Exploiting reductive evolution in Mycobacterium ulcerans to develop a single-dose cure for Buruli ulcer

Sangeeta, Susan Thomas

2021

Sangeeta, S. T. (2021). Exploiting reductive evolution in Mycobacterium ulcerans to develop a single-dose cure for Buruli ulcer. Doctoral thesis, Nanyang Technological University, Singapore. <https://hdl.handle.net/10356/152336>

<https://hdl.handle.net/10356/152336>

<https://doi.org/10.32657/10356/152336>

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**EXPLOITING REDUCTIVE
EVOLUTION IN *MYCOBACTERIUM
ULCERANS* TO DEVELOP A SINGLE-
DOSE CURE FOR BURULI ULCER**

SANGEETA SUSAN THOMAS

**Interdisciplinary Graduate School
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A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of
Doctor of Philosophy

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

02 / 06 / 2021

Date

Sangeeta Susan Thomas

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

02 / 06 / 2021

Date

Kevin Pethe

Authorship Attribution Statements

This thesis contains material from 2 papers published in the following peer-reviewed journals where I was the first author.

1) Chapter 2, excluding unpublished data where indicated, is published as 'Scherr N, Bieri R, **Thomas SS**, et al. Targeting the *Mycobacterium ulcerans* cytochrome bc1:aa3 for the treatment of Buruli ulcer. Nat Commun. 2018;9(1):5370. Published 2018 Dec 18. doi:10.1038/s41467-018-07804-8. This work was also accepted and presented at the 'Microbial Pathogenesis and Host response' conference held at Cold Spring Harbor Laboratory, New York, September 2019.

2) The contributions of the co-authors are as follows:

- Associate Professor Kevin Pethe provided the initial project direction and edited the manuscript drafts.
- Nicole Scherr, Raphael Bierri and I prepared the manuscript drafts and are credited as equal contributors to the paper. The manuscript was revised by Professor Kevin Pethe.
- I co-designed and performed the following experiments in the study with help from Nitin Pal Kalia:- MIC determination with OD measurement, ATP and OCR measurements, the methylene blue experiment following expression of *M. ulcerans* *cydAB* in *M. bovis* BCG, and selection of the spontaneous resistant mutants to Q203. We interpreted the results together.
- Nicole Scherr, Raphael Bierri, Paul Schneide, Marie-Thérèse Ruf, Araceli Lamelas, Marcel Tanner, and Gerd Pluschke designed and

conducted the resazurin based assays and the MBC experiment published in this paper.

- The *in vivo* experiments were codesigned and conducted by Nicole Scherr, Raphael Bierri, Aurélie Chauffour, Paul Schneide, Marie-Thérèse Ruf, Araceli Lamelas, Marcel Tanner, Matthias Witschel, Vincent Jarlier, and Gerd Pluschke.
- Professor Gerhard Gruber and Malathy S. S. Manimekalai provided the protein modelling data.
- Norihisa Ishii and Koichi Suzuki provided the *M. ulcerans* stains from Japan (strains S1324, S1325 and S1326). Professor Gerd Pluschke provided us the *M. ulcerans* strains from Cameroon (strains S1013, S1213 and S1298).
- Garrett C. Moraski and Marvin J. Miller provided the IPA candidate drugs ND-10885 and ND-11176.

.

- 3) Chapter 3 is published as '**Thomas SS**, Kalia NP, Ruf M-T, Pluschke G, Pethe K. 2020. Toward a single-dose cure for Buruli ulcer. Antimicrob Agents Chemother 64:e00727-20. doi.org/10.1128/AAC.00727-20'

The contributions of the co-authors are as follows:

- I co-designed the project with Professor Kevin Pethe.
- I co-designed the workflow from experimental timeline to final termination of the *in vivo* experiment along with Professor Kevin Pethe.
- I prepared the application forms for the institutional IACUC approval.

- I prepared the drug formulations, and the solid media plates for final CFU counting.
- Nitin Pal Kalia and I performed all oral gavaging of mice, monitoring, and final sacrifice of all mice used in the experiment.
- Marie-Thérèse Ruf and Professor Gerd Pluschke performed all histopathological processing of tissues with fixation, staining and microscopy of tissue. All tissue microscopy analysis and interpretation were conducted by Marie-Thérèse Ruf and Professor Gerd Pluschke.
- I prepared the manuscript drafts. Professor Kevin Pethe revised the drafts.

02 / 06 / 2021

Date

Sangeeta Susan Thomas

Acknowledgements

I would like to thank the Interdisciplinary Graduate School for granting me this opportunity to explore my academic ambitions. Also, I would like to thank Lee Kong Chian School of Medicine and the supporting staff for hosting me and always ensuring the smooth operation of the laboratories.

I would like to express my deep and sincere gratitude to my supervisor, Professor Kevin Pethe, who readily agreed to provide me with this opportunity to be part of his research team and guided me throughout this journey of scientifically exploring and presenting my work. It was a great privilege and honour to work and study under his guidance. I would also like to thank my co-supervisor, Professor Loo Say Chye Joachim, and mentor Professor Yeo Tsin Wen, who agreed to meet with me at regular intervals and provided valuable feedback on the progress of my studies.

I would like to express my sincerest gratitude to my laboratory mates cum friends at the Lee Kong Chian School of Medicine, who supported me with positive energy and provided me with many joyful endearing moments that I will cherish.

Last but not least, I would like to acknowledge with gratitude the support, love, and encouragement from my family, without whom this would not have been possible.

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List of publications

Scherr, N., Bieri, R., **Thomas, S.S.** *et al.* Targeting the *Mycobacterium ulcerans* cytochrome *bc₁:aa₃* for the treatment of Buruli ulcer. *Nat Commun* **9**, 5370 (2018). <https://doi.org/10.1038/s41467-018-07804-8>

Thomas SS, Kalia NP, Ruf M-T, Pluschke G, Pethe K. 2020. Toward a single-dose cure for Buruli ulcer. *Antimicrob Agents Chemother* 64:e00727-20. <https://doi.org/10.1128/AAC.00727-20>.

Summary

Mycobacteria form one of the most successful human pathogens, with *Mycobacterium tuberculosis* causing tuberculosis and *Mycobacterium leprae* causing leprosy. Following the effective implementation of the BCG vaccine and the rigorous and conscious effort taken globally to curb tuberculosis and leprosy, infections due to non-tuberculous mycobacteria (NTM) are now gaining prominence worldwide. Some of the predominant NTM infections include the skin ulcers caused by *Mycobacterium ulcerans* and lung infections due to *Mycobacterium abscessus* and *Mycobacterium avium* complex (MAC) agents.

Buruli ulcer, a slowly progressing ulcerative disease of the skin caused by *M. ulcerans*, is the third most common mycobacterial disease. The disease affects mainly children in regions of West Africa and now is seeing a resurgence in regions of South-East Australia. For several years, the disease was considered a neglected tropical disease, and a suboptimal treatment regimen comprising of daily oral rifampicin and streptomycin injections over 8 weeks has hindered effective control of the disease. Moreover, drug toxicity issues such as ototoxicity and renal failure make the treatment regimen very difficult to follow through for the patients.

On consideration of drug repurposed for NTM from the drug pipeline for *M. tuberculosis*, Q203 (Telacebec) was found to be an ideal drug candidate for *M. ulcerans*. Q203 is a clinical-stage drug candidate for *M. tuberculosis* that targets the respiratory cytochrome *bcc:aa₃*, a terminal electron acceptor in the critical oxidative phosphorylation (OxPhos) pathway. In silico studies have shown that the Q203 binding site (QcrB domain of the cytochrome *bcc:aa₃*) was conserved

in both *M. tuberculosis* and *M. ulcerans*. However, in *M. tuberculosis*, Q203 is rendered bacteriostatic due to the rescue action of the alternate terminal oxidase, cytochrome *bd* oxidase. Contrarily, *M. ulcerans* lacks a functional cytochrome *bd* oxidase due to a non-sense mutation as part of drastic genome downsizing in its evolutionary diversification from *Mycobacterium marinum*. Hence, *M. ulcerans* is solely dependent on cytochrome *bcc:aa₃* as a terminal electron acceptor, and in this scenario Q203 might be an exceptionally fitting drug candidate for *M. ulcerans*.

In this study, the cytochrome *bd* oxidase of *M. ulcerans* was confirmed to be inactive using a methylene blue-based functional assay. *M. ulcerans* was found to be hyper susceptible to Q203 with potency in sub-nanomolar ranges (MIC₅₀: 0.2-0.9 nM) as opposed to *M. tuberculosis* where MIC₅₀ of Q203 was 1.5-2.8 nM. The drastic reduction in both ATP levels and Oxygen Consumption Rate on exposure to Q203 validated the action of the drug on the OxPhos pathway. A first attempt at raising drug resistant mutants in *M. ulcerans* in the laboratory was successful in this study. Low resistance rates (5.6×10^{-9}) to Q203 was demonstrated for *M. ulcerans* and all the resistant isolates had mutations mapped to the *qcrB* gene.

Previously, *in vivo* studies have shown remarkable sterilising action of Q203 on *M. ulcerans* in the foot-pad infection model in mice compared to the current clinical standards of rifampicin and streptomycin. In our study, we were able to validate the use of a single dose of Q203 to completely clear infection in the mouse foot-pad infection model. Marked oedema and histopathological evidence of disease were significantly reduced with Q203 treatment alone.

In conclusion, the reductive evolution of *M. ulcerans* has driven the organism to hypersusceptibility to Q203 due to an exclusive dependence of the organism on one terminal electron acceptor in the quintessential OxPhos pathway. This was successfully translated to achieve a single dose cure in the Buruli ulcer mouse model of infection with no relapse.

List of abbreviations

#

16srRNA: 16S ribosomal RNA

A

ABSL-2: Animal biosafety level -2

Acetyl CoA: Acetyl coenzyme A

ADS: Albumin-Dextrose-Saline

AFB: Acid Fast Bacilli

AIDS: Acquired Immunodeficiency Syndrome

ATP: Adenosine triphosphate

AUC: Area under the curve

B

BCG: Bacillus Calmette-Guerin

BDQ: Bedaquiline

BPaL: Bedaquiline + Pretomanid + Linezolid

BSA: Bovine serum albumin

BU: Buruli ulcer

C

Cla: Clarithromycin

CO₂: Carbon-di-oxide

COPD: Chronic Obstructive Pulmonary Disease

CF: Cystic fibrosis

CFU: Colony Forming Units

D

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DRC: Democratic Republic of Congo

E

EDTA: Ethylenediaminetetraacetic acid

EMP pathway: Embden–Meyerhof–Parnas pathway

ETC: Electron transport chain

F

FADH₂: Flavin adenine dinucleotide

FDA: Food and Drug Administration

G

GFP: Green fluorescent protein

H

H⁺: Hydrogen ion

H₂O: Water

HCV: Hepatitis C Virus

HIV: Human immunodeficiency virus

I

IACUC: Institutional Animal Care and Use Committee

IPA: Imidazopyridine amide

K

kB: Kilobase

M

MAC: *Mycobacterium avium* complex

MD: Maryland

MDR: Multi-drug resistant

MIC: Minimum inhibitory concentration

MK: menaquinone pool

MO: Missouri

MOTT: Mycobacteria Other Than TB

Mtb: *Mycobacterium tuberculosis*

N

NACLAR: National Advisory Committee for Laboratory Animal Research

NADH₂: Nicotinamide-adenine-dinucleotide dihydrogen

NADPH: Nicotinamide adenine dinucleotide phosphate

NDH: NADH dehydrogenase

NJ: New Jersey

nM: Nanomolar

NTM: Non-tuberculous mycobacteria

NTU: Nanyang Technological University

O

OADC: Oleic acid Albumin Dextrose Catalase

OCR: Oxygen consumption rate

OD: Optical Density

P

PA-824: Pretomanid

PBS: Phosphate buffered saline

PCR: Polymerase Chain Reaction

PMF: Proton motive force

R

Rif: Rifampicin

RNA: Ribonucleic acid

Rpm: rotations per minute

S

SDH: Succinate dehydrogenase

T

TB: Tuberculosis

TCA: Tricyclic acid

TNF: Tumour necrosis factor

U

USA: United States of America

W

WHO: World Health Organization

X

XDR: Extensively drug resistant

XF: Seahorse extracellular flux analyser

– CHAPTER 1 –

Introduction

Introduction

1.1 Mycobacteria

Mycobacteriaceae is a distinct family in the genus of *Actinobacteria*, harbouring close to 200 species of *Mycobacteria*^{1, 2}. The family derives its name from the Greek prefix *myco-* meaning fungus-like, alluding to its mould-like growth on the surface of cultures. Another distinctive property of the family is the presence of mycolic acid in their cell wall, conferring it a waxy nature and acid resistance³. Acid fastness has been exploited to develop specific stains, such as the Ziehl-Neelsen staining technique whereby the organism appears to stand out as pink beaded rods against a blue background of other bacteria and cellular debris⁴.

Mycobacteria are non-spore-forming, non-flagellated bacilli^{5, 6}. They are known for their typical slow-growing nature and requirement for specialised medium for growth in the laboratory⁷. Members differ in several traits, natural habitat, and pathogenic potential. A handful are distinct pathogens, and others are opportunistic pathogens. Some of the mycobacteria are just saprophytes with no pathogenic potential described so far¹.

Among the mycobacteria species, the most important and notorious member is *Mycobacterium tuberculosis* (Mtb), the etiological agent of human tuberculosis (TB). WHO estimates that approximately 10 million people contracted TB in 2017, with approximately 1.4 million people succumbing to the illness in that year alone⁸. Plagued by difficulties such as delayed, complicated diagnosis and long treatment strategies, the battle against

tuberculosis has been painfully arduous with only successful control of the disease in developed countries⁹. New threats like drug resistance and startling synergy with HIV infection has rendered the fight even more difficult^{10, 11}.

As the fight against TB escalates, one of the more successful battles has been against leprosy. Leprosy, caused by *Mycobacterium leprae*, is a chronic granulomatous infection affecting the skin and peripheral nervous system¹². The disease is notably associated with deforming skin lesions and debilitating lesions of limbs and has incited social stigma and isolation, even in biblical times^{13, 14}. With stigma induced isolation, slow spread of disease, and an effective treatment regimen, the global burden of leprosy is low and even receding at 210,942 cases in 2017 as opposed to 214,783 cases in 2016^{15, 16}. Other than these two main pathogens, all the other mycobacteria are grouped under non-tuberculous mycobacteria.

1.2 Non-tuberculous mycobacteria

Non-tuberculous mycobacteria (NTM), also known as atypical mycobacteria or MOTT (mycobacteria other than TB), greatly vary in their habitat, pathogenicity, and significance^{17, 18}. They are often environmental organisms found in water and soil¹⁹. The most popular classification for them is the Runyon classification based on growth rate and pigment production²⁰. Although nowadays, this method has been replaced in diagnostic laboratories by biochemical or molecular techniques, the broad classification of NTM into slow growing (>7 days to produce colonies on solid medium) and rapid growers (<= 7 days) is still practical^{2, 21}.

The epidemiology of NTM associated infections vary. Organisms like those belonging to the *Mycobacterium avium* complex (MAC) or *Mycobacterium abscessus* have been implicated in infections globally, whereas organisms like *Mycobacterium ulcerans* and *Mycobacterium mageritense* occupy specific geographical niches^{21, 22}.

The infections caused by NTM are also varied, ranging from localised pulmonary or skin and soft tissue infections to disseminated systemic disease²³. Pulmonary infections are mostly seen in the elderly (>65 years) or patients with pre-existing chronic conditions such as bronchiectasis, COPD, or in post-TB patients. Cystic fibrosis patients are at increased risk of infection with *M. abscessus*²⁴. MAC infections are often associated with chronic cough and non-specific symptoms in slender, elderly women²⁵. The common NTM associated with skin infections are *M. ulcerans*, *M. marinum*, and *M. fortuitum*²⁶. *M. scrofulaceum* and *M. mageritense* have been associated with cervical lymphadenitis, and members of MAC have caused disseminated disease in HIV-seropositive individuals²⁷.

NTM infections often result in chronic diseases with complex and largely ineffective treatment regimen. Most of the NTM are naturally resistant to first-line anti-TB drugs. The drug regimen for treating these infections is repurposed and usually poorly tolerated, long and complicated. Symptoms of NTM infection are often non-specific and may mimic TB²³. In such settings, a misdiagnosis can often mean a worsening of patient condition despite initiating therapy. A point in case is *M. kansasii*, which causes pulmonary

infection resembling TB, but is resistant to pyrazinamide, a first-line anti-TB drug²⁸.

