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Discovery of OJT008 as a Novel Inhibitor of Mycobacterium tuberculosis

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DISCOVERY OF OJT008 AS A NOVEL INHIBITOR OF *MYCOBACTERIUM TUBERCULOSIS*

DISSERTATION

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By

Collins Chidi Onyenaka, B. Pharm.

Texas Southern University, 2022.

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Despite recent progress in the diagnosis of Tuberculosis (TB), the chemotherapeutic management of TB is still challenging. *Mycobacterium tuberculosis* (*Mtb*) is the etiological agent of TB, and TB is classified as the 13th leading cause of death globally [WHO 2021]. 558,000 people were reported to develop multi-drug resistant TB globally [WHO 2020]. Our research focuses on targeting Methionine Aminopeptidase (MetAP), an essential protein for the viability of Mtb. MetAP is a metalloprotease that catalyzes the removal of N-terminal methionine (NME) during translation of protein [Giglione et al., 2003]. This essential role of MetAPs makes this enzyme an auspicious target for the development of novel therapeutic agents for the treatment of TB. Mtb possesses two MetAP1 isoforms: MtMetAP1a and MtMetAP1c, which are vital for Mtb viability, hence a promising chemotherapeutic target for Mtb infection [Zhang et al., 2009; Olaleye et al., 2010; Griffin et al., 2011; Vanunu et al., 2019]. In our study, we cloned, overexpressed recombinant MtMetAP1c, and investigated the *in vitro* inhibitory effect of OJT008 on cobalt and nickel ion activated MtMetAP1c. The compound's potency against replicating and multidrug-resistant (MDR) Mtb strains was also investigated. The induction of the

overexpressed recombinant MtMetAP1c was optimized at hours with a final concentration of 1mM Isopropyl β-D-1-thiogalactopyranoside. The average yield for MtMetAP1c was 4.65 mg/L of Escherichia coli culture. A preliminary MtMetAP1c metal dependency screen showed optimum activation with nickel and cobalt ions at 100μM. The half-maximal inhibitory concentration (IC_{50}) values of OJT008 against MtMetAP1c activated with $CoCl_2$ and $NiCl_2$ were in the micromolar range. Our *in silico* study showed OJT008 strongly binds to both metal activated MtMetAP1c, as evidenced by strong molecular interactions and higher binding score thereby corroborating our result. Thus, validating the pharmacophore's metal specificity. The potency of OJT008 against both active and multidrug-resistant (MDR) *Mtb* was in the low micromolar concentrations, correlating well with our biochemical data on MtMetAP1c inhibition. These results suggest that OJT008 is a potential lead compound for the pre-clinical development of novel small molecules for the therapeutic management of TB.

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DEDICATION

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CHAPTER 1

INTRODUCTION

1.1 Tuberculosis disease

Tuberculosis (TB) is documented as one of the oldest infections [Tornheim et al., 2019] known to have affected humans for thousands of years [Hershkovitz et al., 2015]. The etiological agent for TB is *Mycobacterium tuberculosis* (*Mtb*) [Gordon et al., 2018, WHO, 2021]. The etiology of the disease was unknown until March 24, 1884, when Dr. Robert Koch reported the discovery of the bacillus name, *Mtb* [Sakula et al.,1982]. The pathogen is characterized by a microscopic appearance in tissues as caseating granulomas. TB may affect the lungs (pulmonary TB) and, in some cases, other parts of the body (extrapulmonary TB) [Lee et al., 2015].

The *Mtb* genus is aerobic, majorly non-motile, and the morphology of the colony formed varies in different species ranging from pigmented to non-pigmented, rough to smooth [Pfyffer et al., 2015]. It has a distinguishable cell wall from a preponderance of prokaryotes and shares prominent features with gram-negative bacteria, albeit a gram-positive organism [Minnikin et al., 1991; Brennan., & Nikaido., 1995]. The primary constituent of the cell wall is the peptidoglycan consisting primarily of N-acetylmuramic acid and N-acetylglucosa

mine alternating polymers [Brennan., & Nikaido., 1995]. About 90% of the world's population infected with TB are adults, of which the predominant sex is the male gender [WHO, 2021]. *Mtb* transmission is airborne, and the droplet nuclei, diameter 1–5 μm, containing *Mtb* from the sneeze or cough of Infectious patients and through complement factors bind to the alveolar macrophage cell wall of the host, reducing the acidity of the cell wall and phagosome of its host cells. As a result, the phagosome maturation is impeded, and this promotes *Mtb* bacilli multiplication and eruption, thereby propagating infection in the host [Raviglione et al., 2018]. There are 2 types of TB conditions: active TB and latent TB. Active TB is characterized by a multiorgan disease condition caused by either reactivation of TB from its latent state, latent TB (LTB) condition, or through *Mtb* primary infection. Inhaled *Mtb* can be killed by the host alveolar macrophage of the host; however, in an immunocompromised state, the host becomes infected with active TB. It affects mainly the lungs- pulmonary TB, but can also affect other parts of the body, extrapulmonary TB, which includes the musculoskeletal system, reproductive system, gastrointestinal system, lymphoreticular system, the liver, and skin [MacPhersonet al., 2020]. The World Health Organization (WHO) reported 10 million people were infected with active tuberculosis in 2020 [WHO, 2021]. Between 5 - 10% of *Mtb* infected patients develop active TB within 2 to 5 years [Comstock et al., 1974; Vynnycky et al., 1997; Anderson et al., 2018]. In the 21st century, "LTB refers to a host who is TB immunoreactive in the absence of TB disease." [Behr et al., 2021]. LTB is asymptomatic in its clinical manifestation, which can only be detected through blood tests

or tuberculin skin tests. Globally, 1 in 4 persons, approximately 2 billion people, are LTB infected [WHO, 2021). Reactivation occurs in immunocompromised people with risk factors such as diabetes, alcohol, smoking, and undernutrition.

Human immunodeficiency virus (HIV)-infected patients also have a higher risk of developing TB [Havlir, D. V., & Barnes., 1999]. LTBI is treated majorly with Rifapentine [Harley et al., 2017]. In the 1990s, an outbreak of drug-resistant TB occurred in New York, killing about 80% of infected hosts [Elander, 2005].

About 0.05 to 5% of TB cases are the extrapulmonary type of infection [Nanda et al., 2011. The major type of extrapulmonary TB includes tubercular Lymphadenitis [Hegde et al., 2014], pleural TB, abdominal TB [Sharma et al., 2004], central Nervous System TB [Zunt et al. 2018], bone and Joint TB [Pigrau-Serrallach et al., 2013], genito-urinary TB, and miliary TB. The three primary diagnoses for TB include tuberculin skin test, chest X-ray, and mycobacterial cultures [Alzayer et al., 2021].

The pillars and components adopted by the WHO member countries to eradicate TB globally include early diagnosis, patient support, and treatment of TB patients, including the drug-resistant TB, management of TB comorbidities, and prophylaxis measures, including vaccination for persons in and from TB endemic regions [WHO, 2021]. Some countries have successfully reduced the TB burden to less than 10 cases and one death out of 100,000 population per annum [WHO, 2021]. However, research work, including discovering new drugs and vaccines, is still needed to reduce the global TB incidence.

1.2 TB as a global health issue

Tuberculosis (TB) is the 13th leading cause of death globally [WHO, 2021]. It is a communicable and highly infectious disease. As of 2020, TB was predicted to be the second top leading source of death arising from single infectious diseases, below the rank of coronavirus disease 2019 (COVID-19) [Glaziou P et al., 2020]. Approximately 25% of the world population is infected with latent TB, approximately 2 billion people [WHO, 2021]. This implies 1 in 4 persons have asymptomatic and non-infectious Tuberculosis (TB) while 5-10% of TB infected patients are at risk of developing active TB. The number of TB-caused deaths recorded in 2020 was higher than in 2019. There was an increase in TB deaths from 1.2 million in 2019 to 1.3 million in 2020. World Health Organization (WHO) modeling projections forecast that the number and incidents and death related to TB will be comparatively higher in 2021 and 2022 [WHO, 2021]. Global TB deaths rose for the first time in a decade in 2020 due to the COVID-19 pandemic. Health care resources, diagnostic tools, lockdown, and funding were diverted from TB management during the COVID-19 crisis [WHO, 2021]. The challenges of controlling TB spread are still globally divided into five major areas: the demand for WHO to increase short course (DOTS) programs, Directly Observed Therapy, insufficient TB diagnostics and treatments, TB-HIV coinfection, and MDR-TB [Murray et al., 2006]. The increase in TB deaths occurred mainly in the 30 high Burden TB Countries [WHO, 2021b]. Although the United States (U.S) TB incidence for 2020 was 2.2 cases per 100,000 persons [Deutsch-Feldmanet al., 2021], the significant risk factor of United States TB cases is the result of non-U.S. birth accounting for greater than 70% of TB cases in the United States [Miramontes et al., 2015]. Although there has been a global reduction of the incidence rate of TB and cases over the

years, the rate of reduction is not sufficient to achieve the End TB Strategy's first milestone: a 20% reduction of the global TB incidence, 35% reduction of TB deaths, from 2015 to 2020 [WHO, 2015]. Currently, an average 2% global decline in TB incidence rate is recorded, which is below the target of 17% average annual TB incidence decline goal from 2020 to 2035 by the WHO End TB Strategy initiative [Stop TB Partnership et al., 2015, Chaw et al., 2020]

The effort to eradicate TB has been challenging due to reduced drug discovery of novel antibiotics to combat TB. Intensified research and innovation are critical to meeting the WHO End TB Strategy targets for 2030 and 2035. The challenges in eradicating TB disease include the emergence of *Mtb resistance* to standard current anti-TB therapy. These include multidrug-resistant, extensively, and totally drug-resistant strains of *Mtb* [Cole et al., 1998; Fauci et al., 2008, Haydel, 2010]. It is crucial to develop antimycobacterial that inhibit novel *Mtb* drug targets and mechanisms of action [Theuretzbacher et al., 2019]. Another factor in obtaining efficient TB medication compliance is considering novel antimycobacterial that will constitute less pill burden and reduce the duration of the drug regimen. The current regimen consists of a cocktail of drugs for drug-sensitive TB treatment for about six months [Munro et al., 2007, WHO, 2021].

Recent molecular technology involving the whole genome sequencing analysis of *Mtb* has improved the tools and strategy in developing novel antimycobacterial agents to combat *Mtb* drug-resistant types [Cole et al., 1998; Sassetti et al., 2003; Abrahams et al., 2012]. Therefore, the need to develop vaccines and novel drugs with a simpler and shorter duration of treatment to treat about 2 billion people infected with *Mtb* is urgent. Since the approval of rifampicin by the United States Food and Drug Administration (FDA) in 1971 [Sensi,

1983], the discovery and development of an antimycobacterial pipeline have experienced a pause. In August 2020, 25 antibiotics were developed for active, multidrug-resistant, and TB infection in phase 1-3 clinical phase [WHO, 2021]. Sixteen of these drugs are new chemical entities, and two have received accelerated regulatory approval. The FDA has approved three drugs, bedaquiline, delamanid, and pretomanid, while 6 are repurposed drugs [WHO, 2020]. Therefore, the need to discover novel pharmacophores that inhibit new drug targets for *Mtb*, have a new mechanism of action, and are potent against drug-resistant *Mtb* is significantly vital.

1.3 Historical background of tuberculosis

TB has plagued humanity from prehistory to history, with *Mtb* suggesting killing more people throughout history than other infectious pathogens. Ancient, excavated writings from India, China, Egypt, Greece, and Babylonia attested to the different names given to TB. The oldest detection of *Mtb* was in the remains of Bison about 17,000 years ago in Wyoming [Rothschild et al., 2001]. However, the knowledge of whether TB originated from bovines and transferred to humans or if both humans and bovines had different TB ancestry was obscure [Pearce-Duvet et al., 2006]. A comparative study of the genes of human and animal *M. tuberculosis* complex (MTBC) suggested animals did not infect humans; however, TB infection could date as far back as the Neolithic revolution period [Comas et al., 2009]. TB Paleopathological proof existed as far back as 8000 BCE with the discovery of bony tuberculosis, during the 5800 BCE Neolithic period, and by 2400 BCE in Egyptian mummies [Herzog, 1998; A Boire, 2013], TB has existed since ancient times [Martini et al., 2020]. Previous postulations that the genus *Mycobacterium* originated more than 150 million years ago [Hayman, 1984]. Gutierrez et al. postulated that the progenitors

of *Mtb* infected prehistoric hominids and were about 3 million years ago present in east Africa [Gutierrez et al., 2005]. Contemporary *Mtb* complex members, including *Mycobacterium africanum, Mycobacterium Bovis,* and *Mycobacterium canettii,* originated from one African progenitor about 13 to 15 millennia ago [Gutierrez et al., 2005; Kapur et al., 1994; Brosch et al., 2002]. The current strains of *Mtb* present are categorized into six major clades, all of which are present in east Africa but scattered across the globe [Gagneux et al., 2006]. However, the archeological evidence to support TB origin from East Africa was lacking, albeit this evidence was documented in Egypt about 5000 years ago. TB characteristic Skeletal abnormalities were seen in Egyptian mummies and portrayed in Egyptian art [Cave et al., 1939, Morse et al., 1964, Morse et al., 1967; Zimmerman et al., 1979]. Current *Mtb* DNA amplification from Egyptian mummified tissues further elucidated the etiological agents for ancient skeletal diseases [Nerlich et al., 1997; Crubézy et al., 1998]. Schachepheth was the old Hebrew word for TB in Leviticus and Deuteronomy in the bible [Daniel et al., 1999]. Ancient migration from Africa [Gibbons et al., 2001] to China, India [Veith et al., 1969], Europe, and the Americas propagated the spread of TB across the globe [Daniet, 2000]. From the ancient empires and kingdoms, antiquity efforts to name and describe TB have ensued. The old Grecian, Father of allopathic medicine, Hippocrates, called TB phthisis and 'consumption' [Meachen, 1936, Daniel, 1997, Herzog et al., 1998] and described it as a disease of dry seasons from the 17th to 18th century. In Europe, it was called the great white death or white plague [Dormandy, 1999; Rene & Dubos, 1953], robber of youth [Segen, 1992], King's evil or Scrofula [Rothman et al.,2001]. During Laennec's era, the high mortality rate of TB in Europe made the disease an epidemic with an annual mortality rate of 1000- 1000 per 100,000 people [Daniel, 2006].

In Wales and England, about 4 million people aged 15 to 34 died from TB between 1851 and 1910 [Welshman, 2012]. By the end of the 1800s in London, 25% of her citizens died of TB. This death number was comparable to North American big cities.

Over the centuries, the cause of TB has been debated. Girolamo Fracastoro (1476-1553) was the first to postulate the cause of TB to an invincible organism which Benjamin Marten 1720 called a microscopic living organism, "animacula," thereby corroborating the previous theory of Anton van Leuwenhoek (1632-1723). Robert Koch received a Nobel Prize Lecture in 1905 for discovering tubercle bacillus as the etiological agent for TB. He acknowledged the importance of social welfare for Germans infected with TB [Koch, 1905]. In addition to Koch's findings in TB discovery Rudolf Ludwig Karl Virchow, an acclaimed father of social and hygiene medicine, 1821-1902, discovered the multifactorial genesis of the TB disease. He found that besides the infection by the pathogen bacillus, other multi factors contribute to the development of active TB status.

1.4 TB in North America continent

The TB incidence in the U. S has experienced a steady decrease since 1993 [Centers for Disease Control and Prevention report 2020]. This trend is because of the nation's Tuberculosis Elimination and Control Programs geared towards diagnosis, treatment, and prevention of TB. An identical trend in TB incidence has equally been reported globally [Kwak et al., 2020]. By 2021 7,163 TB cases in the District of Columbia (DC) and 50 states were reported by CDC's National Tuberculosis Surveillance System in the United States [Deutsch-Feldman et al., 2021]. The observed cases are a 20% reduction in the TB cases reported in 2020 (TB incidence: 2.2 cases per 100,000 persons) as compared to the previous

2019 (TB incidence: 2.7 cases per 100, 000 persons) [Deutsch-Feldman et al., 2021] Figure 1. Less than 1 case per 1,000,000 population is the TB elimination threshold, approximately 330 cases of TB per year based on the current U.S population. The U.S annual TB annual decline is still insufficient to achieve the WHO new global strategy (End TB) Figure 2

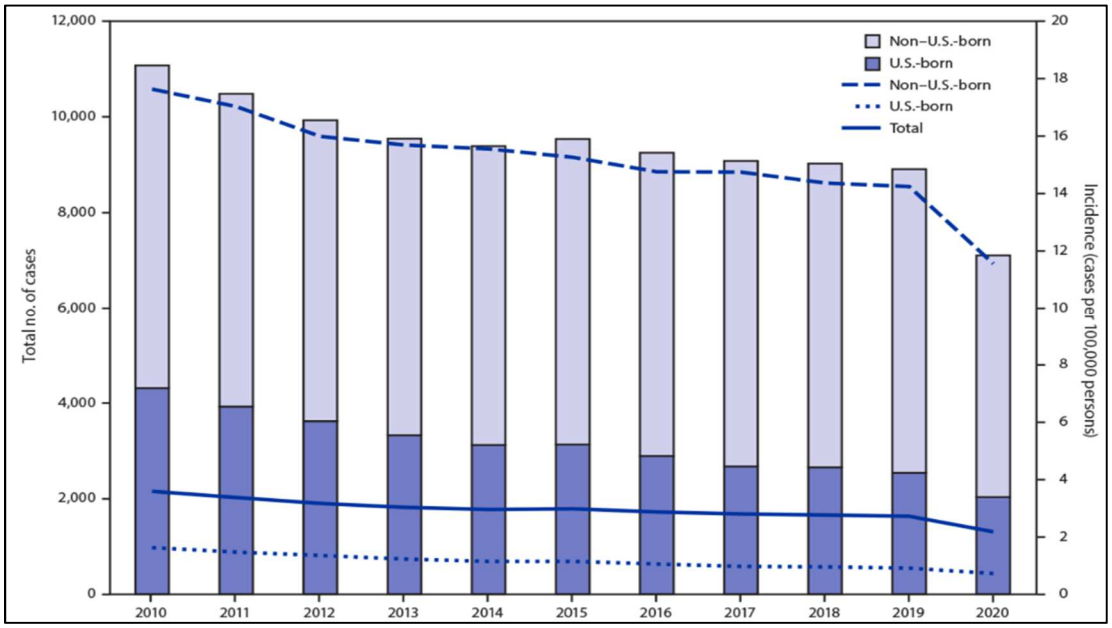


Figure 1: Tuberculosis disease cases and incidence, by birth origin, — United States, 2010–2020.

Adapted from Deutsch-Feldman, M., Pratt, R. H., Price, S. F., Tsang, C. A., & Self, J. L. (2021). Tuberculosis — United States, 2020. MMWR Surveillance Summaries, 70(12), 409–414. https://doi.org/10.15585/mmwr.mm7012a1

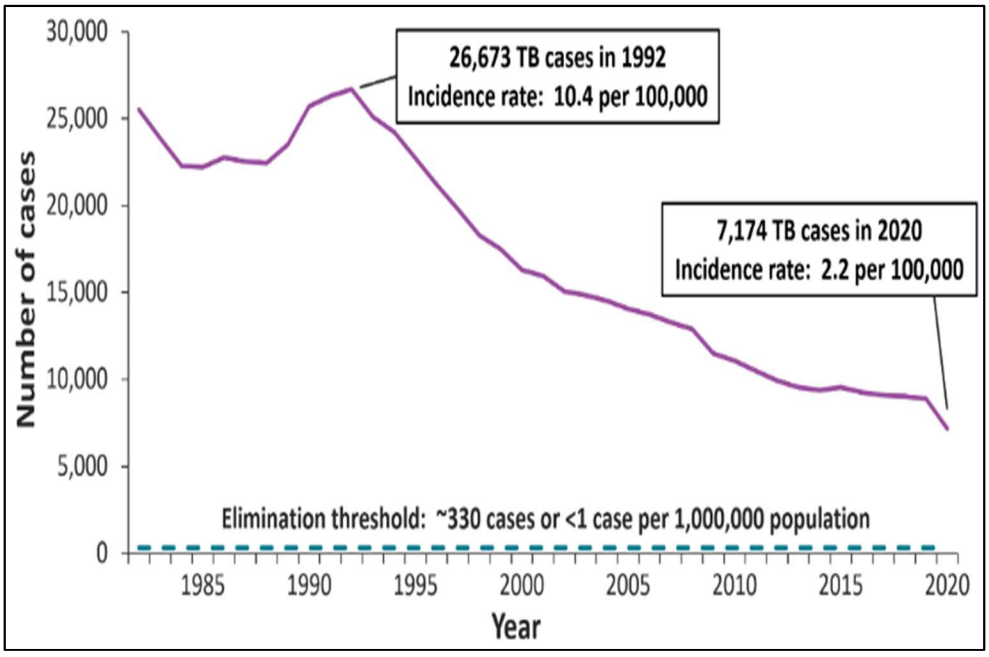


Figure 2. Progress Towards TB Elimination, United States, 1982–2020 [CDC, 2020]

Adapted from Centers for Disease Control and Prevention. (2021). Tuberculosis in the United States 2020 Slide Set. https://www.cdc.gov/tb/statistics/surv/surv2020/default.htm

An average 2-3% annual decrease in TB incidence [CDC, 2019]. Mid-year U.S. Census Bureau population in the United States 8 states recorded an increase in TB cases, and no changes were reported in 3 states, while 39 states and DC reported a decrease. East North central had the largest TB incidence decrease (−25%) concerning the geographical locations. Alaska reported the highest incidence (7.9 cases per 100,000 persons) and California reported the highest cases (1,703) in 2020 [Deutsch-Feldman et al., 2021]. According to the federal guidelines, TB case and incidence reports were categorized under birth origin and self-acclaimed race/ ethnicity, United States, 2017–2020. Under birth of origin, in 2019 and 2020 consecutively, 70% of TB cases were reported amongst non–U.S.-

born persons. The TB incidence in both non- U.S born persons (14.2 to 11.5 cases per 100,000 persons) and U.S born (0.9 to 0.7 cases per 100,000 persons) decreased from 2019 to 2020 [Deutsch-Feldman et al., 2021]. 10 % of all non–U.S.-born persons with TB cases in 2020 received a diagnosis less than a year during their stay in the United States, comparatively lower than the mean 16% value from 2015–to 2019. The change in TB incidence is indicative of a change in the pattern of immigration into the United States in 2020.

The changes in immigration and travel patterns during 2020 could not effectively explain the trend of TB cases decreasing. Nonetheless, the high proportion of TB cases reported by people living in the United States for more than ten years [Tsang et al., 2020] and the significant decrease in TB cases for both U.S born and non-U. S born people. This decrease may be attributed to the implemented protocols designed to reduce the spread of COVID-19 globally, indirectly reducing TB spread in 2020. Another plausible reason is the delayed or under-diagnosed TB cases arising from reluctance to seek medical treatment during COVID-19 [Czeisler et al., 2020].

The success of TB eradication in the United States experienced a deceleration in recent years which is compounded by the pressure on present public health care, control services, and prevention arising from the COVID-19 pandemic. 85% of TB diseases in the United States were discovered through genotyping to be through the reactivation of latent TB [Yuen et al., 2016]. Findings from genotyping of *Mtb* isolates suggest that an estimated 85% of TB cases in the U.S are attributed to reactivation of latent infection with *Mtb* that was acquired >2 years prior [Mirzazadeh et al., 2021].

A study by Mirzazadeh et al. showed that the effective way of preventing TB disease in the United States is through the treatment of LTBI [Mirzazadeh et al., 2021]. There is a need to increase the number of United States TB control efforts. A 1% decline in TB cases were recorded in the United States from 2018 (9,021 cases) to 2019 (8,920), a fourth consecutive year decrease. Nonetheless, the TB elimination goal of 1 case per 1 million people remains far from being attained. The TB rate (2.7 per 100,000) as of 2020 in the U.S is 27 times higher than the global target [CDC, 2020].

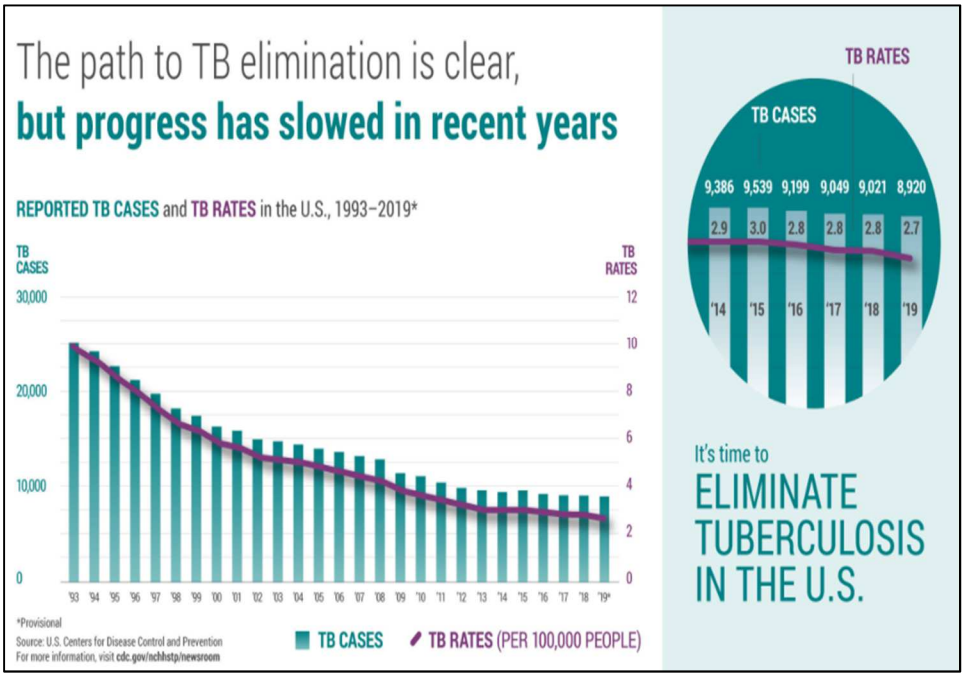


Figure 3. New CDC data highlight the need to strengthen the U.S.

