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***REVIEW ARTICLE***

# The Aspartate Metabolism Pathway: The Achilles' Heel of *Mycobacterium tuberculosis?*

**Michael Jonathan[1], Bobby P. Sulistyo [1*]**

*[1]Department of Biomedicine, Indonesia International Institute for Life-Sciences (i3L), Jakarta, Indonesia*

**corresponding author: bobby.sulistyo@i3l.ac.id*

**A B S T R A C T**

Globally, research has been conducted to find potential new avenues of drug discovery for treating tuberculosis infection. This endless 'arms race' is due to the bacteria's ability to develop resistance to already established antibiotic regimens. Various pathways within *Mycobacterium tuberculosis* are being extensively studied to open new possibilities in drug development, one of which is the aspartate metabolism pathway. This amino acid pathway has proven to be pivotal for the survival of *M. tuberculosis* both *in vitro* and *in vivo*. Furthermore, this pathway is absent in humans, making it a very promising candidate for further research and development in drug discovery. However, most inhibitors of this pathway discussed in this review only made it until the *in silico* stage, with only few being tested *in vitro* for their anti-tuberculosis activity. This review will discuss said attempts to suggest inhibitors that are effective against the critical enzymes that work within this pathway. The inhibitors reviewed in this paper are synthetic and derived from natural sources. The multitude of proposed inhibitors and the various enzymes that they are able to inhibit proves that this pathway has potential that is yet to be explored further.

**K E Y W O R D S**

*Mycobacterium tuberculosis; aspartate metabolism; anti-tuberculosis; drug discovery*

*H I G H L I G H T S*

- The aspartate metabolism pathway is important to the survival and pathogenesis of *M. tuberculosis.*
- This pathway is absent in humans, making it a promising drug target.
- Preliminary *in silico* data has shown that numerous targets within this metabolism pathway can be inhibited.
- However, there is a possibility of *M. tuberculosis* mutating to be resistant to these drugs.

## INTRODUCTION

*Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB). It is the deadliest human pathogen ever to exist, having killed more than one billion lives over the past two hundred years (Paulson, 2013), a feat that has only been recently thwarted by SARS-CoV-2, which became the leading infectious killer based on the global tuberculosis report by the WHO (2021).

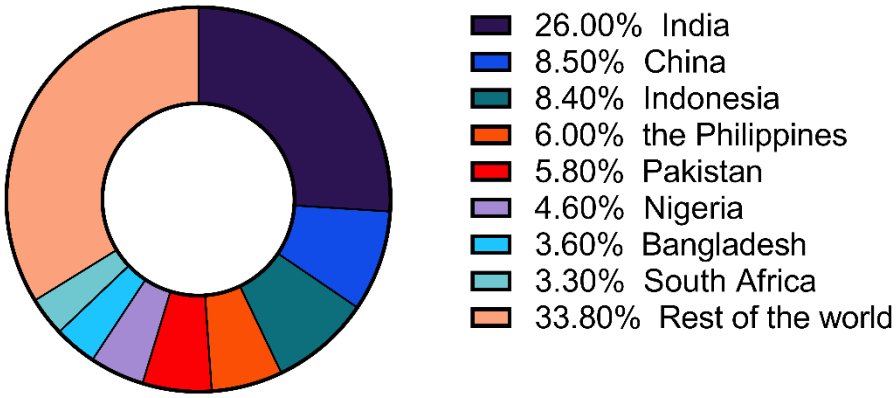


**Figure 1.** The Distribution of Case Burden of Tuberculosis in 2020. The annual TB report of 2021 reported the distribution of TB cases throughout the world and found that there were 8 countries that accounted for two-thirds of the TB cases worldwide.

According to the global tuberculosis report by the WHO (2021), 9.9 million people worldwide fell ill with TB in 2020 alone, with 1.3 million having died because of it. Based on the same annual report, Indonesia ranks second among eight countries that contributed to two-thirds of the total global cases of TB (Figure 1). Through the Indonesia National TB program, efforts are being made in Indonesia to eliminate TB by 2030 and eradicate it by 2050.