Diagnosis of NTM infection, especially pulmonary disease, is complicated. Due to the environmental presence of NTM, it is often difficult to clinically distinguish a pathogenic association of the organism to the disease state, colonisation, or even accidental environmental contamination. Clinical, microbiological, and radiological evidence is required to reach a definitive diagnosis of NTM infection²³. The criteria also emphasise an exclusion of other diagnoses, most importantly, TB²³.

Unlike TB, NTM pulmonary infections are not notifiable. However, several studies have shown that the incidence of NTM is steadily increasing worldwide. In the United States, NTM is now more prevalent than TB and is growing every year at a rate of 8%, with 80% of infections caused by MAC^{29, 30}. A similar trend has been noted worldwide with increased isolation of NTM, even in suspected TB cases³¹⁻³⁴.

Several factors may be contributing to the rise in prevalence of NTM. Raised awareness may account for the increasing incidence and visibility of NTM infections in literature. Efforts to identify NTM species in clinical specimen have intensified, and novel genotypic methods such as 16S ribosomal RNA sequence analysis have contributed to the boom in the field^{1, 23}. An ageing demographic and use of novel anti-inflammatory drugs like anti-TNF factors has also increased the proportion of susceptible population^{35, 36}. Also, the possibility of augmented virulence of NTM cannot be ruled out due to the current worrying trends in manmade circumstances such as rampant

chlorination of water sources and inadvertent use of antibiotics in agriculture and farming³⁷⁻³⁹.

With increasing incidence of NTM infections, a better understanding of NTM, its pathogenesis, host interactions, and environmental adaptations is imperative. All these issues underscore the need for more research into NTM.

1.3 Buruli ulcer

Buruli ulcer is one of the lesser-known bacterial skin infections caused by an NTM, *Mycobacterium ulcerans*. It is a chronic and slowly progressing ulcerating disease of the skin and subcutaneous tissue, often accompanied by debilitating deformities⁴⁰.

The disease was first officially reported in 1948 by P MacCallum et al. from a cluster of cases in Australia. This group pointed out thick clusters of acid-fast bacilli from histopathology sections and prudently noted the lack of characteristic features of a tubercle, thus reporting the disease as a new mycobacterial infection⁴¹. The name, *M. ulcerans*, appeared first in a publication by Australian virologist, Frank Fenner who later successfully developed the mouse footpad animal model of infection for *M. ulcerans*⁴².

Separately, large ulcers consistent with these reports were already described in regions of Congo by Albert Cook and Kleinschmidt before 1948⁴³. The disease derives its current name from the Buruli county of Uganda, where the disease was described⁴⁴. Other names used to identify the disease include Bairnsdale ulcer, Daintree ulcer, and Searle ulcer in Australia, Tora and

Mexican ulcer in Mexico, and Kasongo ulcer, Kakerifu ulcer, or Mputa ya Luaka in Africa⁴⁵.

1.3.1 Epidemiology

Although Buruli ulcer has been reported from 33 countries around the world in all the continents, the disease is mostly restricted to a handful of tropical countries in Africa and coastal regions of Australia⁴⁶. Historically, an increased incidence of disease in the 1980s led the WHO to launch the Global Buruli Ulcer initiative in 1998, followed by the first International Conference on Buruli ulcer control in Yamoussoukro, Cote d'Ivoire⁴⁷. This helped to draw the attention of medical and research communities, and the disease was recognised as an emerging public health problem with notifiable status.

An average of 5,000 cases was reported every year from 2004 to 2010, after which a steady decline in cases was noted, with numbers falling to 1,864 cases in 2016⁴⁸. However, an increase in numbers was noted in the following years, with a total of 2,713 cases recorded in 2018⁴⁰. Also, the actual number of cases may be higher as underreporting is an issue in regions of Africa, where the affected population may lack access to health care facilities. An extensive door to door survey of 39,000 households in the Democratic Republic of Congo revealed that only 18 out of 259 active cases (7%) reported to a health care facility⁴⁹. Similarly, active case searches such as those conducted in endemic regions of Cameroon, Liberia, and Nigeria identified 436, 21, and 14 cases, respectively, all of which may have gone undetected⁵⁰⁻⁵².

Currently, the disease burden is highest in countries of West and Central Africa, namely, Cote D'Ivoire, Benin, Ghana, and Cameroon⁴⁸. An alarming rise in cases was recorded in Australia, with a total caseload of 358 in 2018 as opposed to 89 cases in 2014⁵³⁻⁵⁵. Disease incidence has significantly increased in the south-eastern state of Victoria in Australia, and an increase in the severity of lesions was also noted⁵⁶. Moreover, an expansion of disease endemicity to adjacent regions was also recorded. The reasons behind this changing epidemiology are not well established, and some of the proposed theories are climate change and human activities accompanying change in population dynamics⁵⁶.

Buruli ulcer follows strict ecological association with riverine areas or low-lying wetlands⁵⁷⁻⁶⁰. This association was highlighted in one of the first epidemiological studies of the disease conducted on Rwandan refugees settled in the Kinyara Refugee settlement near River Nile in Uganda⁶¹. While at Kinyara, the community experienced a steep rise in cases, which was followed by a sharp drop in cases when they relocated to another settlement in Kyangwali four years later. This stark contrast in disease occurrence was one of the first convicting evidence of the geographical specificity of the disease⁶¹. Studies have also shown that interventions to the soil and landscape such as deforestation, recently introduced agricultural and irrigation activities can result in surges of the disease⁶²⁻⁶⁴.

The transmission of the disease remains an enigma. Person to person transmission has been largely ruled out in various studies^{57, 65-67}. Two strong contending theories are direct environmental transmission and vector-borne transmission^{67, 68}. PCR and culture positivity have established the presence of *M. ulcerans* in water, soil, aquatic plants, water bugs, mosquitoes, possums, and other vertebrate animals⁶⁹⁻⁷². However, deducing a rational transmission pathway has been plagued with difficulties such as the low case incidence, the long incubation period of the disease, and variable presence and habits of possible vectors. As of now, no consensus has been reached on a plausible transmission mechanism, thereby placing a roadblock on the possible measures that can be adopted to prevent the disease.

No age group is exempt from disease, and gender differences in prevalence are not evident. However, in endemic regions of Africa, approximately 48 % of affected individuals are children below 15 years of age^{40, 73}. In these areas, where social stigmas are prevalent, treatment may get delayed, and the affected children often face a future of severe deformities that significantly impairs their quality of life. This places a heavy burden on an already impoverished society. In Australia, the higher incidence is noted in patients more than 65 years of age, implying waning immunity may play a role in the pathogenesis of the disease⁷⁴.

1.3.2 Clinical features

The lesions from Buruli ulcer can be distinguished as three stages; a) The pre-ulcerative stage, b) the ulcerative lesion, and c) scars⁷⁵. The pre-ulcerative

lesions are most commonly painless nodules, usually less than 5 cm in diameter. Other less common lesions include papules, plaques, and oedematous lesions. The lesion gradually progresses into an ulcer with the classical undermined edges. WHO classifies the lesions into 3 categories⁷⁶.

- a) Category 1: Single nodules, plaques, and ulcers less than or equal to 5 cm.
- b) Category 2: Single ulcer measuring 5-15 cm and oedematous lesions.
- c) Category 3: Single ulcer measuring more than 15 cm, multiple ulcers, ulcers involving the joints or critical areas such as head and neck, genitalia, osteomyelitis.

Slow progression, analgesia, and low incidence of secondary bacterial infections are notable characteristics of the ulcer⁷⁷. Almost 80% of lesions are located on the limbs, mainly in the lower extremities. This may be attributed to the fact that the organism grows only at 28 – 32 °C^{78, 79}.

The progression of the ulcers can be varied. Some ulcers remain small and even persist for several years. Spontaneous healing is noted for one-third of Category 1 lesions^{68, 80}. In other cases, the ulcers progress to large areas of the body that subsequently heal with deformities and/ or contractures. Some of them require extensive debridement and sometimes amputation⁸¹. Scarring with functional limitation is estimated to occur in 11-50% of cases, and physiotherapy is now accepted as an often integral part of therapy^{82, 83}. The persistent functional disability that remains after ulcer healing is notorious

among the afflicted. This has been aptly reflected in one of the names given to the ulcer “juwe okoro” or “bile okoro,” meaning ‘the ulcer that heals in vain’⁸⁴. Scarring can lead to disfigurement, impaired mobility, and reduced elasticity making it more prone to cracking and injuries⁷⁶. A rare complication is the development of squamous cell carcinoma at scar site^{85, 86}.

Osteomyelitis of underlying bone can occur in up to 15% of untreated cases⁸⁷. In the case of oedematous lesions, progression can be fast and may be accompanied by pain and low-grade fever⁵³. This may be attributed to the amount and type of mycolactones, a major toxin and virulence factor produced by the infecting strain^{88, 89}. Large, disseminated ulcers are seen in patients coinfecting with HIV^{90, 91}.

1.3.3 Diagnosis and treatment

Diagnosis of Buruli ulcer is often made clinically, especially in disease-endemic regions of Africa where access to a clinical microbiology laboratory may not be feasible. The four mainstays of laboratory diagnosis include acid-fast staining, culture, histopathology, and PCR. WHO recommends the use of two separate tests to confirm the diagnosis⁹².

In disease-endemic areas with convincing clinical evidence, direct microscopic evidence of acid-fast bacilli using Ziehl-Neelsen staining is deemed the most practical approach^{92, 93}. The sensitivity of the method is 40-80 %⁹². Culture confirmation of specimen can entail prolonged incubation (6-12 weeks) on Lowenstein-Jensen medium and has a lower sensitivity of about

40-60 %⁹². Histopathological evaluation can correctly diagnose over 90 % of cases⁹². It, however, requires more extensive sample preparation and the services of an experienced, dedicated histopathologist⁹⁴. Similarly, although PCR also has high sensitivity rates of over 90 %, the technique can be difficult to perform in the field with its need for stringent laboratory facilities, stable electricity supplies, and qualified lab staff^{92, 93, 95}.

The treatment pathway for Buruli ulcer has constantly been evolving. Until 2004, surgical debridement supplemented with skin grafts was the mainstay of treatment for Buruli ulcer. In poor resource settings, primary surgical treatment could be burdensome with its need for surgical facilities and a dedicated team of surgeons and anesthesiologists. Moreover, the treatment entailed an average hospital stay of 3 months and recurrence rates of 16 % to 28 %⁹⁶⁻¹⁰⁰. These two factors were major deterrents in public confidence in surgical treatment.

In 2004, the use of antibiotics for Buruli ulcer was promoted with the release of the WHO guidance manual on *Provisional guidance on the role of specific antibiotics in the management of Mycobacterium ulcerans disease*⁷⁷. A continuous and strict regimen of oral rifampicin (10 mg/kg) and streptomycin (15 mg/kg) injections for 8 weeks was determined to be the standard¹⁰¹. Compared to surgery, this method has very few recurrences (0-2 %), and the cost of treatment was significantly less, especially with a decreased stay at hospital¹⁰². Antimicrobial therapy alone was deduced to be enough for the treatment of Category 1 lesions. Surgery may be necessary for Category 2

and 3 lesions and was often reserved for after completion of the antibiotic course. Supplementary treatment such as adequate wound cleaning, physiotherapy for involved joints, and good scar management ensure good recovery⁷⁶.

Antimicrobial therapy, however, also has many drawbacks. Although rare, long term administration of rifampicin entails the risk of hepatitis, shock, and acute renal failure¹⁰³⁻¹⁰⁶. Daily intramuscular injections of streptomycin are a major limitation of the current treatment regimen. The injections are painful, and a daily visit to a health care facility for 56 days can be a major impediment to the delivery of continuous treatment. Moreover, streptomycin is toxic, carrying a major risk of developing ototoxicity (0.5-5 % of treated patients) and renal toxicity (5-10 % of treated patients)¹⁰⁷. Streptomycin is also contraindicated in pregnant women and in patients who were previously treated with aminoglycosides since the toxicity of streptomycin, an aminoglycoside, is cumulative^{108, 109}.

The search for an alternate oral drug regimen yielded clarithromycin¹¹⁰⁻¹¹⁴. Clarithromycin is an effective and convenient oral drug alternative to streptomycin. Several recent studies have proven that the efficiency of a rifampicin-clarithromycin oral drug regimen is similar to that of the rifampicin-streptomycin regimen in both Australia as well as regions of Africa¹¹⁵⁻¹²⁰. The regimen again involves a long term eight-week administration of the drug combination.

Critical evaluation of the drug regimen available now yields two main faults. The long duration of treatment and the continued reliance of the regimen on rifampicin as a backbone for the treatment are concerns that need to be addressed. Duration of treatment highly influences the efficiency of a treatment regimen. Compliance to a long duration drug regimen is a major issue in treatment logistics and has been extensively studied¹²¹⁻¹²³. Non-compliance results in suboptimal antimicrobial concentration in affected individuals and often paves the way for the development of resistance and disease recurrence¹²⁴⁻¹²⁷.

Often, an efficiency gap exists between health system effectiveness and field conditions in drug delivery^{128, 129}. Health system effectiveness is often measured during clinical trials and is held in controlled settings. Here efficient monitoring and steady availability of drugs ensure good efficiency. However, in field conditions with imperfect monitoring and unsteady supply of drugs, the effectiveness wanes. Even with a short three-day drug regimen for a critical affliction like malaria, efficiency drop of 58-67% was observed^{128, 130-132}.

The effectiveness gap for Buruli ulcer was never measured, but certainly, the odds are stacked against an efficient field condition. A longer treatment duration of 8 weeks, less awareness of the disease, lower systemic effects of the disease wherein the patients are less likely to be driven to take medication will affect effectiveness even more. Moreover, significantly more children are affected in the African continent, and the social conditions prevailing in

rural regions of the disease often deter patients and caregivers from continuing with a continuous drug regimen of 8 weeks¹³³.

The other drawback is that the bactericidal efficiency of the regimen rides almost entirely on rifampicin. Currently, no good replacement exists for rifampicin. Overall, the development of more efficacious and less toxic drugs will improve the clinical management of Buruli ulcer.

1.4 *Mycobacterium ulcerans* – the organism

M. ulcerans has been determined to be the causative agent of Buruli ulcer. It is considered a slow-growing member of the NTM¹³⁴. In fact, the organism is extremely slow-growing with an average replication time of 4.8 days +/- 0.3 days¹³⁵. This poses several problems like contaminant overgrowth, especially on a solid medium like Middlebrook 7H10, where solid colonies are seen after 2 -3 months of incubation^{136, 137}. Optimal growth is seen at a temperature range of 28 -32°C^{138, 139}. It can be stained with the classical acid-fast staining techniques used for visualising mycobacteria and is often the more convenient method of detecting the organism in diagnostic skin biopsies or swabs¹⁴⁰.

1.4.1 Evolution of *M. ulcerans* from *Mycobacterium marinum*

Among the slow-growing mycobacteria, *M. ulcerans* and *M. marinum* form the closest relatives to Mtb based on DNA-DNA hybridisation studies, 16S rRNA sequencing and gas chromatography of fatty acids and alcohols¹⁴¹. The

closest relative of *M. ulcerans* is now identified to be *M. marinum*^{134, 141, 142}. 16S rRNA studies have shown a 99.6% identity between *M. ulcerans* and *M. marinum*¹³⁴.

M. marinum is a free-living NTM that is distinctively associated with cutaneous granulomas or shallow ulcers that result after exposure of traumatised skin or cuts to water from swimming pools, fish tanks or natural reservoirs^{143, 144}. This association has given the disease the name ‘swimming pool/fish tank granuloma’¹⁴⁵. *M. marinum* is also the closest genetic relative of Mtb¹⁴⁶ and granulomas produced by *M. marinum* in zebrafish has been utilised as a surrogate platform for researching the host-pathogen relationship of Mtb in host tissue tuberculomas¹⁴⁷.

Even though *M. ulcerans* and *M. marinum* share a close genetic similarity and both are skin pathogens in humans, they are characteristically different phenotypically. Table 1.1 highlights the phenotypic differences between *M. marinum* and *M. ulcerans*. Whole genomic sequencing of *M. ulcerans*, and genomic comparison studies with *M. marinum* has helped to identify that *M. ulcerans* has evolutionarily diversified away from a precursor *M. marinum*-like parent strain¹⁴⁸.