Adapted from Centers for Disease Control and Prevention. (2020). Progress Toward Tuberculosis Elimination Has Slowed in Recent Years – March 25, 2021. Retrieved from https://www.cdc.gov/nchhstp/newsroom/multimedia-resources/tb-multimedia-resources.html

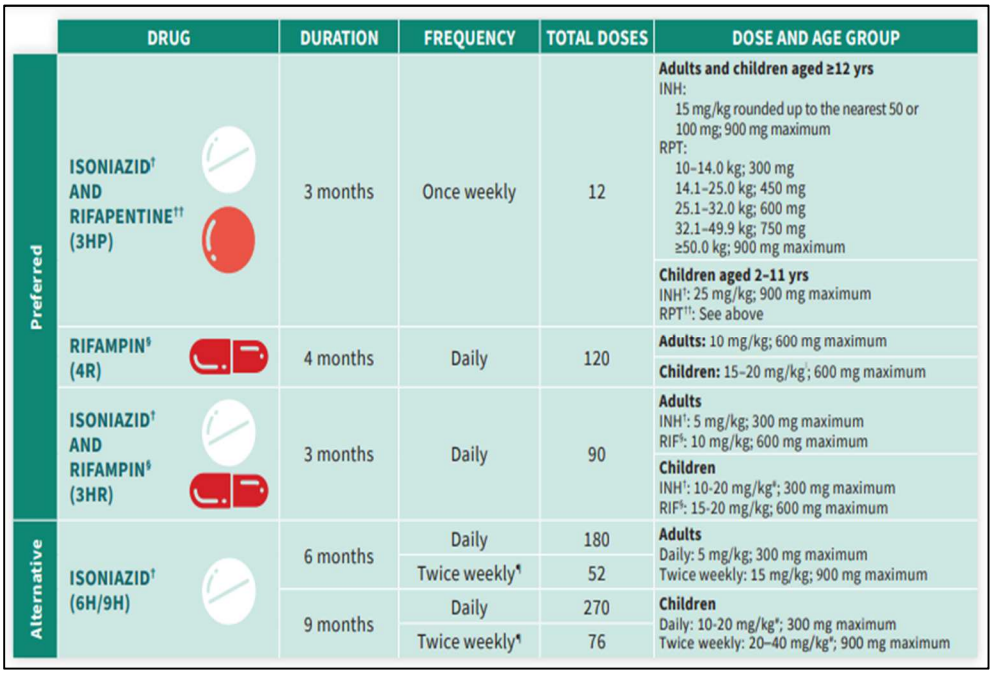
About 13 million people are estimated to have LTBI in the United States in 2020, with an estimate of 10% progressing from latent to active TB in their lifetime [CDC, 2021]. Ten

drugs have been approved for TB treatment in the United States by the FDA. By 2018, the treatment regimen for LTBI recommended by CDC in the United States includes rifapentine, isoniazid, and rifampin [CDC, 2018].

TB drug resistance is a concern both globally and in the United States. Fewer than 100 cases of MDR-TB and XDR-TB. Four hundred fifty-six cases of isoniazid resistance were reported in the United States in 2020, of which 9.5% of cases were recorded amongst non-U.S.–born persons and 4.8% of cases among the U.S.-born population Figure 2. [CDC, 2020a]. Only 1 case of XDR-TB was reported in the United States.

1.4.1 Latent Tuberculosis Infection Treatment Regimens

Fifty-six cases of MDR-TB were reported for 2020 in the United States: 0.2% and 1.3% of the MDR-TB cases were reported from U.S.-born and non-U.S.–born populations [CDC, 2020b].



	DRUG	DURATION	FREQUENCY	TOTAL DOSES	DOSE AND AGE GROUP
Preferred	ISONIAZID† AND RIFAPENTINE†† (3HP)	3 months	Once weekly	12	**Adults and children aged ≥12 yrs** INH: 15 mg/kg rounded up to the nearest 50 or 100 mg; 900 mg maximum RPT: 10–14.0 kg; 300 mg 14.1–25.0 kg; 450 mg 25.1–32.0 kg; 600 mg 32.1–49.9 kg; 750 mg ≥50.0 kg; 900 mg maximum
					Children aged 2–11 yrs INH†: 25 mg/kg; 900 mg maximum RPT††: See above
	RIFAMPIN§ (4R)	4 months	Daily	120	**Adults:** 10 mg/kg; 600 mg maximum
					Children: 15–20 mg/kg; 600 mg maximum
	ISONIAZID† AND RIFAMPIN§ (3HR)	3 months	Daily	90	**Adults** INH†: 5 mg/kg; 300 mg maximum RIF§: 10 mg/kg; 600 mg maximum
					Children INH†: 10-20 mg/kg*; 300 mg maximum RIF§: 15-20 mg/kg; 600 mg maximum
Alternative	ISONIAZID† (6H/9H)	6 months	Daily	180	**Adults** Daily: 5 mg/kg; 300 mg maximum Twice weekly: 15 mg/kg; 900 mg maximum
			Twice weekly¶	52	
		9 months	Daily	270	**Children** Daily: 10-20 mg/kg*; 300 mg maximum Twice weekly: 20–40 mg/kg*; 900 mg maximum
			Twice weekly¶	76	

Figure 4. Latent tuberculosis infection treatment regimens. Choosing the Right Latent TB Infection Treatment Regimen [CDC, 2021].

Adapted from Centers for Disease Control and Prevention. (2021). Treatment Regimens for Latent TB Infection (LTBI). Retrieved from

https://www.cdc.gov/tb/topic/treatment/ltbi.htm

TB is reported in the United States; however, the economic cost, duration to complete an anti-TB regimen, shrug adverse and side effects remain a burden. Eradication of TB in the United States is to have 1 case of TB per 1 million of her population. CDC's commitment to completely eradicate TB in the United States includes expanding diagnosis and drug treatment initiatives for LBTI patient**s.**

1.5 Current TB drug therapy

Current TB drug therapy consists of a combination of several drugs with a treatment duration of about six months for Drug sensitive TB type to a range of 6-20 months for

MDR-TB or/and RR-TB. Efficacious TB drugs were first discovered and developed in the 1940s with a recommended treatment regimen of 6 months for the four first-line anti-TB drugs: isoniazid, rifampicin, ethambutol, pyrazinamide, and streptomycin. The FDA-approved antitubercular medications for *Mtb* infections include ethambutol, pyrazinamide, rifampicin, and isoniazid [Ben Amar et al., 2015].

Table 1. Current antituberculosis drugs

	Intensive Phase		Continuation Phase				
Regimen	Drug[a]	Interval and Dose[b] (Minimum Duration)	Drugs	Interval and Dose[b, c] (Minimum Duration)	Range of Total Doses	Comments[c,d]	Regimen Effectiveness
1	INH RIF PZA EMB	7 d/wk for 56 doses (8 wk), or 5 d/wk for 40 doses (8 wk)	INH RIF	7 d/wk for 126 doses (18 wk), or 5 d/wk for 90 doses (18 wk)	182–130	This is the preferred regimen for patients with newly diagnosed pulmonary tuberculosis.	Greater
2	INH RIF PZA EMB	7 d/wk for 56 doses (8 wk), or 5 d/wk for 40 doses (8 wk)	INH RIF	3 times weekly for 54 doses (18 wk)	110–94	Preferred alternative regimen in situations in which more frequent DOT during continuation phase is difficult to achieve.	
3	INH RIF PZA EMB	3 times weekly for 24 doses (8 wk)	INH RIF	3 times weekly for 54 doses (18 wk)	78	Use regimen with caution in patients with HIV and/or cavitary disease. Missed doses can lead to treatment failure, relapse, and acquired drug resistance.	
4	INH RIF PZA EMB	7 d/wk for 14 doses then twice weekly for 12 doses[e]	INH RIF	Twice weekly for 36 doses (18 wk)	62	Do not use twice-weekly regimens in HIV-infected patients or patients with smear-positive and/or cavitary disease. If doses are missed, then therapy is equivalent to once weekly, which is inferior.	Lesser

Abbreviations: DOT, directly observed therapy; EMB, ethambutol; HIV, human immunodeficiency virus; INH, isoniazid; PZA, pyrazinamide; RIF, rifampin

Adapted from "Official American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America Clinical Practice Guidelines: Treatment of Drug-Susceptible Tuberculosis. Clinical Infectious Diseases", by Nahid, P., Dorman, S. E., Alipanah, N., Barry, P. M., Brozek, J. L., Cattamanchi, A., … Vernon, A. October 2016. Oxford University press. https://doi.org/10.1093/cid/ciw376

The duration of the drug regimen depends on the type of TB infection: latent or active TB [Parekh et al., 2013]. MDR-TB is a terrifying complication in TB drug treatment [Unissa et al., 2016]. MDR-TB is peculiar to its resistance to first-line anti-TB: isoniazid and rifampicin [Unissa et al., 2016], and its therapy is steadily evolving [Bloom et al., 2017].

The common second-line TB drugs recommended for MDR-TB include capreomycin, amikacin, and kanamycin injectable [Bloomet al., 2017]. The following fluoroquinolones are also prescribed as second-line TB therapy for MDR-TB: gatifloxacin, levofloxacin, and moxifloxacin [Berning, 2001, Moadebi et al., 2007]. The recent FDA- approved drugs for MDR-TB bedaquiline, pretomanid, and linezolid [Barryet al., 2017; Mase et al., 2020; Andrei et al. 2019; Riccardi et al., 2017]. The treatment regimen for MDR-TB has a longer duration than the First-line TB therapy, is more expensive, and poses the dangers of drug side effects.

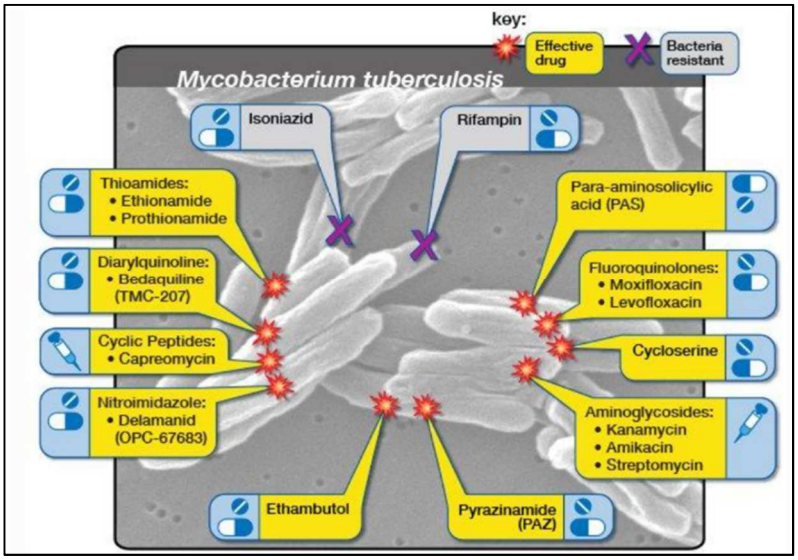


Figure 5. Second-Line Treatments and Multidrug-Resistant Tuberculosis. Adapted from "Tuberculosis Drugs and Mechanisms of Action" by the National Institute of Allergy and Infectious Diseases. 2016. https://www.niaid.nih.gov/diseases-conditions/tbdrugs

Extensively multi-drug resistant tuberculosis (XDR-TB) is another rare but complicated type of TB resistant to medications resistant to first-line TB medications: Isoniazid and

rifampicin, one of the three aminoglycosides from the second-line medication and one of the fluoroquinolones [Cang et al., 2013; Dheda et al., 2017].

Several anti-TB drugs are currently under development (Table 2.) The NAID is currently supporting the development of Pretomanid, Linezolid, SQ-109, Sutezolid, and Meropenems. New Treatment regimens consisting of drug combinations for drug-resistant and drug-sensitive TB are under phase II and phase III trials**.**

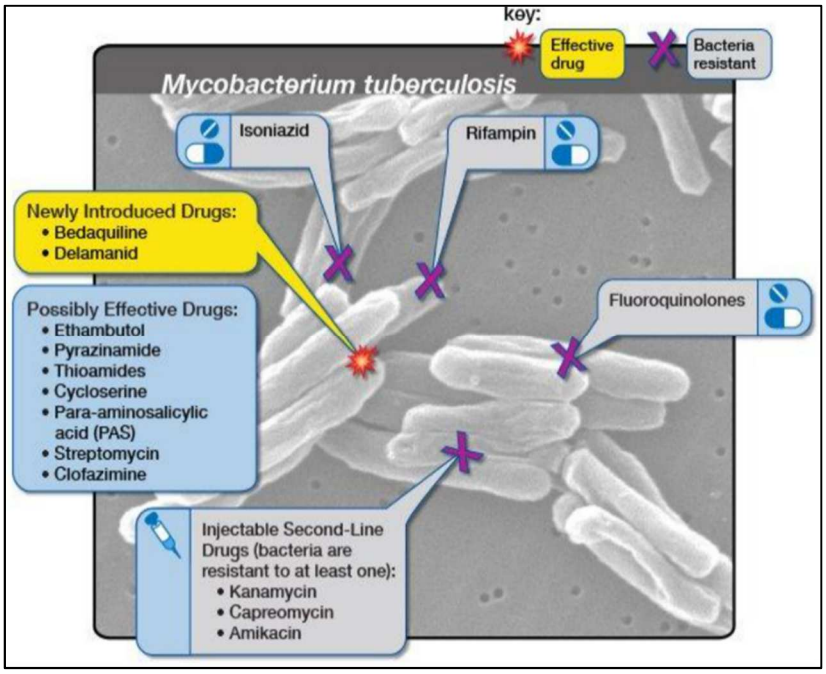


Figure 6. **Extensively Drug-Resistant Tuberculosis (XDR TB) - Options for Treatment [CDC, 2020].** Adapted from "Tuberculosis Drugs and Mechanisms of Action" by the National Institute of Allergy and Infectious Diseases. 2016. Retrieved from https://www.niaid.nih.gov/diseases-conditions/tbdrugs

Table 2. New *Mtb* drugs and their mechanisms of action in different clinical trial phases [Wang et al., 2021]

Drug classification	Groups	Anti-TB drugs	Mechanism of action	Target TB	Efficacy	Mechanism of resistance	References
Cell envelope synthesis inhibitor	Peptidoglycan	Cycloserine	Inhibit 2 enzymes forming D-alanine residues	MDR, XDR	High	Mutations in *alrA*	Patel et al., 2012
		Terizidone	Cycloserine derivative	MDR, XDR	High	Non	Galietti et al., 1991
	Arabinogalactan	Ethambutol	Inhibiting arabinosyltransferase, arabinose acceptor	Active	Low	*embCAB* operon	Telenti et al., 1997; Wolucka, 2008
	Mycolic acid	Isoniazid	Activation katG enzyme and inhibits inhA gene	Active, latent	High	Mutations in *katG* and *inhA* genes	Vilche'ze and Jacobs, 2007; Riccardi et al., 2009
		Triclosan	Inhibits the inhA enzyme without activation katG	MDR	Low	–	Wang et al., 2004; Freundlich et al., 2009
		Pyridomycin	Inhibits the inhA enzyme	MDR, XDR	High	Mutations in *inha*	Hartkoorn et al., 2012
		Ethionamide	Inhibits InhA by enzyme ethA	MDR, XDR	High	Mutations in *inha*, *etha*	Wolff and Nguyen, 2012
		Prothionamide	Inhibits InhA by enzyme ethA	MDR, XDR	High	Mutations in *etha*	Wang et al., 2007
		Thiocarlide	Inhibiting synthesis of oleic acid	MDR, XDR	High	Mutations in *ethA*	Phetsuksiri et al., 2003
		Delamanid	Releasing Nitric oxide by Ddn enzyme	Active, latent, MDR, XDR	High	Non	Gler et al., 2012; Zhang et al., 2013
		SQ109	Membrane transporter *MmpL3*	Active, latent	High	Non	Owens et al., 2013
Protein synthesis inhibitor	Aminoglycosides	Streptomycin	Bind to 30S subunit of ribosome	MDR, XDR	High	Mutation in *rpsl*	Honort and Cole, 1994
		Amikacin	Bind to 30S subunit of ribosome	MDR, XDR	High	Mutation in *rrs*	Sowajassatakul et al., 2014
		Kanamycin	Bind to 30S subunit of ribosome	MDR, XDR	High	Mutation in *rrs*	Sowajassatakul et al., 2014
	Oxazolidone	Linezolid	Bind to 50S subunit of ribosome	MDR, XDR	High	Mutation in *G2576T*(23S)	Scheetz et al., 2008
		Sutezolid	Bind to 50S subunit of ribosome	MDR, XDR	High	Non	Zumla et al., 2015
	Peptidoglycan	Capreomycin	Peptidoglycan Breakdown	MDR, XDR	High	Mutation in *tlyA*	Chen et al., 2003
Nucleic acid inhibitor	Rifamycins	Rifampicin	RNA polymerase inhibitor	Active, latent	Low	Mutations in *rpoB* gene	Sensi, 1983; Telenti et al., 1993
		Rifapentine	RNA polymerase inhibitor (b-subunit)	Active, latent/HIV	High	Mutations in *rpoB* gene	Chan et al., 2014
		Rifabutin	RNA polymerase inhibitor (b-subunit)	Active, latent/HIV	High	Mutations in *rpoB* gene	Yan et al., 2015
		Rifalazil	RNA polymerase inhibitor (b-subunit)	Active, latent/HIV	High	Mutations in *rpoB* gene	Saribaş et al., 2003
	PAS	PAS	Folic acid synth inhibitor	MDR, XDR	High	Mutations in *thyA*	Patel et al., 2012
	Quinolones	Levofloxacin	DNA gyrase inhibitor	MDR, XDR	High	Mutations in *gyrA*	Pranger et al., 2011
		Moxifloxacin	DNA gyrase inhibitor	MDR, XDR	High	Mutations in *gyrA*	Pranger et al., 2011
New drugs		Bedaquiline	Inhibiting ATP synthase enzyme	Active, MDR, dorment,XDR	High	Mutations in *atpE* gene	Chan et al., 2013; Chahine et al., 2014
		Pyrazinamide	Interferes with binding to mRNA	Active,MDR	High	Mutations in *RpsA, pncA*	Zhang et al., 2003; Shi et al., 2011
		Clofazimine	Inhibits DNA replication	MDR, XDR/HIV	High	Mutations in *rv0678*	Arbiser and Moschella, 1995

PAS:para-aminosalicylic acid

Adapted from "Addressing the Challenges of Tuberculosis: A Brief Historical Account" by Al-Humadi, H. W., Al-Saigh, R. J., & Al-Humadi, A. W. (2017, September 26), Frontiers in Pharmacology. Frontiers Media S.A. Retreived from

https://doi.org/10.3389/fphar.2017.00689

The mechanism of action of TB drugs varies as the drugs inhibit different targets found in *Mtb* biology. These include inhibition of nucleic acid synthesis, protein synthesis, and cell wall synthesis.

There are challenges to using second-line anti-TB drugs for MDR-TB [Iseman, 1993]. The challenges exist with the use of current antitubercular drugs. The non-compliance to treatment due to the long duration of the regimen, high pill burden, and complexity of treatment leads to suboptimal efficacy of drugs resulting from drug resistant cases [Karumbi et al., 2015]. Secondly, the adverse drug reaction from TB medications also

contributes to the non-adherence to the drug [Chan et al., 2002]. TB-HIV co-infection cases constitute a challenge in TB eradication due to high pill count, additive toxicity profile, and drug-drug interaction between rifampicin and the antiretrovirals, posing the challenge of medication adherence [Narita et al., 1998]. The countries with limited resources, where a higher number of MDR-TB cases exist, are unable to procure second-line anti-TB medication because of its high cost in comparison to first-line anti-TB drugs.

1.6 Current global status of drug-resistant TB

According to WHO, cases of drug-resistant TB are categorized into 5: isoniazid-resistant TB, MDR-TB, RR-TB, XDR-TB, and pre-extensively drug-resistant TB (Pre-XDR-TB) [WHO TB global report 2021]. Totally drug-resistant TB (TDR-TB) is another type of drug-resistant TB that the WHO does not recognize.

TB resistance to the first-line anti-TB isoniazid, isoniazid-resistant TB, is the most common type of drug-resistant TB case globally. *Mtb* isoniazid-resistant strains usually have mutations of ndh, katG, kasA, ahpC, and inhA [Silva et al., 2003]. WHO reports 7.2% average isoniazid-resistant TB and 11.6% in previously treated and current treated TB cases [WHO, 2019]. Sadly, although the global burden of Isoniazid-resistant TB has been decreasing over the years, this trend is present in the United States despite the decreasing case of TB. [Hoopes et al., 2008]. WHO data reported these regions in the world to have the following percentage of Isoniazid-resistant TB from their total TB cases from 1994 to 2008: 30% in Eastern Europe, 14% in West/Central Europe, and 14% in Africa [Jenkins et al., 2011]. National surveys conducted in South Korea for over 20 years revealed an estimated prevalence of Isoniazid (INH)-resistant TB in 10% of new TB cases and greater

than 30% in the past treated TB cases [Kim et al., 1992, Bai et al., 2007; Kim et al., 2019]. The treatment outcomes for INH-resistant TB using first-line TB medication were poor. WHO published meta-analyses and introduced two guidelines on the treatment of INH-resistant TB in 2019: 1.) 6 months regimen with Levofloxacin-Pyrazinamide-rifampicin-ethambutol 2.) no addition of streptomycin or other amikacin, kanamycin, and capreomycin injectables to the treatment [WHO, 2018].

1. Multidrug resistant tuberculosis

MDR-TB is a type of TB in which the infection is resistant to at least Isoniazid and rifampicin, the first-line anti-TB drugs. MDR-TB can be treated using second-line TB treatment; however, the cost of the treatment is expensive (greater than US$ 1000 per patient), toxic side effects, and the duration of the treatment regimen is long (about two years) [Falzon et al., 2017]. The global reduction of TB incidence from 2015 to 2019 was reported to be 9%. Globally, about 465 000 (range, 400 000–535 000) people were reported with the case of rifampicin-resistant TB (RR-TB), of which 78% of the cases were estimated to be MDR-TB. The MDR-TB global cases in 2019 are 361,000 [WHO, 2020]. WHO recommended a reduction in the duration of the MDR-TB dosing regimen to 9-12 months [Van Deun et al., 2010; Piubello et al., 2014; Kuaban et al., 2015 Trébucq et al., 2018; Nunn et al., 2020; Khan et al., 2017, Adidi et al., 2020]. The latest WHO report states a 57% treatment success rate for MDR-TB treatment globally [WHO, 2020]. A reduction of 15% occurred for people infected with MDR-TB or rifampicin resistant-TB (RR-TB) enrolled for treatment from 2019 (177,100) to 2020 (150,359) [WHO, 2021]. According to a WHO report carried out in 105 countries, 20.1% of average MDR-TB patients were recorded in 2020 [WHO, 2020]. World Health Organization (WHO). A total of 206,030

global cases for MDR-TB/RR-TB in 2019 were diagnosed and reported to WHO and the national TB programs from 6 WHO countries: Africa (29,155), the Americas (4,979), Eastern Mediterranean (6,328), Europe (47,936), South-East Asia (86,623), Western Pacific (31,009) [WHO, 2021]. About 50% of global cases of MDR/RR-TB were in the following geographical location: Russian Federation (8%), China (14%), and India (27%) [WHO, 2020].

2. Rifampicin-resistant TB

RR-TB is a subset of MDR-TB. Drug susceptible TB is sensitive to rifampicin treatment, a potent bactericidal anti-TB due to its effect on penetrating TB bacilli [Lohrasbiet al., 2018]. The most prevalent mechanism of resistance of *Mtb* to rifampicin treatment is through the mutation of the rpoB gene [Gamboa et al.,1998; Kocagoz et al., 2005]. About 465 000 (range, 400 000–535 000) cases were reported globally to develop rifampicin-resistant TB (RR-TB) [WHO, 2021]. WHO has established a list of the high burden countries for 2021- 2025 for TB cases, HIV- associated TB, and MDR/RR-TB. Thirty countries were recorded to have a high MDR/RR-TB burden. [WHO, 2021].

3. Extensively drug-resistant tuberculosis (XDR-TB)

WHO revised the definition of XDR-TB in 2021 as 'TB caused by *Mtb* strains that fulfill the definition of MDR/RR-TB and which are also resistant to any fluoroquinolone and at least one Group A drug '[WHO announces updated definitions of extensively drug-resistant tuberculosis. https://www.who.int/news/item/27-01-2021-who-announces-updated-definitions-of-extensively-drug-resistant-tuberculosis]. This new definition was updated in October 2020 and became applicable from January 2021 [WHO, 2021c].

WHO also revised the definition of pre-XDR-TB as follows: ‘TB caused by *Mycobacterium tuberculosis* (M. tuberculosis) strains that fulfill the definition of multidrug-resistant and rifampicin-resistant TB (MDR/RR-TB) and which are also resistant to any fluoroquinolone’. This new definition was updated in October 2020 and became applicable from January 2021 [WHO, 2021c].

XDR-TB is a more severe case of MDR-TB with a lower treatment success rate. A total of 12,350 global cases for XDR-TB in 2019 were diagnosed and reported to WHO and the national TB programs from the 6 WHO countries: Africa (618), the Americas (138), Eastern Mediterranean (73), Europe (8,560), South-East Asia (2,444), Western Pacific (517) [WHO TB global report, 2020]. In 2019 FDA approved a combination of Pretomanid, bedaquiline, and linezolid to treat XDR-TB cases in specific populations [FDA news release, 2019]. Previous reports have shown that men are more susceptible to XDR-TB than women [Flor de Lima et al., 2014]. The strategy to eradicate XDR-TB stipulates that countries enforce their national TB programs and improve health practitioners' care of TB patients following the TB care international standards [WHO, 2014].

