Review Article

The tryptophan biosynthetic pathway is essential for *Mycobacterium tuberculosis* to cause disease

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*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), is the most significant cause of death from a single infectious agent worldwide. Antibiotic-resistant strains of *M. tuberculosis* represent a threat to effective treatment, and the long duration, toxicity and complexity of current chemotherapy for antibiotic-resistant disease presents a need for new therapeutic approaches with novel modes of action. *M. tuberculosis* is an intracellular pathogen that must survive phagocytosis by macrophages, dendritic cells or neutrophils to establish an infection. The tryptophan biosynthetic pathway is required for bacterial survival in the phagosome, presenting a target for new classes of antitubercular compound. The enzymes responsible for the six catalytic steps that produce tryptophan from chorismate have all been characterised in *M. tuberculosis*, and inhibitors have been described for some of the steps. The innate immune system depletes cellular tryptophan in response to infection in order to inhibit microbial growth, and this effect is likely to be important for the efficacy of tryptophan biosynthesis inhibitors as new antibiotics. Allosteric inhibitors of both the first and final enzymes in the pathway have proven effective, including by a metabolite produced by the gut biota, raising the intriguing possibility that the modulation of tryptophan biosynthesis may be a natural inter-bacterial competition strategy.

Introduction

In many bacteria, the amino acid tryptophan is synthesised *de novo*, starting from chorismate. In contrast, many pathogenic micro-organisms are auxotrophic for tryptophan production, and this biosynthetic deficit is exploited by the mammalian immune system: intracellular pathogens are starved of tryptophan by the action of the degradative enzyme indoleamine 2,3-dioxygenase (IDO). This review will focus on the role of tryptophan biosynthesis in the development of disease by *Mycobacterium tuberculosis*, the causative agent of TB, which is an intracellular pathogen naturally prototrophic for tryptophan. Auxotropic mutants of *M. tuberculosis* are unable to establish an infection in mice, establishing tryptophan biosynthesis as an essential *in vivo* activity of the bacterium that is required for pathogenesis. Subsequent work has identified many inhibitors of various steps of the biosynthetic pathway, some of which show *in vivo* efficacy in animal models of disease, demonstrating that the tryptophan biosynthetic pathway could potentially be utilised as a target for new antibiotics active against *M. tuberculosis*. However, more experiments will need to be performed to complete the validation of the pathways as a viable target for effective therapy, given the complexity of the human disease.

*M. tuberculosis* is a globally significant human pathogen for which new therapies are required

The World Health Organization (WHO) rates tuberculosis (TB) as the most significant cause of death from a single infectious agent worldwide, ranking it above the human immunodeficiency virus (HIV) as the deadliest human communicable disease. Approximately 1.45 million people are estimated to have died from TB in 2018, the most recent year for which figures are available [1]. Drug-sensitive
forms of the disease have a high cure-rate: 85% of cases can be treated successfully with the current standard therapeutic regimen, which consists of four antibiotics (isoniazid and ethambutol, which both inhibit cell wall biosynthesis; rifampicin, which inhibits mRNA production; and pyrazinamide, which is thought to inhibit coenzyme A biosynthesis). The overall TB mortality rate has been slowly declining in recent years, but there remains a substantial concern that the continued presence of multidrug-resistant (MDR) strains of \textit{M. tuberculosis}, defined as those resistant to both isoniazid and rifampicin, may reverse this progress. It is estimated that \(\sim 400\,000\) people were diagnosed with MDR-TB in 2018. MDR-TB is more complicated and slower to treat, and requires the use of second-line antibiotics, many of which have significant side effects, for up to 20 months. The success rate for MDR-TB treatment is only 56%, leading to an estimated total death toll of \(\sim 200\,000\) people in 2018. Extensively drug-resistant TB (XDR-TB) is defined as MDR-TB plus resistance to two of the second-line drugs; the treatment success rate for these strains drops further to only 39% [1].

