Biopharmaceutical optimization of antibiotic therapy for the treatment of Mycobacterium abscessus pulmonary infections : interest of nebulization and antibiotic combinations

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3. **Shachi Mehta***, Nicolas Grégoire, Sandrine Marchand, William Couet, Julien M Buyck. Assessment of *in vitro* efficacy of cefoxitin and amikacin in combination using modelling approach against *Mycobacterium abscessus.* In manuscript.
4. **Shachi Mehta***, Hariyanto Ih, Blandine Rammaert, William Couet, Sandrine Marchand, Julien M Buyck. *In vitro* evaluation of novel bi- or tri-combination against clinical isolates of *Mycobacterium abscessus*. In manuscript.

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1. **Shachi Mehta,** Julien Buyck, Isabelle Lamarche, Christophe Adier, Nicolas Grégoire, William Couet, Sandrine Marchand. 2018. Pulmonary pharmacokinetics of amikacin and cefoxitin after nebulisation in rats. Poster presented at the 28th European Congress of Clinical Microbiology and Infectious Diseases in Madrid, Spain (poster P2204).
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8. **Shachi Mehta**, Blandine Rammaert, William Couet, Sandrine Marchand, Julien Buyck. *In vitro* evaluation of novel bi- or tri-antibiotic combinations against clinical isolates of *Mycobacterium abscessus*. Poster presentation at 39ème Réunion Interdisciplinaire de Chimiothérapie Anti-Infectieuse (RICAI), France (2019).

List of abbreviations

AFB	:	Acid-fast bacilli
ATS	:	American thoracic society
AST	:	Antimicrobial susceptibility test
AMK	:	Amikacin
AVI	:	Avibactam
BAL	:	Broncho alveolar lavage
BTS	:	British thoracic society
BCS	:	Biopharmaceutical drug classification system
CB	:	Checkerboard
CLR	:	Clarithromycin
CLO	:	Clofazimine
CIP	:	Ciprofloxacin
CF	:	Cystic fibrosis
COPD	:	Chronic obstructive pulmonary disease
CFTR	:	Cystic fibrosis transmembrane conductance regulator
erm	:	Erythromycin ribosomal methylase
ECFS	:	European cystic fibrosis society
E-test	:	Epsilometer test
ELF	:	Epithelial lining fluid
FIC	:	Fractional inhibitory concentration
FOX	:	Cefoxitin
GPLs	:	Glycopeptidolipids
HIV	:	Human immunodeficiency virus

HRCT	:	High resolution computed tomography scan
IFN-γ	:	Interferon-gamma
IL	:	Interleukin
IPL	:	Isolated perfused lung
IV	:	Intravenous
IMI	:	Imipenem
LRTIs	:	Lower respiratory tract infections
LZD	:	Linezolid
MAC	:	*Mycobacterium avium complex*
MABC	:	*Mycobacterium abscessus complex*
MGIT	:	Mycobacteria Growth Indicator Tube
MALDI-TOF	:	Matrix-Assisted Laser Desorption Ionization-Time of Flight
MsrA	:	Methionine sulfoxide reducer A
MICs	:	Minimum inhibitory concentrations
MCBT	:	Multiple-combination bactericidal antimicrobial testing
MXF	:	Moxifloxacin
NTM	:	Non-tuberculous mycobacterial
NTM-PD	:	Non-tuberculous mycobacterial – pulmonary disease
NEB	:	Nebulization
OADC	:	Oleic acid, bovine albumin, dextrose, catalase
PAPs	:	Population Analysis profiles
PE	:	Proline-glutamate proteins
PPE	:	Proline-proline-glutamate
pBCS	:	Pulmonary biopharmaceutical drug classification system

PCR	:	Polymerase chain reaction
PK	:	Pharmacokinetics
PD	:	Pharmacodynamics
RGM	:	Rapid growing mycobacteria
RIF	:	Rifampicin
RFB	:	Rifabutin
sodA	:	Superoxide dismutase
TGC	:	Tigecycline
TKC	:	Time-kill curves
TB	:	Tuberculosis
URTIs	:	Upper Respiratory tract infections
WHO	:	World health organization

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INTRODUCTION

In most of the countries, pulmonary infections are being very common and potentially the most severe. According to the report of European Lung Foundation, pulmonary infections are responsible for one of six deaths all around the world. They are also leading responsible cause of death in children less than 5 years old and second cause of death in adults worldwide (Stover & Litwin, 2014). Several pulmonary infections can be caused by smoking, alcoholism, immunosuppression and also different micro-bacterial and viral causes. Approximately 1.9 million children die every year because of acute pulmonary infections in Asian and African countries (Anders *et al.*, 2015). According to the Cystic Fibrosis Foundation Patient Registry (CF foundation Patient Registry, 2016), more than 70,000 people are estimated to be affected annually by pulmonary infections known as cystic fibrosis (CF) worldwide & now days, the main cause of most of the infections are mycobacteria, a pathogen which may cause human death and much other bacterial diseases. There are mainly pulmonary tuberculosis and non-tuberculous mycobacterial pulmonary diseases **(NTM-PD),** which are being the most frequent manifestation involving different types of mycobacteria having epidemiological variations geographically (Ferro, van Ingen, Wattenberg, van Soolingen, & Mouton, 2015). For these infections, antimicrobial resistance, often driven by inappropriate use of antibiotic, is a worldwide growing problem with considerable cost. Another undesirable development is the increased use of broad-spectrum antibiotics. Daily decision of when to prescribe antibiotics for these infections constitute a significant part in the burden of antibiotic use that drives antibiotic resistance. Moreover, it will take more than 10 years to find out a new antibiotic molecule for the treatment, it is very crucial to maintain or increase effectiveness of existing antibiotics until then. Improving antimicrobial stewardship is therefore the most important part these days.

Now days, ***Mycobacterium abscessus*** is one of the major cause for more than 80% of infections caused by rapid growing mycobacteria worldwide. This is a rapidly emerging pathogen, responsible for soft and skin tissue infections, several extrapulmonary infections and especially pulmonary infections, in immunocompromised patients with existing disease like cystic fibrosis (CF) (Lee *et al.*, 2015; Mougari *et al.*, 2016; Nessar, Cambau, Reyrat, Murray, & Gicquel, 2012). The infection with this bacterium is assumed to driven from an environmental reservoir and is associated with poor treatment outcomes. The routine treatment includes clarithromycin co-administered with one aminoglycoside, (i.e. amikacin) and one β-lactam (i.e. cefoxitin or imipenem). However, because of more than 80% isolates are being clarithromycin-resistance, the treatment efficacy is becoming more questionable. Cefoxitin and amikacin are the most

effective antibiotics amongst all other active antibiotics against *M. abscessus* (Ferro, van Ingen, *et al.,* 2015; Greendyke & Byrd, 2008; Lefebvre *et al.,* 2016; Lerat *et al.*, 2014; Soroka *et al.*, 2014), but as these both antibiotics are given by intravenous (IV) administration, they are frequently associated with systemic toxicity and also it is difficult to achieve high concentration into the lungs by IV administration. In order to maximize efficacy of cefoxitin and amikacin in local respiratory tract infections, their targeting into the lungs could be interesting. Antibiotics administered directly into the lungs allows to achieve high local and low systemic exposure to drugs, with expected reduced systemic toxicity (Fernandes & Vanbever, 2009a) and rapid clinical response. Untill now, tobramycin, aztreonam and colistin have been approved for nebulization. Several molecules like gentamicin and amikacin have also shown their ability to be the good candidates for direct pulmonary delivery for the treatment of pulmonary infections (Boisson *et al.,* 2018; Marchand *et al.,* 2018). Also, inhaled amikacin is being recommended for the treatment of NTM - pulmonary disease (NTM-PD) (CF foundation, 2017; Haworth *et al.*, 2017). On the other hand, for the treatment of *M. abscessus* pulmonary infections, since the monotherapy is not efficient, and development of mutational resistance is often, it is always advisable to combine 2 or 3 antibiotics to abate their side effects and resistance. Although the use of two or three antibiotics is accepted as appropriate treatment option for patients with *M. abscessus* pulmonary infection, this approach has not widely been tested *in vitro* or *in vivo*. Only few antibiotics have been investigated, where clofazimine, tigecycline, linezolid, moxifloxacin, rifabutin, etc. have shown good activity against this infection.

LITERATURE REVIEW

1. *Mycobacterium abscessus complex (MABC):* an emerging pathogen

1.1 The genus *Mycobacterium*

Mycobacterial infections are among the leading causes of chronic human infections. The genus *Mycobacterium* contains Gram-positive aerobic to microaerophilic bacteria belonging to the family *Mycobacteriaceae* (Lee *et al*., 2015) and is one of several mycolic-acid-containing genera within the order *Actinomycetes*, comprises of more than 190 different species till date (Parte, 2018). The species are mainly divided into three groups (Lee *et al.,* 2015). *Mycobacterium tuberculosis* is the causative agent for tuberculosis (TB) and *Mycobacterium leprae* causes leprosy, a skin disease. Only few years after the discovery of TB, many other species were described, which were formerly known as "Nontuberculosis mycobacteria (NTM)" (Table 1). NTM denotes all the species of mycobacteria, which causes human infections other than TB, leprosy and are generally resistant to most of the anti-tuberculosis agents (Table 1).

TB is the key issue to public health worldwide. In 2016, it has been reported that 10.4 million people were estimated to be affected by TB mainly in African countries and in some Asian countries, which account for more than 80% of global TB burden (World Health Organisation, 2017). Though, the burden of TB has started to decline slowly by means of effective diagnosis and treatment, whereas pulmonary infections caused by NTM are being increased frequently throughout the world (World Health Organisation, 2017). NTM can be classified into two categories, based on their visible colony formation time duration on the solid media: slow-growing mycobacteria (SGM) and rapid growing mycobacteria (RGM) (Figure I). The required bacterial time interval for colony formation less than 7 days, are referred as RGM and others are SGM (Kim *et al.*, 2013). In fact, RGM grow significantly slower than typical bacteria, as NTMs often have generation time of one day or more in rich media, instead of half to one hour for other bacteria.

Table I: Characteristics of atypical mycobacteria compared to other mycobacteria

	Nontuberculous mycobacteria	***Mycobacterium tuberculosis***	***Mycobacterium leprae***
Rodents	Non-pathogenic	Pathogenic	Pathogenic
Disease	Opportunistic	Tuberculous	Leprosy
Antibiotics **Anti-tuberculosis agents**	Insusceptible	Susceptible	Susceptible

In 1959, Runyon also suggested the NTM classification system based on their colony morphology, growth rates and pigmentation, however this classification is rarely being used.

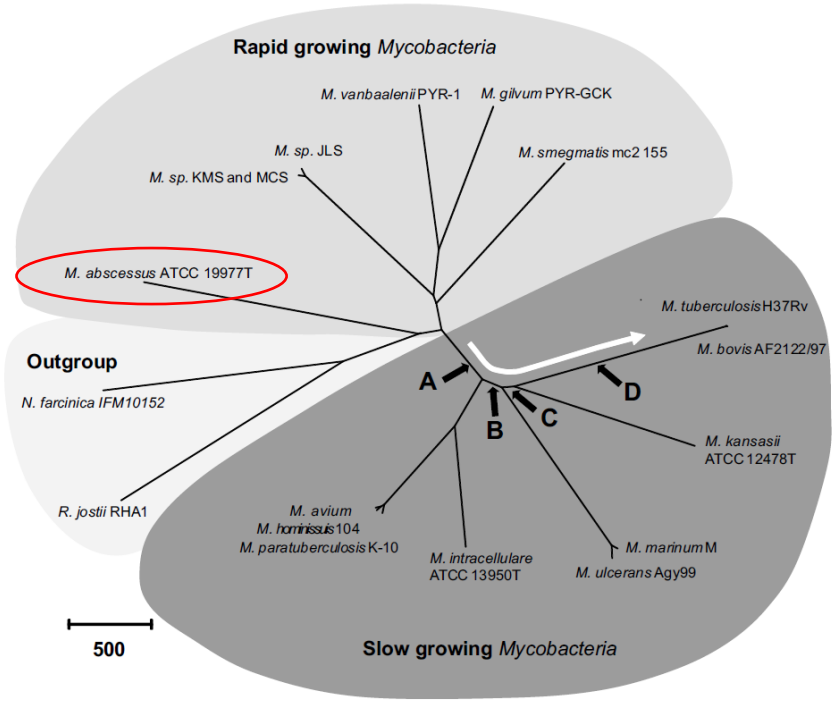

Figure I: Phylogeny of genus *Mycobacterium* and schematic representation of various mycobacteria by group

(Veyrier, Pletzer, Turenne, & Behr, 2009)

Mycobacterial infections are geographically distributed worldwide. *M. abscessus, M. chelonae* and *M. fortuitum* are generally found in the environment like soil and water (Table II) (Falkinham, 2002) but can also be opportunistic pathogen that can cause not only pulmonary

infections, but also skin and soft tissue infections. Some NTMs like *M. abscessus* are known to cause tuberculosis-like lesions in humans.

Table II: Major mycobacterial infections in human
adapted from (Falkinham, 2002; Medjahed, Gaillard, & Reyrat, 2010)

Causative agent	**Reservoir**	**Geographical distribution**	**Clinical manifestation**
Slow growing mycobacteria			
M. tuberculosis	Humans	Worldwide	Pulmonary and extra-pulmonary tuberculosis
M. leprae	Humans	South Asia, Africa, Brazil	Leprosy
MAC	Soil, water and animals	Worldwide	Pulmonary disease, hot tub lung, disseminated disease
Rapid growing mycobacteria			
M. abscessus	Soil and water	Worldwide	Pulmonary disease, soft tissue, skin and bone infections, disseminated disease
M. chelonae	Soil, water and animals	Worldwide	soft tissue, skin and bone infections, disseminated disease, keratitis
M. fortuitum	Soil, water	Worldwide	soft tissue, skin and bone infections

The mycobacterial cell wall is extremely complex and is composed of mycolic acids, waxes, glycolipids, peptidoglycan and arabinogalactan, which plays crucial role in growth, survival and pathogenesis of mycobacteria. Mycobacteria are generally considered as Gram-positive bacteria, but can also be put in Gram-negative bacterial group as it shares characteristics of both Gram-positive and Gram-negative bacteria (Chiaradia *et al.,* 2017). The mycobacterial cell wall has also high lipid content, which accounts for approx. 60% of cell wall weight, compared to other Gram-positive and Gram-negative bacteria where lipid content accounts for only 5% and 10% of cell wall weight respectively. This complexity explains the tendency of clumps formation in NTM (Falkinham, 2002). The role of the complex hydrophobic cell wall of mycobacteria has been widely studied including its characteristic of acid-fastness, high lipid content, slow growth rate to understand the poor diffusion of many antibiotics (Chiaradia *et al.,* 2017; Falkinham, 2002).

1.2 History of *MABC*

Mycobacterium abscessus was first isolated from knee in 1952 and then in 1953, Moore and Frerichs first described this human pathogen (Lee *et al.,* 2015). *M. abscessus* is a rapid growing mycobacteria, also highly acknowledged as a prime cause of pulmonary infections in Cystic Fibrosis (CF) patients (David E. Griffith *et al*., 2007; Simons *et al.*, 2011; Skolnik, Kirkpatrick, & Quon, 2016). Because of its propensity to develop multiple drug resistance, *M. abscessus* has been termed as "New antibiotic Nightmare" (Nessar *et al.*, 2012).

M. abscessus and *M. chelonae* were first considered as a same species until 1992, since *M. abscessus* was determined as an individual species. Then *M. massiliense* and *M. bolletii* were identified but they were considered to come from the same species known as *MABC.* Finally, in 2013, *MABC* was divided into 3 subspecies by genome comparison: *M. abscessus subs. abscessus, M. abscessus subs. massiliense, M. abscessus subs. bolletii* (Figure III) (Tortoli *et al.*, 2016). All three subspecies lead to different treatment outcomes and resistance profiles because of different gene patterns (Lee *et al.*, 2015)

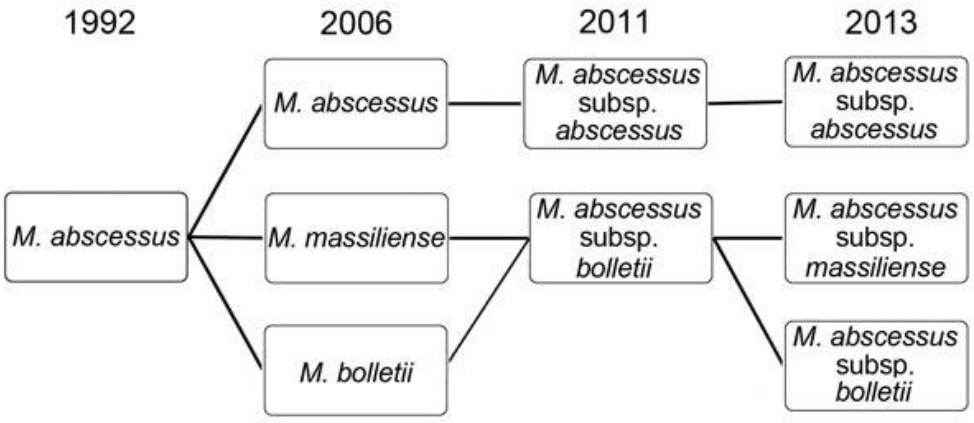

Figure II: Serial Changes in the nomenclature and taxonomic classification of *MABC* (Lee *et al.*, 2015)

1.3 Ecology and epidemiology

M. abscessus generally resides locally in respiratory tract. Immunocompromised patients or the patients with underlying situation such as CF can be infected by this pathogen. Infection is sporadic or epidemic with or without healthcare-related risk factors (Mougari *et al.*, 2016). The main source of these infections are aqueous environments, soil and animals (Falkinham, 2010). Generally, bathroom showers have been reported as a prime source of exposure to aerosolized NTM, as aerosolized droplets are small enough to enter in the alveoli and cause pulmonary infections (Falkinham, Iseman, de Haas, & van Soolingen, 2008). NTM can be present in

livestock, which represents one more health risk to the community (Kazwala, Kusiluka, Sinclair, Sharp, & Daborn, 2006; Mdegela *et al.*, 2004). Various NTMs have been isolated from wild and domestic animals like pigs and cattles (Falkinham, 2002; Muwonge *et al.*, 2010). In addition to this, fishing, aquaculture and leisure activities may induce the exposure to several NTM infections. The evidence of human-to-human transmission for NTM infection has not been well studies (David E. Griffith *et al.*, 2007; Johnson & Odell, 2014), but Bryant *et al.* demonstrated the possibility of human-to-human transmission of *M. abscessus* in CF patients (Bryant *et al.*, 2016).

1.3.1 Geographical distribution

Existence of NTM species varies with the geographical distribution, however *MAC* and *M. abscessus* are often isolated in pulmonary infections worldwide (Johnson & Odell, 2014). A collaborative study from NTM-NET stated the geographical distribution of NTM isolations in 2008 (Hoefsloot *et al.*, 2013). The relative proportions of *MABC* infection cases differ between geographical regions, with 52% in France, 56% in UK and 45% in USA (Mougari *et al.*, 2016). In Asia, *MABC* is responsible for more than 69% pulmonary infections (Simons *et al.*, 2011). In general, *MABC* is predominating worldwide as a cause of NTM-PD after MAC (van Ingen *et al.*, 2017). The geographical distribution of three subspecies of *MABC* has been studied in several individual studies (Table III).

Table III: Distribution of the three subspecies *M. abscessus*, *M. massiliense* and *M. bolletii* within the *MABC* in 11 studies (2008-2016)
adapted from (W.-J. Koh, Stout, & Yew, 2014)

Country	No. of patients	*M. abscessus*	*M. bolletii*	*M. massiliense*	Study year
South Korea	126	53%	2%	45%	2008
USA	40	67.5%	5%	27.5%	2009
Netherlands	39	64%	15%	21%	2009
France	50	60%	18%	22%	2009
South Korea	158	44%	1%	55%	2011
Japan	102	71%	3%	26%	2012
Taiwan	79	43%	1%	56%	2013
Japan	143	63%	2%	35%	2013
South Korea	404	50%	1%	49%	2014
Ireland	36	78%	0%	22%	2016

1.3.2 Incidence and prevalence

The incidence and prevalence of *M. abscessus* infections has strongly been increasing during last several years, possibly due to improved bacterial isolation and identification tools. The annual prevalence of NTM infections from respiratory specimens is 14.1 to 22.2 per 100,000 patients in Canada, while 0.7 to 7.0 cases per 100,000 patients in Europe (Stout, Koh, & Yew, 2016) and 0.9 to 1.3 cases per 100,000 patients in United states and Japan (Morimoto *et al.*, 2017). *MAC* is more often associated with older patients having CF, while *M. abscessus* is frequently seen in younger patients having severe pulmonary infections (Skolnik *et al.*, 2016).

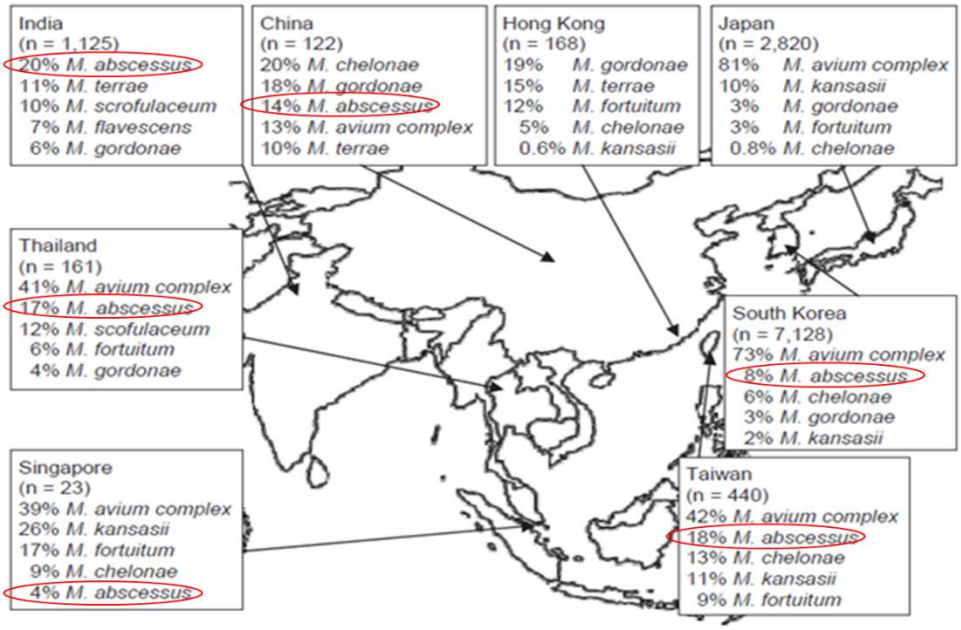

Figure III: Prevalence of *M. abscessus* in Asian countries from pulmonary specimens (Simons *et al.*, 2011)

Table IV describes the prevalence of various NTM infections including *M. abscessus* infection in patients having cystic fibrosis worldwide for last 20 years. In France, 0.73 cases of NTM-pulmonary diseases (NTM-PD) per 100,000 patients per year have been reported (Dailloux *et al.*, 2006). Moreover, cystic fibrosis (CF) has been widely associated with an increased prevalence of NTM infections (Olivier *et al.*, 2003). *M. abscessus* accounts for 5-20% of NTM infections, but *M. abscessus* represents up to 80% of RGM isolates in NTM-PD. In India, *M. abscessus* is the most common NTM responsible for NTM-PD. Currently, *MABC* are spreading widely in East Asia (Figure III) (Simons *et al.*, 2011). Preliminary studies have shown that *MABC* stands second after *MAC* in prevalence among patients with CF and the third most common NTM pulmonary pathogen in the United states after *MAC* and *M. kansasii* (David E. Griffith *et al.*, 2007).

Table IV: Prevalence of NTM infection in patients with cystic fibrosis during last several years worldwide adapted from (Jordan *et al.*, 2007)

Country	Study year	No. of patients	Age of persons affected	Prevalence of NTM	Causative NTM species
Brazil	Segal *et al.* 1998	40	4 months to 25 years	15%	*MAC*
Canada	Radhakrishnan *et al.* 2009	98	6 to 18 years	6.1%	***M. abscessus*** and *MAC*
England	Torres *et al.* 1998	372	9 to 25 years	3.8%	*M. fortuitum*, *MAC*, *M. chelonae*, *M. malmoense*, and *M. kansasii*
France	Fauroux *et al.* 1997	106	1 to 18 years	6.6%	*M. xenopi*, *M. chelonae*, ***M. abscessus*** and *M. fortuitum*
	Sermet-Gaudelus *et al.* 2003	298	2 months to 32 years	9.8%	***M. abscessus***, *M. gordonae*, *MAC*, *M. fortuitum* and *M. kansasii*
	Pierre-Audigier *et al.* 2005	385	1 to 24 years	8.1%	***M. abscessus***, *MAC*, and *M. kansasii*
Germany	Bange *et al.* 2001	214	21 to 35 years	7%	***M. abscessus***, *M. intracellare*, *MAC*, *M. simiae* and *M. interjectum*
Israel	Levy *et al.* 2008	186	10 to 30 years	22.6%	***M. abscessus***, *M. simiae*, *MAC*, *M. fortuitum* and other
Scandinavian countries	Qvist *et al.* 2015	1270	13 to 29 years	3% to 28%	***M. abscessus*** and *MAC*
UK	Seddon *et al.* 2013	7000	Children and adults	3.3% - 5%	Not determined
USA	Kilby *et al.* 1992	87	18 to 64 years	19.5%	*MAC*, *M. chelonae* and *M. fortuitum*
	Aitken *et al.* 1993	64	17 to 50 years	12.5%	*MAC* and *M. fortuitum*
	Olivier *et al.* 2003	986	10 to 40 years	13%	*MAC*, ***M. abscessus***, *M. gordonae*, *M. kansasii* and other
	Esther *et al.* 2005	431	Children < 12 years	4%	***M. abscessus*** and *MAC*

1.4 Pathogenesis and pathophysiology

Despite since the last decade *M.abscessus* infections are on rise (Lee *et al*., 2015), the infectious dose to induce an infection is yet unknown and also the potential to cause infections by different NTM species is highly variable and the mechanisms behind that are not well explained (Johnson & Odell, 2014). *M. abscessus* is an intracellular pathogen able to reside and replicate in macrophages and forms biofilms in human lung that may explain this incurable pulmonary infection or treatment failure (Mougari *et al.,* 2016).

M. abscessus shares also a large number of common features with *M. tuberculosis.* As an example, *M. abscessus* possesses virulence factors involved in intracellular parasitism. It consists of proline-glutamate proteins (PE) and proline-proline-glutamate (PPE), MCE (Mammalian Cell Entry) and Yrbe proteins that allow mycobacteria to penetrate host cells, as well as LpqH-like proteins that modulate host response. The study of pathophysiological mechanisms of *M. abscessus* infections has progressed a great deal by means of the murine experimental model. Indeed, it has shown that infection leads to histopathological damage closely mimicking those observed in humans, including caseous lesions (Rottman *et al.*, 2007).

This bacterial genome is 5 megabases (Mb) long, which is close to that of *M. tuberculosis* with the length of 4.4 Mb. The pathogenicity of this bacteria can better be understood by sequencing of *M. abscessus* genome. Furthermore, the resistance to many antibiotics makes the genetic manipulation of this pathogen very difficult (Mougari *et al.,* 2016).

1.5 Identification, sample collection and diagnosis

The major challenge is to identify patients having *M. abscessus* infection, as it resembles *M. tuberculosis* in terms of acid-fast bacilli (AFB), Gram-staining and symptoms (Floto *et al.,* 2016), although chest radiography and verification of AFB should be done at least three times. Generally, sputum samples are collected, but in case of chronic pulmonary infections, cough swabs, broncho alveolar lavage (BAL), cough plate, oropharyngeal culture can be used (Floto *et al.,* 2016). Modern laboratory culture techniques and molecular identifications provide rapid diagnostic. Recently, Studies have shown that mass spectrometry (MALDI-TOF: Matrix-Assisted Laser Desorption Ionization-Time of Flight) could be used in clinical microbiology for the identification of mycobacteria from a solid culture medium. In case of *M. abscessus* isolation from the environment, techniques used for isolation involve filtration or centrifugation prior to culture in appropriate media (Mougari *et al.,* 2016).

1.6 Colony morphology

M. abscessus is an emerging opportunistic pathogen with the length of 1.0- to 6.0-μm and diameter of 0.2- to 0.5-μm having a bacillus shape, sometimes curved at the extremities. *M. abscessus* can grow on the solid agar as either rough non-biofilm forming colonies or smooth biofilm forming colonies (Figure IV) (A.-L. Roux *et al.*, 2016).