However, this program is met with several challenges, one of which is the appearance of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB strains (Sakamoto et al., 2019). In Indonesia, there were 24 000 cases of MDR-TB in 2019 (WHO, 2020), and the number of incidences has relatively been unchanged throughout the years. Drug resistance in *M. tuberculosis* has been observed since the discovery of the first antibiotic to treat TB, streptomycin (Pyle, 1947). Several drugs have been developed and used to treat TB infection, and *M. tuberculosis* has also developed resistance to these drugs, creating strains of *M. tuberculosis* that are resistant to one or more anti-TB drugs (Figure 2). Therefore, numerous kinds of research are being conducted to probe into other pathways that exist within *M. tuberculosis* that serve as possible foundations for the development of anti-tuberculosis drugs. One such example is the amino acid metabolism pathway of *M. tuberculosis*.

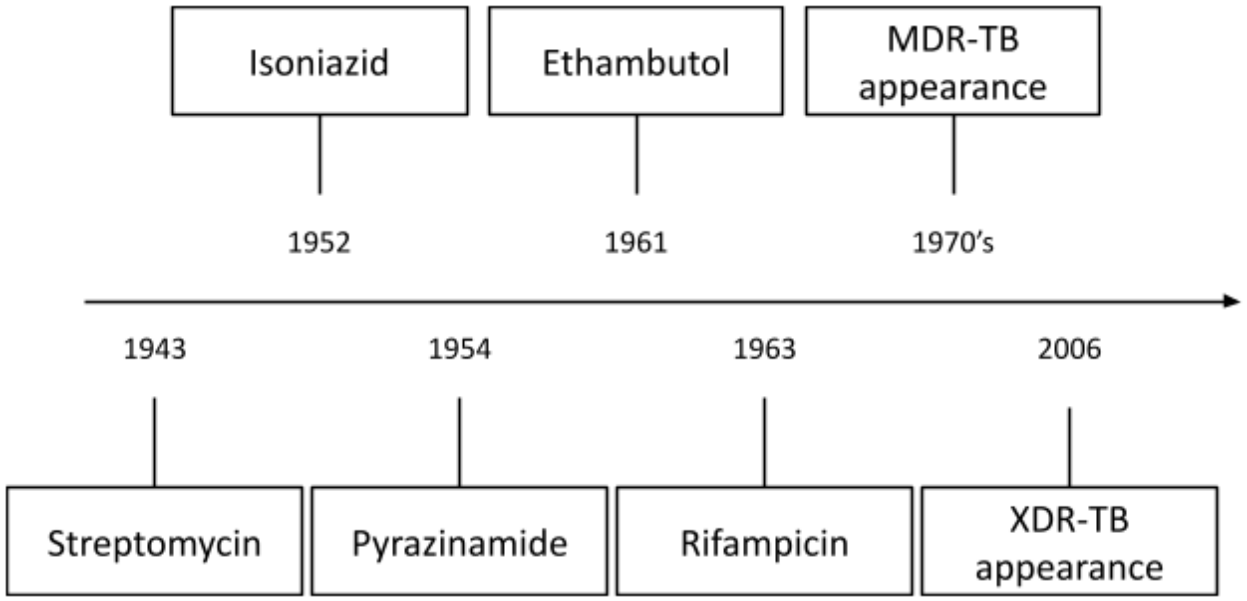


**Figure 2.** The History of TB drug development and appearance of drug-resistant TB. Adapted from Drapal and Fraser (2019) and Vasava et al. (2017).

The aim of this review article is to highlight and discuss one particular amino acid metabolism pathway of *M. tuberculosis*, the aspartate pathway. In particular, this review will discuss the various enzymes in this pathway that may become potential targets for future anti-TB treatment. In addition, this review will also discuss recent research that have suggested potential inhibitors of this pathway, both synthetic and natural. Finally, this review aims to increase awareness regarding the possible untapped potential of the aspartate pathway and pave the way forward for developing novel anti-tuberculosis drugs.