The main challenges to the effective current and future treatment of TB include the duration, toxicity and complexity of the required drug regimens, especially for second-line therapies. All of these factors negatively affect patient compliance, which is turn drives the further selection of drug-resistant strains of \textit{M. tuberculosis}. Hence, despite the development of new effective drugs such as the ATP synthase inhibitor bedaquiline and the mycolic acid biosynthesis inhibitor delamanid [2], there remains a pressing need for new drugs that are more effective, more affordable and less toxic, and that require shorter treatment times.

\textbf{\textit{M. tuberculosis} as an intracellular pathogen}

Person-to-person spread of \textit{M. tuberculosis} occurs via aerosol transmission [3]. Once it has been inhaled into the lower respiratory tract, the bacterium is engulfed by phagocytic cells, primarily by alveolar macrophages but also by dendritic cells and neutrophils [4]. The phagosomes containing the bacteria would normally then undergo a process referred to as phagosome maturation, which includes acidification of the phagocytic vacuole and eventual fusion with the lysosome. However, \textit{M. tuberculosis} has been observed to arrest this process and survive within non-acidified phagosomes [5]. The prevailing paradigm has, therefore, been that the bacterium primarily resides within arrested macrophage phagosomes, and various studies have visualised it in this cellular location using electron microscopy, from the 1970s onwards, e.g. [6]. An alternative view, first put forward in the 1980s [7] is that the bacterium is able to escape into the cytoplasm [8]. The situation is complex and dynamic (reviewed in [4]), but an essential point for understanding the significance of tryptophan biosynthesis to the progression of disease is that the bacterium must survive the harsh intravacuolar environment, and this initial bottleneck plays a critical part in modifying bacterial metabolism [9].

\textbf{The tryptophan biosynthetic pathway in \textit{M. tuberculosis}}

The bacterial tryptophan biosynthetic pathway comprises six catalytic steps, starting from the key metabolic intermediate chorismite (Figure 1) [10]. The seven enzymes responsible and the genes that encode them are represented by the letters A–G, assigned based on the gene order in \textit{Escherichia coli} [11,12]. The first committed step in the biosynthesis is the conversion of chorismate to anthranilate, which is catalysed by the enzyme anthranilate synthase (AS). Anthranilate synthase is a functional heterodimer composed of AS-I (TrpE), which catalyses the production of anthranilate from chorismate and ammonia, and AS-II (TrpG), a glutamine amidotransferase (GAT) that provides the required ammonia by converting glutamine to glutamate. Anthranilate phosphoribosyltransferase (AnPRT; TrpD) then transfers a 5′-phospho-ribose unit from phosphoribosyl pyrophosphate (PRPP) onto the amino group of anthranilate to produce phosphoribosylanthranilate (PRA). The ribose ring of PRA is opened by PRA isomerase (PRAI; TrpF) to produce the isomer 1-carboxyphenylamino-1′-deoxyribulose-5′-phosphate. A ring closure reaction catalysed by indole-3-glycerol phosphate synthase (IGPS; TrpC) forms the characteristic indole heterocyclic ring system. Finally, the heterotetrameric enzyme tryptophan synthase (TrpAB) cleaves the indole ring from the glycerol phosphate backbone, and forms the final amino acid product by condensation of the indole moiety with serine. AllostERIC regulation of the pathway is provided by feedback inhibition of tryptophan binding to a regulatory site in the TrpE protein [13,14].

Once the complete genome sequence of \textit{M. tuberculosis} was published in 1998 [15], the open reading frames (ORFs) that encode the enzymes of the tryptophan biosynthetic pathway could be assigned by sequence comparison. However, not all of the enzymes in the pathway could be unambiguously identified from sequence information alone, and unlike the genetic arrangement in \textit{E. coli}, the ORFs are not arranged in a single operon. Consequently, subsequent biochemical confirmation was required to establish which ORFs encode the enzymes in the pathway.
Two ORFs, Rv1609 and Rv2386c, were initially identified as TrpE candidates, but biochemical and structural analysis showed that Rv2386c in fact encodes a salicylate synthase [16], confirming Rv1609 as TrpE. The identity of TrpG proved more elusive, but of the four potential GATs (Rv0013, Rv0788, Rv1602 and Rv2604c), only Rv0013 forms a functional AS complex with Rv1609 [17]. However, the interaction between the two subunits is too weak for the complex to be crystallised, implying that Rv0013 may promiscuously provide GAT activity to other biosynthetic pathways, as is the case in Bacillus subtilis, where a single GAT functions in the synthesis of both tryptophan and p-aminobenzoate [18].