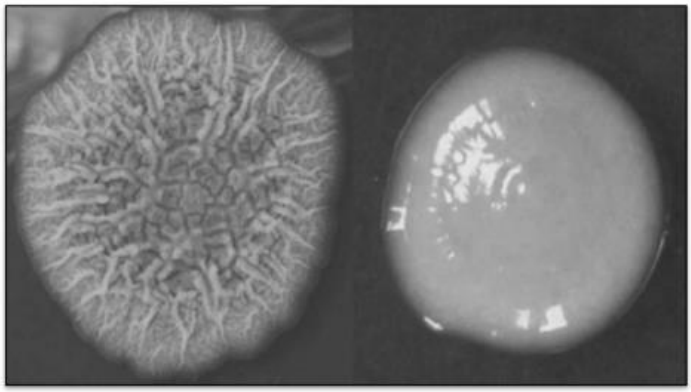

Figure IV: Smooth (right) and rough (left) colonies of *M. abscessus* on 7H11 agar (Kai *et al.*, 2014)

M. abscessus strains are frequently reported to undergo rough (R) to smooth (S) morphotype transition during the course of infection. The conversion of S to R and R to S is related with the loss of a surface GLPs. The S form of *M. abscessus* would produce GLP in certain habitats, allowing it to colonize through biofilm formation. On the other hand, for an effective invasion of the human host, *M. abscessus* could switch to the R-form and no longer produce GLP (Jönsson *et al.*, 2007). The mucosal S form of *M. abscessus* is able to produce glycopeptidolipids, surface lipids, while the R form does not produce it (Figure V). As a result, R forms are associated with more severe clinical forms than with S forms (A. Roux, Hermann, Gaillard, & Rottman, 2010).

1.7 Infections caused by *MABC*

NTMs are responsible for chronic pulmonary, skin and soft tissue infections, disseminated lesions and rare infections of central nervous system (Table V) (Lee *et al.*, 2015). Though, the respiratory tract, skin and soft tissues are the most frequent sites for the infections caused by *M. abscessus.*

Table V: Infections caused by NTM (other species than mentioned here may cause disease) (Johnson & Odell, 2014; Piersimoni & Scarparo, 2009)

Infection	**Species responsible**
Chronic pulmonary disease	*M.kansasii, MAC, M. fortuitum, M. xenopi* and ***M. abscessus***
Skin and soft tissue infections	*M. marinum, M. fortuitum, M. chelonae, M. ulcerans,* and ***M. abscessus***
Extrapulmonary infections (Local lymphadenitis, Bone and joint infections)	*MAC, M. bohemicum, M. lentiflavum, M. genavense, M. fortuitum, M. heckeshornense, M. kansasii, M. malmoense, MAC, M. xenopi,* ***M. abscessus,*** *etc.*

1.7.1 Skin and soft tissue infections

The most common soft tissue infections caused by *M. abscessus* are often associated with iatrogenic action and direct inoculation of *M. abscessus*, usually in immunocompetent individuals. In majority of cases, it is caused by subcutaneous or intramuscular administration of injectable solutions infected with *M. abscessus* or reuse of soiled material (Petrini, 2006).

1.7.2 Extrapulmonary infections

Almost half of the extrapulmonary infections caused by *M. abscessus* are postoperative infections. In France, 7 reported cases out of 20, of NTM infections were related to beauty care (Mougari *et al.,* 2016). *M. abscessus* disseminated infections usually occur in patients with underlying free-standing, acquired immunodeficiency (autoimmune disease, cancers) or in transplant patients. Several types of extrapulmonary infections such as vertebral osteomyelitis, pleural empyema, peritonitis, keratitis and endocarditis have been reported. These infections could be associated with acquired impairments of IL-12 or low CD4 levels (David E. Griffith *et al.,* 2007).

1.7.3 Pulmonary infection

The majority of *M. abscessus* pulmonary infections occur in patients with underlying conditions such as bronchial dilatation, CF, TB or COPD (David E. Griffith *et al.,* 2007). If chronic pulmonary infections are the major manifestation of *M. abscessus* infections, it is also the first RGM involved in acute pulmonary infections (Lerat *et al.,* 2014). *M. abscessus* and *MAC* represent more than 95% of NTM-PD in CF patients. In USA, in a retrospective study of the 154 cases of rapid growing mycobacterial pulmonary infection, 82% were due to *M. abscessus* (David E. Griffith *et al.,* 2007). These infections can also affect previously healthy subjects in

30% of cases, those are generally non-smokers Caucasian women over the age of 60. Table VI represents the global prevalence of *M. abscessus* infection in CF patients from various studies.

M. abscessus may persist silently for years and even decades in the human host. An American retrospective study involving 146 patients (Chalermskulrat *et al.,* 2006), who benefited from lung transplantation due to end-stage CF shows a pre-occurrence of *M. abscessus* (8%). After transplantation, the prevalence of NTM is low (3.4%) but is higher if *M. abscessus* was present before transplantation. This is the only NTM species significantly related to a post-transplant infectious course, with fatal graft failure directly from mycobacterial disease (Chalermskulrat *et al.,* 2006). The similarity of infectious specimens found before and after transplantation indicates that there are certainly other reservoirs of *M. abscessus* beyond the respiratory tract in CF patients.

Table VI: Prospective prevalence studies for NTM-PD in CF patients, for which *M. abscessus* has been identified at least in one patient (Laencina, 2018)

Country	Duration	No. of patients	Prevalence
USA	1992	87	19.5%
	21 centers	986	1-24%
	1992-2004	55	32.7%
	1999-2002	114	6.1%
	2000-2007	1216	11%
	2010-2011	18003	0-28%
	2006-2012	33653	12%
Denmark	1987-1988	185	1.6%
	1974-2014	432	13.4%
France	1995-1996	106	6.6%
	1996-1999	298	9.8%
	CF center	385	8.1%
	2001-2003	262	6.1%
	CF centers	385	8.1%
	2009-2014	401	12%
Germany	1997-1999	214	7%
Spain	1997-2001	28	25%
	2002-2012	44	0-33%
Sweden	1997-2005	140	10%
Brazil	2003-2004	54	11%
	2009-2012	129	7.75%
Israel	2001-2003	186	22.6%
	2002-2011	90	14.5%

- **Cystic fibrosis and associated infections**

In 1938, Dr. Dorothy Hansine Andersen, American pathologist described the disease, named "cystic fibrosis of the pancreas" based on the autopsy finding of children that died because of malnutrition. While other physicians of that era, referred this disease as "mucoviscidosis", as it occurred because of thickening of mucous. Cystic fibrosis (CF) is the most common autosomal transmission genetic disease spreading tremendously worldwide, which happens due to an abnormality of the cystic fibrosis transmembrane conductance regulator (CFTR) protein involved in regulation streams of chlorine, sodium and water at the transmembrane level (Vankeerberghen, Cuppens, & Cassiman, 2002). CF affects the respiratory, digestive and reproductive systems involving thick mucus linings production in the lungs, which can lead to fatal pulmonary diseases. The global prevalence of this genetic diseases at birth is approximately 10 per 1000 (World Health Organisation, 2017). In European Union, one in 2000-3000 new born is found to be affected by CF and in USA, one in every 3,500 births (World Health Organisation, 2017). The evolution is peppered with colonization and recurrent respiratory infections cause pulmonary destruction and insufficiency, which condition into the prognosis. The bacteria responsible for colonization are initially *Staphylococcus aureus* and *Haemophilus influenzae* in young people, then *Pseudomonas aeruginosa,* becomes dominant in adult patients (Fujita *et al.,* 2014). Now days, the prevalence of NTM-PD has been increasing especially in CF patients with the overall prevalence varying from 6% to 13% (Martiniano, Nick, & Daley, 2016; Olivier *et al.,* 2003).

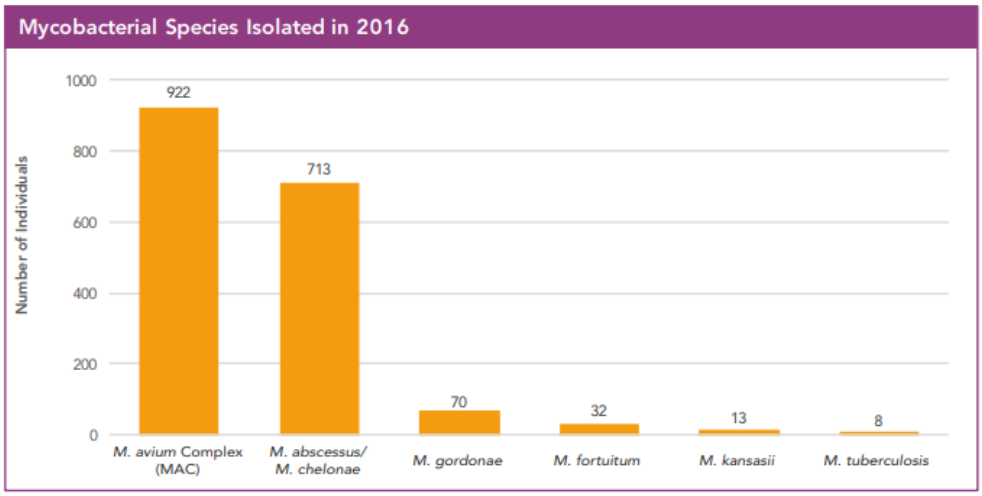

Figure V: Prevalence of NTM infection in patients with CF in 2016 (CF foundation Patient Registry, 2016)

The two most frequently identified mycobacteria in CF patients belong to the *MAC* and *MABC* (Figure V). According to the CF foundation Patient Registry, the proportions of colonized patients with NTMs were13% in 2016, compared with 10.1% in 2010 (CF foundation Patient Registry, 2016). The infections and study vary according to country and age. The gravity of NTM-PD is variable depending on the pathogenicity of the strain as well as according to factors specific to the host.

CF is often associated with NTM-PD, but other than NTM-PD, different NTMs are responsible for different types of disease (Falkinham, 2002; Piersimoni & Scarparo, 2009). Pulmonary infections caused by *M. abscessus* is considered as a major obstacle to lung transplantation, which is often the only chance of survival for some CF patients. It is also recognized to have high contraindication to lung transplantation, as many cases of fatal post-transplant infections have been recorded (Gilljam, Scherstén, Silverborn, Jönsson, & Ericsson Hollsing, 2010). On other hand, some lung transplant studies with short and long-term success rates are also being reported recently (Qvist *et al.,* 2015). Despite all, in some cases, lung transplant is the only option left (David E. Griffith *et al.,* 2007), especially in the case of localized or excavated damage. This resection can be total or partial but only for patients who have a forced vital capacity of more than 30%. In one retrospective study (D. E. Griffith & Wallace, 1996), 7 out of the 10 patients with NTM eradication had benefited from a surgical trial associated with appropriate antibiotic therapy, as suggested by WHO. Similarly, a Survival Study was conducted by Camargos *et al.,* with 21 patients having CF (mean age of 8.09 +/- 4.4 years), operated between 1988 and 2003, and followed up to 2004. Eleven years after resection, the probability of survival was 93.8% (Camargos *et al.,* 2008). A case of fatal pulmonary infection caused by *M. abscessus* in a young patient with CF who had undergone a lung transplant showed the possibility of disseminated post-transplant mycobacterial infection with isolates of bacteria in samples blood (Sanguinetti *et al.,* 2001). Similarly, a more recent study shows that two patients colonized by *M. abscessus* have also developed disseminated post-transplantation infections (Jönsson *et al.*, 2007). Based on the data discussed above for lung transplantation, there are so many controversies regarding positive or negative outcomes, in fact no detailed recommendations are available. So, there is an urgent need for high quality clinical data to inform decision-making (Tissot, Thomas, Corris, & Brodlie, 2018).

2. Antimicrobial resistance of *M. abscessus*: current status and major challenges to treat pulmonary infections

After a critical shift towards macrolide-based multi regimen treatment in 1990s instead of using anti-TB regimens, not much has been accomplished in the treatment of *M. abscessus* pulmonary infections. Whereas, the incidence rate of pulmonary infections caused by *M. abscessus* is increasing at an alarming rate, resistance to antibiotics is also of major concern leading to the treatment failure or poor treatment outcomes in many countries (David E. Griffith *et al.*, 2007; Mougari *et al.*, 2016; Nessar *et al.*, 2012; van Ingen *et al*., 2017).

2.1 Antibiotic susceptibility and efficacy

M. abscessus strains are characterized by a natural multidrug resistance not only to the anti-tuberculous agents but also to almost usable antibiotics (Nessar *et al.*, 2012). However, *M. abscessus* are naturally susceptible to certain β-lactams (cefoxitin, imipenem), amikacin and clarithromycin. Among the new molecules, tigecycline has shown good potency in *in vitro* activity against several *M. abscessus* isolates (Ferro, Srivastava, *et al.*, 2016c). Most isolates are resistant to doxycycline, minocycline and sulfamethoxazole. Few drugs showing *in vitro* activity against *M. abscessus* are mentioned in Table VII.

Moreover, assessing *in vitro* susceptibility is very difficult and can result in inconsistent results. The *in vitro* susceptibility can be determined by broth micro or macro dilution method, as per CLSI guidelines M24-2 (NCCLS, 2003); however susceptibility breakpoints are only determined by CLSI, not by EUCAST yet. Antimicrobial susceptibility testing (AST) can also be done using E-test, agar diffusion or disk diffusion method. Amikacin, cefoxitin, and imipenem are three most potent intravenous antibiotics *in vitro* against *M. abscessus* with MICs lower than serum peak concentrations (Brown-Elliott, Nash, & Wallace, 2012).

Table VII: Antibiotic susceptibility defined by MICs against *M. abscessus* adapted from (Nessar *et al.*, 2012)

Antibiotics	MIC range (mg/L)	% of susceptible strains	Susceptibility breakpoints (S-I-R)*
Antibiotics with high % susceptibility			
Tigecycline	≤0.06-1	100	ND†
Clofazimine	0.25-1	99	ND†
Clarithromycin	0.03-16	83-99	≤2-4-≥8
Amikacin	0.25-≥128	87-94	≤16-32-≥64
Antibiotics potentially active but large variation in susceptibility according to studies			
Cefoxitin	16-128	11-99	≤16-64-≥128
Tobramycin	8-≥128	36-95	≤4-8-≥16
Antibiotics with median activity			
Imipenem	1-64	8-55	≤4-8-≥16
Ciprofloxacin	0.016-8	44-57	≤1-2-≥4
Moxifloxacin	2-32	73	≤1-2-≥4
Antibiotics rarely active			
Linezolid	0.5-128	23	≤8-16-≥32
Doxycycline	0.06->128	5-8	ND†
Minocycline	0.25->64	5	ND†
Tetracycline	4->128	10	ND†
Sulfamethoxazole	4-256	1-12	ND†
*(S-I-R) represents susceptible, intermediate and resistance criteria for antibiotics †ND: not defined			

Clarithromycin is an oral antibiotic considered to be the most active against several clinical isolates and was the molecule of choice up to the description of macrolide inducible resistance (David E. Griffith *et al.*, 2007; Nessar *et al.*, 2012). Treatment recommendation is to use antibiotics in combination. Despite of using the antibiotics with shown highest activity against *M. abscessus*, clinical efficacy of this multidrug therapy is still controversial, with success for some and failure for other patients (Nessar *et al.*, 2012).

2.2 Resistance mechanism

M. abscessus are probably the most antibiotic resistant emerging pathogen among all RGM. This resistance is a result of complex interaction between natural, inducible and mutational resistance acquired during antibiotic exposure. However, using several antibiotics in combination can contest the antibiotic resistance. Knowledge of these resistance mechanisms is very important in selecting and optimizing therapeutic regimens. Figure VI represents the simplified overview of resistance mechanisms.

- **Natural resistance:**

Many mechanisms including slow growth, highly lipophilic and impermeable cell wall, mechanisms that control cell wall content, porin numbers, efflux pumps, various modifying or inhibiting enzymes of antibiotics contribute to the natural resistance of complex mycobacteria to antibiotics. The mycobacterial cell envelop plays an important role in protecting the cell against toxic extracellular compounds. Furthermore, *M. abscessus* produces enzymes which can degrade or modify antibiotics, like β-lactams and then results into antibiotic inactivation. For example, expression of β-lactamase and rifampicin ADP-ribosyltransferases lead to natural resistance respectively to β-lactam antibiotics and rifampicin. *M. abscessus* expresses *whiB* transcriptional regulators, which are induced by antibiotic use. The expression or activation of some efflux pumps could play a role in antibiotic resistance (Nessar *et al.*, 2012; van Ingen, Boeree, van Soolingen, & Mouton, 2012). Other possible mechanisms for natural resistance are described in Table VIII.

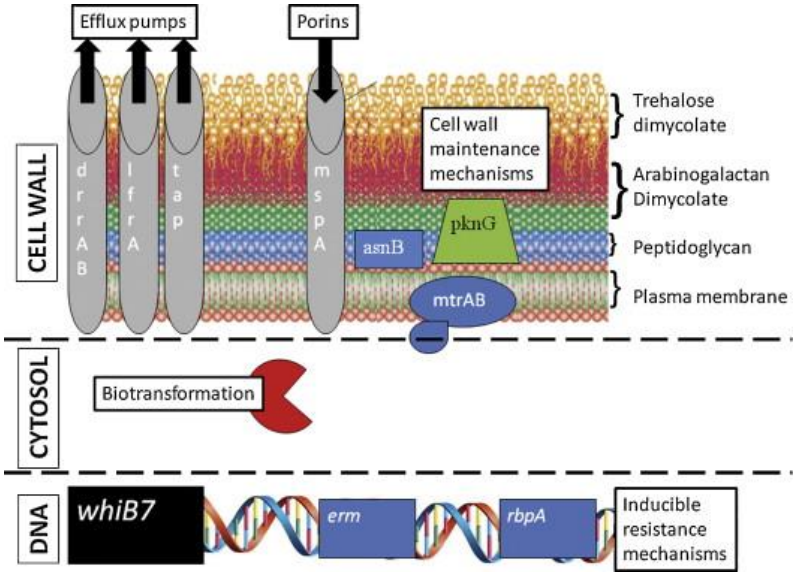

Figure VI: Important role of mycobacterial cell wall in resistance mechanism (Van Ingen, Boeree, *et al., 2012)*

- **Acquired resistance:**

The acquired resistance is due to spontaneous mutations, affecting the key targets of antibiotics. For example, acquired resistance in aminoglycosides occurs due to involvement of *"rrs"* gene and 16sRNA protein with various mutations. In case of macrolides, *"rrl"* gene and 23sRNA protein are involved and for fluoroquinolones, *"gyrA"* and *"gyrB"* genes are responsible. Generally, alteration in the functional chromosomal gene represents the prime mechanism for acquired resistance, however other mechanisms may involve (Nessar *et al.*, 2012).

Table VIII: Mechanism of resistance in *M. abscessus*
(Millar & Moore, 2019; Nessar et al., 2012; Ripoll et al., 2009)

Antibiotics	Mechanism of action	Genes associated with resistance	Proteins involved in resistance
Natural resistance			
Aminoglycosides	Drug absorption is prevented by selective cell wall permeability or antibiotics are modified by enzymes Inhibition of protein synthesis	*MAB_4395, MAB_0327, MAB_0951, MAB_3637c, MAB_4910c, MAB_4395* 30S ribosomal unit (16S rRNA)	Aminoglycoside 2-N-acetyltransferase Aminoglycoside phosphotransferases
β-lactams	Antibiotics are degraded by enzymes Inhibition of cell wall synthesis	*MAB_2875*	β-lactamase Bla_{mab} penicillin-binding protein
Rifampicin	Antibiotics are degraded by enzymes Inhibition of transcription	*MAB_0951*	Rifampicin ADP-ribosyltransferase β-subunit of RNA polymerase
Macrolides	Enzymes modifies the structure of the target	*erm (41)* *MAB_2297*	23sRNA methyltransferases
Ethambutol	Mutation in genes	*embB in ERDR*	Arabinosyl transferase
Other molecules	Efflux pumps export drugs to the outside of bacteria	Distributed in genome	ABC transporters of MmpL
Acquired resistance			
Aminoglycosides	Inhibit protein synthesis	*rrs*	16s RNA
Macrolides	Inhibit drug attachment to rRNA	*rrl*	23s RNA
Fluoroquinolones	Gene polymorphism	*gyrA*	Gyrase A subunit

A macrolide-inducible resistance gene, *erm (41)* has been described, which confers clarithromycin resistance by methylation of 23S rRNA and thus impairs the binding of the antibiotic to its target. The expression and the regulation of this *erm (41)* gene are different according to the subspecies within the complex. In *M. massiliense*, the *erm (41)* gene contains two deletions which no longer allow its expression, making this subspecies sensitive to clarithromycin (Nash, Brown-Elliott, & Wallace, 2009). These differences in susceptibility to antibiotics in the *M. abscessus* are one of the major source of the debate about their separation into subspecies (Won-Jung Koh *et al.,* 2011). Regarding the molecular detection of antibiotic susceptibility, there is no commercial test available for detecting *M. abscessus*-resistant mutations to antibiotics. Detection of macrolide resistance is achieved by partial sequestration of the 23R rRNA and gene *erm (41)* and the detection of aminoglycoside resistance by sequencing the 16S rRNA encoding gene (van Ingen *et al.*, 2012).

2.3 Studies showing drug activity against *M. abscessus*

M.abscessus is notoriously difficult to treat even after a long 12 months multi-drug regimen therapies (Medjahed *et al.*, 2010). Most experts recommend treatment up to 12 months or until achieving negative culture in terms to measure treatment efficacy. The choice of antibiotics for treatment is guided by MICs determination in a liquid medium (David E. Griffith *et al.*, 2007). Recommended treatment is associated with the co-administration of oral linezolid, moxifloxacin, ciprofloxacin, clarithromycin combined with IV administration of amikacin, tigecycline, cefoxitin, imipenem (Brown-Elliott *et al.*, 2012). Among these available antibiotics, amikacin, cefoxitin, imipenem and clarithromycin are the most effective. Furthermore, ATS recommends an initial treatment in combination of several antibiotics: clarithromycin or azithromycin should be combined with one or more parenteral agents: amikacin and / or cefoxitin (or imipenem) at least for 8 weeks prior to oral clarithromycin alone (David E. Griffith *et al.*, 2007). In less severe forms or in people who cannot tolerate treatment, less intensive oral or parenteral macrolide medications may be suggested to control symptoms and progression of infection (Brown-Elliott *et al.,* 2012; David E. Griffith *et al.,* 2007). For patients having macrolide intolerance or resistance, experts recommend a combination of parenteral and oral antibiotics based on *in vitro* activity (Brown-Elliott *et al.*, 2012). Linezolid, rifabutin, fluoroquinolones and tigecycline are alternative molecules but their efficacy has not been fully evaluated and the lack of effective antibiotic treatment is often associated with a high mortality rate in these patients. In general, antibiotic treatment for pulmonary infections is not standardized yet.

Macrolide-inducible resistance may explain the lack of efficacy of antibiotic therapy including a macrolide against *M. abscessus* infections. Sometimes, the antimicrobial treatment is accompanied by adverse effects that exacerbate the severity of the disease. This phenomenon is also known as treatment paradox, which is already observed while taking anti-tuberculosis drugs (Breen *et al.*, 2004) and complicates the management of infection following the inefficacy of treatment and / or side effects of some antibiotics. Overall, with current therapeutic options, *M. abscessus* pulmonary infections are often chronic and incurable for many patients, which can explain high treatment failure rate (David E. Griffith *et al.*, 2007). Another explanation for treatment failure of the standard recommended combination of amikacin, cefoxitin and clarithromycin may be related to antibiotic concentrations in biofilms and macrophages, below bactericidal concentrations (Greendyke & Byrd, 2008).

Therapeutic options are insufficient for a moment and therefore, other parameters such as clinical improvement and / or regression of pulmonary infiltrates and / or a decrease in the number of positive cultures from respiratory tests are also recommended. Also, recommended treatment has never been proved to be significant and is often associated with poor outcomes. In addition to this, comparatively slowly growing mycobacteria and their associated longer incubation periods may lead to think about the *in vitro* stability of the tested antibiotics. The most frequently used β-lactam antibiotics are known to have limited *in vitro* stability, which may explain their moderate *in vitro* activity (Rominski, Schulthess, Müller, Keller, & Sander, 2017; Schoutrop *et al.*, 2018). Here, the data regarding the antibiotic activity against *M. abscessus* by means of *in vitro, in vivo* and clinical studies are scarce and are compiled in Table IX.

Table IX: *In vitro* and *in vivo* studies showing antibiotic activity against *M. abscessus*

Antibiotics	**Type of study**	**Outcomes**	**References**
Several combinations	*In vitro* (FIC index)	Combination of amikacin and cefoxitin showed no synergy; combination of imipenem with clarithromycin, levofloxacin or amikacin was indifference while combination of imipenem with tobramycin, minocycline or moxifloxacin was antagonistic	(Miyasaka *et al*., 2007)
Several combinations	*In vitro* (FIC index)	Combination of clarithromycin and linezolid was the best	(Cremades *et al.*, 2009)
Tigecycline Clarithromycin Amikacin	*In vitro* (FIC index)	Combination of tigecycline and clarithromycin was synergistic against 80.6% isolates	(Huang *et al.*, 2013)
Clofazimine Tigecycline	*In vitro* (FIC index)	Clofazimine and tigecycline combination was synergistic against 19 isolates	(Singh, Bouzinbi, Chaturvedi, Godreuil, & Kremer, 2014)
Tigecycline Moxifloxacin Amikacin	*In vitro* (Time-kill)	Lack of bactericidal activity by each antibiotic	(Maurer *et al.*, 2014)
Rifampicin Penems	*In vitro* (Time-kill)	Rifampicin in combination with doripenem was much active than rifampicin combined with biapenem	(Kaushik *et al*., 2015)
Ceftaroline Avibactam	*In vitro* (MIC)	Ceftaroline was active as cefoxitin but only in absence of β-lactamase; Ceftaroline-avibactam combination inhibited growth at potentially achievable drug concentrations	(Dubée, Soroka, *et al.*, 2015)
Cefoxitin Amikacin Clarithromycin	*In vitro* (Time-kill)	Amikacin showed highest activity followed by clarithromycin and cefoxitin	(Ferro, van Ingen, *et al.*, 2015)

Clofazimine Amikacin Clarithromycin	*In vitro* (Time-kill)	Clofazimine prevented the regrowth of *M. abscessus* exposed to amikacin and clarithromycin	(Ferro, Meletiadis, *et al.*, 2016)
Tedizolid	*In vitro* (MIC)	Potent than linezolid	(Brown-Elliott & Wallace, 2017)
Vancomycin Clarithromycin	*In vitro* (FIC index)	Combination was synergistic	(Mukherjee, Wu, Teo, & Dick, 2017)
Rifabutin	*In vitro* (MIC)	Rifabutin was active against clarithromycin resistant isolates	(Aziz *et al.*, 2017)
Rifabutin Clarithromycin Tigecycline	*In vitro* (Time-kill)	Triple combination of rifabutin, tigecycline and clarithromycin was synergistic	(Pryjma, Burian, & Thompson, 2018)
Teicoplanin – Tigecycline	*In vitro* study	Synergistic activity during checkerboard titration assay	(Dinah B. Aziz, Teo, Dartois, & Dick, 2018)
Rifabutin Avibactam	*In vitro* (Time-kill) and intracellular	Rifabutin alone was bacteriostatic, but addition of imipenem and avibactam increased killing activity	(Le Run, Arthur, & Mainardi, 2018)
Amikacin Cefoxitin Clarithromycin	Biofilms	MICs for amikacin and clarithromycin were out of range of the achievable peak serum concentrations; cefoxitin was inactive	(Greendyke & Byrd, 2008)
Moxifloxacin	*In vitro*, intracellular and *in vivo* mouse model	Moxifloxacin combined with clarithromycin was antagonistic	(Choi *et al.*, 2012)
Amoxicillin Avibactam	*In vitro*, intracellular and *in vivo* zebrafish model	β-lactamase inhibited by avibactam	(Dubée, Bernut, *et al.*, 2015)

Cefoxitin Imipenem	*In vitro* and intracellular	MIC for imipenem was lower compared to cefoxitin but number of imipenem-resistant isolates were higher according to CLSI breakpoints MICs of both antibiotics were higher for rough morphotype than smooth.	(Lavollay *et al.*, 2014; Lefebvre *et al*., 2016)
Amikacin	Hollow fiber model	Limited efficacy	(Ferro, Srivastava, *et al.*, 2015)
Amikacin Cefoxitin Clarithromycin	Hollow fiber model	Standard triple combination failed quickly	(Ferro, Srivastava, *et al.*, 2016a)
Tigecycline	Hollow fiber model	Most active single agent	(Ferro, Srivastava, *et al.*, 2016c)
Moxifloxacin	Hollow fiber model	Poor efficacy	(Ferro, Srivastava, *et al.*, 2016b)
Linezolid Tigecycline	*In vivo* drosophila model	Tigecycline and linezolid was the most active combination, by means of prolonging the survival of infected flies	(Oh, Moon, Park, Kwon, & Jang, 2014)
Clarithromycin Imipenem	*In vivo* zebrafish model	Increased embryo survival in dose-dependent manner	(Bernut *et al.*, 2014)
Amikacin Cefoxitin Tigecycline Bedaquiline Clarithromycin	*In vivo* mouse model	Cefoxitin was the most active, by improving survival and reducing bacterial load; Bedaquiline was not active; Tigecycline showed bactericidal activity Triple drug combination including cefoxitin, amikacin and clarithromycin was active as cefoxitin alone	(Lerat *et al*., 2014)
Clarithromycin Clofazimine Bedaquiline	*In vivo* mouse model	Clofazimine in combination with Bedaquiline reduced the bacterial loads in various organs	(Obregón-Henao *et al.*, 2015)

Combined treatment	Clinical study Comparing *subs. abscessus* and *subs. massiliense*	Response rates were much higher with clarithromycin containing regimens, in the patients with *subs. massiliense* than *subs. abscessus,* where this less activity in *subs. abscessus* can be explained by inducible clarithromycin resistance	(Won-Jung Koh *et al.*, 2011; A.-L. Roux *et al.*, 2015)
Tigecycline	Clinical study Salvage treatment	Tigecycline is the useful addition to currently available antibiotics for the patients with difficult-to-treat infections	(Wallace et al., 2014)
Clarithromycin Amikacin Tigecycline	A retrospective study	In *M. abscessus* pulmonary infections, 15.7% mortality and 33.3% treatment failure were observed. This treatment failure was associated with the macrolide resistance.	(Sfeir *et al.*, 2018)
Imipenem/ cilastatin Amikacin	A case study with the history of *M. tuberculosis* and *M. abscessus* pulmonary infection	Survival with symptomatic improvements, no relapse	(Sugino *et al*., 2009)
Clarithromycin Rifampicin Ethambutol	A case study with *M. abscessus* infection for 10 years	Death	(Haverkamp *et al.*, 2012)
Surgical resection followed by amikacin Cefoxitin and clarithromycin	A case study with pulmonary sequestration	Survival with no relapse	(Won-Jung Koh, Hong, Kim, Ahn, & Han, 2012)
Cefoxitin Amikacin Clarithromycin Moxifloxacin	A retrospective study	Some patients had clinical and radiological improvement, while in some, no improvement was observed. One patient died and new pulmonary lesions were seen in five patients	(Duan *et al*., 2013)

Moreover, bedaquiline alone was shown to be active against this bacteria (Aguilar-Ayala *et al.*, 2017; Brown-Elliott & Wallace, 2019; Vesenbeckh *et al.*, 2017), but recently Lindman and Dick showed that the addition of bedaquiline to B-lactams may negatively affect the treatment outcomes (Lindman & Dick, 2019). Most recently, Ganapathy *et al.* mentioned about repositioning rifamycins, especially rifabutin against *M. abscessus* lung diseases (Ganapathy, Dartois, & Dick, 2019). Rifamycins are the only class to sterilize caseum and granulomas, where these bacteria reside. Rifabutin is the rifamycin not only active *in vitro* (Dinah Binte Aziz *et al.*, 2017; Le Run *et al.*, 2018; Pryjma *et al.*, 2018) but also *in vivo* in a macrophage infection model (Le Run *et al.*, 2018). Consistent with its higher potency, rifabutin accumulates inside macrophages at higher levels, defining its bactericidal activity against *M. abscessus* (Ganapathy *et al.*, 2019). Furthermore, rifabutin shows synergy with amikacin and cefoxitin, the most useful antibiotics in this bacterial infection. Rifabutin enhances the potency of amikacin by reducing induction of Eis2 and enhances cefoxitin activity by aiding its diffusion across cell wall barrier (Ganapathy *et al.*, 2019).

