

# Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/77078/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Ferrari, Valentina ORCID: <https://orcid.org/0000-0003-1895-8906> and Serpi, Michaela ORCID: <https://orcid.org/0000-0002-6162-7910> 2015. Nucleoside analogs and tuberculosis: new weapons against an old enemy. *Future Medicinal Chemistry* 7 (3) , pp. 291-314. 10.4155/fmc.14.166 file

Publishers page: <http://dx.doi.org/10.4155/fmc.14.166>  
< <http://dx.doi.org/10.4155/fmc.14.166> >

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies.

See

<http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



# Nucleoside analogues and tuberculosis: new weapons against an old enemy

Valentina Ferrari<sup>\*,1</sup> & Michaela Serpi<sup>1</sup>

<sup>1</sup>School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, King Edward VII Avenue, Cardiff CF10 3NB, UK.

\* Author for correspondence:

ferrariv@cardiff.ac.uk

## Abstract

Purine and pyrimidine nucleoside and nucleotide analogues have been extensively studied as anticancer and antiviral agents. In addition to this, they have recently shown great potential against *Mycobacterium Tuberculosis*, the causative agent of Tuberculosis (TB). TB ranks as the tenth most common cause of death in the world. The current treatment for TB infection is limited by side effects and cost of the drugs and most importantly by the development of resistance to the therapy. Therefore the development of novel drugs, capable of overcoming the drawbacks of the existing treatments, has become the focus of many research programs. In parallel to that, a tremendous effort has been made to elucidate the unique metabolism of this pathogen with the aim to identify new possible targets.

This review presents the state of the art in nucleoside and nucleotide analogues in the treatment of TB. In particular, we report on the inhibitory activity of this class of compounds, both in enzymatic and whole-cell assays, providing a brief insight to which reported target these novel compounds are hitting.

## Introduction

In 1882, Robert Koch identified *Mycobacterium tuberculosis* (*M.tb*) as the etiological agent of human tuberculosis (TB) [1-2]. *M.tb* is part of the *Mycobacterium tuberculosis* complex within the *Mycobacterium* genus along with *M. africanum*, *M. bovis*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. canettii* and *M. mungi* [3].

### ■ Key term

*Mycobacterium tuberculosis* is a Gram-positive acid fast bacterium with a G + C-rich genome and a cell envelope containing an additional layer beyond the peptidoglycan that is exceptionally rich in unusual lipids, glycolipids and polysaccharides. Novel biosynthetic pathways generate cell-wall components such as mycolic acids, mycocerosic acid, phenolthiocerol, lipoarabinomannan and arabinogalactan, and several of these may contribute to mycobacterial longevity, trigger inflammatory host reactions and act in pathogenesis.

The World Health Organisation (WHO) indicates TB as a global emergency, estimating that in 2011 there were 8.7 million new cases and 1.4 million deaths due to the infection, including 350,000 deaths associated with a HIV co-infection [4].

Sub-Saharan Africa has the highest rates per capita of TB, while India, China, South Africa and the Russian Federation share 60% of all TB cases worldwide [4].

TB predominantly affects the lung although extra pulmonary forms exist and may affect the central nervous system, urogenital tract, digestive system and cutis [5]. All types of mycobacterial infections are initiated by the inhalation of viable bacilli contained in droplets exhaled by patients with active disease [6]; the progression and resolution of the disease is divided into different stages, in which the bacteria are first disseminated by lymphatic circulation to regional lymph nodes in the lung and then spread to extra pulmonary areas leading to secondary manifestations such as tuberculosis meningitis, pleural inflammation (pleurisy) and bones and joints lesions [5,7]. In the pulmonary manifestation, mycobacteria are taken up by macrophages and carried to the lung where additional immune cells are recruited [8] and the immune system organises structures called granuloma, pathological hallmarks of TB, whose aim is to contain the infection, making the chemotherapeutic eradication extremely difficult, due to

the sequestration of bacilli within shielded lesion compartments [9-11]. These pulmonary lesions evolve to necrotising granulomas containing a caseum core resulting from lysis of host and bacterial cells, and surrounded by fibroblasts layers [9].

This stage of TB infection is called latent phase and is not infectious [12]. However the progression from latent to active TB may occur at any time, from soon after infection to many years later and can be triggered by different factors [13]. Patient groups at higher risk include those infected with HIV, children younger than 5 years of age and people who are receiving immunosuppressive drug therapy following organ transplantation [14-15].

## Current treatment

Drug	Main Affected Pathway	Reference
<b>First-line Drugs</b>		
Isoniazid	Mycolic acid synthesis	[16,17]
Rifamicins (Rifampicin, Rifapentine, Rifabutine)	RNA transcription	[18]
Pyrazinamide	RNA trans-translation/fatty acid synthesis	[19,20]
Ethambutol	Arabinogalactan biosynthesis	[21]
<b>Second-line drugs</b>		
Injectable aminoglycosides (Streptomycin, Kanamycin, Amikacin)	Protein synthesis	[22,23]
Injectable polypeptides (Capreomycin, Viomycin)	Protein synthesis	[24]
Oral and injectable fluoroquinolones (Ofloxacin, Ciprofloxacin, Levofloxacin, Moxifloxacin, Gatifloxacin)	DNA supercoiling	[25]
<i>Para</i> -aminosalicylic acid	Folate biosynthesis	[26]
Terizidone	Cell wall synthesis	[27]
Ethionamide Prothionamide	Mycolic acid biosynthesis	[28,29]
Thioacetazone	Mycolic acid biosynthesis	[30]
Linezolid	Protein synthesis	[31]
Cycloserine	Peptidoglycan synthesis	[32]
<b>Third-line drugs</b>		
Clofazimine	DNA synthesis	[33]

Amoxicillin plus Clavulanate, Imipenem plus Ciliastatin	Cell wall synthesis	[34,35]
Clarithromycin	Protein synthesis	[36]

**Table 1.** Classification and main targets of first, second and third line treatments of TB.

Streptomycin, discovered in 1946, was the first drug found to successfully treat TB [37]. Therapy for drug-sensitive TB has evolved leading to the establishment of a first two month-long “intensive phase” requiring a combination of four different drugs among the first line treatments: isoniazid, rifampin, pyrazinamide, ethambutol (Table 1) [3,38]. The following “continuation stage” requires administration of isoniazid and rifampin for four months, completing the treatment [3,38,39]. Within the first few weeks of this drugs combination the patient loses infectiousness, although the remaining months are crucial to eradicate the slow growing fraction of the bacilli. This treatment is recommended for drug-susceptible TB cases and achieves cure rates of >95% when administered under directly observed therapy (DOT) [40-41]. Important side effects, such as hepatotoxicity, in some cases force the termination of the cure; this, together with lengthy cure duration, is responsible for patient’s non-compliance to the treatment [42-45]. HIV and TB co-infection in a patient is the worst case scenario, due to pharmacokinetic drug-drug interactions causing loss of efficacy and increase in toxicity [3,46,47]. These issues led to the emergence of *M.tb* multidrug-resistant strains (MDR-TB), resistant at least to isoniazid and rifampin [48,49]. The treatment of MDR-TB includes at least four second line drugs whose anti-tuberculosis effectiveness was proven (Table 1), over a period of 18-24 months [3,48,50]. Resistance to treatment increased with the emergence of extensively drug-resistant tuberculosis (XDR-TB) which does not respond to most of the first and second-line anti-TB drugs and requires the use of third line anti-TB drugs for longer periods of time (Table 1) [51,52].

Agents used for the treatment of MDR-TB and XDR-TB cause more severe side effects than first line anti-TB drugs, including nephrotoxicity, ototoxicity, hepatotoxicity and dysglycaemia [37,45,49]. Statistics show MDR-TB is now global, especially affecting the developing countries with an estimated 500,000 cases reported in 2011 [4]. XDR-TB, on the other hand, has been reported in 92 countries [4]. The cost of treating MDR-TB is up to 200 fold higher than the treatment of drug-sensitive TB, often requiring up to two years of treatment, daily injections and

in-patient care [53]. Moreover, the achievement of universal access to MDR-TB and XDR-TB treatment and preventive therapy can prove logistically and economically prohibitive [4]. The vaccine Bacille Calmette-Guérin (BCG) is being used today to prevent TB in infants [54]. But while BCG is the most widely used vaccine in the world, it has not successfully eliminated the disease due to its limited efficacy and potential side effects [54]. In summary, progress in the knowledge of the pathology and bacteriology of TB could potentially help achieve global control of this epidemic by discovering new therapeutic targets and introducing new agents that could ameliorate the current treatment of TB by:

- shortening treatment duration
- eradicating MDR and XDR strains
- increasing patient compliance to the treatment (reducing the daily pill burden and dosing frequency)
- reducing toxicities
- allowing optimal treatment of potential HIV co-infection [45].

## **Nucleosides analogues as anti-mycobacterial agents**

### **■ Key term**

**Nucleoside analogues** are synthetically modified nucleosides that generally exert their therapeutic activity after intracellular phosphorylation to nucleotide and act as antimetabolites, mimicking their natural counterparts and altering the processes in which they are involved.

Nucleosides and nucleotides are essential for many cellular functions, including the storage of genetic information, gene expression, energy metabolism and cell signaling. These compounds have already proven their importance as anticancer and antiviral agents [55]. In addition, these derivatives have shown moderate to good activity against several bacterial strains. This review will focus on the anti-mycobacterial potential of nucleoside analogues.

A thorough understanding of the enzymes involved in mycobacterial purine and pyrimidine metabolic pathways led to the identification of nucleoside analogues that potently and selectively inhibit *M.tb* viability.

### ■ Key terms

**Nucleotide salvage pathway** is a pathway in which purine and pyrimidine nucleotides are synthesized from bases and nucleosides that are released during degradation of RNA and DNA. This recovery process is important in those organs that cannot undergo nucleotide *de novo* synthesis.

**Nucleotide *de novo* pathway** is a pathway in which the nucleotides are assembled from simpler starting materials such as amino acids, at the expense of ATP. The *framework* for a pyrimidine base is assembled first and then attached to a ribose, while purine nucleotides are synthesized piece by piece directly onto a ribose-based structure.

Within the purine nucleoside group, few agents were identified as inhibitors of enzymes involved in the purines *de novo* and salvage pathways [56], such as hypoxanthine-guanine phosphoribosyltransferase, purine nucleoside phosphorylase and 5-amino-imidazole-4-carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase. Some classes of purine nucleoside derivatives are also able to interfere with enzymes specifically expressed in *M.tb* [57]. Among these are aryl/acyl adenylating enzymes such as biotin protein ligase (BIR A) and aminoacyl tRNA synthase (aaRSs) and enzymes responsible for siderophore (MtbA), pantothenate (PANC) and mycothiol MshC biosynthesis, the activity of which has been proven essential for *M.tb* virulence.

The alternative pyrimidine nucleoside structures, on the other hand, represent a crucial class of inhibitors of thymidylate synthase (ThyX) and thymidine monophosphate kinase (TMPKmt) enzymes, involved in pyrimidine nucleoside metabolism [58].

This review covers the medicinal chemistry of anti-mycobacterial nucleoside analogues, including their enzymatic inhibition and/or *in vitro* anti-mycobacterial activity. The activity of some analogues whose target has yet to be identified will be reported in the last section.

The identification of multiple targets for nucleoside analogues treatment of TB and the desperate need for new anti-TB therapies support the potential of such molecules as new anti-mycobacterial candidates.

## **Nucleoside inhibitors of enzymes involved in purine metabolism**

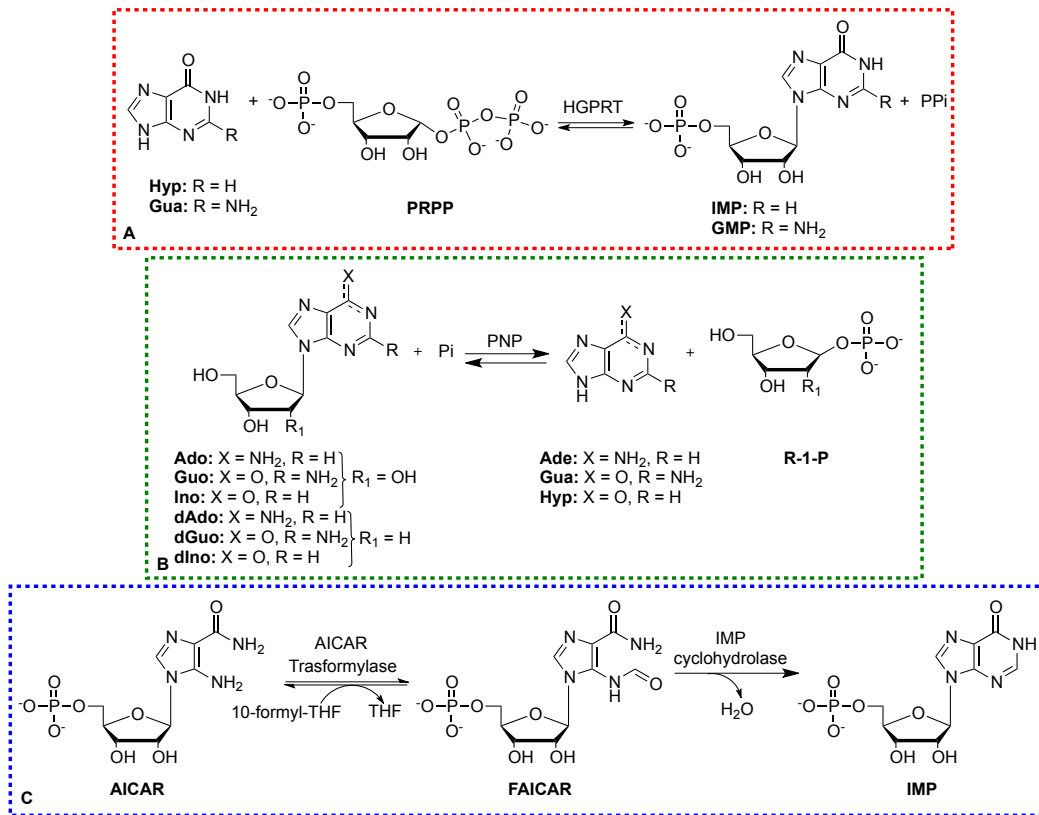
Purine metabolism is conserved among eukaryotic and prokaryotic organisms. Evidence shows that mycobacterial genome encodes for enzymes involved in the *de novo* and salvage synthesis of purine nucleoside monophosphate. However it is still unclear when the switch between one and the other occurs. The exploitation of the differences between human and mycobacterial enzymatic mechanisms and structures has helped identifying some potential anti-mycobacterial agents.