Rv1603 encodes a bifunctional protein that has both PRAI and N°-[5'-phosphoribosyl]formimino]-5-aminomidazole-4-carboxamide ribonucleotide (ProFAR) isomerase activity thus functioning as both TrpF and as HisA in the histidine biosynthetic pathway [19]. The predicted TrpA (Rv1613), TrpB (Rv1612), TrpC (Rv1611) and TrpD (Rv2192c) proteins have all been heterologously expressed and characterised, and demonstrate the expected biochemical activities in vitro [20–24], and all the enzymes in the pathway have been structurally characterised apart from TrpG (Table 1).

**Figure 1. The tryptophan biosynthetic pathway in *M. tuberculosis*.** The steps in the biosynthesis of tryptophan are shown in black. The points of action in the pathway of the inhibitors BRD4592 and IPA and the antimetabolite 5-fluoroanthranilate are shown in red.

**Tryptophan biosynthesis is essential for host colonisation by *M. tuberculosis***

*M. tuberculosis* is fully competent for tryptophan biosynthesis, but its critical dependence on this biosynthetic capability during infection was only revealed when auxotrophic knockout strains were investigated for their potential as vaccine candidates [25]. An *M. tuberculosis* strain with a defined mutation in the *trpD* gene

| Gene name | ORF number* | Enzyme activity | PDB code(s)              |
|-----------|-------------|-----------------|-------------------------|
| TrpA      | Rv1613      | Tryptophan synthase α subunit | TrpA/TrpB complexes: 5ccw, 5tcj, 5tch, 5tci, 5tcf, 5tgc, 6uap, 6ub9, 6e9p, 6dwe |
| TrpB      | Rv1612      | Tryptophan synthase β subunit | TrpB: 2o2e, 2o2j |
| TrpC      | Rv1611      | Indole-3-glycerol phosphate synthase | 3qja, 3t40, 3t44, 3t55, 3t78, 4fb7 |
| TrpD      | Rv2192c     | Anthranilate phosphoribosyl transferase | 1zw, 2bpq, 3r88, 3twp, 3uw1, 4j1, 4giu, 4gkm, 4m0r, 4n5v, 4n8q, 4n93, 4owu, 4oww, 4owm, 4own, 4owo, 4owq, 4ows, 5bty, 5bne, 5c1r, 5c2l, 5c7s |
| TrpE      | Rv1609      | Anthranilate synthase | 5cwa |
| TrpF      | Rv1603      | Phosphoribosyl-anthranilate isomerase | 2y85, 2y88, 2y89, 3zs4 |
| TrpG      | Rv0013      | Glutamine amidotransferase | |

* `Rv` numbers are used to designate ORFs in the *M. tuberculosis* reference genome, as the first sequenced genome was from the virulent H37Rv laboratory strain of the bacterium; Rv numbers suffixed with `c` indicate that the ORF is encoded on the complementary strand of the genome, relative to the designated start point for ORF numbering, which is the gene dnaA.

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(Rv2192c), which effectively disrupts tryptophan biosynthesis, failed to cause disease in mice. Tryptophan biosynthesis, therefore, appears to be required for TB disease formation.

The essentiality of biosynthetic pathways for some other amino acids, for example, leucine [26], arginine [27], lysine [28] and methionine [29], for in vivo growth and disease formation by M. tuberculosis has also been demonstrated. This implies that the bacterium is required to survive in niches with limited access to amino acids during the establishment of an infection, presumably including the phagosomes of macrophages in the lungs.

The tryptophan auxotrophic strain of M. tuberculosis made by knocking out the trpD gene was strikingly avirulent, even in severe, combined immune-deficient (SCID) mice, which lack a functional adaptive immune system. Not only did these mice survive infection, they also cleared the auxotrophic bacteria from their lungs to the limit of detection. This implies that suitable inhibitors of tryptophan biosynthesis may act as effective antibiotics, at least during the initiation of infection. However, it remained unproven from these data whether an established infection by biosynthetically intact wild-type bacteria could be effectively killed via this approach, as although tryptophan biosynthesis is required to establish infection, it may not be critical to maintain an existing or ongoing infection.