Besides new antibiotic treatment, alternative strategies have been proposed. Recently, the first use of bacteriophages for the treatment of a CF patient infected with *M. abscessus subs. Massilience* resulted in clinical improvement (Dedrick *et al.*, 2019). Of interest, a recent publication examining the interaction between spices and antibiotic resistance in *M. abscessus* showed the no growth of this bacteria on the spice enriched media (Millar & Moore, 2019). Several plant extracts from natural products have also shown antimicrobial activity against *M. abscessus*, for example nanoemulsion of *Cymbopogon flexuosus*. Several studies also mentioned bactericidal mechanism of action or good intracellular activity of some novel drugs/compounds like nitrogen heterocycles derivatives, diphenyleneiodonium chloride, indole-2-carboxamides, etc. against *M. abscessus* (Millar & Moore, 2019). Nitric oxide-donor modified from a natural biopolymer that releases nitric oxide spontaneously in solution (BIOC51) significantly reduced *M. abscessus* level *in vitro* (Banaschewski & Hofmann, 2019). In all cases, to know the efficient bactericidal concentration, it is necessary to understand the pharmacokinetics and pharmacodynamics of respective antibiotics in order to optimize their efficacy.

3. *Pharmacokinetics/Pharmacodynamics* of antibiotics: to bring new insights into the treatment of *M. abscessus* pulmonary infections

As previously discussed, pulmonary infections caused by *M. abscessus* are notoriously difficult to treat and no standard treatment is available. Thus, to understand the therapeutic efficacy or failure of antibiotics against these infections, it is necessary to understand the activity and distribution of antibiotics within lungs in accordance with lung pathophysiology. In other words, the therapeutic effectiveness of an antibiotic is related to lung physiology and physicochemical properties of antibiotics. In addition to this, to understand the control of free antibiotic concentration at infection site, the effectiveness of treatment and the concentration-effect relationship, it is important to understand pharmacokinetics and pharmacodynamics (PK/PD) of antibiotics. Different approaches can be used to understand PK/PD relationship of antibiotics, by which one can optimize the treatment and maximize the bactericidal effect with limiting the toxicities. This topic will lead to the understanding of lung pathophysiology, drug distribution in lungs, impact of route of administration and the general PK/PD of an antibiotic when administered alone and in combination.

3.1 Pulmonary drug delivery

The lungs are becoming an important portal for drug delivery in case of mycobacterial infection because of several limitations of oral and parenteral route of administration. The infection site which can be alveolar macrophages can be targeted by this drug delivery (Banaschewski & Hofmann, 2019; Misra *et al.*, 2011). To understand the importance of antibiotic inhalation, its distribution, efficacy and bioavailability at target site (Pham, Fattal, & Tsapis, 2015), it is necessary to first understand lung's pathophysiology.

3.1.1 Lungs and their pathophysiology

The lungs are very sensitive organ to the direct attacks of the external environment. Indeed, the lung has a unique situation in the body because it has an extremely developed vascular network. They are composed of airways and alveolar region, where airway region includes trachea, bronchi and bronchioles. The human bronchial tree is covered with epithelial cells. Being a protective coating to airways, mucus is involved in the mucociliary clearance. Importantly, defects in the structure and function of epithelium airways are responsible for many of lung disorders. The alveolar region consists alveolar ducts and alveolar sacs, showing the greatest importance in drug diffusion. The alveolar epithelium composed of pneumocytes type I and II. Type I pneumocytes are very thin (0.05 µm) and cover >90% of the alveolar surface while type II cells are small and compact. They produce lung surfactant and act as progenitors for type I

cells (Fernandes & Vanbever, 2009b). The pseudostratified large airways epithelium becomes ciliated, columnar and cuboidal in the small airways. Epithelial lining fluid (ELF) is a covering fluid of epithelium in lungs, which is presumed site of infections for pulmonary infections (Kuti & Nicolau, 2015).

In general, pulmonary infections affect one or more bronchopulmonary segments. Therefore, to treat pulmonary infections, aerosol delivery of an antibiotic is receiving a great interest as it could provide an administrative advantage achieving higher drug concentration at the site of infection and lower systemic exposure and toxicity.

3.1.2 Drug distribution in lungs

As explained above, to understand therapeutic effectiveness of an antibiotic in lungs, it is necessary to understand drug distribution in lungs alike the pathophysiology of lungs. In 1955, Amidon and co-workers established Biopharmaceutical drug classification system (BCS) in four categories based on drug dissolution and gastrointestinal permeability. The main goal was to predict *in vivo* pharmacokinetics of antibiotics based on importance of solubility and permeability on drug absorption (Amidon, Lennernäs, Shah, & Crison, 1995). The BCS is categorized into four classes as mentioned on Figure VII: Class I (High solubility, High permeability), Class II (Low solubility, High permeability), Class III (High solubility, Low permeability) & Class IV (Low solubility, low permeability).

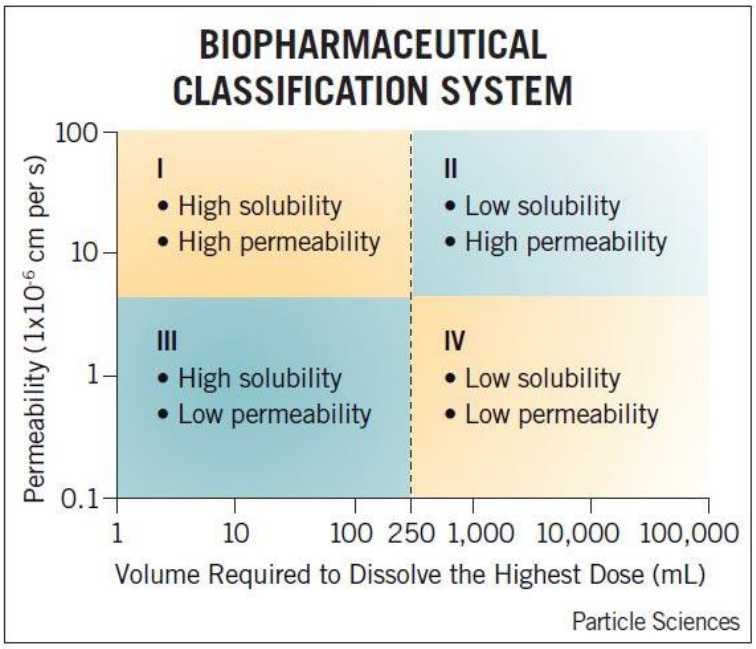

Figure VII: Biopharmaceutical classification system after oral drug delivery

- **pBCS for drug absorption in lungs:**

Biopharmaceutical classification was developed for an early prediction of oral drug absorption based on solubility and permeability, but no such class exists on drug behavior in lungs, only one paper is published explaining pulmonary BCS (pBCS) (Eixarch, Haltner-Ukomadu, Beisswenger, & Bock, 2010). To understand this behavior, lung physiology and the providence of inhaled antibiotics, their particle size, dissolution and permeability characteristics and their interplay with PK and PD should be understood (Hastedt *et al.,* 2016). The respiratory tract is currently considered as an alternative to gastrointestinal drug delivery system and useful to deliver drugs for pulmonary and non-pulmonary disease. Classically, aerosolized antibiotics are designed to treat lung disease, and typically not intended for systemic advantage (Hastedt *et al.*, 2016). Many antibiotics are delivered directly to the respiratory system and the main advantages of this delivery are reduced side effects and an immediate onset of action. It allows large surface area for drug absorption, high blood flow and absence of first pass metabolism being characteristic for the lungs (Fernandes & Vanbever, 2009b). However, lipophilicity and solubility of drugs may affect the penetration and its behavior in deep lungs.

The drug distribution in the lungs can be evaluated by several methods like, *in vitro, ex vivo* and *in vivo.*

- ***In vitro* study**

In vitro study of lungs involves lung epithelial cells and allow to study diffusion and transporter system, drug-drug interaction, transport mechanism and structure permeability relationship. Cell culture models are obtained from primary cells that are originated from continuous cell lines (Fernandes & Vanbever, 2009a). After among different types of cell lines like calu-3, A549, BEAS-2B, NCI-H441 cells, calu-3 is the most used cell model for drug transport studies (Bosquillon, Madlova, Patel, Clear, & Forbes, 2017). They are readily available, easy to culture and robust.

- ***Ex vivo* study**

Ex vivo study, which is also known as isolated perfused lung (IPL), is performed to evaluate pulmonary uptake and drug metabolism. Animal lungs are isolated and maintained at 37°C. Perfusion is done using buffer solution. Here, antibiotics can be administered using intratracheal route or by injection in perfusate solution and then drug absorption in the lungs without any interference of other organs is measured (Fernandes & Vanbever, 2009b). Several authors

already tested this model and evaluated IPL-*in vivo* correlation with *in vivo* rat lung absorption study (Tronde *et al.*, 2002). However, this method requires efficient surgical skills and has a short viability time of 2-3 hours for physiological conditions (Fernandes & Vanbever, 2009b).

Different techniques used to calculate drug concentration in lungs are mentioned in table X.

- ***In vivo* study**

Before delivering new drugs to human lungs, it is necessary to carry out the evaluation of that drug in animals. *In vivo* studies are generally performed in small rodents to predict drug distribution in the human lungs. Drugs can be administered by passive inhalation or directly into the lungs using liquid or powder form. Passive inhalation is the technique where aerosolized drugs are delivered using an aerosolization chamber in the whole body of an animal, generally mice. But this technique is associated with poor outcome reported by low amount of drug delivered to the lungs. Another technique is direct intratracheal administration of drugs with precise dose for administration directly in trachea, which is the precise technique with good success rate. Drug distribution is measured using concentration in ELF and plasma after pulmonary administration. Then pharmacokinetic profile of the antibiotic is compared with different administration (Fernandes & Vanbever, 2009b). ELF measurement is performed through BAL by injecting normal saline solution into the lungs and then retrieving. This BAL method can be useful in animals and humans both, but during the washing process, it may change the composition of ELF and during BAL realization, cellular lysis to macrophages may occur which may lead to release of cell content into ELF. So, it could bias the results. Antibiotic concentration measurement in lung tissue can also be done using lung resection, homogenization and then drug extraction using adequate solvents (Dhanani *et al.*, 2010). Microdialysis using a microdialysis probe is also one more technique for antibiotic concentration measurement in lung tissues (Marchand, Chauzy, Dahyot-Fizelier, & Couet, 2016). Lung microdialysis has been adopted for several antimicrobials such as cefaclor, imipenem, gatifloxacin and levofloxacin in animals and cefpirome, piperacillin, tazobactam, meropenem and levofloxacin in humans.

Table X: Different techniques to measure antimicrobial concentration in lungs
adapted from (Dhanani *et al.*, 2010)

Technique	**Biological sample**	**Invasiveness**	**Technical complexity**	**Advantage**	**Disadvantage**
Lung tissue homogenate	Tissue	Invasive	Low	Easy technique	Cannot measure different concentration in different compartments
Sputum	Sputum	Non-invasive	Low	Frequent sampling	Not representative of target site
BAL	Bronchial and alveolar lining fluid	Semi-invasive	Low	Easy sampling and closer to target site	Cell lysis alters ELF composition
ELF analysis	ELF	Invasive	Low	Easy sampling in mechanically ventilated patients	Invasive and dilution problems
Bronchial biopsy	Tissue	Invasive	Low	Provides local tissue levels	Average of different tissue compartments
Micro-dialysis	Interstitium liquid	Invasive	High	More frequent sampling over days	Cannot be used for lipid-soluble drugs or antimicrobials acting intracellularly
Ex vivo	IPL	Non-invasive	High	Easy sampling of perfusate and lavage fluid	Complex technique, and short viability time

To decide the best method to be used for evaluation of drug distribution in the lungs, several points should be noted: (1) the evaluation of intrapulmonary distribution of antibiotics *in vivo* is a difficult task, (2) the measurement of whole-tissue drug concentration is not recommended, (3) lung microdialysis is difficult for routine application and requires efficient skills. Considering all points discussed above, BAL is assumed to be the most common and relevant method for the investigation of *in vivo* distribution of antibiotics within lung, despite of having several limitations. In BAL method, urea is used as a dilution marker, and using urea concentration in BAL and plasma, the antibiotic concentration and their exposure in ELF and plasma are being calculated after IV and NEB administration.

However, extrapolation of results is not straightforward. The advantage of route of administration can be assessed using targeting advantage (TA) of NEB (Yapa *et al.*, 2014). TA provides an opportunity for quantitative assessment of the benefits of local delivery (NEB) over systemic (IV) administration, is calculated as below:

$$TA = \frac{(AUC_{ELF}/Dose)_{NEB}}{(AUC_{ELF}/Dose)_{IV}}$$

Moreover, PK behavior of an antibiotic is related with its permeability, meaning that high permeability compounds do not exhibit advantage of route of administration, showing TA < 100, while low permeability compounds generally show elevated ELF concentration after NEB than after IV administration, showing TA > 100. Biopharmaceutical characterization of several antibiotics after NEB, using BAL method is explained in Table XI. Based on these investigations, the potential advantage of NEB over IV administration for several antibiotics can be understood, though extra studies are needed to extrapolate these data in clinical settings.

Table XI: Biopharmaceutical characterization of several antibiotics after nebulization

(Galindo Bedor *et al.*, 2016; Gontijo, Brillault, *et al.*, 2014; Gontijo, Grégoire, *et al*., 2014; Marchand *et al.*, 2018, 2015; Marchand, Grégoire, *et al.*, 2016)

Antibiotics (class)	**Available as aerosols**	**TA**	
Ciprofloxacin (FQ)		1.2	**No impact of route of administration**
Levofloxacin (FQ)	Quinsair®	1	
Moxifloxacin (FQ)		0.95	
Linezolid (oxazolidinone)		0.80	
Thiamphenicol (phenicol)		7.0	
chloramphenicol (phenicol)		1.05	
Colistin (polymyxin)	Colimycin®, Colobreathe®	636	**High impact of route of administration**
Aztreonam (monobactam)	Cayston®	2761	
Oseltamivir carboxylate		576	
Tobramycin (aminoglycoside)	TOBI®, TOBI® Podhaler™	315	
Gentamycin (aminoglycoside)		162	

3.1.3 Inhaled antibiotics currently used or recommended for the treatment

In the previous topic, lack of efficacy of existing antibiotics and treatment failure has been explained. On the other hand, the potential advantage of several antibiotics using NEB has also been demonstrated. This discussion opens a new door to think about repurposing the existing antibiotics with different route of administration i.e. nebulization. Also, the use of inhaled antibiotics in combination with antibiotics such as, minocycline, linezolid, clofazimine and moxifloxacin has been recommended by British thoracic society (BTS) (Maselli, Keyt, & Restrepo, 2017), CFF and European Cystic Fibrosis Society (ECFS) (Floto *et al.*, 2016) for the treatment of NTM-PD in CF patients.

20 patients having bronchiectasis were treated with inhaled amikacin, with initial dose of 250 mg once daily, followed by 250 mg once per two weeks, up to 60 months for refractory NTM-PD (Maselli *et al.*, 2017) caused by *M. abscessus* and *MAC* (Olivier *et al.*, 2014). 25% were being treated with 45% improved symptomatology. This study highlighted the fact that with inhaled amikacin treatment outcomes may improvise but how to lessen the side effects is still challenging. Moreover, the activity of an inhaled formulation of liposomal amikacin in an *in vitro* and *in vivo* murine model of NTM infection was investigated by Rose *et al.*, where they reported that this formulation was efficient with less adverse effects (Rose, Neville, Gupta, &

Bermudez, 2014). Several case studies also suggest the use of inhaled amikacin for treatment of NTM-PD (Olivier *et al.*, 2017). Most recently, efficacy of inhaled liposomal ciprofloxacin has been studied (Bermudez, Blanchard, Hauck, & Gonda, 2015), where this formulation has found to be effective *in vitro* and *in vivo* by means of reduction in biofilm formation. But inhaled liposomal ciprofloxacin formulation was not effective in changing bacterial load in mice. Thus, further *in vivo* studies are necessary to include data regarding inhaled ciprofloxacin.

Apart from inhaled antibiotics, adjuvant treatments such as inhaled interferon-γ can also be effective against NTM-PD associated with functional interferon-γ deficiency, as in one study it resulted in rapid and sustained clearance of the organism from the airways and stabilization of lung function (Hallstrand, Ochs, Zhu, & Liles, 2004). One more interest has been developed of a novel inhaled nitric oxide gas formulation, Thiolanox® for the treatment of CF patients, which is in clinical trial phase II. This treatment significantly reduced *M. abscessus* and consequently reduced pulmonary inflammation and increased lung function during phase I clinical study (Millar & Moore, 2019). In conclusion, data are very limited regarding the use of inhaled antibiotics for the treatment of NTM-PD.

3.2 PK/PD of antibiotics

In general, PK is "how the body handles the antibiotic" and PD is "how the antibiotic affects the body". PK and PD attempt to relate the antibiotic interaction to a biological environment. These both represents the key components in the modern drug development. A basic understanding of drug absorption, distribution and elimination, and the relationship between kinetics and dynamics, and the underlying mathematics, is a fundamental aspect of PK/PD modeling.

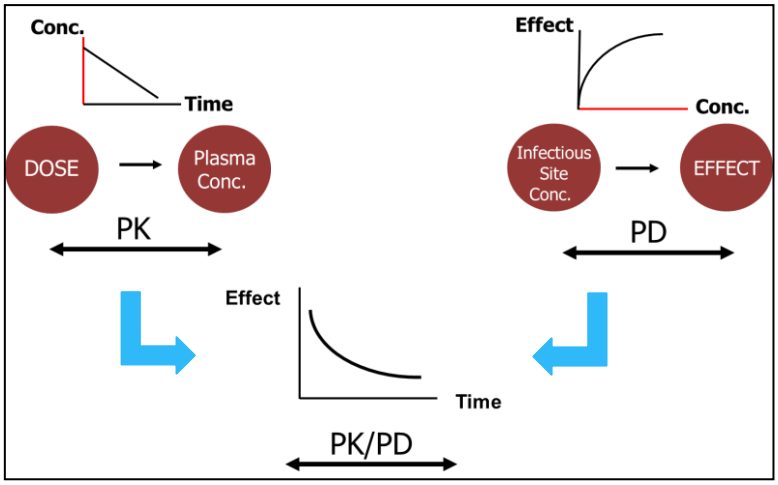

Figure VIII: Schematic presentation of the relationship between pharmacokinetics, pharmacodynamics and PK/PD (Derendorf & Meibohm, 1999)

3.2.1 Pharmacokinetic (PK) parameters of antibiotic

PK describes the link between the dose of the antibiotic and the change in the concentration (C) over time (t) in the body. In a simplest case, this concentration is estimated to decline from an initial concentration (C0) and can be determined using one-compartment PK model as shown in equation 1,

$$C(t) = C_0 \times e^{-k_e \times t} \quad (1)$$

Here, k_e represents elimination rate constant. The half-life can be determined using k_e, where $t_{1/2} = \frac{\ln(2)}{k_e}$. However, shape of the PK profile depends on the dose of the antibiotic, route of administration, and disposition of drug. The elimination rate constant can also be derived from apparent volume of distribution (V_d) and clearance (CL), using $k_e = CL/V_d$.

In some cases, because of more complex distribution and drug disposition, the concentration-time profile should better be described by two-compartmental model, considering central and peripheral compartment. The total antibiotic exposure is generally described as the area under the curve (AUC), the application of AUC is often denoted as Non-Compartmental Analysis (NCA). Many antibiotics bind to plasma proteins, which affects the drug disposition. Whereas, only unbound antibiotic concentration can distribute, eliminate and interact at target site.

- **Pharmacokinetics in animals**

Animal studies help to determine the exposure of the antibiotic at target site and characterize the PK/PD relationship. However, PK characteristics of animals and humans are not similar, and therefore the experimental data obtained from the animal studies cannot be extrapolated directly in humans. Also, presence of infections and host immune system can affect the PK characteristics. Several techniques to study PK characteristics in animals have already been discussed earlier.

3.2.2 Pharmacodynamic (PD) parameters of antibiotic

PD describes the relationship between antibiotic concentration and effect at target site. The effect variable (E) at given time (t) is measured by a function of its value without antibiotic (E_0) and the antibiotic concentration (C). This PK/PD relationship can be described by the sigmoidal E_{max} model as shown in equation 2,

$$E(t) = E_0 + \frac{E_{max} \times C(t)^{\gamma}}{EC_{50}^{\gamma} + C(t)^{\gamma}} \quad (2)$$

Where, E_{max} is the maximum effect achieved by the antibiotic, EC_{50} is the antibiotic concentration at half of the maximum effect can be achieved and γ is the Hill factor, which determines the steepness. E_{max} is the one of the most popular PD models. This model depends on the effect of an antibiotic on the bacteria that means either the antibiotic can inhibit the bacterial growth or stimulate the bacterial killing in a system.

3.2.2.1 Minimum Inhibitory concentration

For many years, minimum inhibitory concentration (MIC) has played a key role for determining bacterial susceptibility to an antibiotic. It is a simple method. In general, broth dilution method is used to determine MIC of an antibiotic by exposing a bacterial inoculum of 5 x 10^5 CFU/mL to a range of the antibiotic concentrations based on two-fold dilution, over an incubation period of 16-20 h (Nielsen & Friberg, 2013). The MIC is then defined as the lowest concentration of antibiotic that under these conditions, inhibits any visible culture of the bacterial strain. The MIC can also be evaluated using agar diffusion and E-test method. The E-test method is much simpler than broth dilution method, but it can only be used against the antibiotics that are supplied by the E-test strip manufacturers.

The MIC is measured as an average effect of a growth and bacterial killing induced by the antibiotic over the time, also is relatively simple parameter to be determined. However, this approach has one limitation that it is a static parameter evaluated only at a given moment and that does not therefore make it possible to characterize the effectiveness of an antibiotic when its concentrations change over time and even MIC doesn't reflect the initial decrease and then regrowth over time.

3.2.2.2 PK/PD indices

The PK/PD index approach has become the gold standard for evaluating PK/PD of antibiotics, and also using this approach dosing regimens can be optimized. The study of the PK/PD relationships of antibiotics led to the definition of three indices based on the MIC of the bacterial strain and three different PK parameters (Mouton & Vinks, 2005). These indices include *f*AUC/MIC, fC_{max}/MIC and $fT_{>MIC}$. The prefix, *f*, indicates free fraction of an antibiotic used. Using these indices, it is also possible to distinguish time-dependent or concentration-dependent antibiotics. For time-dependent antibiotics such as, β-lactams, the effectiveness of these antibiotics is related with the time during which free antibiotic concentrations are greater than MIC ($fT_{>MIC}$), and for concentration-dependent antibiotics, such as aminoglycosides, effectiveness depends on the maximum free concentration as a function of time on MIC (fC_{max}/MIC). The ratio of area under the curve of free concentrations as a function of time on MIC can be described by PK/PD index, *f*AUC/MIC (Barger, Fuhst, & Wiedemann, 2003). Therefore, in order to optimize the dosing regimen, one can conclude that if the antibiotic is time-dependent, the strategy should to maintain free antibiotic concentration above MIC for as long as possible, whereas, if the antibiotic is concentration-dependent, the strategy should be to achieve maximum free concentration sufficiently high compared to MIC (Nielsen & Friberg, 2013).

In general, determination of the PK/PD correlated with antimicrobial efficacy is often based on animal models. Typically, mice are infected with some bacterial inoculum in the thigh or lung, following to one or more antibiotic treatment. Antibiotic concentrations are determined using collected blood samples. An approach of plotting a CFU variation over time (generally, CFU are counted at a given time, e.g. 24 h) versus the magnitude of these PK/PD indices, is used to determine the best PK/PD index for several antibiotic-bacteria combination. This index can be determined by fitting a sigmoidal E_{max} model (Equation 3). The index for which the coefficient of determination (r^2) is the highest is then selected.

$$E = E_0 - \frac{PD_{max} \times X^{\gamma}}{EX_{50}^{\gamma} + X^{\gamma}} \quad (2)$$

Where E is the bacterial density at a given time (expressed as log10 CFU / mL), E0 is the bacterial density at this time in the absence of antibiotic, X is one of the 3 PK / PD indices defined above, PD_{max} represents the maximum effect obtained when the increase in exposure no longer leads to bacterial destruction, EX_{50} is the value of X necessary to reach 50% of PD_{max} and γ is the sigmoidicity parameter.

