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Digest

Mycobacterial tryptophan biosynthesis: A promising target for tuberculosis drug development?

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ABSTRACT

The biosynthetic pathways of amino acids are attractive targets for drug development against pathogens with an intracellular behavior like *M. tuberculosis* (*Mtb*). Indeed, while in the macrophages *Mtb* has restricted access to amino acids such as tryptophan (Trp). Auxotrophic *Mtb* strains, with mutations in the Trp biosynthetic pathway, showed reduced intracellular survival in cultured human and murine macrophages and failed to cause the disease in immunocompetent and immunocompromised mice. Herein we present recent efforts in the discovery of Trp biosynthesis inhibitors.

Introduction

Tuberculosis (TB) is one of the most significant causes of death worldwide from a single infectious agent; the World Health Organization (WHO) estimates that in 2017 1.3 million people died from TB and that there were 10.0 million new cases. Thanks to currently available therapies, mortality has been falling in recent years with estimated 53 million saved lives between 2000 and 2017. However, the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* (*Mtb*) strains is a major concern that might reverse these progresses. WHO estimates 558 000 new cases with resistance to rifampicin (RIF) (RR-TB) in 2017, of which 82% had MDR-TB, defined as TB that is resistant to isoniazid (INH) and RIF. 8.5% of MDR-TB cases had XDR-TB, classified as being resistant to INH and RIF in addition to any fluoroquinolone and injectable second-line drugs.[1,2] Therefore, to develop new drugs acting upon novel mechanisms of action is a high priority in the global health agenda.

The biosynthetic pathways of amino acids are attractive targets for drug development against pathogens with an intracellular behavior like *Mtb*. Indeed, while in the macrophages *Mtb* has restricted access to amino acids such as tryptophan (Trp). Auxotrophic *Mtb* strains, with mutations in the Trp biosynthetic pathway, showed reduced intracellular survival in cultured human and murine macrophages and failed to cause the disease in immunocompetent and immunocompromised mice.[3] Moreover, it has been demonstrated that one of the host immune response after *Mtb* infection, is Trp starvation driven by CD4 T cells.[4] In the case of Trp, macrophages express indoleamine 2,3-dioxygenase (IDO) that catabolizes Trp to kynureine and other metabolites. It has been demonstrated that IDO is one of the most induced genes in both human and mice macrophages infected with *Mtb*,[5,6] and high levels of IDO were detected in sputum samples in a cohort of TB patients.[5,7] These results combined with the fact that Trp is an essential amino-acid for humans highlight this pathway as an attractive target for TB drug development.

Anthranilate synthase (AS), a heterodimeric enzyme, catalyzes the first committed step in the Trp synthesis (Fig. 1) in *Mtb* converting chorismate, the final product of the shikimate pathway,[8] to anthranilate. AS comprises two functional components: AS-I (TrpE) that catalyzes the synthesis of anthranilate from chorismate and ammonia, and AS-II (TrpG) that provides ammonia. The two enzymes form a functional complex and AS-II provides ammonia from glutamine hydrolysis.[9–11] Tryptophan controls its own synthesis by inhibiting TrpE as a cooperative allosteric inhibitor.[9,12]

The biosynthetic pathway continues with the reaction between anthranilate and 5′-phosphoribosyl-1′-pyrophosphate (PRPP) to produce phosphoribosylanthranilate (PRA), through inversion of stereochemistry at the anomeric carbon.[13] This step is catalyzed by anthranilate phosphoribosyltransferase (AnPRT, TrpD). AnPRT is a homodimeric enzyme that requires Mg^{2+} for catalysis.[14,15] PRA undergoes isomerization to 1-(2-carboxy)phenylamino 1′-deoxyribulose-5′-phosphate (CdRP),[16] which is then converted to the product indole-3-glycerol phosphate (IGP) through a ring-closure reaction by releasing CO_2 and H_2O.[17] These two steps are catalyzed by TrpF and TrpC, respectively. The last two steps of the pathway are catalyzed by tryptophan synthase (TrpAB), a bi-enzyme complex consisting of two protein chains, α (TrpA) and β (TrpB) operating as a linear αββα

