitor NXL104

Although still in the preclinical phase, 1,3-benzothiazin-4-ones are an extremely potent bactericidal class of antimycobacterial agents. *S*-Isomer of 2-(2-methyl-1,4-dioxa-8-azaspiro[4.5]decan-8-yl)-8-nitro-6-(trifluoromethyl)-4*H*-benzo[*e*][1,3]thiazin-4-one, BZT043 (Figure 9), exhibited very low MIC against *M. tuberculosis* from 0.001 μg/mL (2.3 nM) and *in vivo* it was active, too. Benzothiazinones target decaprenylphosphoryl-β-D-ribose 2´-epimerase necessary for the biosynthesis of arabinogalactan. The proposed mechanism assumes a reduction of the nitro group to a nitroso which reacts with the cysteine fragments of this enzyme[18,36,61]. This inhibition abolishes the epimerization of decaprenylphosphoryl ribose to decaprenylphosphoryl arabinose, a key precursor for the synthesis of the cell wall arabinans, thus provoking cell lysis[61]. BTZ043 maintained a similar potency against drug-sensitive as well as MDR and XDR isolates and showed the intracellular activity within macrophages[7,18]. Subpopulations with reduced metabolism were less sensitive. BTZ043 is a candidate for a combination therapy of all forms of TB[61].
Also 3,5-dinitrobenzamides were identified being very potent inhibitors of this enzyme with similar mechanism of the action and results. *N*-[2-(Benzyloxy)ethyl]-3,5-dinitrobenzamide (Figure 10) and *N*-[2-(4-methoxyphenoxy)ethyl]-3,5-dinitrobenzamide (Figure 11) were assayed being superior with MIC against drug-susceptible, single drug-resistant, MDR and XDR strains in the range of 0.02 – 0.38 μM; paradoxically, resistant ones showed a higher sensitivity[62].

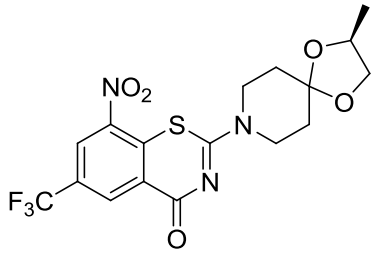


Figure 9. BZT043

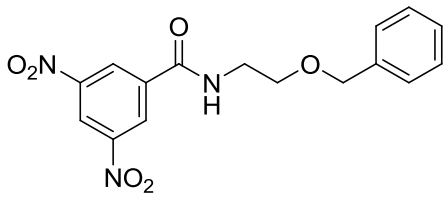


Figure 10. *N*-(2-(Benzyloxy)ethyl)-3,5-dinitrobenzamide

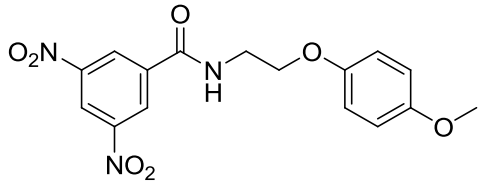


Figure 11. *N*-(2-(4-Methoxyphenoxy)ethyl)-3,5-dinitrobenzamide

DC-159a (7-((*S*)-7-amino-7-methyl-5-azaspiro[2.4]heptan-5-yl)-6-fluoro-1-((2*S*)-2-fluoro-cyclopropyl)-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; Figure 12) is a newer broad-spectrum 8-methoxyfluoroquinolone with a promising antimycobacterial *in vitro* potency even manifold surpassing levofloxacin, moxifloxacin and gatifloxacin against both drug-sensitive and resistant *M. tuberculosis* (MIC of 0.06 μg/mL) with retained activity towards quinolone-resistant ones with MIC_{90} up to 0.5 μg/mL. DC-159a inhibits a growing phase, non-replicating mycobacteria and nontuberculous mycobacterial species. The order of susceptibility of atypical mycobacteria was *M. kansasii* > *M. fortuitum* > *M. avium* > *M. intracellulare* > *M. chelonae* > *M. abscessus*. This compound is still in the preclinical development stage[36,63]. DC-159a pharmacokinetics shares a similarity with moxifloxacin, although it is more active than moxifloxacin not only *in vitro*, also in the initial and continuation phases of the treatment of murine TB model. DC-159a may be useful for shortening the duration of TB treatment[64]. Another quinolone now in the advanced preclinical evaluation is TBK-613[28].

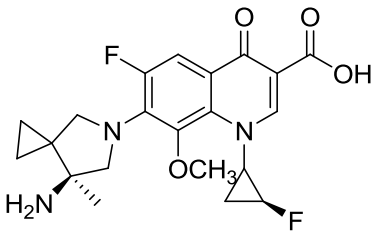


Figure 12. DC-159a

A novel highly active bacteriostatic tetracycline tigecycline with a broad spectrum was determined for its activity on fifty clinical isolates of *M. tuberculosis* with different resistance profile including twenty MDR-TB strains. Tigecycline produced MIC values ranging from 8 to 64 μg/mL. Although no break point value is defined, it disappointed expectations because of poor *in vitro* effectiveness, in contrast to e.g. oxazolidinones. Some rapidly growing nontuberculous mycobacteria are more susceptible[65].

There has been shown some evidence that cotrimoxazole (trimethoprim-sulfamethoxazole) inhibits the growth of *M. tuberculosis* including MDR-TB strains. In all four MDR isolates, at least 80% of growth was inhibited at MIC of ≤ 1/19 μg/mL of trimethoprim-sulfamethoxazole; virtually, two strains of them were susceptible to the concentration of ≤ 0.5/9.0 μg/mL (MIC_{99})[66].

Other known clinically used drug with the reported promising anti-MDR-TB activity is azole antimycotic agent econazole. MIC_{90} and minimum bactericidal concentration

(>99.99) against all evaluated MDR strains were found being very low, 0.120 – 0.125 μg/mL and 0.125 – 0.150 μg/mL, respectively, despite of resistance patterns. These results indicate multiple targets for econazole in *M. tuberculosis*, as it has been shown by *in vitro* binding to various cytochrome P450 enzymes[67].

An unconventional approach to improve the tolerability of thioamides used in MDR-TB treatment is based on the co-administration of positive regulators of their activation. Thioamide prodrugs are activated by monooxygenase EthA; the production of this enzyme is physiologically repressed. When repressor protein function is abolished by the presence of known inhibitors BDM31343 (3-oxo-3-{4-[3-(thiophen-2-yl)-1,2,4-oxadiazol-5-yl]piperidin-1-yl}propanenitrile; Figure 13) or 2-phenylethyl butyrate (Figure 14), an increased sensitivity of *M. tuberculosis* and *M. avium* to ETH was described; better tolerability may be another advantage[18,68].

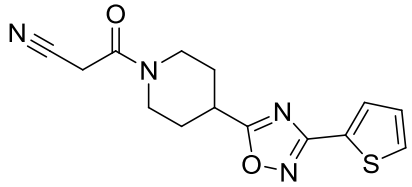


Figure 13. BDM31343

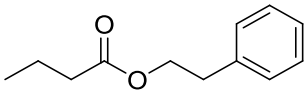


Figure 14. 2-Phenylethyl butyrate

The synergistic drug combination may also bring a progress in the TB therapy. Spectinomycin, an aminocyclitol with only weak intrinsic antimycobacterial potency, was shown to share synergy with RIF, macrolides and azalides, azoles, telmisartan, clioquinol or butyrophenone antipsychotics. Additionally, butyrophenones, telmisartan, azoles and clioquinol may promote the antimicrobial activity of capreomycin, clofazimine or RIF[69].

Protease inhibitors, topoisomerase I, malate synthase, gyrases, folate, RNA polymerase, energy metabolism, menaquinone, InhA inhibitors and diarylquinolines, riminophenazines, nitroimidazoles or pyrazinamide analogues belong to other intensively studied chemical groups of TB Alliance portfolio[36].

3.2.1.1 Targeting

The current first-line TB therapy affects only a very few essential functions – mycolic acid biosynthesis (INH), RNA synthesis (RIF), FAS I (PZA), arabinogalactan and lipoarabinomannan biosynthesis (EMB), proteosynthesis (STM) or damages membrane proton motive force (PZA). Identification of new unique pathways that are required for the bacterial growth and persistence should provide additional novel targets for the rational drug design with the respect of the goals of the novel drug development[36]. The knowledge

about complete genome sequence information of *M. tuberculosis* and the broad spectrum of molecular biology and genetics tools and methods enables and supports searching for new targets. These advancements have been expected to bring satisfactory outcomes in the discovering of new drugs almost immediately; unfortunately this has not been yet fulfilled[7,8]. Genome-derived target-based approaches have had little success in the discovering of antibacterial agents in general. Of course, the essentiality of a target is indispensable, but it does not ensure its drugability. We are still not able to identify inhibitors with drug-like properties for many essential targets. Isocitrate lyase may represent such one; some its inhibitors have been found but without drugability[8].

There exist many of potential and verified targets in mycobacteria cells, for some of them are known inhibitors. An overview of mycobacterial enzymes as perspective targets bring e.g. reviews of Lamichhane[7], Kaneko et al.[36], Mdluli and Spigelman[70] and some of them, which inhibitors were verified for drug-resistant TB, are reported in our review[16]. Some functions or pathways seem to be more important and convenient, especially those being unique for mycobacteria and not present in eukaryotes – e.g. glyoxalate shunt, gluconeogenesis (especially phosphoenolpyruvate carboxykinase, which inhibition attenuates virulence), ATP synthesis and electron transport chain or cholesterol metabolism necessary for the survival of *M. tuberculosis* in infected macrophages and for the maintenance of persistence infection[36].

Targeting on the unique mycobacterial cell wall biosynthesis may be focused on the synthesis of its three main components – peptidoglycan (e.g. alanine racemase and D-alanine-D-alanine ligase affected by cycloserine), arabinogalactan (arabinofuranosyl transferase, ribosyltransferase, decaprenylphosphoryl-β-D-ribose 2´-epimerase, UDP-galactopyranose mutase, galactofuranosyl transferase or dTDP-6-deoxy-L-lyxo-4-hexulose reductase producing dTDP-rhamnose) and mycolic acids (e.g. enzymes of FAS I and FAS II like InhA, target of INH, FabH, MabA, even methyltransferases blocked by *S*-adenosylmethionine analogues, Pks13, acyl-AMP ligase, acyl-CoA carboxylase, cyclopropanation process etc.)[7,70].

When the synthesis of some amino acids is deleted, *M. tuberculosis* infection will be attenuated. Targeting amino acid biosynthesis due to inhibition of many enzymes like dihydrodipicolinate reductase, acetolactate synthase, ATP phosphoribosyl transferase, aminotransferases or shikimate pathway is attractive for antibiotic development. Targeting cofactors biosynthesis represents other site of the action. Folate derivatives are cofactors utilized in the biosynthesis of essential molecules including purines, pyrimidines and

amino acids. Dihydropteroate synthase and dihydrofolate reductase function elimination disrupts these processes. Targeting of pantothenate or riboflavin pathways enzymes (pantothenate kinase and lumazine synthase, respectively) may be promising, as well as adenosine-5´-phosphosulphate reductase catalyzing essentially the first step of reductive sulphur assimilation. Mycothiol, which protects mycobacteria from the toxicity of antibiotics and oxidative stress, menaquinone, non-mevalonate terpenoid pathways, nucleic acids biosynthesis established novel potential enzymatic sources for drug-mediated inhibition, for example ribonucleotide reductases, thymidinemonophosphate kinase, DNA ligases or topoisomerase II (gyrase; the site of quinolone action). Some regulatory proteins have been shown to be essential for the growth of *M. tuberculosis*. Their knock out would produce a pleiotropic disruption of whole network of proteins under the influence of the regulator[7,70]. Proteosynthesis might be affected by the inhibition of single enzymes such as deformylase or methionine aminopeptidases which remove formylated methionine at the amino terminus of translated proteins[7].

Another enzyme being on the interest is maltosyltransferase GlgE essential for the utilization of maltose-1-phosphate to elongate 1,4-glucan chains. The accumulation of maltose-1-phosphate will be toxic, "self-poisoning" for cells[7].

Targeting of dormant mycobacteria is an important challenge with theoretically great merits. Inhibition of isocitrate lyase, proteasome complex, L,D-transpeptidase, DosR (DevR) or CarD may be such potential targets[7].

The glyoxylate shunt of *M. tuberculosis* encodes the glyoxylate cycle, an anaplerotic pathway that bypasses the tricarboxylic acid cycle and replenishes tricarboxylic acid cycle intermediates during growth on fatty acids. It is composed of two enzymes, isocitrate lyase(s) (ICL) and a malate synthase. The loss of both Icl1 and Icl2 results in the grow stop on fatty acids and unables to establish either chronic or acute infection. While mycobacteria utilize fatty acids as a carbon source during an infection, these enzymes are ideal candidates for the development of treatment shortening drugs[36,71]. While the isocitrate lyase inhibition may be a perspective target for combat to latent form of TB, we summarized recent advances about its inhibition especially by newly synthesized compounds. The review entitled "Advances in Mycobacterial Isocitrate Lyase Targeting"[72] (**Appendix III**) is now processed for Medicinal Research Reviews.

The inhibition of proteasome activity increased the susceptibility of mycobacteria to reactive nitrogen intermediates produced by macrophages during infection; limited virulence and decreased proliferation being as results. Probably the proteasome complex is

responsible for the degradation of toxic oxidized proteins. It is targeted by oxathiazol-2-ones at the catalytically active threonine residues; these molecules expressed an activity against a non-replicating population[7].

A loss of L,D-transpeptidase attenuates the ability of *M. tuberculosis* to persist, grow and be virulent. This enzyme catalyzes crosslinkages generating in the peptide chains of the peptidoglycan layer in different way than classical D,D-transpeptidases affected by β-lactams. DosR (DevR) is produced in a higher amount during inconvenient conditions. This molecule binds to the DNA major groove, although it is not essential. Increased nitric oxide or hypoxia are sensed and transduced *via* DosR (DevR), which leads to the physiological adaptation; it could serve as a dormancy regulator. CarD interacts with RNA polymerase β-subunit and regulates stringent response, it is necessary for viability and its depletion blocks the mycobacterial proliferation[7].

3.2.2 Some Hot Topics of the New Therapeutics Development

3.2.2.1 Shortening of the Therapy and the Treatment of the Latent Tuberculosis

This aim requires an introduction of at least one new molecule into standard regimen; the improved activity against persisting *M. tuberculosis* should be included to a quicker eradication of the infectious mycobacteria.

Of greatest importance there are new drugs that shorten the overall duration of therapy. Although four-drug regimen recommended for most forms of TB requires six months of treatment, most of the benefit from treatment comes during the first two months. During this time, the bacterial burden is greatly reduced. The next continuation is required to eliminate persisting bacilli and decrease the risk of relapse. A potent sterilizing drug that shortens treatment to two months or less will be of a great benefit[22].

For example, bactericidal fluoroquinolones like moxifloxacin may be such a promise agent due to its good activity against persistent forms. The most convenient combination of moxifloxacin, RIF and PZA reduced the time needed to eradicate *M. tuberculosis* from the lungs of infected mice by up to two months when compared with the triple combination of INH, RIF and PZA. The findings suggest that this regimen has the potential to short the duration of therapy needed to cure TB[73], as well as moxifloxacin, rifapentine and PZA surpassed the regimen with replaced moxifloxacin by INH at 10 – 12 weeks. Its addition to the standard regimen improves the efficacy of culture conversion from 61 % to 82 % after six weeks[15]. Also ofloxacin or gatifloxacin can be used to shorten the duration of the TB

treatment[20]. Gatifloxacin and moxifloxacin are tried to replace EMB in the standard regimen[50]. If the phase III trials demonstrate safety and efficacy, a 4-month, fluoroquinolone-based treatment for drug-sensitive tuberculosis could be registered for use by 2015[51].

Approved azole antimycotic agents econazole and clotrimazole have also been shown to be effective against persistent/latent TB *in vitro* at ≥ 0.6 μg/mL as well as in a murine model[74].

Another possibility brings nitroimidazole PA-824, now in clinical testing, which displayed significant bactericidal activity against non-replicating persistent mycobacteria. The combination of PA-824, moxifloxacin and PZA was superior to the standard regimen of INH, RIF and PZA[20]. The mechanism of the action responsible for the killing of non-replicating organisms consists in generation of reactive nitrogen intermediates by one metabolite[15]. Both PA-824 and OPC-67683 also displayed the ability, when combined with the RIF and PZA, to decrease the time to sterilization of the lungs by at least two months[50,51].

TMC207 exhibited a very good late bactericidal activity superior to RIF, which suggests possible killing of non-replicating mycobacteria leading to the potential to reduce the continuation phase length[15].

New rifamycine derivatives (like rifapentine) may play potentially an important role in the reduction of the time necessary for the complete cure[20]. Daily or three times weekly rifapentine may dramatically shorten the treatment course, when combined for 10 – 12 weeks with PZA and either INH or moxifloxacin. It suggests that more frequently dosed rifapentine may be more active against persistent mycobacteria than RIF[15]. It has been investigated if high-dosed RIF might contribute to shortening the treatment with the conclusion that higher than standard RIF dosing improved culture conversion rate. High-dosed rifampicin is being investigated in several clinical trials because it has become clear that the optimal dose was never established; higher doses of the rifamycins have the potential to further shorten the duration of TB treatment. However, it should be necessary to examine the safety, pharmacokinetics and efficacy because of possible adverse effects[15,36,51].

Dormant *M. tuberculosis* is inhibited at least partly by other antituberculosis drugs – amikacin, capreomycin and metronidazole. The four-combinations of RIF, moxifloxacin, metronidazole and amikacin/capreomycin were assayed having a significant activity against different populations of dormant mycobacteria[75]. The role and potency

of metronidazole against non-replicating subpopulation of *M. tuberculosis* is still unclear and controversial[15].

Thirty one established antimicrobial agents were determined for their activity to non-replicating mycobacteria. Except drugs reported previously in this chapter, most of the agents acting on the 30S ribosome (aminoglycosides and polypeptides, fusidic acid) demonstrated either modest or potent activity (without minocycline). In contrast, among agents affecting 50S subunit of ribosome, only 11,12-carbazate-substituted macrolide RU66252 demonstrated a potent activity, whereas linezolid, lincomycin, clindamycin and partly clarithromycin avoided any activity. Salicylanilide derivative niclosamide exhibited a very good activity against non-replicating mycobacteria being markedly more active than metronidazole, furazolidone and nitrofurantoin. Also clofazimine displays a potent activity. Cell wall inhibitors (INH, EMB, vancomycin, tazobactam, cycloserine, thioacetazone) were inactive, although very active against growing mycobacterial subpopulation[76]. Nitazoxanide, a known salicylamide agent with a potent activity against various viruses and anaerobic bacteria[77], was described to kill non-replicating mycobacteria[78].

3.2.2.2 Immunotherapy

The potential of the immunotherapy in the treatment of mycobacterial infections now deserves greater consideration to improve the therapy especially of drug-resistant strains. Mycobacteria impair the host immune response and the incorporation of immunomodulators in the treatment of TB may be quite a bit advantageous option. It has been suggested that *M. tuberculosis* is pathogenic not because it fails to elicit a sufficient Th1 response, but rather that the Th1 response is inhibited by an inappropriate Th2 component, which could act to weaken the protective response resulting in failure to control bacterial growth and leading to immunopathology[79].

The cytokines are very powerful immunomodulators and different studies have shown their involvement during mycobacterial infection. Th1 response, the intracellular cell mediated immunity, is vital for protection against TB because mycobacteria are intracellular pathogens. It implicates that cytokines promoting Th1 response like interleukin-12 (IL-12) should provide a better protection. During TB infection, IL-12 promotes the secretion of proinflammatory cytokines of interferon-gamma (IFN-γ), tumour necrosis factor α (TNF-α) and granulocyte macrophage colony-stimulating factor, all of them polarise the immune response to Th1. IL-12 plays a crucial role in the resistance to mycobacterial infection. The exogenous administration of IL-12 in mice showed the enhanced resistance

towards the intravenously infection of *M. tuberculosis*. However, non-specific mode of the action, tissue toxicity and induction of autoimmunity are two major disadvantages limiting the therapeutic use of IL-12 besides a high cost[19,79].

IL-2 based therapy is aimed at increasing immune activation and therefore enhancement of the mycobactericidal response, as demonstrated for mice where exogenous IL-2 inhibits mycobacterial growth, possibly *via* macrophage activation or the development of cytotoxic T cells[79]. IL-10 is an immunosuppressive Th2 cytokine which down-regulates Th1 activation. Anti-IL-10 receptor antibody has been shown to inhibit IL-10 activity *in vivo*[79]. The elevation of IL-4 has been implicated as a potential virulence factor and its higher levels correlate with mycobacterial load. Additionally, IL-4 may play a role in tissue destruction and/or cell death during *M. tuberculosis* infection[80]. When mycobacteria infect the macrophages, the immune response is switched and thereby able to survive within the cell. The murine macrophages showed an increased production of IL-10 which suppresses the production of IL-12[19]. There is the second way how to intervene into immune response, i.e. inhibition of immunosuppressive cytokines which reduce macrophage activity[79].

IFN-γ is another crucial immunomodulator. It facilitates elimination of mycobacteria *via* activation of macrophages. Defects in the IFN-γ functions result in an extreme susceptibility to mycobacterial infection. A number of clinical trials as an adjunct to chemotherapy for pulmonary TB have been undertaken, although with variable outcomes. Aerosolized IFN-γ and subcutaneous administration of IL-2 have been shown to have a bacteriologic effect in patients with MDR-TB and clinical trials of these agents are underway[19,22,79]. The addition of IFN-γ aerosol to failing second-line therapy led to a significant improvement of symptoms and clinical markers at MDR-TB patients[15].

TNF-α displays a central role in the host response to *M. tuberculosis*. TNF-α activates macrophages to liberate nitric oxide synthase-2 which is responsible for killing the intracellular mycobacteria. The TNF-α deficient mice showed a higher susceptibility towards TB[19,79]. TNF-α has also been shown to be responsible for much of the resultant immunopathology, thus emphasizing the insecure balance between protective immunity and immunopathology[79]. Supporting this claim, TNF-α is thought to be associated with fever, necrosis and weight loss. In HIV-infected patients, TB leads to excess production of TNF-α, which in turn might accelerate HIV replication and hasten the development of AIDS[22].