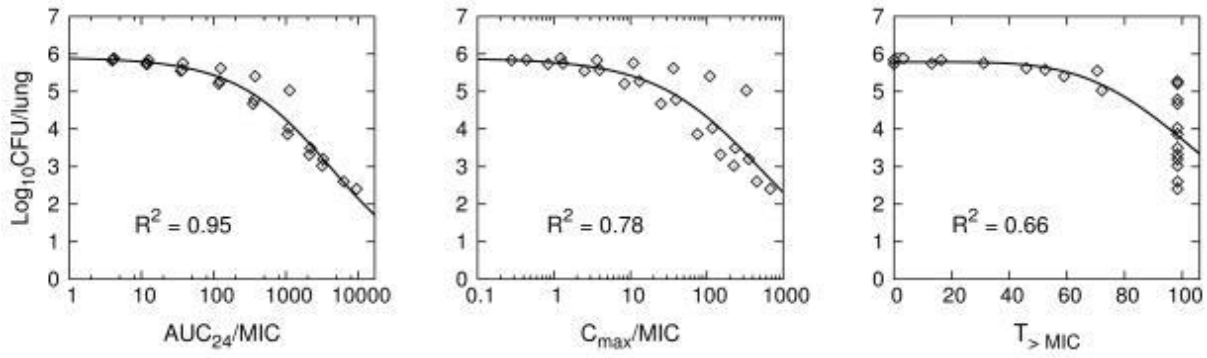

Figure IX: Illustration of relationship between PK/PD indices and $\log_{10}$ CFU in lungs of *M. tuberculosis* infected BALB/c mice after 3 days of treatment with rifampicin

Individual data points (◊) were generated using PK/PD model, with each time point obtained from the simulation of total lung CFU. Corresponding PK/PD indices were calculated from simulated plasma concentrations (Lyons & Lenaerts, 2015).

Figure VIII illustrates the relation between PK/PD indices and the number of *M. tuberculosis* in the lungs of mice. However, none of indices fit perfect with observations. The shape of the concentration-time profile can be different based on the target tissue compared with plasma. Despite of being recommended by regulatory agencies that PK/PD indices play important role in determining the optimal dosing regimen, this approach has several limitations. All indices rely on MIC value and so that it is assumed that MIC remains constant throughout the treatment period. It can also be said that best fit PK/PD index can depend on the half-life of the antibiotic (Nielsen, Cars, & Friberg, 2011) and this is the reason why PK/PD index differs among population with different elimination capacity. Furthermore, if bacteria are exposed to insufficient antibiotic concentration, they are likely to adapt and develop resistance, which may result in a regrowth after initial decay suggesting a variation of the efficacy over time (Nielsen & Friberg, 2013).

3.2.3 *In vitro* data interpretation using PK/PD modeling

In vitro experiments are generally easy to perform, allow to study the full effective concentration range and also helps to characterize PK/PD relationship. However, modeling can be used to optimize *in vivo* and *in vitro* studies and thereby, to minimize the use of animal experiments and clinical trials. But *in vitro* studies have one disadvantage that the situations for bacterial growth and killing are not similar between *in vitro* and *in vivo* studies.

Despite of using *in vitro* system, PK profile simulated for the respective antibiotic can be evaluated by calculating antibiotic concentrations using validated analytical method. And after, more accurate antibiotic exposure characterization can be determined using PK model. All these can then be modeled using PK/PD modelling approach.

3.2.3.1 PK/PD of single antibiotic

In vitro studies for single antibiotic include MIC determination or time-kill experiments to determine the bacterial count over time. The drug exposure in such experiments may be static or it can mimic the dynamic change in concentration-time profile of the antibiotic.

A PK/PD model can be developed from *in vitro* study data characterizing a submodel either considering bacterial growth inhibition with natural killing or simulating bacterial death (PD model), a submodel demonstrating concentration versus time profile (PK model), or a submodel characterizing full PK and PD model (PK/PD model).

A. PD model

In this section, different ways to include antibiotic effect and their assumptions are being illustrated considering various types of resistance like presence of heterogenous populations.

The simplest model involves a single bacterial compartment *(B)* following first order-rate for bacterial growth *(k_{growth})* and death *(k_{death})*. The variation in bacterial count over time observed during time-kill experiments can be explained as below:

$$\frac{dB}{dt} = k_{growth} \times B - k_{death} \times B \qquad (3)$$

In case of control experiments (i.e. no exposure to antibiotic), the net result of bacterial growth rate and death can be explained by, $k_{net} = k_{growth} - k_{death}$. The mean generation time (MGT), the doubling time of bacterial culture is generally calculated using net growth rate, $MGT = \frac{\ln(2)}{k_{net}}$. In

absence of antibiotics, bacteria are assumed to grow until reaching a plateau, and this can be described by a logistic growth function (Mouton & Vinks, 2005) according to,

$$\frac{dB}{dt} = k_{net} \times \left(1 - \frac{B}{B_{max}}\right) \times B \quad (4)$$

Where, B_{max} is the maximum bacterial count reached the plateau.

B. PK model

The change in antibiotic concentration over time within the experimental period can be described using PK model. In general, during time-kill experiments, the concentration of antibiotic is expected to be constant. However, some antibiotics such as β-lactams degrade under *in vitro* experimental conditions, which results in simultaneous decrease of antibiotic concentration over time. This degradation is often expected to follow zero- (Eq. 5) or first-order process (Eq. 6) according to their degradation rate constants (k_{deg}).

$$\frac{dC}{dt} = -k_{deg} \quad (5)$$

$$\frac{dC}{dt} = -k_{deg} \times C \quad (6)$$

In dynamic experimental setup, the decrease in the concentration of an antibiotic over time can be simulated using first-order elimination rate constant (k_e) as below:

$$\frac{dC}{dt} = -k_e \times C \quad (7)$$

C. PK/PD model

The bacterial submodel and PK model are combined to drive the full PK/PD model and equations are introduced to characterize the antibiotic effect on bacteria. In general, antibiotic effect is modeled using an E_{max} or a sigmoidal E_{max} model (Eq. 2). The antibiotic effect can be assumed to either inhibit the bacterial growth (Eq. 8) or to simulate the bacterial kill (Eq. 9 and 10). The effect can be modelled as proportional (Eq. 9) or additive (Eq. 10).

$$\frac{dB}{dt} = k_{growth} \times \left(1 - \frac{E_{max} \times C_{(t)}^{\gamma}}{EC_{50}^{\gamma} + C_{(t)}^{\gamma}}\right) \times B - k_{death} \times B \quad (8)$$

$$\frac{dB}{dt} = k_{growth} \times B - k_{death} \times \left(1 + \frac{E_{max} \times C_{(t)}^{\gamma}}{EC_{50}^{\gamma} + C_{(t)}^{\gamma}}\right) \times B \quad (9)$$

$$\frac{dB}{dt} = k_{growth} \times B - k_{death} \times B - \left(\frac{E_{max} \times C_{(t)}^{\gamma}}{EC_{50}^{\gamma} + C_{(t)}^{\gamma}}\right) \times B \quad (10)$$

In a proportional effect model, the effect is estimated as a fractional increase in bacterial death rate, whereas in an additive effect model, the effect is the bacterial kill rate constant imposed by the antibiotic treatment.

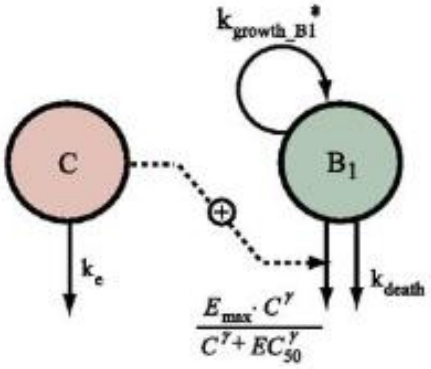

Figure X: Schematic illustration of a PK/PD model, with an antibiotic assumed to enhance bacterial kill rate

*The logistic growth was estimated according to $k_{growth} \times \left(1 - \frac{B}{B_{max}}\right)$. C represents antibiotic compartment, B_1 is the bacterial compartment, k_e represents elimination rate following first order process. k_{growth} and k_{death} are rate constants for bacterial growth and death, respectively. E_{max} is the maximum achievable effect, EC_{50} is the antibiotic concentration producing 50% E_{max} and γ is the sigmoidity factor (Nielsen & Friberg, 2013).

PK/PD models can also include various resistance mechanisms. If the mechanism of resistance is known, the appropriate modelling structure can be chosen. In case of pre-existence of two bacterial subpopulations, two different bacterial compartments representing antibiotic-sensitive and antibiotic-resistant population, with two different antibiotic susceptibilities should be assumed. In general, the dynamics for the emergence of resistance can be described by mathematical PK/PD models and thereby these models can also help to design the dosing regimens by which development of resistance can be minimized. In addition to this, the presence of persistence bacteria and adaptive resistance also can be modelled using such type of PK/PD models.

3.2.3.2 PK/PD of antibiotics in combination

Semi-mechanistic PK/PD modelling can be used for such studies considering concentration patterns as a function of time and their interaction on the bacterial growth and death over time. The interaction data can be used to develop a PK/PD model and study the effects of different dosing regimens. In case of combinations, PK/PD relationship can be described in various ways. For example, two antibiotics have different mode of actions in which one is acting on inhibition bacterial growth while other is stimulating bacterial kill or both antibiotics have identical mode of actions.

A. Checkerboard and time-kill kinetics analysis

As the prevalence of multidrug resistance is on rise, the use of drug combinations is growing to increase the therapeutic advantage of existing antibiotics. To determine the efficacy of combined antibiotics, checkerboard and time-kill kinetics analysis are the most useful approaches. However, time-kill experiments for combinations can be tiresome as drug administration sequence, dose range, dosing interval, drug-drug interactions should be investigated. But in such cases, dynamic systems like hollow fiber model allow to investigate difference in PK properties of both antibiotics including different half-lives, elimination rate etc. Synergy, additivity and antagonism are the main terms used to describe interactions between antibiotics. When the combined effect is greater than the effect observed with single antibiotic, the effect is synergistic, whereas if the combined effect is less than the observed effect with single antibiotic, it is called antagonism. However, this interpretation is not so simple as several definitions of synergy exists.

The synergy between two antibiotics is often determined using checkerboard method, where each antibiotic is combined in 96-well plates and then bacterial inoculum is added following appropriate incubation. At last the turbidity of each well is assessed visually. Then the interpretation of results can be done using isobologram or calculating fractional inhibitory concentration index (FICi). FICi can be calculated by comparing MIC of each antibiotic alone (MIC_A and MIC_B) and in combination ($MIC_{A/B}$ and $MIC_{B/A}$) as below:

$$FICi = \frac{MIC_{A/B}}{MIC_A} + \frac{MIC_{B/A}}{MIC_B} \quad (3)$$

The FIC index of ≤ 0.5 is then interpreted as synergy, $0.5 < FIC < 4.0$ as additive and ≥ 4 as antagonism (Hsieh, Yu, Yu, & Chow, 1993; Sopirala *et al.,* 2010). Although the checkerboard

technique and the determination of the FIC index seem simple, completely different conclusions can be made regarding the nature of the interaction between two antibiotics.

The similar study for combined antibiotics can also be done using time-kill experiments to study the variation in bacterial count over time using appropriate concentration of each antibiotic and drug-drug interactions can be studied. However, such experiments take longer and usually, only a small number of combinations can be tested.

B. A less-model dependent approach

GG Rao *et al.* (Rao, Li, Garonzik, Nation, & Forrest, 2018) developed a new and easy to perform approach to analyse the experimental data to quantify variation in bacterial load (CFU) over time. This method also allows to interpret the initial experimental data to be modelled. For each combination (including control), the total bacterial exposure using area under the curve for CFU (AUCFU) is calculated for the period of the experiment and then the log ratio (LR) of AUCFU of the antibiotic combination compared to control is calculated using:

$$LR = log_{10}\left(\frac{AUCFU_{combination}}{AUCFU_{control}}\right) \quad (8)$$

Here, log ratio is the logs of area under curve, not the CFU. Hence, an LR of "static" regimen compared to control is calculated. LR of -1 or -2 indicates the reduced exposure of combinations compared to control, LR of 0 indicates no reduction in CFU compared to control, while LR of -2 to -4 are required to be equivalent to a "static" regimen.

This approach is very useful in studying drug combinations, and then the next approach of using mechanistic modelling can be used to study mechanisms of these interactions.

C. Interaction models

In case of mechanistic modelling different approaches can be applied. For example, when antibiotics are used in combinations, the assumptions are based on the hypothesis that either one antibiotic stimulates bacterial death and another inhibits bacterial growth, or both antibiotics stimulate bacterial death or both antibiotics inhibit bacterial growth. In a simplest case, it can be assumed that antibiotic A does not affect the efficacy of antibiotic B and Vice versa, which means that the effect of both antibiotics is independent. There are different ways to determine bacterial kill due to each associated antibiotic to achieve the combined antibacterial effect. All this interaction models can be used alone or in combination with PK model to characterize the PK/PD relationship against various bacterial strains. There are several

interaction models, which are used to characterize PD interactions between antimicrobial agents in combination. An ideal approach to develop a model would include the full time-course of effect and accommodate those effects within subpopulations and PD differences between antibiotics.

In general, a basic interaction model is simply to add the effect of each antibiotic used in combination:

$$E_{AB} = E_A + E_B \tag{9}$$

Where, E_A and E_B are the effects of antibiotic A and B, respectively. E_{AB} is the effect observed for the combination of both antibiotics A and B. This approach can also be described as **Linear interaction effect or Response additivity model** (Foucquier & Guedj, 2015). The corresponding combination index (CI) can be calculated using:

$$CI = \frac{E_A + E_B}{E_{AB}} \tag{10}$$

Using this combination index, corresponding effect of antibiotics in combination can be expected. When the observed effect (E_{AB}) is greater than expected (the sum of the effects observed with both antibiotics alone, $E_A + E_B$), the interaction index will be less than unity and the combination is referred as "synergistic". Vice versa, if the observed effect is less than expected, the interaction index will be greater than one and the combinations are classified as "antagonistic" (Figure X). Two reference models have widely been used to describe the joint activity of two antibiotics: The Bliss Independence model (Bliss, 1939) and the Loewe additivity model (Loewe, 1953). Both models use different assumptions for synergy interpretation.

(A) Additivity (B) Bliss Independence

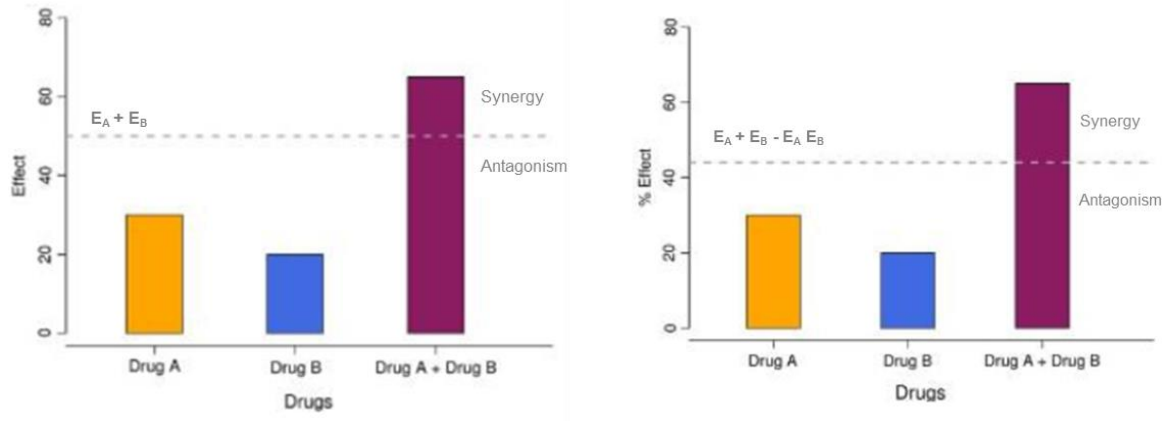

Figure XI: Illustration of Response Additivity and Bliss Independence model Based on $E_A = 30$, $E_B = 20$ and $E_{AB} = 65$ (Foucquier & Guedj, 2015).

Bliss independence model (Bliss, 1939) is based on the assumption that both antibiotics act independently, meaning that they have different mechanism of action in a way that they do not interfere with each other, but contribute to a common result as calculated using equation 10 and then resulting combination index can be calculated using equation 11:

$$E_{AB} = E_A + E_B - E_A \times E_B \quad (10)$$

$$CI = \frac{E_A + E_B - E_{AB}}{E_{AB}} \quad (11)$$

Where, $0 \leq E_A \leq 1$ and $0 \leq E_B \leq 1$ meaning that the individual and combined effects are expressed as probabilities and therefore considered between 0 and 1 (Figure X).

Loewe additivity model uses the probabilistic theory that the antibiotics used in combination act using identical mechanism of action. This is a concentration (dose)-based approach, in contrast to the Bliss independence model which is an effect-based approach. In other words, based on the Bliss independence, "the effects of antibiotics used in combination are additive", whereas based on Loewe additivity, "doses are additive" (Rao *et al.*, 2018). Loewe additivity can be used in case of similar E_{max}, E_0 and H, but different drug susceptibilities (EC_{50}). Loewe additivity relies on dose equivalence and the sham combination principle. Dose equivalence principle is based on the hypothesis that to produce an effect E, dose a of antibiotic A is equivalent to dose b_a of antibiotic B and vice versa. The shame combination principle assumes

that the dose b_a of antibiotic B can be added to any other dose b of antibiotic B to produce the additive effect of the combination. According to this approach, the additive effect of both antibiotics depends on the individual concentration-effect curves of each antibiotic and it can be expressed as:

$$E_{AB} = Effect_{(a+b)} = E_A(a + a_b) = E_B(b_a + b) \quad (12)$$

Where, $(a + a_b)$ refers to the concentration of antibiotic A and $(b + b_a)$ refers to the concentration of antibiotic B producing the same effect E_{AB}. The assumption made by this model is that the antibiotics have constant potency ratio $\left(R = \frac{A}{B}\right)$, hence, concentration-effect curves are parallel on a log-dose scale and have equal individual maximum effects (Foucquier & Guedj, 2015). Therefore, the combination index to evaluate the interaction between antibiotics using Loewe additivity assumption can be expressed as:

$$CI = \frac{a}{A} + \frac{b}{B} \quad (13)$$

As explained earlier, if CI=1, the interaction is additive. If CI<1, the concentrations a and b producing some effect in combination are lower than expected, the interaction considered as "synergistic" and on the opposite side, if CI>1, the interaction id predicted to be described as "antagonistic".

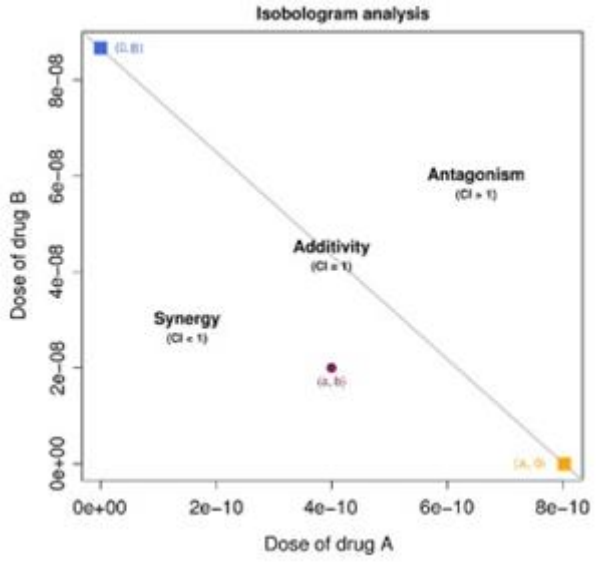

Figure XII: Illustration of Loewe additivity

Isobologram analysis for the combination effect E. The individual concentrations A_E and B_E are used to draw the line of additivity. The localization of experimental concentrations (a, b) needed for the combination effect with respect to the line of additivity can be translated in to the respective additive, synergistic or antagonistic effect (Foucquier & Guedj, 2015).

Loewe Additivity model enables the isobologram analysis. As an example, to produce an effect E using combination of concentration a of antibiotic A and concentration b of antibiotic B, in case of CI = 1, the equation, $\frac{a}{A} + \frac{b}{B} = 1$, determines that all concentrations of antibiotics A and B which produce combination effect $E_{AB,}$ that can be drawn as an additive isobole (Figure XII). Here, by localizing the experimental points of (a, b) corresponding to the concentration to produce E_{AB}, the respective additive, synergistic or antagonistic effects can be determined.

However, both models have several limitations: (1) Loewe model cannot accommodate antibiotics with very different E_{max} or H values. This model specially depends on the dose-effect relationship to calculate effective doses for a given effect and if dose-effect curve is not available or difficult to model, this model becomes unusable (Foucquier & Guedj, 2015)

(2) Using Bliss independence model, sometimes it is difficult to verify the hypothesis of independence of mechanism of actions as the search for synergistic combination often involves molecules with complex or unknown mechanism of actions. Also, Bliss independence model applied only to the effects expressed as probabilities between 0 and 1.

The major difference between Bliss independence and Loewe additivity is based on the definition of “no interaction” and “additivity” between two antibiotics when used in combination (Rao *et al.*, 2018).

OBJECTIVE OF THE STUDY

M. abscessus is a dreadful and arduous to treat mycobacterial pathogen with a high level of innate resistance to most commercially available antibiotics, including the antituberculous agents. A number of studies focusing on *in vitro* and *in vivo* treatment have demonstrated major role of intravenous cefoxitin and amikacin in the efficient treatment outcomes (Czaja *et al.*, 2014; Dubée, Bernut, *et al.*, 2015; Greendyke & Byrd, 2008; Jeon *et al.*, 2009; Lavollay *et al.*, 2014; Lefebvre *et al.*, 2016), however, systemic side effects and resistance are major limitations. The main objective of this study was to draw an attention towards re-use of existing antimicrobials such as cefoxitin and amikacin, with pulmonary route of administration and PK/PD modeling to rationalize the *M. abscessus* treatment. And then find a replacement to the third antibiotic macrolides leading to drug resistance and treatment failure. This PhD has been developed in two complementary axes:

1. Pharmacokinetics of cefoxitin and amikacin has been studied in order to characterize their efficacy after NEB. Consequently, the potential for pulmonary administration of both molecules has been evaluated. However, β-lactams are known for their limited *in vitro* stability, therefore this problem was considered to evaluate by studying its *in vitro* PD alone and in combination. Then, PK/PD type modeling approach was adapted for cefoxitin considering its *in vitro* degradation over time.

2. In addition to this, combination of two (amikacin combined with cefoxitin) and then, by adding a third antimycobacterial agent using *in vitro* experiments has never been evaluated in depth. Macrolide is generally prescribed after treatment completion using IV amikacin or cefoxitin, but in this situations macrolide resistance is the biggest challenge as 80% *M. abscessus* strains are macrolide resistance (David E. Griffith et al., 2007; Nessar et al., 2012; van Ingen, Ferro, Hoefsloot, Boeree, & van Soolingen, 2013). So, in such situation, several questions arise:

- In the ATS recommended gold standard therapy, is it a good idea to include nebulized cefoxitin and amikacin alone or in combination?
- Will it be possible to lessen the side effects, fight against resistance and improve the treatment outcomes by NEB?
- Does triple-combinations are more effective than bi-combinations or monotherapy?

In order to reevaluate the use of cefoxitin and amikacin via pulmonary administration for local pulmonary infections, and to understand drug distribution after NEB and IV administration, PK of cefoxitin and amikacin were studied in healthy rats. Due to the lab expertise, the BAL technique was the preferred method to measure the drug alveolar content. In addition to this, *in vitro* efficacy of cefoxitin, amikacin and several other molecules were studies alone and in combination for the treatment of infections caused by *M. abscessus* and then these results were evaluated using PK/PD type of modeling approach.

The scheme of this thesis work is reported on figure XIII.

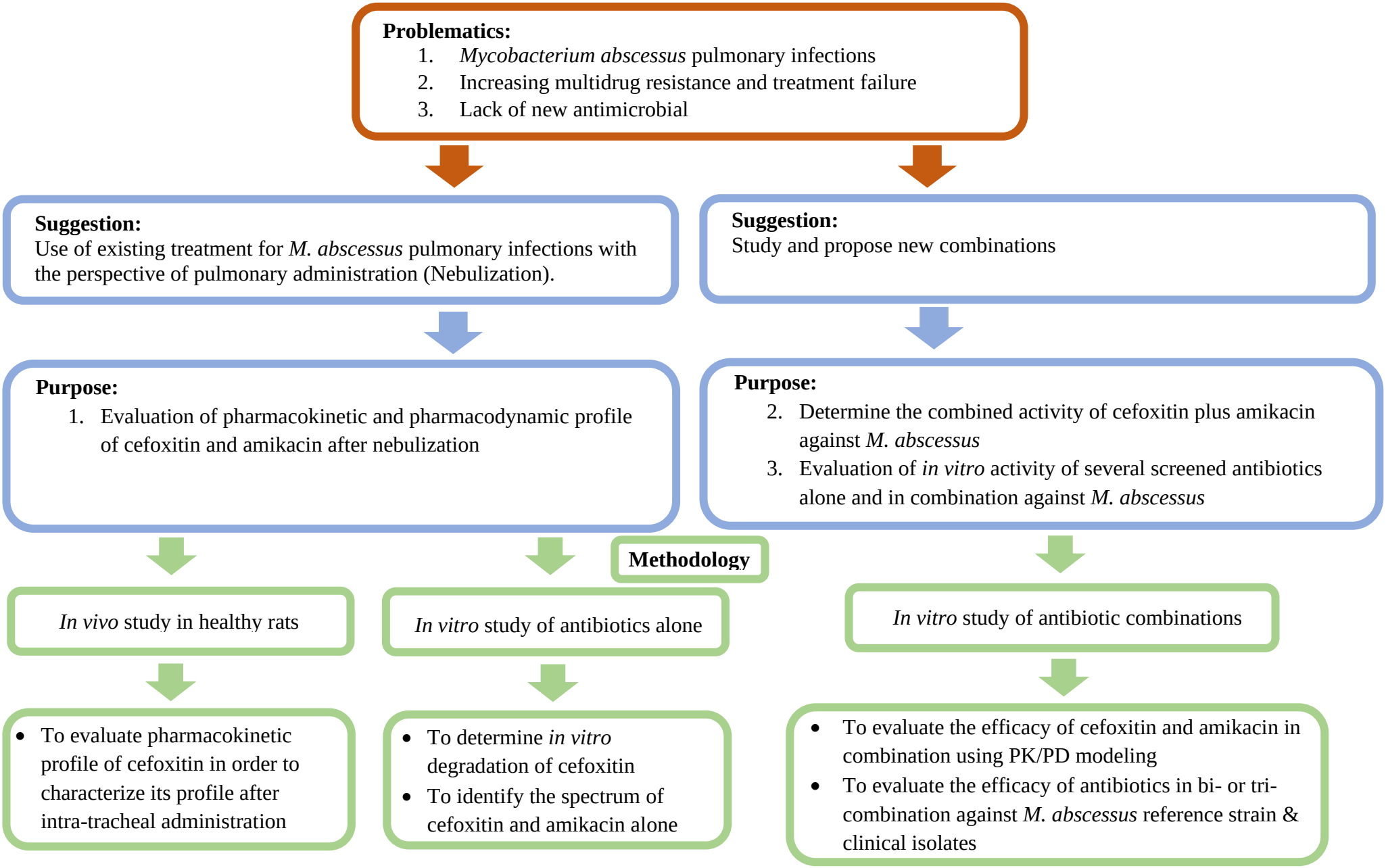

Figure XIII: *Scheme of Research*

EXPERIMENTAL WORK

The PhD experimental work was mainly divided into two axes:

4.1 PK/PD and nebulization as a potent new insight for treatment of *M. abscessus*

4.2 Antibiotic combinations

4.1 PK/PD and nebulization as a potent new insight for treatment of *M. abscessus*

Article 1: Biopharmaceutical Characterization of Nebulized Antimicrobial Agents in Rats: 6. Aminoglycosides.

Résume en français :

Les comportements pharmacocinétiques de l'amikacine et de la gentamicine après la nébulisation ont été déterminés en comparant les concentrations dans le plasma et le liquide épithélium pulmonaire (ELF) chez le rat après administration intratrachéale et intraveineuse. Les AUCs pour l'ELF étaient 874 et 162 fois plus élevées après nébulisation qu'après administration intraveineuse pour l'amikacine et la gentamicine, respectivement. Même si les deux molécules semblent être de bonnes candidates à la nébulisation, ces résultats démontrent un « targetting advantage » de la nébulisation beaucoup plus importante pour l'amikacine que pour la gentamicine.

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PHARMACOLOGY

Biopharmaceutical Characterization of Nebulized Antimicrobial Agents in Rats: 6. Aminoglycosides

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ABSTRACT Amikacin and gentamicin pharmacokinetic behaviors after nebulization were determined by comparing plasma and pulmonary epithelial lining fluid (ELF) concentrations in rats after intratracheal and intravenous administrations. ELF areas under concentration-time curve were 874 and 162 times higher after nebulization than after intravenous administration for amikacin and gentamicin, respectively. Even if both molecules appear to be good candidates for nebulization, these results demonstrate a much higher targeting advantage of nebulization for amikacin than for gentamicin.

