Nucleoside analogues and tuberculosis: new weapons against an old enemy

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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. pinnipedi, M. canetti* 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**

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

<table>
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<tr>
<th>Drug Combination</th>
<th>Target</th>
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<tr>
<td>Amoxicillin plus Clavulanate, Imipenem plus Cilastatin</td>
<td>Cell wall synthesis</td>
<td>[34,35]</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>Protein synthesis</td>
<td>[36]</td>
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**Table 1.** Classification and main targets of first, second and third line treatments of TB.
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**

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 syderophore (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 catalyzed by ATIC enzyme.


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 \textit{Escherichia coli} (\textit{EcHGPRT}) and mycobacterial HGPRT (\textit{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 \textit{EcHGPRT}, with \textit{K}_i 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 \( \text{EcHGPRT} \) and \( \text{MtHGPRT} \) could translate into a potential \( \text{MtHGPRT} \) inhibitory activity of compounds 1-3 and 7, Keough \textit{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 \( \text{EcHGPRT} \), \( \text{MtPNP} \) and ATIC.](image)

\( A \). Acyclic nucleoside phosphonates 1-3 (\( \text{EcHGPRT} \) inhibitors) and relative prodrugs 4-6.
\( B \). Acyclic nucleoside phosphate 7 (\( \text{EcHGPRT} \) inhibitor) and relative prodrug 8.
\( C \). Acyclovir 9 (\( \text{MtPNP} \) 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

\( \text{MIC} \) (minimum inhibitory concentration) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism usually by the 50\% (\( \text{MIC}_{50} \)), 90\% (\( \text{MIC}_{90} \)) or
99% (MIC$_{99}$), after overnight incubation.

EC$_{50}$ (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.tbc* and resulted in minimum inhibitory concentration values (MIC$_{90}$) ranging between 20 and 75 µM. On the other hand, none of the parent compounds (1-3 and 7) exhibited antimycobacterial activity at concentrations ≥ 250 µM, validating the prodrug strategy as a method to target *M.tbc*. Cytotoxic evaluation in human lung carcinoma cells (A549) resulted in EC$_{50}$ > 100 µM, leading to a selectivity index [cytotoxicity/MIC$_{50}$] ≥ 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.tbc* 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.tbc* 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 µM) (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]. Crystalllographic 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 HsPNP do not seem to retain importance for the binding with MtPNP. Such differences could be exploited for the drug-design of novel and selective MtPNP 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^{10}-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 (hATIC) is a dimer containing two distinct functional domains in each monomer. Evidence shows that hATIC 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 undegoes 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 MtATIC 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, MtATIC contained two independent ATIC molecules organized as tightly associated dimers. However, the MtATIC dimer was found fundamentally different to the human enzyme, with the two trasformyl and cyclohydrolase domains rotated by 90°. The origin of this difference lies in the connecting peptide. During these crystallographic studies an adventitius 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 MtATIC active site, strongly suggesting it could be an effective inhibitor.
of this enzyme. These results and the observed differences between the crystal structures of hATIC and MtbATIC 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 savage 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 γ-phosphate group from adenosine triphosphate (ATP), releasing adenosine diphosphate (ADP) (Figure 3) [66].

![Enzymatic reaction catalyzed by ADK.](image)

**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$_{99}$ value of 3.0 µg/mL (Figure 4). Mycobacterial ADK (*MtADK*) and human ADK (*hADK*) 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 *MtADK*. 
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$_{50}$ = 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$_{99}$ 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.tbc* 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.
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 salycilate 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*<sub>i</sub> = 7 nM. Furthermore, it displays potent activity in a whole cell assay against H37Rv mycobacterial strain under iron-limiting conditions, resulting in MIC<sub>50</sub> = 0.39 µM, 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*<sub>i</sub> = 0.27 nM, MIC<sub>99</sub> = 0.049
µM) (Figure 6A) [74]. Introduction of a fluorine substitution in the para position on the salicylate aromatic ring (17) led to an improved MIC<sub>99</sub> = 0.0098 µM and a K<sub>i</sub> = 0.012 µM 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 <i>in vitro</i> antitubercular activity [76]. Compound 18, bearing a 2-phenyl substituted triazole in position 2 of the nucleobase, was the most potent (K<sub>i</sub> = 3.23 nM, MIC<sub>99</sub> = 3.13 µM), selective (active only in iron-deficient conditions) and least cytotoxic derivative of the series (CC<sub>50</sub> > 100 µM in VERO cells) (Figure 6A) [77].

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<sub>i</sub> = 0.12 µM) (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 MIC<sub>50</sub> value higher than 50 µM.

**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.
**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.

**Adenylation 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 adenylation 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 β-alanine (β-Ala) by adelynating enzyme PanC to afford pantothenic acid (vitamin B₅) (Figure 5D).