Another way of the immunotherapy is application of simple molecules or preparations capable of doing the immunomodulation task as adjunct therapy. They are cheaper, without

potential to cause potentially serious adverse effects. This group of agents is represented by ATP and its analogues, picolinic acid, imidazoquinoline S28463, an analogue of imiquimod, diethyldithocarbamate, galactosylceramide, calcitriol and other vitamin D derivatives, glutathione and its precursor *N*-acetylcysteine, Chinese traditional medicines etc. Mechanisms of the action are unique and complex for each agent[19,79]. Dipeptide γ-glutamyltryptophan (Figure 15) boosts the patient's immune response to promote clearance of the bacteria. This molecule might represent a useful component of a combination therapy because of avoiding the mycobacterial resistance spread due to indirect mechanism of the action[18,81].

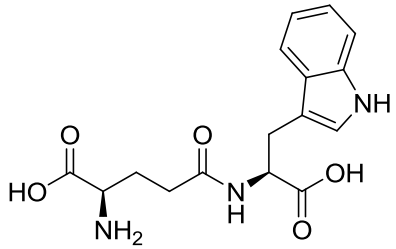


Figure 15. γ-Glutamyltryptophan

Immunotherapy could be realized by mycobacteria, especially non-pathogenic environmenal saprophyte *Mycobacterium vaccae* species. It can protect against TB by inducing Th1 recognition and *M. vaccae* has been found to have extremely potent immunological effects including down regulation of Th2 activity. Early studies suggested that its use as an adjunct to antituberculosis drugs is beneficial; however newer findings have been conducted with seemingly conflicting outcomes. Overall, the greatest benefits of *M. vaccae* appear when drug treatment was suboptimal or patients have a chronic or drug-resistant disease[79].

Further clinical established immune modulators are corticosteroids as inhibitors of proinflammatory cytokines, particularly TNF-α. Their use as an adjunctive therapy for TB is well documented for extra-pulmonary forms, where they have been shown to reduce the inflammatory response, reduce fibrosis and improve survival. More recently, corticosteroids have been shown to be beneficial with concomitant antituberculous chemotherapy because they may improve drug penetration into the infected tissues and reduce the conversion of the bacterial population in the latent form. Similarly, thalidomide as immune regulating agent may be of potential use as an adjunct to the treatment of extra-pulmonary or severe TB[79].

Another therapeutic intervention consists of application of antibodies, although the concept of antibody involvement in response to intracellular pathogens remains somewhat

controversial. However, antibody may play a role in the protective response to *M. tuberculosis* e.g. at the time of initial infection and during death of infected cells[79].

Serum therapy and use of polyclonal preparations has led to inconsistent outcomes. Most investigations into serum therapy for TB have used immune serum, raised in animals by immunizing with mycobacteria or their components. In sum, antibodies to mycobacterial polysaccharides may affect the course of infection. Mycobacteria-specific antibodies are capable to enhance both innate and cell-mediated responses to mycobacterial infection. Polyclonal antibody preparation (human gamma globulin) application resulted in reduced colonization of the lungs. Passively administered monoclonal antibodies have provided an increasing body of evidence that antibody; possibly *via* interfering with some extracellular stage of infection, they can influence the fate of the pathogen[79].

3.2.2.3 Vaccine Development

Though the topics connected with vaccination are an integral part of immunotherapy, they were earmarked into a separate part due to their importance and therapeutic and/or preventive perspectives.

Some problems with current BCG vaccines were mentioned at chapter 3.1.2. In order to have a long-lasting protective immunity, new vaccines should combine selected antigens with potent adjuvants to stimulate the appropriate immunological pathway. An increase in Th1 response and suppression of Th2 response are requested for mycobacteria. The ideal vaccine would contain immunogens inducing the formation of granulomas without necrosis. TB immunity is local and the consequences of a lesion depend on the nature of attracted T cells to the lesion. If the attracted cells are Th2, there is a lesion-necrosis evolution and disease progression; if, however, the attracted cells are Th1, the bacteria are destroyed, there is a granuloma formation and both lesion and disease suffer from regression[3]. However, clinical trials of new vaccines are very expensive.

It is necessary to turn BCG or mycobacterial molecules more immunogenic. Even though BCG was originally developed for oral immunization, this delivery route was gradually replaced to its current intradermal use among most nations. An effort was retained on research of the BCG Moreau Rio de Janeiro oral vaccine suggested being extremely immunogenic and causing negligible side effects. Anyhow, in order to develop alternative mycobacterial vaccines to BCG, it becomes necessary to identify all species of the

Mycobacterium genera that can induce an appropriate and being favourable to cloning and expression of a wide spectrum of antigens[3].

DNA vaccine is a popular area, where microbial DNA sequences can be used as target vaccines. Once injected into animal muscle cells, the plasmid is transcribed to RNA and the cells express the recombinant protein. DNA vaccines expressing a range of mycobacterial antigens combination have been found to be protective against subsequent mycobacterial infection when administered prophylactic; when administered post-infection, they may boost the production of protective T cells and thereby to improve the ongoing immune response. DNA vaccination can convert an inefficient bacteriostatic response to an effective bactericidal response and is found to booster a protective Th1 cellular response. DNA vaccines have used the mycolyl-transferase Ag85 enzyme which has demonstrated promising results in short and long term assays and the heat shock proteins from *M. leprae* showed to be effective in both prophylactic and immunotherapeutic form but when derived from *M. tuberculosis*, they showed no protection. Since one third of the world population is infected with *M. tuberculosis*, two types of vaccines seem to be necessary: one to prevent the invasion of the pathogen and the other to eradicate the already present infection. The administration of DNA vaccine in combination with conventional chemotherapy might stimulate the immune response to eliminate persistent bacilli[3,79].

Although immunotherapy utilizing crude extracts of *M. tuberculosis* is not plausible, as it results in necrosis and disease exacerbation, a recent study has assessed the efficacy of a fragmented *M. tuberculosis* cell formulation as a vaccine following chemotherapy in a murine model of chronic tuberculosis. This vaccine is composed of fragmented *M. tuberculosis* cells, which have been detoxified and are delivered in liposomes. Three doses administered two weeks apart following chemotherapy showed to have bactericidal activity, reducing the bacterial burden when compared to chemotherapy alone. It may boost the existing immune response and induce new responses against other mycobacterial antigens which may favour bacterial elimination, moreover without any toxicity[79].

3.2.2.4 Improvement of Drug Delivery Systems

One attitude how to improve compliance with the anti-TB treatment consists in the reducing of the daily pill burden if shortening of the therapy is unavailable. This goal is achievable by the incorporating sophisticated formulation techniques. Application of micro or nanoparticles can greatly enhance the bioavailability of used drugs and thereby reduce

the frequency of dosing, even up to intermittently once weekly or every ten days when drug reservoirs with slowly releasing will be gained[19]. Besides sustained release and doses reducing, this formulation might bring other potential advantages like the possibility of various application routes, reduction in the adverse effects, drug interactions or/and targeting drug-resistant and latent bacteria or specific sites and tissues; thus also improved therapeutic index. The choice of carrier, large-scale production (e.g. residual organic solvents from the syntheses could be a potential problem), stability and toxicity of the formulation are some of the major issues that merit immediate attention and resolution. INH, PZA, RIF, EMB, ETH, quinolones or clofazimine separately or in the mutual combinations are antituberculosis drugs with reported such evaluated drug delivery forms compositions. Oral and parenteral route of application have been enabled including inhalation. Interestingly, STM has also been formulated into an oral suitable form by nanoencapsulation[82,83].

Many synthetic as well as natural materials are used to prepare these particles – poly (DL-lactide-co-glycolide), poly glycolic acid, poly lactic acid, poly (methyl methacrylate), poly (vinylchloride), poly (ε-caprolactone), Eudragit, *N*-methylpyrrolidone, poly (vinylalcohol), liposomes, polyalkylcyanoacrylates, solid lipids, alginic acid, carbomers, chitosan, gelatine, cyclodextrines etc. The particles in the size range of 1 – 10 μm are predominantly taken up by macrophages and the incorporation of the immune modulator can enhance the effectiveness. There exists an intention of expedient specific targeting with antibodies against the specific macrophage surface markers and the mannose receptor[19,82,83]. Polymeric drug carriers can be attached to cytoadhesive ligands like lectins binding to epithelial surfaces through specific receptors. The choice of appropriate excipients can help to address nano or microparticles to the brain to treat cerebral form of TB[82]. Nanoemulsions, water nanoparticle suspension, micelles, niosomes, dendrimers represent further described drug delivery systems for antituberculosis drugs[83].

Direct pulmonary delivery represents another challenge and it has been studied largely. For example, yet various respirable forms of RIF comprise nano or microparticles, liposomal form, liquid and dry powder inhalation formulations[84]. Inhalable nanoparticles seem to be better for mucosal adherence, particle(s) delivery, high lung bioavailability, rapid absorption, no first pass effect and slow clearance resulting in reduced systemic side effects. Additionally, nanoparticles are efficiently taken up by phagocytic cells, alveolar macrophages which maintain *M. tuberculosis* infection. The direct administration to epithelial cells enables the lowering of drug doses. PAS and capreomycin are drugs

formulated for pulmonary delivery[82,83]. However, a more sceptic point of view was already published[85]. There are some impeding factors of the inhaled TB therapy – lack of access of inhaled particles to poorly-aerated areas of the lung (problematic especially for aminoglycosides), limited penetration through biofilm formed by extracellular bacilli, selection of resistant mycobacteria due to exposure to low concentrations of antimycobacterial agents, induction of undesired immunopathological reactions in the airways and anomalies in antigen processing and presentation of vaccines delivered directly to the lungs. Despite of these problems and fortunately, there are ways how to overcome the above mentioned factors.

3.3 Examples of the Biological Activity of Salicylanilide Esters

3.3.1 Some Notes about Salicylanilide Activity

Salicylanilides (2-hydroxy-*N*-(phenyl)benzamides; Figure 16) are a group having a wide range of biological activities; they have been studied on the interest of medicinal chemistry for a long time for many interesting effects[86]. Closantel, rafoxanide, niclosamide and salantel are established agents in human and/or veterinary medicine belonging to the most known members of this group. For example, salicylanilides act as uncouplers on biomembranes, affect productions of interleukins and regulate an immune response, show analgesic and anti-inflammatory properties, influence ion channels. Moreover, salicylanilides affect more molecular targets with the chance to be potentially useful in cancer therapy[77,87]. Some salicylanilide derivatives expressed moderate hypoglycaemic activity[88] or mild inhibition of aldose reductase-2 activity[89]. They can also influence the metabotropic glutamate receptors[90,91].

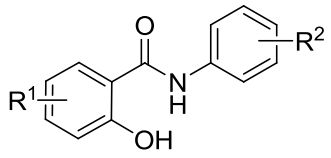


Figure 16. General structure of salicylanilides (2-hydroxy-*N*-(phenyl)benzamides)

Importantly, salicylanilide derivatives are known for their long time ago discovered activity against different bacteria, fungi and human and veterinarian protozoan parasites. They are still investigated and modified for antimycobacterial, antifungal and antibacterial activities, especially against Gram-positive strains[77,87]. These activities are the most important for the aims of this thesis.

We reviewed the antiviral activity of salicylanilides, their esters and partly salicylamides in the paper entitled "Antiviral Activity of Substituted Salicylanilides – a Review"[77] (**Appendix IV**) because we expected, on the base of structure similarity, that our salicylanilide derivatives may share such a type of activity.

The exact mechanism of salicylanilides action as the antimicrobial agents is not still clearly established; however more aspects have been published. We discuss and outline them in our review. Salicylanilides have been demonstrated to inhibit the two-component regulatory systems necessary for the maintenance of cell homeostasis, energy metabolism mainly due to uncoupling of mitochondrial oxidative phosphorylation, they cause membranes damage resulting in the disrupting of their function as the semi-permeable barrier, protein denaturation, some enzyme inhibition, chelation of certain metals.

Salicylanilides may inhibit the energy-dependent cellular uptake of inorganic phosphate and amino acids, incorporation of glucose, nucleic bases and amino acids and release amino acids from the cell pool. Recent studies revealed a blockade of the type III secretion system, which is a virulence factor of many bacteria[92].

In 2010, another new salicylanilide mechanism of the action against *Staphylococcus aureus* (including methicillin-resistant strains, MRSA) was described consisting in the disrupting of transglycosylase. Salicylanilide-based core inhibited the lipid II polymerization and the moenomycin-binding activities of transglycosylase. Bi-functional transglycosylase localised at the outer face of the membrane is essential for the cell wall biosynthesis *via* peptidoglycan formation[93].

Some structure-activity relationships were obtained. Most of salicylanilides bearing an extra hydrophobic group(s) like an aryl group (substituted phenyl, naphtyl or heteroaryl) on the aniline ring showed a complete inhibitory activity. *N*-[5-(4-Chlorobenzoyl)-2-(4-chlorophenoxy)phenyl]-2-hydroxy-3,5-diiodobenzamide (Figure 17) was found being the most potent inhibitor with IC_{50} of 9.3 μM. Salicylanilides containing only two aromatic rings with H, halogen(s), OH, methyl, nitro groups on the aniline ring avoided transglycosylase inhibition, although possessing a very good antimicrobial activity. The presence of the hydroxyl moiety at the position 2 on the salicylic ring plays an essential role for the enzyme inhibition; its elimination or methylation resulted in a loss of activity, as well as a replacement of the amide moiety by imine or amine[93].

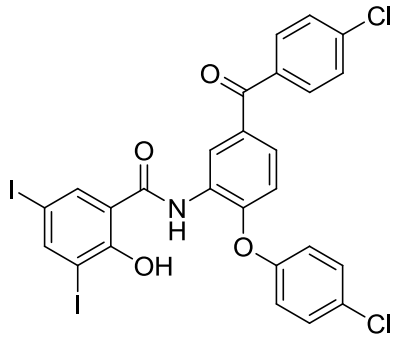


Figure 17. *N*-[5-(4-Chlorobenzoyl)-2-(4-chlorophenoxy)phenyl]-2-hydroxy-3,5-diiodobenzamide

Bulky salicylanilides exhibiting an excellent activity towards transglycosylase from *S. aureus* inhibited also transglycosylase from *Escherichia coli*, but surprisingly not the enzyme from *Mycobacterium tuberculosis*. All evaluated salicylanilide derivatives affect the growth of both drug-sensitive and MRSA strains and *M. smegmatis* with MIC in the range of $\leq 0.03 - 8.0$ μg/mL. Interestingly, salicylanilides with an aryl moiety on the aniline ring with enhanced activity towards transglycosylase show reduced antibacterial

activities, too. Since this enzyme is on the outer surface of bacteria, the permeability through cell membrane does not appear being an important factor for the difference. Additionally, the growth of *S. aureus* was blocked at the concentration values much lower than is IC_{90} for transglycosylase[93]. Both these findings implicate known fact that salicylanilides affect the other targets to exert antimicrobial properties.

D-Alanine-D-alanine ligase is an essential enzyme in both Gram-negative and Gram-positive bacteria cell wall biosynthesis affected e.g. by cycloserine. It may be an important target in the development of new antimicrobial agents. Three salicylanilide derivatives were synthesized and evaluated as potential analogues of leflunomide metabolite LFM-A13 (Figure 18). The inhibitory activity against D-alanine-D-alanine ligase was retained by the salicylanilide derivative *N*-(2,5-dibromophenyl)-2-hydroxybenzamide (Figure 19). In the enzyme inhibition assay, D-alanine-D-alanine ligase in the presence of this inhibitor exhibited approximately 65% residual activity, while LFM-A13 only 41% at 2 mM concentration[94].

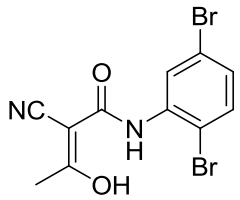


Figure 18. LFM-A13

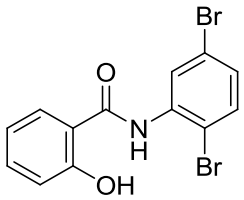


Figure 19. *N*-(2,5-Dibromophenyl)-2-hydroxybenzamide

Two salicylanilide derivatives, 2-hydroxy-*N*-(4-(3-tosylureido)phenyl)benzamide (Figure 20) and (*E*)-4-(4-(2-hydroxybenzamido)phenylamino)-4-oxobut-2-enoic acid (Figure 21), were screened and revealed their activity against sortase A from *S. aureus* with IC_{50} of 226 and 125 μM, respectively. Bacterial sortases are cysteine transpeptidases participating in the secretion and anchoring of many cell-wall proteins at Gram-positive bacteria. Additionally, sortases control the cellular location of multiple virulence factors[95].

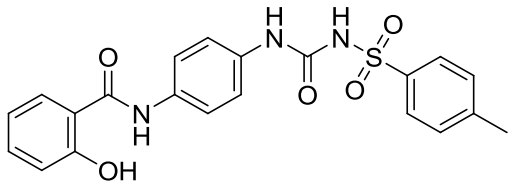


Figure 20. 2-Hydroxy-*N*-(4-(3-tosylureido)phenyl)benzamide

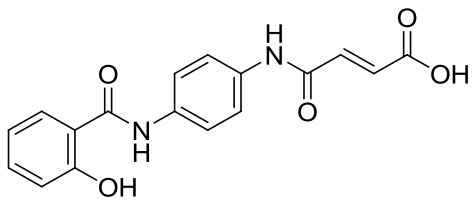


Figure 21. (*E*)-4-(4-(2-Hydroxybenzamido)phenylamino)-4-oxobut-2-enoic acid

It seems to be very likely that they as relatively small and simple molecules affect a wide range of microorganisms by affecting more cellular targets, structures and functions. However, this mechanism of the action versatility may cause a certain toxic effect on mammalian cells.

Various toxic impacts and side effects have been reported – adverse effects on the genetic code and DNA (the genotoxicity may be due to the presence of the halogen and nitro groups), haemolytic activity, membrane damage and peculiar toxic consequences of polyhalogenated salicylanilides particularly those containing bromine like dibromsalan or tribromsalan after the sun exposure[92].

3.3.2 Prodrugs and Salicylanilide Esters

The concept of prodrug formation has been used to improve various undesirable properties of used drugs since the late 19th century, although the term prodrugs or proagents was firstly introduced by Adrien Albert in 1958. Prodrugs are defined as reversible inert chemical derivatives of pharmacologically active agents that must undergo a conversion *in vivo* to release the parent drug and exert a therapeutic effect(s)[92,96].

In most cases, prodrugs are simple chemical derivatives that are only a few steps away from the parent drug. However, some prodrugs lack an obvious carrier but instead result from a molecular modification of the prodrug itself, which generates a new active compound[96]. "Classical" prodrugs called carrier-linked prodrugs are typically esters activated by an enzymatic and/or non-enzymatic hydrolysis. Many chemical groups and moieties can be useful in prodrug formation. A reliable well-designed carrier-prodrug must generate the active form rapidly to ensure stable effective drug levels at the site of the action prior prodrug metabolism[97]. The carrier-linked prodrug may further be classified into four groups[98]:

- double prodrugs or pro-prodrugs, where a prodrug is further derivatized in a fashion so that only enzymatic conversion to prodrug is possible before the latter can cleave to release the active drug,
- macromolecular prodrugs with polysaccharides, dextrans, cyclodextrins, proteins, peptides and polymers as carriers,
- site-specific prodrugs where a carrier acts as a transporter of the active drug to a specific targeted site,
- mutual prodrugs (see lower).

An ideal carrier should not be toxic, immunogenic and antigenic and should not be accumulated. From the "chemical point of view", it should be stable to chemical and physical manipulation and it should be easily characterized[98].

Distinct kinds of prodrugs are bioprecursors that do not have a carrier; but they need a biotransformation *in vivo* as the substrates for the metabolizing enzymes to generate the desired effects. Illustratively, PA-824 is an example of such a prodrug[97].

Mutual prodrugs are a special type of prodrugs comprised of two or more pharmacological active drugs where one is the carrier of the other instead of some inactive molecule[97]. The carrier may have the same biological action as the parent drug and therefore it produces synergistic action, or the carrier may have some additional biological action lacking in the parent drug, or the carrier might help to target specific site of the action or to improve site specificity. The carrier drug may be used to overcome side effects of the parent drug. Mutual prodrug design is not really different from the general drug discovery process. It is a very potent area of research contributing to human therapy improvement[98]. Additionally, mutual prodrugs may be active *in vitro*. Both active moieties may be coupled directly mostly *via* covalent bond or indirectly by a cleavable spacer. Mutual prodrugs surpassed the co-administration of both drugs separately; if two drugs are administered simultaneously they may not be absorbed or transported to the target site, but the mutual prodrugs eliminate this disadvantages as well as provide similar ratio of the distribution and elimination of both components. The term "codrug" may be used instead of mutual prodrug, e.g. in the review of Das et al.[99] For example, scientific and some therapeutic successes has been reached in the case of non-steroidal anti-inflammatory drugs or antimicrobial agents (sultamicillin, mutual prodrugs of INH, PAS and EMB or fluoroquinolones)[98,99].

We designed some salicylanilide ester series as potential mutual prodrugs – salicylanilide pyrazinoates as potential antituberculosis agents with an excellent outcome and salicylanilide benzoates which failed with respect to the intended purpose of the synthesis of potent antifungal agents.

In fact, approximately 20 % of all small molecular drugs approved during the period 2000 to 2008 were prodrugs[96]. The classification of prodrugs involves main points of view[92]:

- according to the property, why is prodrug designed (prodrugs with improved bioavailability, absorption, permeability, toxicity, longer half-time, higher specificity, stability, with disguised inconvenient organoleptic properties, specific distribution or targeting etc.),

- based on therapeutic purposes,
- based on the types of chemical linkage of moiety/carriers modifying the active drug,
- pharmacokinetic properties classification based on the site of conversion.