## THE ASPARTATE METABOLISM PATHWAY

The aspartate metabolism pathways is vital for the synthesis of several essential amino acids for *M. tuberculosis*: isoleucine, threonine, lysine, and methionine (Hasenoehrl et al., 2019). This pathway is a promising avenue for looking for potential drug targets since it is absent in humans (Viola, 2001). The process starts with oxaloacetate produced from the tricarboxylic acid (TCA) cycle (Figure 3), which is converted into aspartate by aspartate aminotransferase (rv3722c). Afterwards, aspartate kinase (ASK*)* converts aspartate into aspartyl-phosphate (AP). The third step is the conversion of AP into aspartate semialdehyde, the first of two intermediates that serve as a branch point in the pathway. Finally, it will be catabolized further to produce either methionine/threonine or lysine, depending on which enzyme catalyzes the reaction (Figure 3).

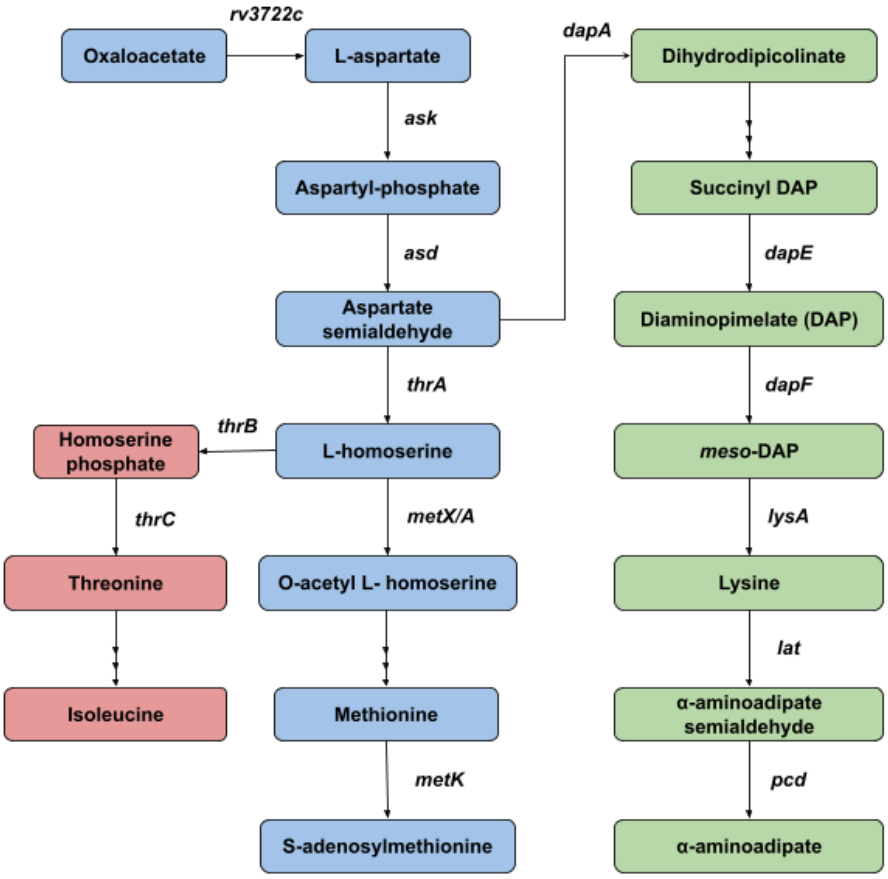


**Figure 3.** The Aspartate metabolism pathway. Oxaloacetate produced from the TCA cycle is converted into aspartate. Aspartate will then be broken down to produce threonine, isoleucine, methionine, and lysine.

Homoserine dehydrogenase (*thrA*) converts aspartate semialdehyde into L-homoserine, leading to the methionine/threonine biosynthesis pathway (Figure 3). L-homoserine serves as the second branch point

that will lead to either methionine biosynthesis or threonine biosynthesis. Homoserine kinase (*thrB*) will lead to the biosynthesis of threonine, whereas homoserine transacetylase (*metX/A*) will lead to the biosynthesis of methionine (Hasenoehrl et al., 2019). It is already well established that methionine is required for the initiation of protein translation since the start codon of mRNA encodes for it (Gualerzi et al., 2000). Therefore, the importance of this amino acid is indisputable. Downstream methionine is S-adenosyl-methionine (SAM), which is an essential cofactor for the methylation of DNA, RNA, proteins, and lipids (Parveen & Cornell, 2011). Specifically for bacteria that possess mycolic acids, SAM is an essential cofactor for the methylation of the mycolic acid cell wall (Daffé et al., 2017).