Three inhibitors that mimic the structure of the adenyate 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 $K_i = 0.22 \mu M$ (Figure 6C). Replacement of the hydroxyl group with an amino group in both compounds 22 ($K_i = 4.0 \mu M$) and 23 ($K_i = 18.0 \mu M$) 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.

Adenylating 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 adenylating 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 ($K_i = 0.3 \mu M$) (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 transferyl enzyme [88].

Biotin protein ligase (BIRA)

The mycobacterial biotin protein ligase regulates lipid methabolism in M. tb trough 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 nucleophillic attack of the biotin carboxylate (Biotin) onto the $\alpha$-phosphate of ATP to yield the acyl-adenylate (Bio-AMP) and 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 ben 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 chemically 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 chemically 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\textsubscript{99} = 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 \textit{de novo} and salvage pyrimidine pathways have been documented in Mycobacteria [92-93]. Thymidylate synthase (ThyX) and thymidylate kinase (TMPKmt) 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.

\textit{Thymidylate synthase (ThyX)}

Thymidylate synthase enzyme (ThyA), was originally discovered as essential for eubacteria, plants and eukaryotic cells, for the \textit{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\textsubscript{5},N\textsubscript{10}-methylene-5,6,7,8-tetrahydrofolate (CH\textsubscript{2}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].
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$_2$THF only as a methylene donor, whereas the hydride donor is in this case represented by reduced flavin adenine dinucleotide (FADH$_2$) (Figure 7B) [96,98]. During the catalysis cycle, nicotinamide adenine dinucleotide phosphate (NADPH) is oxidized to NADP$^+$ allowing the regeneration of FADH$_2$ 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$_{50}$ = 0.57 µM, although also inhibiting ThyA at a similar concentration (IC$_{50}$ = 0.29 µM) [97].
**Key term**

IC$_{50}$ (half maximal inhibitory concentration) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function.

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**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’-deoxyuridin-5’-monophosphate analogues, with an IC$_{50}$ 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 antimycobrial 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$_{90}$ = 62.5 and 125 mg/L against a drug sensitive strain of $M.tib$ (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 (TMPKmt)**

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 $M.tib$ isoform TMPKmt. In fact, TMPKmt 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 µM) (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 µM (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<sub>mt</sub> enzyme in this series (K<sub>i</sub> ranging from 10.5 to 15 µM) (Figure 11A) [113].

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<sub>mt</sub> inhibitors, showing K<sub>i</sub> values of respectively 13.5 µM, 3.5 µM and 37 µM (Figure 11B) [113].

The selectivity most likely arises from the unique flexibility of the sugar ring within the TMPK<sub>mt</sub> 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<sub>mt</sub> 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<sub>mt</sub> K<sub>i</sub> value of 2.3 µM [114]. The same compound was also selected to be tested for in vitro whole cell assay, resulting in a weak inhibitor of <i>M.tb</i> growth (MIC<sub>99</sub> = 100 µM), without showing any additional cytotoxicity against VERO cell lines up to a concentration of 500 µg/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 β-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-β-nucleoside led to the inversion of the nucleoside configuration from β to α, switching the 3’-substituent with the 5’-group (48, Figure 11C).

5’-modified-α-analogues showed consistently higher activities compared to the β-counterparts. Accompanying this, lipophilic and electron-withdrawing phenyl substituents notably increased the inhibitory activity. Molecular modelling confirmed that the superiority of α-analogues depended on a more favourable arrangement inside TMPKmt binding pocket. Compound 49 showed the most promising activity with a Ki value of 0.6 µM, a selectivity index versus TMPKh of 600 and a good growth inhibition of M.bovis (MIC99 = 20 µg/mL) (Figure 11D). The activity of 49 on M.tb growth reached a maximum of 39% at a concentration of 6.25 µg/mL.

In a following study, a successful investigation of acyclic nucleoside analogues as TMPKmt inhibitors was conducted by Familiar et al. [116]. From an initial screening this research group identified the thymidine acyclic analogue 50 as a micromolar TMPKmt inhibitor (Ki = 42 µM) (Figure 36).

![Figure 12. TMPKmt inhibitors 50-52.](image)

 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 (Ki = 0.42 µM and Ki = 0.27 µM, respectively) (Figure 12). Unfortunately no antimycobacterial activity was detected against M.bovis BCG and M.tb up to a concentration of 7.5 µg/mL and 32 µg/mL, respectively.
Nucleoside analogues with unclear target.

Some initial studies report the synthesis of nucleoside analogues and their evaluation as antimycobacterial 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 µM, similarly to the positive control rifampicin (MIC<sub>90</sub> = 2 µg/mL) (Figure 13A) [117].