KEYWORDS aminoglycosides, biopharmaceutics, nebulization

Respiratory infections in mechanically ventilated patients are frequent (1). Although parenteral administration of aminoglycosides constitutes the standard route of administration, nebulization (NEB) is being used more and more frequently, and tobramycin (TOB) and amikacin (AMK) are the most commonly prescribed agents for NEB (1). Compared with fluoroquinolones, which present high membrane permeability (2), the difficulty of aminoglycosides to permeate membranes may allow for high intrapulmonary concentrations to be obtained by patients after NEB. This was observed in rats, with a ratio of TOB area under the epithelial lining fluid (ELF) concentration-time curve (AUC_{ELF}) over plasma AUC of 222 after NEB (3). The aim of this new study was to extend this finding to two other aminoglycoside antibiotics, AMK and gentamicin (GEN), using the same standardized protocol (3).

GEN and AMK for parenteral administration were used for all experiments (GEN sulfate Panpharma solution 80 mg · ml^{-1}, expressed in base, and AMK Mylan 500 mg powder). Animal experiments were conducted in compliance with European Community directive 2010/63/EU after approval by the local ethics committee (COMETHEA) and were registered by the French Ministry of Higher Education and Research under authorization number 2015070211159865. Male Sprague Dawley rats ($n = 49$ for GEN and 40 for AMK; mean weight, 300 g) from Janvier Laboratories (Le Genest-St-Isle, France) were used for the experiments. As previously described, they were divided in two groups corresponding to the route of administration for each molecule (intravenous [i.v.] or NEB) (3, 4). GEN and AMK were administered under anesthesia by i.v. bolus in the tail vein (1 ml) or by intratracheal NEB (100 μl) with a microsprayer 1A-1B (Penn-Century, Wyndmoor, PA) at doses commonly used in clinical practice, after correction for body weight, and equal to 8 and 30 mg · kg^{-1}, respectively. Bronchoalveolar lavage (BAL) fluid and blood sampling were performed until 4 h after administration (4 or 5 sampling times [0.5, 1, 2, 3, 4 h] and 4 to 7 rats per sampling time). Assays

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were conducted by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The mass spectrometer was operated in the positive mode, and ions were analyzed by multiple-reaction monitoring. An Alliance Waters 2695 system coupled with a Quattro micro API tandem mass spectrometer (Waters SAS, Saint Quentin en Yvelines, France) was used to perform GEN analysis in plasma and BAL fluid, using a previously described method with limited modifications (5). Determination of AMK concentrations in plasma and BAL fluid was performed using a method previously developed by KCAS Bioanalytical and Biomarker Services (Shawnee, Kansas). The system was composed of a Shimadzu LC module (Nexera XR; Shimadzu France, Marne la Vallée, France) coupled with an API 3000 mass spectrometer (Sciex; Life Sciences, Les Ulis, France). For both molecules, the same concentrations were used for BAL fluid and plasma standard curves (0.005 to 2 $\mu g \cdot ml^{-1}$); BAL fluid samples were injected directly, whereas plasma samples were deproteinized with trichloroacetic acid 10%. Intra- and interday variabilities were evaluated at four levels of concentration (limit of quantification [LOQ], 2 or 3 times the LOQ, an intermediate concentration in the standard curve, and 75% of the highest standard curve concentration). Precision and accuracy of <15% was tolerated, with the exception of 20% at the LOQ. Urea concentrations in plasma and BAL fluid were measured as previously described (4, 6). ELF antibiotic concentrations were calculated from the measured BAL fluid concentration after correction for dilution by the urea method (4, 6). For each compound, plasma and ELF concentrations versus time were analyzed simultaneously after i.v. and NEB administration with the nonlinear mixed-effect method using S-ADAPT software (7). Plasma protein binding of AMK and GEN was assumed to be negligible. The final structural pharmacokinetic model was the same for both molecules, i.e., a central compartment from which drugs were eliminated and two ELF compartments connected by two distribution clearances, one between plasma and the first ELF compartment and the other between the two ELF compartments. In accordance with previous observations with the Penn-Century microsprayer, complete bioavailability after NEB was hypothesized and was fixed to 100% (3, 8). Areas under plasma and ELF concentration-time curves from time zero to infinity (AUC_{plasma}, AUC_{ELF}) and elimination half-lives were estimated from the model (Berkeley Madonna, version 8.3.18, University of California). To compare pulmonary drug exposure after NEB versus after i.v. administration, the targeting advantage of NEB was estimated by the ratio of AUC_{ELF} after NEB to AUC_{ELF} after i.v. corrected for doses (9).

After i.v. bolus, typical plasma elimination half-lives of AMK and GEN were 0.40 and 0.49 h, respectively. Half-lives in ELF were at least 3 times longer than in plasma (2.1 and 1.6 h, respectively), suggesting that elimination from ELF is distribution-rate limited for both molecules (Fig. 1). Because complete bioavailability after NEB was hypothesized, AMK and GEN plasma concentrations were virtually similar regardless of the route of administration. Data were also in accordance with passive diffusion between plasma and ELF, since the ratios of ELF to plasma AUCs after i.v. administration were estimated to be 1.0 for both molecules. After NEB, the maximal plasma concentration (0.5 h) was observed at the first time of sampling (Fig. 1), and the ratio of mean ELF to plasma concentration was ~5 times higher for AMK than for GEN (871 versus 160) (Table 1). Accordingly, ratios of AUC_{ELF} after NEB to AUC_{ELF} after i.v. corrected for doses were estimated by the model to be 874 and 162 for AMK and GEN, respectively (Table 1), attesting to a high targeting advantage with NEB for both molecules. We previously conducted the same study with TOB, but a slightly different model, where an influx clearance from the ELF1 compartment to a central compartment, was used for data fitting (3). To facilitate comparisons, the previous data were reanalyzed using the same model as for AMK and GEN, which led to virtually similar parameter values in particular AUCs. New fits are presented in Fig. 1; the targeting advantage of NEB over TOB was estimated to be 315 (Table 1). From a pharmacokinetic point of view, AMK presents a targeting advantage for NEB over i.v. administration that was 2.8 and 5.4 times higher than those of TOB and GEN, respectively. These observations do not correspond to differences in physiochemical characteristics between aminoglycosides. Indeed, all aminoglycosides are small molecules (molecular weight, 585.6 $g \cdot mol^{-1}$ for AMK, 477.6

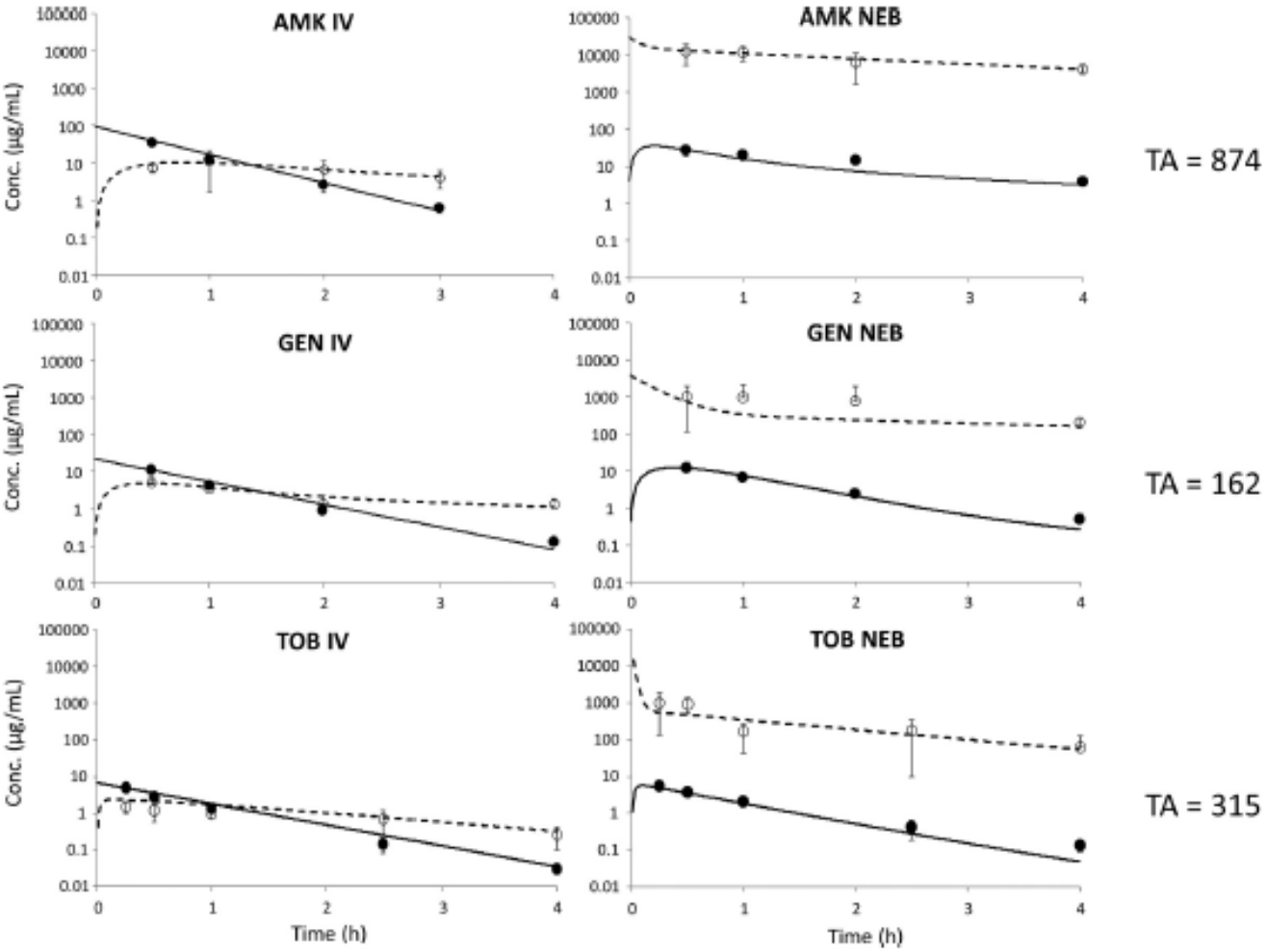

FIG 1 Predicted concentration-time profiles of AMK, GEN, and TOB in plasma (solid line) and ELF (dashed line) from simultaneous plasma and ELF pharmacokinetic modeling, after i.v. administration (left panel) and intratracheal nebulization (right panel) of GEN (8 mg · kg^{-1}), AMK (30 mg · kg^{-1}), and TOB (3 mg · kg^{-1}). Closed and open symbols represent experimental mean ± SD concentrations in plasma and ELF, respectively. TOB data obtained previously were reanalyzed using the same model as for AMK and GEN (3). TA, targeting advantage of NEB, corresponding to AUC_{ELF} after NEB over AUC_{ELF} after i.v. corrected for doses.

g · mol^{-1} for GEN, and 467.5 g · mol^{-1} for TOB), polycationic at a physiological pH (5 net charges for GEN and TOB and 4 charges for AMK), and do not show significant differences in log D values (−8.27 for GEN, −9.81 for AMK, and −9.45 for TOB) (Chemspider, www.chemspider.com). However, GEN solubility in water is at least 3 times lower than the solubility of AMK and TOB (12.6, 49.7, and 53.7 mg · ml^{-1}, respectively) (Drugbank, www.drugbank.ca); but considering concentrations in ELF, this

TABLE 1 AMK, GEN, and TOB results when administered intravenously or via nebulizer

	AMK		GEN		TOB	
Parameter[a]	I.v.	NEB	I.v.	NEB	I.v.	NEB
AUC_{plasma} (μg · h/ml)	53.7	54.5	15.9	16.6	5.1	5.1
AUC_{ELF} (μg · h/ml)	53.5	47,479	15.9	2,656	5.1	1,618
AUC_{ELF}/AUC_{plasma}	1	871	1	160	1	315
TA	874		162		315	

[a]Area under the concentration-time curve from 0 to infinity in plasma (AUC_{plasma}) and in ELF (AUC_{ELF}) after i.v. administration and intratracheal NEB of GEN, AMK, and TOB at 8, 30, and 3 mg · kg^{-1}, respectively. TA, targeting advantage of NEB estimated by ratio of AUC_{ELF} after NEB and i.v. corrected for doses. TOB data obtained previously were reanalyzed using the same model as for AMK and GEN (3).

parameter does not explain differences in the pharmacokinetic behavior of aminoglycosides after NEB (Fig. 1).

In conclusion, these results cannot be directly extrapolated to the clinical setting, and the choice among aminoglycosides depends on resistance patterns of pathogens. Yet, this new investigation clearly demonstrates a much greater, although unexplained, targeting advantage of NEB for AMK over GEN, with TOB being intermediate. Moreover, this study brings additional information for compiling a biopharmaceutical classification of nebulized antimicrobial agents, especially in this category of low-permeability molecules.

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REFERENCES

1. Sole-Lleonart C, Roberts JA, Chastre J, Poulakou G, Palmer LB, Blot S, Felton T, Bassetti M, Luyt CE, Pereira JM, Riera J, Welte T, Qiu H, Rouby JJ, Rello J, ESGCIP Investigators. 2016. Global survey on nebulization of antimicrobial agents in mechanically ventilated patients: a call for international guidelines. Clin Microbiol Infect 22:359–364. https://doi.org/10.1016/j.cmi.2015.12.016.
2. Gontijo AV, Brillault J, Gregoire N, Lamarche I, Gobin P, Couet W, Marchand S. 2014. Biopharmaceutical characterization of nebulized antimicrobial agents in rats: 1. Ciprofloxacin, moxifloxacin, and grepafloxacin. Antimicrob Agents Chemother 58:3942–3949. https://doi.org/10.1128/AAC.02818-14.
3. Marchand S, Gregoire N, Brillault J, Lamarche I, Gobin P, Couet W. 2015. Biopharmaceutical characterization of nebulized antimicrobial agents in rats: 3. Tobramycin. Antimicrob Agents Chemother 59:6646–6647. https://doi.org/10.1128/AAC.01647-15.
4. Gontijo AV, Gregoire N, Lamarche I, Gobin P, Couet W, Marchand S. 2014. Biopharmaceutical characterization of nebulized antimicrobial agents in rats: 2. Colistin. Antimicrob Agents Chemother 58:3950–3956. https://doi.org/10.1128/AAC.02819-14.
5. Heller DN, Peggins JO, Nochetto CB, Smith ML, Chiesa OA, Moulton K. 2005. LC/MS/MS measurement of gentamicin in bovine plasma, urine, milk, and biopsy samples taken from kidneys of standing animals. J Chromatogr B Analyt Technol Biomed Life Sci 821:22–30. https://doi.org/10.1016/j.jchromb.2005.04.015.
6. Marchand S, Gobin P, Brillault J, Baptista S, Adier C, Olivier JC, Mimoz O, Couet W. 2010. Aerosol therapy with colistin methanesulfonate: a biopharmaceutical issue illustrated in rats. Antimicrob Agents Chemother 54:3702–3707. https://doi.org/10.1128/AAC.00411-10.
7. Bulitta JB, Bingolbali A, Shin BS, Landersdorfer CB. 2011. Development of a new pre- and post-processing tool (SADAPT-TRAN) for nonlinear mixed-effects modeling in S-ADAPT. AAPS J 13:201–211. https://doi.org/10.1208/s12248-011-9257-x.
8. Marchand S, Gregoire N, Brillault J, Lamarche I, Gobin P, Couet W. 2016. Biopharmaceutical characterization of nebulized antimicrobial agents in rats: 4. Aztreonam. Antimicrob Agents Chemother 60:3196–3198. https://doi.org/10.1128/AAC.00165-16.
9. Yapa SWS, Li J, Patel K, Wilson JW, Dooley MJ, George J, Clark D, Poole S, Williams E, Porter CJ, Nation RL, McIntosh MP. 2014. Pulmonary and systemic pharmacokinetics of inhaled and intravenous colistin methanesulfonate in cystic fibrosis patients: targeting advantage of inhalational administration. Antimicrob Agents Chemother 58:2570–2579. https://doi.org/10.1128/AAC.01705-13.

Article 2: Preclinical pharmacokinetic and pharmacodynamic data to support cefoxitin nebulization for the treatment of Mycobacterium abscessus

Résume en français :

Mycobacterium abscessus est responsable d'infections pulmonaires chroniques difficiles à traiter en clinique. Les schémas posologiques actuels, y compris l'administration parentérale de céfoxitine en association avec l'amikacine et la clarithromycine, soulèvent des problèmes d'observance et sont souvent associés à un échec élevé et au développement de résistances. L'administration d'aérosols de céfoxitine pourrait être une alternative intéressante afin d'obtenir des concentrations élevées au site infectieux et de limiter la toxicité. La céfoxitine a été administré à des rats sains par bolus intraveineux ou nébulisation intratrachéale, et les concentrations ont été déterminées dans le plasma et le liquide épithélial pulmonaire (ELF) par chromatographie liquide et spectrométrie de masse tandem. Après administration intrapulmonaire, l'aire sous la courbe pour la céfoxitine dans l'ELF était 1 147 fois supérieure à celle du plasma, ce qui indique que cette voie d'administration offre un avantage biopharmaceutique sur l'administration intraveineuse. L'activité antimicrobienne de céfoxitine a été étudiée à l'aide de courbes de bactéricidies combinées à une approche de modélisation de type pharmacocinétique/pharmacodynamique (PK/PD) afin de tenir compte de son instabilité *in vitro* qui empêche la détermination précise de la CMI. Les données sur la dégradation *in vitro* de céfoxitine ont été intégrées dans un modèle comprenant une sous-population de bactéries sensibles (S) et résistantes (R), et un effet inhibition de croissance de céfoxitine. Les IC_{50} ont été estimées à 16,2 et 252 mg/litre pour les sous-populations S et R, respectivement. Ces résultats suggèrent que les schémas posologiques parentéraux pour la céfoxitine utilisés chez les patients pour le traitement de *M. abscessus* ne sont pas suffisants pour réduire la charge bactérienne et que la nébulisation offre un avantage potentiel qui doit être étudié plus en détails.

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PHARMACOLOGY

Preclinical Pharmacokinetic and Pharmacodynamic Data To Support Cefoxitin Nebulization for the Treatment of *Mycobacterium abscessus*

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ABSTRACT *Mycobacterium abscessus* is responsible for difficult-to-treat chronic pulmonary infections in humans. Current regimens, including parenteral administrations of cefoxitin (FOX) in combination with amikacin and clarithromycin, raise compliance problems and are frequently associated with high failure and development of resistance. Aerosol delivery of FOX could be an interesting alternative. FOX was administered to healthy rats by intravenous bolus or intratracheal nebulization, and concentrations were determined in plasma and epithelial lining fluid (ELF) by liquid chromatography-tandem mass spectrometry. After intrapulmonary administration, the FOX area under the curve within ELF was 1,147 times higher than that in plasma, indicating that this route of administration offers a biopharmaceutical advantage over intravenous administration. FOX antimicrobial activity was investigated using time-kill curves combined with a pharmacokinetic/pharmacodynamic (PK/PD) type modeling approach in order to account for its *in vitro* instability that precludes precise determination of MIC. Time-kill data were adequately described by a model including *in vitro* degradation, a sensitive (S) and a resistant (R) bacteria subpopulation, logistic growth, and a maximal inhibition-type growth inhibition effect of FOX. Median inhibitory concentrations were estimated at 16.2 and 252 mg/liter for the S and R subpopulations, respectively. These findings suggest that parenteral FOX dosing regimens used in patients for the treatment of *M. abscessus* are not sufficient to reduce the bacterial burden and that FOX nebulization offers a potential advantage that needs to be further investigated.

KEYWORDS *Mycobacterium abscessus*, cefoxitin, nebulization, pharmacokinetics-pharmacodynamics

Mycobacterium abscessus is the most frequent rapidly growing mycobacteria in human pathology (1). This emerging pathogen is mainly responsible for chronic pulmonary infections in patients with cystic fibrosis (1) and is considered a "new antibiotic nightmare" because of its intrinsic resistance to a broad range of antibiotics, including classical antituberculous agents such as ethambutol, pyrazinamide, and isoniazid (2). Presently there is no reliable antibiotic treatment to cure *M. abscessus* pulmonary infections (2–4). In fact, treatment for pulmonary infections caused by *M. abscessus* is not well standardized yet (4, 5). It consists of intravenous (i.v.) administration of amikacin (AMK) and cefoxitin (FOX) in combination with oral administration of clarithromycin (CLR) for several months (4). Unfortunately, this treatment is associated

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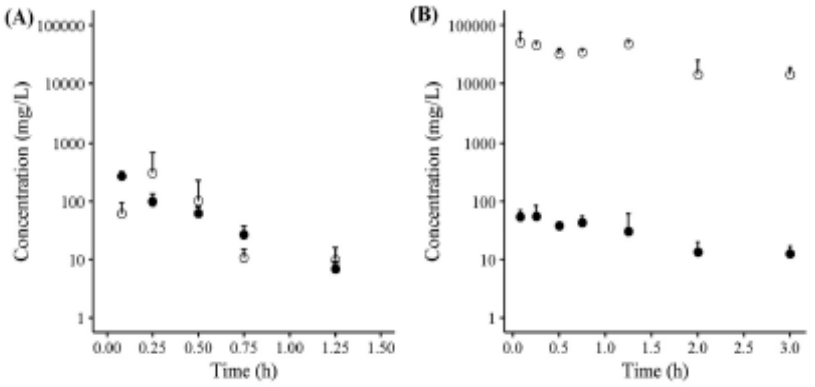

FIG 1 Observed mean concentration ± SD versus time profiles of FOX following i.v. (A) and NEB (B) treatment. Closed symbols correspond to the total plasma concentrations, and open symbols represent ELF concentrations.

with high failure rates, showing infection relapse or death (6). Furthermore, long-term treatments, from several months to a year, with parenterally administered antibiotics are not only challenging and relatively costly but also responsible for low compliance (4). In this context, alternative routes of administration, such as aerosol delivery, should be considered. Nebulization (NEB) is more convenient than i.v. administration and results in higher lung concentrations, and higher efficacy, along with limited systemic side effects, may be achieved (7).

A recent series of well-controlled experiments in healthy rats have shown that antibiotics, such as fluoroquinolones (8), with high membrane permeability are rapidly absorbed after NEB, whereas compounds with much lower membrane permeability, such as colistin (CST) (9), aztreonam (ATM) (10), tobramycin (TOB) (11), gentamicin (GEN), and AMK (12), are slowly absorbed after NEB, leading to high sustained local concentrations, and much higher pulmonary epithelial lining fluid (ELF) concentrations of GEN after NEB than i.v. administration have recently been reported in critically ill patients (13). A simple rule to be considered is that antibiotics that cannot be administered orally, because of poor oral bioavailability due to limited membrane permeability, are, for that same reason, the best candidates for aerosol delivery to treat pulmonary infections. Interestingly, FOX and AMK are rather hydrophilic and are not substantially absorbed after oral administration because of their limited membrane permeability. All of these parameters make FOX as well as AMK good candidates for NEB (12). However, if FOX and AMK present similarities in terms of pharmacokinetics (PK), including low volume of distribution and mostly renal elimination, they do not share similarities in terms of pharmacodynamics (PD). Aminoglycosides, including AMK, are considered to be concentration dependent, whereas β-lactam antibiotics such as FOX are usually supposed to exhibit time-dependent activity (14). Moreover, characterization of FOX activity against *M. abscessus* is made difficult due to its rapid degradation (15, 16) and has been reported only on rare occasions (17–19). Therefore, the objective of this study was first to compare the intrapulmonary PK of FOX after NEB and i.v. administration to healthy rats and then to characterize its *in vitro* PD against a selected strain of *M. abscessus*.

RESULTS

Pharmacokinetics in healthy rats. FOX concentration-time profiles after i.v. administration and NEB are presented in Fig. 1. After i.v. administration, FOX concentrations were almost superimposed in plasma and ELF except at early times due to distribution within ELF. Accordingly, FOX exposure in ELF and plasma was comparable (mean areas under unbound concentration-time curve from time zero to infinity in plasma [$AUC_{u,plasma}$] and ELF [$AUC_{u,ELF}$] of 107 h·μg/ml and 103 h·μg/ml, respectively),

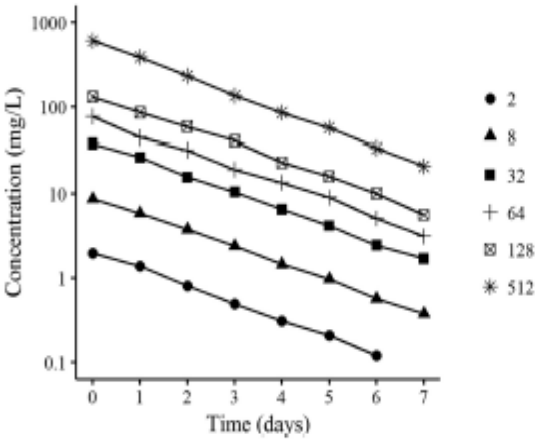

FIG 2 Observed concentration versus time profiles of FOX in 7H9 broth. Initial concentrations of FOX are in mg/liter and indicated by different symbols.

corresponding to a ratio of 1.04. Estimated elimination half-lives in ELF ($t_{1/2}$ = 0.19 h) and plasma ($t_{1/2}$ = 0.23 h) were also virtually identical. After NEB, FOX concentrations were much higher within ELF than in plasma, with $AUC_{u,ELF}$ of 119,289 h·μg/ml and $AUC_{u,plasma}$ of 104 h·μg/ml, corresponding to a ratio of 1,147. Noticeably, $AUC_{u,plasma}$ was identical after NEB and i.v. administration, but the $AUC_{u,ELF}$ was 1,113-fold higher after NEB than after i.v. administration. Again, ELF and plasma concentrations decreased approximately in parallel with time after NEB (Fig. 1), with corresponding half-lives estimated at 1.54 h and 1.23 h, respectively, which is at least 6 times longer than that after i.v. administration.

***In vitro* FOX degradation.** FOX degradation followed first-order kinetics with a half-life estimated at 1.5 days (Fig. 2).

***In vitro* pharmacodynamics. (i) Time-kill kinetics assay.** The first series of time-kill experiments showed no effect for initial FOX concentrations equal to or lower than 16 mg/liter. An initial CFU decay followed by regrowth was observed at day 2 for initial concentrations equal to 32 mg/liter and at day 6 for initial concentrations equal to 64 mg/liter. A decay without regrowth over 8 days was observed for initial concentrations above 128 mg/liter (Fig. 3A). The second series of time-kill kinetics showed that a CFU decay followed by a regrowth occurs for initial FOX concentrations between 12

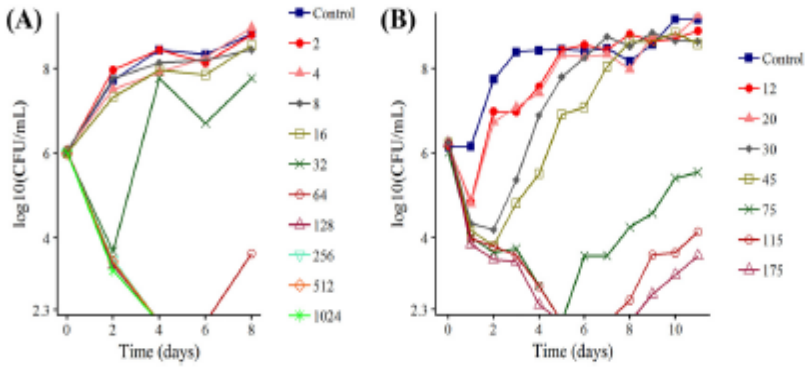

FIG 3 Representative results of FOX time-kill curves against *M. abscessus* CIP 104536 strain. Initial concentrations of FOX are in mg/liter and indicated by different symbols. The ordinate shows the change in the number of CFU (log_{10} scale) per ml of broth. The limit of quantification was 200 CFU/ml (2.3 in log_{10}). (A) First series of experiments. (B) Second series of experiments.

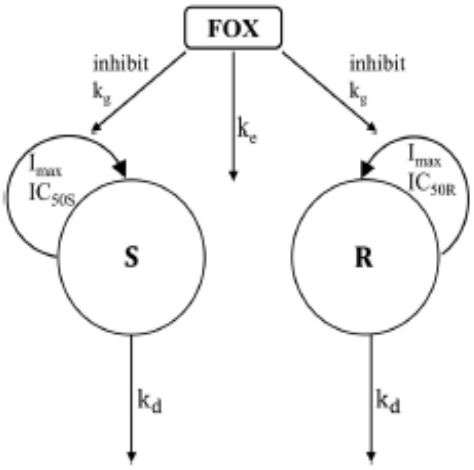

FIG 4 Schematic diagram of the final PK/PD type model. Bacteria multiplied with a first-order rate constant (K_g) in the susceptible (S) and resistant (R) bacterial compartment, and all bacteria had natural death rates (K_d). The cefoxitin compartment (FOX), with a first-order elimination rate (ke), was driving to the bacterial growth inhibition following an I_{max} model.

and 175 mg/liter and that time to regrowth increased with the initial FOX concentrations (Fig. 3B).