### ***Hypoxanthine–guanine phosphoribosyltransferase***

Phosphoribosyltransferase enzymes (PRTs) are expressed in the purine salvage pathway. They are essential for the reproduction and survival of different pathogens, such as *M.tb* [59]. Within this family of enzymes, hypoxanthine-guanine phosphoribosyltransferase (HGPRT) was identified as an interesting target for the development of new anti-mycobacterial agents [60].

*M.tb* is able to internalise 6-oxopurines hypoxanthine (Hyp) and guanine (Gua) from the extracellular medium. Subsequently, HGPRT catalyses the  $Mg^{2+}$ -dependent reversible transfer of the 5-phosphoribosyl group from 5-phosphoribose-1-pyrophosphate (PRPP) to the N9 position of 6-oxopurines (Hyp and Gua), to form respectively inosine monophosphate (IMP) and guanosine monophosphate (GMP) with the release of inorganic pyrophosphate (PPi) (Figure 1A).





**Figure 1.**

**A.** Enzymatic reaction catalyzed by hypoxanthine-guanine phosphoribosyl transferase.

**B.** Enzymatic reaction catalyzed by purine nucleoside phosphorylase.

**C.** Enzymatic reaction catalysed by ATIC enzyme.

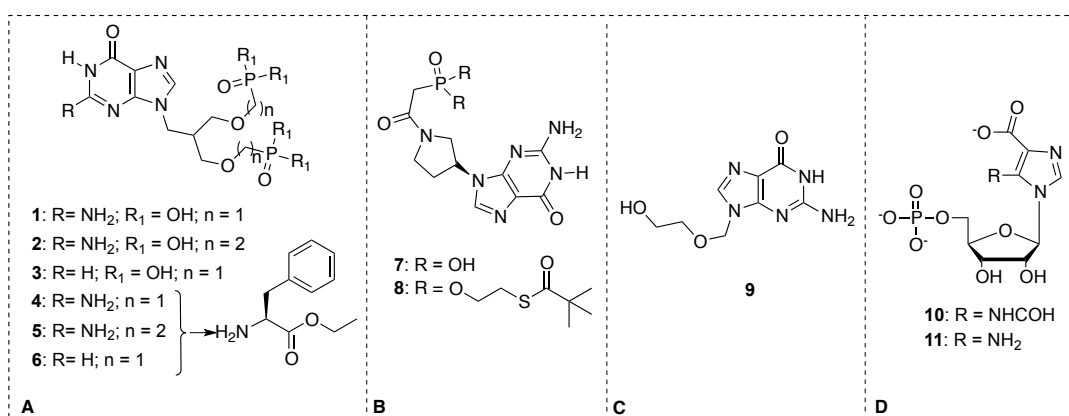
(**Hyp** = hypoxanthine, **Gua** = guanine, **PRPP** = 5-phosphoribose-1-pyrophosphate, **HGPRT** = hypoxanthine-guanine phosphoribosyltransferase, **IMP** = inosine monophosphate, **GMP** = guanosine monophosphate, **PPI** = inorganic pyrophosphate, **Ado** = adenosine, **Guo** = guanosine, **Ino** = inosine, **dAdo** = 2'-deoxyadenosine, **dGuo** = 2'-deoxyguanosine, **dIno** = 2'-deoxyinosine, **PNP** = Purine nucleoside phosphorylase, **Ade** = adenine, **Gua** = guanine, **Hyp** = hypoxanthine, **R-1-P** = ribose-1-phosphate, **AICAR** = 5-aminoimidazole-4-carboxamide; **FAICAR** = 5-formylaminoimidazole-4-carboxamide ribonucleotide, **10-Formyl-THF** = N<sup>10</sup>-formyltetrahydrofolate; **THF** = tetrahydrofolate).

The low sequence homology (24% assigned by pairwise amino acid sequence alignment) between human and mycobacterial HGPRT isoforms may indicate no conservation of key catalytic residues, resulting in distinct steady-state kinetic constant and enzymatic mechanism. On the other hand, the sequence alignment between HGPRT expressed in *Escherichia coli* (*EcHGPRT*) and mycobacterial HGPRT (*MtHGPRT*) shows that these enzymes share 50% identity and 71% similarity in the amino acid residues present in the binding site. Nucleoside phosphonates **1-3** and **7** have been reported to be good inhibitors of *EcHGPRT*, with K<sub>i</sub> values as low as 10 nM (Figure 2A,B) [61].

### ■ Key term

$K_i$  (inhibitory constant) is the concentration of inhibitor required in order to decrease the maximal rate of the reaction by half.

To understand whether the mentioned structure similarity between *Ec*HGPRT and *Mt*HGPRT could translate into a potential *Mt*HGPRT inhibitory activity of compounds **1-3** and **7**, Keough *et al.* described the synthesis and anti-mycobacterial evaluation of different prodrugs (**4-6** and **8**) of the already identified acyclic nucleoside phosphonates (**1-3** and **7**) (Figure 2A,B) [61].



**Figure 2.** Inhibitors of *Ec*HGPRT, *Mt*PNP and ATIC.

**A.** Acyclic nucleoside phosphonates **1-3** (*Ec*HGPRT inhibitors) and relative prodrugs **4-6**.

**B.** Acyclic nucleoside phosphonate **7** (*Ec*HGPRT inhibitor) and relative prodrug **8**.

**C.** Acyclovir **9** (*Mt*PNP inhibitor).

**D.** 4-carboxy-5-formylaminoimidazole ribonucleotide (CFAIR, **10**) and 4-carboxy-5-aminoimidazole ribonucleotide (CAIR, **11**), an ATIC inhibitor.

The common chemical structure of these prodrugs (**4-6** and **8**) is based on the covalent attachment of hydrophobic groups to the phosphoric oxygen atoms (Figure 2A,2B). The purpose of these attachments is to mask the negative charges of the phosphonate groups and thus increase cell permeability. Once within the microbial cells, these moieties are thought to be hydrolysed to release the active species.

### ■ Key terms

**MIC** (minimum inhibitory concentration) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism usually by the 50% (MIC<sub>50</sub>), 90% (MIC<sub>90</sub>) or

99% (MIC<sub>99</sub>), after overnight incubation.

EC<sub>50</sub> (half maximal effective concentration) refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after a specified exposure time.

Prodrugs (**4-6** and **8**) were assayed *in vitro* against *M.tb* and resulted in minimum inhibitory concentration values (MIC<sub>90</sub>) ranging between 20 and 75  $\mu$ M. On the other hand, none of the parent compounds (**1-3** and **7**) exhibited antimycobacterial activity at concentrations  $\geq$  250  $\mu$ M, validating the prodrug strategy as a method to target *M.tb*. Cytotoxic evaluation in human lung carcinoma cells (A549) resulted in EC<sub>50</sub> > 100  $\mu$ M, leading to a selectivity index [cytotoxicity/MIC<sub>50</sub>]  $\geq$  17, suggesting a potential therapeutic window.

### ***Purine nucleoside phosphorylase***

Purine nucleoside phosphorylase (PNP) is responsible for catalyzing the reversible phosphorolysis of purine nucleosides (adenosine: Ado, guanosine: Guo, inosine: Ino) and 2'-deoxyribonucleosides (deoxyadenosine: dAdo, deoxyguanosine: dGuo, deoxyinosine: dIno), in the presence of Pi, generating ribose-1-phosphate (R-1-P) and the corresponding purine base (adenine: Ade, guanine: Gua, hypoxanthine: Hyp) (Figure 1B). *M.tb* was proven to encode for a specific isoform of this enzyme (*MtPNP*), in particular during the latent stages of its life cycle [62]. Therefore, targeting this enzyme could potentially eradicate *M.tb* during this phase.

A number of research groups have dedicated their efforts to determine specificity of substrates, kinetic mechanism, and three-dimensional structures for *MtPNP* in comparison to the human PNP (*HsPNP*) [63].

No specific *MtPNP* inhibitor has been identified so far. 9-(2-Hydroxy-ethoxy-methyl)guanine (acyclovir, **9**) is an acyclic nucleoside analogue endowed with anti-herpetic activity, which has also shown a moderate *HsPNP* inhibitory activity ( $K_i = 91 \mu$ M) (Figure 2C) [63]. Data from acyclovir inhibition studies demonstrate that acyclovir is also a competitive inhibitor of *MtPNP*, with a  $K_i$  value of 150 nM [63].

Crystallographic studies of *MtPNP* in complex with acyclovir and phosphate group allowed the comparison between *MtPNP* and *HsPNP* active sites.

These studies also showed that some crucial residues for the proper ligand binding into *Hs*PNP do not seem to retain importance for the binding with *Mt*PNP. Such differences could be exploited for the drug-design of novel and selective *Mt*PNP inhibitors.

### ***Inosine monophosphate synthase (ATIC or PurH)***

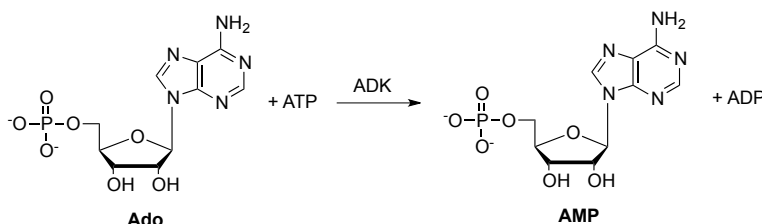
The last two steps to the *de novo* synthesis of IMP are catalyzed by the bifunctional enzyme 5-amino-imidazole-4-carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase, also known as ATIC or PurH [64]. This enzyme catalyses the transfer of a formyl group from N<sup>10</sup>-formyltetrahydrofolate (10-formyl-THF) to the 5-amino group of the substrate 5-aminoimidazole-4-carboxamide (AICAR) to give 5-formylaminoimidazole-4-carboxamide ribonucleotide (FAICAR) and cyclisation of FAICAR to give IMP (Figure 1C) [65].

Human ATIC (*h*ATIC) is a dimer containing two distinct functional domains in each monomer. Evidence shows that *h*ATIC exhibits half-of-site reactivity. The THF-dependent formyl transfer reaction takes place in the C-terminal domain of only one monomer, generating FAICAR that undergoes cyclisation in the N-terminal domain of the same monomer. Interestingly, there is no evidence of any tunnel connecting these two active sites. In 2011 Baker *et al.* expressed the *Mt*ATIC enzyme in *E. coli* and crystallised it in complex with the substrate AICAR, without the addition of any ligands [65]. From X-ray analysis it was found that, similarly to the human isoform, *Mt*ATIC contained two independent ATIC molecules organized as tightly associated dimers. However, the *Mt*ATIC dimer was found fundamentally different to the human enzyme, with the two transformyl and cyclohydrolase domains rotated by 90°. The origin of this difference lies in the connecting peptide. During these crystallographic studies an adventitious nucleoside, identified as 4-carboxy-5-formylaminoimidazole ribonucleotide (CFAIR, **10**), was found in the cyclohydrolase binding site (Figure 2D); CFAIR structural similarity to carboxyaminoimidazole ribonucleotide CAIR (**11**) (Figure 2D), an earlier intermediate in the *de novo* IMP synthesis, already recognised as a 10 µM inhibitor of ATIC, suggests that CFAIR could be produced by formylation of CAIR by ATIC under the conditions of overexpression in *E. coli*. Although **10** was never synthesized, crystallisation studies show this nucleoside is capable of favorable interactions within the *Mt*ATIC active site, strongly suggesting it could be an effective inhibitor

of this enzyme. These results and the observed differences between the crystal structures of *h*ATIC and *Mtb*ATIC constitute an excellent starting point for the development of more potent inhibitors of this enzyme family.

### *Adenosine Kinase*

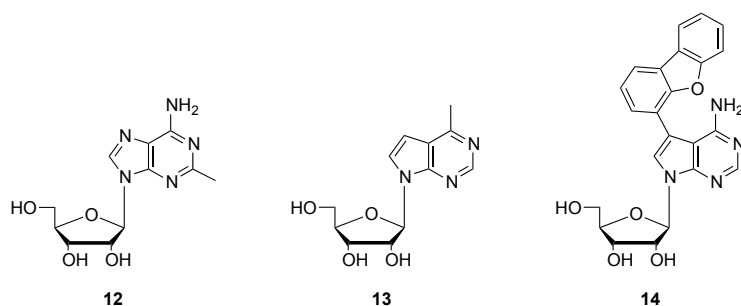
Among all the enzymes from the *M.tb* purine salvage pathway, adenosine kinase (ADK) is considered another promising target for drug development. ADK catalyses the phosphorylation of adenosine (Ado) to adenosine monophosphate (AMP) through a phosphoryl-transfer reaction of the  $\gamma$ -phosphate group from adenosine triphosphate (ATP), releasing adenosine diphosphate (ADP) (Figure 3) [66].



**FIGURE 3.** Enzymatic reaction catalyzed by ADK.

**Ado** = adenosine, **ATP** = adenosine triphosphate; **ADK** = adenosine kinase, **AMP** = adenosine monophosphate, **ADP** = adenosine diphosphate.

ADK is present in fungi, plants and parasites but is not commonly found in bacteria. The expression of ADK has been confirmed in *M.tb* and biochemical characterization indicates that this enzyme shares low structural similarity and different mechanism of action compared to the human isoform [66]. 2-Methyladenosine (**12**) demonstrated promising anti-mycobacterial activity, with an *in vitro* MIC<sub>99</sub> value of 3.0  $\mu\text{g}/\text{mL}$  (Figure 4). Mycobacterial ADK (*Mt*ADK) and human ADK (*h*ADK) exhibited different affinities for **12**, suggesting a potential difference in the substrate-binding sites of these enzymes [67]. The anti-mycobacterial activity of **12** was suggested to follow its phosphorylation and incorporation into the RNA. The selective activity of **12** against *M.tb* has prompted the synthesis of nucleoside analogues that could potentially inhibit *Mt*ADK.



