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The role of Copper (II) in nitrogen metabolism in *Mycobacterium tuberculosis*

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†This work is dedicated to the memory of our dear colleague Professor Juliano Rosa de Menezes Vicenti, who passed away in 2022.

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ABSTRACT

In addition to carbon, nitrogen plays a fundamental role as a constituent of biomolecules such as amino acids, nucleotides, cell wall components and organic cofactors. Compared to carbon metabolism, the acquisition and assimilation processes are used by pathogens to sequester nitrogen from their recently emerged host. *Mycobacterium tuberculosis* can use multiple nitrogen sources for *in vitro* growth, including it appears to play the fundamental role in the alkalinization of the intramacrophagic environment ensuring the survival of the bacillus in the host during infection. In addition, copper (Cu), to be considered essential micronutrients has been proposed as an important metal in infection caused by *M. tuberculosis*, especially as a urease enzyme inhibitor. So, this study evaluated the nitrogen metabolism in the modulation of copper activity in growth kinetics of *M. tuberculosis* exposed to different nitrogen sources and the minimum inhibitory concentration of copper with and without the presence of these compounds. Considering the sources for the acquisition of nitrogen by *M. tuberculosis*, there was a change in the growth kinetics when exposed to glutamic acid, urea, L-arginine and ammonium sulfamate. In addition, glutamic acid and urea, despite being the preferred sources of nitrogen for the maintenance of the bacteria, modulate the antimicrobial action of Copper (II), suggesting competition for the site of action between them. In this way, the development of compounds, such as metallic ones, could be an interesting alternative in the development of new therapeutic alternatives with new mycobacterial targets, including against the latent bacillus.

O papel do Cobre (II) no metabolismo do nitrogênio em *Mycobacterium tuberculosis*

Palavras-chave:
ureia; cobre; antimicobacteriano; latente; tuberculose

RESUMO

Além do carbono, o nitrogênio desempenha um papel fundamental como constituinte de biomoléculas, como aminoácidos, nucleotídeos, componentes da parede celular e cofatores orgânicos. Em comparação com o metabolismo do carbono, os processos de aquisição e assimilação são usados por patógenos para sequestrar nitrogênio de seu hospedeiro recém-emergido. *Mycobacterium tuberculosis* pode usar múltiplas fontes de nitrogênio para o crescimento *in vitro*, inclusive parece desempenhar o papel fundamental na alcalinização do ambiente intramacrofágico, garantindo a sobrevivência do bacilo no hospedeiro durante a infecção. Além disso, o cobre (Cu), por ser considerado micronutriente essencial, tem sido proposto como metal importante na infecção causada pelo *M. tuberculosis*, especialmente como inibidor da enzima urease. Assim, este estudo avaliou a modulação da atividade do cobre na cinética do crescimento de *M. tuberculosis* exposto a diferentes fontes de nitrogênio e a concentração inibitória mínima de cobre com e sem a presença desses compostos. Considerando as fontes de aquisição de nitrogênio por *M. tuberculosis*, houve alteração na cinética de crescimento quando expostos ao ácido glutâmico, ureia, L-arginina e sulfamato de amônio. Além disso, o ácido glutâmico e a ureia, apesar de serem as fontes preferenciais de nitrogênio para a manutenção das bactérias, modulam a ação antimicrobiana do Cu (II), sugerindo competição pelo local de ação entre eles. Desta forma, o desenvolvimento de compostos, como os metálicos, poderia ser uma alternativa interessante no desenvolvimento de novas alternativas terapêuticas com novos alvos micobacterianos, inclusive contra o bacilo latente.

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1. Introduction

Mycobacterium tuberculosis is a prototrophic bacteria and can assimilate inorganic compounds as nitrogen sources for the synthesis of all its nitrogenous constituents. Like other intracellular pathogens, nitrogen metabolism is clearly central to the life cycle of *M. tuberculosis*, but the primary sources of nitrogen that are used by the bacteria *in vivo*, as well as the possible interaction between mycobacteria and nitrogen metabolism of the host, begin to be investigated more recently [1–6]. Nitrogen plays a fundamental role as a constituent of biomolecules such as amino acids, nucleotides, cell wall components and organic cofactors. In all living organisms, central nitrogen metabolism must be regulated to balance metabolite synthesis with absorption from exogenous sources and to ensure survival during nitrogen deprivation conditions [1].