Table 1.1 Phenotypic differences between *M. marinum* and *M. ulcerans*

Characteristics	<i>M. marinum</i>	<i>M. ulcerans</i>
Geographical distribution	Worldwide	Highly restricted niches – wetlands, slow moving rivers
Isolation from environment	Extensively and easily isolated from multiple aquatic species and environmental sources.	Extremely difficult and rare isolation from environment. Several aquatic insects have been implicated as hosts mainly based on DNA isolation.
Mode of transmission	Clear association between skin cuts and infected swimming pools	Remains an enigma with possibility of cuts, abrasion, and insect bites in the transmission cycle
Growth characteristics	Faster growth with doubling time of 6 – 11 hours	Extremely slow growth; Doubling time ranges from 48 hours to 4-8 days
Infection	Limited, granulomatous skin infection	Large ulcers
Treatment	Most anti-TB agents can be used	8 weeks of treatment with Rifampicin and Streptomycin/Clarithromycin

The evolutionary divergence of *M. ulcerans* from *M. marinum* has been extensively studied. The trigger for the initiation of evolutionary diversification of *M. ulcerans* has been the acquisition of a massive plasmid, pMUM001¹⁴⁹. The 174 kB plasmid codes for the major virulence factor of *M. ulcerans*, the toxin mycolactone^{148, 150}. Mycolactone is cytotoxic,

immunomodulatory, and induces analgesia^{151, 152}. The cytotoxic nature of the toxin helps in the formation of the characteristic deep necrotic ulcers of Buruli ulcer. The impaired uptake by phagocytes due to the immunomodulatory effect results in large clusters of extracellular bacilli in infection foci^{153, 154}. Analgesia is a characteristic feature of early lesions and may contribute to the initial apathy in patients in reporting to a healthcare centre. Initially, analgesia was thought to be due to nerve damage induced by mycolactone, but now it has been deciphered to be a hyperpolarising action of mycolactone on neurons^{153, 155, 156}.

Apart from plasmid acquisition, *M. ulcerans* has undergone a massive genome reduction with loss of up to 1,000 kB of genetic information, probably to adapt to a new lifestyle¹⁵⁷. The exact environment that the organism is adapting to is not known yet, with the extremely slow growth rate of the organism being a hinderance to its isolation from environmental samples. Clues from the genes lost points to the organism adapting to a new host where the organism does not require the genes required to survive in the harsh aquatic environment as that of *M. marinum*. Among the genome reduction strategies observed, the main one is the acquisition of the insertion elements IS2404 and IS2606, which has resulted in the disruption of multiple genes^{157, 158}. Table 1.2 highlights the main genetic differences between *M. marinum* and *M. ulcerans*. The strains listed for comparison in the table are the *M. ulcerans* AGY99 (GenBank (Assembly:GCA_000013925.2) and the *M. marinum* M (GenBank (Assembly:GCA_000018345.1) strains. The *M. ulcerans* AGY99 strain was isolated from an ulcerative lesion on the right elbow of a female

patient from the Ga district of Ghana in 1999. The *M. marinum* M strain was isolated from a patient with fish tank granuloma in San Francisco in 1992. The table was adapted from the genomic comparison data published by Demangel et al¹⁵⁹.

Table 1.2 Genomic reduction strategies of *M. ulcerans* and differences between *M. ulcerans* and *M. marinum* genotypically.

Characteristics	<i>M. marinum</i>	<i>M. ulcerans</i>
Genomic size and arrangement	Circular chromosome; 6,637 kb	Circular chromosome; 5632 kb
No: of functional genes	5,424	4,160
Number of pseudogenes	65	771
No: of insertion sequences	7 insertion sequences (Max: 7 copies of Myma01 and Mym02)	IS2404 (213 copies); IS2606 (91 copies)

1.4.2 *M. ulcerans* – lineages and genomic diversification.

Evidence of ongoing genomic reduction and diversification of *M. ulcerans* was studied by comparing the genomes of 30 *M. ulcerans* isolates obtained from several regions of the world. Large sequence polymorphisms in the strains have helped in the delineation of the isolates into two distinct lineages: Ancestral and classical^{160, 161}.

The ancestral lineage bears a closer resemblance to the parental *M. marinum* genome with fewer genomic rearrangements. Infections with the ancestral strain are often sporadic and less virulent¹⁶². They are mainly reported from Japan, Mexico, and South America¹⁶³. The classical lineage has been

estimated to have diverged from the ancestral lineage about 400,000 years ago and carries more extensive genomic rearrangements and more genomic loss compared to the parental *M. marinum*¹⁶⁴. Infection with strains belonging to the classical lineage is more common, severe, and responsible for the major burden of disease in Africa and Australia – the two main epicentres of the disease¹⁶⁵.

1.5 Bioenergetics in mycobacteria – a new hotspot in TB drug research

Respiration and the bioenergetic machinery of mycobacteria were recognised as a novel and efficient drug target space following the identification of bedaquiline (BDQ) as a potent antituberculous agent¹⁶⁶. BDQ, now marketed as Sirturo® acts on the F₁F₀-ATP synthase enzyme, a crucial enzyme that catalyses the formation of ATP in the metabolic pathway¹⁶⁶.

Adaptability and flexibility in metabolism and energy production are crucial in mycobacteria, as they inhabit diverse environments with versatile nutrient availabilities. They can also adeptly shift from actively replicating to dormant, non-replicating states¹⁶⁷⁻¹⁶⁹. Transitions between host environments and replication states can stress metabolism and energy production. Respiratory resilience is manifested in the ability of the organism to modulate enzyme expression in the multiple parallel and alternate pathways¹⁷⁰⁻¹⁷². Through genomic sequencing, manipulations, and metabolomics, the critical metabolic pathways and enzymes essential for the survival of Mtb have been studied extensively^{171, 173, 174}.

1.5.1 Respiratory pathway of mycobacteria

Energy production begins with the breakdown of complex carbon sources such as glucose, fatty acids, and/or proteins. Glucose undergoes lysis through either the glycolysis (EMP pathway), pentose phosphate pathway, or the Entner-Duoderoff pathway. Fatty acids are processed through beta-oxidation. The end product of these pathways produces either pyruvate or acetyl CoA that enters the tricyclic acid cycle (TCA)¹⁷⁵. TCA is a key metabolic pathway that is the entry point for several substrates like succinate and generates numerous intermediates that are absorbed into cell anabolic pathways. The main product of the pathway is carbon dioxide and reduced nicotinamide-adenine-dinucleotide (NADH). A small amount of ATP is generated in these steps as well and has been referred to as substrate-level phosphorylation¹⁷⁶.

In aerobic conditions, the final and critical phase of energy production is catalysed by the oxidative phosphorylation (OxPhos) pathway (Fig 1.1)¹⁷⁷. Here, electron-rich metabolic by-products: NADH or FADH₂ donates electrons into the electron transport chain (ETC) using the primary dehydrogenases that leads to the reduction of menaquinone^{178, 179}. The menaquinone/menaquinol pool is the central link between the electron-donating and accepting reactions¹⁸⁰. The terminal oxidoreductases reoxidise the reduced menaquinol and catalyse the transfer of the electron to oxygen with the formation of H₂O^{171, 178, 181}. While transporting electrons along this chain, the primary dehydrogenases and terminal oxidoreductases play the vital role of pumping protons (H⁺) to the extracellular space, thereby creating the crucial electrochemical gradient build-up across the inner cell

membrane¹⁷¹. This vital biological phenomenon is referred to as the proton motive force (PMF), and it generates the energy needed for ATP synthesis¹⁸².

In mycobacteria, the first reaction of the ETC is primarily borne by the NADH dehydrogenase (NDH) enzyme. Mtb harbour one copy of the proton pumping NDH type I and two copies of the non-proton pumping NDH type II^{183, 184}. An alternate dehydrogenase is the succinate dehydrogenase (SDH) that uses succinate, an intermediate of the TCA cycle, thereby linking central carbon metabolism to the OxPhos pathway. In Mtb, two types of SDH are seen: SDH1 and SDH2. SDH1 is upregulated in energy-deficient conditions, and SDH2 has been recognised to be upregulated in hypoxic conditions^{171, 185}. In keeping with the need for adaptability to hypoxia or starvation, alternate electron donors and dehydrogenases have been described for Mtb, such as proline dehydrogenase, alanine dehydrogenase, hydrogenases, carbon monoxide dehydrogenases etc^{171, 185, 186}.

Menaquinone is the sole lipid-soluble electron carrier in the mycobacterial ETC^{180, 187}. The menaquinol can be oxidised by two terminal oxidoreductases in mycobacteria. The predominant terminal oxidoreductase is the cytochrome *bcc:aa₃* (cyt-*bcc:aa₃*) super complex. This heme-copper respiratory oxidase enzyme is a supercomplex and consists of two main subunits: the cytochrome *bcc* (Complex III) comprising of QcrC, QcrA, and QcrB and the cytochrome *aa₃* subunit (Complex IV) comprising of CtaC, CtaD, CtaE and CtaF^{178, 188, 189}. For every 2 electrons transferred through the complexes, 6 protons are pumped to the extracellular space making cyt-*bcc:aa₃* a highly energy

efficient terminal oxidase¹⁷¹. Attempted gene knockout studies have proven the essentiality of the enzyme in mycobacteria^{178, 188, 190}.

Cytochrome *bd* oxidase (cyt-*bd*) is an alternate terminal oxidoreductase and has been described only in prokaryotes¹⁹¹. Mycobacterial cyt-*bd* comprises of two functional subunits Cyt A and Cyt B coded by *cydA* and *cydB* respectively. *cydDC*, an incompletely studied possible cytochrome ABC transporter, appears with *cydAB* in a single operon (*cydABDC*) in mycobacteria^{181, 192}. Being non-proton pumping, the cyt-*bd* is less energy efficient than cyt-*bcc:aa₃*. It has been described to be upregulated in microaerophilic conditions or when cytochrome *bcc:aa₃* is incapacitated^{173, 193, 194}. The enzyme is non-critical in mycobacteria and is not strictly essential for surviving in nutrient starvation or in the mouse model¹⁷³.

In hypoxia, i.e., the absence of oxygen, nitrate, and fumarate molecules can act as terminal electron acceptors (Fig 1.1). The enzymes that catalyse these reactions are nitrate reductase, coded by *narGHJI*, and fumarate reductase coded by *frdABCD*¹⁷¹. These genes have been shown to be non-essential in Mtb in two separate studies except for the *frdA* gene in Mtb^{195, 196}. Moreover, even though these enzymes are expressed in hypoxia, they are unable to sustain growth and replication of the cells and are probably only used to maintain the redox potential of the cell¹⁷¹.

Figure 1.1 Components and organisation of the electron transport chain of mycobacteria (Fig adapted from Cook *et al.*¹⁷¹)

ATP is synthesised by the membrane-bound F_1F_0 -ATP synthase, encoded by the *atpBEFHAGDC* operon¹⁷¹. In the setting of a high PMF (and low intracellular ATP), the enzyme facilitates the influx of H^+ into the cell¹⁹⁷. This powers a rotary-catalysis process that enables the phosphorylation of ADP, resulting in the synthesis of the high energy phosphate bond of ATP. The enzyme is indispensable for optimal growth in both Mtb and *M. smegmatis*^{198, 199}.

1.5.2 Oxidative phosphorylation as a drug target space

BDQ was the first drug shown to have potent activity against the mycobacterial respiratory chain. The compound was developed and refined by Janssen Pharmaceuticals (Titusville, NJ, USA) and was discovered from a whole-cell screen using *M. smegmatis*¹⁶⁶. The molecule harbours a

quinolinic central heterocyclic nucleus with alcohol and amine side chains. It was shown to act on the F_1F_0 -ATP synthase, the critical enzyme involved in the synthesis of ATP^{166, 200, 201}. Due to its unique mode of action and good potency in both drug-sensitive and resistant TB, it obtained accelerated and conditional FDA approval in the USA in 2012 for drug-resistant TB²⁰². Several years and clinical trials later, BDQ is still a successful drug candidate credited with shortening drug therapy for multi and extremely drug-resistant TB (MDR and XDR TB)^{203, 204}.

With the unprecedented success of BDQ, drug discovery efforts were refocused on the respiratory pathways of mycobacteria. Groundwork with mutagenesis studies had already demonstrated the essentiality of the OxPhos pathway, and it was already proved that substrate-level phosphorylation alone was insufficient for Mtb survival^{198, 199}.

Besides being the energy powerhouse of the cell, the OxPhos pathway also maintains a PMF, which is essential for several activities of the cell²⁰⁵. Targeting the OxPhos pathway obliterates this membrane potential. Efflux pumps mediating drug resistance for several anti-TB drugs is an energy-dependent process. Hence targeting energy production in this context will reduce the efficacy of efflux pumps and thereby impeding resistance to many drugs. This paves the way for excellent synergy in drug combination therapies²⁰⁶.

Several screens have yielded new drugs, and several old drugs like clofazimine have been repurposed for their action on the OxPhos pathway²⁰⁷⁻²¹⁰. Nitroimidazoles like delamanid (Deltysba) and pretomanid (PA-824) have received regulatory approvals and have proven activity against respiratory cytochromes²¹¹. Despite the conservation of OxPhos pathway elements between prokaryotes and eukaryotes, the clinically approved drugs have all demonstrated an excellent safety profile. This eases safety concerns, which were a valid cause of concern in the initial stages of research efforts for discovering antimicrobials targeting this pathway^{207, 211}.

1.5.3 Q203

Q203 (Telacebec), an imidazopyridine amide, was developed as an anti-TB drug after lead optimisation from a high throughput phenotypic screen conducted using Mtb H37Rv-GFP infected macrophage cell line²⁰⁵. Q203 acts on *cyt-bcc:aa₃*, the predominant terminal oxidoreductase of the OxPhos pathway. The compound has exquisite potency against Mtb with an MIC₅₀ (Minimum inhibitory concentration of drug required to achieve a half-maximal inhibition of growth) in the range of 1.5-2.8 nM, almost 10 times lower than BDQ (MIC₅₀ of 42-133 nM in Mtb). The drug has favourable pharmacokinetic properties in mice with a terminal half-life of 23 hours, good volume of distribution, and excellent tissue penetration^{173, 205}.

Q203 interferes with the function of the *cyt-bcc:aa₃* complex by binding on the QcrB subunit of the cytochrome *bcc*^{205, 212}. It is the first compound acting on the *cyt-bcc:aa₃* in Mtb to have completed Phase 2 clinical trials²¹³. An

excellent safety profile was noted in humans in the Phase 1 study with no adverse effects noted, even up to 800 mg of single-dose oral administration²¹³. Several imidazopyridine compounds, structural analogues of Q203, and other compounds acting on *cyt-bcc:aa₃* are now in various phases of drug development²¹⁴⁻²¹⁷.

Although Q203 has an excellent growth inhibitory action, it is not bactericidal. In a recent study, it was demonstrated that the redundant action of *cyt-bd* oxidase could bypass the loss of function of *cyt-bcc:aa₃* elicited with Q203 *in vitro* and *in vivo*¹⁷³. Bactericidal action of Q203 is demonstrated in a *Mtb* strain with a genetic knockout of the *cyt-bd* oxidase¹⁷³. In this scenario, synergistic action is anticipated for Q203 with chemical inhibition of *cyt-bd* oxidase. Studies on inhibition of *cyt-bd* oxidase is now gaining momentum with the more insights gained into the detrimental role played by the enzyme in environmental stress, which may be more reflective of *in vivo* conditions²¹⁸.

1.5.4 Targeting the OxPhos pathway of NTM

With significant funding dedicated to anti-TB drug research, several lead compounds are now being developed for MDR and XDR TB. One advantage of this research is the spill over utility of these candidates for NTM diseases. Many of the diseases caused by NTM are neglected tropical diseases or even designated orphan diseases and may not have adequate funding to promote independent drug research. Thus, repurposing TB drug candidates for NTM

serves as an affordable, time-saving bypass into drug development for NTM²¹⁹.

Targeting energy metabolism in NTM is rapidly expanding due to the novelty of the drug target and the shared, conserved energy metabolism pathways in mycobacteria. BDQ was already established to inhibit the growth of *M. abscessus* and *M. avium*^{166, 220, 221}. However, Q203 was shown to have no activity in *M. abscessus*²²². The varied actions of different drug candidates on NTM highlights the versatility of metabolic pathways. Moreover, sustaining growth in different environments puts pressure to modify metabolic pathways according to each organism's environmental, nutritional availabilities.

The use of energy metabolic inhibitors in NTM is a novel and promising field. More scientific studies into these pathways will provide clues to metabolic adaptations of the numerous NTM and help us to identify drug candidates suitable for each strain.

1.6 Hypothesis

Among the novel drug candidates targeting the OxPhos pathway of Mtb, Telacebec (Q203), an imidazopyridine amide (IPA), is a promising new clinical-stage oral drug candidate (Fig 2.3)^{205, 223, 224}. It acts on the terminal electron acceptor *cyt-bcc:aa₃* of the OxPhos pathway and has MIC₅₀ values in the range of 1-5 nM in Mtb²⁰⁵. Q203 recently completed Phase 2 clinical

trials, demonstrating an excellent safety profile and some degree of efficacy in human subjects²¹³.