Relatively novel trend in the prodrug design is to create a prodrug useful in the targeting of specific site or molecule by considering enzyme-substrate specificity or carrier-substrate specificity to overcome various undesirable drug properties. Poorly absorption can be alleviated by the prodrugs resembling nutrients structurally and being absorbed by specific carrier proteins. In this case, the by-products will not be toxic, when amino acid, nucleoside, peptide and organic anionic transporters would be involved. The coupling of L-amino acids, including L-valine, L-leucine, L-phenylalanine, L-tyrosine or short peptides with antiviral agents has been investigated successfully. Interestingly, an N-terminal amino group and a C-terminal carboxyl group do not appear to be critical requirements for the peptide transporters, although modification of these groups generally diminishes the affinity to the transporters[97].

The prodrug development is a part of rational drug design, albeit many of these substances have also been discovered unintentionally. The esterification of salicylanilides is not only an approach of the last years – for example, biological active salicylanilide carbonates or simple esters were published in 70s and 80s. Due to the presence of aromatic hydroxyl group in the salicylanilide scaffold, they could be considered to be phenols, despite of the chemical nomenclature and hydroxyl group non-priority[92]. This phenolic group presents a convenient opportunity for the prodrug formation to improve the pharmaceutical, pharmacokinetic and/or pharmacodynamic properties[92,100].

Phenolic drugs usually present limited bioavailability and thus lower effectivity. The problems bring their possible metabolic instability including extensive first-pass metabolism, solubility limitations, non-lipophilic character and poor passage through biomembranes. The phenolic prodrugs design may lead to the orally administrated drug avoiding liver and intestine degradation, promoted membrane permeability or lower toxicity. As a case in point, the increased lipophilicity may result in targeting of the intestinal lymphatic transport or central nervous system delivery. The commonest types of phenolic prodrugs are esters, sulphates, carbamates, carbonates and ethers[100]. In general, the hydroxyl group plays an important role in the phenols´ activity by binding to an active site *via* ionic or hydrogen bonding. Esters are the most common prodrugs (approximately 50 % of all marketed prodrugs) and they are easily hydrolyzed by various and ubiquitous esterases or non-enzymatic. The carbonate function is highly sensitive to hydrolysis and

due to its instability carbonates are not often used in the design of prodrugs, whereas carbamates are known to be stable in acidic or basic media, and consequently should be less sensitive to enzymatic hydrolysis[97].

When applied these facts on the salicylanilides, *O*-salicylanilide esters are probably a temporary transport forms which take beneficial physico-chemical properties. Salicylanilide esters are hydrolyzed in plasma (esters with *N*-protected amino acids in a few minutes or half an hour and carbamates about 50 hours at neutral and slightly basic pH). Moreover, when compared with parent phenolic molecules, in more cases salicylanilide esters exhibited better antimicrobial activity. It is supposed that hydrophobicity is one of the factors influencing the biological activity of salicylanilides. Although phenolic group seems to be necessary for the antimicrobial activity, it could have irritative properties and is responsible for the uncoupling activity. Its temporary masking by esterification could be advantageous in a high activity, a better bioavailability, facile passing through the cell walls and biomembranes and at a lower toxicity. When *N*-protected natural α-amino acids are used for esterification, they could facilitate the targeting as a drug delivery system, as pointed in this chapter[92].

However, it not still clear, if *O*-salicylanilide esters act as salicylanilide prodrugs in the strict sense of their definition, i.e. with no or only very low *in vitro* activity and that they cleave the acid and therefore they are only temporary transport form – for this hypothesis witness the fact that they are hydrolyzed in plasma[92]. Other question is, if they could take effect as new chemical entities without necessary transformation to salicylanilides with free phenolic group, at least some portion of all administered molecules. This hypothesis is supported by the finding that salicylanilide esters showed different *in vitro* antibacterial and antifungal activity when compared with "free" salicylanilides – in most cases significantly higher and in some cases the same or slightly worse. The fact, that salicylanilides with methylated hydroxyl (2-methoxy-*N*-(phenyl)benzamides) showed antimycobacterial activity[101] could be considered as supporting for the second hypothesis. Nevertheless, it is not known, if these benzamide derivatives without free hydroxyl group act by the same way as salicylanilides and their esters. Additionally, their metabolism *in vivo* has not been evaluated, although it is possible to hypothesize that 2-methoxy-*N*-(phenyl)benzamides are dealkylated in organism to form "free" salicylanilides. Some ether phenolic prodrugs have been developed[100]. The next studies concerned on these problems are required.

Salicylanilide esters have exhibited a significant antimycobacterial activity including atypical strains and MDR- and XDR-TB strain, in some cases comparable or better than INH, an antibacterial activity especially against Gram-positive strains, variable antifungal properties, which are summarized in our review titled "Salicylanilide Ester Prodrugs as Potential Antimicrobial Agents – A Review"[92] (**Appendix V**). For example, next biological activities of salicylanilide esters have been referred – photosynthesis inhibition activity on spinach chloroplasts[102], antiviral activity against hepatitis B[103], herpes[104] or HIV-1[105] viruses, molluscicidal[106,107], filaricidal[108], anthelmintic[109] and antimalarial[110] acting, they showed analgesic and anti-inflammatory properties[111,112,113,114], anticancer potency[115,116], hypoglycaemic action[117,118], anti-allergy activity[119]. Salicylanilide esters also have been revealed to inhibit plasminogen activator inhibitor-1[120], NF-κB activation with a potentially broad spectrum of therapeutic impacts[121,122], modulate glutamate receptors[123].
Both salicylanilide phosphates and sulphates exhibited inhibition of NF-κB[124] and sulphates also the inhibition of cathepsin D, an enzyme implicated in the pathology of Alzheimer's disease as well as breast and ovarian cancer[125].

4. Experimental Part

The experimental part of the thesis combines two approaches – firstly comments to the published or submitted papers (e.g. chapters 4.2 and 4.3.1) and, secondly, the complete description of the synthesis, physico-chemical properties and/or biological activity (chapters 4.3.2.1, 4.3.3, 4.3.4.1 and 4.4).

4.1 General Methods

All used reagents and solvents were purchased from commercial sources (mostly Sigma-Aldrich, Penta Chemicals, Lach-Ner) and they were used without a further purification. Reactions were monitored by thin layer chromatography, plates were coated with 0.2 mm of silica gel 60 F254 (Merck) and were visualized by UV irradiation (254 nm). The next mobile phases have been used widely – toluene/ethyl-acetate 9:1 and 4:1 (for both esters and amides), toluene/methanol 9:1 (for esters) and dichloromethane/methanol 7:1 (for sulfonamide derivatives).

All synthesized compounds were characterized. Melting points were determined on a Melting Point machine B-540 (Büchi) apparatus using open capillaries and they are uncorrected. Elemental analysis (C, H, N) were performed by Mrs. Věnceslava Hronová (Department of Pharmaceutical Chemistry and Drug Control, Faculty of Pharmacy in Hradec Králové) on an automatic microanalyser CHNS-O CE instrument (FISONS EA 1110, Italy).

Infrared spectra (ATR or KBr pellets) were recorded by Mrs. Iva Vencovská and Mrs. Hana Jílková-Mikešová (Department of Inorganic and Organic Chemistry, Faculty of Pharmacy in Hradec Králové) on a FT-IR spectrometer Nicolet 6700 FT-IR in the range of 400 – 4000 cm^{-1}.

The NMR spectra were measured (Assoc. Prof. PharmDr. Jiří Kuneš, CSc., Department of Inorganic and Organic Chemistry, Faculty of Pharmacy in Hradec Králové) in dimethylsulfoxide DMSO-*d6* solutions or in deuterochloroform at ambient temperature on a Varian Mercury – Vxbb 300 (300 MHz for ^{1}H and 75.5 MHz for ^{13}C; Varian Comp. Palo Alto, USA) or on a Varian VNMR S500 (500 MHz for ^{1}H and 125 MHz for ^{13}C; Varian Comp. Palo Alto, USA). The chemical shifts δ are given in ppm, related to tetramethylsilane as an internal standard. The coupling constants (*J*) are reported in Hz.

Optical activities of the chiral derivatives were measured on a polarimeter ADP 220 BS (Bellingham Stanley Ltd., the United Kingdom) by Mrs. Dana Cardová, Department of Inorganic and Organic Chemistry, Faculty of Pharmacy in Hradec Králové.

The log*P* values, that are the logarithm of the partition coefficient for octan-1-ol/water, were calculated using the program CS ChemOffice Ultra version 11.0 (CambridgeSoft, USA). The chemical nomenclature was adjusted to this program, too.

The *in vitro* antimycobacterial susceptibility was determined in the Laboratory for Mycobacterial Diagnostics and Tuberculosis, Institute of Public Health in Ostrava (Dr. Vít Ulmann and especially RNDr. Jiřina Stolaříková). The standard panel of the mycobacterial strains involves *M. tuberculosis* 331/88 ($H_{37}Rv$, dilution of the strain 10^{-3}), *M. avium* 330/88 resistant to INH, RIF, EMB and ofloxacin in the dilution of 10^{-5} and *M. kansasii* (235/80, dilution 10^{-4}) from the Czech National Collection of Type Cultures (CNCTC) and one clinically isolated strain of *M. kansasii* 6509/96 (dilution 10^{-5}). The most active compounds were evaluated towards five MDR-TB strains and one XDR-TB.

The *in vitro* both antibacterial and antifungal activities were assayed by Mrs. Ida Dufková at the Department of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Králové. The standard panel of the bacterial pathogens includes four Gram-positive and four Gram-negative strains: *Staphylococcus aureus* CCM 4516/08, methicillin-resistant *Staphylococcus aureus* H 5996/08 (MRSA), *Staphylococcus epidermidis* H 6966/08, *Enterococcus* sp. J 14365/08; *Escherichia coli* CCM 4517, *Klebsiella pneumoniae* D 11750/08, ESBL-positive *Klebsiella pneumoniae* J 14368/08 and *Pseudomonas aeruginosa* CCM 1961. Antifungal activity was determined *in vitro* against four yeast strains (*Candida albicans* ATCC 44859, *Candida tropicalis* 156, *Candida krusei* E28, *Candida glabrata* 20/I) and four moulds – *Trichosporon asahii* (formerly *T. beigelii*) 1188, *Aspergillus fumigatus* 231, *Lichtheimia* (formerly called *Absidia*) *corymbifera* 272 and *Trichophyton mentagrophytes* 445.

Other special methods and assays are described either in the text of this thesis or in the attached papers.

4.2 A Study of Salicylanilide Esters Reactivity

Imramovský et al.[126] engaged in the synthesis and biological evaluation of the salicylanilide esters with physiological *N*-protected α-amino acids. In some cases, instead of expected salicylanilide esters, which were obtained when bulkier amino acids were

used, benzoxazepines were formed as a result of a seven-*exo*-trig cyclization in the case of *N*-Cbz glycine and alanine. The mechanism of the cyclization was proposed, too. Additionally, it was supposed, that the unexpected formation of the molecules called "diamide" during treatment of the hydrobromic salt of salicylanilide amino acid esters by triethylamine, where initially only deprotonization of the salt was demanded, undergoes the rearrangement by the similar way (see Figure 22). The deprotected free amino group should immediately attack the amidic carbonyl and form a cyclic seven-membered ester which is attacked and opened by in the previous step cleaved aniline to produce the "diamide".

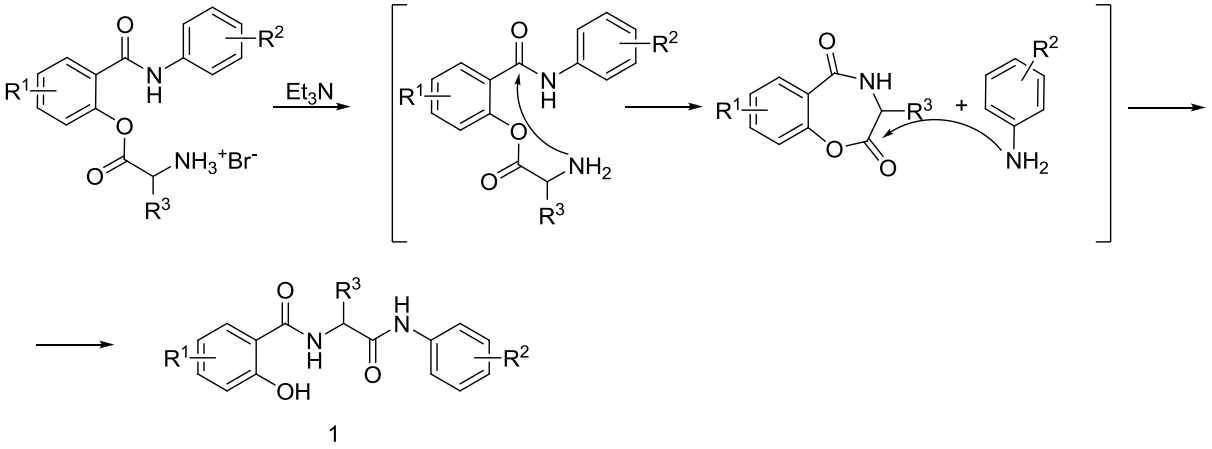


Figure 22. The first proposed mechanism of the rearrangement producing "diamides" (according to[126])

However, this rearrangement has been studied further to clear some circumstances and questions. In the paper "An unprecedented rearrangement of salicylanilide derivatives: imidazolinone intermediate formation"[127] (**Appendix VI**) we demonstrated that the rearrangement is independent on the presence and the position of the chlorine on the salicylic ring and the hydrobromide salts of the esters will rearrange despite the fact there are 3-chlorine, 4-chlorine, 4-methyl or 4-methoxy moieties on the aniline ring. To confirm the proposed mechanism, we have carried out experiments, where anilines bearing a stronger nucleophilic substituent at position 4 were added to the reaction mixture. No rearranged "diamides" containing the added anilines were isolated, only the product containing the original aniline moiety. Therefore we proposed an alternative mechanism of this rearrangement via five-membered imidazolinone ring (see Figure 23), supported by the isolation and next characterization of the dehydrated forms of these intermediates.

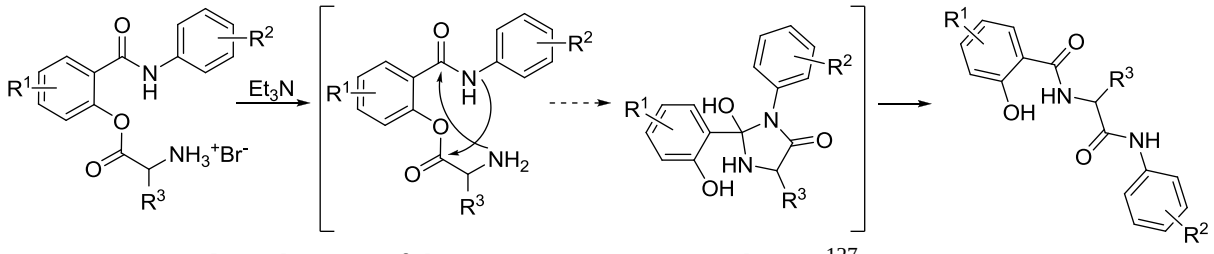


Figure 23. Novel mechanism of the rearrangement (according to[127])

The antimycobacterial, antibacterial and antifungal activities of four final "diamides" derived from L-valine (R^3 = isopropyl) were assayed – see Table 5.

Table 5. Antimycobacterial activity of four L-valine based "diamides"

			MIC [μmol/L]									
	R^1	R^2	*M. tuberculosis* 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96		
			14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
1a	5-Cl	3-Cl	32	62.5	125	125	62.5	62.5	125	32	62.5	62.5
1b	H	4-Cl	62.5	62.5	62.5	125	16	32	125	16	62.5	62.5
1c	H	4-OCH_3	125	125	250*	500*	125	250	250*	32	125	250*
1d	H	4-CH_3	125*	250*	250*	500*	125	250*	250*	125	250*	250*
INH			0.5	0.5	>250	>250	>250	>250	>250	2	4	8

*: at presented concentration the grow of the strain was observed, at duplex concentration there was present a precipitate and/or a turbidity, therefore it was not possible to determine exact MIC value

Although four compounds are not sufficient for structure-activity analyses, it is obvious that the presence of one or two chlorines in the molecule of "diamide" positively modulates the activity. (*S*)-5-Chloro-*N*-(1-(4-chlorophenylamino)-3-methyl-1-oxobutan-2-yl)-2-hydroxybenzamide **1a** was the most active against *M. tuberculosis* (32/62.5 μmol/L) and (*S*)-*N*-(1-(4-chlorophenylamino)-3-methyl-1-oxobutan-2-yl)-2-hydroxybenzamide **1b** towards atypical mycobacteria at 16 – 125 μmol/L. The 4-methylaniline derivative **1d** expressed the lowest *in vitro* efficacy. In general, these "diamides" displayed a mediocre rate of the antimycobacterial activity.

Three "diamides" were inactive against eight commonly tested bacterial strains at the concentration of 125 μmol/L; only 4-chloroaniline derivative **1b** inhibits the growth of *S. aureus*, methicillin-resistant *S. aureus* and *Enterococcus sp.* at 125 μmol/L. At 125 μmol/L, all "diamides" possessed no antifungal potency.

In the second paper dealing with this topic called "Synthetic Route for the Preparation of 2-Hydroxy-*N*-[1-(2-hydroxyphenylamino)-1-oxoalkan-2-yl]benzamides"[128] (**Appendix VII**), we confirmed that the rearrangement is a general process despite the type of amino acid used for the esterification (e.g. heterocyclic amino acid L-tryptophan, BOC-protected

amino acids, non-physiological amino acids L-cyclohexylalanine and L-2-(*tert*-butyl)glycine) and of the salicylanilides substitution pattern (aniline ring substituted by e.g. methyl, bromine, nitro or trifluoromethyl groups).

Some of "diamides" were described having mainly moderate antimycobacterial, antibacterial (Gram-positive strains), antifungal properties and they inhibited photosynthetic electron transport in spinach[129] or they may antagonize formyl peptide receptor-1 of human neutrophils and macrophages with potential anti-inflammatory acting[130].

4.3 Synthesis and Evaluation of the Antimicrobial Activity of the Salicylanilide Esters and Related Compounds

The aim of this part of the thesis has been to synthesize a large amount of salicylanilide derivatives, mainly esters, to evaluate the *in vitro* activity towards various mycobacteria, bacteria and fungi and to analyze the structure-activity relationships. That´s why we have chosen relatively simple derivatives and not exacting synthetic methods and approaches to obtain more compounds.

4.3.1 Salicylanilide Esters with *N*-Acetyl-L-phenylalanine

Based on the fact, that salicylanilide esters with various *N*-Cbz α-amino acids exhibited a good activity against *M. tuberculosis* (1 – 16 μmol/L[131] or 99% inhibition at 3.13 μg/mL[132]) and pathogenic fungi (MIC ≥ 1.95 μmol/L[132]), we decided to synthesize and evaluated a series of salicylanilide esters with lipophilic *N*-protected amino acid, because the direct esterification by "free" amino acids has brought a lot of problems. Lipophilicity of salicylanilide esters was described being one of the factors influencing activity against mycobacteria, although no clear dependency was described[131,132,133].

L-Phenylalanine is one of the more lipophilic amino acid (calculated log*P* of -1.49) and the acetylation of α-amino group increases sharply the lipophilicity (log*P* 0.71), but not as much as Z- or BOC-protecting groups.

The theoretical background, synthesis, results and discussion are summarized in the paper "New amino acid esters of salicylanilides active against MDR-TB and other microbes"[87] (**Appendix VIII**). During this work, we obtained the most active reported salicylanilide ester derivatives towards drug-sensitive *M. tuberculosis* with MIC from 0.25 μmol/L. MDR- and XDR-TB strain were affected from 1 μmol/L; unfortunately, lower

concentrations were not assayed. Some derivatives exhibited low MIC towards Gram-positive (MIC ≥ 0.98 μmol/L) and fungal strains (≥ 3.9 μmol/L). These derivatives showed no sharp cytotoxicity, selectivity index ranges approximately from 6 to 195.

4.3.2 Salicylanilide Benzoates

4.3.2.1 Antimycobacterial Activity

Recently, some substituted benzoic acid esters with substituted phenols displayed mild antimycobacterial properties against typical and atypical species[134,135]. It was one of the reasons for the design and synthesis of a series of new salicylanilide benzoates as potential antimycobacterial agents with improved activity.

Our paper brings the synthesis and properties of salicylanilide benzoates; it concerns with their antibacterial and antifungal properties[136]. All eighteen newly synthesized esters were evaluated for their activity against four mycobacterial strains. Results are summarized in the Table 6. INH, PAS and benzoic acid were selected as referential drugs.

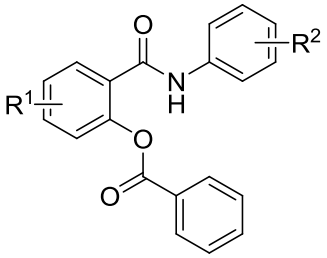
All benzoates exhibited a significant activity against drug-sensitive strain of *M. tuberculosis* at relatively low concentrations, for three derivatives with MIC ≤ 1 μmol/L. Salicylic ring 4-chloroderivatives provide mostly a major benefit than 5-chloro ones. The contribution of the moieties on the aniline ring is according to decreased potency: 4-CF_3 ≥ 3,4-dichloro > 3-CF_3 (limited data) ≈ 4-Br > 3-Cl = 3-Br > 4-Cl > 3-F > 4-F. The benzoylation provided derivatives with noticeably improved activity when compared to parent salicylanilides[137] – e.g. even eight times for **1m**.

M. avium showed the highest level of the resistance with MIC from 4 – 16 μmol/L. Both strains of *M. kansasii* were affected by salicylanilide benzoates at quite small concentrations up to 8 μmol/L. 4-CF_3, 3-CF_3, 3,4-dichloro and 4-Br are more convenient moieties as aniline substitution patterns. Surprisingly, when 5-chloro-2-hydroxy-*N*-(substituted phenyl)benzamides are esterified, the activity does not change, whereas masking of phenolic group of 4-chloro-2-hydroxy-*N*-(substituted phenyl)benzamides results in the molecules mostly with improved *in vitro* inhibition activity (up to four times). Benzoic acid revealed at the experimental conditions only very weak inhibition of *M. kansasii* (MIC ≥ 250 μmol/L).