Ammonium (NH^{4+}), in addition to being the main source for nitrogen acquisition in bacteria, is the first one incorporated to glutamate and glutamine, which function as primary nitrogen donors for the synthesis of nitrogen-containing molecules. [1–6]. Ammonium assimilation occurs mainly through the glutamine oxoglutarate aminotransferase pathway, while the glutamate dehydrogenase pathway is mainly catabolic and results in glutamate degradation [2,3]. In addition to ammonium, the use of nitrate in *M. tuberculosis* has been widely studied, especially in the context of its capacity to survive under hypoxia condition. Nitrates protect *M. tuberculosis* from hypoxic stress by replacing oxygen as the terminal electron acceptor in the respiratory chain [1,7].

Recently, urea has been shown to support the growth of *M. tuberculosis in vitro* when it is the unique nitrogen source, with a notable increase in urease activity under nitrogen-limiting conditions in vitro. Urease catalyzes the hydrolysis of urea, resulting in the formation of ammonia, which can be used by the bacteria as a source for nitrogen assimilation and synthesis of essential compounds, such as amino acids [1,2,8].

Together, these data show that *M. tuberculosis* can use multiple nitrogen sources for *in vitro* growth, but amino acids appear to be the most efficient source. A well-known example of an amino acid that strongly affects host interactions with intracellular pathogens is arginine. Arginine is used as a nutrient by pathogens, but it is also used by phagocytic cells as a substrate for Inducible nitric oxide synthase (iNOS) to generate nitric oxide [4].

In addition, metals such as copper (Cu), in addition to being considered essential micronutrients for mammals, have been proposed as important in host immune system mechanisms that help fight bacterial pathogens, due to the interaction between free Cu(I) and peroxide hydrogen, making this a very important metal in infection caused by *M. tuberculosis*, for example. During *M. tuberculosis* infection, infected macrophages trigger an oxidative burst that releases hydrogen peroxide, which diffuses through the bacterial membranes. In addition, *M. tuberculosis* infection induces the production of interferon-gamma (IFN-γ), which causes the expression of copper transporters in macrophages that direct copper to phagosomes containing the bacteria. Furthermore, copper contributes to the defense of the immune system by increasing the production of hydroxyl radicals from hydrogen peroxide, which are highly reactive and toxic to the bacteria [9].

In this sense, recent studies have pointed out that the activity of metallic compounds containing copper could be associated with a series of mechanisms, among which we highlight the action of urea amidohydrolases (ureases), which have been widely distributed among bacteria [8,10–12]. In *M. tuberculosis*, this enzyme plays a fundamental role in the alkalinization of the intramacrophagic environment and acts as a source of nitrogen that guarantees the survival of the bacillus in the intramacrophagic environment during infection [13]. Therefore, urease inhibitors, such as metals, have been proposed as promising antimicrobials for the treatment of bacterial infections that use this

enzyme in host-pathogen relationships in some essential metabolic route, in addition to the synthesis of mycobacterial nitrogen itself [11,12,14]. This study evaluated nitrogen metabolism using organic and inorganic substrates in the modulation of copper activity against *M. tuberculosis.* Thus, a deeper understanding of the aspects of *M. tuberculosis* related to copper in nitrogen metabolism may contribute to the identification of new therapeutic alternatives and targets, aiming to improve the treatment of tuberculosis and mitigate the impacts and global problems related to infections caused by this bacterium.

2. Material and Methods

1.1 Strain and Copper Prepare

Mycobacterium tuberculosis $H_{37}Rv$ (ATCC 27294), from the bank of Núcleo de Pesquisa em Microbiologia Médica (NUPEMM) - Faculdade de Medicina of Universidade Federal do Rio Grande – FURG, was cultivated on Ogawa-Kudoh medium for up to 21 days, at 37 ºC in aerobiosis and, salt of copper (II) (Sigma-Aldrich, St. Louis, Mo) chloride dihydrate was diluted in 5 mL of water for the experiments.

2.2 Growth media

The liquid media used for *M. tuberculosis* growth were: (a) commercially available MiddleBrook 7H9 media (**BD** Difco™) enriched with 10% OADC (oleic acid, dextrose, catalase; **BD** BBL™); (b) Sauton Minimal medium (4 g/L L-asparagine, 0.5 g/L magnesium sulfate, 0.5 g/L dipotassium phosphate, 2 g/L citric acid, 0.05 g/L ferric ammonium citrate. pH 7.0; Labsynth) supplemented with 6% glycerol (v/v), 0,1% ZnSo4 and 0,1% de albumin (Labsynth). Separately, the Sauton Minimal medium was supplemented with nitrogen source (Labsynth)of interest: urea (3.5 mM and 71.4 mM), glutamic acid (0.05%), ammonium sulfate (0.05%), L-arginine (3.5 mM).