4. Totally drug-resistant TB (TDR-TB)

In 2009, a type of drug-resistant TB was termed totally drug-resistant TB (TDR-TB). *In vitro* studies showed TDR-TB resistant to first line and second-line TB drugs [Ali et al., 2009]. The WHO consultative meeting does not recognize TDR-TB due to the difficulty in the *in vitro* drug susceptibility testing (DST) and the correlation between DST and clinical outcome [WHO, 2008]. Some published works reported TDR-TB as XDR-TB or

XXDR-TB because the approved Bedaquiline and Delamanid may be used to treat TDR-TB [Parida et al., 2015]. The cases of TDR-TB are scarcely documented globally; however, it has been discovered in Italy [Migliori et al., 2007; Parida et al., 2015], Iran [Velayati et al., 2009], India [Udwadia et al., 2012], South Africa [Klopper et al., 2013].

The emergence of drug-resistant TB incidence globally has renewed the interest in the global TB discovery and development field. With the appearance of new drug-resistant TB, the testing and drug therapy for TB patients is becoming progressively difficult. The need to use potent antibiotics comes with the disadvantages of toxicity, such as thrombocytopenia, hepatitis, and death. This challenge has motivated the government and pharmaceutical industries to discover novel anti-TB.

1.7 Global new TB drug pipeline 2021

The pipeline for novel antitubercular drugs as of August 2020 comprises 22 drugs in clinical trials: phases I, II, and III Figure 7. The drugs include 13 newly discovered compounds: delpazolid, GSK-3036656, macozinone, sutezolid, SQ109, TBI-223, TBAJ-876, TBI-166, BTZ-043, OPC-167832, SPR720, Q203, and TBA-7371. In addition, for two drugs, delamanid and bedaquiline, regulatory approval was recently accelerated by the FDA. Levofloxacin, moxifloxacin, clofazimine, linezolid, a high dose of rifapentine, and rifampicin are six clinically approved antimicrobials currently undergoing testing to repurpose for TB treatment. Everolimus, auranofin, and CC-11050 (AMG 634). Studies on novel TB regimens are currently under trial [WHO TB global report, 2020]. Due to the increase in the MDR-TB, XDR-TB, and TDR-TB globally, the focus has recently been centered on host-directed therapies as a treatment option [Zumla et al., 2015].

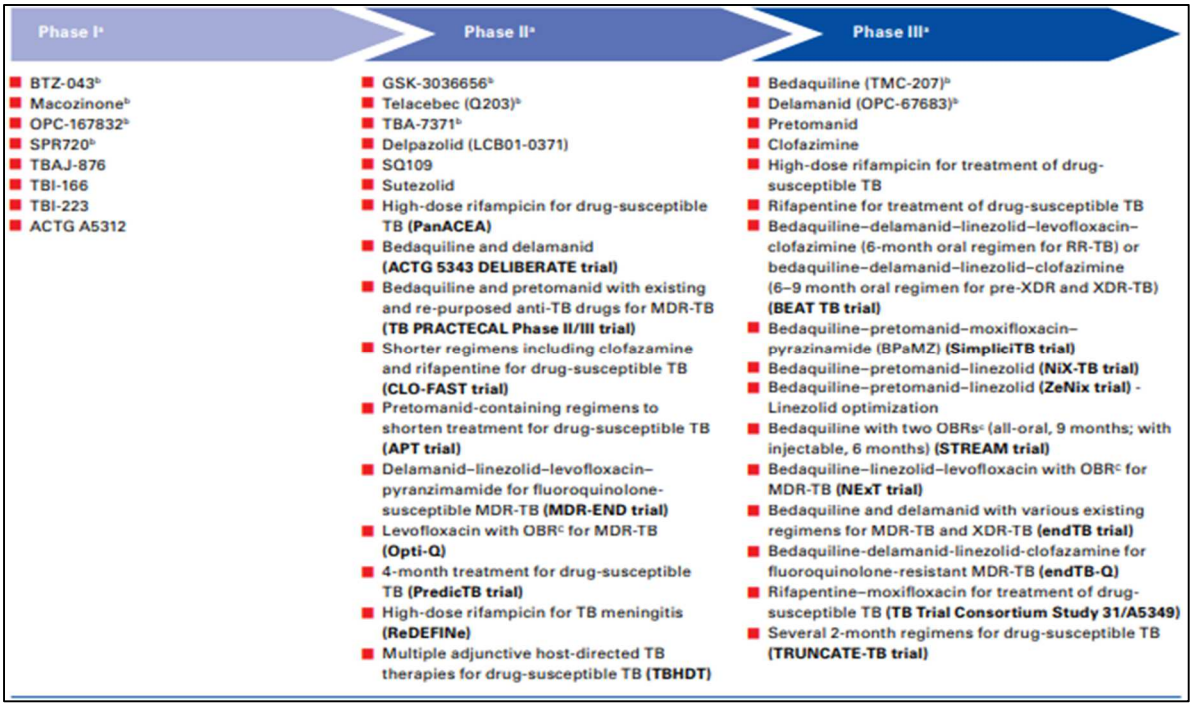


Figure 7. The global clinical development pipeline for new anti-TB drugs and drug regimens to treat TB disease [WHO, 2020]. ACTG A5343 DELIBERATE, ACTG A5312, APT trial, BEAT TB, CLO-FAST trial, endTB, MDR-END, endTB-Q, NeXT, NiX-TB, ZeNix, SimpliciTB, PredictTB trial, TB-PRACTECAL, STREAM, TBHDT trial, and TRUNCATE-TB.

Adapted from "The global clinical development pipeline for new anti-TB drugs and drug regimens to treat TB disease, August 2020" Global tuberculosis report 2020, WHO. (2020), P. 183. Retrieved from https://www.who.int/publications/i/item/9789240013131

1.8 Statement of the Thesis Problem

The challenges involved in the treatment of TB includes poor adherence to the current first-line TB regimen [Chan et al., 2002; Munro et al., 2007]. The rise in MDR-TB and XDR-TB strains of *Mtb* has reduced the effect of current treatment options [Fauci et al., 2008; Cole et al., 1998]. The high cost of treatment of MDR-TB strain is another hurdle in TB drug treatment [Marks et al., 2014]. In addition, current FDA-approved anti-TB drugs; pretomanid, bedaquiline, and delamanid, have been discovered to have serious adverse effects on patients and the cost for procurement of these drugs is expensive. Futhermore, Mtb strains resistant to the current FDA-approved TB drugs have emerged [Bloemberg et al., 2015]. In light of the stated challenges in TB therapy, it is, therefore, crucial to discover promising agents that meet the innovative standard of newly developed antibacterial agents: A new chemical class, a new mechanism of action, a new drug target, absence of cross-resistance with existing antibiotics [WHO 2017].

1.9 Specific aims of the Thesis Problem

1. Aim

To characterize OJT008 as a novel antitubercular agent through its inhibition of nickel and cobalt activated MtMetAP1c enzyme.

Specific Objectives

1. To investigate the in vitro inhibitory effect of OJT008 against nickel and cobalt activated Mtb Methionine aminopeptidase 1c (MtMetAP1c) using a both genetic and biochemical approach

2. To evaluate the inhibitory mechanism of OJT008 against MtMetAP1c using a computational study approach
3. To investigate the effect of OJT008 against drug-sensitive and multidrug-resistant type *Mtb* using a microbiological approach

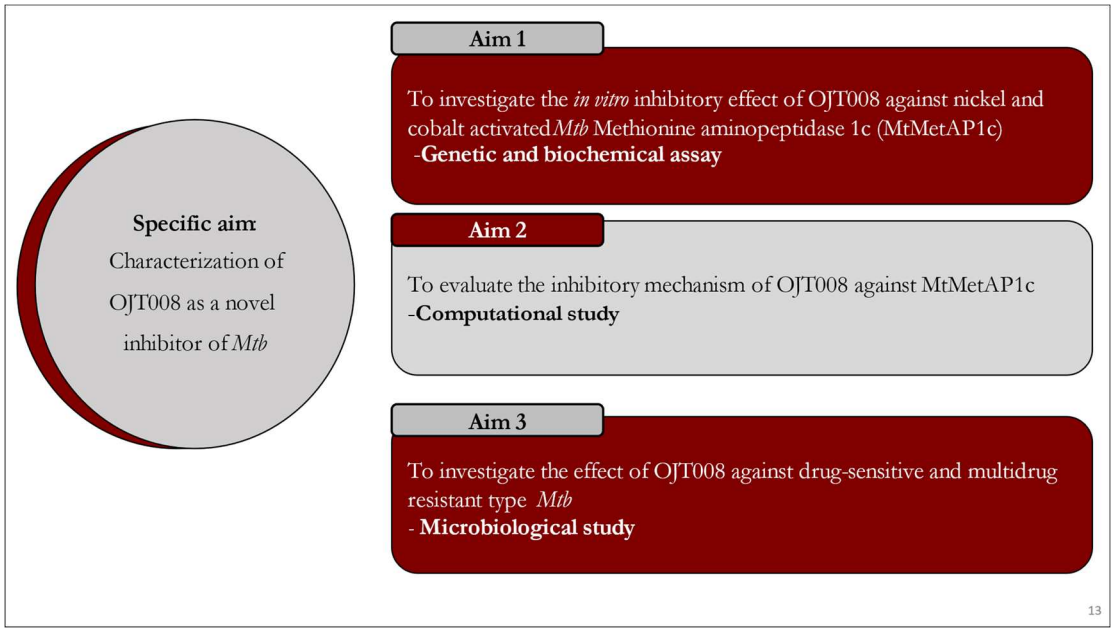


Figure 8. Specific Aims of the Thesis Problem

CHAPTER 2

LITERARY REVIEW

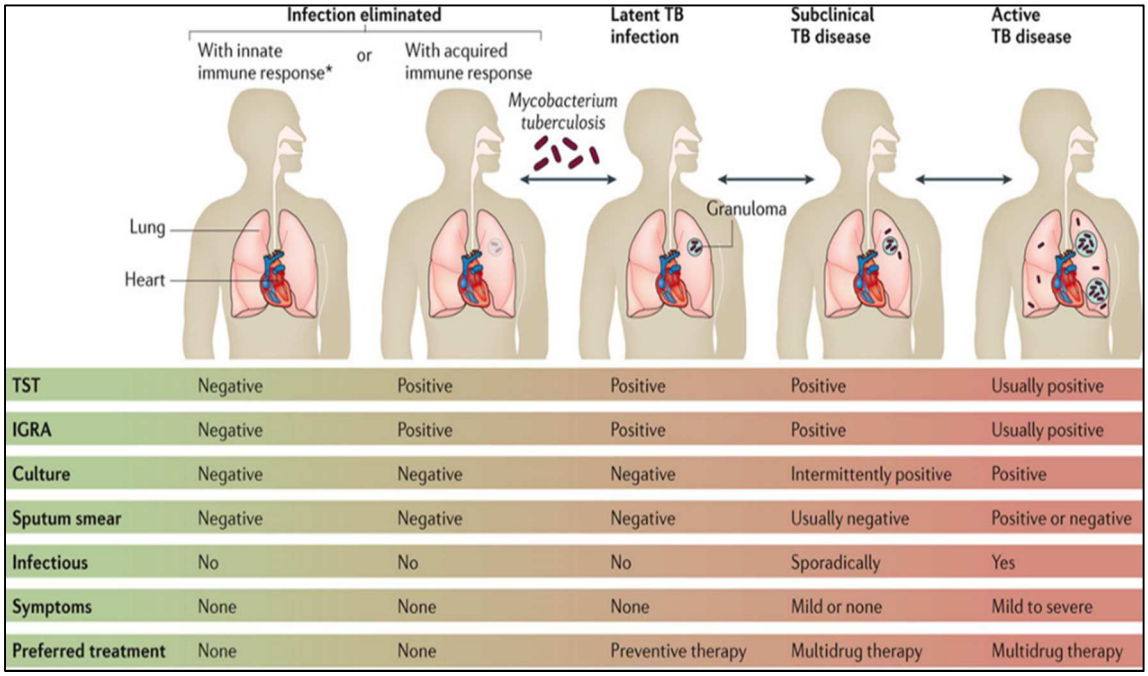
2.1 TB

TB is an airborne communicable disease, and before the emergence of COVID-19 was the leading cause of death arising from a single infection globally [WHO Global TB Report 2021]. TB takes about 1-2 years to develop in 5% of its human host after *Mtb* infection [Thomas et al., 2003]. A German physician discovered *Mycobacterium tuberculosis* complex is the causative organism for an airborne infectious disease called TB [Sakula et al., 1982; Ryan et al., 2004]. The airborne characteristics of TB were confirmed in the 1950s by Wells and Rile [Riley et al., 1962; Riley et al., 1995]. Although *Mtb* primarily infects its host's pulmonary glands, it is also discovered to affect other parts of the human body and presents a different spectrum of presentation from asymptomatic to severe cases of infection [Barry et al., 2009; Esmail et al., 2014]. A continuum exists between the spectrum of *Mtb* infection and active TB. Based on public health and clinical perspective, TB can be categorized into two types: LTBI, which is asymptomatic and noncontagious, and active TB, which is symptomatic and infectious [Lee, 2016]. TB is one of the major prevalent infectious diseases, besides HIV/AIDS, Malaria, and recently COVID-19, globally. 25% of people worldwide are infected with latent TB, and 1.5 million people will die from TB in 2020. Adults comprise 90% of TB infected people, which

predominantly male globally [WHO 2021]. About 10 million people are ill with active TB [WHO TB global report 2021]. 5-10% of *Mtb* infected people develop LTBI within 2-5 years [Anderson et al., 2018]. In both low and middle-income countries, TB remains the primary factor for mortality and morbidity [Su et al., 2020]. The mode of TB transmission is through respiratory nuclei droplets from active pulmonary TB infected persons. Such nucleus droplets contain about 1 to 10 TB Bacilli and can be suspended in the air for hours before being inhaled into the lungs' alveoli. About 40,000 droplets are released from a single sneeze [Cole et al., 1998], and each droplet, no fewer than 10 TB bacilli [Nicas et al., 2005], can transmit TB disease. The TB bacilli are protected from environmental stress, dehydration, oxygen, and injury by respiratory secretions surrounding the nuclei droplet, and the half-life of TB bacilli suspended in the air is about 6 hours [Loudon et al., 1969]. *Mtb* is not as infectious as other respiratory pathogens like varicella-zoster virus and measles. A TB infected individual can transmit the infection to 3 to 10 people per annum [Van Leth et al., 2008]. The virulence of a TB disease results from the infectiousness or virulence of TB bacilli, bacilli load inhaled, environmental stresses, the strength of host's immune system, and other socio-economic factors [Hill et al., 2004]. There are two major types of TB: active TB disease and latent TB infection. TB infected people are classified based on the clinical state as either active TB disease or latent TB infection (LTBI). Latent tuberculosis infection (LTBI) is a type of TB in which the infected person does not manifest any clinical symptoms, microbiological evidence, and radiological abnormality [Thomas

et al., 2003]. LTBI is a state of continuous stimulation of *Mtb* antigens without a clinical manifestation of active TB [WHO, 2015]. Another type of TB disease is active TB which arises from the reactivation of subclinical latent TB; therefore, LTBI infected people are a major reservoir pool of active TB cases [Shea et al., 2014]. LTB refers to a host who is TB immunoreactive in the absence of TB disease." [Behr et al., 2021]. TB exists as a dynamic progression [Young et al., 2008; Barry et al., 2009] from TB infection to active TB disease, thereby classifying TB into active TB disease and LTBI Figure 9. Infected individuals can change from one spectrum type of TB to another based on their immunity state and present comorbidity state. TB exists as a progressive continuum from one end of the spectrum, Mtb infection, to another end, active TB disease. Exposure of the lungs to Mtb droplets is either eliminated from the host through its innate or acquired immune system. Expulsion of the Mtb bacilli through innate immunity without T and B cell priming results in a negative diagnosis result for both interferon-γ release assay (IGRA) results and tuberculin skin test (TST). Elimination of Mtb through acquired immunity will result in a positive or negative IGRA or TST depending on whether T- cells were primed [Barry et al., 2009; Esmail et al., 2014]. In both previous cases, the people's culture and sputum smear tests are negative, with no symptoms, noninfectious, and there is no preferred LTBI treatment. Some people develop a robust memory T cell after eliminating Mtb bacilli are both positive to TST and IGRA. In the case of LTBI, the bacteria are not eliminated. It remains in a quiescent state with no clinical manifestation, positive to both TSA and IGRA, noninfectious, negative to culture, sputum smear, and benefit from LTBI therapy consisting of 6–9 months of

isoniazid. The subclinical TB type exists in which is the patients are asymptomatic, infectious, and positive to culture tests [Barry et al., 2009; Dowdy et al., 2013.]



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

Figure 9. The spectrum of TB — from *Mycobacterium tuberculosis* infection to active (pulmonary) TB disease.

Adapted from "Tuberculosis" by Pai, M., Behr, M. A., Dowdy, D., Dheda, K., Divangahi, M., Boehme, C. C., … Raviglione, M. (2016, October 27). Nature Reviews Disease Primers. Nature Publishing Group. Retrieved from https://doi.org/10.1038/nrdp.2016.76

TB disease occurs when *Mtb* bacillus is spread from a TB sick person and is spread through expulsion from the lungs into the air. *Mycobacterium* is the phylum from the genus *Actinobacteria* and the Mycobacteriaceae. About 190 species belong to the genus *Actinobacteria* [King et al., 2017]. There are three groups of human pathogenic Mycobacteria: *Mycobacterium tuberculosis* complex (MTC), Nontuberculous mycobacteria, and *Mycobacterium leprae* and *M. lepromatosis*. *Mycobacterium*

tuberculosis complex, which includes *Mtb*, *M. Africanum*, *M. Carnettii,* M. bovis, *M. Mungi*. The Nontuberculous mycobacteria are divided into slow and fast-growing Mycobacteria [Franco-Paredes et al., 2018]. The most clinically important *mycobacterium* species infect humans. *Mycobacterium bovis* is a slow-growing species that affects both cattle and humans, while another rare *Mycobacterium* that affects humans in central Africa is *Mycobacterium africanum* [Franco-Paredes et al., 2018]. MTC comprises the species *M. africanum*, vaccine strain M. bovis BCG (Bacille Calmette-Guérin), M. bovis, *M. microti*, *Mtb*, *M. pinnipedii, M. canettii, Mycobacterium orygis,* and *Mycobacterium suricattae* [Brosch et al., 2002; Huard et al., 2006; Alexander et al., 2010]. These species cause TB in animals and humans. Most human TB disease is caused by *Mtb* or a related species, *Mycobacterium africanum.* Fewer cases are attributed *to M. tuberculosis* complex (MTC), such as *Mycobacterium caprae or Mycobacterium bovis* [Bos et al., 2014]

Transmission of *Mtb* infection [Firdessa et al., 2013] and the reactivation of LTBI [Reed et al., 2009] are the major causes of TB disease. The genesis of TB disease occurs when *Mtb* enters the alveoli sacs and proliferates within the endosome of the host's macrophages [Houben et al., 2006; Queval et al., 2017]. Attempts by the host macrophage to phagocytosis the foreign *Mtb* bacilli occurs. The bacterium is temporarily stored as a phagosome by the macrophage, which further forms a phagolysosome. In the phagolysosome, the white cells attempt to destroy the *Mtb* cells by utilizing acid and reactive oxygen species. This attempt by the macrophage is unsuccessful due to the thick mycolic acid encapsulating *Mtb.* As a result, *Mtb* cells reproduce in the macrophage and eventually destroy the host immune system. The upper action of the lower lobe of the lungs, known as the Ghon focus, is the focal site for the *Mtb* infection [Kumar, V., & Robbins, S. L., 2007]. The upper section of the lungs known as Simon focus can also be infected by

Mtb [Khan et al., 2011] which is spread through the blood circulatory system. The infection can also spread to the brain, kidneys, bones, and peripheral lymph nodes [Khan et al., 2011]. TB is also termed the granulomatous inflammatory disease. An aggregation of B lymphocytes, T lymphocytes, macrophages, fibroblasts, and epithelioid cells creates the granulomas, which eventually form large multinucleated cells in the alveolar space. The granuloma obstructs dissemination of the *Mtb* infection and fosters interaction between the pathogen and host immune cells [Jacques et al., 2003]. However, studies have shown *Mtb* utilizes granulomas to evade the host's immune system surveillance, thereby suppressing the host immune system [Bozzano et al., 2014].

The inhaled *Mtb* droplets evade the protective mechanisms of the bronchi due to the size of the droplet and enter the alveolar space, where it is counteracted by alveolar macrophages. Inadequate defense by the alveolar macrophage allows *Mtb* to invade the interstitial tissues of the lungs through the infected macrophage migration to the parenchymatous tissues of the lungs or the *Mtb* directly infecting the epithelial lining of the alveolar. Eventually, either infected monocytes [Hoopes et al., 2008] or infected dendritic cells [Samstein et al., 2013] transfer *Mtb* to be primed by T cells in the lymph nodes. Further recruitment of immune cells such as T-cells and B-cells into the parenchyma of the lungs creates a granuloma. A spectrum of granuloma is found in both latent and active TB. The proper functioning of the granuloma determines the resultant type of TB disease [Flynn et al.,2011]. Caseous granuloma is formed in latent TB infection containing *Mtb* from spreading. Active TB is formed when the growing bacteria load becomes too

large for granulomas to contain the pathogen [Lin et al., 2014], allowing *Mtb* to circulate through the bloodstream to other parts of the body. This is an infectious stage called the active TB disease.

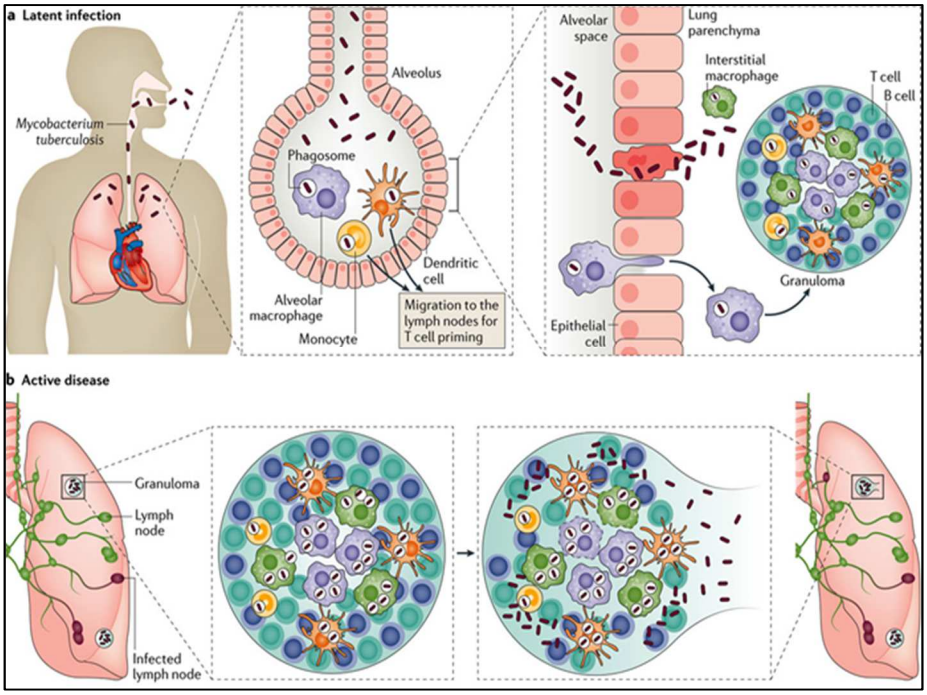


Figure 10. Mycobacterium tuberculosis infection.

Adapted from "Tuberculosis" by Pai, M., Behr, M. A., Dowdy, D., Dheda, K., Divangahi, M., Boehme, C. C., … Raviglione, M. (2016, October 27). Nature Reviews Disease Primers. Nature Publishing Group. Retrieved from https://doi.org/10.1038/nrdp.2016.76

2.2 TB Drug Discovery

The WHO's global TB program collaborates with many research and development stakeholders and Stop TB partnership groups on novel diagnostics, vaccines, and new anti-TB to keep track of the development of novel TB tools. Progress and setbacks in the

research and development of TB tools are recorded annually in the "Global tuberculosis report." The objective of anti-TB discovery is to develop new TB drug regimens and TB drug discovery that are more efficacious, potent, safe, and accessible to all patient populations [Mdluli et al., 2015]. The commercial viability for anti-TB drugs is low; therefore, the focus in this area is minimal. Providentially, non-government and government organizations have taken steps to correct the situation by creating programs such as the Global Alliance for TB Drug Development (TB Alliance). TB Alliance forms a unification of governments, non-government organizations, industries, and an estimated 30 partners around the globe to fast-track the discovery and development of cost-effective antitubercular drugs [Global Alliance for TB Drug Development (TB Alliance) Active Since 2000]. Various consortia have been developed, including Orchid, the Lilly Early TB Drug Discovery Initiative, More Medicines for TB (MM4TB), anti-TB Drug Accelerator. The Gates Foundation and the National Institutes of Health (NIH), funded by the government and academic laboratories, are also involved in translational research to discover TB drugs. Bedaquiline was approved by FDA on 28 December 2012 [US Food and Drug Administration (FDA), 2012; Mahajan et al., 2013], and delamanid was approved in December 2013 by the European Medicines Agency and the Japanese regulatory authorities [European Medicines Agency, 2014]. Both drugs are recommended for MDR-TB drug therapy. Current drug discovery studies have not provided sufficient hit-lead generation to develop antimycobacterial, which shortens TB therapy duration, reduces the TB drug regimen, and ultimately changes the paradigm of TB treatment. The

efficient utilization of current molecular biology in whole-genome sequencing of *Mtb* genes since 1998 [Cole et al., 1998] has accelerated the progress in TB drug discovery. Essential *Mtb* genes *in vitro* and *in vivo* [Sassetti et al., 2003], genome-wide DNA microarrays study to elucidate specific gene expression under different conditions

[Sherman et al., 2001; Schnappinger et al., 2003; Zhang et al., 2012], and the determination of novel drug targets by elucidating mutated genes in drug-resistant mutants [Abrahams et al., 2012; Loerger et al., 2013] were discovered through the knowledge of *Mtb* whole genome. Deleting specific genes through the molecular technique of gene knockdown has helped in *vitro* and *in vivo* validation of novel drug targets [Park et al., 2011].