**Figure 4.** *MtADK* inhibitors and antimycobacterial compounds **12-14**.

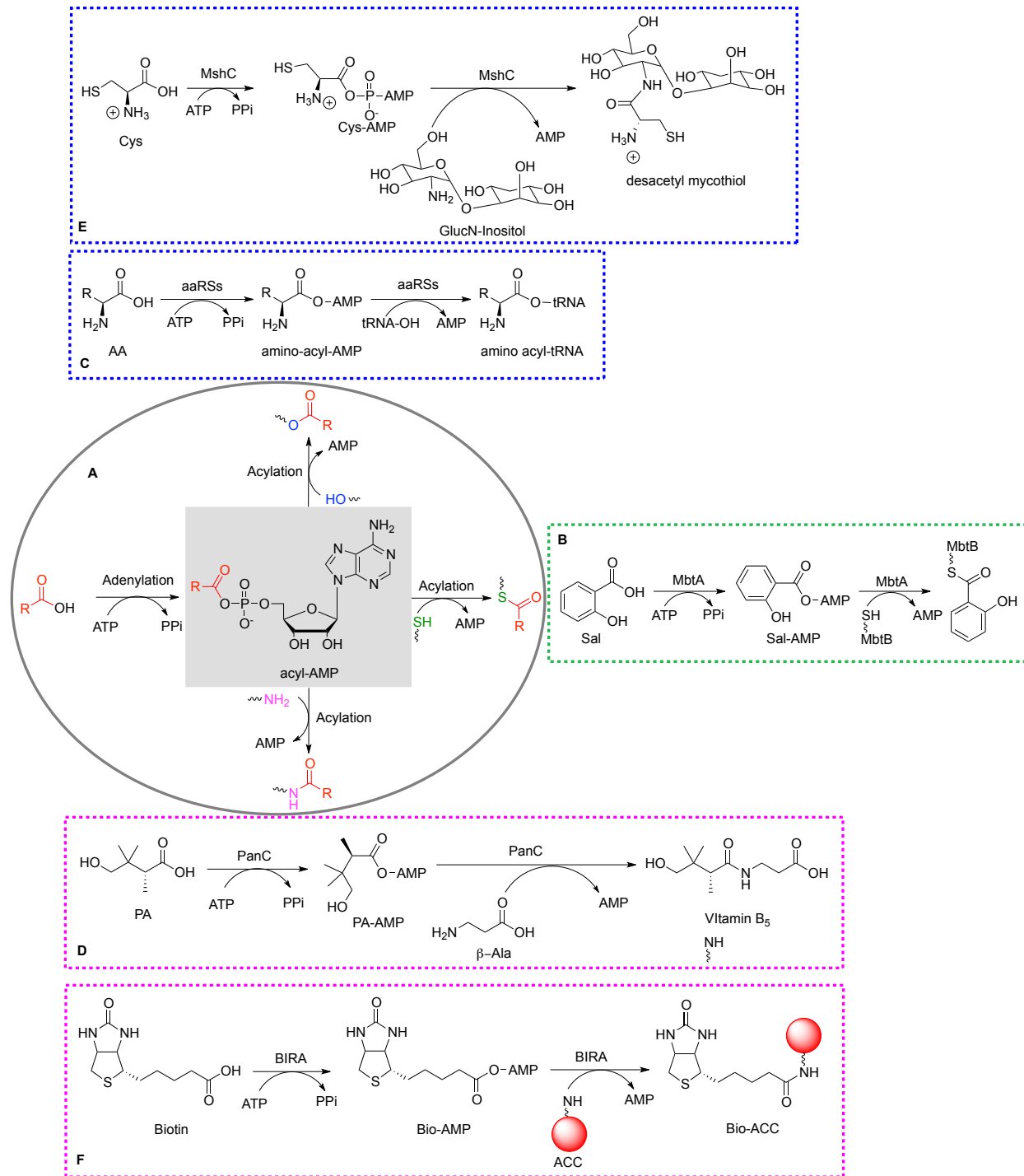
Among this class of compounds, several 6-substituted-7-deazapurine ribonucleosides proved to be strong and selective inhibitors of *MtADK* [68]. However most of these compounds resulted as inactive in whole cell assays, except 6-methyl-7-deazapurine ribonucleoside (**13**) that showed potent antimycobacterial activity against *Mycobacterium bovis* (*M. bovis*, MIC<sub>50</sub> = 0.3 μM) (Figure 4). Surprisingly, **13** showed the highest cytotoxicity and the weakest *in vitro* *MtADK* inhibition, which may indicate an alternative mode of action. More recently, a new series of 7-(het)aryl-7-deazaadenine ribonucleosides showed specific *MtADK* inhibition, along with micromolar *in vitro* activity and low cytotoxicity [69]. Among this series, the dibenzofuran derivative **14** showed the best therapeutic index, with submicromolar *MtADK* inhibitory activity and a MIC<sub>99</sub> value of 0.19 μM against *M. bovis* (Figure 4).

### ***Adenylating enzyme (AEs)***

Adenylating enzymes (AEs) are essential to all living organisms. They take part in protein synthesis, glycolysis, lipid metabolism, and cofactors biosynthesis (biotin, coenzyme A, and nicotinamide adenine dinucleotide).

*M.tb* encodes for more than 60 adenylating enzymes, many of which are reported to be essential for the bacterial virulence, and therefore make attractive targets for the development of effective anti-mycobacterial agents [57].

These enzymes catalyse the transfer of an acyl moiety to the phosphate group of AMP, generating acyl adenylate (acyl-AMP). In a second step, the acyl group is then transferred to different nucleophilic moieties such as alcohol, thiol, and amino groups (Figure 5A).



**Figure 5.**

**A.** General enzymatic reactions, catalyzed by adenylating enzymes from *M. tb*.

**B.** Enzymatic reaction catalyzed by MbtA.

**C.** Enzymatic reaction, catalyzed by aaRSs.

**D.** Enzymatic reaction catalyzed by PanC.

**E.** Enzymatic reaction catalyzed by MshC.

**F.** Enzymatic reaction catalyzed by BIR A.

(**Sal** = salicylic acid, **ATP** = adenosine monophosphate, **PPi** = inorganic pyrophosphate, **Sal-AMP** = 5'-O-salicyl adenosine monophosphate, **AA** = amino acid, **amino-acyl-AMP** = 5'-O-aminoacyl adenosine monophosphate, **PA** = pantoic acid, **PA-AMP** = 5'-O-pantyl adenosine monophosphate,  **$\beta$ -Ala** = beta alanine, **Cys** = Cysteine, **Cys-AMP** = cysteinyl adenosine monophosphate, **GluN-Inositol** = Glucosamine-inositol disaccharide, **Bio-AMP** = Biotinyl adenylate, **ACC** = acyl coenzyme A carboxylase, **Bio-ACC** = Biotinyl acyl coenzyme A carboxylase).

### ***Aryl acid adenylating enzyme (MbtA)***

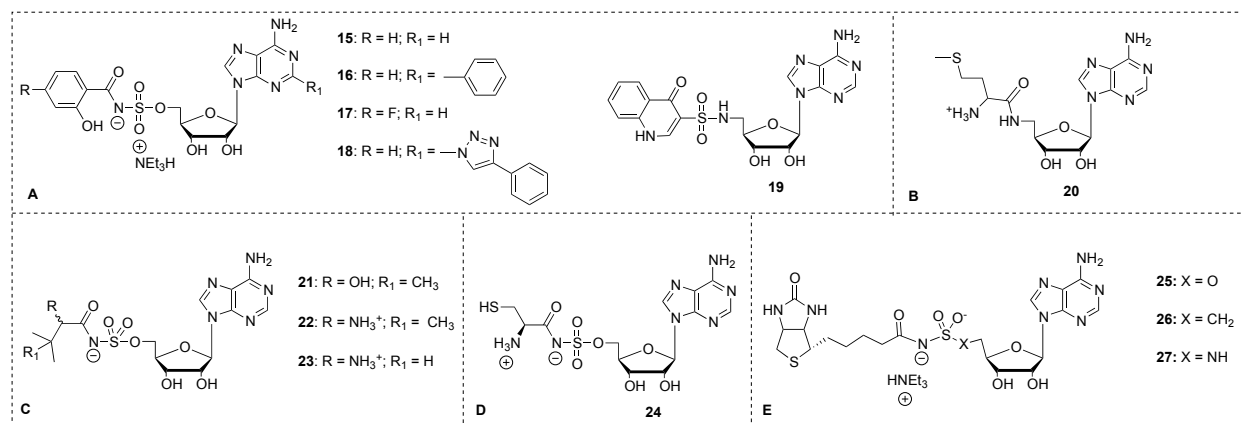
Mycobactins, also known as siderophores, are small iron-chelating molecules synthesised by *M.tb* in order to overcome the lack of readily available iron by chelating the metal from host serum or tissues [70]. Disruption of genes involved in mycobactin biosynthesis results in weakening of *M.tb* virulence and leads to its inability to replicate *in vitro* and grow within pulmonary macrophagic environment, unless chemically complemented with exogenous mycobactin [71]. Targeting mycobactin biosynthesis may therefore represent a novel strategy for the development of anti-mycobacterial agents.

Four adenylating enzymes are part of a multi-enzyme complex, which is responsible for mycobactin biosynthesis [72]. The aryl acid adenylating enzyme (MbtA) is the most characterised of these enzymes and is responsible for initiating the biosynthesis of mycobactins. This enzyme catalyses firstly the adenylation of salicylic acid (Sal) to 5'-O-salicyl adenosine monophosphate (Sal-AMP) and secondly the transfer of the salicylate group onto a thiol containing residue on the carrier protein MbtB (Figure 5B). Following these steps, a series of further steps involving the other three adenylating enzymes lead to the formation and release of the mycobactins.

5'-O-[N-(salicyl)sulfamoyl]adenosine (**15**) is the first rationally designed inhibitor of MbtA, and its structure mimics the natural intermediate Sal-AMP, with the hydrolytically labile acyl-phosphate moiety replaced by a more stable acyl-sulfamate linker (Figure 6A) [73]. **15** is a potent MbtA inhibitor with a  $K_i = 7$  nM. Furthermore, it displays potent activity in a whole cell assay against H37Rv mycobacterial strain under iron-limiting conditions, resulting in  $MIC_{50} = 0.39$   $\mu$ M, therefore competing with the first-line clinical agent isoniazid. Structure activity relationship studies were carried out to investigate whether modifications on linker, aryl, glycosyl or nucleobase moieties could lead to an advantage in terms of anti-mycobacterial activity. Modification of the nucleobase portion of **15** by introduction of a phenyl substituent in position 2 (**16**) resulted in a notable increase in MtbA inhibition ( $K_i = 0.27$  nM,  $MIC_{99} = 0.049$



$\mu\text{M}$ ) (Figure 6A) [74]. Introduction of a fluorine substitution in the para position on the salicylate aromatic ring (**17**) led to an improved  $\text{MIC}_{99} = 0.0098 \mu\text{M}$  and a  $K_i = 0.012 \mu\text{M}$  on the enzyme (Figure 6A) [75]. Modifications on the sugar moiety improved the binding affinity for the enzyme but surprisingly led to a decrease in the *in vitro* antitubercular activity [76]. Compound **18**, bearing a 2-phenyl substituted triazole in position 2 of the nucleobase, was the most potent ( $K_i = 3.23 \text{ nM}$ ,  $\text{MIC}_{99} = 3.13 \mu\text{M}$ ), selective (active only in iron-deficient conditions) and least cytotoxic derivative of the series ( $\text{CC}_{50} > 100 \mu\text{M}$  in VERO cells) (Figure 6A) [77].



**Figure 6.** Inhibitors of MtbA, MtbB, aaRSs, PanC, MshC and BIRA.

- A.** MtbA bisubstrate inhibitors and antimycobacterial compounds **15-18**, MtbA bisubstrate inhibitor **19**.  
**B.** aaRSs bisubstrate inhibitor **20**.  
**C.** PanC bisubstrate inhibitors **21-23**.  
**D.** MshC bisubstrate inhibitor **24**.  
**E.** BIRA bisubstrate inhibitors and antimycobacterial compounds **25-27**.

Conformationally restricted analogues of Sal-AMS were designed to improve oral bioavailability by removal of the two rotatable bonds and the charged sulfamate moiety [78]. However, biochemical studies showed that the negative charge on Sal-AMS structure is crucial to maintain potent activity. In fact, compounds lacking an ionizable function displayed significantly reduced potency. On the contrary derivative **19**, containing an ionizable NH moiety at N-1 position on the quinolone portion, was only 18-fold less active as MtbA inhibitor than Sal-AMS ( $K_i = 0.12 \mu\text{M}$ ) (Figure 6A). It was hypothesised that the loss of potency would be counterbalance by its substantially improved physicochemical properties. However **19**, despite showing significant biochemical potency, proved inactive in the whole-cell assay with a  $\text{MIC}_{50}$  value higher than  $50 \mu\text{M}$ .

### ***Aminoacyl tRNA transferase (aaRSs)***

*M.tb* encodes for all the different aminoacyl tRNA transferase enzymes (aaRSs), whose purpose within RNA biosynthesis is the activation of each amino acid and its association with its cognate tRNA molecule [79-80].

Each aaRSs is specific for its corresponding amino acid and catalyses at first its conversion into acyl adenylate (amino-acyl-AMP), using ATP as AMP donor and releasing PPI. Consequently, the enzyme catalyses the amino acid transfer onto an alcoholic residue on the appropriate tRNA generating acylated tRNA (amino-acyl-tRNA) (Figure 5C).

The evolutionary divergence between eukariotic and prokaryotic aaRSs has made selective inhibition of these enzymes possible, whereas the high conservation of aaRSs in prokaryotes opens the possibility for the development of broad-spectrum drug. Inhibitors of aaRSs have been extensively reported and reviewed.

The bisubstrate inhibitor **20** as been reported as a moderate mycobacterial methionyl-tRNA synthase inhibitor (Figure 6B) [81-82]. Unfortunately this compound did not show activity in *in vitro* whole cell assays.

### ***Adenylating enzyme pantothenate C (PanC)***

Pantothenate or otherwise known as vitamin B5 is a component of coenzyme A, an essential cofactor in the central pathway of cellular respiration and lipid metabolism [83]. *M.tb* as many other bacteria encodes four enzymes (PanA-D) responsible for the synthesis of pantothenate. Among them PanC is the best biochemically and structurally characterized adenylating enzyme in *M.tb*. Genetic studies have shown that the pantothenate mutant strain of *M.tb*, lacking the PanC gene are not capable to establish virulence in a mouse model of infection [84]. Strains defective of both PanC and PanD are indeed under consideration as human vaccine candidate for TB [85].

In the pantothenate biosynthesis, pantoic acid (PA) is condensed to  $\beta$ -alanine ( $\beta$ -Ala) by adenylating enzyme PanC to afford pantothenic acid (vitamin B<sub>5</sub>) (Figure 5D).

Three inhibitors that mimic the structure of the adenylate intermediate in the enzymes catalytic

mechanism, have been described [86]. Compound **21** resembles the 5'-*O*-panthyl adenosine monophosphate (PA-AMP) most closely and was evaluated as the diastereoisomeric mixture at the C-2 hydroxy position of the pantoate moiety, showing a  $K_i = 0.22 \mu\text{M}$  (Figure 6C). Replacement of the hydroxyl group with an amino group in both compounds **22** ( $K_i = 4.0 \mu\text{M}$ ) and **23** ( $K_i = 18.0 \mu\text{M}$ ) lead to derivatives from 18 to 80 fold less active than **21** (Figure 6C). Unfortunately, the anti-mycobacterial activities for these compounds have been not reported.