(ii) Time-kill modeling. Initially the first series of time-kill kinetics experiments was analyzed using a growth inhibition I_{max} (maximal inhibition) model, with Hill coefficient, with a single homogenous population of bacteria (see the supplemental material). After pooling the two time-kill data sets, a growth inhibition I_{max} model with two subpopulations (Fig. 4), susceptible (S) and resistant (R), best described the experimental data. Visual predictive checks (VPCs) with observed and simulated CFU, with 80% prediction interval, show that model predictions fit the experimental data well, except at a 45-mg/liter initial FOX concentration (Fig. 5). Pharmacodynamics (PD) parameter estimates are presented in Table 1. Noticeably, the difference in susceptibilities of the two bacterial subpopulations is reflected by a 50% inhibitory concentration for the resistant subpopulation (IC_{50R}) 15-fold higher than the IC_{50} for the susceptible subpopulation (IC_{50S}).

(iii) Simulations of CFU versus time profiles without FOX degradation. According to simulations, FOX has no effect on both the S and R subpopulations at a concentration equal to 10 mg/liter and an effect on the S subpopulation only at concentrations equal to 20, 100, or 200 mg/liter (Fig. 6). FOX has no effect on the R subpopulation at concentrations of 20 and 100 mg/liter but has an effect followed by regrowth at a concentration of 200 mg/liter. Complete bacterial killing is expected at 300 mg/liter.

DISCUSSION

The initial PK part of this study has clearly demonstrated a major effect of the route of administration on FOX concentrations within ELF. The targeting advantage (TA) provided by NEB, corresponding to the ratio of $AUC_{u,ELF}$ after NEB versus after i.v. administration and reflecting the relative increased FOX exposure within ELF after NEB, was close to a thousand (TA of 1,113). This high TA is consistent with values previously reported under similar experimental conditions: 242, 2,673, 874, and 162 for TOB (11), ATM (10), AMK, and GEN (12), which are all antibiotics with low membrane permeability precluding oral administration. By comparison, for ciprofloxacin (CIP) and moxifloxacin (MXF), two fluoroquinolone antibiotics with high membrane permeability allowing oral administration, the ratio of $AUC_{u,ELF}$ after NEB versus that after i.v. administration were close to unity (TA of 1.2 for CIP and TA of 0.95 for MXF) (8), suggesting limited, if any,

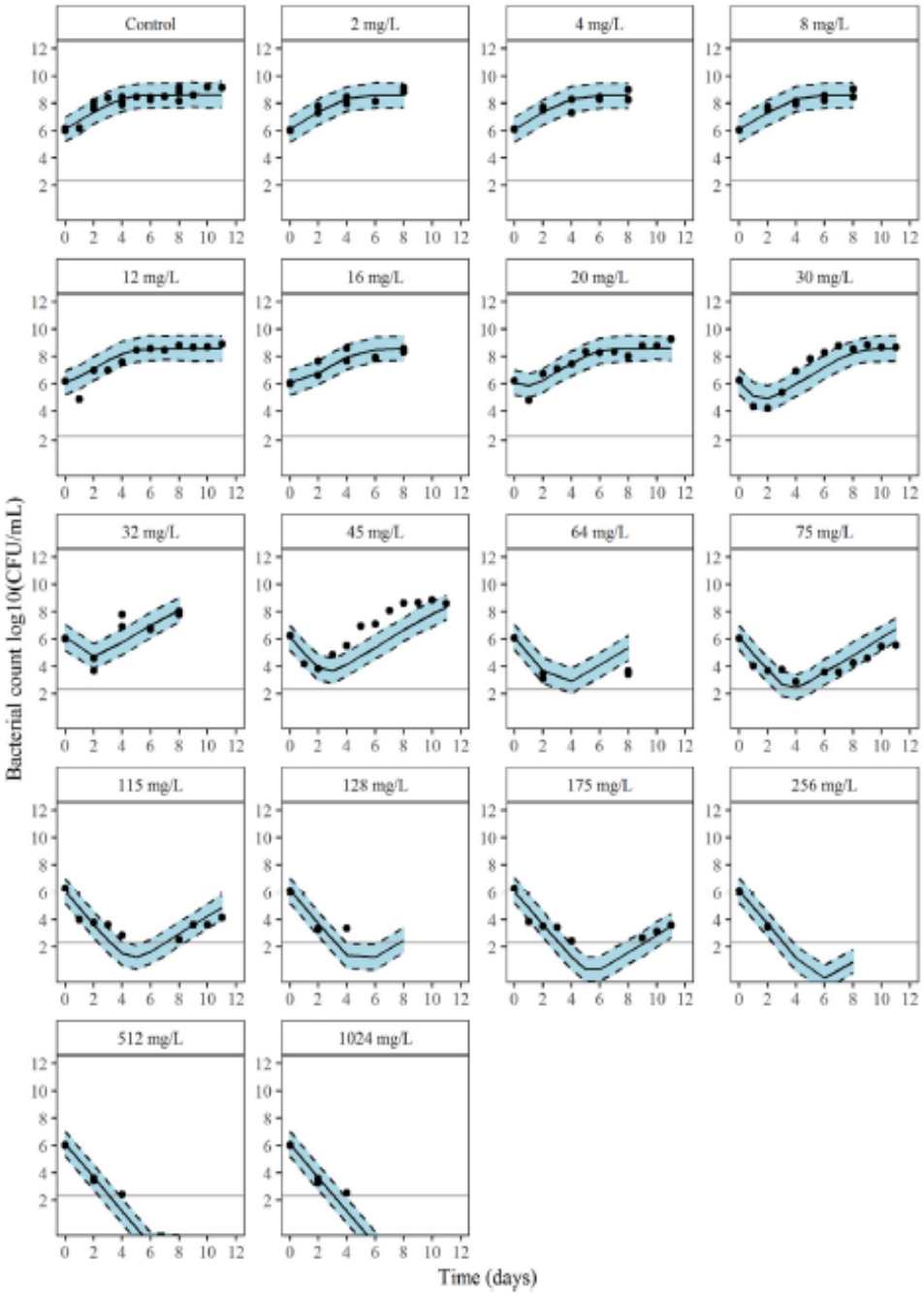

FIG 5 Visual predictive checks (VPCs) for the final PK/PD type model of FOX against *M. abscessus* CIP 104536 with observed bacterial counts (circles), medians (black continuous line), and 80% prediction intervals (black dotted line) of simulated data. Plots include growth control and experimental data by time-kill kinetics. The indicated concentrations are the initial FOX concentrations. The line shows the limit of quantification (200 CFU/ml).

biopharmaceutical advantage of NEB. However, TA values determined in rats after intratracheal administration with the Penn-Century microsprayer cannot be directly extrapolated to humans. Indeed, using this mode of administration, virtually 100% of the dose is absorbed and systemically bioavailable, leading to comparable plasma AUCs

TABLE 1 PD parameter estimations, derived from the growth inhibition model fitted to time-kill kinetics assay

Parameter	Explanation	Estimation (% RSE[a])
LGINOC (log_{10}CFU/ml)	Initial bacterial density	6.1 (1)
K_g (day^{-1})	Bacterial growth rate constant	4.3 (6)
B_{max} (log_{10}CFU/ml)	Bacterial count in stationary phase	9.05 (1)
K_d (day^{-1})	Bacterial death rate constant	2.83 (7)
I_{max}	Maximum fractional reduction of growth by FOX	1 (fixed)
IC_{50S} (mg/liter)	FOX concentration that results in 50% of I_{max} for susceptible subpopulation	16.2 (11)
IC_{50R} (mg/liter)	FOX concentration that results in 50% of I_{max} for resistant subpopulation	252 (20)
K_e (day^{-1})	Degradation rate constant for FOX followed by first-order process	0.438 (fixed)
MUTF	Mutation frequency of bacteria	−9.66 (6)
γ	Hill factor for growth inhibition due to drug activity	4.8 (39)

[a]RSE, relative standard errors.

independent of the route of administration (8–12). This characteristic, which was confirmed in the present study ($AUC_{u,plasma}$ of 103 h·μg/ml after i.v. and 104 h·μg/ml after NEB treatment), does not reflect the clinical setting, with only a small fraction of the dose being absorbed after aerosolization. This can be illustrated by comparing results obtained after nebulization of GEN in rats (12) and patients (13). Comparison of $AUC_{u,plasma}$ values in patients indicates that GEN systemic exposure was close to 5% after NEB compared with i.v. administration.

The PD of FOX, and in particular the potential advantage provided by NEB, not in terms of increased exposure at the infection site but rather in terms of antimicrobial efficacy, was investigated during the second part of this study. Our time-kill results are consistent with those obtained during previous studies over this relatively limited period of time (18, 19). FOX is supposed to be a time-dependent antibiotic, and accordingly it is recommended to maintain concentrations higher than the MIC with no need for high peak concentrations. However, characterization of *in vitro* FOX activity against mycobacterium species is made difficult for two different reasons. Although *M.*

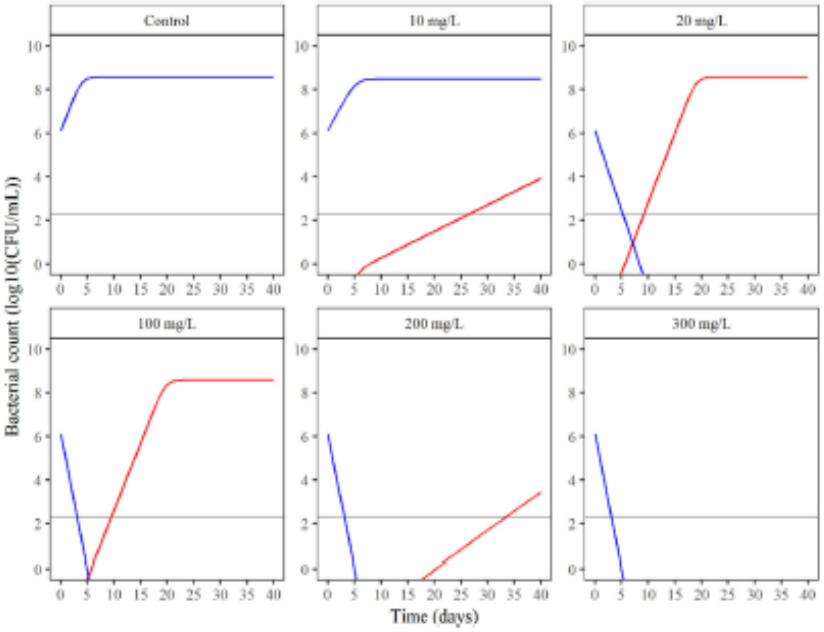

FIG 6 Simulations of CFU counts versus time at several FOX concentrations without considering FOX degradation, using the final PK/PD type model (2 subpopulations) of FOX against *M. abscessus* CIP 104536. Red lines represent the amount of resistant bacteria, and blue lines represent the amount of susceptible bacteria. Horizontal lines show the limit of quantification (200 CFU/ml).

abscessus is considered to be a rapidly growing mycobacterial species, compared with other mycobacteria, such as *M. tuberculosis*, experiments take longer with mycobacterium species than with other bacteria. As an example, MIC determination is conducted over 3 days for *M. abscessus* compared with 1 day for most bacteria. In addition, FOX is degraded within 7H9 broth with an elimination half-life close to 1.5 days, meaning that after 3 days, FOX concentration within the liquid medium would only be 25% of the initial concentration. Actually, FOX degradation within liquid medium was initially documented by Oberholtzer and Brenner (20) and then identified as a real problem for MIC determination by Rominski et al. (15). In fact, when an apparent MIC is reported at 8 mg/liter, it should be noted that this corresponds to the initial FOX concentration, which, after 3 days, or 2 half-lives, should be down to 2 mg/liter. Therefore, the intermediate FOX concentration at 4 mg/liter would better reflect the "true" MIC. In order to take into account this degradation, especially for interpretation of time-kill kinetics experiments conducted over time periods longer than a week, a PK/PD type modeling approach was used. The fact that FOX concentrations decay with time due to *in vitro* first-order degradation was taken into account just as if this was due to *in vivo* elimination. However, the *in vitro* degradation variability was negligible, and the degradation half-life was set at 1.5 days for the PK/PD type modeling, conducted in two separate steps.

Initially, time-kill experiments were conducted at 11 different initial concentrations, from 2 to 1,024 mg/liter, corresponding to multiple values of the apparent MIC, as commonly done (here we used 0.25 to 128 times the MIC), plus a control (Fig. 3). There was virtually no effect at initial concentrations equal to or lower than 16 mg/liter. When the initial concentration was equal to 32 mg/liter, an initial decay of CFU was observed, followed by regrowth after 2 days. For initial concentrations equal to or higher than 64 mg/liter, a CFU decay was observed with no regrowth after 2 days. The question was to determine whether regrowth observed at the intermediate FOX concentration could be explained by its degradation, starting from 32 mg/liter and dropping down to 16 mg/liter and 8 mg/liter after 1.5 and 3 days, respectively. A PK/PD type model was used to answer this question. With this type of model, it can be considered that the antibiotic acts by inhibiting bacterial growth (bacteriostatic effect) or by stimulating bacterial kill (bactericidal effect). Since, according to Lavollay et al. (17) and Greendyke and Byrd (21), FOX is supposed to possess bacteriostatic activity against *M. abscessus*, a growth inhibition PD model was selected with a single homogenous bacterial population. This model provided reasonably good data fitting (see the supplemental material), but noticeably, using the same strain (CIP 104536), Ferro et al. have identified a FOX-resistant subpopulation, preexistent at time zero (22), suggesting that the PK/PD model with a single homogenous bacterial population is not appropriate. However, our initial data set, with regrowth observed at only one FOX concentration, was probably not sufficiently informative to support the superiority of two subpopulations (S and R) versus one population PD model. Therefore, we decided to initiate a second series of time-kill experiments with more intermediate initial FOX concentrations, i.e., susceptible enough to provide initial CFU decay followed by regrowth. Noticeably, according to the one-population model, regrowth should always start at the same FOX concentration (the nadir). Therefore, time to nadir should increase with the initial FOX concentration, more precisely by 1.5 days, corresponding to FOX degradation half-life, each time the initial concentration is increased by twofold. The effect of initial FOX concentration on time to nadir would not be the same in the presence of one and two subpopulations. The supplementary intermediate FOX concentrations used for this second time-kill set of experiments (12, 20, 30, 45, 75, 115, and 175 mg/liter), the frequency of CFU determinations (every day instead of every 2 days), and the experiment duration (increased from 8 to 11 days) were selected in order to allow discrimination between one- and two-population models.

Using the whole data set (initial and second series of time-kill experiments), the two-subpopulation model best described experimental data, as illustrated in Fig. 5.

However, direct interpretation of this figure is difficult, since FOX degradation contributes to regrowth as well as the existence of an R subpopulation. In fact, time-kill experiments are frequently referred to as "static" conditions because they use several different antibiotic concentrations but are stable over time, as opposed to "dynamic" conditions, such as hollow-fiber experiments, in which the medium is replaced in order to let antibiotic concentration decay with time according to 1st-order kinetics, with an elimination half-life selected to mimic *in vivo* PK.

One point of interest in the PK/PD type model was that it allowed simulations of CFU versus time profiles, keeping FOX concentrations constant with time (Fig. 6). It was also possible to simulate the effect of FOX on each subpopulation. Due to the high γ value ($\gamma = 4.8$), the model suggests that a slight change in FOX concentrations around the IC_{50S} (16.2 mg/liter) has dramatic consequences on FOX antimicrobial effect on the S subpopulation. Accordingly, no effect would be expected at a FOX concentration equal to 10 mg/liter, but a marked initial decay of CFU with time with no regrowth is predicted at a FOX concentration equal to 20 mg/liter (Fig. 6). Thus, as far as FOX concentrations remain low compared with the IC_{50R} (252 mg/liter), regrowth of the R subpopulation should be observed at later times (Fig. 6).

Interestingly, after systemic administration at the usual dose (2 g) in patients, FOX total plasma concentrations reach values of up to 200 mg/liter (23), which, considering that the unbound fraction is close to 25% (24), corresponds to maximum unbound plasma concentrations on the order of 50 mg/liter. In this range of values, unbound FOX concentrations should demonstrate antimicrobial efficacy only against the susceptible *M. abscessus* population. Therefore, the possibility of achieving much higher FOX concentrations at the infection site (lung ELF) after nebulization, as suggested in this study using noninfected rats, may offer an opportunity to provide antimicrobial efficacy against the R subpopulation as well.

However, extrapolation of these new data to the clinical setting must be done extremely carefully. First, from a PK standpoint, nebulization with the Penn-Century microsprayer allows good control of the dose, which is of interest for the biopharmaceutical characterization of nebulized antibiotics (8–12, 25) but which does not reflect the clinical setting. Furthermore, on top of potential between-species differences, *M. abscessus* produces biofilm (21), and lung infection induces changes in lung physiology. These phenomena may have an effect on FOX membrane permeability and therefore on lung PK that is not reflected using healthy rats. Second, from a PD point of view, most of our findings rely on a model with a simple two-subpopulation model, which, as previously stated, may be too simplistic. Another limitation of these *in vitro* experiments is that they do not take into consideration the distribution of *M. abscessus* within macrophages or the limited FOX intracellular distribution (19). Finally, in clinical practice *M. abscessus* infections are treated by several antibiotics in combination. This obviously has major consequences in terms of antimicrobial efficacy, which was not addressed here.

In conclusion, the PK/PD type modeling approach developed in this study enabled correction for FOX degradation and, therefore, characterization of the effect-concentration relationship, as is usually done by time-kill experiments. Moreover, combined with the possibility of reaching much higher FOX lung ELF concentrations after nebulization than traditional parenteral administration, it provides evidence to support a potential therapeutic advantage of this route of administration. Furthermore, as far as these high FOX ELF concentrations would not be responsible for undesirable effects, combined with the slow elimination half-life of FOX after NEB, they would allow us to maintain the effect maximum for a long period of time postnebulization. In other words, PK/PD characteristics of FOX may allow spaced nebulization. However, numerous complementary experiments would be necessary to confirm the potential therapeutic advantage of FOX NEB for the treatment of *M. abscessus* infections.

MATERIALS AND METHODS

Antibiotics. FOX sodium salt was obtained from Panpharma (Luitré, France) for *in vivo* and *in vitro* experiments and from Sigma (Saint-Quentin-Fallavier, France) for analytical purposes. Cefuroxime (CXM) was obtained from Aprokam (Clermont-Ferrand, France).

Pharmacokinetics in healthy rats. (I) Administration and sampling. Animal experiments were conducted in compliance with EC Directive 2010/63/EU after approval by the local ethics committee (COMETHEA) and were registered by the French Ministry of Higher Education and Research under authorization number 2015070211159865. Male Sprague Dawley rats ($n = 59$; mean weight of rats, 300 g) from Charles River Laboratories (Saint Germain Nuelles, France) were used for experiments. All rats were divided in two groups corresponding to route of administration (i.v. or NEB) (11). On day 1 of the experiment, FOX sodium salt solutions were prepared in 0.9% NaCl at a concentration of 300 mg/ml for NEB and 30 mg/ml for i.v. administrations. As previously described (9), FOX was administered under isoflurane anesthesia either by i.v. bolus in the tail vein (1 ml) or by intratracheal NEB (100 μl) using a 1A-1B Penn-Century microsprayer (Wyndmoor, USA) at doses commonly used in clinical practices after correction for body weight, close to 90 mg/kg of body weight (23). Bronchoalveolar lavage (BAL) fluid and blood sampling was performed as previously described (8) at various times until 1.25 h after i.v. and 3 h and NEB administration (0.08, 0.25, 0.5, 0.75, and 1.25 h for i.v. and 0.08, 0.25, 0.5, 0.75, 1.25, 2, and 3 h for NEB; 4 to 5 rats were included per time point).

(II) FOX analytical assay. The FOX analytical assay was conducted by liquid chromatography-tandem mass spectroscopy (LC-MS/MS). The system included a Shimadzu high-performance liquid chromatography (HPLC) module coupled with an API 3500 mass spectrometer (Sciex, Les Ulis, France). An XBridge amide column (3.5 μm; 50- by 2.1-mm inside diameter; Waters, Saint-Quentin en Yvelines, France) was used, and a gradient mobile phase composed of 5 mM ammonium formate and acetonitrile (70:30 [vol/vol]) with 0.01% of formic acid was delivered at 0.4 ml/min. The mass spectrometer was operated in the negative mode. Ions were analyzed by multiple-reaction monitoring (MRM). CXM was used as an internal standard. The transitions were *m/z* 426/156 for FOX and 423/207 for CXM. The standard curve of 0, 0.05, 0.1, 0.5, 1, 5, 15, and 20 mg/liter was performed for BAL fluid and plasma samples. Three levels of concentrations (0.1, 1, and 15 mg/liter) were tested for intraday variability with precision and accuracy of <15% ($n = 18$ per medium). The between-day variability was studied at 0.1, 1, and 15 mg/liter with a precision and bias of <15% ($n = 6$). The urea concentrations in plasma and BAL fluid samples were measured as previously described (9).

(III) Data analysis. Noncompartmental PK analysis was conducted from time-averaged unbound FOX concentrations. FOX protein binding was fixed at 25% in plasma (24) and considered to be negligible within ELF, in which protein concentration is 10 times lower than that in plasma (26). Mean areas under unbound concentrations versus time from time zero to infinity were estimated using a trapezoidal method with extrapolation to infinity in plasma ($AUC_{u,plasma}$) and ELF ($AUC_{u,ELF}$) using Phoenix WinNonlin 7.0 software (Certara, St. Louis, MO). Elimination rate constants, ke, in plasma and ELF were estimated by least-squares fit of data points (log concentration-time) in the terminal phase of decline. Corresponding apparent elimination half-lives ($t_{1/2}$) were estimated as 0.693/ke. The $AUC_{u,ELF}/AUC_{u,plasma}$ ratios were compared after NEB and i.v. administration, and the TA of NEB compared to that of i.v. was estimated from the ratio of $AUC_{u,ELF}$ after NEB versus i.v. administration (27). Results are presented as means ± standard deviations (SD).

***In vitro* FOX degradation.** For FOX degradation in 7H9 broth, a 10-mg/ml stock solution of FOX sodium salt in water was prepared and stored at −80°C until being thawed for preparing working solutions after appropriate dilutions in 7H9 broth. To evaluate the degradation of FOX in 7H9 broth, individual tubes of 20 ml of 7H9 broth containing 2, 8, 32, 64, 128, and 512 mg/liter of FOX as an initial concentration were inoculated with the bacterial suspension (~1×10^6 CFU/ml) and incubated at 35°C ± 2°C. Samples were collected daily for up to 8 days. FOX concentrations then were measured by the LC-MS/MS analytical method as explained above.

***In vitro* pharmacodynamics. (I) Bacterial strain and suspension preparation.** *M. abscessus* subsp. *abscessus* reference strain CIP 104536 (Collection of Institute Pasteur, Paris, France) was used. Stock vials were conserved at −80°C in Middlebrook 7H9 broth (referred to as 7H9 broth; BD, BBL, Sparks, MD, USA) with 10% oleic acid-bovine albumin-dextrose-catalase (OADC) growth supplement (BD, BBL, Sparks, MD, USA) and 20% glycerol (Carl Roth GmbH Co. KG, Karlsruhe, Germany). *M. abscessus* was grown on Middlebrook 7H11 agar plates (referred to as 7H11 agar plates) with 10% OADC growth supplement and 0.5% glycerol at 30°C for 3 to 5 days. For each experiment, the mycobacterial inoculum was prepared freshly according to CLSI guidelines (28). Briefly, colonies from agar plates were transferred into a hemodialysis tube with 5 to 6 sterile glass beads of 3 nm and then vortexed for 1 min. One ml of sterile water then was added and the mixture incubated for 15 min at room temperature. The bacterial suspension was adjusted to an optical density at 600 nm of 0.10 to 0.15 (~10^8 CFU/ml). Finally, the suspension was diluted to 1/100 to obtain an ~1×10^6 CFU/ml final concentration in appropriate media.

(II) Time-kill kinetics assay. Individual tubes of 20 ml of 7H9 broth containing 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1,024 mg/liter of FOX as an initial concentration and growth control (CTL) were inoculated with the bacterial suspension (~1×10^6 CFU/ml) and incubated at 35°C ± 2°C under shaking conditions (150 rpm) for up to 8 days. To quantify bacteria at defined time intervals (0, 2, 4, 6, and 8 days), 100-μl samples were taken and diluted serially when appropriate in phosphate-buffered saline (PBS; pH 7.4; Gibco by Life Technologies, France). Samples were then plated on 7H11 agar plates for further CFU counting after 3 to 5 days of incubation at 35°C ± 2°C. The theoretical detection limit was set to 200 CFU/ml, i.e., 2.3 $\log_{10}$ CFU/ml. A second series of experiments was carried out using FOX initial

concentrations of 12, 20, 30, 45, 75, 115, and 175 mg/liter and a growth control using a protocol similar to that explained above. Numbers of CFU were determined daily for up to 11 days.

(iii) Time-kill modeling. CFU counts and FOX concentrations over time were analyzed separately using a nonlinear fixed-effects PK/PD type modeling approach. Parameters were estimated using the first-order conditional estimation (FOCE) method and Laplacian option available in NONMEM version 7.4.1 (Icon Development Solutions Ellicott City, MD, USA). NONMEM project management was made easier using Pirana (29), and CFU counts below the limit of quantification (200 CFU/ml) were handled using Beal's M3 method (30). The degradation of FOX (C) during the experiment was modelled as a first-order process:

$$C = C_0 e^{-K_e \times t} \quad (1)$$

Where C_0 is the initial concentration of FOX (mg/liter) spiked in the tube at time zero, K_e is the first-order degradation rate constant (days^{-1}), and t is the corresponding elimination half-life, equal to $0.693/K_e$ (days). Two bacterial subpopulations were considered: susceptible (S) and resistant (R) bacteria. In the absence of FOX, they were assumed to grow until reaching a plateau, and this was described by a logistic growth function. The effect of FOX on *M. abscessus* CIP 104536 was modelled as an inhibition of bacterial growth, with an I_{max} model and different IC_{50} for each subpopulation. The structure of the PD model is presented in Fig. 5. The differential equations describing variations of susceptible bacterial counts (S) and resistant bacterial counts (R) over time are presented below:

$$\frac{dS}{dt} = K_g \times \left(1 - \frac{B}{10^{B_{max}}}\right) \times \left(1 - \frac{I_{max} \times C^{\gamma}}{IC_{50S}^{\gamma} + C^{\gamma}}\right) \times S - K_d \times S \quad (2)$$

$$\frac{dR}{dt} = K_g \times \left(1 - \frac{B}{10^{B_{max}}}\right) \times \left(1 - \frac{I_{max} \times C^{\gamma}}{IC_{50R}^{\gamma} + C^{\gamma}}\right) \times R - K_d \times R \quad (3)$$

where $B = S + R$ is the total bacterial count (CFU/ml), K_g (day^{-1}) is the bacterial growth rate, K_d (day^{-1}) is the bacterial natural death rate, B_{max} ($\log_{10}$ CFU/ml) is the maximum population size supported by the environment, I_{max} is the maximal inhibition, which was supposed to be total inhibition (I_{max} fixed to 1), and IC_{50S} and IC_{50R} (mg/liter) are the concentrations of FOX for which the effect was 50% of I_{max} for susceptible and resistant bacteria, respectively. The residual variability was described by an additive error model on a $\log_{10}$ scale for bacterial count data and by a proportional error model for concentrations of FOX data. No interexperimental variability was estimated on the parameters because we assumed that it would not be distinguishable from residual variability. Model performance was assessed by the evaluation of the goodness-of-fit plots (e.g., observation versus predictions) and objective function value (OFV). The model was evaluated by performing VPCs with stratification on the type of experiments and FOX concentration. All observations were plotted and overlaid with the median, and 80% prediction intervals were obtained by performing 1,000 simulations of the final model with the original data set as the input.