2.2.1 Repurposed drug candidates for TB treatment

Drug therapy for TB has been adopted for eradicating TB infection for the past 70 years; however, the duration of the antimycobacterial regimen constitutes TB drug resistance emergence. Over the years, the impediment in TB therapy due to drug-resistant *Mtb* strains has posed severe public and health challenges. The strategy of repurposing existing clinically approved antibiotics for treating TB disease circumvents the bottlenecks in anti-TB drug development. Repurposing existing drugs for TB treatment is a promising strategy for eradicating both TB diseases and drug-resistant TB infections. The strategy of repurposing existing clinically approved antibiotics for TB disease treatment reduces the bottlenecks in anti-TB drug development. Novel compounds such as delamanid, bedaquiline, and pretomanid have been discovered to improve the therapeutic outcome of

MDR-TB and XDR-TB [Mahajan, 2013; Ferlazzo et al., 2018]. However, clinical failure has already been discovered in TB therapy [Olayanju et al., 2018]. Repurposing existing drugs has the advantage of being cost-effective and a shorter time of approval for human consumption. Another merit of this approach is that existing lead compounds can be modified through structural modification or computer-based design with a high-throughput approach [Pushpakom et al., 2018]. The following are repurposed drug candidates currently investigated for TB drug therapy.

1. Nonsteroidal anti-inflammatory drugs

Ebselen and Diminazene aceturate are both Nonsteroidal anti-inflammatory drugs being repurposed for TB treatment. Diminazene are indicated for trypanosomiasis and babesiosis [Da Silva Oliveira et al., 2015]. Cappoen et al. performed an anti TB activity screen of 26 compounds with a 1,3-diaryltriazenes structure. These compounds were structurally modified to obtain three lead analogs with a potential for antitubercular activity [Cappoen et al., 2014]. Ebselen is indicated for cytoprotective, anti-atherosclerotic, anti-inflammatory, and antioxidant properties [Scheweet al., 1995]. Padiadpu et al., through a combination of whole-genome sequencing and phenotypic profiling, discovered isoniazid and ebselen, among which are potent against *Mtb* H37Rv and three other strains resistant to drug therapy, MYC431, JAL2287, and BND3200 [Padiadpu et al., 2016].

2. **Antimalarial**

Artemisinin is an antimalarial extract from the Chinese shrub *Artemisia annua*. Together with Its derivative, Artesunate has been proven to have potent antimalarial activity and more pharmacological properties [White et al., 1997]. Choi et al. postulated the effectiveness of artemisinin in blocking or modulating the proliferation of *Mtb*. This hypothesis was investigated and confirmed by using several anti-TB indicator assays, thereby proving the efficacy of artemisinin has a potent effect against *Mtb* activity [Choi et al., 2017]. Chloroquine is a known antimalarial drug [Baruah et al., 2018]. Clearance of isoniazid by efflux pump, breast cancer resistance protein-1, is inhibited by chloroquine, therefore increasing the potency of standard antitubercular drugs [Matt et al., 2017]. The mechanism of chloroquine in inhibiting phagosomal acidification increases the susceptibility of drug-sensitive *Mtb* to drug treatment, decreases post-drug treatment relapse in the BALB/c mouse model, and alleviates lung pathology after TB infection. Chloroquine reduces the duration of TB treatment and increases the efficacy of anti -TB [Mishra et al., 2019]. Bermudez et al. 2014 discovered Mefloquine has a potent bactericidal effect against Erdman and *Mtb* H37Rv strains in unfavorable conditions and in macrophages [Bermudez et al., 2014]. Mefloquine and oxazolidine-derivative compounds were discovered by Rodrigues et al. in 2016 to be active in TB infected murine model [Rodrigues-Junior et al., 2016]

3. Antibiotics

In vitro studies by Kaushik et al. discovered the potency of Biapenem, a drug candidate under trial for treating *pseudomonas aeruginosa* [Chen et al., 1994], and Tebipenem, a drug indicated for respiratory tract infections [Sato et al., 2008] against drug-susceptible *Mtb* H37Rv with a minimum inhibitory concentration (MIC_{90}) of 2.5 - 5 µg/mL [Kaushik et al., 2015] and 1.25- 2.5 µg/mL [Kaushik et al., 2015] respectively with a reported 4-fold reduction of minimum Inhibitory Concentration (MIC) values for both carbapenems with the addition of clavulanate. *In vivo* Study by Kumar et al. [Kumar et al., 2017] also determined the potency of biapenem, or in combination with rifampicin, in *Mtb* infected BALB/c mice. They discovered the biapenem reduced the lesion in the lungs of the infected mice at a 90% minimum inhibitory concentration (MIC_{90}) by inhibiting nitrocefin hydrolysis by the activity of L, D-transpeptidase, and LdtMt2 [Guo et al., 2019; [Kumar et al., 2017]. An *in vivo* study by Kumar et al. also showed that Tebipenem inhibited LdtMt1 and LdtMt2 hydrolysis of nitrocefin [Guo et al., 2019].

4. Antiviral drugs

Isoprinosine is indicated for treating subacute sclerosing panencephalitis [Campoli-Richards et al., 1986]. The antiviral drug stimulates cytokine, thereby playing an important function in the *Mtb* host immune resistance. Mishra et al. hypothesized Isoprinosine's immunostimulatory effect warrants the use of the drug as a prophylactic for TB treatment [Mishra et al.,2018].

5. Cardiovascular drugs

Verapamil is a selective calcium channel blocker clinically approved for managing hypertension, angina, and arrhythmia. It inhibits the P-glycoprotein transporter, thereby modulating the absorption of drugs into cancer cells. As such, it is used in combination with antineoplastics to prevent resistance to cancer drugs. It is hypothesized that alteration of membrane efflux pump would reduce the viability of intracellular functioning *Mtb.* Verapamil, therefore, can inhibit the viability of *Mtb* intracellularly and boost the efficacy of other anti-TB when combined [Adams et al., 2011; Adams et al., 2014]. *In vivo* studies have demonstrated the efficacy of the combination of verapamil and rifampicin treatment increases the potency of other antitubercular drugs [Canezin et al., 2018]. Ghajav et al. study also further validated the efficacy of verapamil in reducing certain strains of *Mtb* efflux pump [Ghajavand et al., 2019].

Simvastatin is a coenzyme A reductase inhibitor indicated for lowering cholesterol levels and management of hypercholesterolemia [Atil et al., 2016]. Skerry et al. postulated that simvastatin could increase the immune response of macrophages, thereby boosting the efficacy of standard anti-TB agents. They discovered that 5µM of simvastatin could enhance the activity of isoniazid in J774 macrophage-like cells. In addition, the group in an *in vivo* study discovered that the advantage of a combinatorial regimen with simvastatin was more effective than rifampicin, isoniazid, or pyrazinamide alone, in killing the *Mtb* bacilli load with a resultant decrease in the colony-forming units, in *Mtb* infected BALB/c mice [Skerry et al., 2014]. A Series of studies by Dutta et al. in 2019 strongly point to

Pravastatin as an effective adjunctive host-directed therapy for TB disease [Duttaet al., 2020]

6. Anticancer drugs

In 2003 FDA approved bortezomib, a proteasome inhibitor, for the treatment of Multiple myeloma [Dou et al., 2014]. A new antimycobacterial screening protocol, which involves both whole-cell and target approach methods, was developed by Moreira et al [Moreira et al., 2015]. Bortezomib was discovered to have potent activity against *Mtb* and *M. smegmatis* through the mechanism of inhibiting caseinolytic protease. The poor pharmacokinetic profile, cost, and prominent adverse effects have limited the utilization of bortezomib for TB therapy [Wilfred et al., 2017]. Gorovoy et al. discovered analogs of boron-containing peptidomimetic compound, with scaffolds identical to bortezomib, to be potent against *Mtb* with a MIC value of <5 µg/mL [Gorovoy et al., 2013]. Elesclomol is an anticancer drug that induces apoptotic cell death of tumor cells through oxidative stress [Kirshner et al., 2008]. Ngwane et al. carried out an in vitro anti-TB activity assay for Elesclomol against *Mtb* H37rv and discovered an IC_{50} of 4µg/mL. They also discovered the drug to be potent against bone marrow macrophages infected with active Mtb by using a checkerboard assay [Ngwane et al., 2019]. Another strategy for combating TB proliferation in infected cells is by an effective modulation of the host's immune response. Metformin is a drug indicated for Type 2 diabetes and hypoglycemia. Metformin directly

inhibits adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) which modulates autophagy [Liu et al., 2014]. Singhal et al. 2014 discovered metformin increases AMPK and blocks MDR-*Mtb*, and *M. tuberculosis* H37Rv strains in human monocyte-derived macrophages and THP-1. In an *in vivo* TB infected mouse model, using just metformin or combined with ethionamide or isoniazid reduces inflammation, improves immune function, and alleviates lung pathology. These results further support the hypothesis of using metformin to modulate the host immune system to enhance the effectiveness of drug-resistant TB therapy [Singhal et al., 2014]. Currently, several studies are being conducted to determine the safety and efficacy of metformin for treating *Mtb* infection [Lachmandas et al., 2019; Park et al., 2019]. Another antidiabetic agent under investigation for TB treatment repurposing purposes includes Bis-biguanide dihydrochloride [Shen et al., 2016]. Other drugs also studied for repurposing for TB treatment include rimonabant, an endocannabinoid receptor antagonist indicated for treating obesity [Fong et al.,2009], and Ramesh et al. studies show the effectiveness in inhibiting *Mtb* H37Rv [Ramesh et al., 2016]. The major limitation in the repurposing drug candidates for TB treatment is that the potency of existing candidates against TB is low, and fewer drug candidates available for repurposing purposes.

2.3 Approaches to Antitubercular drug discovery

The burden of TB disease and the emergence of MDR-TB and XDR-TB strains have posed challenges to the efficacy of existing anti-TB drugs hence the urgency to discover novel

drugs to add to the current treatment strategy. Both traditional and innovative approaches are being adopted in the discovery of novel anti-TB drugs [Migliori et al., 2020]. There are two major approaches to anti-TB drug discovery> target-based approach, and the whole cell-based approach recently added to the methods is the target mechanism-based whole-cell screening.

Currently, 23 drug candidates are in the TB drug discovery pipeline with studies on effective anti-TB combination regimens for treating drug-susceptible, XDR-TB, and XDR-TB strains [WHO global TB report, 2020]. It is essential to discover novel drug candidates that require a lesser duration of treatment (the least duration for current TB treatment is six months), are affordable, safe, and will pose no drug-drug adverse interaction when co-administered with current antitubercular drugs [WHO global TB report, 2020]. The major challenges in the rate of discovering drug candidates against *Mtb* are primarily due to the slow-growing nature of the pathogen, existing in different growth phases [Smith et al., 2013; Gomez et al., 2015], and existence in granulomas and macrophages [Hussain Bhat et al., 2015].

2.3.1 Target-based approach for the discovery of novel TB drugs

This approach is also termed 'reverse pharmacology.' It is an HTS of studied molecular targets to determine drug candidates that inhibit or induce the target. In some cases, the knowledge of the molecular targets can also be understood by the phenotypic approach to drug discovery [Lage et al., 2018]. Genomic and recombinant technology is utilized in the

Target-based drug discovery approach. The whole *Mtb* genome sequencing expedited the discovery of novel *Mtb* drug targets. It starts with the knowledge of the drug target and ends with the discovering an effective drug candidate that efficiently inhibits the molecular target. The knowledge of a molecular target avails the utilization of mutational analysis and computational modeling to determine the interaction of drug candidates with the target. Despite the challenges characterized by this approach, it remains a critical aspect of determining if drug candidates can be translated to the preclinical phase of drug study [Hughes et al., 2011]. The classic TB targets for drug discovery are peptides and proteins expressed in critical biological pathways essential for *Mtb* viability, these proteins are absent in humans, or the nucleotide sequence of the *Mtb* protein is less identical to the human homolog. Several *Mtb* enzymes hypothesized to be promising targets for TB drug candidates based on the essentiality of these enzymes for the viability of *Mtb* are currently under investigation. Examples of these targets are Enoyl-acyl carrier protein reductase [Shirude et al., 2013; Sullivan et al., 2016], N-Acetylglucosamine-1-phosphate uridyltransferase [Li et al., 2011; Rani et al., 2015], Decaprenylphosphoryl-D-ribose 20-epimerase [Batt et al., 2012], Maltosyltransferase GlgE [Veleti et al., 2014], Pantothenate synthetase [Samala et al., 2013], and methionine aminopeptidase 1 [Olaleye et al., 2010; Olaleye et al., 2011; John et al., 2016]

2.3.2 Phenotypic based approach for the discovery of novel TB drug

This is also called whole-cell screens, and the approach involves administering a compound or drug candidate to a cell or pathogen and observing the effect or phenotypic change of the organism after treatment. The phenotypic-based approach is a fast way to determine *Mtb* inhibitors [Ballel et al., 2013; Rebollo-Lopez et al., 2015] by utilizing thousands of drug and compound candidates from libraries unearthed from high-throughput screens (HTS) [Ananthan et al., 2009]. This approach paves the way for the discovery of anti-TB drugs that inhibit both *Mtb* pathways and targets. This approach circumvents the challenges arising from the permeability of drug candidates into cell membranes and has the added advantage of determining the cytotoxicity of screened compounds. In the past, most documented research on whole-cell screens was carried out in aerobic culture conditions, which are favorable for replication of *Mtb* strain. Recently innovative phenotypic drug screens have been designed to simulate the in vivo condition of host cells infected with *Mtb*, for example, the screen of *Mtb* infected macrophages [Christophe et al., 2010]. This approach is relevant in discovering drug candidates that are potent against persisters. However, the lack of knowledge of the specific drug target of the bacteria poses a limitation of this method. Whole-genome sequencing of *Mtb* in 1998 has led to a promising outlook in the target-based screen approach [Mayer., & Hamilton, 2010]. The phenotypic high throughput screen of libraries of TB drug candidates aided the discovery of pretomanid [Stover et al., 2000], bedaquiline [Protopopova et al., 2005], Q203 [Pethe et al., 2013], and

delamanid [Matsumpoto et al., 2006] which are under clinical trial [WHO TB global report, 2020]. An aspect of this dissertation project involves the phenotypic-based approach.

Another approach recently adopted is the target mechanism-based whole-cell screening. In the past, the discovery of antibacterial agents adopted the biochemical assay methods to investigate the interaction of novel pharmacophores recombinant purified protein, and less focus was on the whole-cell approach. This approach has not been quite productive in discovering antibacterial compounds that the inhibitory effect can be translated into potency against whole-cell models. These challenges are major because of the permeability of the poor compound to the cell membrane. This has prompted studies to be shifted to the whole-cell approach method. However, this method's main limitation is the lack of knowledge of the cellular target before the drug screen. Another novel approach called the target mechanism-based whole-cell screening is a synergy of both approaches: target-based and whole cell-based, which maximizes the merits of both approaches. Moreira et al. [Moreira et al., 2017] adopted this method by repurposing the clinically approved bortezomib for *Mtb* therapy

2.4 **Methionine aminopeptidase; a promising chemotherapeutic target**

Methionine aminopeptidases (MetAP) is a universal enzyme essential for the viability of *Mtb* [Olaleye et al., 2010]. The protease is a dinuclear metalloprotease that catalyzes the excision of N-terminal methionine (NME) from nascent proteins from the ribosome during protein translation [Lowther & Matthews, 2000; Giglione et al., 2003; Olaleye et al., 2009].

NME is required for subcellular localization, protein-protein interaction, stability, and protein degradation of some proteins [Giglione et al., 2004; Krishna et al., 1983].

The biological function of MetAP in *Mtb* was identified by Olaleye, Ph.D., MSc, through a combination of genetic and chemical approaches. MetAP was reported to be essential in *Salmonella typhimurium* [Miller et al., 1989], *Escherichia coli* (*E. coli*) [Chang et al., 1989], and yeast *Saccharomyces cerevisiae* [Chang et al., 1992; Li, X., & Chang et al., 1995]. In the NME pathway, MetAP removes the N-terminal methionine from nascent proteins after the activity of Peptide deformylase (PDF) [Giglione et al., 2004, Lowther., & Matthews,2000]. NME occurs in 55-70% of nascent proteins [Giglione et al., 2004]. PDF was initially reasoned to be a promising drug target; however, the essentiality of PDF was refuted due to the ability of organisms to mutate the formyltransferase gene [Solbiati et al., 1999, Mazel et al., 2004]. The critical function of MetAP in all life forms has made it a promising target for discovering novel antibacterial agents, including drug-resistant TB [Vaughan et al., 2012, Olaleye et al., 2010]. MetAP has been discovered as an attractive target for the discovery of drugs for the treatment of diseases such as cancer, rheumatoid arthritis, parasitic and fungal infections [Griffith et al., 1997; Sin et al., 1997; Zhang et al., 2002; Bernier et al., 2004; Han et al., 2018; Rodriguez et al., 2020]. MetAP exists in 2 types, MetAP1 and MetAP2, and are differentiated by inserted polypeptide located in the catalytic domain of MetAP2 [Arfin et al., 1985]. MetAP1 exists as four isoforms: MetAP1a, MetAP1b [Addlagatta et al., 2005], [Giglione et al., 2000], MetAP1c, MetAP1d [Chen et al., 2006], and MtMetAP1n, [Bala et al., 2019]. MetAP1b and MetAP1c are

distinguished from MetAP1a by the presence of N-terminal extension in the subtypes. X-ray structures of apo and holo form of MetAP1c revealed conserved proline residues in the N- terminal extension, which is absent in MetAP1a [Addlagatta et al., 2005]. The biological role of MetAP in bacteria shows the enzyme as a promising drug target.

2.4.1 *Mtb* Methionine aminopeptidase (MtMetAP)

Mtb differs from most prokaryotes in possessing two isoforms of *Mycobacterium tuberculosis* methionine aminopeptidase (MtMetAP1); MtMetAP1a and MtMetAP1c, which both share 33% identity [Sherman et al., 1985] but differs by the presence of 40 amino acid residues in the N-terminal of MtMetAP1c which is relevant for ribosomal interaction [Addlagatta et al., 2005].

Olaleye et al. generated both MtMetAP1a and MtMetAP1c and performed a target validation study to determine to function of MtMetAP1s in *Mtb* [Olaleye et al., 2010].

They discovered MtMetAP1a is essential for the viability of *Mtb*. The mutated type of MtMetAP1a phenotypically manifested slow growth of *Mtb*. The result signified MtMetAP1a as a promising chemotherapeutic target for *Mtb* treatment [Olaleye et al., 2010]. A previous transcriptional comparative analysis study by Zhang et al. showed a difference in the transcript level of *mapA* (MtMetAP1a) and *map*B (MtMetAP1c) transcript level during the exponential and stationary expression level in *Escherichia coli* (e. coli)

culture. They suggested the biological role of the enzymes in the growth phases of *Mtb* [Zhang et al., 2009]. mapA was expressed more in the log phase while mapB's expression level was higher in the stationary phase, thereby suggesting the different roles of MtMetAP1a and MtMetAP1c in the growth phase of *Mtb* [Zhang et al., 2009]. The transcriptional level of mapA was comparatively higher in the stationary phase compared to the bacteria culture's log phase. *map*B transcript level was discovered to be higher in the log phase in comparison to the log phase. The results suggest the different roles of mtMetAP1a and MtMetAP1c in the growth phase of *Mtb* [Zhang et al., 2009]. Olaleye et al. utilized both target-based and phenotypic drug discovery approaches to discover TB drug candidates. This was achieved by utilizing a chromogenic substrate enzymatic assay adapted from Zhou et al. [Zhou et al., 2000] to study the effect of a library of compounds against both MtMetAP1a and MtMetAP1c and discovered promising lead compounds [Olaleye et al., 2010, 2011, 2012, 2016].

2.4.2 MtMetAP: a dinuclear metalloprotease

MetAP, a dinuclear metalloprotease, and divalent metal ions directly participate in the removal of N-terminal methionine from nascent polypeptides by MetAP. The NME process is activated by divalent metal ions such as Ni^{2+}, Fe $^{2+}$, Co^{2+}, Mn^{2+}, and Zn^{2+} [D' Souza et al., 2007; Lowther et al., 2009]. The proteolytic activity of MetAP is inhibited by Ethylenediaminetetraacetic acid (EDTA), further validating the metalloprotease nature of MetAP [Arie et al., 1987]. MetAP1 proteins are activated by cobalt or zinc metals for their

activity [Arfin et al., 1985]. MetAP utilizes conserved amino acid residues to create a scaffold having the capacity to bind at most two metal ions [Lowther et al., 2002]. *E.coli* MetAP was discovered to be activated by Mn(II), Ni(II), Fe(II), Zn(II),and Co(II) [D' Souza et al., 2007]. MetAPs are metalloproteases that are activated by the following divalent metals, Mn (II), Co (II), and Fe (II) as cofactors to activate their inherent catalytic activity [Zhang et al., 2009; Olaleye et al., 2010; John et al., 2016]. The metal required for the activity of MtMetAP1 in its native physiological form has been a subject of debate.

A supplemental work by Olaleye et al. [Olaleye et al., 2010] in which the metal: cobalt (Co) and manganese (Mn), the dependence of apo of MtMetAP1a and MtMetAP1c screen were conducted. MtMetAP1 was discovered to be dependent on both metals for their inherent activity in a concentration-dependent manner. In this dissertation work. We conducted preliminary work to investigate the dependence of the holo from MtMetAP1c on Nickel and cobalt.

2.4.3 MtMetAP1c essential for the viability of *Mtb*

The essential nature of both MtMetAP1a and MtMetAP1c have been studied by different research groups. Investigations of promising MetAP1 inhibitors for *Mtb* metalloprotease have been conducted over the years. Olaleye et al. adopted both genetic and chemical approaches in determining the essentiality of either MtMetAP1a or MtMetAP1c in *Mtb* growth [Olaleye et al., 2010]. Overexpression of either isoform conferred resistance of *Mtb* to screened inhibitors, and knockdown of the MtMetAP1a gene decreased the growth of

Mtb. However, the viability of *Mtb* growth is moderately affected by the knockdown of *mapB,* suggesting its nonessential role in *Mtb* [Olaleye et al., 2010]. Additional studies have proven the importance of MtMetAP1c in *Mtb* viability. Results from a Transcriptional comparative analysis by Xuelian Zhang et al. [Zhang et al., 2009] showed the mapA and *map*B transcripts are expressed more in the exponential and lag phase, respectively, in an *E.coli* growth. These findings suggest the different roles both genes play in the viability of *Mtb*. The life cycle of *Mtb* may very well require more than one MtMetAP1 to catalysis the co-translational modification. A comparative genomic study of the roles of genes in *Mtb* species by Ribeiro-Guimarães et al. showed that both MtMetAP1a and MtMetAP1c are essential for the survival and pathogenicity of *Mtb* [Ribeiro-Guimarães et al., 2007]. The findings of Miriam et al. suggested the essential role of *map*B for *Mtb* viability [Vanunu et al., 2019]. Furthermore, studies have shown that MtMetAP1c displayed activity in a wider pH range compared to MtMetAP1a, having about 60% of its activity intact at a pH as low as 6.5. This characteristic suggests the role of MtMetAP1c in *Mtb* in an acidic condition such as in the host macrophage

A high-density transposon-mutant library analysis by Christopher et al. suggested *map*B as essential for *Mtb* growth and survival [Griffin et al., 2011]. In this dissertation project, we endeavored to characterize OJT008 activity against MtMetAP1c based on the viability of *Mtb* by MtMetAP1c from a literature review.

2.4.4 Essentiality of divalent metal ions for MtMetAP1 activity and discovery of *Mtb* MtMetAP1 inhibitors

A study by Zhang et al. showed MtMetAP1c catalysis is increased magnesium (II) ion, Manganese (II) ion, Cobalt (II) ion, and zinc (II) ion. They discovered that the activity of the enzyme is reduced by Ni^{2+}, Cu^{2+}, Fe^{2+}. Zhuang et al. discovered the different binding affinities of various inhibitors against MetAPs activated by various metals [Ye et al., 2003; Ye et al., 2004]. Nickel, on the other hand, is known to be an excellent MtMetAP1c cofactor [Lu et al., 2010]. Zhang's study used metallated enzymes, not in its apoenzyme; therefore, the inhibitory effect of Ni^{2+}, Cu^{2+}, Fe^{2+} was probably due to competition of the metal added with the active site metal already in place. *In silico* approach in the drug discovery approach, discovered compounds have varying binding affinities to MetAP with different metals at the active site [Wang et al., 2003; Zhuang et al., 2004]. The two types of MyMetAP1 are activated by Fe^{2+}, Ni^{2+}, Co^{2+}, and $Fe^{2+}$for its NME activity Fe^{2+} [Lu., & Ye., et al., 2010a], Zhuang et al., 2010]. Current MetAP inhibitors were characterized using MetAP in its Co^{2+}form. However, recently discovered MetAP1 inhibitors, albeit potent against in vitro bacterial MetAPs, displayed a less significant potency against the corresponding bacterial activities [Luo et al.,2003; Douangamath et al., 2004]. It was discovered that inhibitors of Co^{2+} forms of MetAP1 might not effectively block the activity of different metal-activated MetAP [Li et al., 2003; Qi-Zhuang et al., 2004]. Cobalt (Co^{2+}) Co (II) are exceptional activators of apo-form MetAPs. Recent MetAP inhibitors were discovered by screening against Co^{2+} activated MetAPs [Oefner et al., 2003; Luo et al.,

2003; Huang et al., 2007]. The challenge of the discrepancy between the *in vitro* and *in vivo* potency of MetAP inhibitors can be because of the difference in the affinity of the MetAP and MtMetAP1 for specific metals in vitro and their native cellular state. The challenge in designing a potent, selective inhibitor of pathogen MetAPs is in discovering the specific divalent metals in the active sites of the metalloproteases.