It could be concluded that benzoylation of salicylic phenolic group in the salicylanilide molecules led to the derivatives predominantly with higher *in vitro* activity. This effect may be due to increased lipophilicity, which ameliorates passing through biomembranes.

With respect to the weak activity of BA against *M. kansasii*, the synergistic action of released "free" salicylanilide and benzoic acid could not be excluded.

Table 6. Antimycobacterial activity of benzoates



			MIC [μmol/L]									
	R^1	R^2	*M. tuberculosis* 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96		
			14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
1a	4-Cl	3-Cl	2	2	**4**	8	**2**	4	8	4	4	8
1b	5-Cl	3-Cl	4	4	8	8	**2**	4	4	4	4	8
1c	4-Cl	4-Cl	4	4	8	16	4	8	8	4	8	8
1d	5-Cl	4-Cl	2	4	**4**	**4**	**2**	**2**	4	**2**	**2**	4
1e	4-Cl	3-Br	2	2	8	16	**2**	4	8	**2**	4	8
1f	5-Cl	3-Br	4	4	4	8	**2**	4	4	**2**	4	4
1g	4-Cl	4-Br	2	2	**4**	8	**2**	4	4	**2**	4	4
1h	5-Cl	4-Br	2	2	**4**	**4**	**2**	4	4	**2**	4	4
1i	4-Cl	3-F	4	4	8	16	**2**	4	8	**2**	4	4
1j	5-Cl	3-F	4	8	8	16	**2**	8	8	**2**	4	8
1k	4-Cl	4-F	4	4	8	16	**2**	8	8	**2**	4	4
1l	5-Cl	4-F	8	8	**4**	8	**2**	4	8	4	8	8
1m	4-Cl	3,4-diCl	**1**	**1**	8	8	**2**	4	4	**1**	**2**	**2**
1n	5-Cl	3,4-diCl	2	2	8	8	**1**	**2**	4	**1**	**2**	4
1o	4-Cl	4-CF_3	**0.5**	**1**	**4**	**4**	**1**	**1**	**1**	**2**	**2**	**2**
1p	5-Cl	4-CF_3	2	2	8	8	**1**	**2**	4	**1**	**2**	4
1q	4-Cl	3-CF_3	2	2	8	16	**2**	4	4	**2**	4	4
1r	4-Br	4-CF_3	**1**	**1**	**4**	**4**	**2**	**2**	**2**	**2**	**2**	**2**
INH	-	-	0.5-1	0.5-1	>250	>250	>250	>250	>250	2	4	4-8
PAS	-	-	62.5	62.5	32	125	125	1000	>1000	32	125	500
BA	-	-	>1000	>1000	>1000	>1000	1000	>1000	>1000	250	1000	1000

One or two best MIC for each strain are bolded.

Additionally, four esters with MIC ≤ 1 μmol/L against any mycobacterial strain were selected for the ability to stop the growth of five MDR and one XDR strain (see Table 7). All exhibited very low MIC (0.25 – 2 μmol/L). Interestingly, in most cases MDR strains are even more sensitive than drug-sensitive one. The susceptibility to salicylanilide benzoates is independent on the resistance patterns; it indicates no cross-resistance with the conventionally used drugs.

Salicylanilide benzoates exhibited higher activity against drug-resistant strains (expressed as MIC) than esters with *N*-acetyl-L-phenylalanine[87].

Table 7. Activity of selected benzoates against MDR strains

	MIC [μmol/L]											
	M. tuberculosis 234/2005		*M. tuberculosis* 53/2009		*M. tuberculosis* Praha 1		*M. tuberculosis* Praha 131		*M. tuberculosis* 7357/1998		*M. tuberculosis* 9449/2006	
	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d
1m	0.25	0.5	0.5	1	0.5	0.5	0.5	1	0.5	0.5	0.5	1
1n	0.25	0.5	1	2	1	1	0.5	1	0.5	1	2	2
1o	0.25	0.25	0.5	1	0.5	0.5	0.25	0.5	0.25	0.5	0.5	0.5
1r	0.5	0.5	1	2	1	1	0.5	1	0.25	0.5	1	1
INH	14.6	14.6	14.6	14.6	14.6	14.6	14.6	14.6	14.6	14.6	58.3	58.3

MDR-TB strains: 234/2005 and 7357/1998 (both resistant to INH, RIF, rifabutine, STM, EMB and ofloxacin); 53/2009 (resistant to INH, RIF, rifabutine, STM, EMB); 9449/2006 resistant to INH, RIF, rifabutine and STM; Praha 1 (resistant to INH, RIF, rifabutine, STM, EMB and clofazimine); Praha 131 (resistant to INH, RIF, rifabutine, STM, EMB, ofloxacin, gentamicin and amikacin; XDR-TB strain).

4.3.2.2 Antibacterial and Antifungal Activity

We expected that salicylanilide benzoates would have antibacterial and antifungal properties similar to other salicylanilide derivatives, especially when benzoic acid is a known non-specific broad-spectrum antimicrobial agent. It blocks the growth of e.g. *Staphylococci*, *Streptococcus*, *Candidae* or the family *Enterobacteriaceae*[138]. The results are reported in the paper called "*In Vitro* Antibacterial and Antifungal Activity of Salicylanilide Benzoates"[136] (**Appendix IX**).

All salicylanilide benzoates displayed *in vitro* activity towards *S. aureus* and MRSA (MIC 0.98 – 125 μmol/L) and with one exception against *S. epidermidis*. *Enterococcus* exhibited the highest rate of insusceptibility from Gram-positive bacteria; it was affected by twelve esters of eighteen at the concentrations ≤ 125 μmol/L[136]. Among four Gram-negative strains, only 4-chloro-2-(3-chlorophenylcarbamoyl)phenyl benzoate showed the inhibition of *Pseudomonas aeruginosa* at 3.91/7.81 μmol/L; other strains were not influenced by these derivatives. At our laboratory investigation conditions, benzoic acid alone did not inhibit the growth of all bacterial as well as fungal strains. In general, salicylanilide benzoates afford a slightly lower MIC than salicylanilide pyrazinoates and more than esters with *N*-acetyl-L-phenylalanine.

Surprisingly, in contrast to the antibacterial activity, salicylanilide benzoates expressed only a mild antifungal potency. Three strains were completely insensitive at 125 μmol/L and only six derivatives displayed certain *in vitro* efficacy. The most active was 5-chloro-2-(3,4-dichlorophenylcarbamoyl)phenyl benzoate with MIC ≥ 3.9 μmol/L). Similarly to

other salicylanilide esters, *Candidae* seem to be more resistant than moulds. We tried to adjust pH at approximately 5 to increase antifungal potency, because benzoic acid is more active at slightly acidic environment. However, the change of pH did not bring lower MIC. Unfortunately, benzoylation of salicylanilide molecules failed with respect to searching of the new antifungal agents.

4.3.3 Salicylanilide 4-(Trifluoromethyl)benzoates

Salicylanilide and their esters bearing on the aniline ring trifluoromethyl moiety, especially on the position 4, have exhibited the higher antimycobacterial activity in all series presented in this work. Moreover, salicylanilides benzoates possess the potent activity especially towards MDR-TB and lipophilicity is one of the most important factors influencing the antimycobacterial acting because of a highly lipophilic mycobacterial cell wall. That is why we selected 4-(trifluoromethyl)benzoic acid for the next series of salicylanilide esters with expected lower MIC.

4-(Trifluoromethyl)benzoates hold more lipophilicity than benzoates – e.g. calculated log*P* for 4-chloro-2-(3-chlorophenylcarbamoyl)phenyl benzoate is 5.44 and 6.36 for 4-chloro-2-(3-chlorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate, respectively.

Synthesis

The esterification was performed using DCC as a condensation and dehydrating agent (see Figure 24). 4-(Trifluoromethyl)benzoic acid and appropriate salicylanilide (both 0.001 mol) were dissolved in dry DMF (15 mL), the solution was cooled to -20 °C and DCC in a mild excess (0.0012 mol) was added in three portions during 1 h. Next the mixture was stirred for 3 h at the same temperature and stored at +4 °C for 48 h. The precipitated by-product *N*,*N*′-dicyclohexylurea was filtered off and the solvent was evaporated *in vacuo*. The rest was dissolved in a small amount of ethyl-acetate and the insoluble portion (another *N*,*N*′-dicyclohexylurea) was filtered off. The filtrate was again evaporated *in vacuo*. The crude product was purified by the repeated crystallization from ethyl-acetate/hexane.

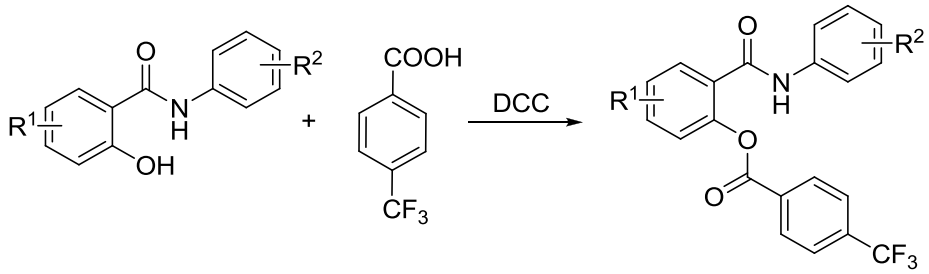


Figure 24. Synthesis of salicylanilide 4-(trifluoromethyl)benzoates (esters: R^1 = 4-Cl, 5-Cl, 4-Br; R^2 = 3-Cl, 4-Cl, 3,4-diCl, 3-Br, 4-Br, 3-F, 4-F, 3-CF_3, 4-CF_3)

A List and Characteristics of salicylanilide 4-(trifluoromethyl)benzoates

4-Chloro-2-(3-chlorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1a**)
White solid; yield 63 %; mp 184-186 °C. IR (ATR): 3308, 2934, 2858, 1718 (CO ester), 1666, 1650, 1588, 1522, 1479, 1452, 1425, 1410, 1323, 1291, 1271, 1205, 1167, 1131, 1113, 1088, 1066, 1015, 861, 766, 693, 680. ^{1}H NMR (300 MHz, DMSO): δ 10.71 (1H, bs, NH), 8.26 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=8.3 Hz, H3´´, H5´´), 7.87 (1H, d, *J*=2.6 Hz, H3), 7.77-7.71 (3H, m, H5, H6, H2´), 7.54 (1H, d, J=8.7 Hz, H6´), 7.30 (1H, t, *J*=8.1 Hz, H5´), 7.11 (1H, ddd, *J*=0.9 Hz, *J*=2.1 Hz, *J*=8.0 Hz, H4´). ^{13}C NMR (75 MHz, DMSO): δ 163.3, 162.9, 146.9, 140.2, 133.7 (q, *J*=32.1 Hz), 133.1, 132.4, 131.9, 130.9, 130.8, 130.7, 130.6, 129.3, 127.9, 126.2 (q, *J*=3.7 Hz), 125.6, 123.8, 123.7 (q, *J*=272.9 Hz), 119.5, 118.4. Anal. Calcd. for $C_{21}H_{12}Cl_2F_3NO_3$ (454.23): C, 55.53; H, 2.66; N, 3.08. Found: C, 55.27; H, 2.80; N, 3.22.

5-Chloro-2-(3-chlorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1b**)
White solid; yield 83 %; mp 165.5-168 °C. IR (ATR): 3284, 2933, 2857, 1738 (CO ester), 1650, 1602, 1593, 1543, 1483, 1451, 1413, 1323, 1259, 1242, 1168, 1130, 1112, 1068, 1016, 860, 699, 673. ^{1}H NMR (300 MHz, DMSO): δ 10.67 (1H, bs, NH), 8.27 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=8.3 Hz, H3´´, H5´´), 7.82 (1H, d, *J*=8.3 Hz, H3), 7.73-7.71 (2H, m, H6, H2´), 7.60 (1H, dd, *J*=8.3 Hz, *J*=2.1, H4), 7.50 (1H, dd, *J*=8.3 Hz, *J*=2.0, H6´), 7.30 (1H, t, *J*=8.1 Hz, H5´), 7.11 (1H, m, H4´). ^{13}C NMR (75 MHz, DMSO): δ 163.4, 163.2, 148.8, 140.3, 136.0, 133.8 (q, *J*=32.1 Hz), 133.1, 132.4, 131.1, 130.9, 130.6, 128.3, 126.7, 126.2 (q, *J*=3.6 Hz), 124.0, 123.9 (q, *J*=272.9 Hz), 123.8, 119.4, 118.4. Anal. Calcd. for $C_{21}H_{12}Cl_2F_3NO_3$ (454.23): C, 55.53; H, 2.66; N, 3.08. Found: C, 55.34; H, 2.85; N, 3.01.

4-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1c**)
White solid; yield 49 %; mp 170-172.5 °C. IR (ATR): 3322, 3073, 2934, 1719 (CO ester), 1667, 1625, 1601, 1547, 1491, 1420, 1399, 1324, 1291, 1244, 1221, 1172, 1134, 1112, 1095, 1067, 1013, 822, 705, 656. ^{1}H NMR (300 MHz, DMSO): δ 10.67 (1H, bs, NH), 8.25 (2H, d, *J*=8.2 Hz, H2´´, H6´´), 7.93 (2H, d, *J*=8.3 Hz, H3´´, H5´´), 7.86 (1H, d, *J*=2.6 Hz, H3), 7.73 (1H, d, *J*=8.8 Hz, H5), 7.62 (1H, d, *J*=8.9 Hz, H6), 7.54-7.40 (2H, m, H2´, H6´), 7.34-7.31 (2H, m, H3´, H5´). ^{13}C NMR (75 MHz, DMSO): δ 163.3, 162.7, 146.9, 137.8, 133.7 (q, *J*=32.0 Hz), 133.2, 130.9, 130.3, 129.2, 128.8, 128.0, 127.8, 126.2 (q, *J*=3.6 Hz), 125.6, 123.9 (q, *J*=272.8 Hz), 122.8, 121.6. Anal. Calcd. for $C_{21}H_{12}Cl_2F_3NO_3$ (454.23): C, 55.53; H, 2.66; N, 3.08. Found: C, 55.40; H, 2.51; N, 3.34.

5-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1d**)
White solid; yield 86 %; mp 170.5-173 °C. IR (ATR): 3305, 2933, 2858, 1737 (CO ester), 1699, 1650, 1601, 1544, 1492, 1452, 1408, 1325, 1258, 1240, 1182, 1157, 1128, 1111,

1097, 1068, 1016, 829, 769, 698, 669. ^{1}H NMR (300 MHz, DMSO): δ 10.63 (1H, bs, NH), 8.26 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.95 (2H, d, *J*=8.3 Hz, H3´´, H5´´), 7.82 (1H, d, *J*=8.3 Hz, H3), 7.72 (1H, d, *J*=2.0 Hz, H6), 7.54-7.40 (3H, m, H4´, H2´, H6´), 7.34-7.30 (2H, m, H3´, H5´). ^{13}C NMR (75 MHz, DMSO): δ 163.6, 163.2, 148.9, 136.1, 133.7 (q, *J*=32.0 Hz), 132.4, 131.1, 130.8, 128.1, 127.9, 126.7, 126.2 (q, *J*=3.6 Hz), 125.5, 124.2, 124.0, 123.9 (q, *J*=272.9 Hz), 119.8. Anal. Calcd. for $C_{21}H_{12}Cl_2F_3NO_3$ (454.23): C, 55.53; H, 2.66; N, 3.08. Found: C, 55.69; H, 2.47; N, 2.86.

4-Chloro-2-(3,4-dichlorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1e**)
White solid; yield 73 %; mp 156-158.5 °C. IR (ATR): 3313, 3079, 2933, 2858, 1718 (CO ester), 1671, 1650, 1627, 1580, 1518, 1478, 1451, 1384, 1323, 1278, 1249, 1204, 1174, 1134, 1107, 1087, 1066, 1016, 862, 823, 767, 696. ^{1}H NMR (300 MHz, DMSO): δ 10.80 (1H, bs, NH), 8.25 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=8.3 Hz, H3´´, H5´´), 7.90-7.87 (2H, m, H2´, H3), 7.75 (1H, dd, *J*=2.6 Hz, *J*=8.7 Hz, H5), 7.61 (1H, d, *J*=8.8 Hz, H6), 7.56-7.52 (2H, m, H5´, H6´). ^{13}C NMR (75 MHz, DMSO): δ 163.3, 162.9, 146.9, 138.9, 133.7 (q, *J*=32.1 Hz), 132.1, 131.1, 130.9, 130.8, 130.7, 130.6, 129.3, 128.7, 127.8, 126.2 (q, *J*=3.7 Hz), 125.7, 123.9 (q, *J*=272.8 Hz), 121.2, 120.0. Anal. Calcd. for $C_{21}H_{11}Cl_3F_3NO_3$ (488.67): C, 51.61; H, 2.27; N, 2.87. Found: C, 51.77; H, 2.41; N, 2.64.

5-Chloro-2-(3,4-dichlorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1f**)
White solid; yield 73 %; mp 144-146 °C. IR (ATR): 3306, 29333, 2858, 1748 (CO ester), 1699, 1649, 1601, 1581, 1525, 1477, 1452, 1409, 1379, 1325, 1261, 1250, 1194, 1164, 1130, 1120, 1068, 1016, 864, 769, 698. ^{1}H NMR (300 MHz, DMSO): δ 10.77 (1H, bs, NH), 8.25 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=8.3 Hz, H3´´, H5´´), 7.91 (1H, s, H2´), 7.82 (1H, d, *J*=8.3 Hz, H3), 7.74 (1H, d, *J*=2.0 Hz, H6), 7.61 (1H, dd, *J*=2.0 Hz, *J*=8.5 Hz, H4), 7.57-7.52 (2H, m, H5´, H6´). ^{13}C NMR (75 MHz, DMSO): δ 163.4, 163.2, 148.8, 138.9, 136.1, 133.7 (q, *J*=31.8 Hz), 132.4, 131.1, 131.0, 130.9, 130.8, 127.9, 126.8, 126.2 (q, *J*=3.7 Hz), 125.6, 124.0, 123.8 (q, *J*=272.3 Hz), 121.1, 120.0. Anal. Calcd. for $C_{21}H_{11}Cl_3F_3NO_3$ (488.67): C, 51.61; H, 2.27; N, 2.87. Found: C, 51.48; H, 2.11; N, 2.98.

2-(3-Bromophenylcarbamoyl)-4-chlorophenyl 4-(trifluoromethyl)benzoate (**1g**)
White solid; yield 49 %; mp 196-198 °C. IR (ATR): 3319, 2932, 2853, 1717 (CO ester), 1666, 1650, 1625, 1573, 1521, 1478, 1451, 1410, 1323, 1291, 1271, 1241, 11167, 1132, 1112, 1088, 1066, 1016, 893, 860, 766, 696, 686. ^{1}H NMR (300 MHz, DMSO): δ 10.68 (1H, bs, NH), 8.26 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.95 (2H, d, *J*=8.2 Hz, H3´´, H5´´), 7.88-7.85 (2H, m, H2´, H6), 7.75 (1H, dd, *J*=2.6 Hz, *J*=8.7 Hz, H5), 7.63 (1H, d, *J*=8.0 Hz, H3), 7.54 (1H, d, *J*=8.7 Hz, H6´), 7.26-7.23 (2H, m, H4´, H5´). ^{13}C NMR (75 MHz, DMSO): δ 163.3, 162.8, 146.9, 140.4, 133.7 (q, *J*=32.1 Hz), 132.4, 131.9, 130.9, 130.9, 130.8, 130.7, 129.3, 126.8, 126.2 (q, *J*=3.7 Hz), 125.6, 123.8 (q, *J*=268.2 Hz), 122.4, 121.6, 118.8. Anal. Calcd. for $C_{21}H_{12}BrClF_3NO_3$ (498.68): C, 50.58; H, 2.43; N, 2.81. Found: C, 50.72; H, 2.19; N, 2.55.

2-(3-Bromophenylcarbamoyl)-5-chlorophenyl 4-(trifluoromethyl)benzoate (**1h**)
White solid; yield 85 %; mp 166-168.5 °C. IR (ATR): 3305, 2933, 2857, 1738 (CO ester), 1650, 1589, 1537, 1479, 1452, 1411, 1323, 1258, 1242, 1169, 1130, 1111, 1068, 1016, 861, 770, 698, 675. ^{1}H NMR (300 MHz, DMSO): δ 10.65 (1H, bs, NH), 8.26 (2H, d, *J*=7.9 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=7.9 Hz, H3´´, H5´´), 7.85 (1H, s, H2´), 7.82 (1H, d, *J*=8.3 Hz, H3), 7.72 (1H, d, *J*=1.9 Hz, H6), 7.65-7.53 (2H, m, H4, H6´), 7.31 (1H, m, H5´), 7.23 (1H, m, H4´). ^{13}C NMR (75 MHz, DMSO): δ 163.4, 163.2, 148.8, 140.4, 136.0, 133.7 (q, *J*=32.3 Hz), 131.1, 130.9, 130.8, 128.2, 127.9, 126.7, 126.6, 126.2 (q, *J*=3.6 Hz), 123.9,

123.4 (q, *J*=272.9 Hz), 122.3, 121.6, 118.8. Anal. Calcd. for $C_{21}H_{12}BrClF_3NO_3$ (498.68): C, 50.58; H, 2.43; N, 2.81. Found: C, 50.46; H, 2.58; N, 2.71.

2-(4-Bromophenylcarbamoyl)-4-chlorophenyl 4-(trifluoromethyl)benzoate (**1i**)
White solid; yield 53 %; mp 173-175.5 °C. IR (ATR): 3329, 2932, 2857, 1717 (CO ester), 1672, 1650, 1523, 1487, 1451, 1394, 1323, 1277, 1242, 1203, 1172, 1133, 1112, 1088, 1066, 1016, 860, 817, 767, 695. ^{1}H NMR (300 MHz, DMSO): δ 10.66 (1H, bs, NH), 8.25 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=8.3 Hz, H3´´, H5´´), 7.86 (1H, d, *J*=2.6 Hz, H6), 7.73 (1H, dd, *J*=2.6 Hz, *J*=8.7 Hz, H5), 7.63 (1H, d, *J*=8.1 Hz, H3), 7.58-7.52 (2H, m, H2´, H6´), 7.47-7.44 (2H, m, H3´, H5´). ^{13}C NMR (75 MHz, DMSO): δ 163.3, 162.7, 146.9, 138.2, 133.6 (q, *J*=32.0 Hz), 132.5, 131.8, 131.7, 130.9, 130.8, 130.6, 129.2, 127.8, 125.6, 126.2 (q, *J*=3.7 Hz), 123.8 (q, *J*=273.0 Hz), 121.9, 115.8. Anal. Calcd. for $C_{21}H_{12}BrClF_3NO_3$ (498.68): C, 50.58; H, 2.43; N, 2.81. Found: C, 50.40; H, 2.67; N, 2.64.