In *Escherichia coli*, three different isoforms of ASK can be inhibited by threonine, methionine, or lysine (Viola, 2001). However, it is apparently not the case for *M. tuberculosis*. Yang et al. (2011) found that the ASK of *M. tuberculosis* is only inhibited by threonine, which is further confirmed by Hasenoehrl et al. (2019). This means that threonine can be considered a vital feedback regulator for the whole aspartate pathway of *M. tuberculosis* on top of protein synthesis.

Coming back to the first branch, the enzymatic action of dihydrodipicolinate synthase (*dapA*) on aspartate semialdehyde leads to lysine metabolism since it converts aspartate semialdehyde into dihydrodipicolinate (Figure 3). Further catabolism processes ensue, which ends in the biosynthesis of lysine (Usha et al., 2016). A precursor for lysine, meso-DAP, is a crucial component for the peptidoglycan cell wall of *M. tuberculosis* (Pavelka & Jacobs, 1996; Meng et al., 2015). The degradation of lysine into α-aminoadipate semialdehyde catalyzed by Lysine ε-aminotransferase (LAT) is pivotal for the establishment of a persistent TB infection. The gene encoding for the enzyme LAT is overexpressed 40-fold in a latent TB infection (LTBI) model (Betts et al., 2002).

## TARGETS OF THE ASPARTATE PATHWAY

**ThrA & ThrB.** Homoserine dehydrogenase (ThrA) and Homoserine kinase (ThrB) are important for methionine and threonine biosynthesis. Hasenoehrl et al. (2019) created several mutants of *M. tuberculosis*. Some of these mutants had their *thrA* or *thrB* gene deleted. The mutants could not establish an infection in immunocompromised mice. Moreover, Hasenoehrl et al. (2019) also demonstrated that *M. tuberculosis* auxotrophic for threonine produced copious amounts of lysine and a catabolite, diaminopimelate (DAP). However, they also found that the mutants are able to respond to this by increasing their lysine degradation and export, suggesting that the bacteria have the ability to respond to this homeostatic imbalance. The deletion of *thrA and/or thrB* will impair the biosynthesis of isoleucine, methionine, and important intermediates such as SAM for *M. tuberculosis*. As previously established, methionine is important to initiate protein translation and threonine is important for the feedback regulation of the entire pathway. Hasenoehrl et al. (2019) demonstrated that the lack of threonine caused a major homeostatic imbalance in the form of lysine buildup, and only when threonine is supplemented will the lysine buildup be reversed. They further speculated that inhibition of threonine biosynthesis coupled with lysine degradation and export inhibition would enhance bactericidal activity.

**MetX/A.** The homoserine transacetylase of *M. tuberculosis* is predicted *in silico* to be highly ligandable (Chaton et al., 2019). Using FTMap algorithm, they managed to find numerous consensus sites (CS) that reside within the active site of MetX. CS denotes the location on the surface of a protein where small molecules are likely to bind. Therefore, numerous presences of CS in the active site substantiate the ligandibility of MetX, which is a promising first step as a target for drug discovery and development. In addition, Berney et al. (2015) found that the deletion of this gene is detrimental to the survival of *M. tuberculosis* because it resulted in the inability to proliferate in primary human macrophages, and an *in vitro* starvation led to the extraordinarily rapid killing. The rate of cell death of this methionine auxotroph is faster than if the bacteria were an auxotroph of tryptophan, pantothenate, leucine, or biotin. This denotes the

crucial role of methionine and SAM in *M. tuberculosis*. Unfortunately, no inhibitors of this enzyme have been discovered, synthesized, or tested. It is interesting to further probe into this as it is already established that this enzyme is both ligandable and pivotal for *M. tuberculosis.*

**Rv3722c.** This aspartate aminotransferase is demonstrated to be important for the survival of *M. tuberculosis.* Jansen et al. (2020) found that Rv3722c is the main aspartate aminotransferase in *M. tuberculosis* as they found no other unannotated aspartate aminotransferases. They also demonstrated that the downregulation of the gene responsible for this enzyme attenuates the virulence of *M. tuberculosis* in macrophages and mice. However, they manipulated the protein stability of Rv3722c by increasing the degradation of Rv3722c, which mimics the inhibition of this enzyme since it resulted in the same outcome of a reduced function. No inhibitors of this enzyme have been discovered nor tested against *M. tuberculosis*.