Several studies have focused on elucidating the inhibitory effect of MetAP1 inhibitors against the enzyme activated by different metals to optimize IC_{50} value [Huang et al., 2007], [Lu et al., 2010], [Lu et al., 2011], [Juhás et al., 2022] and [Lu et al., 2012]. Jing Ping et al. discovered Nickel as an excellent activator of MtMetAP1c activity [Lu et al., 2010b] discovered 1,2,4-Triazole Based Inhibitors inhibit the activity of MtMetAP1 by chelating Ni^{2+} at the active site of MtMetAP. Aryl Carboxylic Acid Inhibitors were discovered to bind specifically to the Mn^{2+}at the active site of *E. coli* MetAP1 [D' Souza et al., 2007]. Therefore, the knowledge of the specific metal(s) in the active site of MtMetAP1, which OJT008 has a high binding affinity as investigated by both in vitro and *in silico* studies, is important.

2.5 OJT008, a MetAP1 inhibitor

OJT008 is a compound purchased from MolPort, Inc. The IUPAC name for the compound is N-benzyl-5-chloro-N,6-dimethyl-2-pyridin-2-ylpyrimidin-4-amine, PubChem Compound CID: 2740173. The compound's protein target stated in the PubChem source is the Metalloprotease M24A subfamily of protease inhibitors. M24A is the family of

methionyl aminopeptidase, one from *E.coli*, metallopeptidase families, that requires cobalt or manganese for its catalytic activity [Neil et al., 2013]. Olaleye et al. screened 175,000 compounds from ASDI Inc. in Johns Hopkins University, against MtMetAP1c at a final concentration of 30 μM. They selected 439 compounds based on a 30-40% inhibition of MtMetAP1c. 81 MetAP1 compounds were discovered by Olaleye et al. which were also potent inhibitors of MtMetAP1s at 10 μM, and 21 of the structural analog's hits were further selected based on their potency against *Mtb*. OJT008 was selected from the analogs [Olaleye et al., 2010], and the compound was discovered and patented by Olaleye et al. as a Methionine Aminopeptidase Inhibitors for Treating Infectious Diseases. Methionine Aminopeptidase Inhibitors for Treating Infectious Diseases

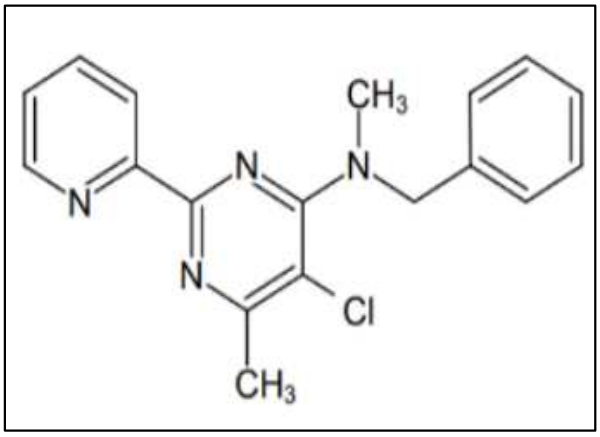


Figure 11. 2-D Structure of 0JT008

Adapted from PubChem, National Library of Medicine. Retrieved from **[**https://pubchem.ncbi.nlm.nih.gov/compound/2740173**]**

In another study, Olaleye et al. screened eight compounds, OJT001- OJT008, to investigate their antileishmanial *in vitro* effect. OJT006, OJT007, and OJT008, which were discovered to be potent against *Leishmania major* (*L. major*) promastigotes and amastigotes infected macrophage cells. OJT008 was previously discovered as a promising chemotherapeutic candidate against cutaneous leishmaniasis infection caused by *L. major* infection by inhibiting the enzyme methionine aminopeptidase1 of Leishmania major. The compound was a potent inhibitor of methionine aminopeptidase 1 of L. major in both *In vitro* and *In vivo* studies. The study also showed the compound is nontoxic to intraperitoneal murine macrophages, which suggests the safety level of the compound [Rodriguez et al., 2020]. In this dissertation project, we sought to characterize OJT008 as a novel inhibitor of *Mtb by determining* the effect against metal-activated MtMetAP1c and potency against both drug-sensitive-*Mtb* and MDR-TB strains of *Mtb.* We further performed an *in silico* study to further investigate the molecular interaction and binding score of OJT008 to both metal-activated MtMetAP1c. Our study validated the pharmacophore's metal specificity.

CHAPTER 3

DESIGN OF THE STUDY

Our study is focused on characterizing the effect of OJT008 against MtMetAP1c and *Mtb* culture. To achieve this objective, we cloned the target gene, cultured and overexpressed the target protein, purified it to near homogeneity, and the effect of OJT008 against metal activated MtMetAP1c activity was determined. An *in silico* study was conducted to investigate the binding characteristics of OJT008 to the divalent metals at the active site of the metalloprotease. We collaborated with Houston Methodist to screen the potency of our compound against both drug-sensitive *Mtb* and MDR- *Mtb* strains. We adopted the genetic cloning technique, molecular technique, biochemical enzymatic assay, computational study, and microbiological approach in this study.

Materials required:

Polymerase chain reaction (PCR) Machine (PTC-200 Peltier Thermal Cycler), Deoxynucleoside triphosphates (dNTPs): dATP, dCTP, dGTP, and dTTP, Taq polymerase from New England Biolabs ®, Genomic DNA from *Mycobacterium tuberculosis* strain H37Rv from ATCC catalog # 25618D-2™, Lot number: 70012901, 10x standard Taq reaction buffer, forward primer: 5'-GCGGGATCCCCTAGTCGTACCGCGCTC-3' (Batch number: WD08128249), reverse primer

5’-GCGTCGAGCTACAGACAGGTCAGGATC-3’ (Batch number: WD08128250) purchased from Sigma-Aldrich®, pET-28a(+) DNA purchased from Novagen (catalog number: 69864-3, Lot number: 3228343, BamHI and XhoI restriction enzyme purchased from Invitrogen®, (6X) Gel Loading Dye purchase from NEB (catalog # B7024S), T4 DNA Ligase Buffer (catalog# M0202S) purchased from NEB, L-Methionine para nitroanilide purchased from sigma Aldrich, CAS Number: 6042-04-2, the Qiagen Plasmid Plus Midi Kit from Qiagen inc, HisPur™ Ni-NTA Resin and isopropyl beta-D-thiogalactopyranoside (IPTG) were purchased from Thermo Fisher Scientific®, Subcloning Efficiency™ DH5α Competent Cells Catalog number: 18265017 purchased from Invitrogen®, One Shot™ BL21 Star™ (DE3) Chemically Competent E. coli, Catalog number: C600003, purchased from Thermofisher scientific, Middlebrook 7H9 broth, a Mtb cultures medium (purchased from Becton Dickinson). OJT008 compound was purchased from Molport® and were prepared in Dimethyl sulfoxide (DMSO) purchased from Sigma Aldrich. Below is the flow chart of my study design.

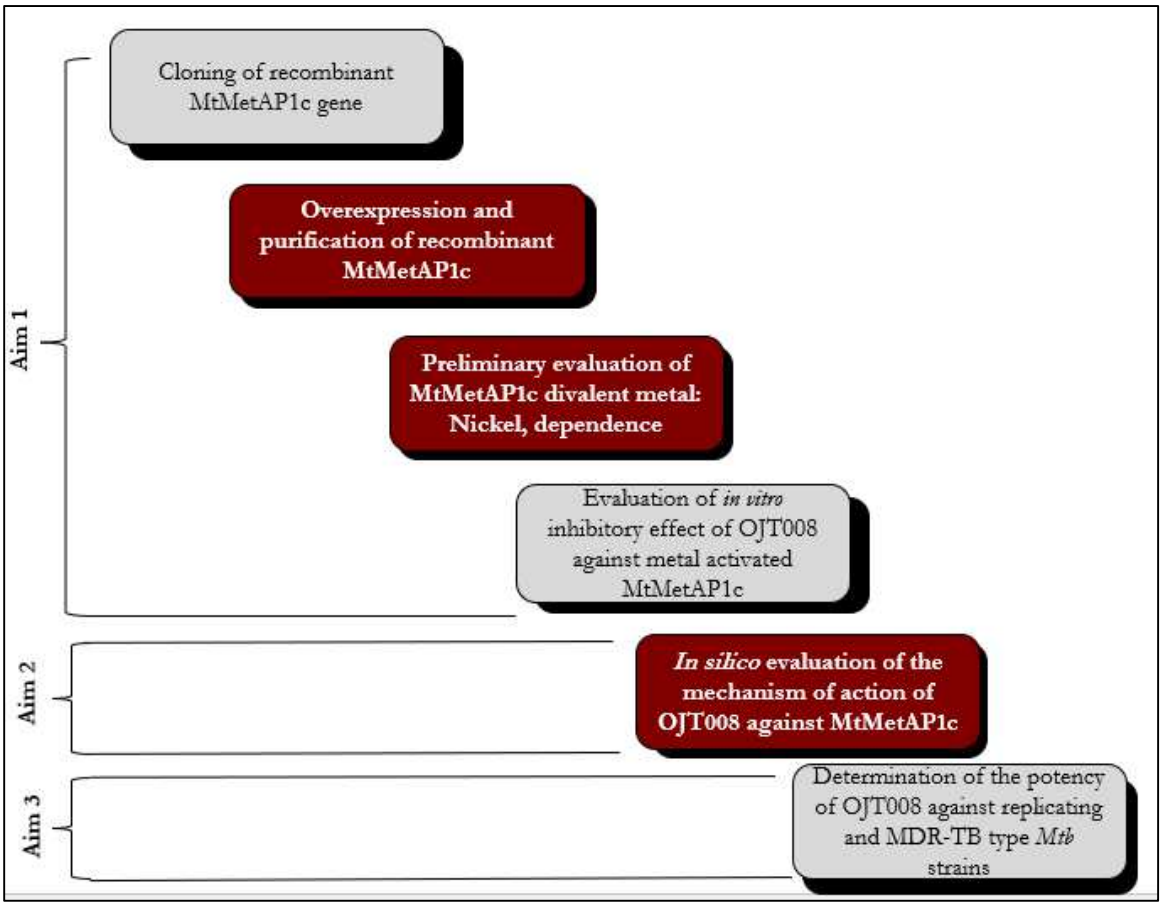


Figure 12: Flow chart of study design

3.1 Cloning and overexpression of MtMetAP1c

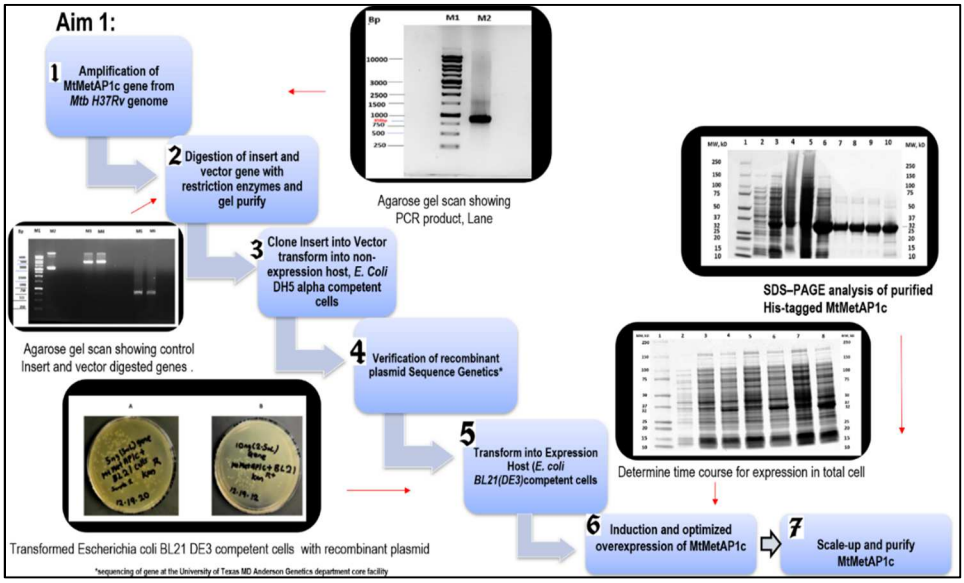


Figure 13. Outline for cloning and overexpression of MtMetAP1c

3.1.1 Amplification of MtMetAP1c gene from *Mtb* H37Rv genome

The study accomplished the aim of cloning, overexpressing, and purifying the expressed recombinant target protein, MtMetAP1c, with the N-terminal end of the sequence tagged with the 6x poly-histidine. The overexpression of MtMetAP1c was achieved by constructing a recombinant pET28a- MtMetAP1c using ligation technique. The amplified DNA produced a prominent band corresponding to the 858bp for MtMetAP1c, corresponding to the 1 kb DNA marker on agarose gel. The protocol was adapted from 'PCR Protocol for Taq DNA Polymerase with Standard Taq Buffer (M0273)- New

England Biolab' (https://www.neb.com/protocols/0001/01/01/taq-dna-polymerase-with-standard-taq-buffer-m0273). MtMetAP1c was polymerase chain reaction amplified from the genomic DNA of *Mtb* H37Rv using the forward and reverse primers;

5'-GCGGGATCCCCTAGTCGTACCGCGCTC-3'

and 5'-GCGTCGAGCTACAGACAGGTCAGGATC-3' respectively. .To prepare a 50µL PCR reaction, the following was prepared by adding the following to the PCR tube to first prepare a 3x mix. 47.625µL of autoclaved water, 7.5 µL (10x)10x Standard taq reaction buffer, 1.5µL of 10mM dNTPs, 1.5µL for both forward and reverse primers, and 0.375 µL of taq DNA polymerase. The mixture is briefly mixed, 20µL of the mix is aliquoted, and 5 µL (500 ng) of the *Mtb* strain H37Rv genome DNA.

Table 3. PCR Reaction setup.

Component	50µL reaction	Final concentration
10x Standard taq reaction buffer	3 x 2.5	1x
10mM dNTPs	3 x 0.5	200 µM
10 µM Forward Primer	3 x 0.5	0.2 µM
10 µM Reverse Primer	3 x 0.5	0.2 µM
Taq DNA Polymerase	3 x 0.125	0.325 units
Nuclease-free water	to 50 µL	to 50 µL

Adapted from PCR Protocol for Taq DNA Polymerase with Standard Taq Buffer (M0273). https://www.neb.com/protocols/0001/01/01/taq-dna-polymerase-with-standard-taq-buffer-m0273

The PCR mixture was placed in the PCR machine and set at a Standard 3-step PCR Cycling with the following steps: initial Denaturation at 94°C for 1 minute (1 cycle), Denaturation at 94°C for 1 minute (35 cycles), annealing at 61°C (35 cycles) (which was calculated using the NEB Tm Calculator, extension at 72 °C for (35 cycles). The mixture was finally held at 4°C. The thermocycling conditions for a PCR protocol is in table 4.

Table 4. The PCR thermocycling conditions

Step	Temperature	Time	Number of cycles
Initial denaturation	94°C	1 minute	1
Denaturation	94°C	1 minute	35
Annealing	61°C	1 minute	35
Extension	72°C	1 minute	35
Final extension	72°C	5 minutes	1
Hold	4°C	infinity	1

Adapted from PCR Protocol for Taq DNA Polymerase with Standard Taq Buffer (M0273). https://www.neb.com/protocols/0001/01/01/taq-dna-polymerase-with-standard-taq-buffer-m0273

DNA agarose gel electrophoresis:

After PCR, a qualitative analysis was performed using the agarose gel electrophoresis. 10µL of PCR sample consisting of 10µL amplified mix and 2µL of (6X) gel Loading dye was loaded on agarose gel and ran with 5µL of 1Kb DNA ladder to confirm amplification of the target gene. The conduction of electrophoresis was at 65V for 40 minutes, and the image was captured with a chemidoc camera.

Purification of amplified target DNA

The amplified genes were purified using QIAquick PCR Purification Kit (50) (cat. nos. 28104), adopting the QIAGEN® PCR purification kit protocol (pages 22-23). 200µL (5 volumes) of Buffer PB was added to 40µL (1 volume) of the PCR reaction and mixed. The mixed sample was added into a QIAquick column and placed in a 2mL collection tube provided. The PCR sample was centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded. 750µL of Buffer PE was added to the QIAquick column and centrifuged for 1 minute. The flow-through was discarded, and the tube was centrifuged for an additional 1 minute. The QIAquick column was placed in a 1.5 ml microcentrifuge tube and eluted with 30 µL Buffer EB. The PCR eluted was collected, and the concentration was determined. The concentration of the eluted PCR mix was 25.7ng/µL, and the volume eluted was 30 µL

3.1.2 Double restriction digestion of vector and purified target DNA

BamhI and XhoI restriction enzymes were used to digest the DNA fragment and vector, pET28a. The New England Biolabs (NEB) protocol was used for the double restriction digestion protocol [https://nebcloner.neb.com/#!/protocol/re/double/BamHI,XhoI]. The digestion of both the target and plasmid were performed in 2 separate reactions. The components were added in the other from top to bottom in Tables 6 A and B.

For the restriction digestion of the eluted PCR mix, 16µL Autoclaved water, 5µL of 10x NEBuffer 3.1, 2µL (40 units) of XhoI, 2µL (40 units) of BamHI, and 25µL (642.5ng) of

PCR mixture (Table 5A) mixed by gently flickering the tube, spun briefly, and incubated for 1 hour at 37°C. The pET28a was digested by adding 40µL Autoclaved water, 5µL of 10x NEBuffer 3.1, 2µL (40 units) of XhoI, 2µL (40 units) of BamHI, and 10µL (500ng) of pET28a (Table 5B) mixed by gently flickering the tube, spun briefly, and incubated for 1 hour at 37°

Table 5. Restriction digestion setup for A. eluted PCR mix and B. pET28a restriction.

A	
Component	**Volume (µL)**
Autoclaved water	40
10x Neb 3.1 buffer	5
BamhI	2 (40 units)
XhoI	2 (40 units)
MtMetAP1c DNA	25 (642.5 ng)

B	
Component	**Volume (µL)**
Autoclaved water	40
10x Neb 3.1 buffer	5
BamhI	2 (40 units)
XhoI	2 (40 units)
pET28a	10 (500 ng)

Adapted from " Restriction Enzyme Double Digestion", NEBcloner New England Biolab Retrieved from https://nebcloner.neb.com/#!/protocol/re/double/BamHI,XhoI

Agarose gel electrophoresis of digested genes:

Digested pET28a and MtMetAP1c were loaded on agarose gel. 10µL of (6X) Gel Loading Dye, and 50µL of each digested DNA. 30µL of each is loaded into two lanes of the agarose gel: Lane 3 and 4: digested pET28a (250ng per lane); Lane 5 and 6: digested MtMetAP1c (321.25ng per lane) . Lane 2: Uncut pET28a (7µL autoclaved water, 2µL of 6x Gel Loading Dye, and 150ng (3µL) of uncut pET28a), Figure 19. The gel ran for 90 minutes at 65 volts.

The next step was to extract the digested vector and target gene from the gel using the Qiagen QIAquick gel extraction kit (cat # 28704) (pages 30 to 32) and adopt the kit's protocol. After electrophoresis, the bands on the gel containing the digested pET28a (M3 &M4) and digested amplified target gene (M5 &M6) was excised from the agarose gel. The excised bands were weighed; 409 mg of (M3 &M4) gel band and 473mg of (M5 &M6) gel band. Three volumes of buffer QG (100mg approximately equals 100µL) that is 1227 µL buffer QG for excised (M3 &M4) and 1419 µL of buffer QG was added to excised (M5 &M6). Both excised gels were incubated at 50°C for 10 minutes to dissolve the gel. One volume of isopropanol was pipetted to both excised gel samples, that is, 409µL of isopropanol for (M3 &M4) dissolved mixture, and 473µL of isopropanol for (M5 &M6) dissolved mixture. Mix and place a QIAquick spin column in a provided 2 ml collection tube. The samples were added to the QIAquick column and then centrifuged at 13,000rpm for 1 minute. The flow-through was discarded and repeated until all the samples were filtered. 0.75 mL of Buffer PE was added into the QIAquick column, and the centrifuge

allowed it to stand for 5 minutes, then centrifuged at 13,000rpm for 1 min. The flow-through was discarded and further centrifuged for another 1 minute at 13,000rpm. The QIAquick column was placed in a 1.5 ml microcentrifuge tube, and the DNA was eluted with 50 µL of Buffer EB and centrifuged in the column for 1 minute. The concentrations of the eluted DNAs were measured using a nanodrop: eluted MtMetAP1c gene: 10.85ng/µL, volume of eluted MtMetAP1c: 28µL and eluted pET28a: 9.4ng/µL, volume of eluted MtMetAP1c: 28µL

3.1.3 Ligation of digested target DNA and vector

Ligation was carried out using two molar ratios of the target DNA to vector: 5:1 and 7:1 adopting the NEB' Ligation Protocol with T4 DNA Ligase (M0202)'. https://www.neb.com/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202. The NEB calculator, 'https://nebiocalculator.neb.com/#!/ligation,' was used to calculate molar ratios. Insert DNA length: 858bp, vector DNA length: 5369bp, vector DNA mass: 100 ng 111.9ng of eluted MtMetAP1c gene mass and 100ng eluted pET28a gene mass was used for the 7:1 molar ratio ligation setup, while 39.95ng of eluted MtMetAP1c gene mass and 50ng eluted pET28a gene mass was used for the 5:1 Molar ratio.

Table 6. Ligation set up for 7:1 DNA molar ratio.

Component	20 µL reaction
T4 DNA Ligase Buffer (10X)	2 µL
MtMetAP1c gene	10.64 µL (111.9 ng)
pET28a	10.63 µL (100 ng)
T4 DNA Ligase	1µL
Nuclease-free water	to 20 µL

Adapted from Ligation Protocol with T4 DNA Ligase (M0202) Retrieved from https://www.neb.com/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202

The outline for components added for the Ligation using a 20 µL reaction for both molar ratios are stated in Tables 7 and 8. The components were gently mixed and centrifuged briefly, and incubated overnight at 16°C

Table 7. Ligation set up for 5:1 DNA molar ratio

Component	20 µL reaction
T4 DNA Ligase Buffer (10X)	2 µL
MtMetAP1c gene	3.68 µL (39.95 ng)
pET28a	5.32 µL (50 ng)
T4 DNA Ligase	1µL
Nuclease-free water	to 20 µL

Adapted from Ligation Protocol with T4 DNA Ligase (M0202). Retrieved from https://www.neb.com/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202

3.1.4 Transformation of *E.coli* cells, non-expression, and expression host, with the recombinant DNA

The recombinant plasmid, pET28a-MtMetAP1c, was first used to transform the non-expression host, Escherichia coli DH5 alpha competent cells, the gene clone was extracted and purified from the non-expression host cell using the The QIAGEN Plasmid Plus Midi Kit and adopting the kit protocol. Both molar ratios of ligated genes, recombinant DNA, were used to transform a non-expression bacteria competent cell, and the Thermo Fisher scientific Transforming Chemically Competent Cells protocol WAS adopted. The competent cells, DH5α Competent Cells, were put into four aliquots of 50 µL and pipetted into four vials left on the ice. For the ligation reaction vials, add 4µL of ligation mix (7:1 molar ratio) and 2µL of ligation mix (5:1 molar ratio). Incubate on ice for 30 minutes. The mix is incubated for 30 seconds in the 42°C water bath then placed on ice after heat shock. 250 µl of pre-warmed Listeria broth (LB) medium was added to each vial and is shaken at 225 rpm for exactly 1 hour at 37°C. 200 µL is plated on an LB agar plate with 50µg/mL Kanamycin (Catalog number: J60540.EQF); the LB agar plate is inverted and incubated at 37°C overnight. For the control setup, 1µL of pET28a for vector control and 2.5µL of pUC19 control in place of the ligated mix. The same procedure is *carried* out for the control setup. The puC19 control mix is spread on an LB Agar plate with 100µg/mL Ampicillin (Catalog number: J63197.EQF)

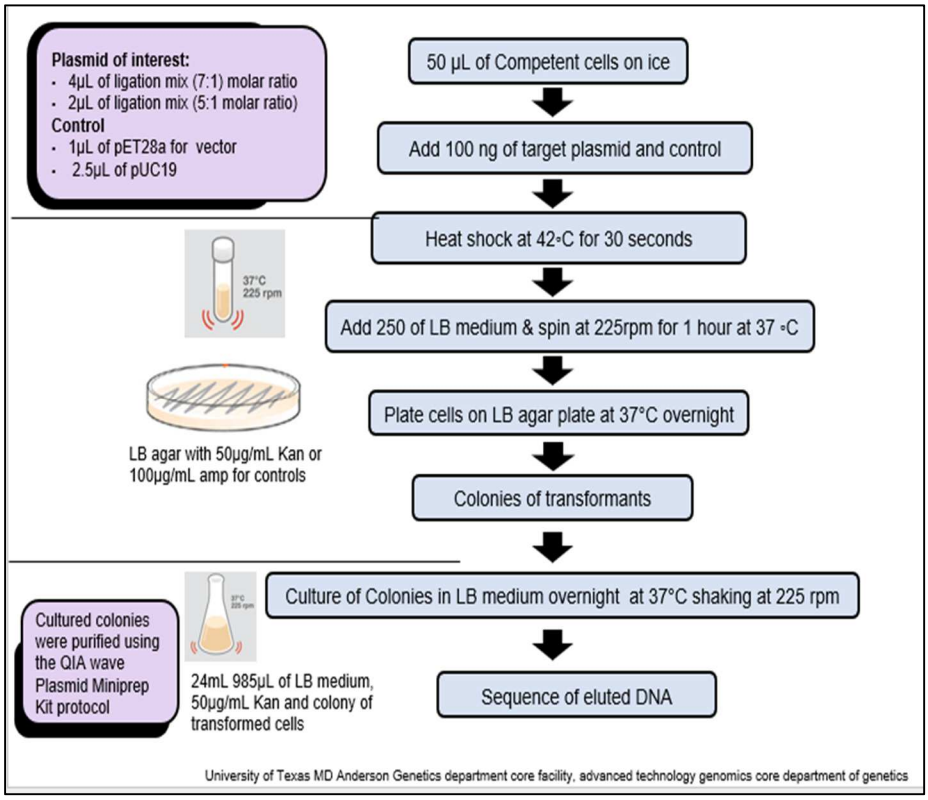


Figure 14. Transformation of non-expression DH5α Competent Cells with the recombinant plasmid. Kan: Kanamycin, amp: ampicillin.