In Mtb, Q203 induces a rapid but partial cellular ATP depletion and sustained growth inhibition. However, the compound is limited in its ability to kill Mtb and is at best bacteriostatic. In an elegant study, Kalia et al., demonstrated that the alternate terminal oxidase *cyt-bd* played a major role in mitigating the bactericidal potency of Q203, likely due to its compensatory action in acting as terminal oxidase when the *cyt bcc-aa₃* is pharmacologically inhibited^{172, 173, 225}. In Mtb cells with genetically deleted *cyt-bd*, Q203 is bactericidal and rapidly cleared *in vivo* infection¹⁷³.

From preliminary genomic evidence in *M. ulcerans* strains belonging to the preponderant classical lineage, there is genetic evidence of loss of *cyt-bd* along with three defined terminal electron acceptors in the OxPhos pathway (Fig 2.3). The sole terminal oxidoreductase is postulated to be the *cyt bcc-aa₃*. From our previous knowledge of the mode of action of Q203 and the unique arrangement of the OxPhos pathway of *M. ulcerans*, we predicted that Q203 might be a potent drug for BU treatment. In this study, I aim to outline the effect of Q203, a *cyt bcc-aa₃* inhibitor in *M. ulcerans*.

Figure 2.3: Repurposing Q203 for *M. ulcerans* rests on the hypothesis that Q203 can effectively bind to the *M. ulcerans* *cyt-bcc:aa₃* to shut down its respiration. *M. ulcerans* is unable to mount any rescue of the OxPhos pathway due to defunct alternate terminal oxidoreductases. In *Mtb*, it has been shown that *cyt-bd* can functionally compensate for the role of *cyt-bcc:aa₃* when inhibited by Q203.

Aims

1. To determine the potency of Q203 in *M. ulcerans* classical and ancient lineages.
2. To determine the potential of a single dose cure for Buruli ulcer in the mouse model of infection.

– CHAPTER 2 –

**Exploiting the reductive evolution of
Mycobacterium ulcerans to repurpose the
tuberculosis drug candidate Q203**

2. Exploiting the reductive evolution of *Mycobacterium ulcerans* to repurpose the tuberculosis drug candidate Q203.

2.1 Introduction

2.1.1 Buruli ulcer

Buruli ulcer (BU) is a slowly progressing necrotising disease of the skin and subcutaneous tissue. The disease initially manifests as subcutaneous nodules and progresses to large ulcers with undermined edges if left untreated (Fig 2.1a). The lesions are most commonly located in the limbs with 80% of lesions localised to the distal extremities of the leg²²⁶. Although mortality is not a major complication of this disease, severe debilitation (Fig 2.1b) as a result of bone and joint involvement can restrict the patient's mobility and quality of life²²⁷.

Figure 2.1. A small Buruli ulcer of lower limb (a) and healing with deforming contractures in the hand (b) of patients from West Africa^{76, 228}.

The disease has been reported in 33 countries around the world and is most commonly found in tropical countries in Africa, and coastal regions of Australia (Fig 2.2a)⁴⁵. In rural Africa, around 50% of infections are children

below 15 years of age⁷⁶. The epidemiology of the disease is constantly evolving worldwide as certain countries of West Africa, like Nigeria and Liberia, is now witnessing a surge in the number of identified cases (Fig 2.2b)^{229, 230}. Of marked concern recently were the reports of BU with increasing severity and incidence emerging from regions of South East Australia (Fig 2.2b)^{231, 232}.

Fig 2.2 a) Epidemiology of Buruli ulcer worldwide reported by WHO²³³. b) Graph depicting the changing epidemiology of the disease in West African countries (DRC: Democratic Republic Of Congo) and Australia from 2013 to 2015, graph created with data from WHO Global Health Observatory Data²³⁰.

2.1.2 *Mycobacterium ulcerans* – origin and evolution of the causative agent

Mycobacterium ulcerans, a slow-growing environmental mycobacterium, is the etiological agent of BU⁴¹. Through extensive genomic studies, it has been determined that *M. ulcerans* has evolved from *Mycobacterium marinum*, an aquatic mycobacterium that can cause granulomas in both fish and humans^{134, 234}. The main genomic distinction of *M. ulcerans* from *M. marinum* is its massive genome reduction of almost 1.1 Mb through the acquisition of insertion elements IS2404 and IS2606 that interrupt genes, pseudogene formation through accumulated mutations, large chromosomal rearrangements, and gene deletions^{157, 159}.

Evidence of ongoing genomic reduction among *M. ulcerans* isolates from various regions of the world has helped to identify and classify two main lineages of the organism¹⁶². The ancient lineage is associated with the milder and sporadic cases in South America, Japan, and China¹⁶⁰. The classical (also known as modern) lineage is responsible for most of the cases in Africa and Australia and has been established to be the more successful pathogen in both severity and number of infections. Classical strains have undergone extensive genomic rearrangement compared to the ancestor *M. marinum*, while the ancient strain is more similar genetically to *M. marinum*¹⁶⁰. Compared to the ancient lineage, the classical lineage has undergone more genomic reduction with distinctively more copies of IS2606 that help in gene disruptions¹⁴⁸.

2.1.3 Treatment of BU – current standards and scope for improvement

Until recently, BU was treated with wide surgical debridement alone. High surgical bed occupancy, coupled with poor public health infrastructure and high relapse rates, prompted clinicians to consider antimicrobial therapy⁹⁶⁻⁹⁸. The initial attempts at anti-microbial therapy were the use of anti-tuberculous and anti-leprosy drugs. Rifampicin, rifabutin, clofazimine, several macrolides, and aminoglycosides were tested for efficacy in the established mouse model of BU infection^{235, 236}. These studies laid the foundation for the current treatment regimen of daily oral rifampicin and intramuscular streptomycin put forward by WHO in 2004 based on interim results of a landmark study by Etuaful et al^{77, 101}.

Although an improvement over surgical approaches, with very little relapse rates^{101, 102, 237}, this regimen entails a number of problems such as long duration of treatment (8 weeks) leading to compliance issues, and potentially severe ototoxicity and renal impairment associated with daily doses of streptomycin^{238, 239}. Daily intramuscular injections of streptomycin for 8 weeks can be painful, inconvenient, and a major cause of dropouts in treatment, especially in children²⁴⁰. Long term rifampicin administration also can cause serious toxicities such as hepatitis and can interfere with numerous other vital medications such as anticoagulants, antifungals, etc^{103, 105}. The current urgent need for BU therapy is an all-oral drug combination regimen with a shorter treatment duration.

Efforts to replace streptomycin with an oral drug alternative yielded clarithromycin, a macrolide agent that is used to treat pulmonary infections caused by other non-tuberculous mycobacteria like *Mycobacterium avium*^{110, 112, 241}. Recently rifampicin-clarithromycin combination for 8 weeks was found to be non-inferior to the current rifampicin-streptomycin combination in human trials conducted in Ghana and Benin¹¹⁸. Although an improvement over daily injections, this regimen still bears a high pill burden with 8 weeks of therapy. Compliance remains an issue, and even more since patients do not report to a healthcare centre for injections¹³³. Moreover, all these regimens still rely on rifampicin as the major sterilising agent. Shorter treatment and alternative drugs are paramount for controlling the disease.

2.1.4 Drug discovery for *M. ulcerans*

Historically, the development of new drugs against *M. ulcerans* has not always been a precise science. A consolidated effort for drug discovery for BU is still lacking and is largely hindered by the exorbitant cost of developing a novel drug candidate, especially for a neglected tropical disease with a very confined geographical occurrence²⁴². Another technical complication is the extremely slow growth rate of *M. ulcerans*, which complicates modern high throughput cell-based drug screens. The strains often have a replication time of 72-96 hours, with colonies often taking up to 3 months to appear on solid agar plates.

Repurposing drug candidates developed for *Mycobacterium tuberculosis* (Mtb) remains a viable option for the pathogen. Despite the process of

reductive evolution, *M. ulcerans* is genetically very close to Mtb¹⁴⁸. The similarity of drug targets can be exploited to cross over drug candidates from the Mtb drug pipeline to *M. ulcerans*^{219, 243}, although successes are still rare. A study describing a resazurin-based assay using 83 lead anti-tuberculous compounds yielded only five compounds with potential against *M. ulcerans*²⁴³. Pretomanid (PA-824), which forms a component of the now approved BPaL regimen for drug-resistant tuberculosis²⁰⁴, failed to kill *M. ulcerans*, with disappointing MIC²⁴⁴. One reason for the limited success may be the loss of activating enzymes in *M. ulcerans* following its reductive evolution from Mtb.

A successful cross over drug was bedaquiline (Sirturo®, BDQ), a strikingly potent antituberculous drug approved by FDA for drug-resistant tuberculosis (TB) in 2012^{166, 202}. BDQ was hailed as a welcome break in the drug discovery efforts for TB after a forty-year dearth in new drugs since the approval of rifampicin in 1963²⁴⁵. *In vitro* studies of BDQ on *M. ulcerans* proved the drug was active with very low MIC reflecting the same activity range as Mtb¹⁶⁶. Its sterilising potential was further validated in *in vivo* BU mouse model studies^{244, 246}.

2.1.5 Oxidative phosphorylation – a potential drug target space

BDQ was distinctly novel in being the first anti-TB drug to act on F₁F₀-ATP synthase, a quintessential enzyme that harvests the potential of the proton motive force (PMF) generated by the oxidative phosphorylation (OxPhos) pathway to synthesise ATP¹⁶⁶. BDQ was key in unmasking the potential of

energy metabolism of mycobacteria as a drug target space²⁰⁷. Several years of research have unearthed numerous compounds targeting the ATP synthesis of mycobacteria, especially the OxPhos pathway²¹¹.

Mutagenesis experiments involving various enzymes of the OxPhos pathway has proven that this pathway is essential for mycobacteria^{188, 207, 247}. Several reasons have been postulated, such as the lack of an NADH-dependent lactate dehydrogenase^{174, 248}, the inability of the massive cell to sustain itself on the ATP generated by substrate-level phosphorylation, or the need for an OxPhos pathway to sustain and maintain a membrane potential¹⁷¹. Predominant targets in the OxPhos pathway are the NADH dehydrogenase type 2 enzyme, menaquinone biosynthesis pathway, the cytochrome *bcc:aa₃* (cyt-*bcc:aa₃*), and cytochrome *bd* oxidase (cyt-*bd*)^{187, 211}.

The cyt-*bcc:aa₃*, a massive heme-copper supercomplex, is the predominant terminal oxidase of the OxPhos pathway. Due to its essentiality, it forms a particularly good drug target, and several compound classes such as imidazopyridine amide (IPA) compounds^{172, 249, 250}, 2-(quinolin-4-yloxy)acetamides²⁵¹, and phenoxyalkylbenzimidazoles²⁵² have been described to impede its action. Even zolpidem (a sedative-hypnotic) and lansoprazole (an antacid) have been shown to act on cyt-*bcc:aa₃*^{217, 253}. Remarkably, all these compounds have been determined to bind to a very specific site of the QcrB protein of the cytochrome *bcc* complex. In fact, due to its susceptibility to numerous drug inhibitors belonging to different chemical classes, QcrB has earned the moniker of being a “promiscuous

target'²¹². Among the compounds, Q203 (Telacebec) belonging to the IPA series is the front runner, having recently completed phase 2 clinical trials against TB^{205, 213}.

With respect to *M. ulcerans*, the bioenergetics pathway may be an attractive drug target space, and BDQ has already proven to be effective in *M. ulcerans*^{166, 244, 254}. The first published whole genome sequence of *M. ulcerans* Agy99 strain belonging to the classical lineage provided insight into how genomic reduction of *M. ulcerans* has affected the respiratory pathways¹⁵⁷. Several similarities between the metabolic pathways of Mtb and *M. ulcerans* was noted with intact glycolysis and pentose phosphate pathways along with conserved systems for utilising fatty acids. However, extensive gene disruption has rendered key enzymes in the Entner Duoderoff pathway and the methyl isocitrate pathway defunct making these pathways nonviable¹⁵⁷.

In the OxPhos pathway, among the numerous primary dehydrogenases, three copies of the NADH dehydrogenases and two copies of the succinate dehydrogenase remain intact. Among the terminal oxidoreductases, *cydA* of cytochrome *bd* oxidase (*cyt-bd*) has been rendered a pseudogene with a point mutation causing a premature stop codon¹⁵⁷. Also, the entire *nar* and *frd* operon coding for nitrate and fumarate reductases, the alternate terminal reductases of menaquinone in conditions of hypoxia are missing from sequenced *M. ulcerans* genome^{157, 159}. The only viable terminal oxidoreductase in this scenario is the cytochrome *bcc:aa3* (*cyt-bcc:aa3*).

Genome reduction has affected the respiratory pathway of *M. ulcerans*. In several key pathways, several redundant enzymes and critical enzymes have been lost. This places the organism at a unique vantage point for drug development since alternate and redundant pathways have been trimmed off in respiration, and the new surge of drugs targeting these pathways can be used to advantage in *M. ulcerans*.

2.3. Materials and Methods

2.3.1 Bacterial strains and culturing conditions:

M. ulcerans clinical strains S1013, an African strain belonging to classical lineage, and S1325, a Japanese strain belonging to ancient lineage, were obtained from Dr. Gerd Pluschke, Swiss Tropical and Public Health Institute. 15 % glycerol stocks of the above strains were prepared and stored at -80 °C. Bacterial strains were cultured in Middlebrook 7H9 liquid medium (Becton Dickson and Company Limited, USA) supplemented with 0.05 % Tween 80, 0.5 % Glycerol, and ADS enrichment to an optical density range of growth from OD_{600nm} of 0.3 to 0.7. The solid medium used for culture was Middlebrook 7H10 or 7H11 medium supplemented with 0.5 % glycerol and OADC enrichment broth (Biomed Diagnostic Private Limited, USA). The incubation temperature was 32 °C for *M. ulcerans*. Due to the slow growth rate of *M. ulcerans* and complete aerobic respiration, the cultures were incubated for two–three weeks with constant shaking to reach the log phase of growth. To avoid contamination with other fast-growing microorganisms, all *M. ulcerans* strains were handled in a dedicated biosafety cabinet reserved for extremely slow-growing mycobacteria.

2.3.2 Antimicrobial agents

Bedaquiline was purchased from Cellagen Technology, San Diego, USA, and Q203 was purchased from GVK Biosciences Private Limited, India. ND-011176 (HT-04) was synthesised by and a gift from Garrett Moraski, University of Montana, USA.

2.3.3 Minimum inhibitory concentration (MIC) Assay

2.3.3.1 Drug intermediate plate

The drugs were dissolved in 90 % DMSO to create stock solutions that were stored at -20 °C. The drug intermediate plates were prepared at 100 times the testing concentration, and the drugs were serially diluted 2-fold and ten times in 90 % DMSO.

A dose-response curve was plotted by charting the fluorescence read with Alamar blue reagent or optical density (OD) measurement against the log of drug concentration. The half-maximal activity (MIC₅₀) was derived from the curve, corresponding to the concentration of the drug that decreases the activity of the organism by half from the baseline in a full dose-response curve. All the MIC assays were conducted in technical triplicates. All assays were repeated at least once.

2.3.3.2 Alamar blue assay

AlamarBlue™ cell viability reagent (ThermoFischer, USA) was used to measure metabolic activity of *M. ulcerans* cells in drug inhibitory assays. The assay works on the principle that the redox dye in the assay kit is reduced in the presence of ongoing metabolic activity. The dye can be used for a fluorometric or colorimetric assessment of metabolic activity.

The organisms to be tested were grown to log phase (OD₆₀₀: 0.3-0.7) in 7H9 medium supplemented with glycerol, Tween 80, and ADS. 200 µl of cultures at OD₆₀₀ of 0.02 was inoculated into each well of 96 well transparent flat plate. 2 µl of the drug was transferred to each corresponding well in the plates. The outer wells were inoculated with 200 µl of autoclaved de-ionised water

to prevent the edge effect. The plates were incubated at 32 °C for 7 days. 20 µl of Alamar blue reagent along with 12.5 µl of filter sterilised Tween 80 was added to each well. The plates were then incubated further at 37 °C for 24 hours, and fluorescence reading (Excitation: 540 nm; Emission: 610 nm) was taken on the Cytation-3 Cell Imaging Multiple-mode reader (BioTeck instruments)

2.3.3.3 Optical density measurement

M. ulcerans cultures were grown in 7H9 medium to reach log phase growth of OD₆₀₀ 0.3 to 0.7. The cultures were resuspended in 7H9 liquid medium (without glycerol) to an OD₆₀₀ of 0.05. 200 µl of cultures was added to each well of a 96 well flat-bottomed transparent plate. 2 µl of the drug was added from the drug intermediate plate at 2-fold serial dilution. The outermost wells were filled with 200 µl of autoclaved DI water. The plates were incubated at 32 °C for 15 days. The optical density absorbance reading at 600 nm was taken on the Cytation-3 Cell Imaging Multiple-mode reader (Biotek Instruments) after mixing the contents of each well by pipetting.