### ***Adenyating enzyme responsible for mycothiol biosynthesis (MshC)***

The mycothiol biosynthetic pathway has been considered attractive for the development of potential antitubercular agents since mycothiol is unique to mycobacteria and essential for their growth [87-88]. The biosynthesis of mycothiol is carried out in five steps, the adenyating enzyme MshC is responsible for the penultimate step that catalyses the ligation of the cysteine (Cys) to the glucosamine-inositol disaccharide (GlcN-Inositol) to generate the desacetyl mycothiol (Figure 5D) [89].

Compound **24** is the only reported MshC inhibitor of this enzyme, having a nucleoside like structure ( $K_i = 0.3 \mu\text{M}$ ) (Figure 6D). While this compound serves as useful tool for mechanistic analysis it was not considered interesting for further development due to its concomitant inhibition of the corresponding human cysteinyl-tRNA transferase enzyme [88].

### ***Biotin protein ligase (BIRA)***

The mycobacterial biotin protein ligase regulates lipid metabolism in *M.tb* through biotinylation of acyl coenzyme A carboxylases, which is involved in lipid biosynthesis [90]. In particular this reaction is accomplished in two steps: in the first half reaction, BIRA binds biotin and ATP and catalyses the nucleophilic attack of the biotin carboxylate (Biotin) onto the  $\alpha$ -phosphate of ATP to yield the acyl-adenylate (Bio-AMP) and  $\text{PPi}$  (Figure 5F). In the second-half reaction BIRA transfer biotin from bio-AMP onto a conserved lysine residue within the acyl coenzyme A carboxylase (ACC) to provide biotinylated-acyl coenzyme A (Figure 5F).

Bistubstrate inhibitors, which mimic the intermediate acyl-adenylate and are therefore capable to interact with both substrate binding pockets of BIRA have been reported [90-91]. Among them is

the Bio-AMP derivative **25**, in which the labile acylphosphate linkage has been replaced with the bioisosteric acylsulfamate, showed an affinity for the enzyme 1.4 fold lower than the endogenous substrate biotin (Figure 6E). This lack of potency was probably due to chemical instability. Replacement of the 5' oxygen atom in **25** with a nitrogen atom or a methylene group produced respectively acylsulfamide **26** and acylsulfonamide **27** with an improved chemical stability (Figure 6E).

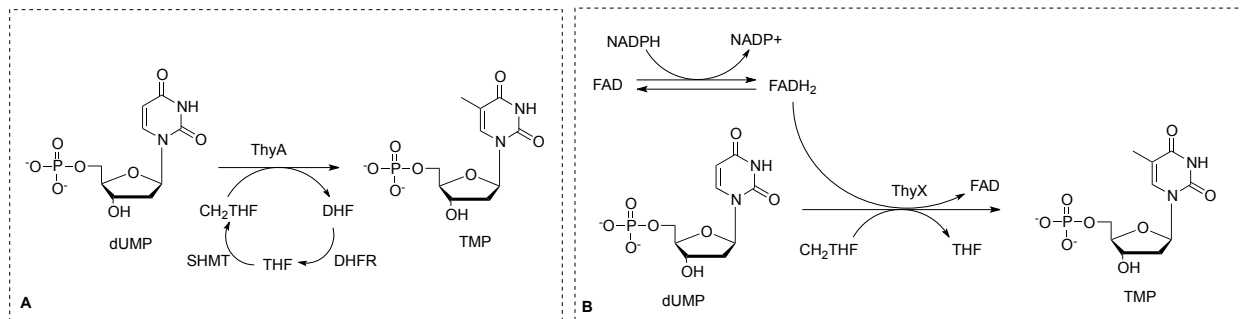
These derivatives with an enzyme affinity 1700 fold higher than biotin, were evaluated in whole-cell assays against drug-sensitive *M.tb* H37Rv strain and as well as a panel of MDR and XDR strains, showing impressive anti-mycobacterial activity (MIC<sub>99</sub> = 3.12 μM and 0.78 μM, respectively for **26** and **27**). These studies show that this class of compounds represents an excellent probe to chemically validate anti-mycobacterial therapeutic development.

## **Nucleoside inhibitors of enzymes involved in pyrimidine metabolism**

The *de novo* and salvage pyrimidine pathways have been documented in Mycobacteria [92-93]. Thymidylate synthase (ThyX) and thymidylate kinase (TMPK<sub>mt</sub>) enzymes were identified as targets for the development of several inhibitors with nucleosidic structure. The following reports the structures and biological data of these agents.

### ***Thymidylate synthase (ThyX)***

Thymidylate synthase enzyme (ThyA), was originally discovered as essential for eubacteria, plants and eukaryotic cells, for the *de novo* synthesis of thymidine monophosphate (TMP) [94]. ThyA catalyses the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to TMP, by means of the cofactor R-N<sub>5</sub>,N<sub>10</sub>-methylene-5,6,7,8-tetrahydrofolate (CH<sub>2</sub>THF) which is in turn converted into 7,8-dihydrofolate (DHF), acting as both a methylene and hydride donor (Figure 7A) [94-95]. The final step of this cycle is the reduction of DHF to tetrahydrofolate (THF) catalysed by dihydrofolate reductase (DHFR), with the concomitant reintroduction of a methylene group by the enzyme serinehydroxymethyl transferase (SHMT) (Figure 7A) [95].



**Figure 7.**

**A.** Enzymatic reaction catalyzed by ThyA enzyme.

**B.** Enzymatic reaction catalyzed by ThyX enzyme.

(**dUMP** = 2'-deoxyuridine-5'-monophosphate, **CH<sub>2</sub>THF** = R-N<sub>5</sub>,N<sub>10</sub>-methylene-5,6,7,8-tetrahydrofolate, **DHF** = 7,8-dihydrofolate, **SHMT** = serinehydroxymethyl transferase, **THF** = tetrahydrofolate, **DHFR** = dihydrofolate reductase, **TMP** = thymidine monophosphate, **FAD** = flavin adenine dinucleotide, **FADH<sub>2</sub>** = reduced flavin adenine dinucleotide, **NADPH** = reduced nicotinamide-adenine dinucleotide phosphate, **NADP<sup>+</sup>** = nicotinamide-adenine dinucleotide phosphate).

In further investigations, several microorganisms were found to lack the genes encoding for ThyA and DHFR but retain viability in thymidine-deficient media. These microorganisms are able to express the ThyX enzyme, a flavin-dependent thymidylate synthase (FDTS). This enzyme and the parent *ThyX* gene are rare in eukaryotes and absent in humans [96-97].

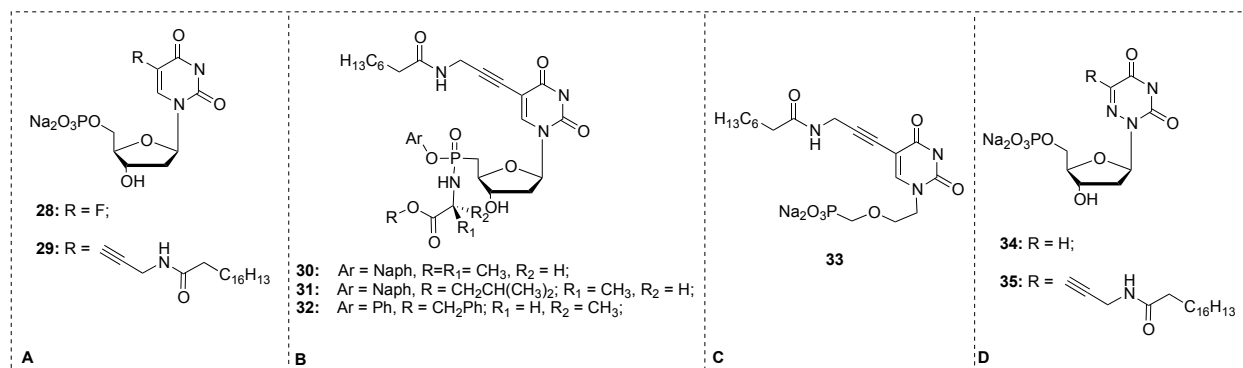
Although both catalyzing the conversion of dUMP to dTMP, ThyX and ThyA share no structural similarity, due to a different catalytic mechanism: ThyX activity depends on CH<sub>2</sub>THF only as a methylene donor, whereas the hydride donor is in this case represented by reduced flavin adenine dinucleotide (FADH<sub>2</sub>) (Figure 7B) [96,98]. During the catalysis cycle, nicotinamide adenine dinucleotide phosphate (NADPH) is oxidized to NADP<sup>+</sup> allowing the regeneration of FADH<sub>2</sub> via reduction of FAD (Figure 7B).

*M.tb* encodes for ThyX, along with ThyA and DHFR enzymes. Despite some studies showing that *M.tb* requires ThyX for survival within the macrophages, it is still unclear when this gene is activated instead of ThyA. Since macrophages represent a reservoir of *M.tb* in the latent form of the disease, selective ThyX inhibitors may have an effect on this phase of the mycobacterial life cycle.

5-Fluoro-2'-deoxyuridine-5'-monophosphate (**28**, Figure 8A), was the firstly reported ThyX inhibitor, with an IC<sub>50</sub> = 0.57 μM, although also inhibiting ThyA at a similar concentration (IC<sub>50</sub> = 0.29 μM) [97].

## ■ Key term

**IC<sub>50</sub>** (half maximal inhibitory concentration) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function.



**Figure 8.**

- A.** ThyX and ThyA inhibitors **28** and **29**.  
**B.** Anti-mycobacterial compounds **30-32**, prodrugs of **29**.  
**C.** ThyX inhibitor **33**.  
**D.** ThyX and ThyA inhibitors **34** and **35**.

The first selective ThyX inhibitors were synthesised by Herdewijn *et al.* who identified *N*-(3-(5-(2'-deoxyuridine-5'-phosphate))prop-2-ynyl)octanamide (**29**, Figure 8A) as the most active candidate in a series of differently C-5 alkynyl-substituted 2'-deoxyuridine-5'-monophosphate analogues, with an IC<sub>50</sub> value of 0.91 μM against ThyX [99]. This compound was also found to be selective for ThyX, showing 92.8 % inhibition at 50 μM compared to only 15.6 % inhibition for ThyA at the same concentration.

Despite the intriguing selectivity of this powerful new ThyX inhibitor, its antimicrobial evaluation was impossible due to its high polarity limiting the mycobacterial cell wall penetration. In 2014 McGuigan *et al.* applied the ProTide prodrug approach to this promising derivative, in order to enhance the drug-like characteristics of this compound [100]. This strategy consists in masking the negative charges on the monophosphate group by two lipophilic moieties: an amino acid ester and an aryloxy group [101]. As a consequence, this prodrug approach increases the possibility of cell penetration through passive diffusion and stability towards dephosphorylation. Enzymatic and spontaneous steps are required then for the intracellular delivery of the monophosphate drug. This strategy was already successfully applied to anticancer [102,103] and antiviral [104] nucleoside analogues. A series of prodrugs of **29** was

prepared. Compounds **30-32** showed moderate anti-mycobacterial activity ranging from MIC<sub>99</sub> = 62.5 and 125 mg/L against a drug sensitive strain of *M.tb* (H37Rv) (Figure 8B) [100].

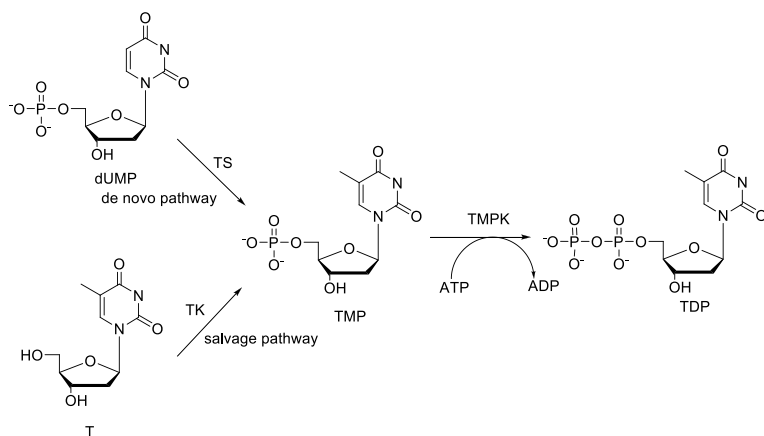
Alternative modifications on the structure of **29** involved the replacement of the sugar monophosphate moiety with a potentially more chemically stable acyclic phosphonate group (Figure 8C) [105]. Only compound **33** was reported to have moderate ThyX inhibitory activity with 43% inhibition of ThyX at a concentration of 50 μM (Figure 8C) [105].

Herdewijn *et al.* synthesised 6-aza-2'-deoxyuridine-5'-monophosphate (**34**) and a series of its C-5 differently substituted analogues and tested these compounds against ThyX and ThyA [106]. Both **34** and one of the most active derivatives **35** (6-aza analogue of compound **29**), showed weak ThyX and ThyA inhibitory activities (% inhibition at 50 μM ranging respectively between 33.5% and 40.9% against ThyX and 0.99% and 13.8% for ThyA) (Figure 8D) [106].

### **Thymidylate kinase (TMPK<sub>mt</sub>)**

Thymidine monophosphate kinase (TMPK) belongs to the superfamily of nucleoside monophosphate kinase (NMPK) enzymes. It catalyses the phosphorylation of TMP to thymidine diphosphate (TDP) by means of ATP as phosphoryl donor. It represents the last specific junction enzyme between the *de novo* and salvage pathways of the synthesis of thymidine triphosphate (TTP). In the *de novo* pathway TMP derives from dUMP via thymidylate synthase (ThyA/ThyX) enzyme; in the salvage pathway thymidine (T) is monophosphorylated by thymidine kinase (TK) to produce TMP (Figure 9) [107,92].

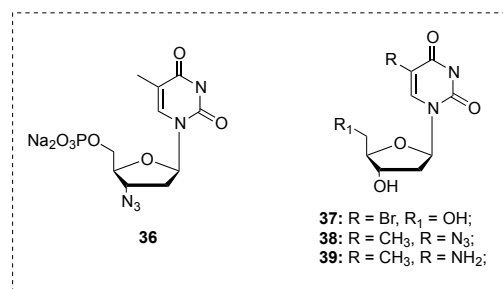
TMPK has already been considered as an interesting target for the synthesis of inhibitors both as anticancer [201] and as antiviral agents [108], and it recently attracted interest also for the development of anti-tubercular agents that could selectively target the *M.tb* isoform TMPK<sub>mt</sub>. In fact, TMPK<sub>mt</sub> appeared to be essential for mycobacterial DNA synthesis and biochemical and structural characterizations revealed only a 22% of sequence homology with the human isozyme (hTMPK) [109].



**Figure 9.** Enzymatic mechanism catalyzed by *TMPKmt*.

(**dUMP** = 2'-deoxyuridine-5'-monophosphate, **T** = thymidine, **TS** = thymidylate synthase, **TK** = thymidine kinase, **TMP** = thymidine monophosphate, **TMPK** = thymidine monophosphate kinase, **ATP** = adenosine triphosphate, **ADP** = adenosine diphosphate, **TDP** = thymidine diphosphate).