**The role of indoleamine 2,3-dioxygenase (IDO-1) in infection by M. tuberculosis and other intracellular pathogens**

One of the innate immune system’s responses to microbial infection is an interferon-γ (IFN-γ)-induced depletion of cellular tryptophan [30]. IFN-γ induces an isofrom of the host enzyme indoleamine 2,3-dioxygenase (IDO-1) that converts tryptophan to N-formylkynurenine, which is further metabolised to kynurenine. This degradative process is known as the kynurenine pathway, is the major route used for tryptophan breakdown in mammals [31], and when strongly induced by IFN-γ, is capable of depleting all of the detectable tryptophan in the lung to the limit of detection. This implies that suitable inhibitors of tryptophan biosynthesis may act as effective antibiotics, at least during the initiation of infection. However, it remained unproven from these data whether an established infection by biosynthetically intact wild-type bacteria could be effectively killed via this approach, as although tryptophan biosynthesis is required to establish infection, it may not be critical to maintain an existing or ongoing infection.

Patients with pulmonary TB show increased IDO-1 activity, decreased tryptophan concentration and increased kynurenine concentration in both serum [41] and pleural fluid [42], suggesting that infection with M. tuberculosis also triggers IFN-γ-induced tryptophan depletion, possibly in a lung-specific fashion. This implies that if the M. tuberculosis tryptophan biosynthetic pathway were to be inhibited, the increased activity of IDO-1 would cause M. tuberculosis cell death through tryptophan starvation, as was seen for F. novicida. However, as M. tuberculosis is prototrophic for tryptophan, elevated IDO-1 activity has little direct effect on the ability of the bacterium to replicate and cause disease. Indeed, IDO-1 activity itself has been used a predictor of death from M. tuberculosis infection [41]. It is possible then that the immunomodulatory effects of kynurenine may actually serve to increase the severity of pulmonary infection. It makes sense, therefore, to consider IDO-1 as a potential target for host-directed therapy against M. tuberculosis. Gautam et al. [43] used the IDO inhibitor D-1-methyl-tryptophan to test this idea in a non-human primate model of TB disease. They found that inhibition of IDO-1 reduced the bacterial burden and severity of disease and increased the proliferation of CD4+ and CD8+ memory T cells. Additionally, IDO-1 inhibition allowed remodelling of granulomas to occur, giving CD4+ T cells greater access to the necrotic, bacteria-rich central region of the granuloma, thus significantly increasing immune-mediated bacterial cell death.

**Inhibitors targeting tryptophan biosynthesis in M. tuberculosis**

Zhang et al. [44] used transposon insertion mutagenesis to confirm that an intact tryptophan biosynthetic pathway is necessary for M. tuberculosis to survive in immunocompetent mice, but not in immunodeficient CD4+ T-cell-deficient mice. They subsequently showed that a tryptophan auxotrophic strain of M. tuberculosis (produced by a deletion of the trpE gene) was susceptible to tryptophan starvation, both in vivo when grown in a defined medium,
and also by IFN-γ-induced, IDO-mediated tryptophan depletion in stimulated macrophages. The in vivo essentiality of tryptophan biosynthesis to M. tuberculosis thus makes the pathway a potential target for new antibiotics with a novel mode of action. Also in its favour is the lack of the equivalent biosynthetic pathway in mammals, which makes host toxicity less likely. These qualities have inspired a range of investigations into identifying inhibitors of the pathway that could potentially function as new antibiotics.

Anthranilate synthase is a member of the structurally conserved chorismate-utilising enzyme (CUE) family. Many inhibitors have been designed against CUE family members, and inhibitors designed against one family member often function against other family members [45], as the active sites of the enzymes are similar. However, almost none of them have achieved better than micromolar potency against their targets [46]. Consistent with this, isochorismate-like inhibitors designed to act against M. tuberculosis salicylate synthase [47–49] were also active against M. tuberculosis anthranilate synthase, albeit with reduced potency [17].