(iv) Simulations of CFU versus time profiles without FOX degradation. In order to evaluate the effect of FOX on *M. abscessus* at constant concentration, the developed PK/PD type model with two subpopulations was used along with PD parameter estimates to simulate the variation in CFU count over time at different concentrations. R package mrgsolve (31) was used to simulate CFU versus time profiles. The final parameter estimates from the current FOX PK/PD type model with two subpopulations were used to simulate the CFU versus time profiles without considering FOX degradation.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02651-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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REFERENCES

1. Roux A-L, Catherinot E, Ripoll F, Soismier N, Macheras E, Ravilly S, Bellis G, Vibet M-A, Le Roux E, Lemonnier L, Gutierrez C, Vincent V, Fauroux B, Rottman M, Guillemot D, Gaillard J-L. 2009. Multicenter study of prevalence of nontuberculous mycobacteria in patients with cystic fibrosis in France. J Clin Microbiol 47:4124–4128. https://doi.org/10.1128/JCM.01257-09.
2. Nessar R, Cambau E, Reyrat JM, Murray A, Gicquel B. 2012. Mycobacterium abscessus: a new antibiotic nightmare. J Antimicrob Chemother 67:810–818. https://doi.org/10.1093/jac/dkr578.
3. Jarand J, Levin A, Zhang L, Huitt G, Mitchell JD, Daley CL. 2011. Clinical and microbiologic outcomes in patients receiving treatment for Mycobacterium abscessus pulmonary disease. Clin Infect Dis 52:565–571. https://doi.org/10.1093/cid/ciq237.
4. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, Holland SM, Horsburgh R, Huitt G, Iademarco MF, Iseman M, Olivier K, Ruoss S, von Reyn CF, Wallace RJ, Winthrop K, ATS Mycobacterial Diseases Subcommittee, American Thoracic Society, Infectious Disease Society of America. 2007. An official ATS/IDSA statement: diagnosis, treat-

ment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med 175:367–416. https://doi.org/10.1164/rccm.200604-571ST.
5. van Ingen J, Ferro BE, Hoefsloot W, Boeree MJ, van Soolingen D. 2013. Drug treatment of pulmonary nontuberculous mycobacterial disease in HIV-negative patients: the evidence. Expert Rev Anti Infect Ther 11:1065–1077. https://doi.org/10.1586/14787210.2013.830413.
6. Sfeir M, Walsh M, Rosa R, Aragon L, Liu SY, Cleary T, Worley M, Frederick C, Abbo LM. 2018. Mycobacterium abscessus complex infections: a retrospective cohort study. Open Forum Infect Dis 5:ofy022. https://doi.org/10.1093/ofid/ofy022.
7. Flume PA, VanDevanter DR. 2015. Clinical applications of pulmonary delivery of antibiotics. Adv Drug Deliv Rev 85:1–6. https://doi.org/10.1016/j.addr.2014.10.009.
8. Gontijo AVL, Brillault J, Grégoire N, Lamarche I, Gobin P, Couet W, Marchand S. 2014. Biopharmaceutical characterization of nebulized antimicrobial agents in rats. 1. Ciprofloxacin, moxifloxacin, and grepafloxacin. Antimicrob Agents Chemother 58:3942–3949. https://doi.org/10.1128/AAC.02818-14.
9. Gontijo AVL, Grégoire N, Lamarche I, Gobin P, Couet W, Marchand S. 2014. Biopharmaceutical characterization of nebulized antimicrobial agents in rats. 2. Colistin. Antimicrob Agents Chemother 58:3950–3956. https://doi.org/10.1128/AAC.02819-14.
10. Marchand S, Grégoire N, Brillault J, Lamarche I, Gobin P, Couet W. 2016. Biopharmaceutical characterization of nebulized antimicrobial agents in rats. 4. Aztreonam. Antimicrob Agents Chemother 60:3196–3198. https://doi.org/10.1128/AAC.00165-16.
11. Marchand S, Grégoire N, Brillault J, Lamarche I, Gobin P, Couet W. 2015. Biopharmaceutical characterization of nebulized antimicrobial agents in rats. 3. Tobramycin. Antimicrob Agents Chemother 59:6646–6647. https://doi.org/10.1128/AAC.01647-15.
12. Marchand S, Boisson M, Mehta S, Adier C, Mimoz O, Grégoire N, Couet W. 2018. Biopharmaceutical characterization of nebulized antimicrobial agents in rats. 6. Aminoglycosides. Antimicrob Agents Chemother 62:e01261-18. https://doi.org/10.1128/AAC.01261-18.
13. Boisson M, Mimoz O, Hadzic M, Marchand S, Adier C, Couet W, Grégoire N. 2018. Pharmacokinetics of intravenous and nebulized gentamicin in critically ill patients. J Antimicrob Chemother 73:2830–2837. https://doi.org/10.1093/jac/dky239.
14. Levison ME, Levison JH. 2009. Pharmacokinetics and pharmacodynamics of antibacterial agents. Infect Dis Clin North Am 23:791–797. https://doi.org/10.1016/j.idc.2009.06.008.
15. Rominski A, Schulthess B, Müller DM, Keller PM, Sander P. 2017. Effect of β-lactamase production and β-lactam instability on MIC testing results for Mycobacterium abscessus. J Antimicrob Chemother 72:3070–3078. https://doi.org/10.1093/jac/dkx284.
16. Schoutrop ELM, Brouwer MAE, Jenniskens JCA, Ferro BE, Mouton JW, Aarnoutse RE, van Ingen J. 2018. The stability of antimycobacterial drugs in media used for drug susceptibility testing. Diagn Microbiol Infect Dis 92:305–308. https://doi.org/10.1016/j.diagmicrobio.2018.06.015.
17. Lavollay M, Dubée V, Heym B, Herrmann J-L, Gaillard J-L, Gutmann L, Arthur M, Mainardi J-L. 2014. In vitro activity of cefoxitin and imipenem against Mycobacterium abscessus complex. Clin Microbiol Infect 20:O297–O300. https://doi.org/10.1111/1469-0691.12405.
18. Ferro BE, van Ingen J, Wattenberg M, van Soolingen D, Mouton JW. 2015. Time-kill kinetics of antibiotics active against rapidly growing mycobacteria. J Antimicrob Chemother 70:811–817. https://doi.org/10.1093/jac/dku431.
19. Lefebvre A-L, Dubée V, Cortes M, Dorchêne D, Arthur M, Mainardi J-L. 2016. Bactericidal and intracellular activity of β-lactams against Mycobacterium abscessus. J Antimicrob Chemother 71:1556–1563. https://doi.org/10.1093/jac/dkw022.
20. Oberholtzer ER, Brenner GS. 1979. Cefoxitin sodium: solution and solid-state chemical stability studies. J Pharm Sci 68:863–866. https://doi.org/10.1002/jps.2600680720.
21. Greendyke R, Byrd TF. 2008. Differential antibiotic susceptibility of Mycobacterium abscessus variants in biofilms and macrophages compared to that of planktonic bacteria. Antimicrob Agents Chemother 52:2019–2026. https://doi.org/10.1128/AAC.00986-07.
22. Ferro BE, Srivastava S, Deshpande D, Pasipanodya JG, van Soolingen D, Mouton JW, van Ingen J, Gumbo T. 2016. Failure of the amikacin, cefoxitin, and clarithromycin combination regimen for treating pulmonary Mycobacterium abscessus infection. Antimicrob Agents Chemother 60:6374–6376. https://doi.org/10.1128/AAC.00990-16.
23. Brunetti L, Kagan L, Forrester G, Aleksunes LM, Lin H, Buyske S, Nahass RG. 2016. Cefoxitin plasma and subcutaneous adipose tissue concentration in patients undergoing sleeve gastrectomy. Clin Ther 38:204–210. https://doi.org/10.1016/j.clinthera.2015.11.009.
24. Schrogie JJ, Rogers JD, Yeh KC, Davies RO, Holmes GI, Skeggs H, Martin CM. 1979. Pharmacokinetics and comparative pharmacology of cefoxitin and cephalosporins. Rev Infect Dis 1:90–98. https://doi.org/10.1093/clinids/1.1.90.
25. Galindo Bedor DC, Marchand S, Lamarche I, Laroche J, Pereira de Santana D, Couet W. 2016. Biopharmaceutical characterization of nebulized antimicrobial agents in rats. 5. Oseltamivir carboxylate. Antimicrob Agents Chemother 60:5085–5087. https://doi.org/10.1128/AAC.00909-16.
26. Zhang R, Ghosh SN, Zhu D, North PE, Fish BL, Morrow NV, Lowry T, Nanchal R, Jacobs ER, Moulder JE, Medhora M. 2008. Structural and functional alterations in the rat lung following whole thoracic irradiation with moderate doses: injury and recovery. Int J Radiat Biol 84:487–497. https://doi.org/10.1080/09553000802078396.
27. Yapa S, Li J, Patel K, Wilson JW, Dooley MJ, George J, Clark D, Poole S, Williams E, Porter CJH, Nation RL, McIntosh MP. 2014. Pulmonary and systemic pharmacokinetics of inhaled and intravenous colistin methanesulfonate in cystic fibrosis patients: targeting advantage of inhalational administration. Antimicrob Agents Chemother 58:2570–2579. https://doi.org/10.1128/AAC.01705-13.
28. National Committee for Clinical Laboratory Standards. 2003. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes; approved standard. NCCLS document M24-A. National Committee for Clinical Laboratory Standards, Wayne, PA.
29. Keizer RJ, van Benten M, Beijnen JH, Schellens JHM, Huitema A. 2011. Piraña and PCluster: a modeling environment and cluster infrastructure for NONMEM. Comput Methods Programs Biomed 101:72–79. https://doi.org/10.1016/j.cmpb.2010.04.018.
30. Beal SL. 2001. Ways to fit a PK model with some data below the quantification limit. J Pharmacokinet Pharmacodyn 28:481–504. https://doi.org/10.1023/A:1012299115260.
31. Baron KT. 2019. mrgsolve: simulate from ODE-based population PK/PD and systems pharmacology models. https://mrgsolve.github.io/.

Supplementary material:

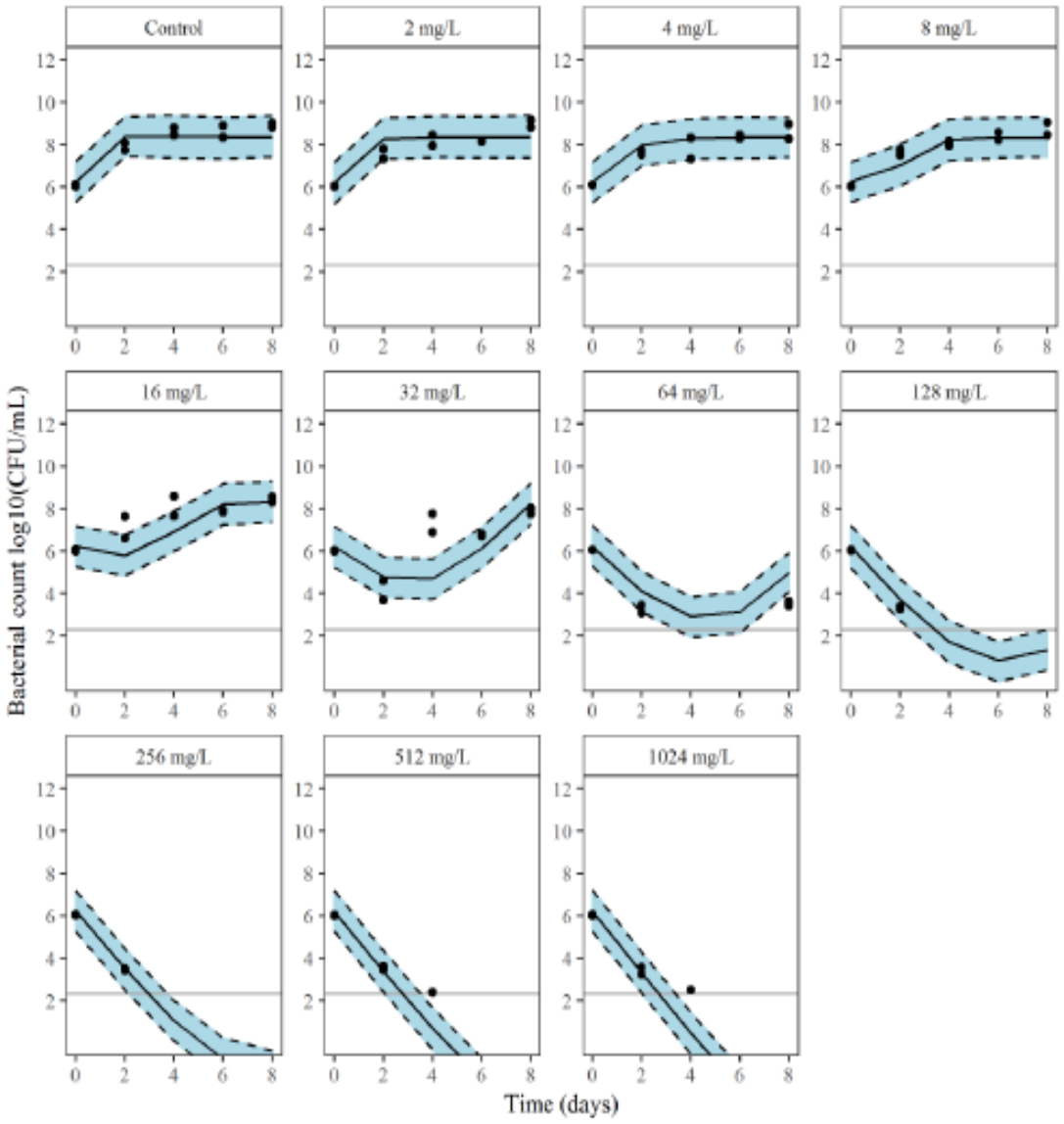

Figure S1: Visual predictive checks (VPCs) for one homogenous population PK/PD type model of cefoxitin against *M. abscessus* CIP 104536 with observed bacterial counts (circles), median (black continuous line) and 80% prediction interval (black dotted line) of simulated data. Plots include growth control and experimental data by time-kill kinetics. The indicated concentration are the initial FOX concentrations. Line shows the limit of quantification (200 CFU/mL).

4.2 Antibiotic combinations

Article 3: Assessment of *in vitro* efficacy of cefoxitin and amikacin in combination using modelling approach against *Mycobacterium abscessus*

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Résume en français :

La prévalence croissante mondiale des infections à *Mycobacterium abscessus* oblige les cliniciens à prescrire des combinaisons d'antibiotiques pour traiter ces bactéries particulièrement résistantes. Le traitement actuel consiste en une association de clarithromycine, d'amikacine et de céfoxitine qui est souvent associé à un fort taux d'échec et au développement de résistances. Actuellement, la méthode d'analyse de l'efficacité d'un traitement antibiotiques en association consiste à comparer l'effet observé à l'effet attendu à l'aide de modèles de référence. Qui permettent différentes interprétation hypothèses d'des synergies. Dans un premier temps, des données expérimentales provenant de m méthodes de checkerboard et de E-test ont été utilisées pour évaluer la synergie entre la céfoxitine et l'amikacine. Dans un deuxième temps, l'interprétation de la synergie a été faite à partir des données expérimentales des courbes de bactéricidies à l'aide d'une méthode indépendante puis un modèle pharmacocinétique/pharmacodynamique mécanistique a été mis au point pour interpréter les données des courbes de bactéricidies de la céfoxitine et de l'amikacine seule ou en association. Les modèles fondés sur des mécanismes fournissent une approche pour décrire l'évolution de l'effet de différents antibiotiques à l'aide de leur mécanisme d'action et de la sensibilité des pathogènes. Pour chaque approche, l'association de céfoxitine et d'amikacine a été montrée additive ou synergique, à différentes concentrations, contre *M. abscessus*. Cette approche permet de comprendre l'efficacité de nouvelles thérapies combinées.

Abstract

The increasing global prevalence of difficult to treat *mycobacterium abscessus* is forcing clinicals to prescribe combination antibiotic regimens to treat such infections. Current combined treatment consisting clarithromycin, amikacin and cefoxitin is often associated with treatment failure and resistance. Currently, the joint activity of a combined regimen is analyzed by comparing the observed effect versus expected effect using reference models. Using such reference models, different assumptions for synergy interpretations are made. In a first example experimental data from checkerboard and E-test method was used to evaluate synergy between cefoxitin and amikacin. In a second example, synergy interpretation was made using a model independent method from the experimental data from time-kill kinetics assay. Then, a pharmacokinetics/pharmacodynamics model was developed using mechanism-based mathematical model and a single agent effect and combined regimen effect data obtained from time-kill kinetics assay evaluating cefoxitin and amikacin regimens against these multidrug resistance bacteria. Mechanism-based models provide an approach for describing time course of effect for different antibiotics using their mechanism of actions and pathogen susceptibilities. Using each approach, combined regimen of cefoxitin and amikacin was additive to synergistic at different concentrations against *M. abscessus*. This approach allows to understand the design of effective combination therapies.

Introduction

The rapid emergence and spread of pulmonary infections due to multidrug-resistant (MDR) *Mycobacterium abscessus* emulates a major health issues (1) in terms of disease burden with diminished antibiotic efficiency and clinical response rate (2). Currently, combined therapy including cefoxitin (FOX), amikacin (AMK) and clarithromycin (CLR) is the recommended therapeutic regimen, but is often associated with CLR based intrinsic resistance (3). On other hand, the world is running out of new antibiotics. To win this battle between shortage of new antibiotics and MDR *M. abscessus* infections, it is essential to develop an interest in repurposing the existing antibiotics using antibiotic combination therapy with strategic route of administration. Combination therapy favors efficacy of antibiotics, diminishes toxicity and reduces therapeutic cost by reducing dosage of each antibiotic (4). Moreover, pre-clinical data of FOX (5) and AMK (6) reported these both antibiotics as a promising agent for nebulization (NEB), which offers to achieve high lung concentration and low plasma concentrations. Hence, despite of using single active antibiotic alone, use of two active nebulized regimes in combination can improve treatment efficacy against *M. abscessus* pulmonary infections as already mentioned (7). While dealing with combination therapies, the ability to qualify the interaction in terms of synergy, additivity and antagonism is necessary. These interactions can be characterized by the comparison of observed effect against expected effect in combination using reference models (2). There are largely two reference models i.e. Bliss Independence model and Loewe Additivity model (2, 8) to define how independent effects combine. Bliss independence model works on assumption that two antibiotics act independently from each other, in contrast Loewe additivity assumes that combined antibiotics acts on the same pathway or target by an identical mechanism of action.

As FOX and AMK are two major backbones of the treatment of infections caused by *M. abscessus* (9, 10), *in vitro* activity of FOX and AMK alone and in combination at various concentrations was compared in this study. A model independent approach was used to compare effects of FOX and AMK in combination to their effects as single agents (2). Moreover, FOX and AMK effects were modelled as single agents and then the effects observed in combinations were compared to the effects expected under both Loewe and Bliss hypothesis. The prime objective of this study was to evaluate whether combination therapy of FOX and AMK is more advantageous than monotherapy or not, using different reference models.

Results

Antimicrobial susceptibility tests: Minimum inhibitory concentrations (MIC) of FOX and AMK were found to be 8 mg/L and 32 mg/L respectively by broth microdilution method and E-test method.

Synergy test and interpretation: The combination of FOX and AMK exhibited additive effect by checkerboard method with FIC value of 1 and also by E-test method for combination with FIC value of 1.375 against *M. abscessus* CIP104536.

Time-kill kinetics assay: For FOX, the killing activity was determined with 50% amount addition each 24 h to compensate the degradation. As an individual agent, FOX showed bacterial killing above 8*MIC concentration and reached detection limit over 8 days (Figure 1A), the concentrations below 2*MIC exhibited immediate regrowth from the beginning, however the FOX concentration at 4*MIC showed bacteriostatic activity. Similarly, AMK exhibited efficient killing above 4*MIC (Figure 1B), and below the concentration at 4*MIC, initial bacterial killing was observed followed by bacterial regrowth. AMK exhibited concentration-dependent activity against *M. abscessus*. For combined regimens, bacterial killing was observed above 2*MIC of each antibiotic in combination. The combined concentrations of both antibiotic above 4*MIC showed complete bacterial killing by reaching the detection limit at day 4 (Figure 2).

Model independent method: Based on *model independent method*, predicted activity of each combined regimen is presented in log ratio in the Table I. The LR of static regimen was found to be 2.2. From predicted activity for each combined regimen compared to reference (growth control) and compared to the highest single agent (AMK), combinations of FOX and AMK below 2*MIC had LR less than -2.2 and combinations of FOX and AMK above 2*MIC had LR higher than -2.2. Thus, it can be concluded that combinations of FOX and AMK above 2*MIC exhibited enhanced effect than "static regimen".

Model dependent approaches: The PK/PD models used to assess FOX and AMK effects as single agents are presented in Figure S1. Observations fitted well both models as illustrated by VPCs presented on Figure S2 for FOX and Figure S3 for AMK. Pharmacodynamics parameters estimated from the final PK/PD model are presented in Table S1. Maximum effect for estimated to be identical for FOX and AMK (I_{max}=1).

CFUs observed when FOX and AMK were in combination overlaid with Bliss independence prediction (orange) or the Loewe additivity prediction (Blue) are presented on Figure 4. As observations were close to prediction for both Loewe and Bliss hypothesis, the interaction between FOX and AMK can be considered as additive over time, for concentrations ranging between 0.5*MIC and 16*MIC.

Discussion

This study provides fundamental new information on the pharmacodynamic relationship between antibiotic concentration and mycobacterial population dynamics for *M. abscessus.* The complete bacterial killing was observed over the 4*MIC concentration of each antibiotic (FOX at 32 mg/L with AMK at 128 mg/L). As recommended by Greendyke *et al.* (7) to use FOX and AMK in combination at high concentration to increase their efficacy at target site and abate the resistance development, this study was performed to evaluate the combined activity of FOX and AMK. This concentration of AMK can only be achieved using nebulization (6) as reported previously in healthy rats. Also, pre-clinical data of FOX confirmed its good ability of being used as nebulization to achieve high lung drug concentration (5). Olivier *et al.* (11) has reported that despite of being use of inhaled amikacin in addition to standard therapy for several non-tuberculosis mycobacterium pulmonary infections, relapse occurred in few patients in their retrospective study, but in some patients, inhaled AMK was proved to be effective and therefore they have suggested to use inhaled AMK in combination with other effective antimycobacterial agent. AMK has also shown good *in vitro* activity against *M. abscessus* in hollow-fiber system (9). Furthermore, bactericidal activity of AMK has been reported at very high concentration of 512 mg/L (or 16*MIC), but this concentration is not achievable in humans (12). Thus, FOX and AMK both antibiotics can be more efficient by nebulized form, to achieve high lung drug concentration at target site as supported by pre-clinical data.

On the other hand, FOX is known for its limited *in vitro* activity with the degradation half-life of 1.5 days at 35±2°C in 7H9 broth and it also parades bacterial killing based on T>MIC, which means that to achieve bacterial killing by FOX, it is necessary to maintain certain concentration for longer period of time. Also, the *in vitro* MIC determination and time-kill data interpretation may be misleading because of its instability as also reported by Rominski *et al.* (13) and Schoutrop *et al.* (14). Therefore, to compensate this degradation, 50% amount of FOX was added each 24h throughout the experiment.

Usually, research in the area of combined regimes has focused on demonstrating superiority of combined regimens over monotherapy and the interpretation of the combined effect can be done using semi-mechanistic PK/PD modeling approach. In the present study, FOX and AMK were combined at various concentrations using different approaches like checkerboard method, E-test method and time-kill kinetics method. But using checkerboard and E-test method, several fixed concentrations can be analyzed at fixed time range while using time-kill kinetics method, assessment of resistance emergence can be performed, which can not be case with other methods. Then, the effect of combination was quantified by comparing the observed combined effect with predicted effect using a model independent LR method, Bliss independence model and Loewe additivity model. The model independent method is based on the calculation of static regimen and then comparing LR of combined effect by the LR of static regimen. Other two models use different assumptions for synergy interpretation, where the main difference is based on the additivity and "no interaction" between two antibiotics in combination. Bliss independence model is based on the assumption that antibiotics have distinct mechanisms of actions or target, antibiotics do not affect each other. Moreover, antibiotic A (FOX) is equally active against *M. abscessus* that are sensitive or resistant to antibiotic B (AMK) and similar for antibiotic B. On the contrary, Loewe additivity model assumes that combined antibiotics have the same mechanism of actions or target, here doses are additive (2). Here, the combined effect was additive by both reference models. Experimental analysis by checkerboard method and E-test method resulted in additive effect, which is consistent with the results obtained by *Ferro et al* (15) for FOX and AMK combination by checkerboard analysis and Lefebvre *et al.*, where FOX was equivalent to AMK and combination of both was not much active that AMK alone.

Despite of showing additive to synergistic effect using combined FOX and AMK, this experimental study, carried out over 8 days, which is comparatively longer than other reported *in vitro* studies (12, 16) has one limitation. FOX and AMK was tested only at fixed-ratio concentrations, which prohibited the investigation of complex pharmacodynamic interactions.

It is noteworthy that the combination effect of FOX and AMK is additive to synergistic but, the addition of both these molecules in nebulized form to the standard therapy or with another oral antibiotic from fluoroquinolones, Oxazolidinone, or clofazimine based on susceptibility testing may warrant a "renaissance" of the treatment of pulmonary infections caused by *M. abscessus.*

Materials and method

Bacterial strain: In this study, *M. abscessus subspecies abscessus* reference strain CIP 104536 (Collection of Institute Pasteur, Paris, France) was used. *M.abscessus* was grown on Middlebrook 7H11 agar plates (referred as 7H11 agar plates) with 10% oleic acid/bovine albumin/dextrose/catalase (OADC) growth supplement (BD, BBL^{TM}, Sparks, MD, USA) and 0.5% glycerol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at 30°C for 3-5 days. Stock vials were presevered at -80°C in Middlebrook 7H9 broth (referred as 7H9 broth) with 10% OADC growth supplement and 20% glycerol and was thawed for each experiment.

Antibiotics: FOX was obtained from Panpharma (Luitré, France) and AMK was obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Aqueous stock solutions were prepared for both antibiotics and stored at -80°C. Prior to each experiment, one aliquot was thawed to prepare the different concentrations to be tested.

Antimicrobial susceptibility tests: MICs were determined by broth microdilution method and epsilometric (E-test) method. In 96-well plates, Cation-adjusted Mueller–Hinton broth 2 (referred as CAMHB II; Sigma-Aldrich, Saint-Quentin-Fallavier France) supplemented with 10% oleic acid/bovine albumin/dextrose/catalase (OADC) growth supplement (BD, BBL^{TM}, Sparks, MD, USA) and 0.5% glycerol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) with a final inoculum of approximately 10^6 CFU/mL was used for broth microdilution method as recommended in CLSI guidelines (17). 7H11 agar plates were inoculated with bacteria was used to place E-test strips of FOX and AMK for MIC determination by E-test method (Figure 5A). Plates were incubated at 30°C for 3 days (17) and the growth was evaluated by visual inspection. All experiments were performed in duplicate.

Synergy test and interpretation: Synergy between FOX and AMK was evaluated using checkerboard, E-test method and time-kill kinetics assay. Checkerboard and E-test method define synergy at a given time while time-kill kinetics assay allow to define synergy over time.

Checkerboard titration assay: The checkerboard titration assay is the two-dimensional representation of the broth microdilution assay for two different antibiotics. Required solutions of FOX and AMK were prepared in 7H9 broth and then antibiotics were added to 96-well plates containing 7H9 broth, each starting at 2-times higher than MICs and serially diluted 2-fold, so all possible 2-fold dilution combinations above and below the respective MICs were represented. Then, 10^6 CFU/mL bacterial inoculum was inoculated into each well. Positive and negative control were included. The cultures were incubated at 30°C (17) and evaluated for

growth by visual inspection after 3 days. The fractional inhibitory concentration (FIC) of each antibiotic in combination was determined as described previously (18). The FIC index was calculated for each combination of antibiotics that inhibited *M. abscessus* growth. FIC index of ≤ 0.5 was interpreted as synergy, $0.5 < FIC < 2.0$ as additive or indifference and ≥ 2 as antagonism.

E-test method: Once MICs for FOX and AMK individually were obtained using E-test method against *M. abscessus* CIP104536, *in vitro* activity of combined FOX and AMK was determined by placing E-test strips on inoculated 7H11 agar plates at 90° angle with the intersection at respective MICs as shown in Figure 5B (19). The agar plates were incubated at 30°C for 3 days. Consequently, MICs for each antibiotic in combination was measured. From the results of MICs obtained with antibitoics alone and in combination, the FIC was calculated and based on the FIC index, effects were interpreted.

Time-kill kinetics assay: For Single antibiotics: As a first set of experiments, time-kill kinetics assays of FOX (with 50% FOX amount addition each 24 h to compensate FOX degradation, as FOX follows first order-degradation with a half-life of 1.5 days (data not shown)) (13, 14) and AMK as monotherapy were performed. Individual tubes containing 20 mL of 7H9 broth for 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 times of MIC of each antibiotic were cultured with the inoculum $1*10^6$ CFU/mL at 35±2°C, under shaking conditions (150 rpm). At defined time intervals (0, 2, 4, 6 and 8 days), the bacterial population was quantified to characterize the effect of the different antibiotics at different concentrations. From each tube, 100 µL samples were taken and required serial dilutions in phosphate buffer saline (PBS pH 7.4, Glibco™, by life technologies, France) were prepared. Volumes of 100 µL from undiluted samples and from each dilution were plated in on 7H11 agar plates for further CFUs counting after 3–5 days of incubation at 35±2°C. The theoretical detection limit was set to 200 CFU/mL (i.e. 2.3 $\log_{10}$ CFU/mL). All experiments were performed in duplicate. *For antibiotics in combination:* As a second set of experiments, following the same procedure of time-kill assay for single antibiotics, combined antibiotics assay having FOX (with 50% FOX amount addition each 24h) in combination with AMK at multiple times of MICs were tested in following combinations: 0.5*MIC of FOX with 0.5*MIC of AMK, 1*MIC of FOX with 1*MIC of AMK, 2*MIC of FOX with 2*MIC of AMK, 4*MIC of FOX with 4*MIC of AMK, 8*MIC of FOX with 8*MIC of AMK and 16*MIC of FOX in combination with 16*MIC of AMK.