2-(4-Bromophenylcarbamoyl)-5-chlorophenyl 4-(trifluoromethyl)benzoate (**1j**)
White solid; yield 72 %; mp 152.5-154.5 °C. IR (ATR): 3291, 2933, 1737 (CO ester), 1650, 1602, 1544, 1489, 1449, 1406, 1325, 1257, 1241, 1182, 1156, 1128, 1111, 1069, 1015, 893, 861, 824, 770, 698, 667. ^{1}H NMR (300 MHz, DMSO): δ 10.63 (1H, bs, NH), 8.25 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=8.3 Hz, H3´´, H5´´), 7.81 (1H, d, *J*=8.3 Hz, H3), 7.71 (1H, d, *J*=2.1 Hz, H6), 7.64-7.55 (3H, m, H4, H2´, H6´), 7.46-7.43 (2H, m, H3´, H5´). ^{13}C NMR (75 MHz, DMSO): δ 163.2, 162.7, 148.8, 138.3, 135.9, 133.7 (q, *J*=32.1 Hz), 132.4, 131.7, 131.0, 130.9, 128.3, 127.9, 126.7, 126.2 (q, *J*=3.7 Hz), 123.9, 123.8 (q, *J*=272.8 Hz), 121.9, 115.7. Anal. Calcd. for $C_{21}H_{12}BrClF_3NO_3$ (498.68): C, 50.58; H, 2.43; N, 2.81. Found: C, 50.36; H, 2.65; N, 2.94.

4-Chloro-2-(3-fluorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1k**)
White solid; yield 76 %; mp 151-153.5 °C. IR (ATR): 3317, 2933, 2857, 1721 (CO ester), 1699, 1650, 1543, 1444, 1410, 1323, 1272, 1236, 1167, 1154, 1130, 1115, 1089, 1065, 1020, 859, 844, 766, 686. ^{1}H NMR (300 MHz, DMSO): δ 10.73 (1H, bs, NH), 8.26 (2H, d, *J*=7.9 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=7.9 Hz, H3´´, H5´´), 7.86 (1H, s, H3), 7.73 (1H, d, *J*=8.5 Hz, H5), 7.64-7.49 (2H, m, H6, H6´), 7.39-7.29 (2H, m, H2´, H5´), 6.88 (1H, t, *J*=8.3 Hz, H4´). ^{13}C NMR (75 MHz, DMSO): δ 163.3, 162.8, 162.1 (d, *J*=241.5 Hz), 146.9, 140.5 (d, *J*=11.1 Hz), 133.7 (q, *J*=31.7 Hz), 132.5, 131.9, 130.9, 130.7, 130.5 (d, *J*=9.4 Hz), 129.2, 127.9, 126.2 (q, *J*=3.6 Hz), 125.6, 123.8 (q, *J*=273.0 Hz), 115.8 (d, *J*=2.8 Hz), 110.6 (d, *J*=21.2 Hz), 106.7 (d, *J*=26.2 Hz). Anal. Calcd. for $C_{21}H_{12}ClF_4NO_3$ (437.77): C, 57.62; H, 2.76; N, 3.20. Found: C, 57.88; H, 2.99; N, 3.03.

5-Chloro-2-(3-fluorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1l**)
White solid; yield 55 %; mp 169-171.5 °C. IR (ATR): 3320, 2932, 2852, 1739 (CO ester), 1651, 1624, 1602, 1569, 1548, 1491, 1414, 1325, 1259, 1242, 1200, 1172, 1138, 1122, 1070, 1016, 861, 770, 700, 669. ^{1}H NMR (300 MHz, DMSO): δ 10.70 (1H, bs, NH), 8.26 (2H, d, *J*=8.0 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=8.1 Hz, H3´´, H5´´), 7.82 (1H, d, *J*=8.3 Hz, H3), 7.72 (1H, d, *J*=1.8 Hz, H6), 7.59 (1H, dd, *J*=1.7 Hz, *J*=8.3 Hz, H4), 7.52 (1H, d, *J*=11.4 Hz, H6´), 7.38-7.28 (2H, m, H2´, H5´), 6.87 (1H, t, *J*=8.4 Hz, H4´). ^{13}C NMR (75 MHz, DMSO): δ 163.4, 163.2, 162.1 (d, *J*=241.5 Hz), 148.8, 140.6 (d, *J*=10.9 Hz), 135.9, 133.7 (q, *J*=32.1 Hz), 132.4, 131.0, 130.9, 130.5 (d, *J*=9.4 Hz), 128.3, 126.7, 126.2 (q, *J*=3.7 Hz), 124.0, 123.8 (q, *J*=272.8 Hz), 115.7 (d, *J*=2.8 Hz), 110.5 (d, *J*=21.0 Hz), 106.7 (d, *J*=26.2 Hz). Anal. Calcd. for $C_{21}H_{12}ClF_4NO_3$ (437.77): C, 57.62; H, 2.76; N, 3.20. Found: C, 57.39; H, 3.02; N, 3.47.

4-Chloro-2-(4-fluorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1m**)
White solid; yield 70 %; mp 181-183.5 °C. IR (ATR): 3329, 2933, 2858, 1721 (CO ester), 1665, 1650, 1572, 1530, 1508, 1479, 1451, 1408, 1323, 1279, 1235, 1167, 1132, 1114, 1087, 1065, 1016, 825, 767, 695. ^{1}H NMR (300 MHz, DMSO): δ 10.58 (1H, bs, NH), 8.25 (2H, d, *J*=7.8 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=7.9 Hz, H3´´, H5´´), 7.85 (1H, d, *J*=2.4 Hz, H3), 7.77-7.73 (1H, dd, *J*=2.4 Hz, *J*=8.7 Hz, H5), 7.64-7.51 (3H, m, H6, H2´, H6´), 7.13-7.09 (2H, m, H3´, H5´). ^{13}C NMR (75 MHz, DMSO): δ 163.3, 162.5, 158.5 (d, *J*=240.6 Hz), 146.9, 135.2 (d, *J*=2.6 Hz), 133.6 (q, *J*=32.0 Hz), 132.5, 131.7, 131.0, 130.9, 129.2, 127.8, 126.2 (q, *J*=3.8 Hz), 125.6, 123.8 (q, *J*=272.9 Hz), 121.9 (d, *J*=7.9 Hz), 115.5 (d, *J*=22.3 Hz). Anal. Calcd. for $C_{21}H_{12}ClF_4NO_3$ (437.77): C, 57.62; H, 2.76; N, 3.20. Found: C, 57.95; H, 2.52; N, 3.26.

5-Chloro-2-(4-fluorophenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1n**)
White solid; yield 72 %; mp 176.5-179 °C. IR (ATR): 3294, 2933, 2857, 1737 (CO ester), 1646, 1602, 1556, 1508, 1452, 1413, 1328, 1260, 1234, 1192, 1156, 1137, 1126, 1111, 1069, 1016, 861, 833, 771, 699, 669. ^{1}H NMR (300 MHz, DMSO): δ 10.55 (1H, bs, NH), 8.26 (2H, d, *J*=7.8 Hz, H2´´, H6´´), 7.94 (2H, d, *J*=7.8 Hz, H3´´, H5´´), 7.81 (1H, d, *J*=8.2 Hz, H3),
7.71 (1H, d, *J*=1.9 Hz, H6), 7.64-7.59 (3H, m, H4, H2´, H6´), 7.13-7.08 (2H, m, H3´, H5´). ^{13}C NMR (75 MHz, DMSO): δ 163.2, 163.0, 158.5 (d, *J*=240.5 Hz), 148.8, 135.7, 135.2 (d, *J*=2.5 Hz), 133.7 (q, *J*=32.2 Hz), 132.5, 131.0, 130.9, 128.5, 127.9, 126.7, 126.2 (q, *J*=3.7 Hz), 123.9, 123.8 (q, *J*=272.9 Hz), 121.8 (d, *J*=7.9 Hz), 115.5 (d, *J*=22.2 Hz). Anal. Calcd. for $C_{21}H_{12}ClF_4NO_3$ (437.77): C, 57.62; H, 2.76; N, 3.20. Found: C, 57.94; H, 2.59; N, 2.94.

4-Chloro-2-(4-(trifluoromethyl)phenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1o**)
White solid; yield 75 %; mp 166.5-169 °C. IR (ATR): 3330, 2933, 2857, 1717 (CO ester), 1676, 1650, 1527, 1478, 1452, 1409, 1322, 1280, 1257, 1237, 1168, 1126, 1110, 1088, 1066, 1017, 861, 843, 767, 697. ^{1}H NMR (300 MHz, DMSO): δ 10.89 (1H, bs, NH), 8.25 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.92 (2H, d, *J*=8.2 Hz, H3´´, H5´´), 7.90 (1H, d, *J*=2.6 Hz, H3), 7.81 (2H, d, *J*=8.5 Hz, H2´, H6´), 7.76 (1H, dd, *J*=2.6 Hz, *J*=8.7 Hz, H5), 7.64 (2H, d, *J*=8.7 Hz, H3´, H5´), 7.55 (1H, d, *J*=8.7 Hz, H6). ^{13}C NMR (75 MHz, DMSO): δ 163.3, 163.1, 147.0, 142.4, 133.7 (q, *J*=32.1 Hz), 132.4, 132.0, 130.9, 130.8, 130.7, 129.3, 127.8, 126.2 (m), 125.6, 124.5 (q, *J*=271.4 Hz), 124.4 (q, *J*=31.8 Hz), 123.9 (q, *J*=272.9 Hz), 119.9. Anal. Calcd. for $C_{22}H_{12}ClF_6NO_3$ (487.78): C, 54.17; H, 2.48; N, 2.87. Found: C, 54.42; H, 2.71; N, 3.14.

5-Chloro-2-(4-(trifluoromethyl)phenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1p**)
White solid; yield 70 %; mp 142-143.5 °C. IR (ATR): 3346, 2932, 2856, 1719 (CO ester), 1671, 1649, 1602, 1524, 1485, 1451, 1407, 1321, 1281, 1251, 1179, 1165, 1120, 1089, 1064, 1017, 863, 843, 824, 761, 697. ^{1}H NMR (300 MHz, DMSO): δ 10.85 (1H, bs, NH), 8.25 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.93 (2H, d, *J*=8.2 Hz, H3´´, H5´´), 7.85 (1H, d, *J*=8.3 Hz, H3), 7.80 (2H, d, *J*=8.3 Hz, H2´, H6´), 7.73 (1H, d, *J*=2.0 Hz, H6), 7.63 (2H, d, *J*=8.6 Hz, H3´, H5´), 7.60 (1H, dd, *J*=2.1 Hz, *J*=8.3 Hz, H4). ^{13}C NMR (75 MHz, DMSO): δ 163.6, 163.2, 148.9, 142.5, 136.1, 133.7 (q, *J*=32.1 Hz), 132.4, 131.1, 130.9, 128.1, 127.8, 126.7, 126.1 (m), 124.4 (q, *J*=271.3 Hz), 124.0, 124.0 (q, *J*=32.0 Hz), 123.8 (q, *J*=273.0 Hz), 119.8. Anal. Calcd. for $C_{22}H_{12}ClF_6NO_3$ (487.78): C, 54.17; H, 2.48; N, 2.87. Found: C, 54.33; H, 2.65; N, 2.69.

4-Chloro-2-(3-(trifluoromethyl)phenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1q**) White solid; yield 68 %; mp 158.5-160.5 °C. IR (ATR): 3306, 2933, 2858, 1719 (CO ester), 1699, 1670, 1650, 1543, 1450, 1409, 1323, 1276, 1236, 1168, 1131, 1108, 1066, 1020, 844, 766, 696, 687. ^{1}H NMR (300 MHz, DMSO): δ 10.84 (1H, bs, NH), 8.26 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 7.92 (2H, d, *J*=8.2 Hz, H3´´, H5´´), 7.90 (1H, s, H2´), 7.86 (1H, d, *J*=8.2 Hz, H3), 7.76 (1H, dd, *J*=2.5 Hz, *J*=8.7 Hz, H5), 7.63 (1H, d, *J*=8.0 Hz, H6), 7.56 (1H, d, *J*=8.7 Hz, H6´), 7.51 (1H, d, *J*=8.0 Hz, H5´), 7.40 (1H, d, *J*=7.8 Hz, H4´). ^{13}C NMR (75 MHz, DMSO): δ 163.3, 163.0, 146.9, 139.5, 133.7 (q, *J*=32.1 Hz), 132.4, 132.0, 130.9, 130.8, 130.7, 130.2, 129.8 (q, *J*=29.8 Hz), 129.3, 127.9, 126.1 (q, *J*=3.7 Hz), 125.5, 124.4 (q, *J*= 263.2 Hz), 123.8 (q, *J*= 272.2 Hz), 123.6, 120.5 (q, *J*=3.7 Hz), 116.2 (q, *J*=3.7 Hz). Anal. Calcd. for $C_{22}H_{12}ClF_6NO_3$ (487.78): C, 54.17; H, 2.48; N, 2.87. Found: C, 54.02; H, 2.57; N, 2.99.

4-Bromo-2-(4-(trifluoromethyl)phenylcarbamoyl)phenyl 4-(trifluoromethyl)benzoate (**1r**) White solid; yield 67 %; mp 162-163.5 °C. IR (ATR): 3325, 2933, 2858, 1717 (CO ester), 1699, 1674, 1650, 1528, 1452, 1408, 1322, 1279, 1236, 1167, 1129, 1110, 1089, 1066, 1017, 844, 766, 687. ^{1}H NMR (300 MHz, DMSO): δ 10.88 (1H, bs, NH), 8.25 (2H, d, *J*=8.1 Hz, H2´´, H6´´), 8.03 (1H, d, *J*=2.4 Hz, H3), 7.92 (2H, d, *J*=8.3 Hz, H3´´, H5´´), 7.88 (1H, dd, *J*=2.6 Hz, *J*=8.8 Hz, H5), 7.80 (2H, d, *J*=8.5 Hz, H2´, H6´), 7.66-7.61 (2H, m, H3´, H5´), 7.48 (1H, d, *J*=8.7 Hz, H6). ^{13}C NMR (75 MHz, DMSO): δ 163.3, 163.0, 147.4, 142.4, 135.0, 133.7 (q, *J*=32.3 Hz), 132.4, 132.0, 131.0, 130.9, 127.8, 126.2 (q, *J*=3.7 Hz), 125.9, 125.5, 124.4 (q, *J*=32.0 Hz), 124.8 (q, *J*=274.1 Hz), 123.5 (q, *J*=268.6 Hz), 119.9, 118.8. Anal. Calcd. for $C_{22}H_{12}BrF_6NO_3$ (532.23): C, 49.65; H, 2.27; N, 2.63. Found: C, 49.43; H, 2.12; N, 2.54.

Biological Activity

These esters were evaluated for their *in vitro* antimycobacterial, antibacterial and antifungal activity. Table 8 summarizes the antimycobacterial activity with INH and PAS as the control compounds.

Additionally, the most active compounds (i.e. with at least one MIC against any strain ≤ 1 μmol/L) were evaluated against six MDR and XDR strains (the dilution 10^{-3}) with different resistance patterns: 7357/1998 and 234/2005 (both resistant to INH, RIF, rifabutine, STM, EMB and ofloxacin); 9449/2006 resistant to INH, RIF, rifabutine and STM; 53/2009 (resistant to INH, RIF, rifabutine, STM, EMB); Praha 1 (resistant to INH, RIF, rifabutine, STM, EMB and clofazimine); and Praha 131 (resistant to INH, RIF, rifabutine, STM, EMB, ofloxacin, gentamicin and amikacin; XDR strain). The following concentrations were used: 1000, 500, 250, 125, 62, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 μmol/L. Other conditions were similar to the testing of standard strains (e.g. ref.[87]). For results see Table 9.

Table 8. Antimycobacterial activity of salicylanilide 4-(trifluoromethyl)benzoates

			MIC [μmol/L]									
	R^1	R^2	*M. tuberculosis* 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96		
			14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
1a	4-Cl	3-Cl	**2**	4	8	16	**0.5**	2	4	**2**	8	8
1b	5-Cl	3-Cl	4	4	8	8	**1**	4	4	**2**	8	8
1c	4-Cl	4-Cl	**2**	**2**	4	8	**1**	2	**2**	**1**	4	4
1d	5-Cl	4-Cl	4	4	4	8	**1**	4	4	**1**	4	4
1e	4-Cl	3,4-diCl	**1**	**1**	4	4	**1**	2	4	**1**	**2**	4
1f	5-Cl	3,4-diCl	**1**	**2**	**2**	8	**1**	2	4	**1**	**2**	4
1g	4-Cl	3-Br	**1**	**2**	**2**	8	2	4	4	**1**	4	4
1h	5-Cl	3-Br	4	8	8	16	4	8	8	**2**	8	8
1i	4-Cl	4-Br	**2**	**2**	8	8	**1**	4	8	**2**	8	8
1j	5-Cl	4-Br	**2**	4	8	8	**1**	4	4	**2**	4	4
1k	4-Cl	3-F	8	8	16	32	8	16	16	8	16	16
1l	5-Cl	3-F	4	4	8	16	4	8	8	8	8	16
1m	4-Cl	4-F	8	8	16	32	4	16	16	8	16	16
1n	5-Cl	4-F	4	8	4	4	2	8	8	4	8	8
1o	4-Cl	4-CF_3	**1**	**1**	**1**	4	**1**	2	2	**1**	**2**	**2**
1p	5-Cl	4-CF_3	**2**	**2**	**2**	8	2	2	4	**1**	4	4
1q	4-Cl	3-CF_3	**2**	**2**	4	16	2	4	8	**1**	4	8
1r	4-Br	4-CF_3	**1**	**1**	**2**	4	**1**	2	4	**1**	**2**	4
INH			0.5-1	0.5-1	>250	>250	>250	>250	>250	2	4	4-8
PAS			62.5	62.5	32	125	125	1000	>1000	32	125	500
CF_3-BA			>1000	>1000	>1000	>1000	1000	>1000	>1000	250	1000	1000

One or two best MIC for each strain are bolded.

Table 9. MIC of salicylanilide 4-(trifluoromethyl)benzoates towards MDR- and XDR-TB

			MIC [μmol/L]											
	R^1	R^2	*M. tuberculosis* 7357/1988		*M. tuberculosis* 9449/2006		*M. tuberculosis* 53/2009		*M. tuberculosis* 234/2005		*M. tuberculosis* Praha 1		*M. tuberculosis* Praha 131	
			14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d
1a	4-Cl	3-Cl	4	4	4	4	4	4	4	4	4	4	4	4
1b	5-Cl	3-Cl	8	8	4	4	8	8	8	8	4	4	8	8
1c	4-Cl	4-Cl	2	2	2	2	4	4	4	4	2	2	2	2
1d	5-Cl	4-Cl	4	4	4	4	4	4	4	4	2	4	2	4
1e	4-Cl	3,4-diCl	**1**	2	2	2	2	4	**1**	2	**1**	2	2	2
1f	5-Cl	3,4-diCl	2	2	2	2	2	2	**1**	2	**1**	2	**1**	2
1g	4-Cl	3-Br	2	2	**1**	2	2	2	**1**	2	**1**	2	**1**	2
1i	4-Cl	4-Br	2	4	2	4	4	4	2	2	2	2	2	2
1j	5-Cl	4-Br	2	2	2	2	2	2	2	2	2	2	**1**	2
1o	4-Cl	4-CF_3	**0.5**	**1**	**1**	**1**	**1**	**1**	**0.5**	**1**	**0.5**	**0.5**	**0.5**	**1**
1r	4-Br	4-CF_3	**1**	**1**	**1**	**1**	**1**	2	**0.5**	**1**	**0.5**	**1**	**0.5**	**1**

MIC values of 1 μmol/L and lower are bolded.

4-(Trifluoromethyl)benzoic acid exhibited only a very mild activity towards *M. kansasii*. Regardless this fact, all of the 4-(trifluoromethyl)benzoates displayed a significant antimycobacterial activity (MIC ≥ 0.5 μmol/L). However they possess potent *in vitro* properties, although sharing a higher lipophilicity, their efficacy did not surpassed salicylanilide benzoates. In general, the most active derivatives beard trifluromethyl, two chlorines and then 4-bromine on the aniline ring; on the other side, 3- or 4-fluorine provides only a minimal benefit. These findings correspond with previously investigated salicylanilide esters. *M. avium* showed the highest MIC values.

Importantly, all evaluated esters showed a significant activity towards MDR- and XDR-TB strains independently of the particular resistances at similar values for each compound. The XDR strain was affected analogously to MDR. Interestingly, in this assay there were not assayed uniquely only esters with the best activity towards *M. tuberculosis* (MIC ≤ 1 μmol/L), even additionally derivatives with "moderate" activity (for salicylanilide esters 2 – 4 μmol/L) being also powerful. All of these derivatives affected the growth of drug-resistant *M. tuberculosis* at the equal or almost equal concentrations as drug-sensitive one. These facts indicate that salicylanilide 4-(trifluoromethyl)benzoates do not share any cross-resistance to conventionally used drugs (INH, rifamycines, EMB, STM, ofloxacin, clofazimine and aminoglycosides) and they may be prospective and perspective agents for combating of drug-resistant TB.

Table 10 reports the antibacterial activity and Table 11 antifungal properties. Remaining esters, of which MIC are not reported, were not possible to evaluate. Probably due to a high lipophilicity, they could not be dissolved in the tested media or they precipitated in them. As well as for salicylanilide benzoates, the change of pH to acidic (~ 5) did not improve the activity significantly. MIC. 4-(Trifluoromethyl)benzoic acid showed no intrinsic inhibitory action.