A series of substrate mimetic inhibitors of M. tuberculosis AnPRT have been synthesised [50–53]. AnPRT has a surprisingly complex substrate-binding pattern, with PRPP binding in the base of the active site cleft, and two molecules of anthranilate binding nearer the mouth [24,52]. The most effective of the inhibitors mimic the ability of the enzyme to bind anthranilate in multiple places in the active site cleft, but still only exhibit micromolar Kᵢ values and show only modest activity against mycobacterial cells [50].

Two derivatised anthranilates, fluorinated at the 5- or 6-positions on the ring, were shown to be effective against M. tuberculosis, both in axenic culture and, with more modest effect, when administered intravenously in a mouse model of TB [44]. The genome of an escape mutant strain showed that a mutation in the trpE gene results in a hypermorphic version of the enzyme that has lost its usual allosteric feedback inhibition [17], confirming tryptophan biosynthesis as the cellular target of these compounds. However, none of the four possible fluorinated anthranilate isomers act as inhibitors of M. tuberculosis AnPRT in vitro; rather they act as substrates, producing fluorinated versions of PRA, which are more stable than the parental compound. The fluorinated PRAs are substrates for E. coli PRAI and IGPS in vitro, resulting in the production of fluoro-indole-3-glycerol phosphate [51]. A subsequent metabolic tracing study [54] showed that anthranilates fluorinated at the 4-, 5- or 6-position are all metabolised by M. tuberculosis to the cognate fluorinated isomers of tryptophan, which subsequently cause cell killing when incorporated into proteins. This generally cytotoxic cellular effect of fluorinated tryptophan has previously been demonstrated in E. coli [55]. Hence, the fluoroanthranilates can be thought of as pro-drugs.

Two phenotypic screens of high-diversity chemical libraries independently identified inhibitors of M. tuberculosis tryptophan synthase that are active against the bacterium in culture (MIC₅₀ values in the 0.5–3 μM range) and also show moderate in vivo efficacy in both zebrafish and mouse models of TB [20,21,56]. Tryptophan synthase is a remarkable and complex enzyme. The α-subunit (TrpA) cleaves indole-3-glycerol phosphate (I3GP) into indole and glyceraldehyde 3-phosphate. The indole is then transported through a 25 Å-long channel to the active site of the β-subunit (TrPB), where it displaces the hydroxyl group of serine using pyridoxal 5’-phosphate (PLP) as cofactor. The identified inhibitors bind in the channel, at the interface between the two subunits, resulting in a complex and potent inhibition of the enzyme. The best characterised of the compounds discovered, BRD4592, was shown to be an uncompetitive inhibitor with respect to I3GP and to be a mainly non-competitive inhibitor with respect to serine. Additionally, BRD4592 promotes a slow allosteric change in the enzyme that traps tryptophan in the active site, effectively inducing product inhibition.

A phenotypic screen of a chemical fragment library identified indole propionic acid (IPA) as active against M. tuberculosis in culture (MIC₅₀ = 68 μM), and also as having modest effect against the bacterium in a mouse model of disease [57]. Intriguingly, IPA is a metabolite produced by several species of bacteria found in the normal gut biota, including species of Clostridium and Pectostreptococcus [58], that is detectable at a concentration of ~80 μM in the peripheral blood of mice whose gut flora contain these species [58]. IPA is an allosteric inhibitor of M. tuberculosis anthranilate synthase, and exerts its antibacterial effect by down-regulating the biosynthetic pathway and preventing the bacterium from making tryptophan [59]. (Interestingly, 5-fluorotryptophan, the metabolic end-product of 5-fluoranthranilate, is also an allosteric inhibitor of anthranilate synthase of similar potency.) The identification of IPA as an inhibitor of mycobacterial tryptophan synthesis is interesting in two respects: first as a starting point for the synthesis of synthetic allosteric inhibitors of the pathway, and secondly as an indication that the inhibition of the pathway may be a natural inter-bacterial competition strategy.
Future challenges to utilising tryptophan biosynthesis as a drug target in *M. tuberculosis*