*TMPKmt* was firstly crystallised in 2001 by Li de la Sierra *et al.* [110]. Soon after, 3'-azido-3'-deoxythymidine 5'-*O*-monophosphate (AZT-MP, **36**) was identified as the very first inhibitor of *TMPKmt* ( $K_i = 10 \mu\text{M}$ ) (Figure 10) [110].



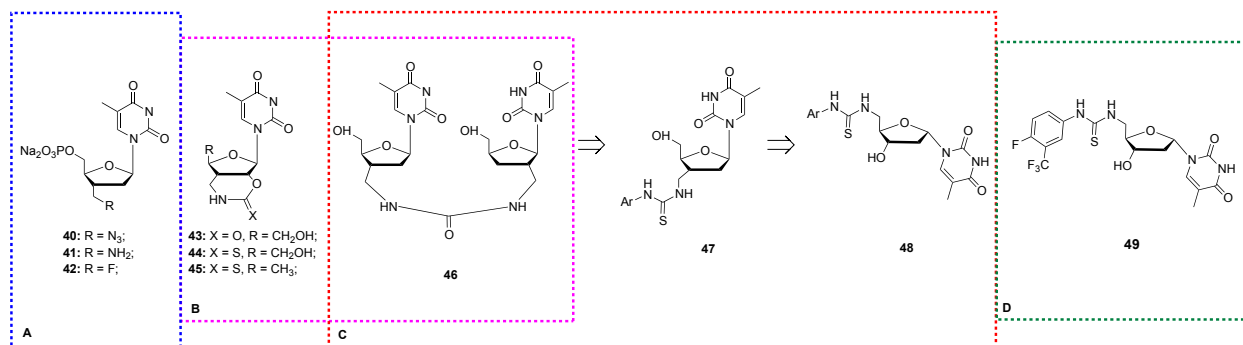
**Figure 10.** *TMPKmt* inhibitor **36-39**.

*TMPKmt* does not phosphorylate AZT-MP, possibly due to an interaction of the terminal nitrogen of the azido group with a residue in the active site of *TMPKmt*. This potentially leads to less efficient binding of ATP, the cofactor, to the active site [111]. Modifications of the structure of compound **36** generally reduced *TMPKmt* inhibitory activity [112]. On the other hand, some nucleoside derivatives, lacking the 5'-monophosphate group, were found to be good *TMPKmt* inhibitors, the better examples being 5-bromo-2'-deoxyuridine (**37**) and 5'-azido (**38**) or 5'-aminothymidine (**39**) whose  $K_i$  were reported to be ranging from 5 to 12  $\mu\text{M}$  (Figure 10).

The synthesis of 3'-branched nucleosides was also considered by Vanheusden *et al.*, in order to investigate the effect of bulky substituents on *TMPKmt* inhibitory activity [113]. The introduction of 3'-azidomethyl- (**40**), 3'-aminomethyl- (**41**) and 3'-fluoromethyl (**42**) substituents



on the thymidylate structure led to the highest affinities for TMPK*mt* enzyme in this series ( $K_i$  ranging from 10.5 to 15  $\mu\text{M}$ ) (Figure 11A) [113].



**Figure 11.**

- A.** TMPK*mt* inhibitors **40-42**
- B.** TMPK*mt* bicyclic inhibitors **43-45** and dinucleoside inhibitor **46**.
- C.** Optimisation of compound **46** to compound **48**.
- D.** TMPK*mt* inhibitor **49**.

Deletion of the phosphate group in this series of compounds resulted in a better selectivity profile, despite the modest affinity loss, producing promising new leads [112].

Bicyclic thymidine analogues **43**, **44** and di-nucleoside **46** were discovered by serendipity and, despite their peculiar structures, these compounds were soon recognised as potent and selective TMPK*mt* inhibitors, showing  $K_i$  values of respectively 13.5  $\mu\text{M}$ , 3.5  $\mu\text{M}$  and 37  $\mu\text{M}$  (Figure 11B) [113].

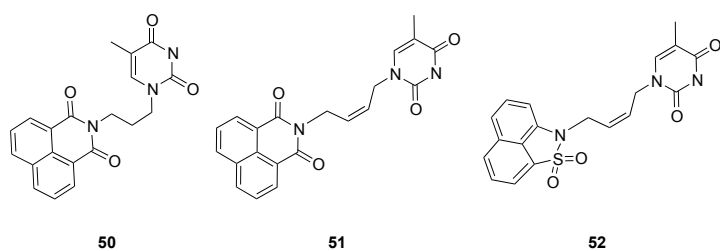
The selectivity most likely arises from the unique flexibility of the sugar ring within the TMPK*mt* active site [113]. The potent inhibitory activity of the di-nucleoside **46** was initially surprising, due to the lack of any binding site for a second nucleoside in TMPK*mt* active site. Competition experiments performed with TMP and ATP, however, subsequently revealed that this compound behaves as a competitive inhibitor of both substrates. In a further study, derivative **45**, lacking of the 5'-hydroxyl group, resulted as the most potent bicyclic analogues, with a TMPK*mt*  $K_i$  value of 2.3  $\mu\text{M}$  [114]. The same compound was also selected to be tested for *in vitro* whole cell assay, resulting in a weak inhibitor of *M.tb* growth ( $\text{MIC}_{99}$  = 100  $\mu\text{M}$ ), without showing any additional cytotoxicity against VERO cell lines up to a concentration of 500  $\mu\text{g/mL}$  [114].

The unexpected activity of di-nucleoside analogue **44** prompted to the synthesis of derivatives that could enhance its inhibitory potency. In the optimisation process, one of the two thymidine

monomers was replaced by different phenyl groups, and the urea linker group was replaced by a thiourea connection (**47**, Figure 11C) [115]. Within this series of 3'-branched  $\beta$ -thymidine derivatives, substituents on the phenyl ring of the 3'-group were found to significantly affect the activity of compounds, with lipophilic and electron-withdrawing groups leading to the highest potencies. A further optimisation of this initial 3'-modified- $\beta$ -nucleoside led to the inversion of the nucleoside configuration from  $\beta$  to  $\alpha$ , switching the 3'-substituent with the 5'-group (**48**, Figure 11C).

5'-modified- $\alpha$ -analogues showed consistently higher activities compared to the  $\beta$ -counterparts. Accompanying this, lipophilic and electron-withdrawing phenyl substituents notably increased the inhibitory activity. Molecular modelling confirmed that the superiority of  $\alpha$ -analogues depended on a more favourable arrangement inside TMPK $_{mt}$  binding pocket. Compound **49** showed the most promising activity with a  $K_i$  value of 0.6  $\mu\text{M}$ , a selectivity index versus TMPK $_{h}$  of 600 and a good growth inhibition of *M.bovis* ( $\text{MIC}_{99} = 20 \mu\text{g/mL}$ ) (Figure 11D). The activity of **49** on *M.tb* growth reached a maximum of 39% at a concentration of 6.25  $\mu\text{g/mL}$ .

In a following study, a successful investigation of acyclic nucleoside analogues as TMPK $_{mt}$  inhibitors was conducted by Familiar *et al.* [116]. From an initial screening this research group identified the thymidine acyclic analogue **50** as a micromolar TMPK $_{mt}$  inhibitor ( $K_i = 42 \mu\text{M}$ ) (Figure 36).



**Figure 12.** TMPK $_{mt}$  inhibitors **50-52**.

Modifications were introduced on the nucleobase moiety, on the distal 1,8-naphthalimide, and on the linker between these two groups. The introduction of a (*Z*)-butenyl linker (**51**) resulted beneficial in terms of activity. The best results were obtained for the naphtholactam containing compound **51** and the naphthosultam **52** ( $K_i = 0.42 \mu\text{M}$  and  $K_i = 0.27 \mu\text{M}$ , respectively) (Figure 12). Unfortunately no antimycobacterial activity was detected against *M.bovis* BCG and *M.tb* up to a concentration of 7.5  $\mu\text{g/mL}$  and 32  $\mu\text{g/mL}$ , respectively.

## Nucleoside analogues with unclear target.

Some initial studies report the synthesis of nucleoside analogues and their evaluation as anti-mycobacterial agents without a clear identification of the intracellular target. An overview about these molecules follows.

Kumar *et al.* were the first research group who synthesised nucleoside analogues and tested them in *in vitro* antimycobacterial activity assays [117]. They prepared unnatural deoxyribose, ribose and dideoxyribose pyrimidine nucleosides to determine the effects of size and electronegativity of groups at C-5, C-2' and C-3' on the antimycobacterial activity against *M. bovis* and *M. avium* [117]. Their hypothesis was that these compounds could interact with mycobacterial enzymes involved in the nucleic acid synthesis as inhibitors or competitive substrates, hence altering DNA or RNA synthesis [117]. Their efforts yielded some selective inhibitors of *M. avium* growth: compounds 5-(1-hydroxyethyl)-2'-deoxyuridine (**53**), 5-(1-fluoro-2-chloroethyl)-2'-deoxyuridine (**54**) and 5-(1-fluoro-2-bromoethyl)-2'-deoxyuridine (**55**) inhibited the *M. avium* growth up to 90% with concentrations of 5  $\mu$ M, similarly to the positive control rifampicin ( $MIC_{90} = 2 \mu$ g/mL) (Figure 13A) [117].

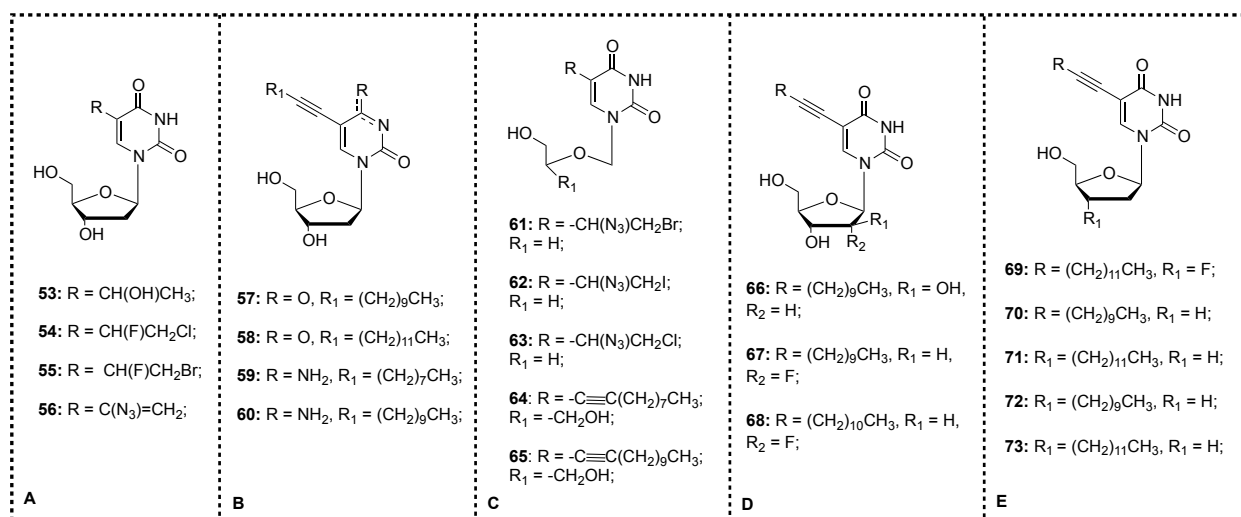


Figure 13. Anti-mycobacterial compounds 53-73.

The introduction of an azidovinyl group in position 5 of 2'-deoxyuridine (**56**) led to moderate activity against *M. avium* ( $MIC_{50} = 1-5 \mu\text{g/mL}$ ), although proving to be surprisingly inactive against *M. bovis* and *M. tb* (Figure 13A) [118].

5-Dodecynyl (**57**, **60**), 5-tetradecynyl (**58**) and 5-decynyl (**59**) derivatives of 2'-deoxyuridine and/or 2'-deoxycytidine showed moderate activity against *M. bovis* growth, with  $MIC_{90}$  values ranging from 10 to 50  $\mu\text{g/mL}$  (Figure 13B), while the introduction of either a ribose sugar moiety or smaller 5-alkynyl chains induced a decrease in antitubercular activity [119].

In an attempt to introduce further variability in the nucleoside scaffold, Kumar *et al.* decided to synthesise nucleoside analogues in which the cyclic carbohydrate moiety is replaced with open-chain "acyclic sugar moieties" [120]. Compounds with similar structure already proved to be effective therapeutic agents, possibly due to the ability of the acyclic side chain to mimic the interactions of the glycosyl portion with the putative target, although retaining flexibility [121]. Moreover this modification stabilizes the nucleoside structure towards phosphorolysis, a catabolic process responsible for the glycosidic bond cleavage and further inactivation of 2'-deoxyuridine analogues [122].

Within this new series, compounds bearing different 5-(1-azido-2-haloethyl)- substituents (**61–63**) on the common 1-(2-hydroxyethoxy)methyl uracil scaffold, were reported to be endowed with moderate activity against *M. tb* ( $MIC_{50} = 10 \mu\text{g/mL}$ ) (Figure 13C) [120]. Similar activity was retained with the introduction of a 1-[(2-hydroxy-1-(hydroxymethoxy)ethoxy)methyl] moiety as acyclic sugar analogue and 5-decynyl and 5-dodecynyl side chain on the uridine structure (**64**, **65**, Figure 13C) [120].

Investigations on other possible sugar modifications that could lead to enhancement in antimycobacterial activity of nucleoside analogues afforded 1- $\beta$ -D-2'-arabinofuranosyl and 1-(2'-deoxy-2'-fluoro-  $\beta$ -D-ribofuranosyl) pyrimidine nucleosides bearing different sets of alkynyl, alkenyl, alkyl and halo substituents at the C-5 position of the uracil [123]. In this series, the 5-alkynyl derivatives proved the most potent anti-mycobacterial activities, the most active structures being 1- $\beta$ -D-2'-arabinofuranosyl-5-dodecynyluracil (**66**), 1-(2'-deoxy-2'-fluoro-  $\beta$ -D-ribofuranosyl)- 5-dodecynyluracil (**67**) and 1-(2'-deoxy-2'-fluoro-  $\beta$ -D-ribofuranosyl)- 5-tetradecynyluracil (**68**), with activity against *M. tb* and *M. bovis* close to that of the reference drug rifampicin ( $MIC_{90} = 1-5 \mu\text{g/mL}$ ), but only low results on *M. avium* growth inhibition ( $MIC_{90} >$

10-50  $\mu\text{M}$ ) (Figure 13D) [123]. Very interestingly, these compounds were also found to retain sensitivity against a rifampicin-resistant strain of *M.tb* H37Rv at similar concentrations [123].