3.1.5 Culturing of transformed non-expression competent cells

The colonies of transformed DH5 alpha competent cells with recombinant genes (MtMetAP1c-pET28a grown on the plates were collected and cultured in LB medium. Two colonies from the 7:1 molar ratio plate and one colony from the 5:1 molar ratio plate. One colony is added to 25mL of sterile LB culture containing 30ug/mL of Kanamycin by adding 15µL of 50 mg/mL kanamycin into 24mL 985µL of LB. The inoculated medium was incubated overnight at 37°C, shaking at 225 rpm. The following morning the culture is

divided into 2, that is, four aliquots of 12mL for the 7:1 molar ratio culture and two aliquots of 12mL for the 5:1 molar ratio culture. The protocol was adapted from 'QIAwave Plasmid Miniprep Handbook. For purification of molecular biology–grade DNA', page 21-24

https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiawave-plasmid-kits/. The overnight culture was centrifuged at 5000 rpm for 10 minutes at 20°C. The bacterial cell pellets obtained were resuspended in 600 µL buffer P1 mixture (10 µL of lyse blue added to 10mL buffer P1: for a final dilution of 1:1000) and agitated until the pellet was mixed. 250 µL of Buffer P2 was added to the cell suspension and mixed by inverting about 6 times. 350 µL of buffer N3 was added to the mixture and mixed by inverting the tube until a colorless suspension was obtained. The suspension was spun for 10 minutes at 13,000rpm, and the filtrate, 800µL, was collected, loaded to the Spin Column, and centrifuged for 1 minute at 13,000rpm. The flow-through was discarded, and 750 µL of Buffer PB was added to the QIAprep 2.0 Spin Column and washed for 1 minute at 13,000rpm. The QIAprep 2.0 Spin Column was added to a 1.5mL Eppendorf tube, and 100 µL Buffer EB was added to the column and allowed to stand for 1 minute before spinning for another 1 minute at 13,000rpm. The eluted plasmid was collected as the flow-through from the column. The concentration of the eluted plasmid derived from the transformed DH5 competent cells was 55.6ng/mL and 108.5ng/mL for both colonies 1 and 2 from the 7:1 molar ratio recombinant plasmid. The concentration from eluted plasmid for colony three from 5:1 molar ratio recombinant plasmid was 100.5ng/mL. 20µL aliquots of the eluted DNA (plasmid) was

sequenced by the University of Texas MD Anderson Genetics department core facility, advanced technology genomics core department of genetics. The sequence result obtained showed that the 5:1 molar ratio recombinant DNA was free of the mutation. This was confirmed by using the NCBI blast software to perform sequence alignment analysis. The mutation-free plasmid was further used to transform an *e. coli* expression host.

3.1.6 Transformation of *E.coli* BL21 (DE3) competent cells with the recombinant DNA

The E. coli expression host, Escherichia coli BL21 DE3, was transformed with the clones of mutant free recombinant plasmid for the overexpression of the MtMetAP1c protein. The purchased kit protocol was adopted for the transformation protocol. Manual: OneShot BL21(DE3), One Shot BL21(DE3) pLysS and One Shot BL21(DE3) pLysE. 'Basic Transformation Procedure' (pages 5- 6)

(https://www.thermofisher.com/order/catalog/product/C600003?SID=srch-srp-C600003)

The eluted plasmid (without mutation) from the 5:1 molar ratio gene was first diluted from 106.5ng/µL to make a final concentration of 1ng/µL and 4ng/µL. 0.275µL of the stock eluted plasmid was added to 29.724µL Buffer EB to produce 1ng/µL concentration, while 0.737µL of stock eluted plasmid was diluted with 19.262µL of buffer EB to produce 4ng/L final concentration. 5µL of 1ng/µLand 2.5µL of 4ng/µL was used to transform 50µL *E. coli* BL21 (DE3) competent cells, respectively. For the control, 1uL (10 pg) of puC19 and

1µL (10 ng) pET28a were added to 50µL of the competent cells. The mixture of gene and *E.coli* cells was gently mixed and incubated on ice for 30 minutes. The mixture was heat-shocked at 42 °C in a water bath for 30 seconds, then quickly incubated on ice 250 µL of pre-warmed LB medium was added to the vial of the mixture and shook at 225rpm for 1 hour at 37°C. 200µL of the bacteria culture transformed with 5ng DNA and 100µL of the bacteria culture transformed with 10 ng DNA is plated on an LB Agar plate with 50µg/mL Kanamycin, respectively. 50µL (500pg) of pUC19 and 100µL (100 ng) of pET28a was spread on an LB agar plate containing 100 µL/mL ampicillin and 50µLug/mL Kanamycin, respectively. The LB agar plates were inverted and incubated overnight at 37°C. A single colony was picked the following morning and stored in 25% glycerol, and kept in -80°C.

3.2 Overexpression and purification of MtMetAP1c

3.2.1 Small scale MtMetAP1c overexpression

A mini-pilot overexpression of the protein to determine the optimum post-induction harvest of cells time was conducted. 4mL 995µL of LB media was pipetted into tubes 1, 2,3,4,5, 6, 7, and 8.

Table 8. Small scale MtMetAP1c overexpression post induction time set up

Tube	Harvest time Post induction (hour)
1	1mL *E. coli* culture at 0 hour prior to induction
2	1mL uninduced *E. coli* culture 2 hours after induction
3	1mL induced *E. coli* culture 2 hours post induction
4	1mL uninduced *E. coli* culture 4 hours after induction
5	1mL induced *E. coli* culture 4 hours post induction
6	uninduced *E. coli* culture 8 hours post induction
7	1mL induced *E. coli* culture 8 hours after induction

Scope BL21 DE3 glycerol stock into a 12mL tube containing 5mL and 30 µg\mL kanamycin. This culture was incubated at 37◦C and shaken in a Brunswick shaker at 275rpm overnight. The following morning 100µL (1:50) of the overnight culture was inoculated into tubes 1, 2, 3, 4, 5, 6, 7, and 8 LB containing 30 µg\mL kanamycin and further incubated at 37◦C, shaking at 275rpm. 1mL of a sample is taken from tube 1 Immediately prior to induction of cells. The growth of the *E.coli* cells was monitored until an optical density ($O.D_{600}$) of 0.6-0.8 was reached. At an $O.D_{600}$ of 0.61. At this $O.D_{600}$ of 0.61, 5µL of 1M Isopropyl ß-D-1-thiogalactopyranoside (IPTG), at 1 mM IPTG final concentration, was added to tubes 3, 5, and 7 and incubated at 16◦C shaking at 280rpm.

1mL of samples was taken from tubes 2 and 3 2 hours after induction. After 4 hours of induction, 1mL samples were taken from tubes 4 and 5. 1 mL sample was taken from tubes 6 and 7 8 hours after induction. The samples were spun at 3500 rpm, the supernatant was discarded, and the pellets were dissolved with 50 µL buffer (50mM HEPES, 100mM NaCl, pH 7.5), vortexed, and centrifuged at 8,100rpm for 2 minutes. 20 µL of 2x Lammelli buffer was added to 20 µL of the cells and heated for 5 minutes at 95◦C. 10 µL of the samples were loaded on a gel, and the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to qualitatively determine the optimum post expression harvest time.

3.2.2 Scale-up of MtMetAP1c overexpression

A single colony of BL21-DE3 cells obtained from small scale culture plate was grown overnight at 37◦C, 275rpm, in 100mL LB containing 30 µg\mL kanamycin. The next morning, the overnight culture was cultured in 4 Liters of LB media containing 30 µg\mL kanamycin at 37◦C at the same speed of rotation. 1 mL sample of the uninduced culture was taken for SDS analysis. The incubated culture was monitored until an $O.D_{600}$ of 0.6-0.8 was reached. The target protein is subsequently induced by the addition of 1mM IPTG final concentration (4mL of 1M IPTG) at an $O.D_{600}$ of 0.678 and further cultured at 16 ◦C for 8 hours at 280 rpm before the harvest of cells. The 8 hours post-induction was selected for the harvest of cells based on previous preliminary mini-pilot overexpression. 1mL of the induced cells was taken for gel analysis, and the induced cells were harvested in 1X

Phosphate-Buffered Saline (PBS). The pellets were incubated in 30mL ice-cold lysis buffer (0.4% Triton X-100, 1xPBS, 10% glycerol, and EDTA-free protease inhibitor tablets, and 20mM Imidazole) and incubated on ice for 1 hour prior to sonication of the cells. The cells were sonicated in a lysis buffer using the Branson SFX250 Sonifier. The cells were sonicated, with 35 cycles for 15 sec with 30 seconds pause on ice between each burst.

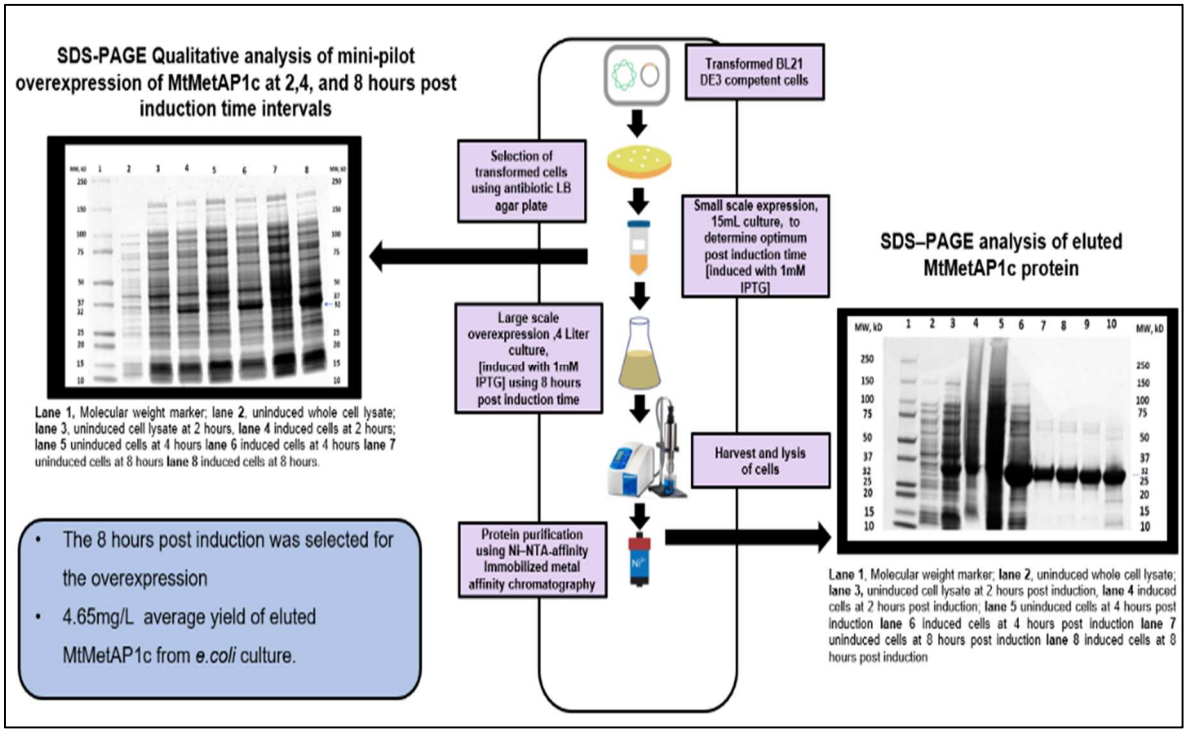


Figure 15. Overview outline for overexpression and purification of MtMetAP1c

The lysate was Centrifuged at 5000g for 1 hour at 4°C to pellet any insoluble material. The supernatant was gently collected without disturbing the pellet. 1mL of both supernatant and pellet was reserved at 4°C for SDS/PAGE analysis. The protocol for the purification was adopted with slight modifications from TALON® Metal Affinity Resins User Manual,

pages 29-31 (http://www.takara.co.kr/file/manual/pdf/pt1320-1.pdf). The harvested cells were in 1X PBS and sonicated in a lysis buffer (0.4% Triton X-100, 1xPBS, 10% glycerol, and EDTA-free protease inhibitor tablets, and 20mM Imidazole. The cell lysate was centrifuged at 5000g for 1 hour and the clarified protein was retrieved for the purification process. The supernatant was further purified through the Immobilized metal affinity chromatography (IMAC) technique. The MtMetAP1c clarified protein was incubated for 1 hour to pre-equilibrate His Pur™ Nickel Nitrilotriacetic Acid (Ni-NTA) Resin, a sample of beads after protein binding was taken for SDS-

PAGE analysis. The beads were washed first and the second time with wash buffer (50 mM HEPES [pH 8.0], 100mM NaCl, 10% glycerol, and 20mM Imidazole), then finally washed the third time with wash buffer (50 mM HEPES [pH 8.0], 100mM NaCl, 10% glycerol, and 40mM Imidazole) and eluted with elution buffer containing 75mM Imidazole Figure 19. The elution was repeated twice. 1mL of each elution and beads after final elution was taken for SDS-GEL analysis. The average yield of MtMetAP1c was determined with the Thermo Scientific NanoDrop UV-Vis spectrophotometer, which was 4.65mg per liter of *E.coli* culture. The efficiency of the expression and purification protocol of the protein was determined by running an SDS-PAGE of the cell lysate and the supernatant. The average yield, quantified with the Thermo Scientific NanoDrop UV-Vis spectrophotometer, was 4.65mg of mtMetAP1c per liter of *E.coli* culture.

3.3 Preliminary evaluation of MtMetAP1c divalent metal dependence

Firstly, the activity of eluted MtMetAP1c was determined. An enzymatic assay screen to determine the NME ability of the cobalt ion activated MtMetAP1c and the concentration of the protease required for optimum activity was performed. The enzymatic assay was a slight modification of the method adopted by Zhou et al and Olaleye et al [Zhou et al., 2000; Olaleye et al., 2010], by using a monopeptide substrate and the assay incubated for 1 hour. The reaction was performed at 37◦C in a 96-well plate and activity monitored on a spectrophotometer at 405 nm. Each well had a total of 100 µL reaction containing 50mM HEPES buffer (pH 8), 100mM NaCl, 15 µM CoCl2, 100 µg/mL BSA, 1mM chromogenic substrate (L-Methionine para nitroanilide) purchased from sigma Aldrich, CAS Number: 6042-04-2. The MtMetAP1c cleaves the L-Methionine para nitroanilide to form methionine and the chromogenic p-Nitroaniline. p-Nitroaniline absorbs the white light spectrum at 405 nm. The 100 µL reaction was incubated for 1 hour at 37°C in a 96-well plate, and activity was monitored on a spectrophotometer [Molecuar Devices SpectraMax iD3 Multi-Mode Microplate Reader] at 405 nm. Each well contains 50mM HEPES, 100mM NaCl buffer (pH 8), 15 µM $CoCl_2$, 100 µg/mL BSA, 1mM chromogenic substrate (L-Methionine para nitroanilide), and purified MtMetAP1c. The concentration range for the enzyme in each plate: 4.19, 8.38, 10.48, 12.58, 16.77, and 20.96 µM (Table 8). A unit of enzyme activity is defined as 1 µM of the substrate product, nitroanilide per minute under the stipulated experimental condition.

Table 9. The concentrations of MtMetAP1c in each assay well for the MtMetAP1c divalent metal dependence

Molecular weight (kDa)	Concentration of MtMetAP1c (µg/uL)	Volume of MtMetAP1c (µL)	Mass of MtMetAP1c (µg)	Assay volume per well (uL)	Concentration of MtMetAP1c per well (µg/uL)	Concentration of MtMetAP1c per well (µM)
32	2.68	5	13.42	100	0.13	4.19
32	2.68	10	26.83	100	0.27	8.38
32	2.68	12.25	33.54	100	0.34	10.48
32	2.68	15	40.25	100	0.40	12.58
32	2.68	20	53.66	100	0.54	16.77
32	2.68	25	67.08	100	0.67	20.96

The absorbance was measured with the spectrophotometer at 405 nm after 1-hour incubation. An enzyme activity calibration curve consisting of the concentration of the enzyme at the x-axis and the corresponding concentration absorbance on the Y-axis was plotted to determine (i) if the enzyme is active (II) the optimum concentration of the enzyme that produces an N-terminal methionine excision from the substrate to produce the chromogenic functional group, p- nitroanilide.

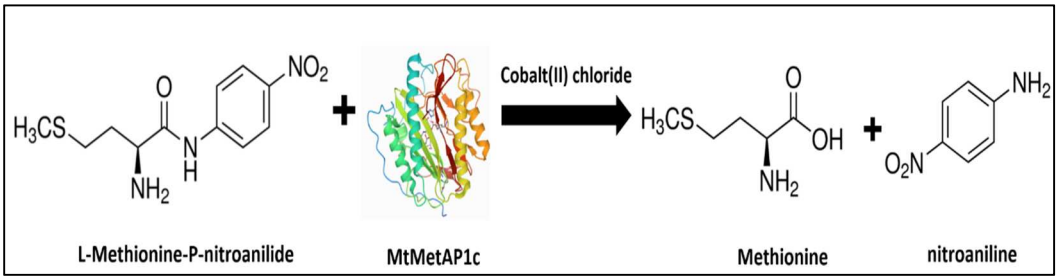


Figure 16. Diagrammatic representation of the in vitro enzymatic assay.

Adapted from "Two continuous spectrophotometric assays for methionine aminopeptidase", by Zhou, Y., Guo, X. C., Yi, T., Yoshimoto, T., & Pei, D. (2000). Analytical Biochemistry, 280(1), 159–165. https://doi.org/10.1006/abio.2000.4513

A preliminary MtMetAP1c metal dependence activity was conducted. The cobalt (II) and nickel (II) ions were determined by assaying the catalytic effect of MtMetAP1c by adapting the same colorimetric assay stated above [Zhou et al., 2000; Olaleye et al., 2010]. The extinction coefficient of p-nitroaniline was 9.96 mM^{-1} cm^{-1} and was used for the calculation of enzyme activity. The activity of MtMetAP1c is expressed as the amount in µM of p-Nitroanilide released per minute per mL protein.

Cobalt (II) chloride anhydrous (catalog number: 7791-13-1) purchased from sigma Aldrich and a stock of 10mM in aqueous solution. A metal was serially diluted in water, and the final concentration of 0.1, 1, 10, 100, 1000, 10,000 µM and volume 10 µL per well. The

specific divalent metal concentrations that catalyzed the highest activity of MtMetAP1c were selected for subsequent in vitro compound inhibitory effect

Table 10. Activation of MtMetAP1c using different concentrations of (A) cobalt (II) chloride and (B) nickel (II) chloride

A			B		
Concentration of $CoCl_2$ (µM)	**Absorbance OD_{600}**	**Enzyme activity (µM mL^{-1} min^{-1})**	**Concentration of $NiCl_2$ (µM)**	**Absorbance OD_{600}**	**Enzyme activity (µM mL^{-1} min^{-1})**
No metal	0.16	72.03	No metal	0.16	87.02
0.1	0.14	62.80	0.1	0.12	52.03
1	0.12	51.32	1	0.13	63.62
10	0.14	62.66	10	0.08	35.50
100	0.61	267.81	100	0.59	258.82
1,000	0.35	152.85	1,000	0.25	108.06
10,000	0.12	53.89	10,000	0.24	105.70

screens. The test wells contain 33.4 µg (10.48 µM) MtMetAP1c, buffer (50mM HEPES, 100mM NaCl, 100 µg/ mL BSA pH 7.5). The microplate was incubated at 37◦C for 1 hour,

the absorbance was measured at 405 nm using a spectrophotometer, and the corresponding enzyme activity was calculated [Table 10 A and 10 B].

Extinction coefficient of p-nitroaniline 9.96 mM^{-1} cm^{1}, pathlength in a 96 well microplate assay can be assumed to be around 2–5 mm (0.5cm)

A plot of the *in vitro* activity of MtMetAP1c against the corresponding $NiCl_2$ was performed. The metal control had no $NiCl_2$, the blank had no enzyme, and the controls were subtracted from the corresponding assay reaction. All experiments were performed in duplicates. An identical preliminary enzyme dependence screen was conducted using Nickel (II) chloride hexahydrate (catalog number: CAS Number: 7791-20-0) as a cofactor. The concentration of the metals that activated MtMetAP1c the most was used to perform the compound inhibitory effect screen.

3.4 Evaluation of *in vitro* inhibitory effect of OJT008 against divalent nickel and cobalt activated MtMetAP1c

The half-maximal inhibitory concentration (IC_{50}) of OJT008 for MtMetAP1c was evaluated. Based on the optimum activity obtained from the enzyme using Nickel chloride and cobalt (II) chloride, MtMetAP1c is activated with 100 µM $CoCl_2$ and 100 µM $NiCl_2$. 50mM stock of the test compound in DMSO was prepared and serially diluted in DMSO to obtain a final concentration of 500 µM to 1 µM in each assay well in a 96 well plate.

The reaction was carried out in triplicates. Each reaction well contains a buffer (100 mM NaCl, 50 mM HEPES buffer (pH 8), 100 µg/mL BSA, and 100 µM of $CoCl_2$. The reaction was incubated for 20 minutes at 37°C, then subsequently incubated with the substrate for an hour at the same temperature. The reaction was monitored at a wavelength of 405 nm. Drug-free and DMSO media were used as controls. The background hydrolysis was corrected, and the data were analyzed with four-parameter (variable slope) equations using GraphPad Prism software. The identical assay was repeated using 100 µM of $NiCl_2$. The reaction was carried out in triplicates.

3.5 *In silico* evaluation of the mechanism of action of OJT008 against MtMetAP1c

Molecular Docking and Simulation

MtMetAP1c Acquisition and Preparation

X-ray crystal structures of Nickel and Cobalt ions bound MtMetAP1c, PDB code: 3IU8 and PDB code: 1YJ3 were obtained from the RSCB Protein Data Bank [Lu et al., 2010b; Addlagatta et al., 2005]. The structures were then prepared on the UCSF Chimera software package [Yang et al., 2012]; the proteins were prepared by removing water molecules, nonstandard naming, and protein residue connectivity. The 3-D structure of OJT008 was prepared on the Avogadro software package [Hanwell et al., 2012].

Molecular Docking

Autodock vina available on Chimera version 1.14 was used for molecular docking analysis (Trott and Olson, 2010), with default docking parameters. Before docking, Gasteiger charges were added to the molecules, and the non-polar hydrogen atoms were merged to

carbon atoms. The molecules were then docked into the proteins' binding pocket, by defining the grid box with a spacing of 1 Å each and size and (18× 31 × 21) and (21 × 27 × 17) pointing in x, y and z directions, respectively. The protonation state of the target was performed before docking calculations. Exhaustiveness number eight was used. The molecular docking scores of OJT008 for both Nickel and Cobalt ions were estimated.