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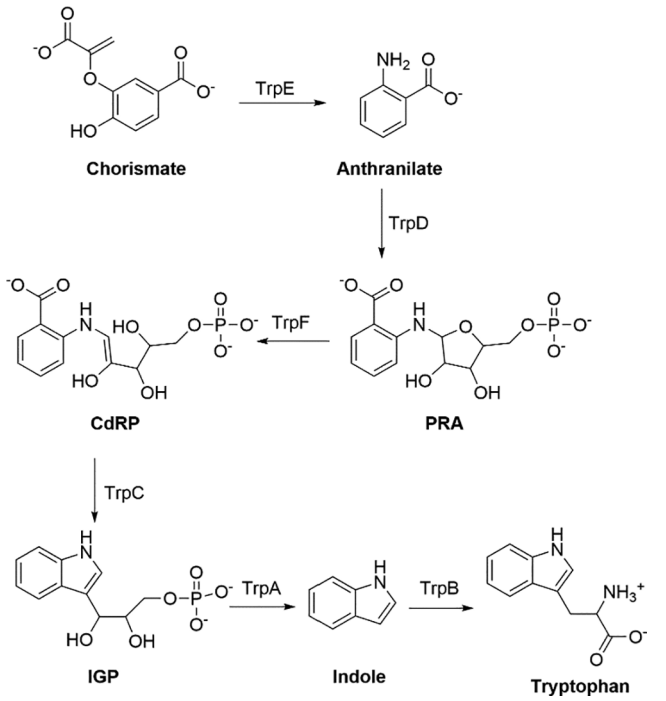


Fig. 1. Tryptophan biosynthetic pathway.

heterotetrameric complex.[18–20] TrpA converts IGP to indole and glyceraldehyde-3-phosphate while TrpB catalyzes the condensation of L-serine and indole to form L-tryptophan in a complicated multistep mechanism.[21]

Tryptophan biosynthesis inhibitors

The first inhibitor of this biosynthetic pathway was discovered by Zhang and collaborators while investigating the CD4 "counteractome", mycobacterial genetic requirements to survive the CD4 response.[4] Through an unbiased genetic screen employing a transposon insertion site-mapping method (TraSH),[22–24] authors found that genes involved in gluconeogenesis and Trp synthesis are present in mycobacteria in response to immune system of immunocompetent mice. To validate Trp biosynthesis as a target for drug discovery, authors tested a panel of anthranilate analogs against *Mtb* in the presence and absence of tryptophan and two compounds, 2-amino-5-fluorobenzoic acid (**5-FABA**) and 2-amino-6-fluorobenzoic acid (**6-FABA**) (Fig. 2), showed a MIC of 5 μM in liquid broth in the absence of Trp, while the addition of Trp to the medium rescued *Mtb* growth.

6-FABA and its ethyl ester also showed a significant reduction in bacterial load in infected mouse spleens (10-fold reduction respect to control). To determine the target of these compounds, resistant mutants were selected and the whole genome was sequenced; a mutation in the *trpE* gene was obtained (F68I). Cloned F68I mutant form of TrpE had a 3-fold increase in activity compared to the wild type TrpE, and it was 50-fold less sensitive to allosteric inhibition by Trp *in vitro*. These data suggested that F68I mutant is a hypermorphic enzyme that confers resistance to **6-FABA** rather than being the target. Later on, Islam et al. demonstrated that fluoro-anthranilates inhibit *Mtb* growth through the

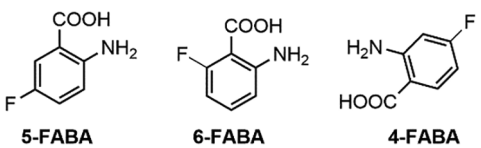


Fig. 2. Chemical structures of **5-FABA, 6-FABA** and **4-FABA**.

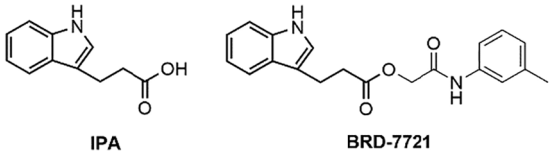


Fig. 3. Chemical structures of **IPA** and **BRD-7721**.

production of fluorinated Trp rather than inhibiting a specific enzyme in the pathway or causing accumulation of a toxic fluorinated intemediate.[25] Authors analyzed the metabolic profile of *Mtb* cells treated with fluoro-anthranilates (**6-FABA**, **5-FABA** and **4-FABA**, Fig. 2) by LC–MS experiments. Indeed, analysis of extracts did not reveal accumulation of any fluorinated intermediates in tryptophan synthesis, instead significant levels of fluoro-tryptophan were observed, suggesting that there was not an inhibition of the conversion of fluoro-anthranilate to fluoro-tryptophan. Addition of Trp to cultures treated with **6-FABA**, **5-FABA** and **4-FABA** rescued *Mtb* growth. Moreover, exposure to **5**- or **6-FABA** inhibited bacterial growth in a dose-dependent manner (growth inhibition at 25 μM for **5-FABA** and 50 μM for **6-FABA**).