![Figure 13. Anti-mycobacterial compounds 53-73.](image-url)
The introduction of an azidovinyl group in position 5 of 2’-deoxyuridine (56) led to moderate activity against *M. avium* (MIC<sub>50</sub> = 1-5 µg/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<sub>90</sub> values ranging from 10 to 50 µg/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<sub>50</sub> = 10 µg/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-β-D-2’-arabinofuranosyl and 1-(2’-deoxy-2’-fluoro- β-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-β-D-2’-arabinofuranosyl-5-dodecynyluracil (66), 1-(2’-deoxy-2’-fluoro- β-D-ribofuranosyl)- 5-dodecynyluracil (67) and 1-(2’-deoxy-2’-fluoro- β-D-ribofuranosyl)- 5-tetradecynyluracil (68), with activity against *M. tb* and *M. bovis* close to that of the reference drug rifampicin (MIC<sub>90</sub> = 1-5 µg/mL), but only low results on *M. avium* growth inhibition (MIC<sub>90</sub> >
10-50 µM) (Figure 13D) [123]. Very interestingly, these compounds were also found to retain sensitivity against a rifampicin-resistant strain of \( M.\text{tb} \) 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 \( M.\text{bovis} \), \( M.\text{tb} \) and \( M.\text{avium} \) (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 µg/mL, approaching the reference drug rifampicin, and also retaining activity towards a rifampicin-resistant strand of \( M.\text{tb} \) [124].

In 2010 the first antimycobacterial \textit{in vivo} data in a mouse model infected with H37Ra \( M.\text{tb} \) strain were published [125]. After synthesis and antimycobacterial \textit{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 \textit{in vitro} activity of this compound (MIC\(_{50}\) = 1-5 µg/mL on \( M.\text{bovis} \), MIC\(_{50}\) = 5 µg/mL on \( M.\text{tb} \)) prompted to \textit{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 \textit{in vitro} antimycobacterial activity against \( M.\text{tb} \) showed an improvement (68-70% inhibition at 10 µg/mL) as compared to 74 (70% inhibition at 50 µg/mL) (Figure 14A) [125]. Compound 76 demonstrated no cytotoxicity in Huh-7 cells up to a concentration of > 200 µg/mL, whereas
compound 75 showed 25% inhibition in cell viability at 200 µg/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 necroscopy 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-arabinofuranosythymine (77) was the most effective agent (MIC<sub>50</sub> = 1 µg/mL) in vitro against wild-type M.<i>tb</i> strain (H37Ra) as well as rifampicin and isoniazid-resistant strain of <i>M.tb</i> (H37Rv) (MIC<sub>50</sub> = 1-2 µg/mL) (Figure 14B) [126]. Most intriguingly, compound 77 also inhibited intracellular <i>M.tb</i> in a human monocytic cell line (THP-1) infected with H37Ra, with a > 90% of reduction of the number of surviving bacteria at a
concen
concentration of 25 µg/mL, demonstrating higher activity against intramacrophagic mycobacteria than extramacrophagic mycobacteria [126]. Moreover, no citotoxicity was found up to the highest concentration of compound tested (CC_{50} > 100-200 µg/mL) [126].

Further investigations generated in 2012 a series of 5-alkyl(or halo)-3’-azido(amo
Further investigations generated in 2012 a series of 5-alkyl(or halo)-3’-azido(amo 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 \( M. avium \) (MIC_{50} ranging from 1 and 5 µg/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-β-D-arabinofuranosyl)-5-ethyluracil provided promising in vitro activity against \( M. bovis \) and \( M. tb \) alone and in combination with isoniazid (MIC_{50} = 5 µg/mL) (Figure 14B).

1-(β-D-arabinofuranosyl)-4-thio-5-hydroxymethyluracil (80) displayed the most potent inhibition of wild-type H37Ra (MIC_{50} = 0.5 µg/mL), rifampicin-resistant H37Rv (MIC_{50} = 1 µg/mL) and \( M. bovis \) (MIC_{50} = 1 µg/mL), along with inhibition of \( M. avium \) (MIC_{50} = 10 µg/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 µg/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 MIC99 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 LD50 ≥ 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 CD50 = 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 \textit{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 \textit{M.\textit{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 \textit{M.\textit{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 \textit{M.\textit{tb}} metabolism, whereas others proves selective toward enzymes from \textit{M.\textit{tb}}, but also expressed by the host. These derivatives may also be active in \textit{M.\textit{tb}} whole-cell assays.
- Some analogues show activity in whole-cell assay but not target has been yet identified for them.

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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.

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


** Describes TB drug discovery and development.


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


*Focuses on the complexity of drug development against TB, describing causes and providing guidelines for lead optimisation programs.*


* Review of the enzymes involved in purine salvage pathway in M.tb, useful for targeted drug development.


106. Köglér 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


**Patent**


**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\textsubscript{50}) or 90% (MIC\textsubscript{90}), after overnight incubation.

**IC\textsubscript{50}** (half maximal inhibitory concentration) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function.