3.6 Determination of the potency of OJT008 against drug-sensitive *Mtb* and Multidrug resistant-*Mtb*

OJT008 (purchased from Molport) potency against both *Mtb* cells, drug-sensitive *Mtb* type; strain CDC 1551 and MDR-TB type; *Mtb* HN 3409 strain, were determined using serial dilution broth. The compound screen was conducted in collaboration with Houston Methodist Research Institute. A 5mL 7H9 medium was modified to produce a working culture medium by pipetting the 4807.5 µL 7H9 medium to 90 µL of 2 % glycerol, 2.5 µL of 0.05% Tween-80. The medium was further modified by adding 10% albumin /dextrose complex. The *Mtb* cells were first grown to an Optical density (O.D) of 1.0. *Mtb* CDC 1551 and *Mtb* HN 3409 were grown to an OD of 1.07 and 1.01, respectively. The culture was further diluted to 1:10 dilutions by adding the 100µL of the *Mtb* culture (O.D of 1) with 900µL of 7H9 medium. The bacteria cells were further diluted to 1:100 dilution with the 7H9 medium by adding 300µL of *Mtb* culture (OD of 1:10) into 2700µL 7H9 medium to produce a 1:100 dilution. OJT008 was serially diluted in DMSO to produce 5, 1, 0.5,

0.25, 0.1, 0.05, 0.025, 0.0125, and 0.00625 mg/ mL. 50 µL of serially diluted OJT008 was added to the culture medium was added to the culture medium described above to produce

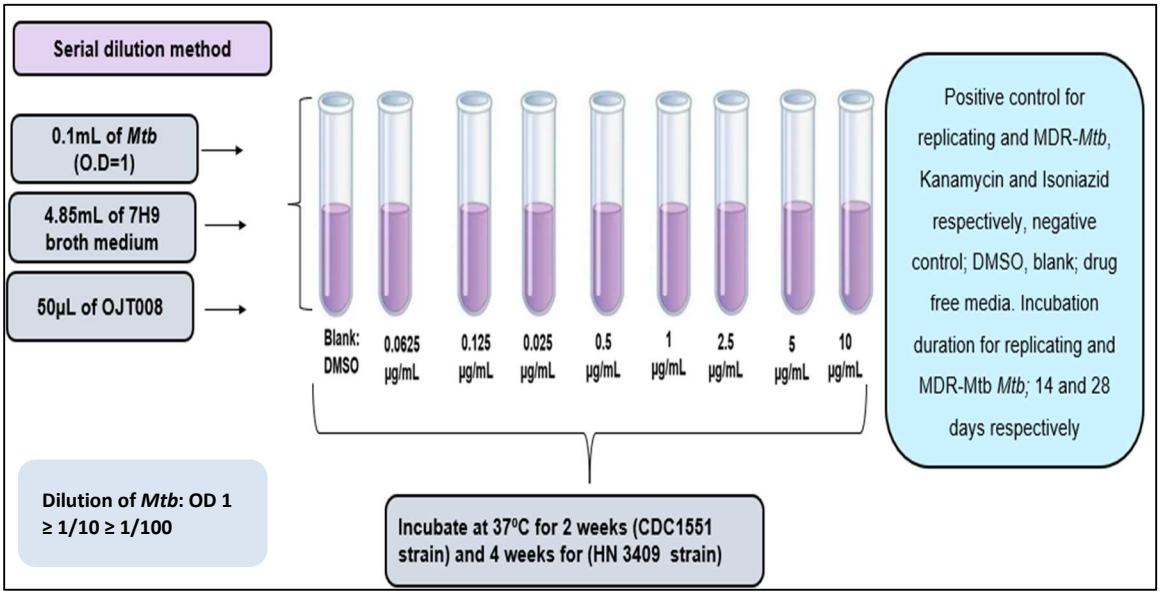


Figure 17. Determination of minimum inhibitory concentration of OJT008 in Multi drug resistant *Mtb*

a corresponding concentration of 50, 10, 5, 2.5, 1, 0.5, 0.25, 0.125, and 0.0625 µg/mL final concentration and incubated at 37°C. Kanamycin and Isoniazid were used as the positive

control for the MDR-TB and drug-sensitive *Mtb* and, respectively. OJT001 was used as a reference control compound. The blank control had 50 µL DMSO without a test compound. The reaction was carried out in duplicates, and CDC 1551 strain was incubated for two weeks while HN 3409 was incubated for four weeks. The incubated culture was visually observed to determine the presence or absence of *Mtb* growth

Table 11. Outline for the setup for compound antibacterial screen

	Compound screen (µL)		**Reference control (µL)**		**Positive control (µL)**		**Negative control (µL)**	
	CDC 1551 strain	**HN 3409 strain**	**CDC 1551 strain**	**HN 3409 strain**	**CDC 1551 strain**	**HN 3409 strain**	**CDC 1551 strain**	**HN 3409 strain**
OJT008	50	50	__	_	__	__	__	__
OJT001	__	__	50	50	50	50	__	__
Kanamycin	__	__	__	__	__	50	__	__
Isoniazid	__	__	__	__	50	__	__	__
7H9 broth	4850	4850	4850	4850	4850	4850	4850	4850
DMSO	__	__	__	__	__	__	50	50
***Mtb* culture**	100	100	100	100	100	100	100	100

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Amplification of MtMetAP1c gene from *Mtb* H37Rv genome

The study accomplished the aim of cloning, overexpressing, and purifying the expressed recombinant target protein, MtMetAP1c, with the N-terminal end of the sequence tagged with the 6x poly-histidine. To successfully characterize OJT008 as an inhibitor of *Mtb,* MtMetAP1c was generated through cloning, and the protease was overexpressed, purified to its homogenous state, and *in vitro* activity determined. The amplified gene produced a prominent band corresponding to the 858bp for MtMetAP1c, corresponding to the 1 kb DNA marker on agarose gel (Figure 18).

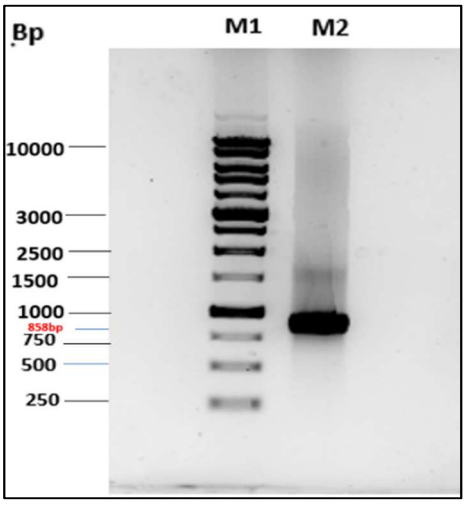


Figure 18. Agarose gel scan showing PCR product

Lane 1 (M1): 5µL of 1Kb DNA ladder, Lane 2 (M2): amplified MtMetAP1c gene.

The overexpression of MtMetAP1c was achieved by constructing a recombinant pET28a-MtMetAP1c using digestion enzymes (Figure 19)

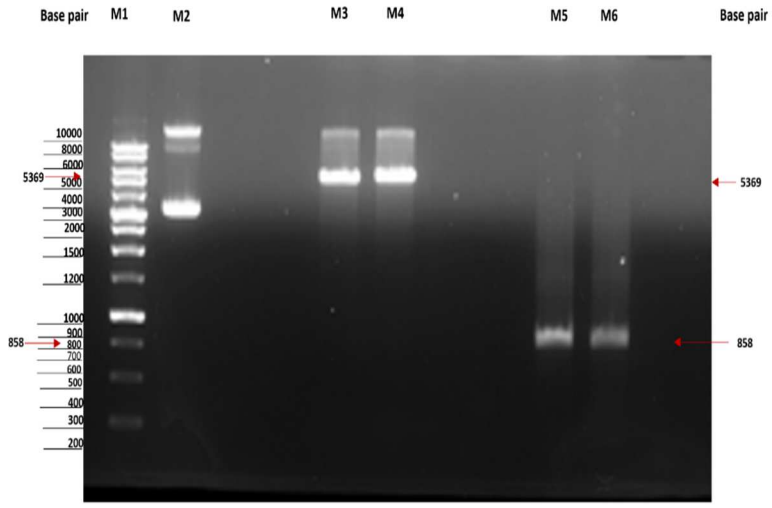


Figure 19. Agarose gel scan showing controls and digested DNA.

Lane 1 (M1):5µL of 1Kb DNA ladder, Lane 2 (M2): undigested vector; pET28a (150 ng), Lane 3 & 4 (M3 &M4): digested vector (250 ng each), Lane 5 & 6 (M5 & M6): digested amplified target gene (321.25ng) each.

The recombinant DNA was successfully used to transform Escherichia coli DH5 alpha competent cells. A colony from the purified recombinant plasmid, 5:1 insert gene to vector molar ratio, was obtained with no mutation.

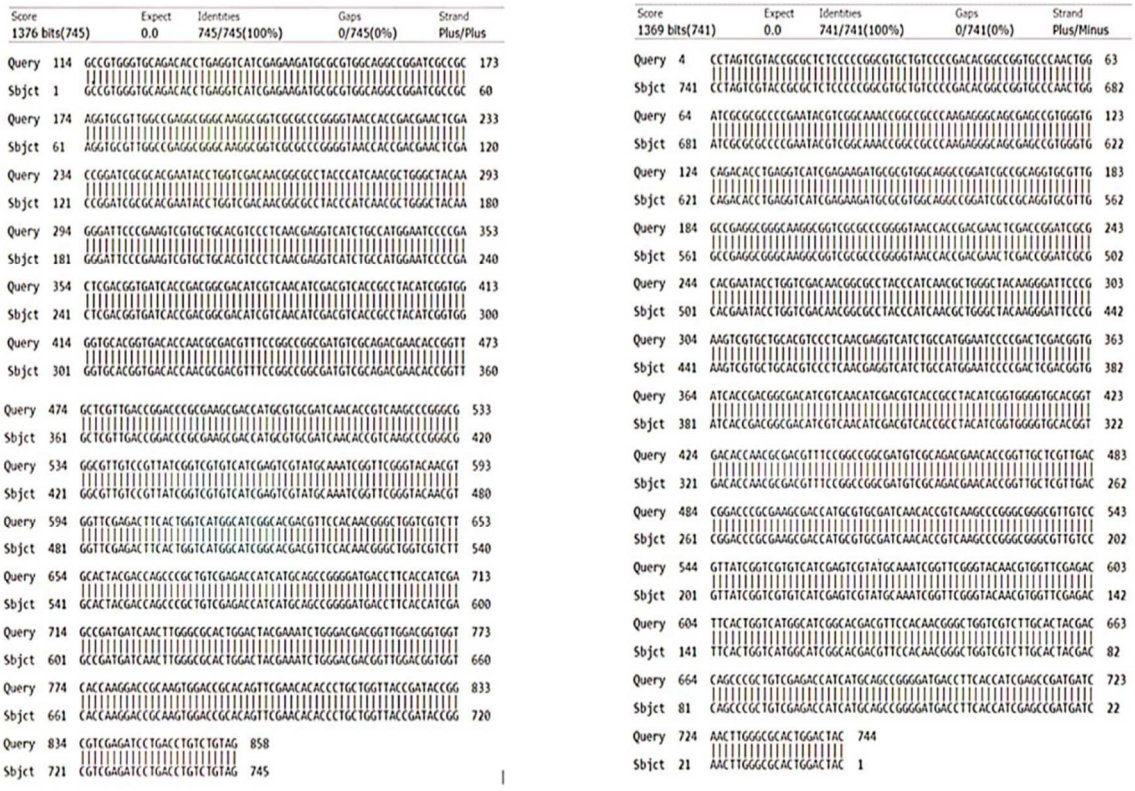


Figure 20. DNA sequence alignment using the NCBI blast software using forward and reverse primers (Left to right): sequencing of the gene at the University of Texas MD Anderson genetics department core facility.

and successfully transformed into *E. coli* BL21 (DE3) competent cells (Figure 21 a and b).

Figure 21. Transformed expression *E. coli* BL21 (DE3) with the recombinant plasmid 5ng and 10ng A. Competent cells transformed with 5ng Recombinant plasmid **B.** Competent cells transformed with 10ng Recombinant plasmid.

The mini pilot overexpression of transformed expression host cells showed an optimized expression 8 hours post induction for cells transformed with pET28a-MtMetAP1c (Figure 22).

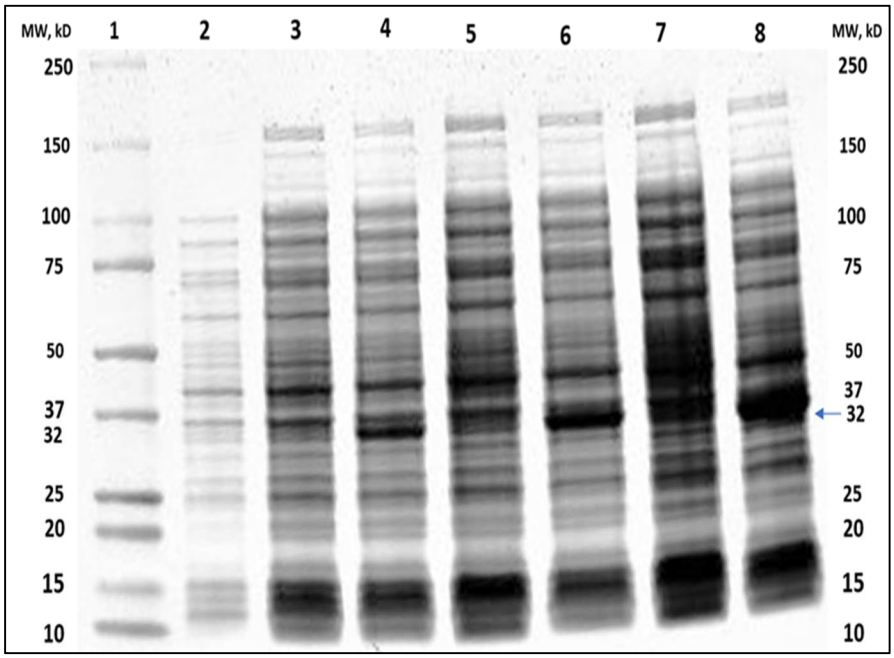


Figure 22. Mini induction pilot of Recombinant MtMetAP1c from *Mtb* gene at different time intervals. Lane 1, Molecular weight marker; lane 2, uninduced whole cell lysate; lane 3, uninduced cell lysate at 2 hours post-induction, lane four induced cells at 2 hours post-induction; lane 5 uninduced cells at 4 hours post-induction lane 6 induced cells at 4 hours post-induction lane 7 uninduced cells at 8 hours post-induction lane eight induced cells at 8 hours post-induction.

4.2 Overexpression and purification of MtMetAP1c

An 8 hour post-induction harvest time of overexpressed MtMetAP1c in *E.coli* BL21 competent cells was required for optimum yield of the enzyme, as confirmed by performing a qualitative SDS-PAGE analysis. A total of 4.65mg of MtMetAP1c was eluted from a liter of *E.coli* culture, and an *in vitro* enzymatic assay showed that the purified MtMetAP1c is its highest activity at 10.48 μM as shown by the nitroanilide production from the chromogenic substrate with the absorbance monitored using a spectrophotometer at 405 nM. The overexpressed protein was purified by IMAC using His Pur™ Ni-NTA Resin. The purified and N-terminally poly-His-tagged MtMetAP1c appeared as a band

corresponding to the 32 kDa on Coomassie blue-stained SDS-polyacrylamide gel (Figure 23). The average yield of MtMetAP1c from 1 liter of the BL21 (DE3) culture was 4.65mg.

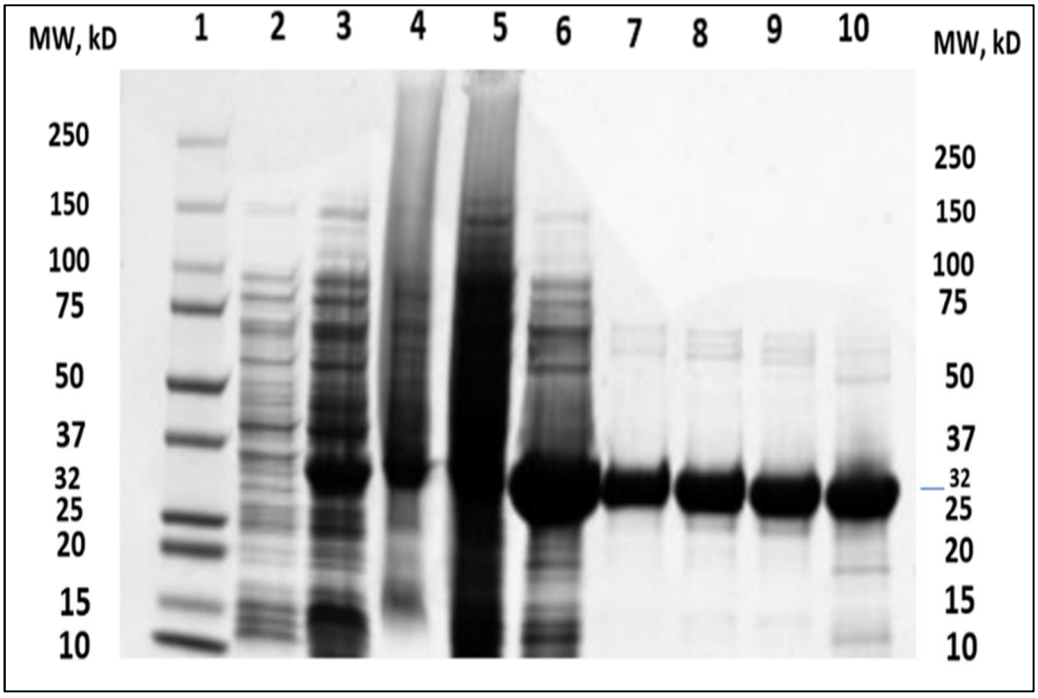


Figure 23. SDS–PAGE analysis of purified His-tagged MtMetAP1c protein using Ni–NTA-affinity chromatography. A. Lane 1, protein weight marker; lane 2, uninduced whole cell lysate; lane 3, induced cell lysate taken after 8 hrs; lane 4, Pellet after sonication; lane 5, Supernatant containing clarified protein after sonication; lane 6, Beads after final binding; lane 7, Beads after final elution; lane 8, Elution 1 (using 75mM Imidazole); lane 9, Elution 2 (using 75mM Imidazole) lane 10, Elution 3 (using 75mM Imidazole).

An 8 hour post-induction harvest time of overexpressed MtMetAP1c in *E.coli* BL21 competent cells was required for optimum yield of the enzyme, as confirmed by performing a qualitative SDS-PAGE analysis. A total of 4.65mg of MtMetAP1c was eluted from a liter of *E.coli* culture, and an *in vitro* enzymatic assay showed that the purified MtMetAP1c is its highest activity at 10.48 μM as shown by the nitroanilide production from the

chromogenic substrate with the absorbance monitored using a spectrophotometer at 405 nM. Based on enzymology studies, different factors contribute to the activity of a protease [Grahame et al., 2015]. In a previous study by Olaleye et al. the concentration of the enzyme required for the optimum activity of MtMetAP1c is 334 nM. In this study, 10.48 µM of MtMetAP1c was required for the optimum *in vitro* NME activity, approximately 31 times higher than the previous studies [Olaleye et al., 2010]. Although the plasmid and *Mtb* genome strain used was different from the study by Olaleye et al. and considering all other physical parameters required for the protease activity were maintained in the laboratory, a study by Zhang et al. suggested that the length of substrate peptide used for the chromogenic assay determines the level of activity of MtMetAP1c [Zhang et al., 2009]. They discovered that MtMetAP1s were more active with tetrapeptide substrate compared to the tripeptide counterpart. Dipeptide substrate, dipeptide, methionine-proline coupled to p-Nitroaniline, was used in Olaleye et al. study [Olaleye et al., 2010], while in this study, a monopeptide chromogenic substrate, L-Methionine ρ -nitroanilide was used for the *in vitro* assay. However, Naanan et al. assay suggested both MtMetAP1a and MtMetAP1c exhibited the highest specific activity when L-Methionine ρ -nitroanilide was used as the substrate [Narayanan et al., 2012]. The specific substrates used with other adopted assay parameters may explain the observed enzyme activity difference.

4.3 Preliminary evaluation of MtMetAP1c divalent metal: Nickel, dependence

In this study, the purified MtMetAP1c generated was discovered to be activated by both Nickel and cobalt (II) ions. The preliminary enzyme metal-dependent screen showed both divalent metal salts, $NiCl_2$ and $CoCl_2$, optimum activation of MtMetAP1c is at the same

concentration of 100 µM and produced approximately the same activity 258.8 µM mL-1 min-1 and 267.8 µM mL^{-1} min^{-1}(Figure 23 a and b).

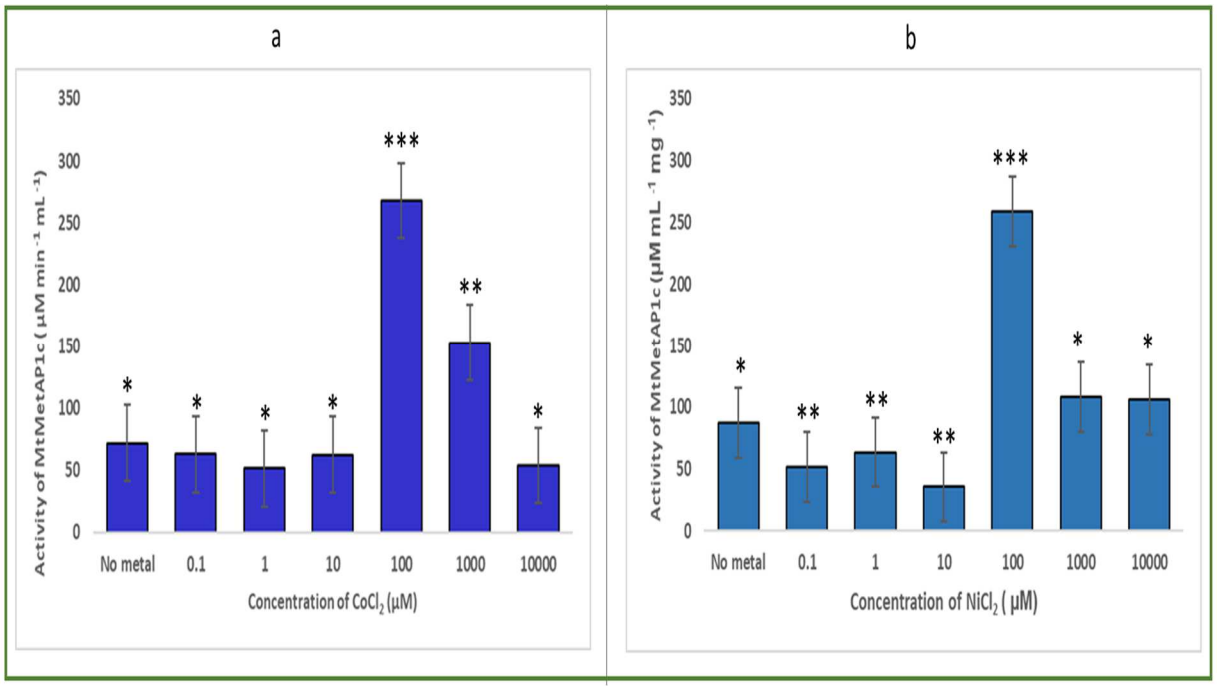


Figure 24. Activity of MtMetAP1c in the presence of different concentrations of a) Cobalt (II) chloride and b) Nickel (II) Chloride

The efficiency of Nickel ions in catalyzing MtMetAP1c is discovered to be significantly lower than that of the Cobalt ions. In addition, this study showed that an increase in the concentrations of the two metals higher than 100 µM inhibits the activity of MtMetAP1c, as evidenced by the decrease in the enzyme's activity.

MetAPs are dinuclear metalloproteases and utilize divalent cofactors for their NME functions. N-terminal methionine processing occurs in about 55-70% of nascent proteins [Giglione et al., 2000]. A study by aerie et al. discovered that the activity of MetAP was inhibited by EDTA, a metal chelating compound, which confirms the metalloprotease

nature of the enzyme [Ben-Bassat et al.,1987]. Purified apo-form MetAP have been proven to be consistently activated by divalent metal ions like Fe^{2+}, Ni^{2+}, Mn^{2+}, and Co^{2+} [Arfin et al., 1995; D'souza et al., 1999; Jing-Ya Li et al.,2003]. *E. coli* MetAPs are activated by metal ions such as Ni^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} [D'souza et al., 2002]. Comparable sizes of the divalent metal's ions have made isomorphous replacement achievable. In another study, Chai et al. showed that cellular *e. coli* Methionine aminopeptidase utilizes Fe (II) for its activation [Chai et al., 2008]. Nonetheless, the specific biological metal (s) required for MetAP catalytic activity is still debatable in contrast to PDF with an established specific physiological metal [Rajagopalan et al.,1997; Rajagopalan et al.,2000]. In previous studies, the cobalt ion was believed to be the best cofactor for MetAP; this postulation was backed by evidence of 2 cobalt (II) ions docked in the active site of the X-ray structure of MetAP [Lowther., & Matthews, 2000]. Subsequently, the majority of discovered MetAP inhibitors were characterized using MetAP activated with Co^{2+}. Paradoxically, a preponderance of pharmacophores selected with the specified cobalt metallated MetAP was highly effective in inhibiting MetAP *in vitro* assays but lacked significant potency against the corresponding bacteria [Luo et al., 2003; Douangamath et al., 2004; Schiffmann et al., 2005].

MtMetAP1s, which are MetAP1 isoforms in *Mtb*, are known to be activated by certain divalent metal ions [Zhang et al., 2009; Olaleye et al.,2010; Lu et al., 2010; Narayanan et al.,2012]. Cobalt and nickel ions, however, have consistently been proven by different groups through reproducible results to activate MtMetAP1 isoforms in their purified apo-enzyme form [Olaleye et al.,2010, Jing-Ping et al., 2010; Narayanan et al., 2012]. The MtMetAP1s and MetAPs from different bacteria cells, metal-dependent screens performed

by different groups, discovered a similar trend of a bell-shaped divalent metal activation curve which is in line with our Preliminary evaluation of MtMetAP1c divalent metal. It is suggested that a progressive increase in the concentration of MtMeAP1c cofactors in the active site causes an inhibitory effect.