2.3 Source of nitrogen by *M. tuberculosis*

To study the preferable source of nitrogen by *M. tuberculosis*, from the culture of the pan-susceptible *M. tuberculosis* $H_{37}Rv$ strain in Ogawa-Kudoh, a cell suspension was prepared according to scale 1 Mc Farland (3×10^8 CFU/mL), and from this suspension, the inoculum was prepared at a ratio of 1:20 in each medium tested, and 100 µL of bacterial inoculum was added to each well of a 96-well microplate [1]. Controls of bacterial growth in 7H9 medium with OADC and sterility were also included. The microplate was incubated at 37 °C, and the results were obtained, in triplicates, measuring the optical density at 600 nm, at the following times: from zero to 192 hours (8 days).

2.4 Antimycobacterial activity of Cu(II)

The minimum inhibitory concentration (MIC) of copper was determined by the Resazurin Microtiter Assay (REMA) [15]. In a 96-well microplate, 100 µL of Cu (II) were diluted (1:2) in 100 µL Middlebrook 7H9 media enriched with 10% OADC (oleic acid, dextrose, catalase), to obtain a final concentration of 200 – 12.5 µg/mL. After the serial dilution, 100 µl of inoculum were added to the test wells. The plate included positive (media plus inoculum) and negative (media only) controls. After seven days of incubation at 37 °C, the optical density was measured for 2 cycles, 25 flashes, 20 µs integration time and gain optimal (100%) at 37 °C (Ex 530 nm/ Em 580 nm), using the Infinite F200

fluorometer (Tecan). MIC was defined as the lowest concentration of a compound capable of inhibiting bacterial growth, as proposed by Halicki et al., 2021 [16].

2.5 Modulation fator

The effect of sources of nitrogen on the minimal inhibitory concentration of Cu (II) against *M. tuberculosis* was determined in a 96-well plate, add 100 µl of the medium in each test well and 100 µl of Cu (II), and perform the serial microdilution (1:2). After the serial dilution, 100 µl of each inoculum (with different medium preparation according to the source of nitrogen tested) were added to the test wells and incubated at 37°C in aerobiosis for seven days. After these, the optical density was measured (Ex 530 nm/ Em 580 nm), using the Infinite F200 fluorometer (Tecan) and the effect of sources of nitrogen in the Cu (II) or the possible competition of the active site, was determined by the modification in MIC values of the metal when exposed to the different substrates presents in culture medium.

2.6 Processing and analysis of data

The experiments were performed in triplicate, and the data were processed according to mean values and standard deviation. For statistical analysis was used one-way ANOVA, followed by Bonferroni's Multiple Comparison Test. Values of $p<0.05$ were considered statistically significant. The graphics were constructed using the software GraphPad Prism 5.00.

3. Results

Considering the sources for nitrogen acquisition by *M. tuberculosis* previously described, different substrates added to a minimal medium were evaluated and made available to the bacteria through aerobic incubation for up to 8 days of exposure. Compared to Sauton medium, a poor medium, all nitrogen sources evaluated influence *M. tuberculosis* at some point on the microorganism's growth curve.

We emphasize, however, that glutamic acid seems to positively influence ($p<0.001$) the kinetics of *M. tuberculosis* at all times evaluated. Interestingly, ammonium sulfamate ($p<0.05$) and arginine reduced growth by up to 68 and 80%, respectively, at the end of 8 days of exposure (Figure 1).