Since the discovery of **6-FABA**, several other Trp biosynthesis inhibitors were discovered. Indole propionic acid (**IPA**, Fig. 3) is the first microbiome-derived metabolite active against TB, suggesting a functional link between human microbiota and tuberculosis.[26] This fragment hit is currently undergoing early clinical development studies for Friedrich's ataxia[27] and was identified by Negatu et al. through a whole-cell phenotypic screening of a fragment library.[28] Its whole-cell activity (MIC_{50} = 68 μM) and low cytotoxicity (CC_{50} > 1000 μM on HepG2 cells), as well as its favorable pharmacokinetic properties, made this fragment hit an ideal starting point for further hit-to-lead development. **IPA** was then selected for *in vivo* studies and proved to be well-tolerated in acute toxicity testing at 100 mg/kg. The pharmacokinetic profiling revealed an acceptable bioavailability (30–33%) and adequate plasma levels, above the MIC_{50} for more than 50% of the dosing interval. Unexpectedly, **IPA** was able to reduce the bacterial burden only in the spleen, with a 7-fold reduction compared to untreated animals. Possible explanations of this surprising organ-specific effect include a differential response to the specific microenvironments and differences in the immune response in the two organs, but a specific reason remains to be determined. However, the fact that a microbiota metabolite could exert anti TB activity raised a whole series of questions concerning the bacterial and host dynamics involved in the mechanism of action of **IPA** and its possible use as a coadjuvant to accelerate TB treatment. It has been recently reported that **IPA** exerts its activity by mimicking Trp and blocking its biosynthesis through inhibition of the TrpE allosteric binding site, consistent with the structural relationships between Trp and **IPA**.[29] Indeed, a high level cross-resistance to **IPA** was observed in fluoro-anthranilate resistant strains harboring mutations in the allosteric binding pocket of TrpE. Interestingly, two additional TrpE-independent mechanisms of resistance were identified through the selection of spontaneous **IPA**-resistant mutants: i) mutations in the Rv0880 transcriptional regulator, which also influences sensitivity to bedaquiline and is likely to be involved in general drug resistance; ii) missense mutations in a cytoplasmatic chorismate mutase. This enzyme catalyzes the synthesis of other aromatic amino acids by using the same substrate of TrpE and its reduced activity is likely to lower the vulnerability of Trp biosynthesis.

Very recently, an **IPA** ester (**BRD-7721**, Fig. 3) was identified by screening a library of 50.000 compounds against pools of strains depleted of essential bacterial targets.[30] Anti-mycobacterial activity of **BRD-7721** (MIC_{90} less than 64 μM) was abrogated upon supplementation of the culture media with tryptophan, confirming the Trp biosynthesis as the target. However, the sentinel gene aroused from the screen was *trpG* instead of *trpE*. Clearly further studies are needed to clarify if **IPA** and **BRD-7721** act by inhibiting the same enzyme.