2.3.4 Intracellular ATP measurement and quantification

Bactiter-Glo™ Microbial Cell Viability Assay (Promega, USA) was used to quantitatively assess the intracellular ATP levels of the organisms tested. The assay works on the principle that the recombinant firefly luciferase enzyme requires ATP as a substrate to convert beetle luciferin to oxyluciferin, a luminescent product. Mid-log-phase cultures of *M. ulcerans* were resuspended to OD₆₀₀ of 0.05. 100 µl of cultures were dispensed into each

well of the 96 well white plate along with 1 µl of test drugs. After incubation of 24 hours at 32 °C, an equal amount of the Bactiter-Glo reagent was added to the wells of the plate. The cultures were incubated with the reagent for ten minutes at room temperature. The luminescence reading was taken on the Cytation-3 Cell Imaging Multiple-mode reader (Biotek Instruments) after applying the luminescence fibre filter.

2.3.5 Oxygen consumption assays

Oxygen is reduced to water in the OxPhos pathway. The amount of oxygen consumed over time (Oxygen consumption rate – OCR) in a closed environment is an indicator of the functionality of the terminal oxidases of the OxPhos pathway.

2.3.5.1 Methylene blue assay

Mid-log phase cultures were resuspended in 7H9 liquid medium to OD₆₀₀ 0.3 and incubated with drugs at the required concentration for 5 hours at 37 °C. 1.8 ml of the incubated cultures were transferred to 2 ml sterile glass bottles. Methylene blue dye at a concentration of 0.001 % was added to each bottle, and the bottles were tightly screwed shut, after which Day 1 pictures were taken. The bottles were then incubated in 1 litre anaerobic tight lockboxes with Anaeropack bags. After 72 hours, the tubes were observed, and pictures were taken. Cultures that have not utilised the oxygen dissolved in the medium remain blue, whereas the cultures which utilised the oxygen in the medium would have decolorized the methylene blue dye to colourless (Fig 2.4). The assay was conducted in technical triplicates and repeated once.

Figure 2.4: Working details and principle of the methylene blue assay. The principle of the methylene blue assay depends on the colour difference in the reduced and oxidised state of the methylene blue dye and the capability of a live organism to respire and deplete the oxygen in a closed environment.

2.3.5.2 Quantitative oxygen consumption assay - OCR measurement using Seahorse XFe96 respirometer.

The Seahorse XFe96 analyzer (Agilent Technologies, Santa Clara, USA) was adapted for use with *M. ulcerans* cultures to measure OCR immediately before and after drug action.

The XFe96 sensor probes were hydrated with sensor calibrant 24 hours prior to the assay as per manufacturer's instructions²⁵⁵ and incubated at 37 °C in a non-CO₂ incubator. Cell culture plates were coated with Cell-Tak at 22.4 µg/ml in 0.1 M Sodium bicarbonate solution and washed twice. All drugs were dissolved at 10 X of the final desired concentration in 7H9 medium. At the time of the assay, the drugs were dispensed into the drug ports of the assay

plate. Mid-log phase cultures were resuspended to OD₆₀₀ of 0.4 in 7H9 medium. 100 µl of culture was into the test well of the Cell culture microplate coated with Cell-Tak and centrifuged at 3800 rpm for 15 minutes at room temperature. Following the centrifuge, 80 µl of 7H9 medium was dispensed into the test wells by carefully pipetting down the sides of the well so as not to dislodge the cells. The four corner calibration wells were filled with 180 µl of 7H9 medium. The cell culture plate was incubated at 32 °C for 20 minutes before loading into the XF analyzer. Reading conditions were set to 20 minutes of calibration followed by OCR read every three minutes after dispensing of drugs. Analysis of results was done on GraphPad Prism 8 software. The assay was conducted in technical triplicates and repeated once.

2.3.6 Time Kill-kinetics assay

M. ulcerans cultures were grown to mid-log phase (OD₆₀₀: 0.4 to 0.6) in Middlebrook 7H9 medium (7H9 supplemented with 0.05 % Tween-80, 0.5 % glycerol, and ADS enrichment), washed twice, and adjusted to OD₆₀₀ 0.005 in 7H9 medium. 1 ml of the adjusted culture was dispensed into each well of a 24-well plate and incubated with 10 µl of the test compounds for 25 days at 32 °C. The assay was conducted in technical triplicates with three aliquots of culture for every antimicrobial concentration. Bacterial viability was determined by Colony Forming Units (CFUs) enumeration on agar plates. CFU enumeration was performed by transferring 100 µl of inoculum from the culture flasks into a single well of a 96-well clear round bottom plate. All samples were serially diluted in Middlebrook 7H9 medium without glycerol, and 50 µl was plated on 7H10/OADC agar quadrant plates. The plates were

incubated at 32 °C for 8-10 weeks. To prevent antibiotic carry-over effect, the cells were washed in 7H9 medium once before plating.

2.3.7 Raising drug-resistant mutants

To selectively enrich *M. ulcerans* mutants resistant to Q203, we plated cultures on a 7H10 medium containing 10 nM (10 x MIC₅₀) of Q203. Approximately 10¹⁰ cells were plated. 100 ml of *M. ulcerans* S1013 strain at OD₆₀₀ 0.4 was centrifuged at 3800 rpm for 10 minutes after dividing the volume equally into four 50 ml Falcon tubes. The deposit was resuspended in 600 µl of 7H9 medium. The deposit was plated on three separate 90 mm 7H10 agar plates containing 10 nM of Q203, each plate getting 200 µl of the resuspended inoculum. A representative sample of cultures was plated on 7H10 quadrant plates, in dilutions, to precisely determine the number of plated bacteria. Plates were covered in a double layer of parafilm, placed in Ziplock bags, and incubated at 32 °C for 8-10 weeks. Colonies were counted and sub-cultured for further analysis in a 7H9 liquid medium. Resistance to Q203 was established by checking MIC using the microbroth dilution technique with OD read at the endpoint. DNA extraction was performed by heating cell pellet resuspended in Tris-EDTA buffer (pH:8) at 95 °C for 10 minutes. The *qcrB* gene was sequenced to identify possible mutations conferring resistance to Q203.

2.4 Results

Repurposing Q203, a novel antituberculous drug, for *M. ulcerans*, opens new avenues of drug discovery for *M. ulcerans*. In addition to testing the potency of Q203 against *M. ulcerans*, we were able to design and establish novel drug testing methods for *M. ulcerans*, an organism that is extremely slow-growing and difficult to handle in the laboratory.

2.4.1 QcrB sequence alignment of *M. tuberculosis* and *M. ulcerans* yields 90% sequence identity.

The binding site of Q203 on the *cyt-bcc:aa₃* super complex of Mtb was determined to be the QcrB protein²⁰⁵. Hence it was imperative to compare the QcrB protein of *M. ulcerans* and Mtb to predict the susceptibility of Q203 to *M. ulcerans*. ExPASy program was used to compare the genes of Mtb and *M. ulcerans* Agy99. Sequences were obtained from PubMed from the fully sequenced Agy99 strain, a representative strain from the classical lineage. *M. ulcerans* Agy99 strain was obtained in 1999 from a lesion of a BU patient residing in Ghana¹⁵⁷.

In comparison, sequence identity of 90.3 % was generated between the two sequences (Fig 2.5). A three-dimensional model comparison also yielded a similar topology for both proteins. An additional 10 amino acids, ²⁰⁵CGGVGDDCTA₂₁₄, were present in the *M. ulcerans* protein subunit. However, on protein model comparison, the additional residue was estimated to lie away from the proposed Q203 binding site. Studies have repeatedly revealed six different sites at the inferable Q203 binding site that can harbour mutations that make QcrB resistant to Q203^{172, 205}. On comparison of those