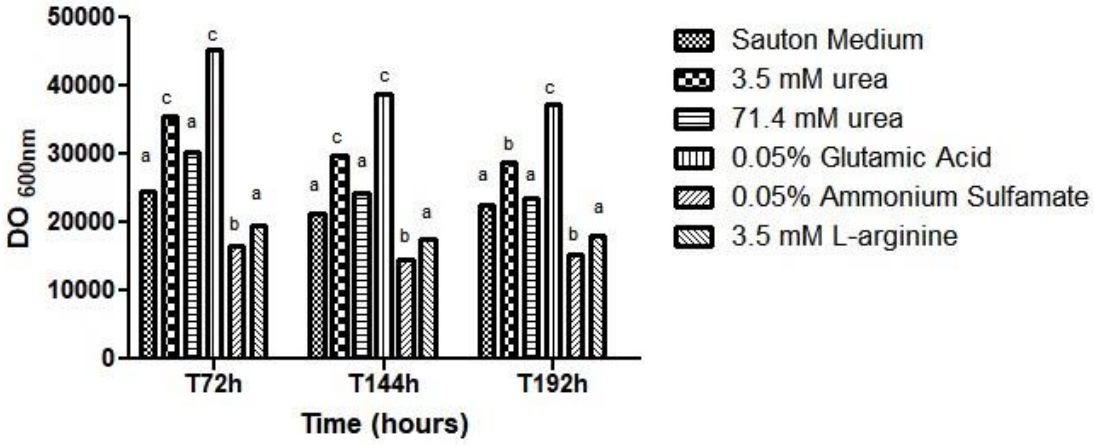

Figure 1 - *M. tuberculosis* growth (expressed by optical density 600nm) under different nitrogen sources: Sauton Minimal medium; 3.5 mM and 71.4 mM urea; 0.05% glutamic acid and ammonium sulfamate; and 3.5 mM L-arginine. Different letters indicate statistically significant differences: a, no difference; b, $p< 0.05$. c, $p< 0.01$).

Therefore, considering urea as one of the preferred substrates and that metals play an important role as inhibitors of this enzyme, the antimycobacterial activity of Cu(II) against $H_{37}Rv$ was evaluated, using the method recommended for evaluating new anti-TB compounds, which uses Middlebrook 7H9 plus OADC as a culture medium, establishing a concentration of 12.5 µg/mL as the minimum concentration capable of inhibiting the growth of the bacillus.

We also emphasize that there is a statistically significant difference ($p<0.05$) between the control and the treatment with 12.5 µg/mL of Cu(II), after seven days of incubation, and, therefore, evaluating the different concentrations of this compound in the seventh day of the experiment, it can be seen that the higher the concentration, the greater the antimicrobial activity of Cu(II) against *M. tuberculosis* (Figure 2).

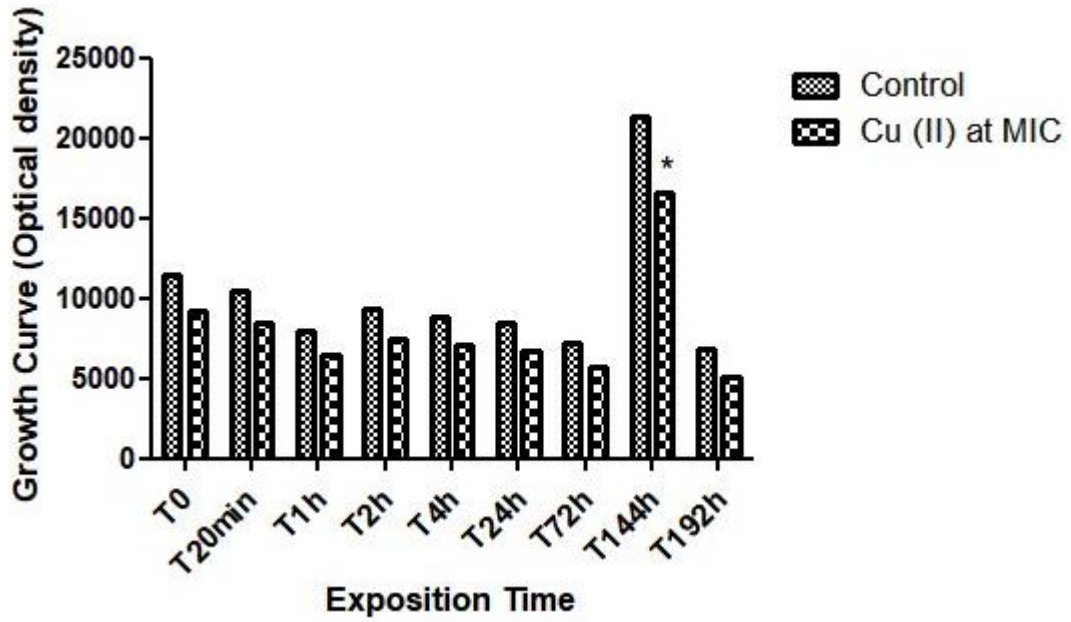

Figure 2 - Antimycobacterial activity of Cu(II) against *M. tuberculosis* after seven days of exposure. Control (without Cu^{2+}); treatment with Cu^{2+} at MIC concentration (12.5 µg/mL). * indicate significant differences between groups ($p<0.001$).