There is now a clear body of data to show that tryptophan auxotroph strains of *M. tuberculosis* fail to establish infections [20,25,44], and that inhibitors of tryptophan synthesis show *in vivo* efficacy against mycobacterial infections in both zebrafish and mice [20,21]. Unlike many other bacteria, tryptophan synthesis is not controlled transcriptionally in mycobacteria [60], and they constitutively express the tryptophan biosynthetic genes, even in the presence of exogenous tryptophan [61]. However, tryptophan inhibits its own biosynthesis via allosteric feedback inhibition of anthranilate synthase: it has been variously estimated that the enzyme is 50% inhibited by a tryptophan concentration of 200 nM [44], 1.5 $\mu$M [59] or 6.3 $\mu$M [17]. Either way, this seems at odds with the reported 20 $\mu$M $K_{m}$ of mammalian IDO-1 [62]; if there is likely to be sufficient residual tryptophan available to inhibit its own synthesis, then the precise mechanism of the observed growth inhibition by exogenous compounds *in vivo* remains unclear. Given the diverse range of microenvironments encountered by *M. tuberculosis* during infection [63], the complete validation of tryptophan biosynthesis as a viable target for effective antibacterial compounds requires further experiments. It will be especially helpful to establish the true *in vivo* concentration of host tryptophan to which *M. tuberculosis* is exposed, the rate of uptake of tryptophan by the bacterial cell, and the relative affinities of transporter(s) involved. An additional challenge in designing drugs that inhibit bacterial tryptophan biosynthesis is the potentially complex results of interfering with tryptophan metabolism in the human gut microbiome, which has recently been recognised to have an important influence on the gut-brain axis, with an associated possible impact on a range of neurological conditions [64].

**Perspectives**

- **Highlight the importance of the field:** It has been recognised for several years that widespread antibiotic resistance has the potential to undermine modern medicine as we know it. TB is the world’s leading cause of death due to a single bacterial infection and the rise of multidrug resistance necessitates the development of new effective treatments that have novel mechanisms of action. Most antibiotics, including those in clinical use and those currently in development, redundantly target a limited range of cellular activities. Hence, the discovery that biosynthetic pathways for key cellular chemicals such as tryptophan are druggable targets, provides fresh avenues for the development of new classes of antibiotic.

- **Summary of the current thinking:** Genetic knockout strains of *M. tuberculosis* have confirmed that the pathway is essential for the bacterium to survive *in vivo* and cause disease in animal models. Inhibitors have been identified for several of the enzymes in the pathway, the most successful of which show good activity against *M. tuberculosis* cells and moderate activity against the bacterium in animal infection models. The most active compounds identified so far have been allosteric inhibitors of the first and final steps in the pathway.

- **Comment on future directions:** Much still needs to be learned about the biological availability of host tryptophan to *M. tuberculosis* at the various stages of disease progression, and whether the bacterium is able to circumvent inhibition of tryptophan biosynthesis by using environmental sources of the amino acid. This is made especially challenging by the range of both intracellular and extracellular microenvironments to which different bacterial subpopulations are exposed during the course of the disease.

**Competing Interests**

The author declares that there are no competing interests associated with the manuscript.
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Abbreviations
AnPRT, anthranilate phosphoribosyl transferase; AS, anthranilate synthase; CUE, chorismate-utilising enzyme; HIV, human immunodeficiency virus; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-γ; IGPS, indole-3-glycerol phosphate synthase; IPA, indole propionic acid; MDR, multidrug-resistant; MIC50, minimum inhibitory concentration required to inhibit the growth of 50% of bacteria; ORF, open reading frame; PLP, pyridoxal 5'-phosphate; PRA, phosphoribosyl-anthranilate; PRAI, phosphoribosyl-anthranilate isomerase; ProFAR, V{[5'-phosphoribosyl[formiminomino]-5-aminomimidazole-4-carboxamide ribonucleotide; PRPP, phosphoribosyl pyrophosphate; SCID, severe, combined immune-deficient; TB, tuberculosis; WHO, World Health Organisation; XDR, extensively drug-resistant.

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