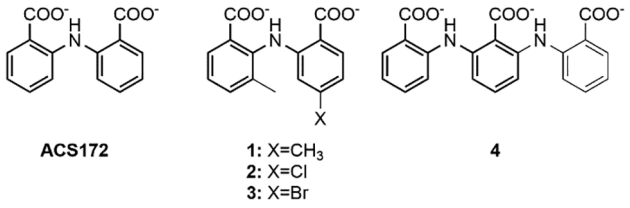


Fig. 4. Chemical structures of **ACS172** and compounds **1, 2, 3** and **4.**

Castell et al. discovered a bi-anthranilate hit compound, 2-(2-carboxyphenylamino)benzoate (**ACS172**, Fig. 4), by screening a library of 165 compounds from the Auckland Cancer Society Research Centre against TrpD.[31] Anthranilate-like compounds were the best inhibitory ones. Among them, **ACS172** demonstrated a competitive inhibition with respect to anthranilate with an inhibition constant of 1.5 ± 0 μM for *Mtb*-TrpD. The X-ray crystallography of *Mtb*-TrpD complexed with **ACS172** and PRPP showed that **ACS172** binding in the S2-S3 site of the enzyme mainly contributes to its inhibitory activity. Moreover, **ACS172** induced growth inhibition in a concentration-dependent manner from 25 to 600 μg/ml when tested against *M. marinum*, a close relative of *Mtb*. In search of more potent inhibitors, Evans et al. explored the structure-activity relationships (SARs) landscape around **ACS172** and prepared several analogues.[32] The first series of benzoic acid derivatives, in which the secondary amine was replaced with ether or thioether bridges, proved to be less potent highlighting the crucial role of the amine for binding to the enzyme. Indeed, the secondary amine group forms a bifurcated hydrogen bond to promote **ACS172** correct conformation in the binding site. Therefore, in the second series of compounds both the secondary amine and the carboxyl groups were left unchanged and **ACS172** scaffold underwent chemical modifications to one ring at each reasonable position. Modifications at position 4 of one ring bearing hydrophobic substituents like halogens or methyl group let to more potent inhibitors with respect to PRPP and similar activity with respect to anthranilate than **ACS172**. The enhancement of potency could be related to more extensive interactions with the residues in the S2-S3 binding site. The last series of compounds comprise 3′-methylated analogues of the most promising derivatives from the second series. Methylated compounds with 5-Me, 5-Cl and 5-Br substitutions (compounds **1**, **2** and **3**, Fig. 4) showed the best IC_{50}^{abs} values (6.8 ± 0.3, 5.8 ± 0.2 and 3.3 ± 0.2 μM respectively) and very low residual activity, suggesting a complete inhibition of *Mtb*-TrpD. The binding modes of the most encouraging analogues from the last series were evaluated by X-ray crystallography, observing that these derivatives bind in the S1-S2 site, instead of the partial occupancy occurring by **ACS172**. Authors suggested that enhancing the steric hindrance with the insertion of 3′-methyl group could induce the more twisted conformation of these compounds, allowing a deeply binding in the S1-S2 site in disfavour of the S2-S3 binding. According to these findings, an extended scaffold comprising three linked anthranilate-like groups was synthesised. Notably, the 2,6-bis-(2-carboxyphenylamino)benzoate (**4**) resulted as the best full inhibitor overall, with an IC_{50}^{abs} of 2.2 ± 0.1 μM and a residual activity of 6 ± 1%. The X-ray crystallography of *Mtb*-TrpD complexed with **4** revealed that the extended scaffold confers to the derivative the ability to bind both the sites S2-S3 and S1-S2, promoting the full occupancy of entire anthranilate binding site. None of these new compounds were evaluated for whole-cell activity.

The azetidine derivative **BRD4592** (Fig. 5) was the first chemical probe targeting TrpAB and was identified through the screening of the Broad Institute diversity-oriented synthetic (DOS) library against log-phase *Mtb* expressing Green Fluorescent Protein (GFP).[33] This compound contains 3 stereocenters, but only the *2R,3S,4R* stereoisomer was active, suggesting target specificity. The *in vitro* profile of this hit was encouraging, with a MIC_{90} in the micromolar both against *Mtb* (MIC_{90} = 3 μM) and clinical isolates, including resistant strains (MIC_{90} ranging from 1.6 to 3 μM), and a CC_{50} higher than 100 μM in HepG2 cells. Spontaneous mutants resistant to **BRD4592** all harbored mutations either in TrpA or TrpB subunits. In addition, the bactericidal activity was abrogated by supplementation of the medium with Trp, confirming that this compound interferes with Trp biosynthesis. The complex and multifaceted **BRD4592** mechanism of inhibition was extensively studied by combining biophysical and structural data. This compound is a mixed-type allosteric inhibitor which binds to a cavity located along the tunnel connecting α- and a β-subunits, stabilizing the interaction between the two and probably blocking the indole shuttling to the β-subunit. Even though the high intrinsic clearance (mouse liver microsomes intrinsic clearance (Cl_{int}) = 336.2 μl/min/mg) of this compound limited *in vivo* studies in mouse models, **BRD4592** proved to be efficacious not only in *Mtb*-infected macrophages (8-fold reduction of *Mtb* growth compared to control), but also in *M. marinum* infected zebrafish embryos (1.5 log reduction compared to control), proving to be active also in models lacking T cells and supporting the hypothesis that its biological activity might be independent of adaptive host immunity.