Then, to assess the role of this antimicrobial agent in nitrogen metabolism in *M. tuberculosis*, we exposed the microorganism to copper MIC in different media with different nitrogen sources, and on the 7th day (when there was significant antimicrobial activity in the REMA assays, Figure 3) and verified that in Sauton medium, ammonium sulfamate and L-arginine, the Cu(II) presented antimicrobial activity similar to that found by REMA, where we used a complex medium containing ammonium sulfate as the main nitrogen source.

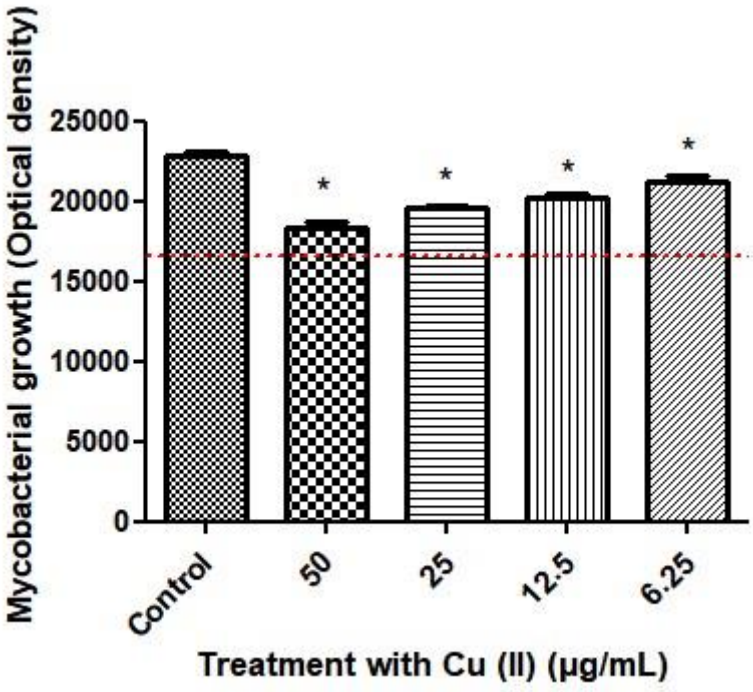

Figure 3 - Modulating activity of different mycobacterial nitrogen sources (Sauton Minimal medium; 3.5 mM and 71.4 mM urea; 0.05% glutamic acid and ammonium sulfamate; and 3.5 mM L-arginine) on the susceptibility profile of *M. tuberculosis* exposed to Cu(II). Red line: MIC of Cu(II) against *M. tuberculosis* according to the REMA method, proposed for the development of new anti-TB, using Middlebrook 7H9 medium supplemented with OADC [15]. *indicate significant differences between groups ($p<0.05$).

Thus, the treatments with ammonium sulfamate and L-arginine maintained the antimicrobial activity of Cu(II), reducing bacillus growth by 1.3 x and 1.2 x when compared to the control (7H9 + OADC medium) and in 1.5 x and 1.3x when compared to Sauton medium (Figure 3).

On the other hand, in assays using urea (3.5 and 71.4 mM) and glutamic acid, the main nitrogen sources used by *M. tuberculosis* in previous assays, Cu(II) seems to lose its antimicrobial action (Figure 3). Especially considering the MIC of Cu(II) in the treatment with glutamic acid, at all times of exposure, in which there is a statistically significant increase in mycobacterial growth ($p<0.05$), indicating that there may be a competition between the metal ion and glutamic acid, for example, by the same site of action.

4. Discussion

Our results first demonstrate that the presence of glutamic acid favored the growth of *M. tuberculosis in vitro* more efficiently than the other substrates evaluated, while

ammonium sulfamate and arginine promoted a marked reduction in the growth of the bacillus. Recently, Agapova et al. (2019), showed that *M. tuberculosis* can capture exogenous amino acids, changing their intracellular concentration, however, they highlighted that other amino acids than arginine (glutamate, aspartate, asparagine and glutamine), which were not evaluated in our study, are the preferred source of nitrogen, even surpassing ammonia itself.

Considering urea as a source of bacterial nitrogen, an inversely proportional relationship between the concentration of the compound and the growth of *M. tuberculosis* was evidenced. The dose of 3.5 mM urea promoted a 20% greater growth than the concentration of 71.4 mM, mainly on the seventh day when the microorganism appears to be at the peak of logarithmic growth. Corroborating the results obtained, Lin et al. (2012) identified that the exogenous concentration of 71.4 mM urea did not result in an increase in growth compared to lower concentrations, however, significant levels of ammonia were observed, reinforcing evidence that under nitrogen-limiting conditions *M. tuberculosis* positively regulates urea hydrolysis and ammonia production. Furthermore, possibly the concentration of 3.5 mM urea could also contribute to the maintenance of the bacillus in a hypoxic-acid *in vitro* model, which is associated with the alkalinization effect of the medium and the intense activity of the urease enzyme.