All soluble derivatives showed a very good activity against Gram-positive cocci, in some cases (**1e**, **1j**, **1p**, particularly **1b**, **1h** and **1k**) even excellent MIC lower than 1 μmol/L despite of the presence of the methicillin-resistance. Based on MIC values, it was possible to hypothesize that these esters, as well as other ones, act mainly as bactericidal agents. MIC towards *S. aureus* was comparable to PNC and, moreover, 4-(trifluoromethyl)benzoates exceeded the activity of PNC for MRSA, *S. epidermidis* and partly for *Enterococcus*.

Table 10. MIC of salicylanilide 4-(trifluoromethyl)benzoates towards selected bacteria

			MIC/IC_{90} [μmol/L]									
	R^1	R^2	*Staphylococcus aureus* CCM 4516/08		*S. aureus* H 5996/08 (MRSA)		*Staphylococcus epidermidis* H 6966/08		*Enterococcus sp.* J 14365/08		*Escherichia coli* CCM 4517	
			24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1b	5-Cl	3-Cl	**0.98**	1.95	**0.98**	1.95	**0.98**	1.95	7.81	15.62	>250	>250
1e	4-Cl	3,4-diCl	**0.49**	**0.98**	**0.49**	**0.98**	**0.49**	**0.98**	**0.49**	3.9	>500	>500
1h	5-Cl	3-Br	**0.98**	**0.98**	**0.49**	**0.98**	**0.49**	**0.98**	31.25	250	>500	>500
1j	5-Cl	4-Br	**0.49**	**0.98**	**0.49**	**0.98**	**0.49**	**0.98**	**1.95**	7.81	250	>250
1k	4-Cl	3-F	3.9	3.9	**0.98**	1.95	3.9	3.9	31.25	>250	>250	>250
1m	4-Cl	4-F	7.81	7.81	15.62	15.62	31.25	>500	500	>500	>500	>500
1o	4-Cl	4-CF_3	3.9	7.81	3.9	7.81	3.9	7.81	7.81	62.5	>125	>125
1p	5-Cl	4-CF_3	**0.49**	**0.98**	**0.49**	**0.98**	**0.49**	**0.98**	**0.49**	3.9	**31.25**	250
PNC	-	-	0.98	0.98	62.5	125	250	250	7.81	15.62	>500	>500

One or two best MIC for each strain are bolded.

Table 11. *In vitro* antifungal activity of salicylanilide 4-(trifluoromethyl)benzoates

	MIC/IC_{80} [μmol/L]															
	Candida albicans		*Candida tropicalis*		*Candida krusei*		*Candida glabrata*		*Trichosporon asahii*		*Aspergillus fumigatus*		*Absidia corymbifera*		*Trichophyton mentagrophytes*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	72 h	120 h
1c	62.5	>250	>250	>250	250	>250	>250	>250	250	>250	>250	>250	250	250	15.62	15.62
1e	>500	>500	**7.81**	**31.25**	**7.81**	**31.25**	>500	>500	>500	>500	>500	>500	>500	>500	15.62	125
1f	>125	>125	>125	>125	62.5	>125	>125	>125	**31.25**	>125	>125	>125	>125	>125	>125	>125
1h	125	>500	>500	>500	125	250	>500	>500	62.5	250	>500	>500	250	>500	**0.98**	1.95
1j	**15.62**	**31.25**	500	>500	**31.25**	62.5	**500**	>500	**15.62**	**31.25**	**15.62**	**31.25**	**7.81**	**15.62**	**0.49**	**0.98**
1k	250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	250	>250	62.5	62.5
1o	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	3.9	15.62
1p	62.5	500	>500	>500	**31.25**	62.5	>500	>500	**15.62**	62.5	**15.62**	**15.62**	**15.62**	**15.62**	**0.49**	1.95
FLU	1.0	2.0	3.0	5.0	>50	>50	22.0	>50	4.00	9.00	>50	>50	>50	>50	17.0	26.0

The best MIC value(s) for each strain is bolded.

Among Gram-negative strains, only *E. coli* possessed a moderate susceptibility from 31.25 μmol/L, whereas both strains of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* showed the completely resistance up to 500 μmol/L.

Some derivatives (**1e** or especially **1j** and **1p**) exhibited a significant activity against fluconazole-resistant *C. krusei*, *A. fumigatus* and *A. corymbifera*. Much more esters overcame fluconazole towards *T. mentagrophytes* (additionally **1c**, **1h**, **1o**). In general, *Candidae* are less susceptible (MIC ≥ 7.81 μmol/L with the superiority of **1e** and **1j**) than filamentous fungi (MIC ≥ 0.49 μmol/L with the superiority of **1j** and **1p**). *Candida*

glabrata is the most resistant strain, almost completely resistant, and on the other pole stays *T. mentagrophytes*.

Due to limited number of successfully assayed esters it is not possible to discuss properly the structure-activity relationships.

Furthermore, although 4-(trifluoromethyl)benzoates failed with respect to find the most antimycobacterial salicylanilide esters, they offer the best MIC against Gram-positive strains from all the compounds presented in this thesis. The antimycotic potency could be denoted being above-average.

4.3.4 Salicylanilide Pyrazinoates

4.3.4.1 Antimycobacterial Activity

Despite the wide use, the mechanism of PZA activity towards mycobacteria is still not fully elucidated. Although FAS I has been proposed as a target of PZA based especially on the investigation of 5-chloropyrazinamide, a subsequent study ended negatively[139]. The conclusions are controversial, the disruption might be a consequence of the inhibition of the membrane synthesis; newly it was found that both PZA and pyrazinoic acid (POA) bind to FAS I, although with different binding sites. POA showed a greater affinity[140].

PZA as a prodrug enters *M. tuberculosis* cell probably by passive diffusion and it is hydrolyzed intracellularly to POA by nicotinamidase/pyrazinamidase (PZase, encoded by *pncA* gene), an enzyme frequently lost in PZA-resistant strains. POA is therefore trapped within the cell as the anion, where it is excreted by a weak efflux pump and passive diffusion. If the extracellular pH is acidic, a small proportion of the POA outside the bacteria will take an uncharged conjugate acid form, which permeates through the membrane easily. Protonated POA is reabsorbed into the bacilli and accumulates there because the efflux pump is inefficient. Small amounts of protonated POA capable of the diffusion across the membrane have been proposed to uncouple the proton gradient, disrupt membrane potential and transport functions, acidify cytoplasm and thereby influence vital enzymatic function[139,141]. However, the molecular target of POA has remained unclear and it has been believed that the mechanism of the action is non-specific. Recently, a new previously unknown target of POA was found – the ribosomal protein S1 involved in protein translation and the ribosome-sparing process of trans-translation[139].

PZA is active only against *M. tuberculosis* complex organisms (*M. tuberculosis, M. africanum* and *M. microti*)[141]. *M. kansasii* is naturally resistant to PZA due to reduced

PZase activity. MIC of PZA and POA for *M. kansasii* were determined being 500 and 125 μg/mL, respectively[142]. There have been inconsistent reports on the susceptibility of *M. tuberculosis* to POA. In some reports, MIC of POA was higher than for PZA, while another group reported comparable MIC[141].

Esters of POA were found to have greater antituberculosis potency than POA *in vitro*, likely due to an increased lipophilicity. The mode of the action is expected to be similar as for PZA and POA. Despite improved anti-tuberculosis activity *in vitro*, efficacy studies in mice have so far failed to demonstrate any favourable activity against *M. tuberculosis*, presumably due to instability of the POA esters *in vivo*[141]. The pyrazinoic acid esters active towards more mycobacterial species have been published, including those with substituted phenols[143,144].

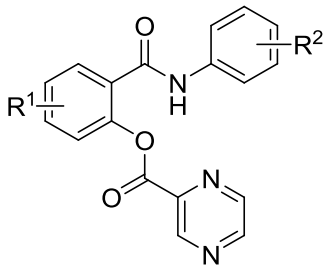
Based on the presented facts, we proposed the salicylanilide esters of pyrazine-2-carboxylic acid, which may be converted *in vivo* into "free" pyrazinoic acid non-enzymatic without PZase to avoid the main reason of the PZA-resistance and to affect naturally PZA-resistant *M. kansasii*. These esters may be considered being prodrug forms of either salicylanilides or pyrazinoic acid, i.e. mutual prodrugs with respect to significant antimycobacterial activity of both individual components. The aromatic esters of POA should by more stable than simple aliphatic ones which are in organisms rapidly hydrolyzed. Additionally, they help to overcome the difficulties with POA passing through mycobacterial cell wall.

The overview, synthesis and chemical characteristics of salicylanilide pyrazinoates are reported in our publication dealing with the antibacterial and antifungal properties of them[145]. Table 12 reveals the antimycobacterial activity of salicylanilide pyrazinoates.

The esterification of salicylanilides by pyrazine-2-carboxylic acid leads to the esters which are more hydrophilic than parent salicylanilides. However, a lot of antituberculosis drugs (typically PZA, INH or aminoglycosides) may be classified as hydrophilic despite the highly lipophilic mycobacterial cell wall.

Salicylanilide pyrazinoates blocked the growth of four mycobacterial strains with MIC from 0.5 up to 16 μmol/L. Salicylanilides bearing trifluoromethyl moiety expressed their highest activity – **1o** and **1r** against *M. tuberculosis* (0.5/1 μmol/L), **1d** for *M. avium* (2 μmol/L) and **1p** for *M. kansasii* (1 – 2 μmol/L). The most important findings are related to the activity towards drug-resistant strains.

Table 12. Antimycobacterial activity of the pyrazinoates



			MIC [μmol/L]									
	R^1	R^2	*M. tuberculosis* 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96		
			14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
1a	4-Cl	3-Cl	2	4	8	8	**2**	4	4	**2**	**2**	4
1b	5-Cl	3-Cl	2	2	**4**	8	**2**	4	4	4	4	8
1c	4-Cl	4-Cl	2	2	**4**	8	**2**	**2**	4	**1**	**2**	4
1d	5-Cl	4-Cl	2	4	**2**	**2**	**2**	**2**	4	**2**	**2**	**2**
1e	4-Cl	3,4-diCl	**1**	**1**	**4**	16	**2**	4	4	**1**	**2**	**2**
1f	5-Cl	3,4-diCl	2	2	8	16	4	4	8	**2**	4	4
1g	4-Cl	3-Br	**1**	2	8	16	4	4	4	**1**	**2**	4
1h	5-Cl	3-Br	2	8	8	16	4	4	4	**2**	4	4
1i	4-Cl	4-Br	2	2	8	8	**2**	**2**	4	**2**	**2**	4
1j	5-Cl	4-Br	2	2	**4**	**4**	4	4	4	**2**	**2**	4
1k	4-Cl	3-F	2	4	8	16	**2**	4	8	4	8	8
1l	5-Cl	3-F	2	8	8	16	4	4	8	4	4	8
1m	4-Cl	4-F	4	8	8	16	4	8	8	4	8	8
1n	5-Cl	4-F	2	8	**2**	**4**	**2**	4	4	**2**	4	8
1o	4-Cl	4-CF_3	**0.5**	**1**	**2**	**4**	**2**	**2**	**2**	**1**	**2**	**2**
1p	5-Cl	4-CF_3	**1**	**1**	**4**	8	**1**	**1**	**2**	**1**	**1**	**1**
1q	4-Cl	3-CF_3	**1**	2	8	32	8	8	8	4	8	8
1r	4-Br	4-CF_3	**0.5**	**1**	**2**	**4**	**2**	**2**	4	**1**	**1**	**2**
INH			0.5-1	0.5-1	>250	>250	>250	>250	>250	2	4	4-8
PAS			62.5	62.5	32	125	125	1000	>1000	32	125	500
POA			1000	>1000	>1000	>1000	250	>1000	>1000	250	1000	1000

One or two best MIC for each strain are bolded.

The most active pyrazinoates **1e**, **1g**, **1o**-**1r** (i.e. with MIC against *M. tuberculosis* being ≤ 1 μmol/L) were evaluated against five MDR- and one XDR-TB strains at the same conditions as salicylanilide benzoates and 4-(trifluoromethyl)benzoates (see Table 13).

Table 13. MIC of salicylanilide pyrazinoates towards MDR- and XDR-TB

			MIC [μmol/L]											
	R^1	R^2	*M. tuberculosis* 7357/1988		*M. tuberculosis* 9449/2006		*M. tuberculosis* 53/2009		*M. tuberculosis* 234/2005		*M. tuberculosis* Praha 1		*M. tuberculosis* Praha 131	
			14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d
1e	4-Cl	3,4-diCl	**0.125**	**0.25**	**0.5**	**0.5**	**0.5**	1	**0.125**	**0.125**	**0.25**	**0.5**	**0.5**	1
1g	4-Cl	3-Br	**0.5**	1	1	1	**0.5**	1	**0.25**	**0.5**	**0.125**	1	**0.25**	1
1o	4-Cl	4-CF_3	**0.25**	**0.25**	**0.125**	**0.25**	**0.125**	**0.25**	**0.25**	**0.25**	**0.125**	**0.125**	**0.125**	**0.125**
1p	5-Cl	4-CF_3	**0.5**	**0.5**	**0.125**	**0.25**	**0.125**	**0.5**	**0.5**	**0.5**	**0.125**	**0.25**	**0.125**	**0.125**
1q	4-Cl	3-CF_3	**0.5**	1	1	2	1	2	1	2	1	1	1	2
1r	4-Br	4-CF_3	**0.125**	**0.25**	**0.5**	**0.5**	**0.5**	1	**0.25**	**0.5**	**0.125**	**0.25**	**0.25**	**0.5**

MIC values and lower than 1 μmol/L are bolded.

Although salicylanilide pyrazinoates possessed the lowest lipophilicity from all evaluated esters (calculated log*P* for POA ester of the 5-chloro-*N*-(3-chlorophenyl)-2-hydroxybenzamide is 3.19 and for benzoate 5.44), pyrazinoates exhibited significantly the highest *in vitro* activity towards MDR and XDR strain from all salicylanilide esters evaluated in this thesis with MIC from 0.125 μmol/L. All drug-resistant strains exhibited a similar susceptibility despite their resistance pattern and salicylanilide pyrazinoates affected their growth at even lower concentrations than for *M. tuberculosis* 331/88. 4-Chloro-2-(4-(trifluoromethyl)phenylcarbamoyl)phenyl pyrazine-2-carboxylate (**1o**) is the most *in vitro* active derivative with MIC of 0.125 – 0.25 μmol/L towards resistant strains.

We presume that this excellent *in vitro* activity may be caused by the molecular synergy or summation of the effects of both salicylanilide and pyrazine-2-carboxylic acid, in which probably the esters are hydrolyzed in mycobacteria. Salicylanilide pyrazinoates affected significantly the growth not only of *M. tuberculosi*s, but of atypical strains reported being resistant to PZA, a prodrug of POA. Hydrophilic POA alone in our assay displayed certain, but just mild MIC values towards *M. kansasii* (≥ 250 μmol/L). However, the assay was realized at the conditions convenient for the MIC determination of INH, salicylanilide derivates and most of potential antimycobacterial agents, but not at specific condition required for a standard PZA susceptibility testing. In our assay, PZA inhibited mycobacteria at the concentration of 125 μmol/L and higher.

4.3.4.2 Antibacterial and Antifungal Activity

Salicylanilide pyrazinoates were also evaluated for their potential to stop *in vitro* the growth of some bacterial and fungal species. The article called "*In vitro* Antibacterial and

Antifungal Activity of Salicylanilide Pyrazine-2-carboxylates"[145] (**Appendix X**) summarizes this field.

Aromatic esters of pyrazine-2-carboxylic acid have been reported to share antimycobacterial activity[143,144], but no references of their activity towards Gram-positive, Gram-negative bacteria and fungi have been published as well as no synthesis *via N,N´*-dicyclohexylcarbodiimide. In our article[145], it was confirmed the possibility of the alternative synthesis by DCC. Salicylanilide pyrazinoates displayed a significant activity against Gram-positive strains from 0.98 μmol/L and all four tested strains (with one exception) were uniformly susceptible. Additionally, we found two esters inhibiting the growth of *Pseudomonas aeruginosa* at 7.81 – 15.62 μmol/L. Concurrently with one salicylanilide benzoate (4-chloro-2-(3-chlorophenylcarbamoyl)phenyl benzoate), it is the first published evidence that such "simple" salicylanilide esters could interfere with the growth of this dangerous pathogen.

One paper described a mild activity of pyrazinoates towards fungi[146], but no series has been published. With one exception, we have demonstrated the activity of presented esters against at least one of four tested moulds with MIC ≥ 1.95 μmol/L and several derivatives affect the growth of *Candidae* at the concentration of 7.81 μmol/L and higher.

When compared to other salicylanilide ester derivatives[92], pyrazinoates inhibit Gram-positive bacteria universally; all molecules are active (in contrast to esters with *N*-acetyl-L-phenylalanine which expressed similar MIC values, but only for some members of the series). Similarly for the antifungal activity, pyrazinoic acid derivatives exceeded *N*-acetyl-L-phenylalanine esters. Other esters with Z-protected amino acids exhibited favourable MIC towards *Candidae* and together worse against filamentous moulds.

4.3.5 Salicylanilide Benzenesulfonates

We intended to evaluate the antimicrobial activity of salicylanilide esters with sulfonic acid in comparison to carboxylic acids esters. We dealt with the counterpart benzoic and benzenesulfonic acid in this study to describe whether sulfonates diminish or increase MIC values against mycobacteria, fungi and Gram-positive and negative bacteria when compared to isosteric carboxylate analogues.

In contrast to other esters, the synthesis was performed using benzenesulfonyl chloride in the presence of a slight triethylamine excess to form salicylanilide salt *in situ*. The methodology based on the DCC-mediated esterification failed, the conversion of the

reactants (salicylanilide and benzenesulfonic acid) to the products was realized only in a very low rate. The multiple extension of the reaction time up to one week, the higher excess of DCC and the higher temperature (up to room temperature) have not brought any significant improvement of the yields. Maybe it is due to hygroscopic properties of benzenesulfonic acid, while the presence of any water impairs DCC-assisted condensation.
Sulfates are slightly less lipophilic than carboxylates – e.g. calculated log*P* for 4-chloro-2-(3-chlorophenylcarbamoyl)phenyl benzoate amount to 5.44 and 5.04 for 4-chloro-2-(3-chlorophenylcarbamoyl)phenyl benzenesulfonate, respectively.
The paper "Antimycobacterial Activity of Salicylanilide Benzenesulfonates"[147] (**Appendix XI**) brings the findings about antimycobacterial activity of them. Although all benzenesulfonates exhibited a significant *in vitro* activity (MIC against *M. tuberculosis* 1 – 125 μmol/L and 2 – 500 μmol/L for atypical mycobacteria), these activities are mostly only moderate, when compared to other salicylanilide esters. Thus benzenesulfonates are at least antimycobacterial potent salicylanilide esters discussed in this thesis. Moreover, only a few of these esters expressed a higher activity than parent "free" salicylanilides[137], some MIC values did not differ for salicylanilides and their benzenesulfonates and even the activity of salicylanilides surpassed those of esters in many cases.
Unhappily, all salicylanilide benzenesulfonates avoided any antibacterial and antifungal activity for standard strains at the concentration of 500 μmol/L or lower. In comparison to the salicylanilide benzoates, their sulphur isosters diminish fully the activity.
In summary, the introduction of the benzenesulfonyl moiety into salicylanilide molecules by the ester bond brings significantly less benefit than other assayed organic acid for every studied activity.

4.3.6 Sulfonamide Derivatives Based on 5-Chlorosalicylic Acid and 5-Chlorosalicylaldehyde Scaffolds

Salicylanilides bearing on the position 4 of the aniline ring an electron-accepting group (e.g. trifluoromethyl, nitro, ethoxycarbonyl) were described possessing certain antimycobacterial activity[137] and sulfonamides exhibited both antibacterial and antimycobacterial potency[148], therefore we decided to design "double" mutual derivatives. From the point of view of sulfonamides, these derivatives are their salicylamides (Figure 25a) and for salicylanilides they contain (*N*-aryl substituted-4-sulfamoyl)anilines (Figure 25b). To evaluate the importance of both amide bridge and salicylic hydroxyl, we

also synthesized derivatives with amide replaced by azomethine bridge (Schiff bases) and without phenolic group, respectively. Additionally, salicylaldehyde-derived Schiff bases were reported as potential antimicrobial agents[149] and the similar derivatives with etherified salicylic hydroxyl showed the antibacterial and antifungal potency[150]. 5-Chlorosalicylic acid and 5-chlorosalicylanilide were selected on the basis of the structure-activity relationships[137,149].

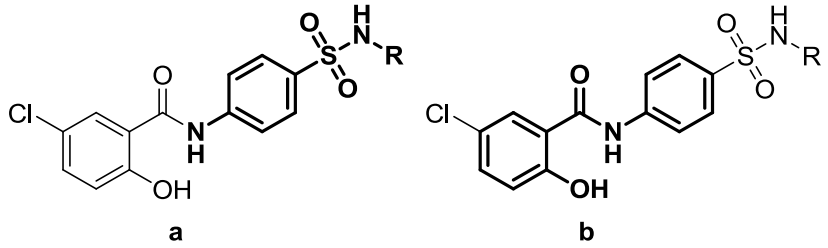


Figure 25. Sulfonamide (a) and salicylanilide (b) core of presented sulfonamide derivatives

In the paper entitled "Antimicrobial Activity of Sulfonamides Containing 5-Chloro-2-hydroxybenzaldehyde and 5-Chloro-2-hydroxybenzoic Acid Scaffold"[148] (**Appendix XII**), sulfathiazole, sulfamethoxazole, sulfamethazine and sulfadiazine were modified to form "double amides" and Schiff bases.

The results revealed interesting relationships. Unfortunately, all derivatives prevented any significant antifungal activity, it is only sporadic. *M. tuberculosis* was more resistant and inhibited at MIC of 32 – 500 μmol/L; the most susceptible were *M. kansasii* strains (MIC ≥ 1 μmol/L). However, all these modification led to the derivatives with less or comparable activity towards *M. tuberculosis* and *M. avium* when compared to starting sulfonamides. The presence of 2-hydroxyl group on the salicylic ring is necessary for the activity only for amides, not for Schiff bases. For the activity against *M. kansasii* this hydroxyl is not necessary and some derivatives exhibited a better activity than unsubstituted sulfonamides. No distinct superiority of Schiff bases or amides was observed[148].