Other C-5-alkynyl uridine derivatives bearing 3'-fluoro-2',3'-dideoxysugars (**69**) and 2',3'-dideoxysugars (**70-73**) ranged from potent to modest inhibitors of *M.bovis*, *M.tb* and *M.avium* (Figure 13E) [124]. Also in this case, the uridine derivatives bearing 5-alkynyl side chains between 10 and 14 carbon atoms in length, showed the best anti-mycobacterial potencies, with  $\text{MIC}_{90}$  ranging from 1 to 2-50  $\mu\text{g}/\text{mL}$ , approaching the reference drug rifampicin, and also retaining activity towards a rifampicin-resistant strand of *M.tb* [124].

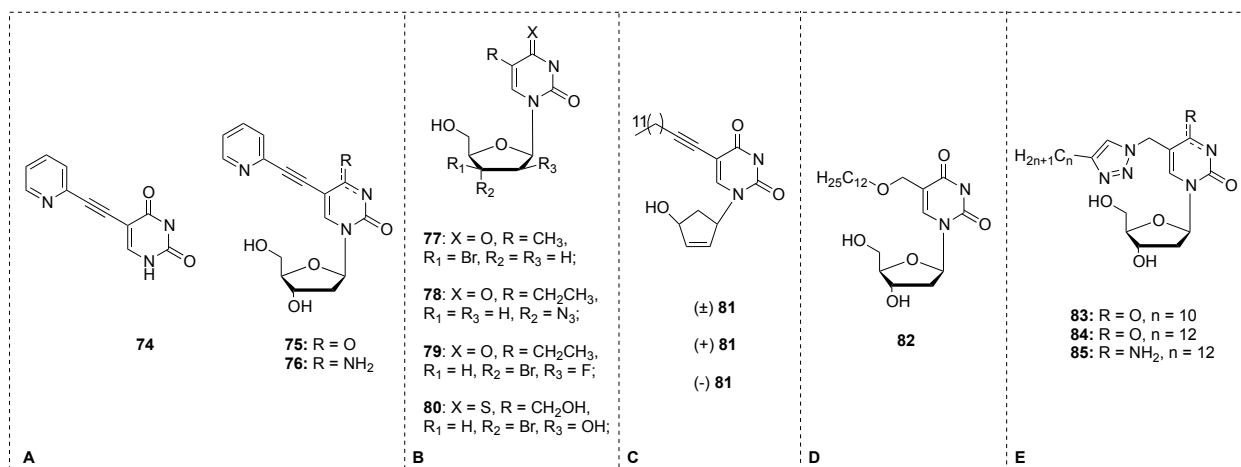
In 2010 the first antimycobacterial *in vivo* data in a mouse model infected with H37Ra *M.tb* strain were published [125]. After synthesis and antimycobacterial *in vitro* evaluation of a novel series of 5-alkyl and 5-alkyl(or aryl)alkynyl pyrimidines, 5-(2-pyridylethynyl)-uracil (**74**) was selected as a potent candidate for further investigations (Figure 14A) [125]. The promising *in vitro* activity of this compound ( $\text{MIC}_{50} = 1-5 \mu\text{g}/\text{mL}$  on *M.bovis*,  $\text{MIC}_{50} = 5 \mu\text{g}/\text{mL}$  on *M.tb*) prompted to *in vivo* evaluation of its efficacy in female BALB/c mice infected with H37Ra at a dose of 50 mg/kg, formulated in 10% DMSO-saline, by the intraperitoneal route. Drug treatment was started five days post infection and was given for 5 weeks (5 days a week). Encouragingly, compound **74** caused considerable reduction of the CFU (colony forming units) counts in the lungs, liver, and spleen of the drug treated animals compared with those of the untreated controls.

#### ■ Key terms

CFU (colony-forming unit) in microbiology is a rough estimate of the number of viable bacteria or fungal cells in a sample. Viable is defined as the ability to multiply via binary fission under the controlled conditions.

However, **74** was found to be much less active than isoniazid administered at 25 mg/kg dose, although no toxicity was observed in any of the treated mice. In the same study, both the 2'-deoxyuridine (**75**) and the 2'-deoxycytidine (**76**) analogues of **74** were synthesized and their *in vitro* antimycobacterial activity against *M.tb* showed an improvement (68-70% inhibition at 10  $\mu\text{g}/\text{mL}$ ) as compared to **74** (70% inhibition at 50  $\mu\text{g}/\text{mL}$ ) (Figure 14A) [125]. Compound **76** demonstrated no cytotoxicity in Huh-7 cells up to a concentration of  $> 200 \mu\text{g}/\text{mL}$ , whereas

compound **75** showed 25% inhibition in cell viability at 200  $\mu\text{g/mL}$ ; therefore, **76** was selected to test its potency in a mouse model infected with H37Ra mycobacterial strain. Drug treatment was carried out similarly to **74**. The efficacy of **76** was compared to that of cycloserine at 50 mg/kg and isoniazid at 25 mg/kg, which were administered as parallel treatment. Compound **76** showed significant activity in three out of five mice (85-90% reduction of the CFU in the lungs) compared to the untreated control group and reduced the bacterial load to 50% in four out of five mice in the liver tissues. In the spleens, compound **76** was slightly less effective, providing 50% inhibition of the CFUs in only three mice. Mice administered with compound **76** at 50 mg/kg for 5 weeks showed no adverse effects in terms of weight loss, behavioral changes, or in the findings of gross necropsy after the mice were euthanized. Although compound **76** was less active than isoniazid both *in vitro* and *in vivo*, it exhibited similar *in vitro* and superior *in vivo* inhibition of mycobacterial replication in all organs (lungs, spleen, liver) as compared to another reference drug cycloserine.



**Figure 14A-D.** Anti-mycobacterial compounds **74-85**.

In another study, various 2'- or 3'-halogeno derivatives of pyrimidine nucleosides containing uracil, 5-fluorouracil and thymine bases were synthesised and evaluated as antimycobacterial agents [126]. 3'-Bromo-3'-deoxy-arabinofuranosylthymine (**77**) was the most effective agent ( $\text{MIC}_{50} = 1 \mu\text{g/mL}$ ) *in vitro* against wild-type *M.tb* strain (H37Ra) as well as rifampicin and isoniazid-resistant strain of *M.tb* (H37Rv) ( $\text{MIC}_{50} = 1\text{-}2 \mu\text{g/mL}$ ) (Figure 14B) [126]. Most intriguingly, compound **77** also inhibited intracellular *M.tb* in a human monocytic cell line (THP-1) infected with H37Ra, with a > 90% of reduction of the number of surviving bacteria at a

concentration of 25  $\mu\text{g/mL}$ , demonstrating higher activity against intramacrophagic mycobacteria than extramacrophagic mycobacteria [126]. Moreover, no cytotoxicity was found up to the highest concentration of compound tested ( $\text{CC}_{50} > 100\text{-}200 \mu\text{g/mL}$ ) [126].

Further investigations generated in 2012 a series of 5-alkyl(or halo)-3'-azido(amino or halo) analogues of pyrimidine nucleosides, of which only 3'-azido-5-ethyl-2',3'-dideoxyuridine (**78**), was found to have significant antimycobacterial activities against *M.bovis*, *M.tb* and *M.avium* ( $\text{MIC}_{50}$  ranging from 1 and 5  $\mu\text{g/mL}$ ) (Figure 14B) [127].

In the same year, Kumar *et al.* reported the synthesis of 5-ethyl and 5-(hydroxymethyl or methoxymethyl) substituted pyrimidine analogues. Among these compounds, the 2'-fluorinated analogue **79**, 1-(3-bromo-2,3-dideoxy-2-fluoro- $\beta$ -D-arabinofuranosyl)-5-ethyluracil provided promising *in vitro* activity against *M.bovis* and *M.tb* alone and in combination with isoniazid ( $\text{MIC}_{50} = 5 \mu\text{g/mL}$ ) (Figure 14B).

1-( $\beta$ -D-arabinofuranosyl)-4-thio-5-hydroxymethyluracil (**80**) displayed the most potent inhibition of wild-type H37Ra ( $\text{MIC}_{50} = 0.5 \mu\text{g/mL}$ ), rifampicin-resistant H37Rv ( $\text{MIC}_{50} = 1 \mu\text{g/mL}$ ) and *M.bovis* ( $\text{MIC}_{50} = 1 \mu\text{g/mL}$ ), along with inhibition of *M.avium* ( $\text{MIC}_{50} = 10 \mu\text{g/mL}$ ) (Figure 14B).

Again in the same year, a different research group published anti-mycobacterial results of a new series of 5'-nor carbocyclic uracil derivatives [128]. Carbocyclic nucleosides, despite the replacement of the O atom by a methylene group, are still recognised by many receptors and enzymes whose natural substrates are nucleosides, and there is evidence of their anticancer and antiviral activity [129]. Moreover, they show increased stability towards phosphorylase- and glycosylase-induced catabolism. 5'-Nor carbocyclic nucleosides lack of the 5'-hydroxyl group, therefore they cannot be phosphorylated by intracellular kinase enzymes, resulting in less toxic compounds and useful tools for understanding whether phosphorylation of the nucleoside analogue is required for antimycobacterial activity. Among this series of 2',3'-dideoxy-2',3'-didehydro-5'-noruridine analogues bearing 4'-hydroxy-2'-cyclopentenyl residues, the racemic 1-[4'-hydroxy-2'-cyclopentenyl-1'-yl]-5-tetradecynyluracil (**81**) completely inhibited the growth of laboratory sensitive H37Rv at a concentration of 10  $\mu\text{g/mL}$ , retaining activity also on MS-115 (resistant to rifampicin, isoniazid, streptomycin, ethambutol, pyrazinamide) (Figure 14C). These results demonstrate that 5-substituted uracil derivatives should have an alternative mechanism of

*M. tuberculosis* inhibition. The activity of the (-) isomer was found to be the highest against the H37Rv strain, however comparable with the racemic mixture.

In 2013, the same research group synthesised a new set of 5-modified derivatives of pyrimidine 2'-deoxynucleosides containing extended alkyloxymethyl or alkyltriazolidomethyl groups [130]. The most active compounds in this series, 5-dodecyloxymethyl-2'-deoxyuridine (**82**), 5-decyltriazolidomethyl-2'-deoxyuridine (**83**), 5-dodecyltriazolidomethyl-2'-deoxyuridine (**84**) and 5-dodecyltriazolidomethyl-2'-deoxycytidine (**85**), inhibited with low MIC<sub>99</sub> values (10-50 µg/mL) the growth of wild-type H37Rv and drug-resistant MS-115 (Figure 14D).

The cytotoxicity of these compounds was assayed in VERO, A549 and Jurkat cell lines, always resulting in LD<sub>50</sub> ≥ 100 µg/mL. The introduction of different substituents in 3' or 5' positions of the sugar moiety (amino-, iodo-, alkylamino-) only slightly affected the antituberculosis activity, although it considerably increased the cytotoxicity (up to CD<sub>50</sub> = 5 µg/mL).

## Future perspective

The need for new anti-TB agents has risen fast in the last few decades, especially due to the emergence of MDR and XDR mycobacterial strains. In order to overcome this issue, interest has grown in anti-mycobacterial research to identify new targets and novel drugs for TB treatment.

Nucleoside analogues represent important agents for the treatment of a range of human diseases and only recently their potential as anti-mycobacterial agents has emerged. Many nucleoside analogues have, in fact, been shown to target enzymes involved in the mycobacterial metabolism. These pathways have never been exploited before for the treatment of TB and therefore may offer an advantage in terms of overcoming resistance. Although still an evolving field of research, the application of nucleoside analogues already shows a great potential to become part of the future of anti-TB therapy. Among this class of compounds, several analogues have been identified as both potent and selective enzyme inhibitors, whereas others have shown potent *in vitro* anti-mycobacterial activity, although their biological targets have not yet been clearly identified.

Future investigations should aim at translating the enzymatic activity of these compounds in antitubercular activity by chemically tuning their pharmacokinetic properties and thus improving



their mycobacterial cell wall penetration. In this context, effective prodrug approaches should be further explored.

With regards to the nucleoside derivatives whose *in vitro* activity has not been paralleled by the identification of the appropriate targets, efforts should be made to connect their activity with the inhibited pathway. This will certainly aid the construction of structure activity relationships in order to improve their anti-TB activity.

The chemistry of nucleoside analogues together with their pharmacokinetic properties and toxicological profiles have been thoroughly investigated in the past century. Therefore it is easy to anticipate that this knowledge will offer many advantages for their development as anti-TB agents.

### Executive summary

#### ***Tuberculosis:***

- TB is an airborne infectious disease caused mostly by *M.tb*.
- It predominantly affects the lungs, but extra pulmonary forms exist.
- Approximately eight million people develop active disease each year and between two and three million cases of active disease result in death.

#### ***Current treatment and drawback:***

- Different regimens based on three lines drugs exist.
- Despite the current treatment TB still remains a global threat, due to the emergence of multi and extensively resistance *M.tb* strains.
- Drug-drug interaction impedes the cure to people with HIV, who are taking antiretroviral therapy, whereas the cost and the length of these therapies limit their coverage for the less-developed countries.
- The side effects range from mild to severe and can sometimes even warrant a change or end of medication.

#### ***Nucleoside and nucleotides analogues***

- Derivatives of natural nucleosides and nucleotides have become cornerstones of treatment for patients with cancer or viral infections. Recently their antituberculosis activity was also recognized.
- Several purine and pyrimidine nucleoside are able to target enzymes specifically involved in *M.tb* metabolism, whereas others proves selective toward enzymes from *M.tb*, but also expressed by the host. These derivatives may also be active in *M.tb* whole-cell assays.
- Some analogues show activity in whole-cell assay but not target has been yet identified for them.

### Financial and competing interest disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. We would like to acknowledge Robert James Gutteridge for helpful discussions during the preparation of this manuscript.

## ■ References

Papers of special note have been highlighted as: \* of interest

1. Kaufmann SHE. Robert Koch's highs and lows in the search for a remedy for tuberculosis. *Nature Med.* Special Web Focus: Tuberculosis (2000).
2. Koch R. die aetiologie der tuberkulose. (*reprint Am. rev. tuberc.* 25, 285-323 1932), 19, 221-230 (1882).
3. Zumla A, Nahid P, Cole ST. Advances in the development of new tuberculosis drugs and treatment regimens. *Nature Rev. Drug Discov.* 12(5), 388-404 (2013).

### \*\* Describes TB drug discovery and development.

4. World Health Organization, Global Tuberculosis Report. (2013).
5. Smith I. Mycobacterium tuberculosis pathogenesis and molecular determinants of virulence. *Clin. Microbiol. Rev.* 16(3), 463-496 (2003).