six sites, no mutations were noted in all six positions in the *M. ulcerans* protein (Fig 2.5).

```

H37Rv_QcrB      1 MSPKLSPPNIGEVLARQAEDIDTRYHPSAALRRQLNKVFPTHWSFLLGEIALYSFVLLI
AGY99_QcrB      1 MSPKLSPPKIGDVLARQAEDIDTRYHPAAALRRQFNKVFPTHWSFLLGEVALYSFIVLLI
*****  ** *****

H37Rv_QcrB     61 TGVYLTLLFFDPSMVDVTYNGVYQPLRGVEMSRAYQSALDISFEVRGGLFVRQIHWWAALM
AGY99_QcrB     61 TGVYLTLLFFDPSMMDVTYNGVYQPLRGVEMSKAYASALDISFEVRGGLFVRQVHWWAALM
*****  *****

H37Rv_QcrB    121 FAAAIMVHLARIFTGAFRRPRETNWVIGSLLLILAMFEGYFGYSLPDDLSSGLGLRAAL
AGY99_QcrB    121 FAAAIMVHLARIFTGAFRRPREANWIIIGSLLLILAMFEGYFGYSLPDDLSSGLGLRAAL
*****  *****

H37Rv_QcrB    181 SSITLGMPVIGTWLHWALFGGDFP-----GTILIPRLYALHILLPGIILALIGL
AGY99_QcrB    181 SSITLGMPVIGTWLHWALFGGDFPCGGVGDDCTAAGYIIPRMYSLHILLPGIILALIGM
*****  *****

H37Rv_QcrB    231 HLALVWFQKHTQFPGPGRTEHNVVGVVRMPVFAFKSGAFFAAIVGVLGLMGGLQINPIW
AGY99_QcrB    241 HMALVWFQKHTQFPGPGRTEHNVVGVVRMPVFAVKSGAFFAAITGVVLGLMGGLQINPIW
* *****

H37Rv_QcrB    291 NLGPYKPSQVSAGSQPDFYMMWTEGLARIWPPWEFYFWHHTIPAPVWVAITMGLVFVLLP
AGY99_QcrB    301 NLGPYKPAHVSAGSQPDFYMMWTEGLARIWPPWEFYFWHHTIPAPVWVALIMGLIIFMLLI
*****  *****

H37Rv_QcrB    351 AYPFLEKRFTGDYAHNLLQRPDPVVRTAIGAMAIAFYMLTLFAMNDIIALKFHISLN
AGY99_QcrB    361 VYPFLEKRFTGDYAHNLLQRPDAPVRTAVGAMAI SFYMLTLFAMNDIIALKFHISLN
*****  *****

H37Rv_QcrB    411 ATTWIGRIGMVILPPFVFITYRWCIGLQSRSDRSVLEHGVTGIKRLPHGAYIELHQPL
AGY99_QcrB    421 ATTWIGRIGMVILPPFVFISYRWSIGLQSRSDRAVLEHGIETGIKRLPHGAYIELHQPL
*****  *****

H37Rv_QcrB    471 GPVDEHGHPIPLQYQGAPLPKRMNKLGSAGSPGSGSFLFADSAAEDAALREAGHAAEQRA
AGY99_QcrB    481 GPVDEHGHPIPLDYQGAPLPKRMNKLGSAGSPGSGSFLTADPASEDAALREAGHAAEHRA
*****  *****

H37Rv_QcrB    531 LAALREHQDSIMGSPDGE
AGY99_QcrB    541 LTALREYQDSLNETSNGE
* ****

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Figure 2.5. Alignment of the *M. tuberculosis* and *M. ulcerans* QcrB. The sequences of QcrB from *M. tuberculosis* H37Rv and *M. ulcerans* Agy99 were aligned using the ExPASy program. The residues in the red boxed region are the ten additional amino acids found in the *M. ulcerans* QcrB. The green boxed residues are the positions where substitutions are reported to be associated with resistance to Q203 and other structural analogues of Q203.

On comparison with four other fully sequenced clinical isolates of *M. ulcerans*, the QcrB protein shares 99 % sequence homology with the Agy99 strain QcrB with all of them sharing homology in the six essential Q203 binding sites. Three of the four strains belonged to the classical lineage, whereas *M. ulcerans* subsp. *shinshuense* belonged to the ancient lineage.

Hence, these observations pointed to the susceptibility of *M. ulcerans* to Q203.

2.4.2 *M. ulcerans* Classical lineage do not express a functional *cyt-bd*

Information gleaned from genomic sequencing of *M. ulcerans* classical strains has pointed to the conversion of *cydA*, a gene responsible for an integral component of the *cyt-bd*, into a pseudogene¹⁵⁷. The *cydA* gene coding for the functional subunit 1 of *cyt-bd* harbours a single nucleotide mutation at position 692 (G>A). This results in premature protein termination of CydA as the tryptophan encoding TGG is converted to a stop codon TAG (W231X). This phenomenon has only been observed in the classical lineage, whereas the ancestral lineage has no such mutation²⁵⁶ (Fig. 2.6). The differential disruption of the *cydA* sequence in classical lineages is in conformation with the general sequence of events where the ancient lineage is genetically closer to the parental *M. marinum* species with fewer genomic disruptions than the classical lineage that emerged later^{148, 162}.

	Leu	Gly	Cys	Trp	Val	Ala	Leu		
682-	CTG	GGG	TGT	TGG	GTT	GCG	TTG	-702	Mtb
682-	CTG	GGG	TGT	TAG	GTG	GCG	TTG	-702	<i>M. ulcerans</i> Classical
682-	CTG	GGG	TGT	TGG	GTG	GCG	TTG	-702	<i>M. ulcerans</i> Ancestral

Figure 2.6: A single-nucleotide polymorphism at position 692 from G to A of the *cydA* gene of the *M. ulcerans* classical lineage leads to the formation of a premature stop codon (in red). The conserved sequences in Mtb and *M. ulcerans* ancestral lineage are highlighted in green.

A functional *cyt-bd* impedes the action of Q203 in Mtb and renders the drug bacteriostatic¹⁷³. To prove the loss of function of the *M. ulcerans* classical lineage *cyt-bd*, a surrogate function detection system for *cyt-bd* from *M. ulcerans* using *M. bovis* BCG strain deficient in the expression of *cyt-bd* (BCG Δ *cydAB*) was developed. Two independent BCG Δ *cydAB* strains were transformed with the *cydAB* genes from *M. ulcerans* Agy99 (representative of classical lineage) and *M. ulcerans subsp. shinshuense* (representative of ancestral lineage), respectively (Fig 2.7). The three strains, BCG Δ *cydAB* :: Agy99 *cydAB*, BCG Δ *cydAB* :: Shinshuense *cydAB* and BCG Δ *cydAB*, were treated with Q203 to inhibit the *cyt-bcc:aa3*. In this setting only a functional *cyt-bd* can rescue electron transfer in the OxPhos pathway and help the organism respire (Fig 2.7). Oxygen consumption, a parameter to measure the OxPhos pathway functioning, was measured using the methylene blue assay in a closed environment.

Figure 2.7 Working principle of the assay designed to test the functionality of the *cydAB* from both lineages of *M. ulcerans*. In a Q203 treated *BCG ΔcydAB* cell, oxidative respiration is rescued by an adequately functioning complemented *cydAB*.

Upon treatment with Q203, oxygen respiration is completely inhibited in the *BCG ΔcydAB* strain (Fig 2.8a), and this has been previously described by Kalia et al¹⁷³. In the Q203-treated *BCG ΔcydAB* strain carrying the ancestral *cydAB* genes (*BCG ΔcydAB* :: Shinshuense *cydAB*), there was the restoration of oxygen respiration, confirming the functionality of *cyt-bd* in the ancestral lineage. Conversely, complementation with the *cydAB* genes of classical strain origin harbouring the *cydA* W231X substitution (*BCG ΔcydAB* :: Agy99 *cydAB*), failed to restore oxygen respiration (Fig 2.8b). As a control, BDQ that targets the mycobacterial F_1F_o -ATP synthase inhibited oxygen consumption in both strains (Fig. 2.8b). These results demonstrated that the

cydA W231X polymorphism in the classical *M. ulcerans* strains has rendered the *cyt-bd* non-functional.

Figure 2.8: Methylene blue assay for detection of oxygen consumption. (a) *M. bovis* BCG $\Delta cydAB$ after treatment with Q203 (100 nM), bedaquiline (BDQ, 1 μ M), and DMSO (-). Q203 inhibits the *cyt-bcc:aa₃*, and the lack of a functional *cyt-bd* renders the organism unable to utilise oxygen in the medium and hence the dye remains blue. (b) *M. bovis* BCG $\Delta cydAB$ complemented with *M. ulcerans* Agy99 *cydAB* (Classical *cydAB*), or *M. ulcerans* shinshuense *cydAB* (Ancestral *cydAB*) is treated with Q203 (100 nM), Bedaquiline (BDQ, 1 μ M) and DMSO (-). Methylene blue is decolorized in tubes with Q203 treated BCG $\Delta cydAB$ complemented with Ancestral *cydAB* indicating the presence of respiring organisms utilizing oxygen in media, whereas Q203 strains complemented with Classical *cydAB* shows blue colour indicating the presence of oxygen in medium, and hence non-respiring organism. As a control, BDQ inhibits respiration in all three strains.

2.4.3 Q203 inhibits metabolic activity in *M. ulcerans* classical strain.

In order to measure the metabolic activity of *M. ulcerans* following Q203 exposure, an Alamar blue (resazurin) based microbroth dilution assay was used²⁵⁷. Resazurin intercalates into the plasma membrane and is reduced by the electron flow of the electron transport chain. The reduced form of the dye, resorufin, is pink and fluorescent. The fluorescence readout can be plotted as a measure of metabolic activity against drug concentration to obtain a dose-

response curve²⁵⁸. Representative strains from both the classical and ancestral lineage were used to detect differences in the action of Q203.

Metabolic activity of *M. ulcerans* classical lineage is significantly decreased with Q203 (Fig 2.9a). A dose-response curve generated with increasing concentrations of Q203 starting from 0.04 nM shows an MIC₅₀ less than 1 nM (0.2 – 0.6 nM). In the ancestral lineage strain of *M. ulcerans*, Q203 showed a very partial restriction of metabolic activity at a similar concentration (Fig 2.9b). In both strains, BDQ critically lowers metabolic activity with similar dose-response curves.

Figure 2.9. Determination of metabolic inhibition of Q203 (blue) and bedaquiline (red) against classical (a) and ancestral lineage (b) of *M. ulcerans*. Q203 and bedaquiline were diluted two-fold ten times in 90% DMSO. Dose range tested for Q203: 20 – 0.04 nM; Bedaquiline: 1000 – 2 nM)

2.4.4 Growth inhibition of *M. ulcerans* with Q203

The microplate Alamar blue assay has been successful in expediting the antimicrobial susceptibility testing in *M. ulcerans*. However, the assay is not a true indicator of growth inhibition, and small molecules can interfere with the dye to produce spurious results²⁵⁹. Microbroth dilution technique has been advocated by CLSI as a method of MIC determination in mycobacteria²⁶⁰. To confirm the potency of Q203 in *M. ulcerans*, a microbroth dilution assay was

adapted for use in *M. ulcerans* with an endpoint readout of OD measurements. Extra precautions such as reserved work surfaces, humidified incubators and continuous monitoring was adopted to prevent contamination or premature drying associated with long incubation periods. Although this assay is a more robust indicator of whole-cell inhibitor effect it can take two weeks for a clear readout.

In the classical microbroth dilution assay, growth inhibition of the classical strain is complete with Q203 (Fig 2.10a) with an MIC₅₀ of 0.2 – 0.9 nM. In the ancient strain, Q203 is less active, demonstrating an MIC₅₀ 4-8-fold higher in the range of 1.7 – 2.6 nM (Table 2.1). Moreover, growth inhibition of the ancestral strain is only partial with Q203, as reflected by the higher bottom plateau observed, even at the highest concentrations of Q203 (Fig 2.10b).

Figure 2.10 Q203 and bedaquiline inhibit *M. ulcerans* growth in a microbroth dilution assay. Q203 was used at a dose range of 20 to 0.04 nM for the classical lineage (a) and a range of 100 to 0.2 nM for the ancestral lineage (b). Bedaquiline was tested at a range of 1000 to 2 nM for both strains.

Table 2.1: MIC₅₀ values of Q203 and BDQ treatment against *M. ulcerans* Classical and Ancestral strain. The values reported are derived from the mean of triplicate measurements.

	<i>M. ulcerans</i>	
	Classical lineage	Ancestral lineage
Q203 (nM)	0.2 - 0.9	1.7 - 3.6
Bedaquiline (nM)	45 - 60	58 - 79

In Mtb, Q203 has an MIC₅₀ of 1.5-2.8 nM^{173, 205}. In the *M. ulcerans* classical lineage, the potency of Q203 is 0.2 -0.9 nM making it an even more potent growth inhibitor in *M. ulcerans* compared to Mtb. The MIC₅₀ of Q203 is 4-8-fold higher in the ancestral lineage harbouring a functional *cyt-bd* oxidase, similar to Mtb. A similar phenomenon was described by Kalia et al.¹⁷³, who was also able to demonstrate a 10-fold lower MIC₅₀ (0.62 +/- 0.01 nM) in the *cyt-bd* deficient H37Rv Mtb strain compared to the parental H37Rv strain (MIC₅₀: 1.45 +/- 0.08 nM). In support, the above finding further corroborates the role assumed by *cyt-bd* with the loss of function of the *cyt-bcc:aa₃* in the presence of Q203.

BDQ demonstrated a similar profile of growth depletion in both the classical and ancient lineages of *M. ulcerans* (Table 2.1). The MIC₅₀ of BDQ in both these strains also mirrors the MIC₅₀ of BDQ in Mtb (MIC₅₀: 42-133 nM)^{166, 173}.

2.4.5 ATP depletion and inhibition of oxygen respiration validates the action of Q203 on the OxPhos pathway

Validation of drug action on the OxPhos pathway relies on its ability to demonstrate a depletion in net ATP levels and a significantly reduced capability to utilise oxygen.

Intracellular ATP levels were measured using BactiterGlo Microbial Cell Viability Assay (Promega), an assay that is well established in mycobacteria^{173, 182}. Rapid and marked ATP depletion is noted with Q203 in the classical *M. ulcerans* strain as opposed to the ancestral strain (Fig 2.11). The blue double-headed arrow in Fig 2.11b emphasises the incomplete ATP depletion noted with Q203 in the ancestral strain. BDQ demonstrated comparable ATP depletion in both strains (Fig 2.11). Sustained ATP depletion for up to 72 hours was noted in the classical strain even with 0.2 nM of Q203 (Fig 2.11c). Similar depletion of ATP was noted with BDQ at 1000 nM. In the ancient strain no notable ATP depletion was appreciated at 5 and 20 nM of Q203 at 24 hours (Fig 2.11d).

Figure 2.11 ATP depletion measured at 24 hours after treatment with Q203 (Test range: 20-0.04 nM) (blue) and bedaquiline (Test range: 1000-2 nM) (red) in the classical strain (a) and the ancestral strain (b). (c) ATP levels of *M. ulcerans* classical strain were measured after 24 and 72 hours of exposure to Q203 at 0.2 and 5 nM and bedaquiline (1000 nM) and that of *M. ulcerans* ancient strain (d) was measured after 24 hours of exposure to Q203 at 5 and 20 nM and bedaquiline at 1000 nM.

Oxygen consumption rate (OCR) of the organism was monitored using the Seahorse XFe96 respirometer, a technology that accurately measures and computes OCR of cultures in real-time. The technology also allows OCR to be measured immediately following the injection of preloaded drugs of interest.

I standardised a protocol for measuring the OCR of *M. ulcerans* by adapting the technique described by Lamprecht et al. in *Mtb*²⁶¹. For initial standardisation the assay was performed with cultures at two concentrations – OD₆₀₀ 0.4, and 0.8. In the optimised assay final run, the cultures were standardized at OD₆₀₀ of 0.4 and readings were taken for ten consecutive time points every three minutes

following the injection of drugs. An immediate, sustained and significant drop in OCR was recorded after injection of Q203 at 100 nM in the classical strain as opposed to the ancient strain (Fig 2.12a,b). Streptomycin at 5 μ m was used as a negative control for the assay and failed to elicit any OCR drop for the classical strain.

Figure 2.12 Q203 inhibits oxygen consumption in *M. ulcerans* classical strain. OCR measured using the Seahorse XFe96 respirometer following injection of drugs: Q203 (100 nM, blue) and Streptomycin (5 μ M, purple) on the classical strain (a) and ancient strain (b). Control (DMSO treated) is represented as yellow in both graphs. The first vertical line represents the injection of the drug into the test well, and the second line represents the time point at which CCCP (an oxidative uncoupler, control) was injected.

The mycobacterial cell is unable to sustain itself from ATP generated from substrate-level phosphorylation alone, and this may be due to an absent NADH dependent lactate dehydrogenase^{174, 248}. OxPhos pathway is the major generator of ATP in a mycobacterial cell and any molecule disrupting the electron flow of the pathway will cause a rapid and substantial drop in ATP. Similarly, with oxygen being the main electron acceptor of the pathway, a noticeable decrease in oxygen consumption by the cell will be inevitably discernible with an OxPhos pathway inhibitor. With breakthrough innovations like Seahorse XFe respirometers, it is now possible to accurately measure real time OCR.

Q203 evokes a rapid and sustainable ATP depletion in the classical strain of *M. ulcerans*. The depletion was comparable to that elicited by BDQ, and a commendable property is that the ATP depletion was elicited at 100 times less concentration than that of BDQ. Like ATP levels, a drastic drop in OCR was elicited with Q203 in the classical strain. The significant action of Q203 on both ATP levels and OCR consumption validates the action of Q203 on the OxPhos pathway of *M. ulcerans*.

The inability of Q203 to demonstrate any significant decline in ATP levels or OCR in the ancestral strain was very evident (Fig 2.11b, 2.12 b). The *cyt-bd* of the ancestral strain has already been demonstrated to be functional and its compensatory action may have rescued any inhibition of *cyt-bcc:aa₃* in this strain.

2.4.6 Q203 analogues also show good potential against *M. ulcerans* classical strain.

Among the enzyme complexes of the OxPhos pathway, *cyt-bcc:aa₃* has emerged as a prominent drug target, with discovery of various chemical class inhibitors in different stages of preclinical and clinical development^{217, 251, 262}. Among the IPA class of compounds, Q203 remains the leading compound in clinical development, having completed safety and efficiency studies for Mtb in clinical studies^{181, 213}. I tested another IPA compound, ND-11176, against *M. ulcerans*. ND-11176 is an advanced preclinical drug candidate belonging to the IPA chemical class acting on the *cyt-bcc:aa₃*.

ND-11176 was found to have comparable activity to Q203 (Fig 2.13) in the *M. ulcerans* classical strain. ND-11176 elicited growth inhibition of *M. ulcerans* in the microbroth dilution assay (Fig 2.13a) and demonstrated a reduction in metabolic activity (Fig 2.13b) in a dose-dependent manner. ATP depletion was complete and was similar to responses by Q203 and BDQ (Fig 2.13c). OCR measured on Seahorse Xfe96 respirometer noted an immediate drop in the OCR immediately after injection for both ND-11176 and Q203 (Fig 2.13d). ND-11176 demonstrated a higher MIC₅₀ than Q203 with values ranging from 12 – 24 nM (Table 2.2).

Figure 2.13 ND-11176 elicited a similar response to Q203 with growth inhibition on (a) MIC microbroth assay, (b) metabolic activity reduction, and (c) ATP depletion in a dose-dependent manner. Dose range tested for Q203 (blue circles, 20 – 0.04 nM), ND-11176 (green triangles, 200 – 0.4 nM) and Bedaquiline (red squares, 1000-2 nM). Changes in (d) OCR were measured on the Seahorse XFe96 respirometer following injection of ND-11176, Q203, Bedaquiline, and DMSO control (purple inverted triangles) represented by the first dotted vertical line. The second line indicates the time point of injection of CCCP, an oxidative uncoupler.

Table 2.2 MIC₅₀ values of ND-11176, Q203, and Bedaquiline in *M. ulcerans* Classical lineage

	MIC ₅₀ (nM)
ND-11176	12 - 24
Q203	0.2 – 0.6
Bedaquiline	37 - 53

ND-11176, a preclinical drug candidate, is an IPA derivative belonging to the same class of compounds as Q203. The compound was able to elicit a similar response to Q203 in the classical lineage for growth inhibition, ATP depletion, and OCR decrease. Q203 seems to be a better candidate among the two, with the potency of Q203 being 100 times better than ND-11176. However, it is worthwhile noting that two separate drugs acting on the *cyt-bcc:aa₃* was able to elicit noticeable metabolic and growth inhibition. It is now possible to say with confidence that the *cyt-bcc:aa₃* holds promises as a drug target for BU treatment.

2.4.7 Q203 is bactericidal to *M. ulcerans* Classical lineage.

Q203 is bacteriostatic in Mtb and bactericidal only in strains in whom the *cyt-bd* oxidase was inactivated¹⁷³. The ability of Q203 and ND-11176 to kill *M. ulcerans* was tested in the time-kill kinetic assay. Both the classical and ancient strains of *M. ulcerans* was exposed to Q203, ND-11176, and BDQ in liquid

cultures and subsets of culture were performed every 7 days onto antibiotic-free 7H10 medium. In keeping with the low MIC₅₀ of Q203 in the classical lineage, it was decided to use Q203 at 2 and 10 nM for the classical lineage. Q203 was used at higher concentrations of 10 and 100 nM for the ancient strain. Given the potency of Q203 in the low nanomolar range, I modified the classical assay by adding one washing step to eliminate the antibiotic before plating on agar media to tide over any antibiotic carryover effect²⁶³.