The situation for other bacteria is quite different. The introduction of both 5-chlorosalicylaldehyde and 5-chlorosalicylic acid resulted in the increased antibacterial activity. The sulfonamide Schiff bases expressed all lower MIC than parent compounds (for both drug-sensitive and methicillin-resistant *S. aureus* 15.62 – 500 μmol/L); salicylamides exhibited lower MIC values. *S. epidermidis*, *Enterococcus* and Gram-negative species are more resistant. Schiff bases without salicylic hydroxyl expressed good activity, whereas removal of 2-hydroxy group from the amide results in total loss of the antibacterial activity. Amides seem to be more beneficial than imines for sulfadiazine and

sulfamethazine; reverse phenomenon was observed for sulfamethoxazole and sulfathiazole[148].

In sum, we obtained derivatives with the poorest activity towards *M. tuberculosis*, but very good against *M. kansasii* and advantageous for *Staphylococcus aureus*. The analyses of the individual contributions of salicylic phenol moiety and both amide and imine bridge to the activities bring interesting findings. The next research may be oriented into synthesis and evaluation of *O*-acetylated esters of sulfonamide salicyl Schiff bases and salicylamides.

4.4 An Enzymatic Inhibition Assays

Based on the facts about enzymatic targeting presented in the chapter 3.2.1.1, we selected two perspective enzyme targets for the *in vitro* evaluation of the most active salicylanilides and salicylanilide esters towards mycobacterial isocitrate lyase and methionine aminopeptidase.

We have struggled to progress the salicylanilide derivatives-oriented development of new antimicrobial agents from whole-cell assays to the identification of one or more exact known molecular target(s).

4.4.1 Inhibition of Isocitrate Lyase

4.4.1.1 Methods

The cloning and expression of recombinant isocitrate lyase (ICL) and enzymatic assay were performed at the Department of Biochemical Sciences, Faculty of Pharmacy in Hradec Králové, Charles University in Prague (Mgr. Eva Novotná, Ph.D.).

The gene encoding the isocitrate lyase was amplified by PCR from *M. tuberculosis* H_{37}Rv genomic DNA. The amplified DNA was digested using *Nde*I and *Hind*III, cloned into the pET28b(+) plasmid and the recombinant plasmid was transformed into *Escherichia coli* HB101. DNA sequencing was used to confirm that the inserted coding sequence had no mutations. For bacterial expression, 25 ml culture volumes were inoculated with BL21(DE3) cells containing the recombinant plasmid and allowed to grow until OD_{595} = 0,6 was achieved. The culture was then induced with 1 mM isopropyl-*β*-D-thiogalactopyranoside and incubated further at 30 °C for 4 hours. The cells were harvested by centrifugation at 4 °C and re-suspended in BugBuster Protein Extraction Reagent (Novagen). Histidine-tagged protein was purified using Äkta purifier (Amersham

Biosciences) and confirmed by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining of the gel.

Isocitrate lyase activity was assayed according to Dixon and Kornberg[151]. The reaction mixture contained 50 mM of potassium phosphate, 4 of mM magnesium chloride, 4 mM of phenylhydrazine, 12 mM of cysteine, 2 mM of (+)-potassium Ds-*threo*-isocitrate and the enzyme in a final volume of 1 ml. Isocitrate cleavage was measured by the change in absorbance at 324 nm associated with the formation of glyoxylate phenylhydrazone (Figure 26). The potential inhibitors dissolved in DMSO (10 µl of 10 mM solution) were added to the mixture of 939 µL reaction buffer and 1 µL of recombinant isocitrate lyase to final concentration of 100 µM. For the reliability of the result, the cleavage reaction and detection was performed at additional different concentration of the substrate (0.4 mM) and Mg^{2+} (0.8 mM).

The inhibitory activity of DMSO alone was measured repeatedly and the detected inhibition was subtracted from the activities of evaluated compounds. 3-Nitropropanoic acid was used as a standard for the comparison.

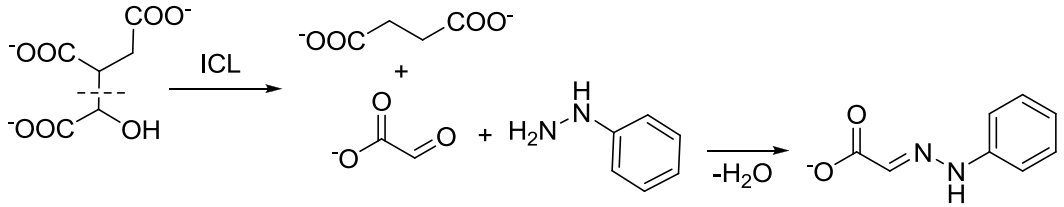


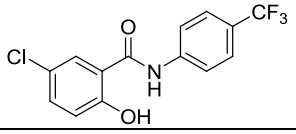
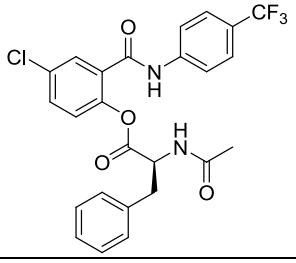
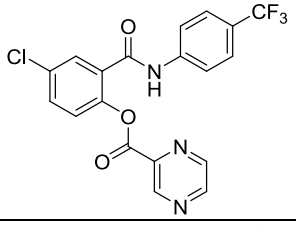
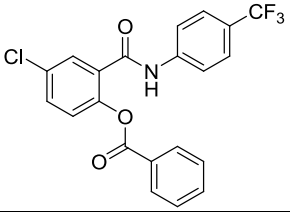
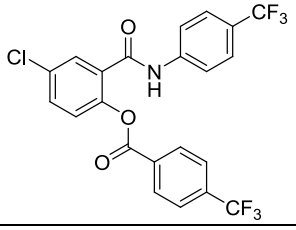
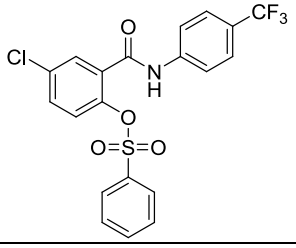
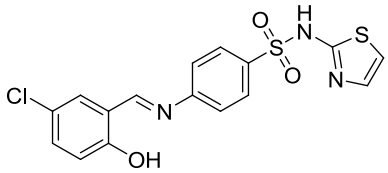
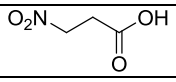
Figure 26. Isocitrate cleavage by ICL and the detection of the arising glyoxylate by phenylhydrazine

4.4.1.2 Results

For the pilot study we have selected 5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide, its five esters and one sulfonamide 5-chlorosalicylaldehyde-based Schiff base derivative presented in this thesis. They are the most antimycobacterial active molecules in their series. At first, this pilot trial should confirm our hypothesis that salicylanilides and related compounds active against mycobacteria *in vitro* in a whole cells assay could affect the function of ICL. Secondly, if the activity will be confirmed, we wanted to evaluate the influence of different carboxylic acid used for the esterification of the anti-ICL activity.

Table 14 summarizes the results.

Table 14. The ICL inhibition by some synthesized compounds

Compound	Rate of the ICL inhibition at 100 μmol/L (± standard deviation)	Calculated log*P*
	59 ± 3.84	3.93
	48 ± 3.24	4.92
	59 ± 5.75	3.55
	< 10	5.80
	< 10	6.72
	< 10	5.40
	8 ± 2.5	4.20
	67 ± 2.68	-0.40

Presented data constitute the first evidence that salicylanilide moiety-based molecules may affect the function of mycobacterial ICL. Significantly, the highest inhibition rate of 59%

was caused by “free” salicylanilide and its pyrazine-2-carboxylate, closely followed by the ester with *N*-acetyl-L-phenylalanine (48%). Especially the inhibition of two first derivatives is almost as high as for 3-nitropropanoic acid (67 %), a known inhibitor. Interestingly, in the antimycobacterial assay 3-nitropropanoic acid expressed no cells growth inhibition at 1 000 μmol/L. The benzoylation, 4-(trifluoromethyl)benzoylation and benzenesulfonation hampered the *in vitro* activity. Based on the limited number of tested compounds till now, it seems that less lipophilic salicylanilide derivatives provide more intense inhibition, although Schiff base avoided almost the activity. Further tests are required to clear and uncover the structure-activity relationships in this field.

Albeit some salicylanilide derivatives exhibited distinct anti-ICL effect, the concentration values for this inhibition are comparatively high. Salicylanilide derivatives inhibit mycobacteria even at the concentration lower than 1 μmol/L, i.e. at one hundred or more times lower concentration than was evaluated towards ICL. Presented data support the fact that salicylanilides share multiple targets and mechanisms of the action. The inhibition of isocitrate lyase may bring desired supportive effect especially in the treatment of latent (dormant) mycobacterial subpopulations and for the killing of the mycobacteria growing on the nutrition poor substrates with the presence of the carbon C_2 sources.

However, presented results represent only preliminary finding, further studies necessary for the evaluation of the structure-activity relationships are in progress.

4.4.2 Inhibition of Methionine Aminopeptidase

Methionine aminopeptidase (MetAP) is a metalloprotease removing the *N*-terminal methionine from nascent proteins during proteosynthesis. In prokaryotes, protein synthesis begins with a *N*-formylated methionine; peptide deformylase catalyzes the removal of the formyl group and then MetAP removes the newly unmasked *N*-terminal methionine. Essential mycobacterial enzyme MetAP1 is another promising target for the development of new antituberculosis agents. Olaleye et al.[152] identified chlorinated 1,4-naphthoquinone core in a high-throughput screening as its inhibitors expressing the activity also in culture for both non-replicating and growing mycobacteria. An ideal drug-candidate should avoid the inhibition of human MetAP1.

Some of 1,4-naphthoquinone bearing substituted phenylamino moiety (Figure 27) were determined being active with IC_{50} at micromolar range and were suggested that

salicylanilide derivatives sharing partial similarity at the aniline part may affect the function of these enzyme family.

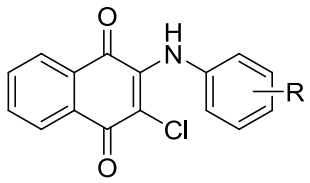


Figure 27. The general structure of 3-(substituted phenylamino)naphtoquinones

4.4.2.1 Methods

The cloning and expression of recombinant methionine aminopeptidase and enzymatic assays were performed at the Department of Pharmacology, Johns Hopkins School of Medicine, Baltimore, USA, under the supervision of Professor Jun O. Liu, Ph.D.

The methodology of the subcloning of the two MetAP from *M. tuberculosis* as well as enzyme assay conditions are described in the report of Prof. Liu and his co-workers[152].

MetAP inhibition assays were performed in duplicate in the presence of 10 μM compounds.

Computed % inhibition values are in relation to the reaction where MetAP and BcProAP were acting on H-Met-Pro-pNA uninhibited.

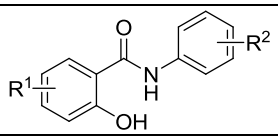
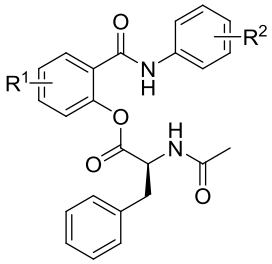
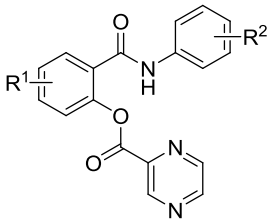
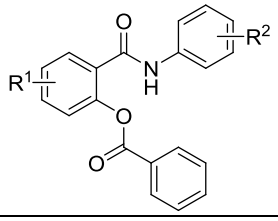
4.4.2.2 Results

The enzymatic assays were performed at the time, when we had completed only the series of "free" salicylanilides, salicylanilide pyrazinoates, benzoates and esters with *N*-acetyl-L-phenylalanine. That is the reason, why other groups were not evaluated, why we revealed only moderate inhibition activity. There were evaluated only esters previously investigated against MDR strains in this assay. For the results see Table 15, which overviews the inhibition rate of both human and mycobacterial MetAP1c at 10 μM concentration of the investigated derivatives.

An IC_{50} (against mycobacterial MetAP1c) measurement was attempted with 4-bromo-2-(4-(trifluoromethyl)phenylcarbamoyl)phenyl pyrazine-2-carboxylate, but unfortunately data did not converge to yield a sigmoidal curve.

While investigated compounds have an activity against the enzymes, their potencies are only moderate, not sufficiently high for detailed assays. All salicylanilides and their esters inhibited at 10 μM both forms of MetAP1c in the range from 5 to 49 %. The benzoylation of salicylanilides led to the derivative with decreased anti-MetAP potency, the influence of the introduction of other acyls is not overly pronounced.

Table 15. The percentage inhibition rate of mycobacterial and human MetAP1c

General structure	R^1	R^2	% inhibition of mycobacterial MetAP1c	% inhibition of human MetAP1c
	5-Cl	4-CF_3	21	48
	5-Cl	3,4-diCl	27	47
	5-Br	4-CF_3	26	49
	4-Cl	4-CF_3	23	44
	4-Cl	3,4-diCl	26	45
	4-Br	4-CF_3	22	42
	4-Cl	4-CF_3	22	42
	4-Cl	3,4-diCl	27	28
	4-Br	4-CF_3	41	27
	4-Cl	3-CF_3	25	16
	4-Cl	4-Br	27	27
	5-Cl	4-CF_3	19	40
	4-Cl	4-CF_3	19	18
	4-Cl	3,4-diCl	5	16
	4-Br	4-CF_3	19	28
	5-Cl	3,4-diCl	16	15
SB9OO64	a positive control		90	-

Regrettably, the majority of the molecules inhibited the human enzyme more strongly than that from *M. tuberculosis*. This action may contribute to the cytotoxicity for human cells. Two derivatives blocked significantly more mycobacterial than human MetAP – 4-bromo-2-[4-(trifluoromethyl)phenylcarbamoyl]phenyl pyrazine-2-carboxylate (the most favourable evaluated molecule) and 4-chloro-2-[3-(trifluoromethyl)phenylcarbamoyl]-phenyl pyrazine-2-carboxylate; four esters expressed the equal or almost equal inhibition values for both enzymes – two benzoates and two pyrazinoates. The esterification by pyrazine-2-carboxylic acid, as the most convenient salicylanilide modification, retains or in one case improves the activity towards mycobacterial enzyme and (with one exception) decreases the inhibition of human MetAP.

Although salicylanilide derivatives affect moderately the function of mycobacterial MetAP, their concentration in the assay was for some derivatives much higher (up to eighty times) than MIC towards *M. tuberculosis* including drug-resistant strains. The explanation of their potent *in vitro* efficacy exclusively due to inhibition of MetAP is

plainly inadequate; however it may contribute to the antimycobacterial activity analogous to ICL.

4.5 Discussion of the Structure-Activity Relationships

4.5.1 Antimycobacterial Activity

All salicylanilide esters exhibited a significant activity against mycobacterial strains at MIC values from 0.125 to 500 μmol/L. In general, salicylanilide esters possess mostly a higher activity than parent salicylanilides towards mycobacteria, especially *M. tuberculosis* and *M. kansasii*, in some cases even up to eight times.

For the substitution of the aniline ring, trifluoromethyl group has brought the maximal benefit, especially on the position 4; its relocation to the *meta*-position slightly reduces the activity, although it remains still above-average. 3,4-Dichloroaniline represents the second most favourable moiety, followed by bromine at positions 3 or 4. Contrarily, fluorine proves the least activity improvement from evaluated substituents in all series. The evaluation of the influence of the position of bromine, chlorine and fluorine on the aniline ring has not revealed distinct conclusion, it is changing for individual ester series and mycobacterial strain or the activities equal roughly themselves.

When concentrated on the salicylic ring, 4-chlorinated and 4-brominated esters (i.e. derived from 5-chloro/bromosalicylic acid) are mostly more convenient for the antimycobacterial activity than 5-chlorinated, relatively independent to the aniline substitution patterns. However, the data about bromine substitution are limited.

In summary, salicylanilide esterification has lead to the derivatives with predominantly stronger activity than parent salicylanilides towards mycobacteria, only with benzenesulfonates being an exception. The benzenesulfonylation has not proved successful. Esters with *N*-acetyl-L-phenylalanine reached the lowest MIC for drug-sensitive *M. tuberculosis* ($\geq$ 0.25 μmol/L), pyrazinoates expressed significantly a higher activity towards MDR and XDR-TB strains with MIC $\geq$ 0.125 μmol/L, followed by benzoates.

The sensitivity of mycobacteria to salicylanilide derivatives decreases in the order *M. tuberculosis* > *M. kansasii* > *M. avium*. Interestingly, in most cases drug-resistant strains of *M. tuberculosis* are even more sensitive than the drug-sensitive one. Based on MIC values, these derivatives act by the bactericidal mechanism of the action.

Salicylanilide esters with *N*-acetyl-L-phenylalanine, benzoic, 4-(trifluoromethyl)benzoic and pyrazine-2-carboxylic exceeded the antimycobacterial activity of salicylanilide acetates[133] and esters with *N*-Cbz amino acids[131,132] published previously. The activity towards MDR-TB of selected benzoates and pyrazinoates are comparable or superior to salicylanilide carbamates, the salicylanilide derivatives with the lowest MIC, which have been published already[92].

Table 16 summarizes the overview of the MIC of salicylanilide esters based on different substitution patterns.

Table 16. An overview of the antimycobacterial activity the group of salicylanilide esters expressed as comprehensive MIC

Derivative		*M. tuberculosis* 331/88	*M. avium* 330/80	*M. kansasii* 235/80	*M. kansasii* 6509/96	MDR-TB	XDR-TB
salicylic ring	aniline ring	MIC range [μmol/L]					
esters with *N*-acetyl-L-phenylalanine							
4-Cl	-	0.25 – 4	4 – 32	0.5 – 8	1 – 8	1 – 4	1 – 2
5-Cl	-	2 – 16	4 – 32	2 – 8	1 – 16	NT	NT
4-Br	-	0.25 – 0.5	4	0.5 – 2	1 – 2	1 – 2	2
-	3-Cl	2 – 4	4 – 32	4 – 8	2 – 8	NT	NT
-	4-Cl	4	8 – 16	4	2 – 16	NT	NT
-	3,4-diCl	0.5 – 2	8 – 16	1 – 4	1 – 4	1 – 4	2
-	4-Br	2 – 4	4 – 8	2 – 4	4 – 8	NT	NT
-	4-CF_3	0.25 – 16	4 – 32	0.5 – 4	1 – 8	1 – 2	1 – 2
benzoates							
4-Cl	-	0.5 – 4	4 – 16	1 – 8	1 – 8	0.25 – 1	0.25 – 1
5-Cl	-	2 – 8	4 – 16	1 – 8	1 – 8	0.25 – 2	0.5 – 1
4-Br	-	1	4	2	2	0.25 – 2	0.5 – 1
-	3-Cl	2 – 4	4 – 8	2 – 8	4 – 8	NT	NT
-	4-Cl	2 – 4	4 – 16	2 – 8	2 – 8	NT	NT
-	3,4-diCl	1 – 2	8	1 – 4	1 – 4	0.25 – 2	0.5 – 1
-	3-Br	2 – 4	4 – 16	2 – 8	2 – 8	NT	NT
-	4-Br	2	4 – 8	2 – 4	2 – 4	NT	NT
-	3-F	4 – 8	8 – 16	2 – 8	2 – 8	NT	NT
-	4-F	4 – 8	4 – 16	2 – 8	2 – 8	NT	NT
-	3-CF_3	2	8 – 16	2 – 4	2 – 4	NT	NT
-	4-CF_3	0.5 – 2	4 – 8	1 – 4	1 – 4	0.25 – 1	0.25 – 1
4-(trifluoromethyl)benzoates							
4-Cl	-	1 – 8	1 – 32	0.5 – 16	1 – 16	0.5 – 4	0.5 – 4
5-Cl	-	1 – 8	2 – 16	1 – 8	1 – 16	1 – 8	1 – 8
4-Br	-	1	2 – 4	1 – 4	1 – 4	0.5 – 2	0.5 – 1
-	3-Cl	2 – 4	8 – 16	0.5 – 4	2 – 8	4 – 8	4 – 8
-	4-Cl	2 – 4	4 – 8	1 – 4	1 – 4	2 – 4	2 – 4
-	3,4-diCl	1 – 2	2 – 8	1 – 4	1 – 4	1 – 2	1 – 2
-	3-Br	1 – 8	2 – 16	2 – 8	1 – 8	1 – 2	1 – 2
-	4-Br	2 – 4	8	1 – 8	2 – 8	1 – 4	1 – 2
-	3-F	4 – 8	8 – 32	4 – 16	8 – 16	NT	NT
-	4-F	4 – 8	4 – 32	2 – 16	4 – 16	NT	NT
-	3-CF_3	2	4 – 16	2 – 8	1 – 8	NT	NT
-	4-CF_3	1 – 2	1 – 8	1 – 4	1 – 4	0.5 – 2	0.5 – 1
pyrazinoates							
4-Cl	-	0.5 – 8	2 – 32	2 – 8	1 – 8	0.125 – 2	0.125 – 2

5-Cl	-	1 – 8	2 – 16	1 – 8	1 – 8	0.125 – 0.5	0.125
4-Br	-	0.5 – 1	2 – 4	2 – 4	1 – 2	0.125 – 1	0.25 – 0.5
-	3-Cl	2 – 4	4 – 8	2 – 4	2 – 8	NT	NT
-	4-Cl	2 – 4	2 – 8	2 – 4	1 – 4	NT	NT
-	3,4-diCl	1 – 2	4 – 16	2 – 8	1 – 4	0.125 – 1	0.5 – 1
-	3-Br	1 – 8	8 – 16	4	1 – 4	0.125 – 1	0.25 – 1
-	4-Br	2	4 – 8	2 – 4	2 – 4	NT	NT
-	3-F	2 – 8	8 – 16	2 – 8	4 – 8	NT	NT
-	4-F	2 – 8	2 – 16	2 – 8	2 – 8	NT	NT
-	3-CF_3	1 – 2	8 – 32	8	4 – 8	0.5 – 2	1 – 2
-	4-CF_3	0.5 – 1	2 – 8	1 – 4	1 – 2	0.125 – 1	0.125 – 0.5
benzenesulfonates							
4-Cl	-	1 – 16	≥ 16	≥ 4	≥ 8	NT	NT
5-Cl	-	≥ 2	≥ 8	≥ 2	≥ 4	NT	NT
-	3-Cl	4 – 16	≥ 32	8 – 16	8 – 500	NT	NT
-	4-Cl	4 – 8	8 – 32	8 – 16	8 – 16	NT	NT
-	3,4-diCl	1 – 8	16 – 62.5	4 – 8	8	NT	NT
-	3-Br	8 – 16	≥ 16	16 – 62.5	8 – 125	NT	NT
-	4-Br	8	8 – 32	8 – 16	4 – 16	NT	NT
-	3-F	16	≥ 500	16 – 32	16 – 125	NT	NT
-	4-F	8 – 16	16 – 32	8 – 32	16	NT	NT
-	3-CF_3	≥ 2	≥ 62.5	≥ 8	≥ 8	NT	NT
-	4-CF_3	1 – 4	≥ 125	≥ 2	≥ 4	NT	NT

NT: not tested

The salicylamides and salicylaldehyde-based Schiff bases of sulfonamides expressed clearly a lower activity towards *M. tuberculosis* and *M. avium*. For *M. tuberculosis* any sulfonamide modification resulted mostly in increased MIC, for *M. avium* this attenuation is not so dramatic, despite the connection by both *via* amidic or azomethine linkage. The presence of phenolic group seems to be necessary for the activity against *M. tuberculosis* and *M. avium* only for amides, not for Schiff bases. The activity against *M. kansasii* does not require phenolic moiety. Sulfathiazole modifications were evaluated to be the most beneficial, while sulfamethazines showed the lowest activity.