The question of whether MtMetAP1a or MtMetAP1c was stripped off every possible such imidazole or metal impurities from the host cell or beads used to purify the protease through dialysis prior to the metal screen determines the true representation of the MtMetAP1-metal screen performed. Hence the apo-form or holo-form of MtMetAP1 is critical for the evaluation of MtMetAP1 divalent metal dependence. In contrast, Zhang et al. reported that Co (II) activated both holoenzyme MtMetAP1a and MtMetAP1c, while nickel (II) and iron (II) significantly inhibited the activity of the enzyme [Zhang et al., 2009]. However, Jing-Ping et al used the purified apo-form of both MtMetAP1s to determine metal dependence for activation and subsequently reported nickel ion to be an effective activator of both MtMetAP1a and MtMetAP1c [Lu & Ye et al., 2010a], which is consistent with other reports in which the apoenzyme form instead of the metallated form, was used. In this study, the two metal ions, Ni^{2+} and Co^{2+}, the optimum concentration required to activate the holo-MtMetAP1c was determined. This screen is a preliminary evaluation of metal dependence as the purified enzyme was not dialyzed to obtain its apo-form prior to performing the metal screen. The obtained concentration of the metal will further determine the evaluation of the OJT008 inhibitory effect against MtMetAP1c using the metal concentration obtained from the screen. However, In a previous study, Narayanan et al reported Ni^{2+} and Co^{2+} as activators of both MtMetAP1a and MtMetAP1c [Narayanan et al.,2012]. They also discovered the maximum *in vitro* activity of apo-form MtMetAP1c

occurred in the presence of 100μM of Ni^{2+} and Co^{2+}, and excess metal ion binding resulted in an approximate reduction of the catalytic effect by half [Narayanan et al., 2012]. This report suggests our results may be less affected, at least, by metal impurities present in the enzyme prior to the preliminary enzyme metal dependence screen. This suggests our holo form MtMetAP1c activity was not affected by the presence of nickel beads used for the enzyme purification. Our results showed a steady decrease in the activity of MtMetAP1c from a concentration of 1000μM. This trend was also discovered by D'souza et al [D'souza et al., 2000]

4.4 Evaluation of *in vitro* inhibitory effect of OJT008 against divalent nickel and cobalt activated MtMetAP1c

The *in vitro* inhibitory effect of OJT008 against the $NiCl_2$ and $CoCl_2$ (at 100μM) activated MtMetAP1c was evaluated (Figure 24). OJT008 inhibited the purified MtMetAP1c with an IC_{50} value in a low micromolar concentration. The measured IC_{50} values of OJT008 against $CoCl_2$ and $NiCl_2$ (100μM) metallated MtMetAP1c are 11.81 μM and 40.12 μM, respectively , Table 11.

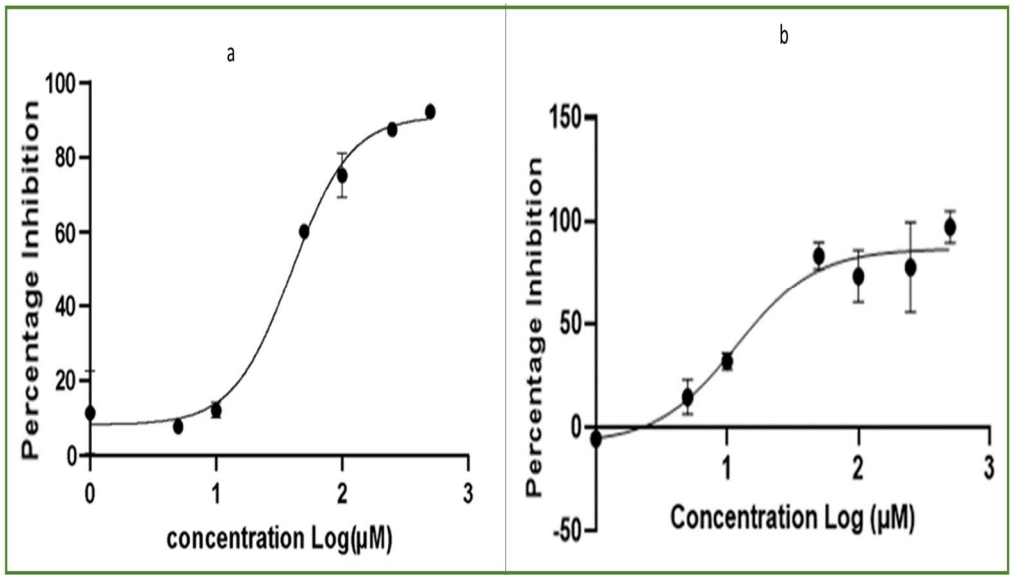


Figure 25. The inhibitory effect of OJT008 against the activity of metal activated MtMetAP1c using a) 100 µM cobalt (II) chloride and b) 100 µM nickel (II) chloride

Table 12. The half-maximal inhibitory concentration of OJT008 Metal-activated MtMetAP1c against 100uM Nickel (ii) chloride and 100µM Cobalt (II) chloride

Inhibitor ID	Chemical structure	MtMetAP1c IC_{50} values	
		100µM $CoCl_2$	**100µM $NiCl_2$**
OJT008		11.81 ± 1.30	40.12 ± 1.12

In this study, we assayed the effect of OJT008 against nickel and cobalt ion activated MtMetAP1c. The IC_{50} OJT008 against the cobalt ion activated MtMetAP1c was approximately 3.5-fold lower than the nickel ion activated MtMetAP1c. The result suggests the pharmacophore comparably binds to cobalt ion in the metalloprotease compared to nickel ion. These findings suggest OJT008 is an effective MtMetAP1c inhibitor.

The challenge posed in the selection and design of MetAP inhibitors is the inadequate information regarding the native metals in the active sites of the metalloprotease. Several reasons exist for the disparity between *in vitro* and *in vivo* potency of a compound which ranges from poor absorption of the compound into the pathogen cells or metabolic processes of the bacteria. Another logical reason for these discrepancies in the potency of MetAP inhibitors is the difference in the specific metals in the purified enzyme and the metals utilized by the microorganism in the native form. Therefore, MetAP inhibitors can be therapeutically useful if the potency is optimized by inhibiting the specific metal in MetAPs in microbes. The reported mechanism of action of a preponderance of clinically approved drugs that target metalloprotease is through the interaction with metal ions in the catalytic site of the enzyme [Mullard et al., 2014; Chen et al., 2019].

Since inhibitors of metalloprotease usually interact with the metal ions sequestered in the active site, MetAP inhibitors have varying potencies against different metallated proteases, as previously proven in *E. coli* MetAP [Ye et al., 2004]. Fe^{2+} was previously determined to be the native metal ion required for the activity of *E. coli* MetAP in *E. coli.* Furthermore, *E.coli* inhibitors selective of the cell's Fe^{2+} metalloform form exhibited a potent antibacterial effect [Chai et al., 2008]. A study by Lu et al. reported MtMetAP1c utilizes Fe (II) in the E. coli cellular environment [Lu et al., 2011]. To date, the specific metal ion

utilized by MtMetAP1a and MtMetAP1c in *Mtb* is unknown. Lu et al. discovered benzamide derivatives 4- 10 effectively inhibited MtMetAP1c by binding to the manganese ion activated MtMetAP1c in a low micromolar concentration range (0.4-1.3 μM) and had no potency with Ni^{2+}-form of *Mt*MetAP1c [Lu et al., 2011]. Olaleye et al. however, discovered 2,3-dibromo-1,4-naphthoquinone [Olaleye et al., 2010], 7-bromo-5-chloroquinolin-8-ol analogues [Olaleye et al., 2011], 2-hydroxy-1-naphthaldehyde isonicotinoyl hydrazone [John et al., 2016] exhibited an IC_{50} of 0.71 ± 0.02 μM, 3.76 mM - 5.44 μM, 5.3 ± 1.8 μM respectively against Co^{2+} activated MtMetAP1c respectively, and the group performed a MtMetAP1s metal dependence screen for cobalt (II) and Nickel(II) and discovered both metals activated MtMetAP1c [Olaleye et al., 2016]. Bhat et al. discovered Co^{2+} and Mn^{2+} displayed the highest activation of MtMetAP1c, and three methanimidamides analogs derived from structure-activity modifications of N'-hydroxy-N-(naphthothiazolyl) methanimidamide were shown to be effective in *in vitro* inhibiting the activity of Co (II) metallated MtMetAP1c, the most potent analog inhibited the cobalt-form of MtMetAP1c at an IC_{50} of 730nM but unfortunately the *in vitro* potency did not translate to a good MIC value against *Mtb* [Bhat et al., 2012]. In another study, Lu et al. investigated the X-ray structures of MtMetAP1c-compound bound complex and discovered compounds 7 and 8 both bound to the Mn^{2+} and both Ni^{2+} and Co^{2+} metallated MtMetAP1c [Lu et al., 2012]. By structurally modifying 3-amino-N-(thiazol-2-yl) pyrazine-2-carboxamides parent compound Juhás et al. discovered the compound analogs are dependent on the cofactor of the enzyme. The findings show different IC_{50} values against the MtMetAP1a activated with cobalt, nickel, and iron ions [Juhás et al., 2022].

These findings support the earlier discussion about the importance of designing a potent MtMetAP1inhibitor based on the knowledge of the specific metal ions that, on the one hand, are specific for the activation of MtMetAP1s and, on the other hand, can potentially interact with prospective inhibitors. OJT008 target tree is the Metalloprotease M24A subfamily of protease inhibitors and was patented by Olaleye et al. as a Methionine Aminopeptidase Inhibitors for Treating Infectious Diseases. Olaleye et al. successfully characterized OJT008 as a potent Antileishmanial Methionine Aminopeptidase 1 Inhibitor. They performed both in vivo and in vivo assays to discover the compound is both effective and potent against methionine aminopeptidase 1 of *L. major,* and OJT008 significantly decreased the footpad parasite load in *L. major* infected mice model. In addition, the safety of the compound was ascertained; OJT008 was non-toxic to intraperitoneal murine macrophages [Rodriguez et al., 2020].

4.5 *In silico* evaluation of the mechanism of action of OJT008 against MtMetAP1c

A Molecular docking (*in silico*) analysis was conducted to evaluate the binding of OJT008 to MtMetAP1c with divalent metal ions (cobalt and nickel) bound to its active site. The binding mode and docking scores at the MtMetAP1c active site were investigated and estimated, respectively.

Table 13. Molecular Docking Score of OJT008 towards the active site of Metal-bound MtMetAP1c

	Co-Enzyme	
	MtMetAP1c+$CoCl_2$	**MtMetAP1c+$NiCl_2$**
Docking Scores (Kcal/mol)	-6.52	-7.01

An insignificant increase in the docking score of the pharmacophore in the presence of cobalt ion (-6.52 Kcal/mol) compared to the docking score in the presence of nickel ion (-7.01 Kcal/mol). The relatively high docking scores above signify the high blinding propensity of OJT008 with MtMetAP1c having the two metal ions in its active site. This confirms the in vitro inhibitory effect of OJT008 against MtMetAP1c. The docking scores portray the change in potential energy when a protein and ligand interact. Conventionally the more negative value of the docking score corresponds to a stronger affinity binding of ligand to protein, while a lesser negative or positive value depicts lower binding of ligand to a protein target.

In this report, an *in silico* evaluation conducted discovered OJT008 interacted with both cofactors and the following amino acid residues; His (100), His (114), Thre (94), His (202), Phe (211), and Cys (105). Recently, computational approach methods have been adopted in the hit identification and lead optimization of drug discovery processes [Patrick et al.,

1998; Langer T., & Hoffmann 2001; Bajorath, 2002]. Receptor-ligand plots were performed to investigate the interactions of the amino acid residues, the metal ions at the active site of the target enzyme molecule, and OJT008 [Kehinde et al., 2019; Shode et al., 2021]. In this project, OJT008 was discovered to be highly sequestered in the highly conserved MetAP pita-bread fold [Lowther., & Matthews., 2000; Lu et al., 2010b] Figure 25.

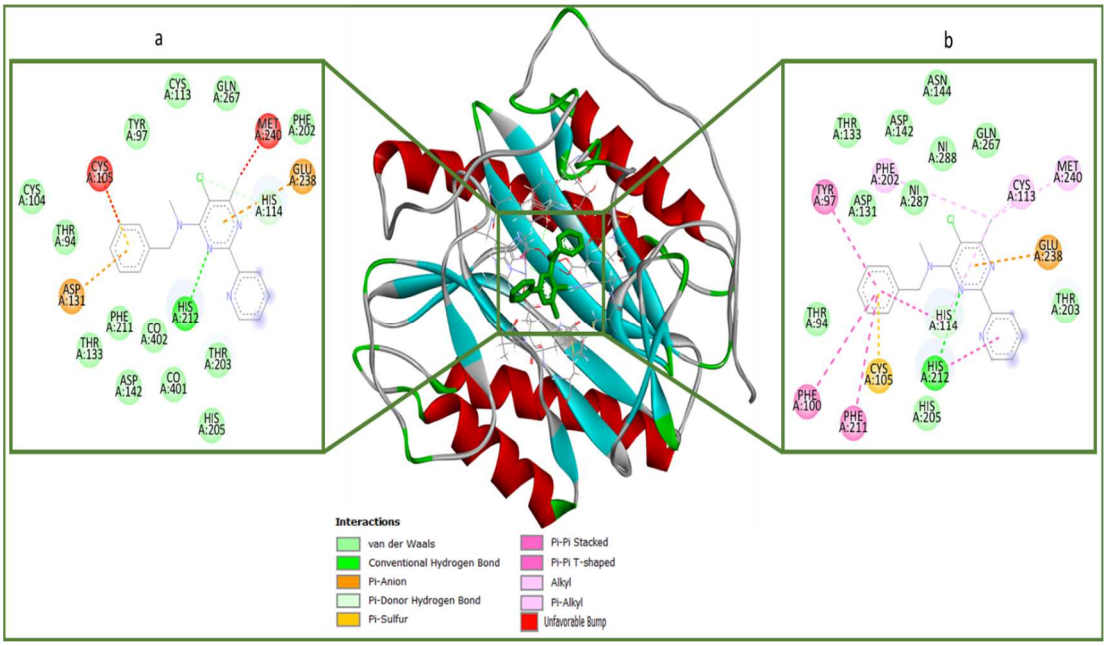


Figure 26. The 2-D visualization plot of MtMetAP1c -OJT008 interactions plots with the following divalent metals bound a) $CoCl_2$ and b) $NiCl_2$ to MtMetAP1c.

Previous studies report MetAP inhibitors interact with divalent metals at the active site of MetAP [Addlagatta et al., 2005; Lu & Ye et al., 2010a; Lu et al., 2010b]. Similarly, this study reported identical interactions of OJT008 with Ni^{2+} and Co^{2+}. The interaction

between the inhibitor, 3-[(4-fluorobenzyl) sulfanyl]-4H-1,2,4-triazole (T03) [Lu et al., 2010] and MtMetAP1c in a previous study were identical to OJT008 interaction with MtMetAP1c reported in this project. The compound displayed similar types of interaction bonds such as hydrogen bond, p-sigma, p-cation, p-Sulfur, p-alkyl, p-p stacked interaction, donor-donor interactions, and Van der Waals (vdW) overlaps) observed [Lu et al., 2010b]. This finding further proves OJT008 an potent inhibitor of MtMetAP1c enzymeIn another study, bengamide derivatives were also discovered to bind to Co^{2+}in the active site of MtMetAP1c.

In the Metalloprotease M24A subfamily of enzyme, a divalent metal like zinc, copper, cobalt, or manganese activates a molecule of water. Also, the divalent metal is held in the active site of the enzyme by majorly three amino acid residues. In the co-catalytic metalloprotease family, five amino acid residues form bonds with two metal ions. The residues are usually Glu, His, Lys, or Asp, and at least one of the amino acid molecules is required for the catalytic function of the protease [Neil et al., 2013]. The MetAPs have a homologous catalytic domain with conserved amino acid residues at their active sites [Lowther & Matthews, 2000]. The five conserved amino acid residues are utilized by MetAPs for metal ion coordination. This peculiarity poses a difficulty in the development of selective MetAP inhibitors. Hit discovery and lead optimization in drug discovery recently adopted the computational approach, which evaluates the complementarity to binding of compounds and drug candidates to active sites of target enzymes. The results from the molecular docking (*in silico*) analysis and protein-ligand interaction plot suggest OJT008 as a potent inhibitor of MtMetAP1c nickel and cobalt ion metalloform.

4.6 **Determination of the potency of OJT008 against drug-sensitive *Mtb* and Multidrug resistant- TB**

In collaboration with Dr. Graviss's laboratory at Houston's Methodist research institute. Texas. OJT008 potency against both drug-sensitive *Mtb* (CDC 1551 strain) and Multidrug-resistant TB *Mtb* (HN 3409 strain). The compound displayed inhibition of the growth of both drug-sensitive *Mtb* CDC 1551 strain and Multidrug-resistant-TB *Mtb* HN 3409 strain at < 0.063 µg/mL Table 13.

Table 14. The potency of OJT008 against drug-sensitive *Mtb* and Multidrug-resistant *Mtb*

	Minimum inhibitory concentration (µg/mL)	
OJT008	**Drug-sensitive *Mtb* (CDC 1551 strain)**	**Multidrug-resistant *Mtb* (HN 3409 strain)**
	<0.063	<0.063

Isoniazid and kanamycin were used in the compound screen as the positive control with a MIC value of 0.2µg/mL and 5.00 µg/mL. In this evaluation, OJT008 MIC values against both drug-sensitive *Mtb* and MDR-*Mtb* (< 0.063 µg/mL = < 0.194 µM) was 207-fold and 61-fold respectively more potent than the IC_{50} values against 100µM $NiCl_2$ and $CoCl_2$ activated MtMetAP1c; IC_{50} for MtMetAP1c-Ni^{2+}metalloform: 40µM and MtMetAP1c-Co^{2+} metalloform: 11 µM. The potency of OJT008 against *Mtb* was higher than it's *in vitro* inhibitory effect against MtMetAP1c. Several studies were conducted to determine the

MIC values of compounds against different types and strains of *Mtb in vitro.* Numerous pyrazine-containing compounds were discovered to have antimycobacterial activity against *Mtb* [Juhás et al., 2020; Jandourek et al., 2017; Semelkováet al., 2017; Zitko et al., 2018; Bouz et al., 2019]. Moderate potency was discovered for pyrazinecarboxamides analogs against *Mtb* [Dolezal et al., 2006]. Olaleye et al. studies discovered promising antimycobacterial compounds against drug-sensitive and non-replicating *Mtb* [Olaleye et al., 2010; Olaleye et al., 2011, John et al., 2016]. Lu et al. discovered that one bengamide derivative exhibited a moderate potency of 50.6 μM against drug-sensitive *Mtb.* Bhat et al. discovered two fractions of bengamide derivatives were potent against both drug-sensitive *Mtb* and non-replicating *Mtb* in the nanomolar concentration range; (SN31863) 0.39 μg/mL and (SN31927) 1.56 μg/mL [Quan et al., 2019]. In some studies, there is a notable potency correlation between the assayed compound *in vitro* effect against the purified MtMetAP and the *Mtb* growth [Olaleye et al., 2010; Olaleye et al., 2011].

In another study compound's MtMetAP1s inhibition does not correlate with MIC values reported [Bhat et al., 2012]. These discrepancies can be attributed to the poor absorption of the compound into the bacteria membrane or the intrinsic efflux mechanism of the cells reducing the intracellular concentration of the inhibitors [Rossi et al., 2006; Nguyen & Pieters et al., 2009].

In the investigation, the OJT008 potency against drug-sensitive *Mtb* and MDR-*Mtb* was 207-fold and 61-fold higher than it's inhibitory effect of the two metalloform MtMetAP1c (MtMetAP1c- Ni^{2+} and MtMetAP1c- Co^{2+} respectively). Interestingly, our results show that OJT008 is 4-fold and 6.6-fold more potent the isoniazid (MIC for *Mtb* CDC1551 strain: 0.25 μg/mL) [Vilchèze et al., 2018] and rifampicin (MIC for *Mtb* CDC1551 strain:

0.41µg/mL) [Yang et al., 2020] which are the first-line TB drug for drug-sensitive *Mtb* respectively. Also, OJT008 was discovered to be about 80-fold (5 µg/mL) more potent than the standard antibiotic for treating MDR-Mtb strain.

The difference in the potency of the compound against the enzyme *in vitro* and in *Mtb* could be attributed to the difference in the substrate present in Mtb cells and the biochemical substrate analog used for the *in vitro* enzymatic assay. A plausible suggestion could be that OJT008 targets another *Mtb* genome, which encodes for about 4,000 genes [Cole et al., 1998], besides the mapB gene in the Mtb types screened. Another explanation for the comparatively lower OJT008 potency against purified holo-form MtMetAP1c could be attributed to the excess metal ions or imidazole impurities inhibitory effect [Zhang et al., 2009]. A possible source of metal impurity could be the Ni-NTA Resin, used to purify the expressed MtMetAP1c and the native metal ions present in the *E.coli* BL21 DE3 competent cells used as the host cell for the overexpression of the target enzyme. In addition, the imidazole, a chelating agent [Yamauchi et al., 1968; Yip., & Hutchens., 1994] used for eluting MtMetAP1c, can also chelate the Ni^{2+} and Co^{2+} used to activate MtMetAP1c. These factors can contribute to the higher representative IC_{50} reported in this project. Although, as stated earlier, Narayanan et al. studies also discovered that 100 µM of both Nickel and cobalt ions produced a maximum activation of the apo-form MtMetAP1c [Narayanan et al., 2012]. Dialysis of the eluted MtMetAP1c should be done to strip the pure enzyme of possible impurities prior to conducting the OJT008 inhibitory effect on the metalloprotease to accurately represent the activity and potency of the compound against the enzyme.

CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Tuberculosis (TB) is classified as the 13th leading cause of death globally [WHO, 2021]. Before COVID-19, TB was the foremost cause of death arising from a single infection globally [WHO, 2021]. An estimated 25% world population is infected with latent TB, which implies 1 in 4 persons have an asymptomatic, non-infectious TB [WHO, 2021]. Approximately 13 million people had latent TB infection in the United States in 2020, and about 10% of this population can develop active TB in their lifetime [CDC,2021]. Despite the advance in TB research, the challenges of eradicating TB disease are further complicated due to the emergence of *Mtb* to resistant standard current anti-TB therapy. These include multidrug-resistant, extensively, and totally drug-resistant strains of *Mtb* [Cole et al., 1998; Fauci et al., 2008; Shelley et al., 2010]. It is crucial to develop antimycobacterial that inhibit novel *Mtb* drug targets and mechanisms of action [Theuretzbacher et al., 2019]. Another factor to consider in obtaining an efficient TB medication compliance is considering novel antimycobacterial that will constitute less pill burden and reduce the duration of the drug regimen. Therefore, the study focused on

characterizing a novel therapeutic agent that will be therapeutically potent against both wild and resistant types of *Mtb*. In this project, a combination of genetic, biochemical

assay, computational, and microbiological approaches to characterize OJT008 as an inhibitor of Mtb targeting MtMetAP1c. This was achieved by evaluating the *in vitro* inhibitory effect of OJT008 against MtMetAP1c, evaluating the mechanism of inhibition of the compound against the protease-activated with divalent metals, and the antimycobacterial effect of OJT008 against drug-sensitive *Mtb* strain and MDR-TB *Mtb* strain was investigated.

In other to successfully determine the inhibitory effect of OJT008 against MtMtAP1c, the target protease was generated in the laboratory. In this study, *map*B was amplified from the *Mtb* H37Rv genome using the PCR technique. Successively, the target gene and vector, pET28a, was successfully digested through a double restriction enzyme digestion protocol. The digested DNAs were prepared in molar DNA ratios of 7:1 and 5:1 and ligated to form the target recombinant DNA using the T4 DNA Ligase. The expression *E.coli* competent cells, DH5 alpha competent cells, were utilized in generating copies of recombinant target genes. The mutant free recombinant plasmid was used to transform an E. coli expression host, *E. coli* BL21 DE3, and the target protease was overexpressed using 1mM IPTG. The expressed protease was purified, and a Preliminary evaluation of MtMetAP1c dependence on divalent metal ion: $Nickel^{2+}$ and $cobalt^{2+}$ was performed. The protease metal

dependence screen showed that an optimum activity of MtMetAP1c was achieved at 100 μM of both divalent metal ions. OJT008 was discovered to inhibit the activity of

We conducted an investigation of the inhibitory effect of OJT008 against the activity of $CoCl_2$ and $NiCl_2$ (100μM) activated MtMetAP1c at a low micromolar concentration. An *in silico* study was studied to determine OJT008's mechanism of inhibition of the metalloform MtMetAP1c. The high negative binding score values depicted the strong inhibiting effect of the compound against nickel and cobalt ion activated MtMetAP1c. The inhibitory effect was also confirmed by receptor-ligand plots, which show a strong molecular interaction of the pharmacophore with the conserved amino acid residue and bound divalent metal at the active site of MtMetAP1c. To determine the potency of OJT008 against both drug sensitive *Mtb* and MDR-TB strain of *Mtb*, we collaborated with the Methodist research institute, Houston. Texas to screen the *in vitro* inhibitory effect of the pharmacophore against the Mtb strains. The compound inhibited the growth of both drug-sensitive *Mtb* CDC 1551 strain and Multidrug-resistant-TB *Mtb* HN 3409 strain at < 0.063 μg/mL. This study shows a promising potency of OJT008 against the MDR-TB, thereby strategically positioning OJT008 as a promising antimycobacterial pharmacophore against TB cases resistant to first line TB treatment. The significance of our study is the potential characterization of a novel chemotherapeutic agent against active and MDR-*Mtb*, thereby overcoming the challenges of the current TB drug regimen. In addition, the compound's utilization of MtMetAP1c as a potential chemotherapeutic target poses the benefits of circumventing the global challenges posed by multidrug-resistant (MDR) and drug-

sensitve TB. Therefore, OJT008 can serve as a propitious lead to discovery and development of novel antimycobacterial agents. This compound will be promising in the treatment of TB and as well serve as a prerequisite towards the development of inhibitors MtMetAP1c upon additional *in vivo* and clinical evaluations. Future consideration includes dialyzing the eluted MtMetAP1c to it's apoenzyme state prior to determining the enzyme metal dependence and the *in vitro* inhibitory effect of the compound against MtMetAP1c. Another recommendation is to perform the MtMetAP1c metal dependence activity with more divalent metals such as manganese, iron, and zinc. In this project, we screened the potency of OJT008 against *Mtb* using a final concentration range of 50 - 0.0625 μg/mL. The discovered MIC values for *Mtb* strains were 0.0625 μg/mL. It is recommended to screen the potency of the compound at a lower concentration should also be used in evaluating the potency against *Mtb* to determine the specific MIC value of the compound. The cytotoxicity and safety profiles of OJT008 against mammalian cells should be conducted. Finally, another recommendation is to conduct a preclinical study to evaluate the potency of OJT008 on both TB drug sensitive and resistant mouse model. Further structural modification of this pharmacophore to increase the *in vitro* and *in vivo* potency of OJT008 is recommended.

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