Anand and Chandra (2014) suggest that among many other drugs, tamoxifen can potentially be repurposed to treat *M. tuberculosis*. The drug is inferred to be able to target multiple proteins of *M. tuberculosis*, one of which is Rv3722c. Tamoxifen itself is a drug for the treatment of breast cancer. Tamoxifen is a nonsteroidal antiestrogen that will bind to estrogen receptors (ER) and prevent the proliferative actions of estrogen on the mammary epithelium, thereby preventing the proliferation of ER-positive breast cancer. The proposed mechanism of action of tamoxifen on *M. tuberculosis* is not yet understood. However, El Arbi et al. (2014) demonstrated that analogs of tamoxifen caused high efflux of $K^+$ and $Na^+$, thereby causing the loss of transmembrane potential and cell death. No other molecular mechanisms of tamoxifen have been elucidated to involve Rv3722c. In their study, they suggested their own approach to the application of polypharmacology in drug discovery. They probed into *M. tuberculosis* as an example of how their approach could be utilized, which led to the aforementioned results. This was further confirmed by the work done by Jang et al. (2015) who tested tamoxifen for its bacteriostatic activity against drug-susceptible, MDR-TB, and XDR-TB. They found that its minimum inhibitory concentration (MIC) in MDR and XDR TB were lower than isoniazid and rifampicin, two drugs that are used as first-line treatments for TB.

**Aspartate Aspartate-β-semialdehyde dehydrogenase** (**ASD).** It is an enzyme that reduces AP into aspartate semialdehyde (Figure 3) (Shafiani et al., 2005). Because the enzyme works upstream of the pathway, inhibiting this enzyme has proven to be fatal for the survival of *M. tuberculosis*. Meng et al. (2015) demonstrated that a reduced expression of ASD caused a marked reduction in cell wall materials and reduced capability in infecting macrophages compared to the wild-type. Khan et al. (2017) conducted mathematical modeling of the metabolic pathways of *M. tuberculosis*. They found that ASD, encoded by the gene *Asd*, has the highest priority in terms of vital *M. tuberculosis* functionality, further substantiating its importance in the survival of *M. tuberculosis*.

Be that as it may, specific inhibitors of ASD are not yet available. Therefore, Meng et al. (2015) used genetic manipulation to modulate the expression of ASD to study the physiological functions of ASD in *M. tuberculosis*. However, there are *in silico* studies that suggest compounds that can inhibit ASD, one of which is a study done by Khan et al. (2017). They conducted a molecular docking study and found that rosmarinic acid, curcumin, and huperzine A are capable of binding to the active site of ASD, signifying that they are potential inhibitors of ASD. Unfortunately, no studies were found that specifically tested the anti-tuberculosis activity of either of these compounds.

Using Autodock, it was found that rosmarinic acid was the finest ASD inhibitor because of its low free energy, followed by curcumin and huperzine A. This is good because lower free energy signifies a higher binding affinity (Du et al., 2016; Morris et al., 1998). Rosmarinic acid also underwent a high degree of hydrogen binding with ASD, second only to curcumin which formed the highest amount of hydrogen binding with ASD. In contrast, huperzine A formed the least amount of hydrogen bonds. A high degree of hydrogen bonding increases the binding affinity as it increases it by an order of magnitude per hydrogen bond formed (Schaeffer, 2008). Taken together, these further warrants *in vitro* testing to test their anti-TB capabilities.

Ajijur et al. (2021) sought compounds that can serve as a competitive inhibitor of ASD. They created a virtual combinatorial library containing a series of synthetic analogs of the substrate of ASD, which is AP. Their combinatorial library resulted in 6000 analogs of AP that were further screened and narrowed down into eight analogs that were then docked using Autodock. They found the two best candidates for ASD inhibitors that exhibited a very low free energy and a modest formation of hydrogen bonds. Both of these analogs were lower compared to the previous study that screened for natural compounds, which signifies a much higher binding affinity. However, these analogs formed fewer hydrogen bonds than the best candidate from the previous study, rosmarinic acid. It must be noted that none of these compounds were brought up for *in vitro* study. Therefore, the good results obtained from this study also warrant further testing on the supposed inhibitory activity.