### \* Extensive review on TB infection, pathology and resistance to the treatment.

6. Smith NH, Hewinson RG, Kremer K, Brosch R, Gordon SV. Myths and misconceptions: the origin and evolution of *Mycobacterium tuberculosis*. *Nature Rev. Microbiol.* 7(7), 537-544 (2009).
7. Wallgren A. The time table of tuberculosis. *Tubercle* 29, 245-251 (1948).
8. Dartois V. The path of anti-tuberculosis drugs: from blood lesions to mycobacterial cells. *Nature Rev. Microbiol.* 12, 159-167 (2014).
9. Lin PL, Ford CB, Coleman MT *et al.* Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. *Nature Med.* 20(1), 75-79 (2014).
10. Russell D, Cardona, PJ, Kim, MJ, Allain, S, Altare, F. Foamy macrophages and the progression of the human tuberculosis granuloma. *Nature Immunol.* 10(9), 943-948 (2009).

11. Dartois V, Barry 3<sup>rd</sup> CE. A medicinal chemists' guide to the unique difficulties of lead optimization for tuberculosis. *Bioorg. Med. Chem. Lett.* 23(17), 4741-4750 (2013).
- \* **Focuses on the complexity of drug development against TB, describing causes and providing guidelines for lead optimisation programs.**
12. Tufariello JM, Chan J, Flynn JL. Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. *Lancet Infect. Dis.* 3(9), 578-590 (2003).
13. Lin PL, Flynn JL. Understanding latent tuberculosis: a moving target. *J. Immunol.* 185, 15-22 (2010).
14. Hauck FR, Neese BH, Panchal AS, El-Amin W. Identification and management of latent tuberculosis infection. *Am. Fam. Physician.* 79(10), 879-886 (2009).
15. LoBue PA, Enarson DA, Thoen TC. Tuberculosis in humans and its epidemiology, diagnosis and treatment in the United States. *Int. J. Tuberc. Lung Dis.* 14(10), 1226-1232 (2010).
16. Timmins GS, Deretic V. Mechanisms of action of isoniazid. *Mol. Microbiol.* 62, 1220–1227 (2006).
17. Suarez J, Ranguelova K, Jarzecki AA *et al.* An oxyferrous heme/protein-based radical intermediate is catalytically competent in the catalase reaction of *Mycobacterium tuberculosis* catalase-peroxidase (KatG). *J. Biol. Chem.* 284, 7017–7029 (2009).
18. Sharma SK, Sharma A, Kadhiravan T, Tharyan P. Rifamycins (rifampicin, rifabutin and rifapentine) compared to isoniazid for preventing tuberculosis in HIV-negative people at risk of active TB. *Cochrane Database Syst Rev.* 5(7) CD007545 (2013).
19. Zhang Y, Mitchison D. The curious characteristics of pyrazinamide: a review. *Int. J. Tuberc. Lung Dis.* 7(1), 6–21 (2003).
20. Scorpio A, Zhang Y. Mutations in PncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat. Med.* 2, 662–667 (1996).
21. Belanger AE, Besra GS, Ford ME *et al.* The embAB genes of *Mycobacterium avium* encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. *Proc. Natl. Acad. Sci.* 93, 11919–11924 (1996).
22. Donald PR, Sirgel FA, Venter A *et al.* The early bactericidal activity of streptomycin. *Int. J. Tuberc. Lung Dis.* 6, 693–8 (2002).

23. Peloquin CA, Berning SE, Nitta AT *et al.* Aminoglycoside toxicity: daily versus thrice-weekly dosing for treatment of mycobacterial diseases. *Clin. Infect. Dis.* 38, 1538–44 (2004).
24. Akbergenov R, Shcherbakov D, Matt *et al.* Molecular basis for the selectivity of antituberculosis compounds capreomycin and viomycin. *Antimicrob. Agents Chemother.* 55(10), 4712–4717 (2011).
25. Takiff H, Guerrero E. Current prospects for the fluoroquinolones as first-line tuberculosis therapy. *Antimicrob Agents Chemother.* 55(12), 5421–5429 (2011).
26. Rengarajan J, Sasseti CM, Naroditskaya V, Sloutsky A, Bloom BR, Rubin EJ. The folate pathway is a target for resistance to the drug *para*-aminosalicylic acid (PAS) in mycobacteria. *Mol. Microbiol.* 53, 275–282 (2004).
27. Dooley KE, Mitnick CD, DeGroot MA *et al.* Old drugs, new purpose: retooling existing drugs for optimized treatment of resistant tuberculosis. *Clin. Infect. Dis.* 55(4), 572–581 (2012).
28. Quémard A, Lanéelle G, Lacave C. Mycolic acid synthesis: a target for ethionamide in mycobacteria? *Antimicrob. Agents Chemother.* 36(6), 1316–1321 (1992).
29. Wang F, Langley R, Gulien G *et al.* Mechanism of thioamide drug action against tuberculosis and leprosy. *J. Exp. Med.* 204(1), 73–78 (2007).
30. Alahari A, Trivelli X, Guérarde Y *et al.* Thiacetazone, an antitubercular drug that inhibits cyclopropanation of cell wall mycolic acids in mycobacteria. *PLoS ONE* 2(12), e1343 (2007).
31. Lee M, Lee J, Carroll MW *et al.* Linezolid for treatment of chronic extensively drug-resistant tuberculosis. *N. Engl. J. Med.* 367(16), 10.1056/NEJMoa1201964 (2012).
32. Prosser GA, S. de Carvalho LP. Reinterpreting the mechanism of inhibition of *Mycobacterium tuberculosis* d-Alanine:d-Alanine ligase by d-cycloserine. *Biochemistry* 52(40), 7145–7149 (2013).
33. Gopal M, Padayatchi N, Metcalfe JZ, O'Donnell MR. Systematic review of clofazimine for the treatment of drug-resistant tuberculosis. *Int. J. Tuberc. Lung Dis.* 17(8), 1001–1007 (2013).
34. Solapure S, Dinesh N, Shandil R *et al.* In vitro and in vivo efficacy of  $\beta$ -lactams against replicating and slowly growing/nonreplicating *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 57(6), 2506–2510 (2013).

35. Chambers HF, Turner J, Schechter GF, Kawamura M, Hopewell PC. Imipenem for Treatment of Tuberculosis in Mice and Humans. *Antimicrob. Agents Chemother.* 49(7), 2816–2821 (2005).
36. Cavalieri SJ, Biehle JR, Sanders WE Jr. Synergistic activities of clarithromycin and antituberculous drugs against multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 39(7), 1542–1545 (1995).
37. Janin YL. Antituberculosis drugs: ten years of research. *Bioorg. Med. Chem.* 15(7), 2479-2513 (2007).
38. Grange JM, Zumla A. Advances in the management of tuberculosis: clinical trials and beyond. *Curr. Opin. Pulm. Med.* 6(3), 193-197 (2000).
39. Treatment of tuberculosis guidelines 4<sup>th</sup> ed. World Health Organization (2010)
40. Dye C, Garnett GP, Sleeman K, Williams BG. Prospects for worldwide tuberculosis control under the WHO DOTS strategy. *Lancet* 352(9144), 1886-1891 (1998).
41. Bastian ILR, Van Deun A, Portaels F. Directly observed treatment, short-course strategy and multidrug-resistant tuberculosis: are any modifications required? *Bulletin of the World Health Organ.* 78(2), 238-251 (2000).
42. Ramappa V, Aithal GP. Hepatotoxicity related to anti-tuberculosis drugs: mechanisms and management. *J. Clin. Exp. Hepat.* 3(1), 37-49 (2013).
43. Shu CC, Lee CH, Lee MC, Wang JY, Yu CJ, Lee LN. Hepatotoxicity due to first-line anti-tuberculosis drugs: a five-year experience in a Taiwan medical centre. *Int. J. Tuberc. Lung Dis.* 17(7), 934-939 (2013).
44. Munro SA, Lewin SA, Smith HJ, Engel ME, Fretheim A, Volmink J. Patient adherence to tuberculosis treatment: a systematic review of qualitative research. *PLoS Med* 4(7), e238 (2007).
45. Koul A, Arnoult E, Lounis N, Guillemont J, Andries K. The challenge of new drug discovery for tuberculosis. *Nature* 469(7331), 483-490 (2011).
46. Fenner L, Gagneux S, Janssens J-P *et al.* Tuberculosis in HIV-negative and HIV-infected patients in a low-incidence country: clinical characteristics and treatment outcomes. *PLoS ONE* 7(3), e34186 (2012).
47. Chang SH, Cataldo JK. A systematic review of global cultural variations in knowledge, attitudes and health responses to tuberculosis stigma. *Int. J. Tuberc. Lung Dis.* 18(2), 168-173 (2014).

48. Ormerod LP. Multidrug-resistant tuberculosis (MDR-TB): epidemiology, prevention and treatment. *Br. Med. Bull.* 73-74(1), 17-24 (2005).
49. Langendam MW, Tiemersma EW, van der Werf MJ, Sandgren A. Adverse events in healthy individuals and MDR-TB contacts treated with anti-tuberculosis drugs potentially effective for preventing development of MDR-TB: a systematic review. *PLoS ONE* 8(1), e53599 (2013).
50. Van der Walt M, Lancaster J, Odendaal R, Davis JG, Shean K, Farley J. Serious treatment related adverse drug reactions amongst anti-retroviral naïve MDR-TB patients. *PLoS ONE* 8(4), e58817 (2013).
51. Philip L. Extensively drug-resistant tuberculosis. *Curr. Opin. Infect. Dis.* 22(2), 167-167 (2009).
52. Shakya N, Garg G, Agrawal B, Kumar R. Chemotherapeutic interventions against tuberculosis. *Pharmaceut.* 5, 690-718 (2012).
53. Owens JP, Fofana MO, Dowdy DW. Cost-effectiveness of novel first-line treatment regimens for tuberculosis. *Intern. J. Tuberc. Lung Dis.* 17(5), 590-596 (2013).
54. Hokey DA, Ginsberg A. The current state of tuberculosis vaccines. *Hum. Vacc. Immunother.* 9(10), 2142-2146 (2013).
55. Jordheim JP, Durantel D, Zoulim FCD. Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. *Nature Rev.* 12, 448-464 (2013).
56. Ducati RG, Breda A, Basso LA, Santos DS. Purine salvage pathway in *Mycobacterium tuberculosis*. *Curr. Med. Chem.* 18, 1258-1275 (2011).
- \* **Review of the enzymes involved in purine salvage pathway in *M.tb*, useful for targeted drug development.**
57. Duckworth PB, Nelson MK, Aldrich CC. Adenylating enzymes in *Mycobacterium tuberculosis* as drug targets. *Curr. Top. Med. Chem.* 12(7), 766-796 (2012).
58. Shmalenyuk ER, Kochetkov SN, Alexandrova LA. Novel inhibitors of *Mycobacterium tuberculosis* growth based on modified pyrimidine nucleosides and their analogues. *Russian Chem. Rev.* 82(9), 896 (2013).
59. Hochstadt J. Hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase from enteric bacteria. *Methods Enzymol.* 51, 549-558 (1978).
60. Biazus G, Schneider CZ, Palma MS, Basso LA, Santos DS. Hypoxanthine-guanine phosphoribosyltransferase from *Mycobacterium tuberculosis* H37Rv: cloning, expression, and biochemical characterization. *Protein Expr. Purif.* 66(2), 185-190 (2009).

61. Keough DT, Hocková D, Rejman D *et al.* Inhibition of the *Escherichia coli* 6-oxopurine phosphoribosyltransferases by nucleoside phosphonates: potential for new antibacterial agents. *J. Med. Chem.* 56(17), 6967-6984 (2013).
62. Shi W, Basso LA, Santos DS *et al.* Structures of purine nucleoside phosphorylase from *Mycobacterium tuberculosis* in complexes with immucillin-H and its pieces. *Biochemistry* 40(28), 8204-8215 (2001).
63. Caceres RA, Timmers LFSM, Ducati RG *et al.* Crystal structure and molecular dynamics studies of purine nucleoside phosphorylase from *Mycobacterium tuberculosis* associated with acyclovir. *Biochimie* 94(1), 155-165 (2012).
64. Elizabeth A. Rayl, and BAM, Beardsley GP. The human purH gene product, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase. Cloning, sequencing, expression, purification, kinetic analysis, and domain mapping. *J. Biol. Chem.* 271, 2225-2233 (1996).
65. Le Nours J, Bulloch EMM, Zhang Z *et al.* Structural analyses of a purine biosynthetic enzyme from *Mycobacterium tuberculosis* reveal a novel bound nucleotide. *J. Biol. Chem.* 286(47), 40706-40716 (2011).
66. Long MC, Escuyer V, Parker WB. Identification and characterization of a unique adenosine kinase from *Mycobacterium tuberculosis*. *J. Bacteriol.* 185(22), 6548-6555 (2003).
67. Barrow EW, Westbrook L, Bansal N *et al.* Antimycobacterial activity of 2-methyladenosine. *J. Antimicrob. Chemother.* 52(5), 801-808 (2003).
68. Perlikova P, Konecny P, Naus P *et al.* 6-Alkyl-, 6-aryl- or 6-hetaryl-7-deazapurine ribonucleosides as inhibitors of human or *Mtb* adenosine kinase and potential antimycobacterial agents. *MedChemComm.* 4(11), 1497-1500 (2013).
69. Snášel J, Nauš P, Dostál J *et al.* Structural basis for inhibition of mycobacterial and human adenosine kinase by 7-substituted 7-(het)aryl-7-deazaadenine ribonucleosides. *J. Med. Chem.* 57(20), 8268-8279 (2014).
70. Gobin J, Moore CH, Reeve JR, Wong DK, Gibson BW, Horwitz MA. Iron acquisition by *Mycobacterium tuberculosis*: isolation and characterization of a family of iron-binding exochelins. *Proc. Nat. Acad. Sci.*, 92(11), 5189-5193 (1995).
71. De Voss JJ, Rutter K, Schroeder BG, Su H, Zhu Y, Barry CE. The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. *Proc. Nat. Acad. Sci.* 97(3), 1252-1257 (2000).