Thus, in our study, the preferential route of *M. tuberculosis* via urea hydrolysis, catalyzed by urease, was evident, since glutamic acid and urea were the substrates that most favored the growth of the bacillus when compared to the control. In addition, it is noteworthy that the conversion of ammonia into L-glutamic acid, an essential amino acid, has been reported as a limiting step in growth rates *M. tuberculosis* [1].

Understanding the different metabolic pathways in *M. tuberculosis* during infection has been critical to developing new therapeutic strategies, and urea has been a target, recently studied for this purpose [17]. In this sense, many studies have proposed which metals have the ability to interact with these enzymes, especially transition metals, such as copper [10,18]. In 2020, our group demonstrated, through *in silico* tests, that metallic compounds presented an important molecular interaction, using the UreA protein (PDB ID 2FVH), from *M. tuberculosis*, and could therefore be considered an interesting target in the search for new compounds with antimycobacterial activity [10].

Copper is an essential element for a wide variety of cellular metabolic processes, being involved in processes related to its redox catalysis capacity or as a carrier of oxygen, especially in the enzyme urea amidohydrolases. Furthermore, previous studies describe that the copper ion could polymerize proteins leading to a reduction in their enzymatic activity, and in the case of ureases, it could be attributed to the blocking of the thiol group in the active site or by binding to its histidine residues [17, 19].

In these sense, when evaluating the antimicrobial activity of Cu(II) against the bacillus using the culture medium supplemented with different sources of nitrogen, maintenance or increase (MIC reduction) of the antimicrobial action of the metal can be observed, however, it seems there was competition for the same molecular target between Cu(II) and urea or glutamic acid, since the presence of these substrates negatively modulated the antimycobacterial action of the evaluated metal.

Previously, Coelho et al. (2020) demonstrated the absence of cytotoxicity of Cu(II) in macrophage cells of the J774A.1 lineage (IC50 > 200 µg/mL), which further corroborates our findings, since even at 12.5 µg/mL, where the compound has pronounced antimycobacterial activity, it maintains the absence of cytotoxicity, maintaining pharmacological safety, with a selectivity index (IC_{50}/MIC) ≥ 16.

Considering the biosynthetic nitrogen route proposed by Gouzy et al. (2014) for *M. tuberculosis*, we can infer that Cu(II) acts both in the early stages of urea uptake and in

the conversion to glutamate, probably due to the inhibitory activity of glutamate dehydrogenase, but also in the conversion of glutamate itself into glutamine, acting on glutamine synthetase, thus inhibiting an important nitrogen acquisition mechanism and inhibiting the growth of the bacillus by depleting cellular nitrogen.

We also emphasize that the mycobacterial nitrogen cycle is essential for the maintenance of the microorganism intramacrophagically, since intracellular *M. tuberculosis* accesses several amino acids that act as important nitrogen donors and uses an important enzymatic apparatus for survival under adverse conditions in regarding environmental acidity and hypoxia, for example, and this could be an important target for the development of new antimicrobials [5].

Different nitrogen sources, such as glutamic acid, ammonium sulfate, and arginine, have been shown to influence the growth of *M. tuberculosis*, and copper has shown inhibitory potential for bacterial growth, suggesting a possible interaction between the different nitrogen sources and copper, evidencing the potential of copper as an antimicrobial agent against *M. tuberculosis*. Therefore, the use of copper-based metallic compounds would favor not only the rational design of compounds with bioactivity against the free-living bacillus, but also latency, one of the main goals proposed by the Stop TB strategy in 2015.

5. Conclusion

Considering that *M. tuberculosis* is a flexible bacterium that is able to metabolize a wide variety of sources for growth, through these analyzes we hypothesize that the bacillus uses glutamic acid as its main source of immediate assimilation, which is an essential amino acid for its metabolism, and that the action of the urease enzyme could further favor bacterial growth since, in addition to using the ammonia itself as a substrate, it can, through the hydrolysis of urea, generate more ammonia and consequently favoring the microorganism with different sources of nitrogen. Furthermore, the antimicrobial activity of Cu(II) seems to be related to the urea cycle, which has been involved in bacterial metabolism, including during intramacrophagic infection.

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