**LAT.** One possible explanation why LAT is important for persistence might be explained in the study by Duan et al. (2016). They found that the expression of *lrpA,* a gene upstream of LAT, is lowered by deleting the LAT gene. *lrpA* encodes for a leucine-responsive regulatory protein that controls many genes during nutrient starvation and the transition to the stationary phase of *M.tuberculosis*, one of which is *lat* (Reddy et al., 2008). The expression of *lrpA* is proportional to the levels of guanosine tetraphosphate (ppGpp). ppGpp is important since it mediates various metabolic alterations in bacteria during a stringent response. Such metabolic alterations result in slowed growth rate and reduced levels of rRNA, tRNA, and protein synthesis. In addition, RNA polymerase activities are modified, the activity of transport systems is reduced, and the metabolism of carbohydrates, amino acids, and phospholipids is also decreased (Cashel et al., 1996). Therefore, ppGpp is important for bacterium to persist in an environment that is otherwise not favorable for them, and *M. tuberculosis* utilizes ppGpp to persist. Parthiban et al. (2016) investigated the degradation pathway of lysine on possible enzymes that can be inhibited. In particular, they designed, synthesized, and tested possible inhibitors for LAT. Out of 21 compounds they synthesized and tested, one compound named 2,20 -oxybis (N’ -(4-fluorobenzylidene) acetohydrazide) showed the most promising inhibitory capability.

This compound has a greater bactericidal capability than rifampicin and isoniazid in a nutrient-starved model of RAW 264.7 cells, a monocyte-macrophage cell line, to mimic a latent TB infection at an equal dose. They also tested the safety of this compound and found that the compound is relatively harmless to eukaryotic cells because the MIC of the compound is lower than the highest dose of the compound that is toxic to eukaryotic cells, further substantiating the benefit of the absence of the aspartate pathway in humans. Achieving a better anti-TB effect on latent TB infection on top of having a good safety profile is a promising trait since LTBI is a severe problem that must be tackled if this disease is to be eradicated.

## POSSIBLE CHALLENGES THAT MIGHT ARISE

*M. tuberculosis* can theoretically become resistant to treatments that attack the aspartate pathway, provided that the fitness cost is not too steep. Fitness cost is a comprehensive measure of the bacteria's ability to survive, reproduce, and undergo transmission (Zhan et al., 2020). It is mutating a particular protein that taxes the ability of *M. tuberculosis* to do the aforementioned processes. However, the fitness cost can be too steep for certain mutations to target, making the mutation lethal.

Therefore, it is important to investigate the fitness cost levied upon *M. tuberculosis* if any target of the aspartate pathway is mutated in response to an inhibitor. Zhan et al. (2020) also pointed out several publications have reported the capability of *M. tuberculosis* to mutate several of its genes to confer resistance to rifampicin, isoniazid, and ethambutol. Despite these mutations reducing the fitness of *M. tuberculosis*, it had developed compensatory mechanisms to negate the decreased fitness due to the mutation. Therefore, it is imperative to also consider whether the same will happen if any target of the aspartate pathway is inhibited.

## CONCLUSION

Recent research in the aspartate metabolism of *M. tuberculosis* has re-established this previously neglected metabolic pathway as a potential target for drug development due to its importance in the survival of acute and chronic *M. tuberculosis* both *in vitro* and *in vivo*. This review has established that the aspartate metabolism pathway is important for the synthesis of crucial amino acids, particularly threonine, methionine, and lysine, along with their intermediates. These products are pivotal for the pathogenesis and survival of *M. tuberculosis*, demonstrated by the incapability of establishing infection in immunocompromised mice. Numerous potential targets within the aspartate metabolism of *M. tuberculosis* exist, and preliminary results *in silico* have demonstrated that these targets could be inhibited. At the same time, some even are shown to have resulted in an antimycobacterial activity *in vitro*. The writers of this review hope that this small discussion could open up a new avenue in drug discovery for the treatment of *M. tuberculosis*.

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