As demonstrated in Fig 2.14, Q203 displayed excellent killing potency in the *M. ulcerans* classical lineage since it induced significant killing even at the lowest dose tested (2 nM). ND-11176 was also able to elicit a cidal action on the classical strain at 50 nM. Conversely, Q203 fails to kill the ancient strain even at higher doses of Q203 at 100 nM. This mirrors the effect elicited by Q203 in *Mtb* and further reinforces the role played by the *cyt-bd* in the rescue of the pathway. BDQ at 500 nM was able to kill *M. ulcerans* classical lineage. However, curiously the drugs fail to kill the ancestral lineage strain and this opens up further questions regarding the role of *cyt-bd* in the pathway. A similar pattern was observed by Berney et al., who demonstrated increased susceptibility to BDQ and rapid killing in a *cyt-bd* knockout strain of *Mtb* H37Rv²⁶⁴.

Figure 2.14 Time kill kinetics of the *M. ulcerans* classical lineage (a) versus ancestral lineage (b) strains. Drugs used were Q203 2 nM (blue circles), Q203 10 nM (red squares), Q203 100 nM (green squares), bedaquiline 500 nM (purple inverted triangles), ND-011176 50 nM (yellow diamonds), and DMSO vehicle control 0.9% (black circles).

2.4.8 Spontaneous mutants resistant to Q203 arise at a low frequency *in vitro* and validate the *cyt-bcc:aa₃* as the primary target in *M. ulcerans*.

A low mutation rate is a desirable characteristic of a potential drug candidate²⁶⁵.

Reports of antibiotic-resistant mutants of *M. ulcerans* are rare, and there were no efforts to derive drug resistant mutants on culture media before our study^{266, 267}.

An attempt was made to determine the spontaneous resistance rate to Q203 on 7H10 agar. *M. ulcerans* classical strain was plated on 7H10 agar plates containing 10 nM of Q203 (~10 x MIC₅₀), and inoculum count was estimated to be 2.5 x 10¹⁰ CFU. Since *M. ulcerans* colonies are extremely slow growing on solid medium, taking up to 12 weeks, measures like using a double layer of parafilm to seal the plates and placing the plates in Ziplock bags inside the incubator was adopted to avoid contamination and premature drying of plates.

After 10 weeks of incubation, we successfully isolated 14 colonies. The mutation rate was determined to be 5.6 x 10⁻⁹, indicating a very low probability of the emergence of resistant mutants. The isolates were tested for MIC to Q203 to

confirm resistance in the microbroth dilution assay format. The mutants isolated registered MIC ten to hundred-fold higher than the parent strain (Fig 2.15).

Figure 2.15 MIC₅₀ of the mutant strains was determined using the biomass detection assay against Q203 (a). Representative strains #1 (red squares), #2 (green triangles), and #8 (purple inverted triangles) were tested in comparison to the parental *M. ulcerans* classical strain (S1013, blue circles).

The QcrB subunit of the cytochrome bcc complex of *cyt-bcc:aa₃* was determined to be the primary binding site of Q203²⁰⁵. In fact, a site close to the menaquinone binding site (Q_p) of the cytochrome bcc was ascertained to be the binding pocket of Q203¹⁸¹. Six distinct single nucleotide polymorphisms mapped to this site has been consistently associated with Q203 resistance in *Mtb*^{181, 205, 251, 262, 268}. Based on this knowledge, the *qcrB* gene of the mutants generated in our study was sequenced. In *Mtb*, T313A of QcrB is the most common polymorphism linked to Q203 resistance²⁰⁵. In our study, mutants with T323A mutation were isolated and these mutants have shown an MIC₅₀ 1000 times (160 – 200 nM) to that of the normal strain (Table 2.3). The additional ten amino acids ²⁰⁵CGGVGDDCTA₂₁₄ in the *M. ulcerans* QcrB accounted for the ten-number shift. The other prominent sites of mutation (M352V, M352T, and W322C) mirrored the mutations in the corresponding gene of *Mtb*¹⁷² (Table 2.3).

Table 2.3: Mutations mapped to the *qcrB* gene of *M. ulcerans* and corresponding amino acid changes in the QcrB protein in the 14 Q203 resistant mutants.

Clones #	<i>qcrB</i> SNP	QcrB substitution
1, 7, 8, 9, 11	A967G	T323A
2, 3, 5, 6, 10, 13, 14	A1054G	M352V
4	T1055C	M352T
12	G966T	W322C

2.5 Discussion

The OxPhos pathway has been deemed essential for the survival and replication of mycobacteria²¹¹. It is now the focus of a targeted drug discovery following the successful application of BDQ and, more recently, delamanid (Deltiba) and pretomanid (PA-824)¹⁶⁶. In *M. ulcerans*, a genome-wide downsizing has left the OxPhos pathway more streamlined with fewer functional enzymes keeping the critical electron transport process operative¹⁵⁷. Our study hoped to utilise this rigidity in the pathway advantageously to repurpose Q203, an antituberculous drug acting on the *cyt-bcc:aa₃* supercomplex.

Genome-wide comparison of different *M. ulcerans* strains has helped to identify its evolutionary diversification from *M. marinum* through a massive genome reduction and acquisition of a toxin coding plasmid^{148, 157}. Continued evaluation has also helped demarcate two different lineages of *M. ulcerans*-classical and ancestral¹⁶¹. The ancestral strains are closer to the parental *M. marinum* genomically, and the classical lineage has undergone even more genomic rearrangements and gene disruptions^{160, 161, 269}. Despite the genome rearrangements, the classical lineage has emerged as the more “successful” pathogen, with it being responsible for the severe cases in Africa and Australia – the two main epicentres of the disease^{160, 269}. In keeping with the trend, gene comparison has revealed that the *cydA* gene, coding for a functional component of the *cyt-bd* terminal oxidase, of the classical lineage is a pseudogene, and that of ancestral lineage is preserved. In this study the functionality of the *cyt-bd* complex in the classical lineage was proven to be

compromised, while the presence of a functional *cyt-bd* was asserted in the ancestral lineage. The methylene blue redox dye-based assay was successfully used to demonstrate the functionality of oxygen consumption in a closed environment by Kalia et al. on *M. bovis* BCG, and Mtb previously^{173, 270}. The same system was successfully adapted in this study to assess the functionality of a non-native gene of interest.

The compensatory role of *cyt-bd* in Mtb treated with Q203 was established by Kalia et al ¹⁷³. Our study was able to delineate this differential action in the natural setting with representative strains from the two different lineages of *M. ulcerans*. Q203 demonstrated excellent potency for *M. ulcerans* classical lineage strains. A profound depletion of ATP and metabolic activity, along with the robust bacteriocidal, was noted with Q203 exposure in the classical lineage. The ancient lineage bearing a functional *cyt-bd* showed limited response to Q203 with only partial loss of ATP and metabolic activity and partial inhibition of growth. Q203 remained bacteriostatic for the ancient lineage strain even on exposure to high amounts of up to 100 nM.

The study has also shed more light on the OxPhos pathway of mycobacteria. The similarities of the pathway were reinforced with Q203 demonstrating a similar action in both Mtb and *M. ulcerans*. As predicted, the differences in killing action of the drug in Mtb and the ancestral lineage of *M. ulcerans* may be traced back to the compensatory action of a functional *cyt-bd* terminal oxidase. Similarities were also noted in the action of BDQ on Mtb and *M. ulcerans*. BDQ was bactericidal for both Mtb and *M. ulcerans* classical

lineage. However, in the ancestral lineage, BDQ remained bacteriostatic. Similarities can be drawn to the rapidly bactericidal action of BDQ on the *cyt-bd* deficient strain of Mtb²⁶⁴. Moreover, BDQ is conspicuously bacteriostatic in predominant NTM, such as *M. abscessus* and *M. avium*, which are notoriously difficult to treat. These findings can help explore the role played by different arms of the complex OxPhos pathway and its ancillary pathways in maintaining homeostasis in the event of disruption of action of its components.

The potential in drugging the OxPhos pathway was further corroborated in our study. The bactericidal actions of IPA compounds Q203 and ND-11176, and BDQ on *M. ulcerans* at the nanomolar concentrations is proof of the potentially lethal action of shutting down the central energy metabolism of bacteria by targeting key molecules of respiration. Even *cyt-bd* is now gaining traction as a promising drug target following its implicated role in affecting the sensitivity of mycobacteria not only to Q203¹⁷³ but also BDQ²⁶⁴ and clofazimine²⁷¹. Combination drug therapy targeting both terminal oxidases is proving to be a viable option for mycobacteria, and our studies in *M. ulcerans* with a natural loss of *cyt-bd* corroborates the wisdom of seeking a drug candidate for *cyt-bd*²¹⁸.

M. ulcerans is notoriously difficult to handle in the laboratory since it is extremely slow growing. However, several laboratory techniques and assays reserved for fast-growing mycobacteria were successfully translated for use in *M. ulcerans* by modulating various elements such as the quantity of

inoculum, incubation conditions, and reserved work surfaces. Moreover, the isolation of raising drug-resistant mutants of *M. ulcerans* on agar plates was the first attempt of its kind to be undertaken in a laboratory. Newer technology such as the Seahorse XFe96 respirometer was also successfully adapted for use in *M. ulcerans*, highlighting the need for innovative research.

– CHAPTER 3 –

Evaluating a single-dose cure for Buruli ulcer with Q203 in the mouse footpad infection model

3. Evaluating a single-dose cure for Buruli ulcer with Q203 in the mouse footpad infection model

3.1 Introduction

3.1.1 Buruli ulcer treatment – current standards and limitations

Buruli ulcer (BU) has slowly evolved from a state of obscurity to that of a disease of re-emerging importance with worldwide recognition. It is now recognised as the third most common mycobacterial disease, and its incidence has surpassed that of tuberculosis and leprosy in severely affected areas⁷⁶. In fact, it has been identified in all continents of the world, with 33 countries reporting disease incidence to WHO²⁷². The African region bears the major brunt of disease burden with 94.9 % of the global burden followed by the Western Pacific region contributing to almost 4.9 %²⁷³.

In rural Africa, public health and social infrastructure is a major determinant of treatment success. Often, misinformation, disrupted socio-economic situation, overburdened public healthcare, and lack of public awareness can interfere with appropriate interventions making it difficult to manage at all stages of prevention, diagnosis, treatment, and rehabilitation²⁴².

Preventive measures for BU are limited. Even though a strong association exists between specific environmental locations and disease incidence, the precise reservoirs of the disease remain unidentified⁶⁹⁻⁷². Hence any solid measures to break the chain of transmission are critically lacking. Timely diagnosis is also severely crippled in heavily affected areas of rural Africa⁷⁶. Lab diagnosis may be affected by the long culture time of 6-12 weeks, the low sensitivity of culture, and unavailability of advanced tests like PCR in rural peripheries⁹³. Late

presentation of the disease is also common with issues like social stigmas, mistaken beliefs of the cause of disease, lack of appreciation of treatment possibilities, and inability to stay in touch with treatment centres²⁷⁴⁻²⁷⁷. Hence the need for timely and complete treatment cannot be overemphasised.

Antimicrobial therapy has evolved to become the backbone of disease management. Initial attempts at drug therapy were marred by inconsistent and inconclusive field trials with rifampicin monotherapy²⁷⁸, clofazimine⁸⁰, cotrimoxazole²⁷⁹, and dapsone²⁸⁰. The need for well-planned and precise drug trials with concise, measurable endpoints was recognised, and the rising number of cases forced WHO authorities to consolidate efforts. The study by Etuaful et al. was viewed as a turning point in disease management when WHO endorsed preliminary results from the trial to formulate a drug regimen of 8 weeks of therapy with rifampicin and streptomycin injections^{77, 101}. This formed the basis for the start of a new era in the management of BU¹⁰².

Although a major step up in disease management, this regimen has troubles of its own. Rifampicin carries the major sterilising activity in the regimen. To date, no replacement drug for rifampicin has been successful for BU. Rifampicin resistant mutants have also been described²⁶⁷.

Streptomycin, the other drug in this two-drug combination therapy, is not an ideal choice. Apart from hypersensitivity, one of the main adverse effects is ototoxicity. Up to 29% of adults and 25% of children developed hearing loss in the BURULICO drug trial conducted in Ghana²⁸¹. Streptomycin is contraindicated in pregnancy due to its potential for ototoxicity in the foetus. Although the least nephrotoxic among the aminoglycosides, up to 14% of adults

also developed signs of nephrotoxicity in the BURULICO trial²⁸¹. Streptomycin injections warrant careful monitoring on the part of the healthcare team that needs to be mindful of potentially toxic drug interactions.

Daily injections in resource-poor settings of rural Africa can put a strain on infrastructure, often leading to cutbacks like needle sharing. This can lead to the unwarranted spread of bloodborne pathogens like HIV, HCV, etc²⁸². Also, streptomycin injections are painful, and often patients can spend long hours commuting to a healthcare facility to receive the injection²⁴⁰. All these factors make the combination a difficult regimen to adhere to and can drive up non-compliance in the treated population.

The need for an oral drug combination was recognised, and several in vivo studies in mice recognised clarithromycin as a good oral drug alternative to streptomycin^{113, 241}. Clinical trials of 4 weeks of rifampicin-clarithromycin treatment in conjunction with 4 weeks of rifampicin-streptomycin concluded that the former is non-inferior to the current standard of care comprising of rifampicin and streptomycin^{283, 284}. The recent study by Phillips RO et al. successfully established the non-inferiority of an 8-week therapy of rifampicin-clarithromycin compared to the standard 8-week rifampicin-streptomycin regimen in a study of 310 participants¹¹⁸.

A clarithromycin based oral drug regimen will improve drug administration and help avert the toxicities associated with streptomycin. However, compliance with therapy may still be an issue, with patients discontinuing medication if not under direct observation¹³³. Studies have shown that even a three-day therapy for malaria suffered an efficiency decline from 98% in field trials to 38% in real-

world settings, owing to a reluctance on the part of patients to continue therapy¹²⁹. Hence, there is a substantial need for a shorter drug regimen for BU.

3.1.2 Q203 – A potent oral drug alternative for BU

The extraordinary potency of Q203 against *M. ulcerans* has been proven *in vitro* and this makes it a good candidate for shorter drug trials. Several studies have established the high efficiency of Q203 in the BU disease model in mice footpads. The technique of the mouse footpad inoculation model was established by Fenner et al. and has been extrapolated successfully for leprosy as well^{285, 286}.

In preliminary *in vivo* drug efficiency studies with Q203, a daily regimen with Q203 at 0.5 mg/kg was administered by oral gavage for 8 weeks after foot pad infection with *M. ulcerans* was established. At the end of four weeks, a 99.99% decrease in CFU counts were noted for the Q203 treatment group and a 1000-fold lower CFU count compared to the control Rifampicin (10mg/kg) plus Streptomycin (150mg/kg) group²⁸⁷.

Further studies have attempted a reduced dose of Q203. A reduced duration regimen of 4 weeks with alternate-day administration of Q203 at 0.5 mg/kg was also shown to be better than rifampicin administration alone. The associated oedema and inflammation of the foot was reduced by as short as 10 days after initiation of therapy, whereas monotherapy with rifampicin was unable to reverse pathology in the control group²⁸⁷.

In yet another study, Q203 was administered at 10 mg/kg in various combinations with rifapentine/bedaquiline (BDQ)/clofazimine for 2 and 4 weeks after the infection was established. The authors have noted a rapid reversal in swelling and bacteriological clearance with two weeks of treatment in Q203 containing

regimen²⁴⁶. Another study investigated the potency of Q203 administered twice a week for 2, 4, and 8 weeks at a dose of 5 mg/kg in the BU mouse model. Complete bacteriological cure was observed in the 8-week regimen²⁸⁸.

All the previous studies have established that Q203 is an extremely potent chemotherapeutic agent with excellent sterilising activity on its own against *M. ulcerans in vivo*. Q203 has a very favourable pharmacokinetic profile for a reduced duration regimen. The drug showcases a long half-life of 23 hours in mice, good oral bioavailability of 24 %, low toxicity profile with no adverse effects at a single dose of 1000 mg/kg, and excellent tissue distribution^{205, 213}.

We hypothesise that the favourable pharmacokinetic profile of Q203, along with its excellent potency against *M. ulcerans*, can be exploited for a single dose cure for BU. A single-dose cure may be the most advantageous for field management of BU, holding potential as a conducive agent for mass drug administration campaigns, which will help to diagnose and treat BU at the same time in the field²⁸⁹.

3.3 Materials and methods

3.3.1 Ethics committee and institutional approval for in vivo study

The study protocol was approved by the Nanyang Technological University-Institutional Animal care and use committee (NTU-IACUC) and assigned project number A18022. Experiments with the mouse footpad infection model were performed by Dr. Nitin Pal Kalia and Dr. Sangeeta Susan Thomas according to the NACLAR guidelines on the care and use of animals for scientific purposes²⁹⁰.

3.3.2 Procurement and housing of mice.

Female Balb/c mice aged 6-8 weeks and an average weight of 18 – 20 grams were procured from *InVivos* Pte Ltd, Singapore. Animals were grouped with a maximum of 5 animals per cage in the ABSL-2 facility of NTU. Cages were maintained with corn cob bedding, and mice were fed a diet of Altromin#1324 maintenance diet for mice.

3.3.3 Infection of mouse footpad

The mouse foot pad infection model for *M. ulcerans* was replicated as per the technique introduced by Fenner et al. and subsequently developed by Shepard et al.^{285, 291}. *M. ulcerans* strain S1013 was used for all *in vivo* experiments. 20 µl of inoculum was injected into the left hind footpad after anaesthetising the mice with intraperitoneal ketamine (100 mg/kg): xylazine (10 mg/kg) injection. Inoculum count was determined by plating in dilutions (10^{-1} to 10^{-8}) on 7H10 quadrant plates. Plates were incubated at 32 °C for three months before counting colony forming units (CFU).

3.3.4 Preparation of drugs

Bedaquiline was purchased from Cellagen Technology, San Diego, USA, and Q203 was purchased from GVK Biosciences Private Limited, India. Rifampicin was purchased from Sigma-Aldrich, Singapore, and clarithromycin was procured from Gold Biotechnology, St Louis, MO. All drugs were reconstituted in 20% 2-hydroxypropyl- β -cyclodextrin (HP- β -CD) solution (Sigma-Aldrich, Singapore)

3.3.5 Oral gavage of drugs

The animals were weighed, and an appropriate volume of the drug was administered by oral gavage using a tuberculin syringe and 22 gauge curved oral gavage needles. Clarithromycin was administered one hour after administration of rifampicin in the control group.

3.3.6 Response to treatment

3.3.6.1 Foot pad thickness measurement

Foot pad thickness was measured at least once every week after infection. Five animals per group were measured using a Vernier caliper.

3.3.6.2 CFU enumeration