Table 17 overviews the overall summarization of MIC related to structure.

Table 17. Accumulated MIC of sulfonamide derivative

Derivative			*M. tuberculosis* 331/88	*M. avium* 330/80	*M. kansasii* 235/80	*M. kansasii* 6509/96
phenyl ring	bridge	sulfonamide	MIC range [μmol/L]			
"free" sulfonamides			32 – 125	32 – 62.5	2 – 125	1 – 32
2-OH-5-Cl	-	-	≥ 32	≥ 32	2 – 250	2 – 250
3-Cl	-	-	≥ 62.5	≥ 32	2 – 8	4 – 8
-	CONH	-	≥ 125	≥ 32	8 – 62.5	4 – 62.5
-	CH=N	-	32 – 500	62.5 – 125	2 – 250	2 – 250
-	-	sulfamethoxazole	≥ 62.5	≥ 32	2 – 16	4 – 8
-	-	sulfadiazine	125 – 250	62.5 – 125	4 – 32	4 – 32
-	-	sulfathiazole	≥ 32	≥ 62.5	2 – 16	2 – 8
-	-	sulfamethazine	125 – 500	32 – 125	16 – 250	16 – 250

4.5.2 Antibacterial and Antifungal Activity

Salicylanilide derivatives presented in this thesis revealed an obvious *in vitro* antibacterial activity. In general, Gram-negative strains exhibited a stronger rate of the resistance than well susceptible Gram-positive strains, especially methicillin-sensitive *S. aureus* and MRSA strain. The antibacterial activity of previously published salicylanilide esters[92] (acetates, amino acid esters and carbamates) was not determined, albeit salicylanilides have been known to hamper the growth especially of Gram-positive bacteria for many years[86,92].

For the antibacterial activity, in general, it seems that the most convenient substituents for the aniline ring are generally 4-CF_3, 4-Cl or bromine. The antibacterial activity is mostly positively modulated by derivatives of 5-chlorosalicylic acid rather than by 4-chlorosalicylic acid (i.e. position 4 and 5 of the esters, respectively); derivatives of 5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide represent an partial exception.

The *in vitro* efficacy for salicylanilide carboxylic acids esters steps down in this order: 4-(trifluoromethyl)benzoates > benzoates > pyrazinoates > esters of *N*-acetyl-L-phenylalanine.

Based on MIC values, we hypothesized that salicylanilides esters act mainly as bactericidal agents against *Staphylococci*, but not clearly towards *Enterococci*. MIC of a wide range of the esters towards *S. aureus* was comparable to PNC and comparable or superior to bacitracin.

A few esters expressed *in vitro* activity towards *Pseudomonas aeruginosa* (one benzoate, two pyrazinoates). The formation of the benzenesulfonic acid esters totally abolished any *in vitro* antibacterial activity.

The introduction of both chlorinated salicylanilide and salicylaldehyde moiety into molecules of all four sulfonamides resulted in the antibacterial activity improvement with salicylamide superiority. MIC of new derivatives are much more lower than of parent sulfonamides. The replacement of 2-hydroxy group from the salicylamide resulted in the total loss of the antibacterial activity; this dependence was not observed for Schiff bases. The mild activity against Gram-negative species was described preferably particularly for the Schiff base derivatives, not for amides.

Although the sulfonamide modifications (their "salicylic" amides and Schiff bases) significantly and promisingly enhance the antibacterial activity, *in vitro* MIC values still remain obviously higher than those revealed for salicylanilide esters.

The antifungal potency is for all salicylanilide derivatives generally worse than antibacterial and antimycobacterial activity when compared on the base of MIC; some esters have demonstrated no activity.

In contrast to the antibacterial acting, the antimycotic activity is positively modulated by the presence of 5-chloro substitution of the salicylic ring. The discussion of the influence of the aniline ring substitution has to surmount due to limited data – two series of esters (benzenesulfonates and benzoates) are for this purpose unusable because of the limited efficacy and only for a few 4-(trifluoromethyl)benzoates it was feasible to determine MIC. With this restriction, the presence of 4-chloro, 4-bromo, 3,4-dichloro or 4-CF_3 moiety enhances the antifungal activity. The comparison of the phenolic hydroxyl modification corroborated that 4-(trifluoromethyl)benzoic acid derivatives are the most favourable, followed by pyrazine-2-carboxylic acid, then by *N*-acetyl-L-phenylalanine and benzoic acid esters. Salicylanilide benzenesulfonates avoided completely the antifungal activity and sulfonamide derivatives showed only a sporadic activity with no clear structure dependence.

It seems to be a general rule that *Candidae* are the most resistant than filamentous fungi for all presented salicylanilide esters. *C. tropicalis* and *C. glabrata* represent the least sensitive strains, when *Trichophyton mentagrophytes* showed the highest susceptibility. It seems that the action towards *Candidae* is only fungistatic, whereas moulds may be killed irreversibly. The acidic pH did not improved MIC of benzoic and 4-(trifluoromethyl)benzoic acids esters.

Unhappily, the antifungal potential of the salicylanilide derivatives presented in this thesis is not as hopeful as in the case of the antimycobacterial and antibacterial (Gram-positive) activities. Presented salicylanilide derivatives revealed overall higher MIC than salicylanilide acetates[133] and heterogeneous with the respect to *N*-Cbz amino acids esters[132].

5. Conclusion

The thesis deals with three main scopes – the topic of tuberculosis treatment, the resistant form of tuberculosis and the development of novel potential agents to combat them, the study of the rearrangement of the salicylanilide esters with amino acids and the salicylanilide molecules modification especially by esterification.

The problematic of the TB treatment still remains one of the urgent health problems of the world. The searching for new antimycobacterial drugs is demanded due to many reasons like the length and not complete efficiency of current antituberculosis therapy, the need of the new remedies to suppress latent form of mycobacterial infections and for concomitant therapy of TB/HIV infections as well as the growing incidence and prevalence of MDR-, XDR- and most recently the most dangerous TDR-TB. The development of new drugs consists of two main approaches – the whole-cells screening of previously synthesized compounds and subsequent identification of appropriate molecular target, or the first identification of suitable target, the design, synthesis and evaluation of potential inhibitors and final whole-cells assay *in vitro*.

In the field of theoretical research, we reviewed the recent advances in the research of new anti-MDR-TB drugs until 2009 in both English and Czech language (**Appendices I** and **II**), we engaged in the area of isocitrate lyase inhibition and inhibitors (**Appendix III**), the biological activity of salicylanilide derivatives – antiviral for salicylanilides and partly salicylamides (**Appendix IV**) and antimicrobial for salicylanilide esters, including notes about salicylanilide mechanism of the action and undesired effects (**Appendix V**).

Experiments have been focused firstly on the study of the rearrangement of the salicylanilide amino acid esters after liberation of the amino group to form "diamides". The mechanism of the rearrangement has been improved and corrected with respect to prior opinion; nowadays it is considered *via* five-membered imidazolinone ring (**Appendix VI**). We also studied the variability and versatility of this rearrangement performed with motley *N*-protected amino acids used for the esterification (**Appendix VII**). The synthetic procedure consists of micro waves-assisted salicylanilide synthesis, esterification using *N*,*N*′-dicyclohexylcarbodiimide as an activating agent, acidolysis (HBr/acetic acid) of the protecting group and then liberation of the amino group by triethylamine leading to the rearrangement and subsequent "diamide" formation.

The second part of the experiments has been aimed to the design, synthesis and evaluation of the new salicylanilide derivatives, especially esters, to find more convenient prodrug

forms especially towards mycobacteria including atypical and multidrug-resistant strains and then against bacteria and fungi, in the case of salicylanilide pyrazinoates being very probably mutual prodrugs. The salicylanilide esters were prepared using proved methodology *via N,N´*-dicyclohexylcarbodiimide as well as *via* direct reaction of the triethylamonnium salt of appropriate salicylanilide with acylchloride. A wide range of synthesized esters displayed a very good or excellent *in vitro* activity towards mycobacteria including MDR-TB, Gram-positive cocci and/or filamentous fungi (**Appendices VIII**, **IX**, **X**, **XI**). Additionally, the experimental part contains unpublished results about characterization of synthesized compounds and their biological activities. The searching for new antimicrobial active molecules not based on the salicylanilide esterification represent sulfonamides with modified primary amino group by 5-chlorosalicylic acid and 5-chlorosalicylaldehyde (**Appendix XII**).

Moreover, there were attempts to find new molecular target(s) for salicylanilides and their esters with proposed enzymes being isocitrate lyase and methionine aminopeptidase with only a partial success.

Salicylanilides represent one promising group of potential antimicrobial agents possessing many interesting pharmacological properties.

In conclusion, the goals of this thesis have been successfully fulfilled and on this basis the further research will continue.

List of Publications and Presentations

This thesis is based on the following publications which are numbered in the text by the Roman numerals I – XII. Here presented ordering retains chronological aspect.

The list of presentations comprises only those developed during the course of Ph.D. study.

A) Published, Accepted and in Progress Publications

1. Vinšová, J.; **Krátký, M.** *Tuberculosis — The Development of New MDR-TB Drugs*. In: Nguy, S.; Kung, Z., editors. Drug-Resistant Tuberculosis: Causes, Diagnosis and Treatments. New York: Nova Science Publishers; 2009, pp. 59-141. ISBN 978-1-60876-055-8.
2. Vinšová, J.; Imramovský, A.; **Krátký, M.**; Férriz, J.M.; Palát, K.; Lyčka, A.; Růžička, A. Unprecedented rearrangement of salicylanilide derivatives: imidazolinone intermediate formation. *Tetrahedron Lett.* 2010, *51*, 23-26. [ISSN 0040-4039, IF_{2009} = 2.660] (**Appendix VI**)
   The participation: synthesis of starting salicylanilides, synthesis, isolation and purification of four salicylanilide esters and corresponding hydrobromide salts and "diamides" (from the total of six), isolation of one imidazolinone intermediate, a partial characterization of synthesized compounds, a share during article writing and processing.
3. **Krátký, M.**; Vinšová, J. Pokroky ve vývoji antituberkulotik působících na multilékově resistentní kmeny. *Chem. Listy* 2010, *104*, 998-1005 plus online supplement. [ISSN 0009-2770, IF_{2009} = 0.717]
   (**Appendix II**)
   The participation: main author, i.e. translation, reduction, re-structuring, improvement and formal changes of the sourcing article text under the supervision of J. Vinšová.
4. Vinšová, J.; **Krátký, M**. *The Development of New MDR-TB Drugs*. New York: Nova Science Publishers; 2010, 1st ed. 98 pp. ISBN 978-1-6168-233-0.
   (**Appendix I**)
   The publication is almost the same as a chapter in the book (publication number 1).
   The participation: main author of the chapters 2.1., 3.6., 4., co-author of the chapters 1., 2.2., 3.1., 3.3., 5., 6., a share on the general adjustment and graphical form.
5. Imramovský, A.; Férriz, J.M.; Pauk, K.; **Krátký, M.**; Vinšová, J. Synthetic Route for the Preparation of 2-Hydroxy-*N*-[1-(2-hydroxyphenylamino)-1-oxoalkan-2-yl]benzamides. *J. Comb. Chem*. 2010, *12*, 414-416. [ISSN 1520-4766, IF_{2009} = 3.450]
   (**Appendix VII**)
   The participation: synthesis of starting salicylanilides, synthesis, isolation and purification of ten salicylanilide esters, corresponding hydrobromide salts and "diamides", evaluation of one-pot synthesis without a previous isolation of salicylanilide esters and hydrobromide salts, a partial characterization of synthesized compounds, a share during article writing and processing.
6. **Krátký, M.**; Vinšová, J.; Buchta, V.; Horvati, K.; Bösze, S.; Stolaříková, J. New amino acid esters of salicylanilides active against MDR-TB and other microbes. *Eur. J. Med. Chem*. 2010, *45*, 6106-6113. [ISSN 0223-5234, IF_{2009} = 3.269] (**Appendix VIII**)
   The participation: synthesis of all starting salicylanilides and esters, their isolation and purification, characterization, management of the biological evaluation, discussion of

the biological activity and structure-activity relationships, main share on the article writing and processing.

7. **Krátký, M.**; Vinšová. J. Antiviral Activity of Substituted Salicylanilides – A Review. *Mini-Rev. Med. Chem.* 2011, *11*, 956-967. [ISSN 1389-5575, IF_{2010} = 2.622] (**Appendix IV**)
   The participation: main author of the whole text under the supervision of J. Vinšová, main share on the article processing.
8. **Krátký, M.**; Vinšová, J. Salicylanilide Ester Prodrugs as Potential Antimicrobial Agents – A Review. *Curr. Pharm. Des.* 2011, *17*, 3494-3505. [ISSN 1381-6128, IF_{2010} = 4.774] (**Appendix V**)
   The participation: main author of the whole text under the supervision of J. Vinšová, main share on the article processing.
9. **Krátký, M.**; Vinšová, J.; Buchta, V. *In vitro* Antibacterial and Antifungal Activity of Salicylanilide Pyrazine-2-carboxylates. *Med. Chem.* 2011, accepted. [ISSN 1573-4064, IF_{2010} = 1.603] (**Appendix X**)
   The participation: synthesis of all starting salicylanilides and esters, their isolation and purification, characterization, discussion of the biological activity and structure-activity relationships, main share on the article writing and processing.
10. **Krátký, M.**; Vinšová, J.; Buchta, V.; Stolaříková, J. Antimicrobial Activity of Sulfonamides Containing 5-Chloro-2-hydroxybenzaldehyde and 5-Chloro-2-hydroxybenzoic Acid Scaffold. *Eur. J. Med. Chem.* 2011, under revision. (**Appendix XII**)
    The participation: synthesis of all amide and Schiff base derivatives, their isolation and purification, characterization; discussion of the biological activity and structure-activity relationships, main share on the article writing and processing.
11. **Krátký, M.**; Vinšová, J.; Buchta, V. *In Vitro* Antibacterial and Antifungal Activity of Salicylanilide Benzoates. *TheScientificWorldJOURNAL* 2011, under revision. (**Appendix IX**)
    The participation: synthesis of all starting salicylanilides and esters, their isolation and purification, characterization, discussion of the biological activity and structure-activity relationships, main share on the article writing and processing.
12. **Krátký, M.**; Vinšová, J.; Guisado Rodriguez, N.; Stolaříková, J. Antimycobacterial Activity of Salicylanilide Benzenesulfonates. *Molecules* 2011, under revision. (**Appendix XI**)
    The participation: synthesis of most starting salicylanilides and esters, their isolation and purification, remaining ones were synthesized by N. Guisado Rodriguez under my direct supervision; their spectral characterization, discussion of the biological activity, main share on the article writing and processing.
13. **Krátký, M.**; Vinšová, J. Advances in Mycobacterial Isocitrate Lyase Targeting. *Med. Res. Rev.* 2011, in progress. (**Appendix III**)
    The participation: main authors of the chapters 2., 3. and 4., a share on the article processing, general adjustment and graphical form.

B) Presentation in Czech Language

1. **Krátký, M.**; Vinšová, J.; Stolaříková, J. *Nová proléčiva antimykobakteriálně aktivních salicylanilidů*. 38. Konference Syntéza a analýza léčiv, 14.-16. 9. 2009, Hradec Králové, ISBN 978-80-7305-0778-8, p. 104. (poster)
2. **Krátký, M.**; Vinšová, J.; Stolaříková, J.; Buchta, V. *Antimikrobiálně aktivní estery salicylanilidů s kyselinou benzoovou*. 39. Konferencia Syntéza a analýza liečiv, 2.-4. 9. 2010, Modra-Harmonia, ISBN 978-80-7399-986-5, p. 86. (poster)

3. Vinšová, J.; **Krátký, M**. *Vývoj antituberkulotik působících na MDR kmeny*. 39. Konferencia Syntéza a analýza liečiv, 2.-4. 9. 2010, Modra-Harmonia, ISBN 978-80-7399-986-5, pp. 28-29. (plenary lecture)
4. **Krátký, M.**; Vinšová, J.; Buchta, V. *Nové salicylanilidové estery s potenciální antibakteriální a antifungální aktivitou*. 45. Pokroky v organické, bioorganické a farmaceutické chemii, 20.-22. 11. 2010, Nymburk, Abstract in *Chem. Listy* 2010, *104*, p. 1097. [ISSN 0009-2770, IF_{2009} = 0.717] (poster)
5. **Krátký, M.**; Stolaříková, J.; Buchta, V.; Vinšová, J. *Recent Advances in the Group of Salicylanilide Esters as Potential Antimicrobial Agents*. 1. Fakultní postgraduální vědecká konference, 1.-2. 2. 2011, Hradec Králové, abstract should be published in *Folia Pharm. Univ. Carol.*, 2011. (lecture)
6. **Krátký, M.**; Vinšová, J.; Buchta, V. *Salicylanilide 4-(trifluoromethyl)benzoates as potential antimicrobial agents*. 40. Syntéza a analýza léčiv, Brno, 12.-13. 9. 2011, ISBN 978-80-7305-593-6, P92. (poster)
7. **Krátký, M.**; Vinšová, J.; Buchta, V.; Stolaříková, J. *Nové deriváty sulfonamidů s 5-chlorsalicyladehydem a kyselinou 5-chlorsalicylovou*. 63. Sjazd chemikov, 5.-9. 9. 2011, Tatranské Matliare. ChemZi 7/11 2011, ISSN 1336-7242, p. 225. (poster).
8. **Krátký, M.**; Vinšová, J.; Stolaříková, J.; Liu, J.O. *Salicylanilidové estery působící na rezistentní mykobakteria*. 46. Pokroky v organické, bioorganické a farmaceutické chemii „Liblice 2011", 11.-13. 11 2011, Lázně Bělohrad. Sborník abstraktů, p. 76. (poster)

C) English Presentations

1. **Krátký, M.**; Stolaříková, J.; Ulmann, V.; Vinšová, J. *New prodrugs of antimycobacterial active salicylanilides*. 1st Turkish-Russian Joint Meeting on Organic and Medicinal Chemistry, 14th-17th October 2009, Antalya, Turkey. Book of Abstracts, pp. 63-64. (poster)
2. **Krátký, M.**; Vinšová, J.; Stolaříková, J. *Multidrug-resistant Tuberculosis and the Development of New Drugs*. 6th Meeting of the European Network of Doctoral Studies in Pharmaceutical Sciences, 16th-18th November 2009, Palermo, Italy. Book of Abstracts, p. 33. (lecture)
3. **Krátký, M.**; Vinšová, J.; Bösze, S.; Stolaříková, J. *Salicylanilide esters and their activity on drug-resistant Mycobacterium sp. strains*. 31st Annual Congress of the European Society of Mycobacteriology, 4th-7th July 2010, Bled, Slovenia. Book of Abstracts, ISBN 978-961-6633-28-4, p. 214. (poster)
4. Imramovský, A.; Pauk, K.; Vinšová, J.; **Krátký, M.** *A New Synthetic Route for the Preparation of 2-Hydroxy-N-(1-(oxo-(phenylamino)-alkan-2-yl)benzamides (salicyldiamides)*. 3rd EuCheMS Chemistry Congress, August 29th – September 2nd 2010, Nürnberg, Germany. Abstract of Papers VIIa/073. (poster)
5. **Krátký, M.**; Stolaříková, J.; Vinšová, J. *Structure-activity relationships of the salicylanilide esters group active against MDR-TB*. 18th Euro QSAR, Discovery Informatics & Drug Design, 19th-24th September 2010, Rhodes, Greece. Book of Abstracts, p. 325. (poster)
6. **Krátký, M.**; Vinšová, J.; Stolaříková, J. *New salicylanilide esters active in vitro against multidrug-resistant tuberculosis*. Italian, Austrian, Czech, Greek, Hungarian, Polish, Slovak, Slovenian VII Joint Meeting on Medicinal Chemistry, June 30th – July 2nd 2011, Catania, Italy. Book of Abstracts, p. 181. (poster)
7. Vinšová, J.; **Krátký, M.**; Stolaříková, J. *Salicylanilide esters active against MDR tuberculosis*. 29th Cyprus-Noordwijkerhout-Camerino Symposium Trends in Drug Research, 2nd-7th October 2011, Limassol, Cyprus. Book of Abstracts, ISBN 978-9963-9615-2-8, p. 83. (poster)

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