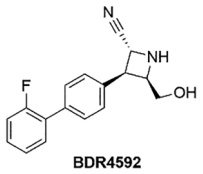


Fig. 5. Chemical structure of **BDR4592.**

Sulfolane **5** and the indoline-5-sulfonamides **6** and **7** (Fig. 6) have been identified by Abrahams et al. through a phenotypic screening aimed at finding novel anti-TB hits.[34] These compounds showed good potencies against *Mtb,* a very clean profile in HepG2 cells, and lipophilicity in the desired range, but suffered from high intrinsic clearance in mouse microsomal fractions (Table 1). While medicinal chemistry efforts to improve compounds **6** and **7** were unsuccessful, compound **5** optimization afforded the improved hits **8** and **9**, which were both progressed to *in vivo* studies (Table 1). Given its enhanced potency, compound **9** was selected despite the low metabolic stability in mouse microsomal fractions and administered subcutaneously to circumvent first pass metabolism.

Disappointingly, it did not show a significant reduction of colony forming units (CFU) in mouse lungs and further experiments would be needed to assess whether such a lack of efficacy could be due only to its lower half life. On the other hand, compound **8** was able to reduce CFU of 1.4 log at 350 mg/kg and emerged as an improved hit for future hit-

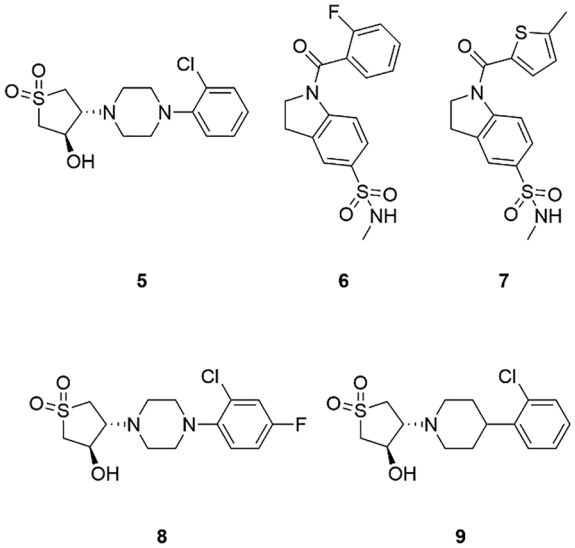
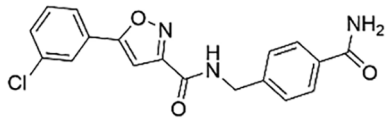


Fig. 6. Chemical structures of compounds **5–9.**

Table 1
Activity against *Mtb*, cytotoxicity against HepG2 cells, lipophilicity and clearance in liver microsomes of compounds **5–9**.

Compound	5	6	7	8	9
MIC H37Rv (μM)	0.76	5.6	1.1	2.25	0.5
HepG2 CC_{50} (μM)	> 50	> 100	> 100	> 100	> 100
ClogP	2.05	1.63	1.69	2.66	2.51
Cl_{int} (ml/min/g)	15.7	39.5	27.1	< 0.5	73.7



10

Fig. 7. Chemical structure of compound **10.**

to-lead development. Spontaneous resistors to **5**, **6** and **7** were then generated in a *M. bovis* BCG (BCG) model and subjected to whole genome sequencing (WGS), which revealed single nucleotide polymorphisms (SNPs) located both to *trpA* and *trpB*, suggesting these compounds act by inhibiting the whole TrpAB complex, rather than the individual subunits. The target was validated both by supplementation and overexpression experiments. Addition of Trp to the culture media restored bacterial growth, confirming that these compounds act by inhibiting Trp biosynthesis. The inhibitors were then evaluated in engineered BCG strains overexpressing components of the *trp* operon. Constructs containing the *trp* operon *(trpEA)*, *trpB*, *trpA* and the mutations *trpB*-N185S and *TrpA*-D64E were then generated by using an overexpression plasmid, being TrpA and TrpB mutations frequent in the sequenced spontaneous resistant mutants and involved in the subunit communication, respectively. Differently from the wild type individual subunits, overexpression of both the whole *trp* operon and the individual mutant subunits increased the MICs, supporting evidences from WGS and confirming that the two structurally distinct inhibitor series target the whole tryptophan synthase complex. TLC, native PAGE and gel filtration-HPLC data were then combined to probe the mechanism of action and prove that these inhibitors reduce the enzymatic activity and enhance the complex formation, probably by stabilizing one conformational state. Interestingly, crystallographic studies suggested that these compounds bind at the interface between the two subunits and might prevent indole shuttling, similarly to **BRD4592**.