Preparation of 7H11 plates for CFU enumeration

Difco Middlebrook 7H11 agar base powder was used to prepare the base agar. After autoclaving, 50 ml of Oleic acid-albumin-dextrose-catalase (OADC) enrichment was added to the cooling liquid medium. The following antibiotics were added to the medium: Nalidixic acid (16 μ g/ml), Amphotericin B (40 μ g/ml), Cycloheximide (30 μ g/ml), and Trimethoprim (25 μ g/ml). The medium was poured into both 90 mm quadrant plates and 90 mm whole plates.

CFU enumeration

Mice were euthanized by CO₂ administration. After immersing the mouse in 70% ethanol, it was placed on dry tissue inside a biosafety cabinet. After 5 minutes, the foot was cut using sterile surgical scissors and cut into four pieces. The pieces (from one foot) were then placed in individual Precellys® MK28 lysing tubes (Bertin Corp, Rockville, MD) containing 1 ml of sterile PBS with 0.05 % Tween 80. The foot tissue was homogenized on the Precellys® Evolution homogenizer (Bertin Corp, Rockville, MD) by pulsing at 5000 rpm for 20 seconds. The cycle was repeated thrice with 30-second pause. The homogenized tissue with PBS was then transferred to a sterile 50 ml conical tube. The MK28 tube was rinsed out in another 1 ml of PBS. The tissue homogenate was centrifuged at 3800 rpm for 10 minutes at room temperature. The supernatant was discarded. 1 ml of fresh PBS was added to the tube. 20 µl of this suspension was used to prepare dilutions from 10⁻¹ to 10⁻⁴ in PBS. 50 µl of the dilution was plated on each quadrant of the 7H11 plate with antibiotics. The rest of the suspension was plated on five 90 mm 7H11 plates equally (200 µl per plate). The plates were covered with parafilm, placed in Ziplock bags, and incubated at 32 °C for ten–twelve weeks.

3.3.6.3 Histopathological evaluation

After euthanizing mice, infected feet were removed using scissors at the ankle joint. The feet were immediately immersed in 10 % formalin and kept at room temperature for 24 hours. Following this, the foot was moved to fresh 10% formalin and stored at 4 °C. The feet were shipped to our collaborating lab in Switzerland, where the histopathological evaluation was conducted after decalcification as per standard protocol²⁹².

3.3.7 Experimental protocol

All animals were inoculated with *M. ulcerans* S1013 strain as per the protocol described above. At week 5 following inoculation, 14 animals were randomly assigned to the treatment categories and 18 mice to the untreated vehicle control group. Treatment protocols were followed as per the dosing regimen described in Table 3.1, for 4 weeks. 7 animals were sacrificed from each treatment category after completion of treatment (Week 9 post infection): 6 feet were used for CFU enumeration, and 1 reserved for histopathology analysis. Relapse after treatment was measured by monitoring animals for 24 weeks after infection (Fig 3.1).

Figure 3.1 Experimental timeline for Phase 2 detailing infection at Week 0, Treatment initiation at Week 5, and first and second sacrifice at Week 9 and Week 24 post infection, respectively.

Table 3.1 Treatment groups

Drug	Dosing regimen	Total number of doses	Dose (mg/kg)
Q203	Weekly dosing	4	5
	Single dose	1	20
Bedaquiline	Single dose	1	20
Rifampicin + Clarithromycin	Daily dosing	20	Rifampicin – 10 Clarithromycin – 100
Untreated control – HP- β -CD	Daily dosing		

3.4 Results

Initial inoculum count injected per foot was determined to be 1.1×10^5 CFU, and treatment was initiated at Week 5 after the infection was established as evidenced by an average footpad swelling of 3.7 mm. Since swelling progressed quite rapidly in the untreated and BDQ single-dose group, the animals were sacrificed by week 8 of infection. After four weeks of therapy, 7 animals from every group were sacrificed for CFU enumeration. At Week 24 (19 weeks post treatment), all animals were euthanised, and feet from 6 animals per group was used for CFU enumeration and 1 reserved for histopathology (Fig 3.1, 3.2).

3.4.1 Mice treated with Q203 based regimen showcased rapid reversal of swelling with outstanding bacteriological clearance.

All mice were monitored for footpad swelling once a week during the entire course of the experiment. An immediate decrease in footpad swelling was seen in the feet of mice administered a single dose of Q203 (20 mg/kg) in the first post treatment measurement taken one week after administration of the drug (Fig 3.2). A similar response is seen even with the first dose of Q203 in the weekly (5mg/kg) dosing regimen (Fig 3.2). In contrast, the control group, rifampicin + clarithromycin, shows a more gradual decline in footpad swelling and never achieves the almost complete reduction seen with Q203 based regimen. On continuous monitoring for 19 weeks post treatment, no increase in footpad swelling was seen with Q203 regimen indicating a relapse was unlikely in these groups. However, an increase in swelling was noted in the rifampicin + clarithromycin control group by 17 weeks post treatment.

Figure 3.2: Rapid and sustained reversal of footpad swelling was observed in all Q203 based regimen. Q203 weekly dose (Q203 4X, 4 doses, purple inverted triangles) and Q203 single dose (Q203 1X, red diamonds) regimen demonstrated better reversal of swelling compared to the control, Rifampicin + Clarithromycin (R + C 20X, 20 doses, brown squares). All animals in the untreated group (blue circles) and bedaquiline single dose (BDQ, green triangles) had to be sacrificed by Week 8 (indicated by a cross) due to unfavourable progression of the disease.

Seven mice from each treatment group were sacrificed after 4 weeks of initiating therapy for CFU enumeration. No CFU's were observed in animals treated with Q203 based regimen, indicating complete sterilisation of the pathogen (Fig 3.3). A second sacrifice was performed at 19 weeks post treatment to look for relapse. No CFU were observed in the Q203 based regimen at the second sacrifice time point as well, indicating complete sterilisation (Fig 3.3).

Figure 3.3 Complete bacteriological sterilisation with no relapse was seen with both single and 4 doses of Q203. 20 doses of Rifampicin + Clarithromycin (Rif + Cla) were unable to clear infection immediately after treatment, and relapse is evident at 19 weeks after treatment completion with an increase in CFU counts. A single dose of bedaquiline (BDQ) was unable to clear the infection, and CFU counts mirror progression in the untreated (Unt). ***P value < 0.0001, when Q203 dosing regimen are compared to rifampicin + clarithromycin

Histopathological analysis of the feet at 4 weeks post treatment corroborated the above evidence. Staining for acid-fast bacilli (AFB) revealed irregularly and faintly stained bacilli in Q203 based regimen, indicating the presence of dead bacilli (Fig. 3.4, Panels 1 and 2). Bundles of AFB with thick and uniform staining, characteristic of live bacilli²⁹², was observed from feet in the untreated group (Fig. 3.4, Panel 4)). An intermediate staining reaction was seen with the rifampicin + clarithromycin group indicating presence of both irregularly stained dead bacilli and uniformly stained live bacilli (Fig 3.4, Panel 3). All the results for the Q203 based regimen were reproduced in a replicate assay, described in supplementary data.

Figure 3.4 Ziehl-Neelsen staining following histopathological analysis of mouse feet after 4 weeks of initiation of treatment (Week 9 post infection) for (1) Q203 Weekly dosing, 4 doses; (2) Q203 Single-dose, (3) Rifampicin + Clarithromycin, Daily dosing 20 doses and (4) Untreated vehicle control. Irregularly stained acid-fast bacilli (AFB) are morphological characteristics of dead bacilli, as seen in Panels 1 and 2. Panel 3 representing Rifampicin + Clarithromycin shows signs of viable bacilli indicated by the darker staining pattern in between irregularly stained bacilli. Regularly stained prominent clumps of brightly stained AFB indicating viable bacilli are seen in the untreated groups.

All three parameters of measurement of pathology in our study indicate that administration of a single dose of Q203 can achieve complete cure in the well-established BU mouse model of infection. Even the reduced dosing strategy of four weekly doses achieves the same result.

3.4.2 Relapse was seen in the rifampicin + clarithromycin treatment group

A slower reduction of swelling was noted with rifampicin + clarithromycin treatment, and complete reversal of swelling was never achieved in the full course of our study (Fig 3.2). All mice in this treatment category grew bacteria on culture when sacrificed immediately after treatment completion (Average CFU: 2.3×10^4 , Range: $6 \times 10^3 - 6 \times 10^4$, Fig 3.3). Evidence of relapse was visible in this group, with an increase in swelling noted by 17 weeks post treatment (Fig 3.2). All 5 feet obtained from mice at 19 weeks post treatment post grew *M. ulcerans* (Average CFU: 4.3×10^5 , range: $3.6 \times 10^4 - 1 \times 10^6$, Fig 3.3). Histopathological

pictures also indicate presence of persisting live bacilli interspersed among possible dead bacilli (Fig 3.4).

The latest clinical trials from a multicentric study in Ghana and Benin was able to show that an 8-week rifampicin + clarithromycin drug combination was non inferior to the current clinical standard of 8 weeks of rifampicin+ streptomycin¹¹⁸. In our study we used an abbreviated rifampicin + clarithromycin regimen (4 weeks) as our control to match the Q203 based regimen administered for a maximum of four weeks.

The clarithromycin-based drug regimen offers the advantage of being a complete oral combination. However, the rifampicin + clarithromycin combination may not be a suitable candidate for abbreviated therapy, as demonstrated by the relapse noted with a four-week therapy in this study. Moreover, the relapse in this group highlights the dangers of an incomplete therapy, which is often a clinical reality, especially in rural and impoverished areas of West Africa.

3.4.3 Administration of a single dose of bedaquiline failed to revert disease progression

BDQ, with excellent *in vivo* and *in vitro* activity on *M. ulcerans*^{244, 286}, was also administered as a single dose. This was unable to yield reversal of the disease. Footpad thickness followed the same trend as that of untreated after a single dose of BDQ (Fig 3.2). For humane reasons, all animals in this group were sacrificed at Week 8 along with the untreated animals. CFU enumeration yielded an average count of 3.2×10^7 (Range: $8.4 \times 10^6 - 5.8 \times 10^7$). This mirrored the average CFU count in the untreated group of 3.5×10^7 (Fig 3.3).

The inability of a single dose of BDQ to reverse pathology as robustly as Q203, despite a favourable pharmacokinetic profile, maybe due to its higher MIC₅₀ compared to Q203²⁸⁷. Combination therapy is the backbone of antimycobacterial therapy. A good drug candidate to pair with a single dose of Q203 would target another enzyme of the metabolic pathway and hence predictably demonstrate a synergistic effect. Considering a single dose of BDQ failed to achieve this, the newer diarylquinolines in the drug pipeline may offer hope.

3.5 Discussion

An improvement in the current therapy for BU is necessary, especially in rural Africa where compliance to a long duration multidrug therapy seriously hinders the disease management²⁸⁶. Serious toxicities of streptomycin are a major deterrent, and the clarithromycin alternative still warrants an 8-week therapy^{118, 281}.

Q203 has impressive potency against *M. ulcerans* due to the organism's exclusive dependence on *cyt-bcc:aa₃* as the sole terminal oxidase. Moreover, the favourable pharmacokinetic profile of Q203 with a long half-life, good oral bioavailability, and low toxicity makes it a good candidate for a single dose therapy^{205, 287}.

As hypothesised, a single dose administration of Q203 at a moderate dose of 20 mg/kg yielded a complete bacteriological cure with no relapse in an established mouse model of BU. The rapid reversal of local inflammation and swelling as early as one-week post drug administration is indicative of the potent bactericidal

activity of Q203. Complete bacteriological sterilisation with no relapse and favourable histopathological evidence all point to the fact that Q203 can be a compelling clinical drug candidate for BU.

An abbreviated therapy of four weeks of rifampicin + clarithromycin failed to cure the mice of infection in our studies. This evidence further underscores the need for a rigorously followed therapy of 8 weeks and the dangers of non-compliance with this therapy.

Moreover, the current regimen of rifampicin and streptomycin has been associated with paradoxical reactions with enlargement of lesions and development of new lesions^{293, 294}. In our mouse model studies, a rapid reversal of inflammatory signs, with decreased swelling and redness, was quite prominent in Q203 based regimen. This may be favourable in clinical trials where paradoxical reactions often deter patients and discourage antimicrobial therapy.

3.6 Supplementary information

Figure S3.1 Replicate assay confirming the exceptional action of Q203 Single dose in a mouse model of Buruli ulcer. The mice were inoculated with 9.8×10^5 CFU, and treatment was initiated at Week 3, owing to the higher inoculum and faster progression of the disease. The Q203 based regimen, both single dose (green triangles) and weekly dose (four doses, red squares), demonstrated rapid reversal of footpad swelling. The mice treated with rifampicin + clarithromycin (20 doses, purple inverted triangle) showed a slower reversal of swelling and never recovered completely to preinfection foot pad thickness. Untreated animals demonstrated progressive illness and were sacrificed at Week 7 for humane reasons (indicated by a cross).

Figure S3.2 Replicate assay confirming the sterilising potential of single-dose and four doses of Q203 compared to 20 doses of rifampicin + clarithromycin (administered over 4 weeks) at 4 and 8 weeks post treatment. ***P value < 0.0001, when Q203 dosing regimen are compared to rifampicin + clarithromycin

– CHAPTER 4 –

Conclusion

4. Conclusion

In our study, we hoped to exploit the genomic reduction of *M. ulcerans* to repurpose an antituberculous drug candidate Q203 for use in treatment of BU. Q203 is a leading clinical stage drug candidate with strong action on the *cyt-bcc:aa₃*²⁰⁵. However, the poor bactericidal action of Q203 in Mtb has been attributed to the presence of a functional *cyt-bd*, an alternate terminal oxidase in the OxPhos pathway¹⁷³. Our study rested on the hypothesis that *M. ulcerans* classical lineage has a more restricted OxPhos pathway due to the loss of the alternate terminal oxidase *cyt-bd*.

4.1 Q203 is an excellent drug for *M. ulcerans*

Several well-established and innovative assays were used in this study to identify and establish that Q203 is an excellent bactericidal drug for *M. ulcerans* classical lineage, the major pathogenic lineage involved in the more severe and widespread cases of BU. The exquisite potency of Q203 on the organism is demonstrated in the ability of the drug to inhibit the growth and kill it at nanomolar concentration. The contrasting suboptimal action of the drug on the ancient lineage strain of *M. ulcerans* with a functional *cyt-bd* establishes the role played by this enzyme in the rescuing the functionality of the OxPhos pathway and viability of the organism when exposed to a *cyt-bcc:aa₃* inhibiting drug.

Q203 has completed Phase 2a clinical trials, and the drug exhibits a favourable toxicity profile. No severe adverse effects were seen with a single dose administration of 800 mg in humans or daily administration of 100 mg for up to 14 days²¹³. Taking advantage of a favourable safety profile, tissue penetration properties, good half-life²⁰⁵ and excellent bactericidal action on *M. ulcerans* at

nanomolar concentrations, we attempted and successfully established a single dose cure for BU in the mouse model. A potential single-dose regimen for BU in the field can be a public health revolution that can help manage the disease especially in rural parts of Africa. Moreover, if a single dose cure is feasible for BU in humans, it will be a forerunner and promising breakthrough for all mycobacterial infections as antimycobacterial therapy always runs from weeks, months or even years.

4.2 The successful application of Q203 validates the OxPhos pathway as a remarkable drug target space for *M. ulcerans*.

The action of Q203 on the OxPhos pathway was validated in the studies that revealed the massive drop in ATP as well as OCR in the organism on being exposed to Q203. The bactericidal action at a similar concentration showcases the potentially lethal action of the drug by acting on the OxPhos pathway. This study has further highlighted the validity of targeting the energy metabolism space in *M. ulcerans* and other mycobacteria.

The exceptional activity on *M. ulcerans* classical lineage is contrasted with the suboptimal activity on a representative strain of the ancestral lineage and has helped to highlight the rescue role played by *cyt-bd*, an alternate terminal oxidase of the OxPhos pathway. This study has shed more light on the OxPhos pathway, both by reiterating the conservation of the general pathway among mycobacteria species and by underscoring the differences among the individual components. Further drug discovery efforts can help spearhead more research into the less understood energy metabolism pathway of mycobacteria, especially the NTM, like *M. abscessus* and *M. avium* that are notoriously difficult to treat.

4.3 Repurposing antituberculous drugs for NTM is a practical and efficient means of drug discovery.

Being a highly funded and sought-after drug research area, many anti-tuberculous drugs are now in the development pipeline. Q203 was discovered and developed as antituberculous drug and has reached late clinical development stages with excellent safety profiles. However, further studies have shown that Q203 may be a suboptimal candidate for TB since it lacks a bactericidal action in *Mtb*. Fortunately, when repurposed for *M. ulcerans*, the drug has displayed exquisite potency *in vitro* and *in vivo*. This underlines the importance of collaborative research and drug repositioning.

The intense efforts allocated for drug discovery in *Mtb* can benefit non-tuberculous mycobacteria (NTM) as well. Although NTM infections are now increasing worldwide and are notoriously difficult to treat, they do not receive the same focus as TB. The successful translation of anti-TB drugs for NTM can be a cost-effective and efficient way of drug discovery for NTM²¹⁹.

4.3 Future work

Q203 has been demonstrated to be a good drug candidate for *M. ulcerans* both *in vitro* and *in vivo* in mice. The drug has also cleared Phase 2 clinical trials in 2020 with an excellent safety profile²¹³. This places the drug at an advantage to move forward into clinical trials for BU and even experiment with reduced dosage and timing as validated by our *in vivo* study.

The study further validates the potential of targeting the *cyt-bd* as a druggable component. Already research has shown how targeting *cyt-bd* might potentiate the action of other drugs like bedaquiline²⁶⁴ and clofazimine²⁷¹ that have known action on energy metabolism. Our study also showed how ancestral lineage strain with a functional *cyt-bd* was able to bypass killing with BDQ.

The action of Q203 on *M. ulcerans* has shed more light on the OxPhos pathway of NTM, and the data collected can be applied for research on other NTM like *M. abscessus* and *M. avium* that are difficult to treat. Hence there is a need for continuous research into discovering more compounds that target the OxPhos pathway and strengthen our armamentarium to treat mycobacterial infections.

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