72. Quadri LEN, Sello J, Keating TA, Weinreb PH, Walsh CT. Identification of a *Mycobacterium tuberculosis* gene cluster encoding the biosynthetic enzymes for assembly of the virulence-conferring siderophore mycobactin. *Chem. Biol.* 5(11), 631-645 (1998).
73. Somu RV, Boshoff H, Qiao C, Bennett EM, Barry CE, Aldrich CC. Rationally designed nucleoside antibiotics that inhibit siderophore biosynthesis of *Mycobacterium tuberculosis*. *J. Med. Chem.* 49(1), 31-34 (2006).
74. Neres J, Labello NP, Somu RV *et al.* Inhibition of siderophore biosynthesis in *Mycobacterium tuberculosis* with nucleoside bisubstrate analogues: structure–activity relationships of the nucleobase domain of 5'-O-[N-(salicyl)sulfamoyl]adenosine. *J. Med. Chem.* 51(17), 5349-5370 (2008).
75. Qiao C, Gupte A, Boshoff HI *et al.* 5'-O-[(N-acyl)sulfamoyl]adenosines as antitubercular agents that inhibit MbtA: an adenylation enzyme required for siderophore biosynthesis of the mycobactins. *J. Med. Chem.* 50(24), 6080-6094 (2007).
76. Somu RV, Wilson DJ, Bennett EM *et al.* Antitubercular nucleosides that inhibit siderophore biosynthesis: SAR of the glycosyl domain. *J. Med. Chem.* 49(26), 7623-7635 (2006).
77. Gupte A, Boshoff HI, Wilson DJ *et al.* Inhibition of siderophore biosynthesis by 2-triazole substituted analogues of 5'-O-[N-(salicyl)sulfamoyl]adenosine: antibacterial nucleosides effective against *Mycobacterium tuberculosis*. *J. Med. Chem.* 51(23), 7495-7507 (2008).
78. Engelhart CA, Aldrich CC. Synthesis of chromone, quinolone, and benzoxazinone sulfonamide nucleosides as conformationally constrained inhibitors of adenylating enzymes required for siderophore biosynthesis. *J. Org. Chem.* 78(15), 7470-7481 (2013).
79. Schimmel P, Tao J, Hill J. Aminoacyl tRNA synthetases as targets for new anti-infectives. *FASEB J.* 12(15), 1599-1609 (1998).
80. Kim S, Lee SW, Choi EC, Choi SY. Aminoacyl-tRNA synthetases and their inhibitors as a novel family of antibiotics. *Appl. Microbiol. Biotech.* 61(4), 278-288 (2003).
81. Ingvarsson H, Jones TA, Unge T. Crystallization of *Mycobacterium smegmatis* methionyl-tRNA synthetase in the presence of methionine and adenosine. *Acta Crystall. Sect. F.* 65(6), 618-620 (2009).
82. Lee J, Kang SU, Kang MK *et al.* Methionyl adenylate analogues as inhibitors of methionyl-tRNA synthetase. *Bioorg. Med. Chem. Lett.* 9(10), 1365-1370 (1999).
83. Webb ME, Smith AG, Abell C. Biosynthesis of pantothenate. *Nat. Prod. Rep.* 21(6), 695-721 (2004).



84. Sambandamurthy VK, Wang X, Chen B *et al.* A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. *Nat. Med.* 8(10), 1171-1174 (2002).
85. Sambandamurthy VK, Derrick SC, Hsu T *et al.* *Mycobacterium tuberculosis*  $\Delta$ RD1  $\Delta$ panCD: a safe and limited replicating mutant strain that protects immunocompetent and immunocompromised mice against experimental tuberculosis. *Vaccine* 24(37–39), 6309-6320 (2006).
86. Ciulli A, Scott DE, Ando M *et al.* Inhibition of *Mycobacterium tuberculosis* pantothenate synthetase by analogues of the reaction intermediate. *ChemBioChem.* 9(16), 2606-2611 (2008).
87. Sareen D, Newton GL, Fahey RC, Buchmeier NA. Mycothiol is essential for growth of *Mycobacterium tuberculosis* Erdman. *J. Bacteriol.* 185, 6736-6740 (2003).
88. Gutierrez-Lugo M-T, Bewley CA. Susceptibility and mode of binding of the *Mycobacterium tuberculosis* cysteinyl transferase mycothiol ligase to tRNA synthetase inhibitors. *Bioorg. Med. Chem. Lett.* 21(8), 2480-2483 (2011).
89. Fan F, Vetting MW, Frantom PA, Blanchard JS. Structures and mechanisms of the mycothiol biosynthetic enzymes. *Curr. Opin. Chem. Biol.* 13(4), 451-459 (2009).
90. Duckworth BP, Geders TW, Tiwari D *et al.* Bisubstrate adenylation inhibitors of biotin protein ligase from *Mycobacterium tuberculosis*. *Chem. Biol.* 18(11), 1432-1441 (2011).
91. Shi C, Tiwari D, Wilson DJ, Seiler CL, Schnappinger D, Aldrich CC. Bisubstrate inhibitors of biotin protein ligase in *Mycobacterium tuberculosis* resistant to cyclonucleoside formation. *ACS Med. Chem. Lett.* 4(12), 1213-1217 (2013).
92. Villela AD S-QZ, Ducati RG, Santos DS, Basso LA. Pyrimidine salvage pathway in *Mycobacterium tuberculosis*. *Curr. Med. Chem.* 18, 1286-1298 (2011).
- \* **Review of the enzymes involved in pyrimidine salvage pathway in *M.tb*, useful for targeted drug development.**
93. Wheeler PR. Biosynthesis and scavenging of pyrimidines by pathogenic mycobacteria. *J. Gen. Microbiol.* 136, 189-201 (1990).
94. Carreras CW, Santi DV. The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* 64, 721-762 (1995).
95. Ostermann N, Schlichting I, Brundiers R *et al.* Insights into the phosphoryltransfer mechanism of human thymidylate kinase gained from crystal structures of enzyme complexes along the reaction coordinate. *Structure* 8(6), 629-642 (2000).

96. Myllykallio H, Lipowski G, Leduc D, Filee J, Forterre P, Liebl U. An alternative flavin-dependent mechanism for thymidylate synthesis. *Science* 105-107. (2002).
97. Graziani S, Xia Y, Gurnon JR *et al.* Functional analysis of FAD-dependent thymidylate synthase ThyX from *Paramecium bursaria* Chlorella Virus-1. *J. Biol. Chem.* 279(52), 54340-54347 (2004).
98. Mathews II, Deacon AM, Canaves JM *et al.* Functional analysis of substrate and cofactor complex structures of a thymidylate synthase-complementing protein. *Structure* 11(6), 677-690 (2003).
99. Kögler M, Vanderhoydonck B, De Jonghe S *et al.* Synthesis and evaluation of 5-substituted 2'-deoxyuridine monophosphate analogues as inhibitors of flavin-dependent thymidylate synthase in *Mycobacterium tuberculosis*. *J. Med. Chem.* 54(13), 4847-4862 (2011).
100. McGuigan C, Derudas M, Gonczy B *et al.* ProTides of N-(3-(5-(2'-deoxyuridine))prop-2-ynyl)octanamide as potential anti-tubercular and anti-viral agents. *Bioorg. Med. Chem.* 22(9), 2816-2824 (2014).
101. Mehellou Y, Balzarini J, McGuigan C. Aryloxy phosphoramidate triesters: a technology for delivering monophosphorylated nucleosides and sugars into cells. *ChemMedChem.* 4(11), 1779-1791 (2009).
102. McGuigan C, Murziani P, Slusarczyk M *et al.* Phosphoramidate proTides of the anticancer agent FUDR successfully deliver the preformed bioactive monophosphate in cells and confer advantage over the parent nucleoside. *J. Med. Chem.* 54(20), 7247-7258 (2011).
103. Slusarczyk M, Lopez MH, Balzarini J *et al.* Application of proTide technology to gemcitabine: a successful approach to overcome the key cancer resistance mechanisms leads to a new agent (NUC-1031) in clinical development. *J. Med. Chem.* 57(4), 1531-1542 (2014).
104. McGuigan C, Madela K, Aljarah M *et al.* Design, synthesis and evaluation of a novel double pro-drug: INX-08189. A new clinical candidate for hepatitis C virus. *Bioorg. Med. Chem. Lett.* 20(16), 4850-4854 (2010).
105. Parchina A, Froeyen M, Margamuljana L *et al.* Discovery of an acyclic nucleoside phosphonate that inhibits *Mycobacterium tuberculosis* ThyX based on the binding mode of a 5-alkynyl substrate analogue. *ChemMedChem.* 8(8), 1373-1383 (2013).
106. Kögler M, Busson R, De Jonghe S *et al.* Synthesis and evaluation of 6-aza-2'-deoxyuridine monophosphate analogs as inhibitors of thymidylate synthases, and as

- substrates or inhibitors of thymidine monophosphate kinase in *Mycobacterium tuberculosis*. *Chem. Biodivers.* 9(3), 536-556 (2012).
107. Sclafani R, Fangman, WL. Yeast gene CD8 encodes thymidylate kinase and is complemented by herpes thymidine kinase gene TK. *Proc. Natl. Acad. Sci. U.S.A.* 81 (1984).
  108. Cui QSW, Luo Y, Tian J *et al.* Thymidylate kinase: an old topic brings new perspectives. *Curr. Med. Chem.* 20(10), 1286-1305 (2013).
  109. Li de la Sierra I, Munier-Lehmann H, Gilles AM *et al.* X-ray structure of TMP kinase from *Mycobacterium tuberculosis* complexed with TMP at 1.95 Å resolution. *J. Mol. Biol.* 311(1), 87-100 (2001).
  110. Munier-Lehmann H, Chaffotte A, Pochet S *et al.* Thymidylate kinase of *Mycobacterium tuberculosis*: a chimera sharing properties common to eukaryotic and bacterial enzymes. *Protein Sci.* 10(6), 1195-1205 (2001).
  111. Vanheusden V, Munier-Lehmann H, Pochet S *et al.* Synthesis and evaluation of thymidine-5'-O-monophosphate analogues as inhibitors of *Mycobacterium tuberculosis* thymidylate kinase. *Bioorg. Med. Chem. Lett.* 12(19), 2695-2698 (2002).
  112. Vanheusden V, Munier-Lehmann H, Froeyen M *et al.* 3'-C-branched-chain-substituted nucleosides and nucleotides as potent inhibitors of *Mycobacterium tuberculosis* thymidine monophosphate kinase. *J. Med. Chem.* 46(18), 3811-3821 (2003).
  113. Vanheusden V, Munier-Lehmann H, Froeyen M *et al.* Discovery of bicyclic thymidine analogues as selective and high-affinity inhibitors of *Mycobacterium tuberculosis* thymidine monophosphate kinase. *J. Med. Chem.* 47(25), 6187-6194 (2004).
  114. Van Daele I, Munier-Lehmann H, Hendrick PMS *et al.* Synthesis and biological evaluation of bicyclic nucleosides as inhibitors of *M. tuberculosis* thymidylate kinase. *ChemMedChem.* 1(10), 1081-1090 (2006).
  115. Van Daele I, Munier-Lehmann H, Froeyen M *et al.* Rational design of 5'-thiourea-substituted  $\alpha$ -thymidine analogues as thymidine monophosphate kinase inhibitors capable of inhibiting mycobacterial growth. *J. Med. Chem.* 50(22), 5281-5292 (2007).
  116. Familiar OM-LH, Negri A, Gago F *et al.* Exploring acyclic nucleoside analogues as inhibitors of *Mycobacterium tuberculosis* thymidylate kinase. *ChemMedChem.* 3(7), 1083-1093 (2008).
  117. Johar M, Manning T, Kunimoto DY *et al.* Synthesis and in vitro anti-mycobacterial activity of 5-substituted pyrimidine nucleosides. *Bioorg. Med. Chem.* 13(24), 6663-6671 (2005).

118. Srivastav NC, Manning T, Kunimoto DY *et al.* In vitro anti-mycobacterial activities of various 2'-deoxyuridine, 2'-arabinouridine and 2'-arabino-fluoro-2'-deoxyuridine analogues: synthesis and biological studies. *J. Med. Chem.* 2(3), 287-293 (2006).
119. Rai D, Johar M, Manning T *et al.* Design and studies of novel 5-substituted alkynylpyrimidine nucleosides as potent inhibitors of Mycobacteria. *J. Med. Chem.* 48(22), 7012-7017 (2005).
120. Srivastav NC, Manning T, Kunimoto DY *et al.* Studies on acyclic pyrimidines as inhibitors of Mycobacteria. *Bioorg. Med. Chem.* 15(5), 2045-2053 (2007).
121. Chu CK, Cutler SJ. Chemistry and antiviral activities of acyclonucleosides. *J. Heterocyclic Chem.* 23(2), 289-319 (1986).
122. Freeman S, Gardiner JM. Acyclic nucleosides as antiviral compounds. *Mol. Biotechnol.* 5(2), 125-137 (1996).
123. Johar M, Manning T, Tse C *et al.* Growth inhibition of Mycobacterium bovis, Mycobacterium tuberculosis and Mycobacterium avium in vitro: effect of 1- $\beta$ -d-2'-arabinofuranosyl and 1-(2'-deoxy-2'-fluoro- $\beta$ -d-2'-ribofuranosyl) pyrimidine nucleoside analogs. *J. Med. Chem.* 50(15), 3696-3705 (2007).
124. Rai D, Johar M, Srivastav NC *et al.* Inhibition of Mycobacterium tuberculosis, Mycobacterium bovis, and Mycobacterium avium by novel dideoxy nucleosides. *J. Med. Chem.* 50(19), 4766-4774 (2007).
125. Srivastav NC, Rai D, Tse C *et al.* Inhibition of mycobacterial replication by pyrimidines possessing various C-5 functionalities and related 2'-deoxynucleoside analogues using in vitro and in vivo models. *J. Med. Chem.* 53(16), 6180-6187 (2010).
126. Shakya N, Srivastav NC, Desroches N *et al.* 3'-Bromo analogues of pyrimidine nucleosides as a new class of potent inhibitors of Mycobacterium tuberculosis. *J. Med. Chem.* 53(10), 4130-4140 (2010).
127. Srivastav NC, Shakya N, Bhavanam S *et al.* Antimycobacterial activities of 5-alkyl (or halo)-3'-substituted pyrimidine nucleoside analogs. *Bioorg. Med. Chem. Lett.* 22(2), 1091-1094 (2012).
128. Matyugina E, Khandazhinskaya A, Chernousova L *et al.* The synthesis and antituberculosis activity of 5'-nor carbocyclic uracil derivatives. *Bioorg. Med. Chem. Lett.* 20(22), 6680-6686 (2012).
129. Jeong LS, Lee JA. Recent advances in the synthesis of the carbocyclic nucleosides as potential antiviral agents. *Antivir. Chem. Chemoth.* 15(5), 235-250 (2004).

130. Shmalenyuk ER, Chernousova LN, Karpenko IL *et al.* Inhibition of Mycobacterium tuberculosis strains H37Rv and MDR MS-115 by a new set of C5 modified pyrimidine nucleosides. *Bioorg. Med. Chem. Lett.* 21(17), 4874-4884 (2013).

■ **Patent**

201. Zee-Fen C, Chun-Mei H, Jim-Min F. Targeting human thymidylate kinase induces DNA repair toxicity in malignant tumor cells. WO 2012072019 A1 (2012).

**MIC** (minimum inhibitory concentration) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism usually by the 50% (MIC<sub>50</sub>) or 90% (MIC<sub>90</sub>), after overnight incubation.

**IC<sub>50</sub>** (half maximal inhibitory concentration) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function.