Novel TrpA inhibitors were also identified through a structure-based virtual screening approach. Naz *et al.* virtually screened the eMolecules (> 6 million compounds) database against a homology model generated for the α-subunit of TrpA. Seven compounds, all bearing a benzamide moiety, had predicted binding energies below 40 kcal/mol and were selected for *in vitro* experimental validation. Compound **10** (Fig. 7) was the only one showing a considerable bactericidal activity (MIC = 6 μg/ml) and was subjected to molecular dynamic simulation to analyze its binding mode. Even though the stability of the **10**-α-subunit complex was predicted over the explored time, the effect of this compound both on the β-subunit and the whole complex was not studied and would be needed to probe its mechanism of action.[35]

Conclusions

Heads of State and government representatives from all United Nations (UN) member states, including those from high burden tuberculosis countries, met in September 2018 for the first UN High-Level Meeting (UNHLM) on tuberculosis and decided that ending tuberculosis is a global priority.[36,37] Unfortunately, finding new anti-TB drugs is challenging because regimens should be all-oral, more effective to shorter treatment durations, less toxic and safe if used together with other medicines. Combined efforts of researchers and pharmaceutical companies have let to the growth of the TB drug pipeline in recent years.[38] Bedaquiline,[39] which targets mycobacterial energy production, and delamanid,[40] which targets cell wall synthesis and energy production have been approved in 2012 and 2014, respectively. Other novel compounds such as the nitroimidazole pretomanid, the oxazolidinones delpazolid, the ethylenediamine SQ-109 and the benzothiazinone PBTZ169 are in phase II or phase III trials. While several repurposed drugs such as linezolid, fluoroquinolones, clofazimine and rifapentine are also being evaluated in phase II and phase III trials for TB.[38,41] Important challenges in anti-TB discovery are finding new compound classes that kill *Mtb* with novel mechanisms of action, rapid bactericidal activity, as well as activity against bacteria in different metabolic states without host toxicity. Although optimization of hits with multiparametric cell based activity might be challenging, the whole-cell based approach still remains predominant in TB drug discovery. Indeed, given the difficulties of translating enzymatic potency into anti-mycobacterial activity, phenotypic methods are the main source for active compounds in this field.[42] Surprisingly, most of the compounds discovered in the last few years repeatedly target cell wall (MmpL3[43,44], DprE1[45,46], FadD32[47,48] and Pks13[49]), while most of the approximately 625 essential *Mtb* genes are unexploited.

Targeting amino acid biosynthesis for TB drug development is still an under-explored field. *Mtb* has restricted access to many amino acids or their intermediary metabolites while in the macrophage phageosome,[4,50] and Trp is one of those. Zhang et al. clearly demonstrated that of course CD4 T cells are demanding in Trp bacterial starvation, but also that there might be other CD4-indipendent mechanisms of Trp starvation, suggesting that targeting this pathway would be effective in treating HIV-positive patients, who lack a normal CD4 compartment.[4] However, Trp is an essential amino acid for mammals present in many tissues.[51] Suzuki et al. demonstrated that patients with pulmonary TB showed significant increases in kyneurine concentrations and IDO activity and significant lower Trp concentrations (but only by about 30%) in serum.[52] Even if, differently from other bacteria *Mtb* synthesizes amino acids regardless of environmental availability,[53,54] the exact mechanism of bacterial growth inhibition by Trp starvation *in vivo* remains unclear especially since bacilli reside in different microenvironments generated in the tubercule lesions and granulomas[55].

Luckily, in the last few years several compounds inhibiting Trp biosynthesis have been discovered, confirming this as a druggable target, even if much still remains to do in order to obtain drug-like compounds. Mostly all the compounds reported in this review show low bactericidal activity and poor drug-like properties and need to be improved.

Acknowledgments

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