Curve fitting and data analysis: For data interpretation of combined regimens in time-kill assays, both model independent (2) and model dependent approaches with Bliss Independence or Loewe Additivity hypothesis were used (2, 8).

Model independent method for data interpretation of combination regimens: For each regimen, total duration of the study, i.e. 8 days. AUCFU were calculated by a trapezoidal method. Effects of FOX and AMK ($AUCFU_{test}$) in combination were compare to different reference regimens using equation 22:

$$\text{LR} = Log_{10}\left[\frac{AUCFU_{test}}{AUCFU_{reference}}\right] \quad (1)$$

As a reference regimen, AUCFU of control (no antibiotic), of a static regimen (CFU constant, *i.e.* equal to the inoculum over the whole assay duration), and of FOX and AMK used as single agents, were considered. A negative value of LR meant that the test regimen (combination) was more effective than the reference regimen. From that approach, synergy was characterized when combination was more effective than the highest single agent.

For model dependent approaches, in a first step data were modelled for single agents and then predictions for combinations were made under either Loewe additivity or Bliss independence hypothesis.

Modelling for single agents. Data were analyzed using a nonlinear mixed-effects PK/PD model. Parameters for each antibiotic were estimated using the first-order conditional estimation (FOCE) method and Laplacian option available in NONMEM version 7.4.1 (Icon Development Solutions Ellicott City, MD, USA, 2009). NONMEM project management was made easier using Pirana (20), CFU counts below the limit of quantification (200 CFU/mL) were handled using Beal's M3 method (21).

Pharmacokinetic modelling

The decrease of FOX concentrations (C_{FOX}) due to degradation during the experiment was modelled independently from pharmacodynamics (CFU) as a first order process:

$$C_{FOX} = C_0 e^{-K_e \times t} \quad (2)$$

Where, C_0 is the initial theoretical concentration of FOX (mg/L) spiked in the tube at time 0 and k_e is the first order degradation rate constant of FOX (day^{-1}).

Pharmacodynamic modelling

In absence of antibiotic, bacteria were assumed to grow until reaching a plateau, and this was described by a logistic growth function. Re-growth of bacteria observed, whether with FOX or AMK, were described by using two bacterial subpopulations with different susceptibilities to single antibiotics. The fraction of bacteria belonging to the "resistant" subpopulation at time 0 ($MUTF_{FOX}$ or $MUTF_{AMK}$ depending on the antibiotic) was estimated. The total bacterial load (B, CFU/mL) was obtained by the summing both subpopulations.

The effect of each antibiotic alone on *M. abscessus* CIP104536 was modelled as an inhibition of bacterial growth, with an I_{max} model, and different IC_{50} for each subpopulation. The structure of the PD model is presented on Figure S1, the differential equations describing variations of susceptible bacterial counts (FOX_S and AMK_S) and resistant bacterial counts (FOX_R and AMK_R) over time are presented below:

$$\frac{dS_{FOX}}{dt} = k_g \times \left(1 - \frac{B}{10^{Bmax}}\right) \times \left(1 - \frac{I_{max} \times C_{FOX}^{\gamma_F}}{IC_{50FOX_S}^{\gamma_F} + C_{FOX}^{\gamma_F}}\right) \times FOX_S - k_d \times FOX_S \quad (3)$$

$$\frac{dR_{FOX}}{dt} = k_g \times \left(1 - \frac{B}{10^{Bmax}}\right) \times \left(1 - \frac{I_{max} \times C_{FOX}^{\gamma_F}}{IC_{50FOX_R}^{\gamma_F} + C_{FOX}^{\gamma_F}}\right) \times FOX_R - k_d \times FOX_R \quad (4)$$

$$\frac{dS_{AMK}}{dt} = k_g \times \left(1 - \frac{B}{10^{Bmax}}\right) \times \left(1 - \frac{I_{max} \times C_{AMK}^{\gamma_A}}{IC_{50AMK_S}^{\gamma_A} + C_{AMK}^{\gamma_A}}\right) \times AMK_S - k_d \times AMK_S \quad (5)$$

$$\frac{dR_{AMK}}{dt} = k_g \times \left(1 - \frac{B}{10^{Bmax}}\right) \times \left(1 - \frac{I_{max} \times C_{AMK}^{\gamma_A}}{IC_{50AMK_R}^{\gamma_A} + C_{AMK}^{\gamma_A}}\right) \times AMK_R - k_d \times AMK_R \quad (6)$$

Where, k_g (day^{-1}) was the bacterial growth rate, k_d (day^{-1}) the bacterial natural death rate, B_{max} ($\log_{10}$CFU/mL) the maximum population size supported by the environment, I_{max} the maximal inhibition, IC_{50FOX_S} and IC_{50FOX_R} (mg/L) were the concentration of FOX for which the effect was 50% of I_{max} for susceptible and resistant bacteria, respectively. IC_{50AMK_S} and IC_{50AMK_R} (mg/L) were the concentration of AMK for which the effect was 50% of I_{max} for susceptible and resistant bacteria, respectively. γ_F and γ_A were the Hill-factors for growth inhibition due to the activity of FOX and AMK, respectively.

The residual variability was described by an additive error model on $\log_{10}$ scale for bacterial count data and by a proportional error model for concentrations of FOX data. No inter-experimental variability was estimated on the parameters.

Discrimination between models was mainly based on the inspection of graphical diagnostics and changes in the objective function value (OFV) provided by NONMEM, which is minus twice the log-likelihood. For a more complicated model to be retained it had to provide a significant improvement over the contending model ($p<0.05$ for nested models) and provide plausible parameter estimates that were not associated with excessively high relative standard errors (RSE). In case of non-nested models, Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values were used to discriminate between models.

Model's performances were assessed by the evaluation of the goodness of fit plots, i.e. observation versus predictions and residuals versus time and predictions. Moreover, the model was evaluated by performing visual predictive checks (VPCs) with stratification on the type of experiments and FOX concentration. For that, observations were plotted and overlaid with the median and 80% prediction intervals obtained by performing 1000 simulations of the final model with original dataset as input.

Predictions in combination. The PD model for the simulation in combination was pooled from two models for FOX and AMK as single agent (Figure 3). It included four bacterial subpopulations; one susceptible to amikacin and susceptible to cefoxitin (AMK_S/FOX_S), one susceptible to amikacin and resistant to cefoxitin (AMK_S/FOX_R), one resistant to amikacin and susceptible to cefoxitin (AMK_R/FOX_S), and one resistant to amikacin and resistant to cefoxitin (AMK_R/FOX_R). The differential equations were as follow:

$$\frac{d_{AMK_S/FOX_S}}{dt} = k_g \times \left(1 - \frac{B}{10^{Bmax}}\right) \times E_{com\_AMK_S/FOX_S} \times A_{AMK_S/FOX_S} - k_g \times A_{AMK_S/FOX_S} \quad (7)$$

$$\frac{d_{AMK_S/FOX_R}}{dt} = k_g \times \left(1 - \frac{B}{10^{Bmax}}\right) \times E_{com\_AMK_S/FOX_R} \times A_{AMK_S/FOX_R} - k_g \times A_{AMK_S/FOX_R} \quad (8)$$

$$\frac{d_{AMK_R/FOX_S}}{dt} = k_g \times \left(1 - \frac{B}{10^{Bmax}}\right) \times E_{com\_AMK_R/FOX_S} \times A_{AMK_R/FOX_S} - k_g \times A_{AMK_R/FOX_S} \quad (9)$$

$$\frac{d_{AMK_R/FOX_R}}{dt} = k_g \times \left(1 - \frac{B}{10^{Bmax}}\right) \times E_{com\_AMK_R/FOX_R} \times A_{AMK_R/FOX_R} - k_g \times A_{AMK_R/FOX_R} \quad (10)$$

Parameter values used for simulation were those estimated for single agents. Effects of FOX and AMK in combination ($E_{com\_AMK/FOX}$) depended on the hypothesis.

For Loewe hypothesis, the maximum effect had to be identical for both drugs, which was verified for FOX and AMK (I_{max}). Effects were as follows:

$$E_{com_{AMK_S/FOX_S}} = 1 - \frac{I_{max} \times \left(\frac{C_{AMK}}{IC_{50AMK_S}} + \frac{C_{FOX}}{IC_{50FOX_S}}\right)^{\gamma}}{1 + \left(\frac{C_{AMK}}{IC_{50AMK_S}} + \frac{C_{FOX}}{IC_{50FOX_S}}\right)^{\gamma}} \quad (11)$$

$$E_{com_{AMK_S/FOX_R}} = 1 - \frac{I_{max} \times \left(\frac{C_{AMK}}{IC_{50AMK_S}} + \frac{C_{FOX}}{IC_{50FOX_R}}\right)^{\gamma}}{1 + \left(\frac{C_{AMK}}{IC_{50AMK_S}} + \frac{C_{FOX}}{IC_{50FOX_R}}\right)^{\gamma}} \quad (12)$$

$$E_{com_{AMK_R/FOX_S}} = 1 - \frac{I_{max} \times \left(\frac{C_{AMK}}{IC_{50AMK_R}} + \frac{C_{FOX}}{IC_{50FOX_S}}\right)^{\gamma}}{1 + \left(\frac{C_{AMK}}{IC_{50AMK_R}} + \frac{C_{FOX}}{IC_{50FOX_S}}\right)^{\gamma}} \quad (13)$$

$$E_{com_{AMK_R/FOX_R}} = 1 - \frac{I_{max} \times \left(\frac{C_{AMK}}{IC_{50AMK_R}} + \frac{C_{FOX}}{IC_{50FOX_R}}\right)^{\gamma}}{1 + \left(\frac{C_{AMK}}{IC_{50AMK_R}} + \frac{C_{FOX}}{IC_{50FOX_R}}\right)^{\gamma}} \quad (14)$$

For Bliss hypothesis:

$$E_{com_{AMK_S/FOX_S}} = 1 - \left(\frac{I_{max_{AMK}} \times C_{AMK}^{\gamma}}{IC_{50AMK_S}^{\gamma} + C_{AMK}^{\gamma}} + \frac{I_{max_{FOX}} \times C_{FOX}^{\gamma}}{IC_{50FOX_S}^{\gamma} + C_{FOX}^{\gamma}} - Min\left(I_{max_{FOX}}; I_{max_{AMK}}\right) \times \frac{C_{AMK}^{\gamma}}{IC_{50AMK_S}^{\gamma} + C_{AMK}^{\gamma}} \times \frac{C_{FOX}^{\gamma}}{IC_{50FOX_S}^{\gamma} + C_{FOX}^{\gamma}}\right) \quad (15)$$

$$E_{com_{AMK_S/FOX_R}} = 1 - \left(\frac{I_{max_{AMK}} \times C_{AMK}^{\gamma}}{IC_{50AMK_S}^{\gamma} + C_{AMK}^{\gamma}} + \frac{I_{max_{FOX}} \times C_{FOX}^{\gamma}}{IC_{50FOX_R}^{\gamma} + C_{FOX}^{\gamma}} - Min\left(I_{max_{FOX}}; I_{max_{AMK}}\right) \times \frac{C_{AMK}^{\gamma}}{IC_{50AMK_S}^{\gamma} + C_{AMK}^{\gamma}} \times \frac{C_{FOX}^{\gamma}}{IC_{50FOX_R}^{\gamma} + C_{FOX}^{\gamma}}\right) \quad (16)$$

$$E_{com_{AMK_R/FOX_S}} = 1 - \left(\frac{I_{max_{AMK}} \times C_{AMK}^{\gamma}}{IC_{50AMK_R}^{\gamma} + C_{AMK}^{\gamma}} + \frac{I_{max_{FOX}} \times C_{FOX}^{\gamma}}{IC_{50FOX_S}^{\gamma} + C_{FOX}^{\gamma}} - Min\left(I_{max_{FOX}}; I_{max_{AMK}}\right) \times \frac{C_{AMK}^{\gamma}}{IC_{50AMK_R}^{\gamma} + C_{AMK}^{\gamma}} \times \frac{C_{FOX}^{\gamma}}{IC_{50FOX_S}^{\gamma} + C_{FOX}^{\gamma}}\right) \quad (17)$$

$$E_{com_{AMK_R/FOX_R}} = 1 - \left(\frac{I_{max_{AMK}} \times C_{AMK}^{\gamma}}{IC_{50AMK_R}^{\gamma} + C_{AMK}^{\gamma}} + \frac{I_{max_{FOX}} \times C_{FOX}^{\gamma}}{IC_{50FOX_R}^{\gamma} + C_{FOX}^{\gamma}} - Min\left(I_{max_{FOX}}; I_{max_{AMK}}\right) \times \frac{C_{AMK}^{\gamma}}{IC_{50AMK_R}^{\gamma} + C_{AMK}^{\gamma}} \times \frac{C_{FOX}^{\gamma}}{IC_{50FOX_R}^{\gamma} + C_{FOX}^{\gamma}}\right) \quad (18)$$

Experimental CFU data in combination were overlaid with simulations under the different hypothesis. When CFU observed in combination were close to Loewe additivity line or Bliss independence line, the combination was considered as additive, whereas when observations were below predictions the interaction was considered as synergistic, and when they were above predictions the interaction was considered as antagonistic.

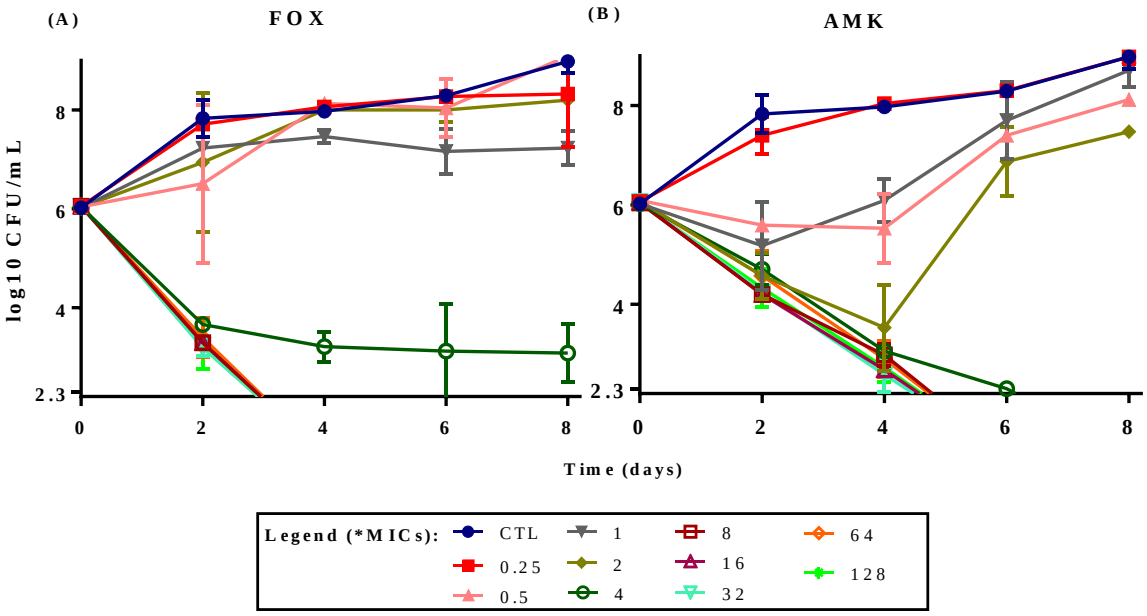

Figure 1: Time-kill curves of *Mycobacterium abscessus* CIP104536 exposed to (A) FOX and (B) AMK. Concentrations of both antibiotics are in multiples of MICs and indicated by different symbols. For cefoxitin, 50% FOX amount was added each 24h to compensate degradation. The ordinate shows the change in the number of CFU (log_{10} scale) per ml of broth. The dotted horizontal line shows the limit of quantification (200 CFU/mL). Results represent mean ± SD of two individual experiments, when not visible, error bars are smaller than the symbols

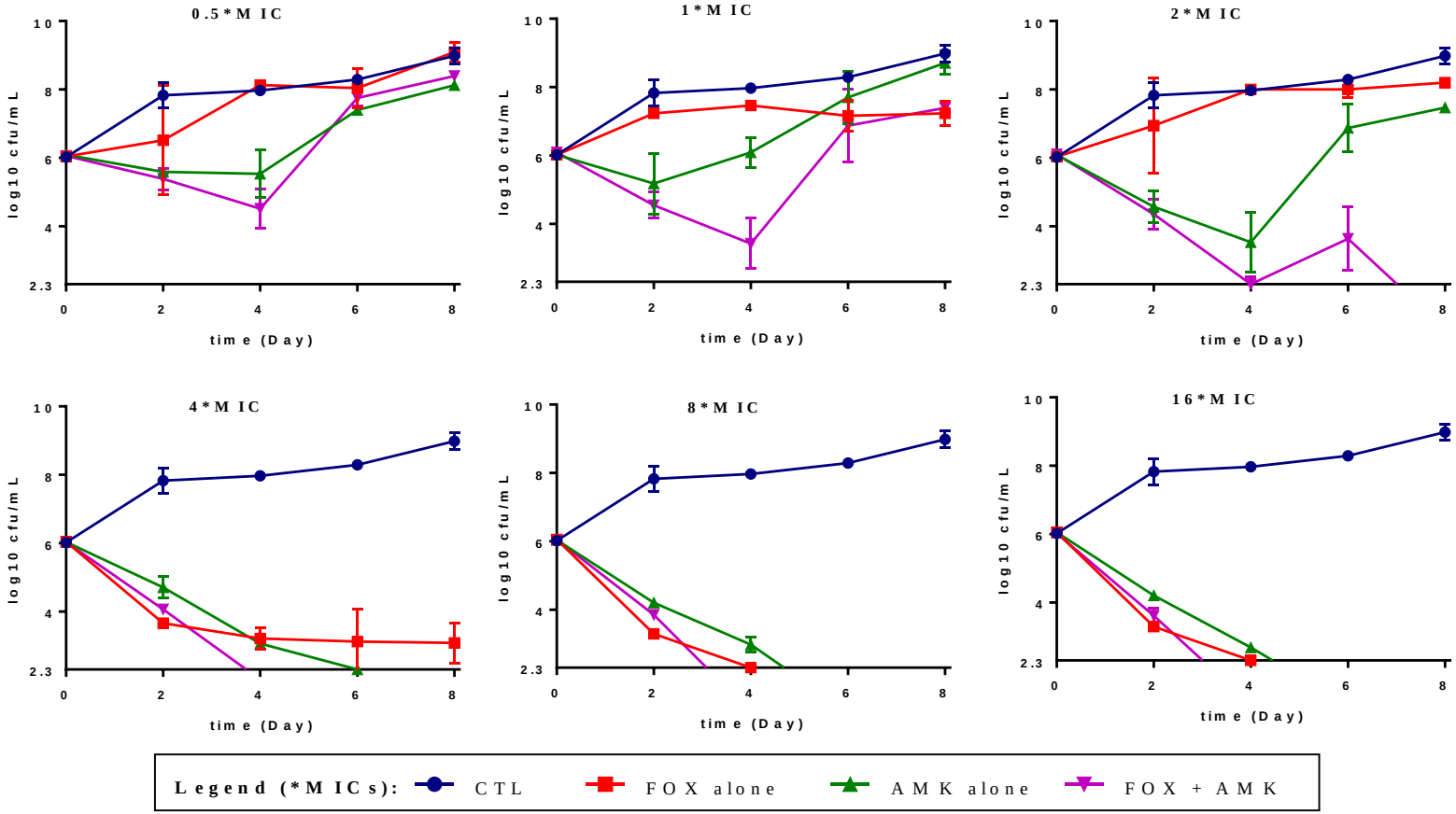

Figure 2: Time-kill curves of *Mycobacterium abscessus* CIP104536 exposed to combined cefoxitin and amikacin at 0.5*MIC, 1*MIC, 2*MIC, 4*MIC, 8*MIC and 16*MIC of each antibiotic. 50% FOX amount was added each 24h to compensate FOX degradation. The dotted horizontal line shows the limit of quantification (200 CFU/mL). Results represent mean ± SD of two individual experiments, when not visible, error bars are smaller than the symbols.

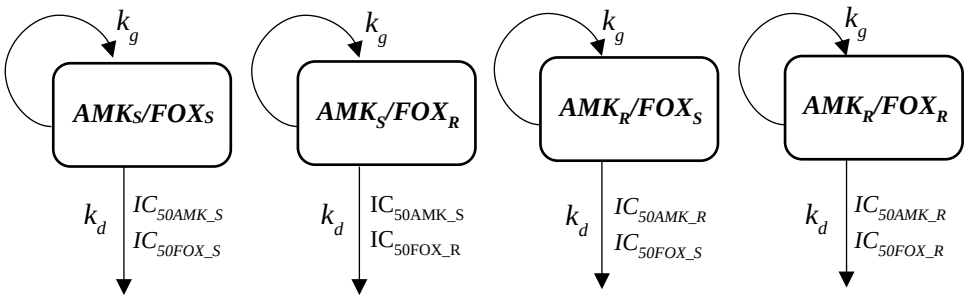

Figure 3: A schematic diagram of the final PK/PD model for FOX and AMK in combination against *M. abscessus*. Model included four subpopulations: one susceptible to AMK and susceptible to FOX (AMK_S/FOX_S), one susceptible to AMK and resistant to FOX (AMK_S/FOX_R), one resistant to AMK and susceptible to FOX (AMK_R/FOX_S), and one resistant to AMK and resistant to FOX (AMK_R/FOX_R). Both antibiotics were modelled as inhibiting k_g. Bacteria multiplied with a first-order rate constant (k_g) in all four compartments. Each subpopulation had different IC_{50} values. All bacteria had natural death-rate (k_d). FOX followed first-order degradation (k_e).

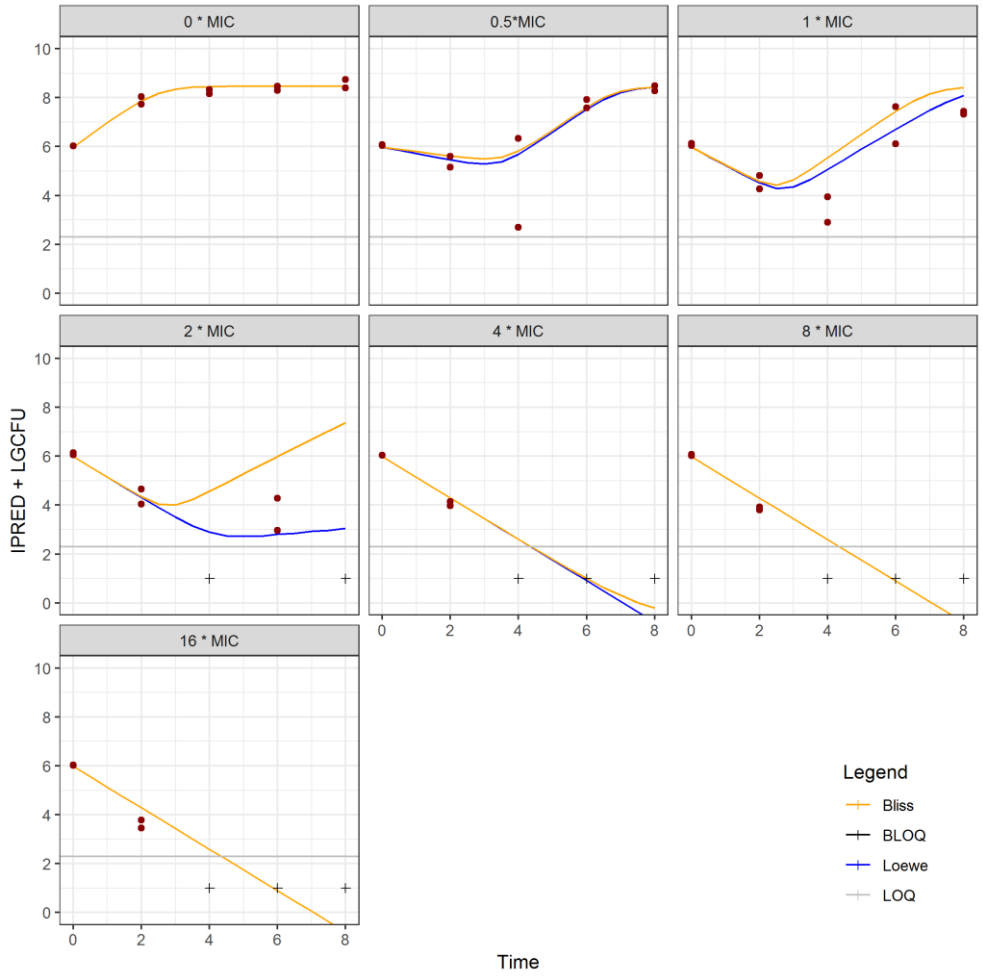

Figure 4: Observed versus fitted time-kill curves of *M. abscessus* CIP104536 exposed to FOX and AMK in combination. All data were simulated assuming Bliss independence (orange line) and Loewe additivity (blue line). Closed symbols represent observed bacterial counts (log_{10}CFU/mL) of combination experiment. The fitted functions are based on the monotherapy estimations and assumptions by independence and additivity.

(A) **(B)**

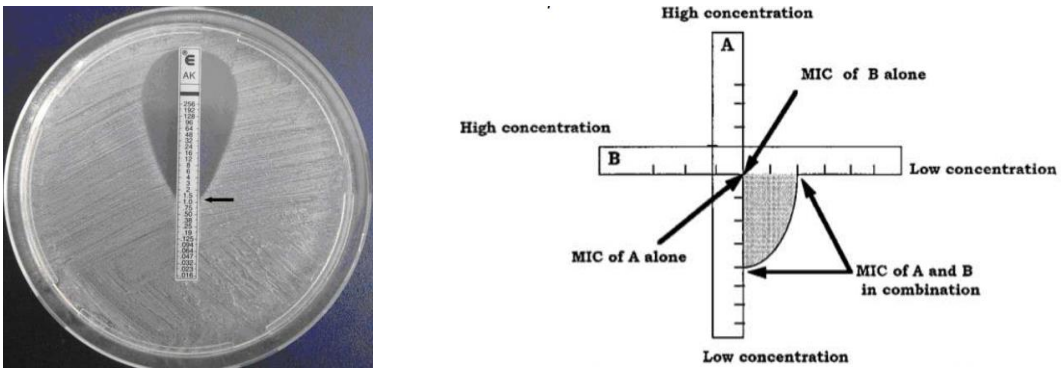

Figure 5: (A) AST by E-test method for single antibiotic, where arrow indicated the value for MIC (B) set up on agar plate for E-test combination testing

Table I: Predicted activity in terms of log ratio for each FOX and AMK combination as compared with growth control & highest single agent

FOX+AMK combination @	Predicted activity in terms of log ratio for FOX+AMK combination as compared to growth control	Predicted activity in terms of log ratio for FOX+AMK combination as compared to highest single agent (AMK)
0.5*MIC	-1	0.30
1*MIC	-1.31	-1.05
2*MIC	-3.02	-1.62
4*MIC	-3.11	-0.04
8*MIC	-3.10	-0.01
16*MIC	-3.13	-0.04

References

1. Lee M-R, Sheng W-H, Hung C-C, Yu C-J, Lee L-N, Hsueh P-R. 2015. Mycobacterium abscessus Complex Infections in Humans. Emerg Infect Dis 21:1638–1646.

2. Rao GG, Li J, Garonzik SM, Nation RL, Forrest A. 2018. Assessment and modelling of antibacterial combination regimens. Clin Microbiol Infect 24:689–696.

3. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, Holland SM, Horsburgh R, Huitt G, Iademarco MF, Iseman M, Olivier K, Ruoss S, von Reyn CF, Wallace RJ, Winthrop K, ATS Mycobacterial Diseases Subcommittee, American Thoracic Society, Infectious Disease Society of America. 2007. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med 175:367–416.

4. Martin-Vicente A, Guarro J, Capilla J. 2017. Does a triple combination have better activity than double combinations against multiresistant fungi? Experimental in vitro evaluation. Int J Antimicrob Agents 49:422–426.

5. Mehta S, Aranzana-Climent V, Rammaert B, Grégoire N, Marchand S, Couet W, Buyck JM. 2019. Preclinical Pharmacokinetic and Pharmacodynamic Data To Support Cefoxitin Nebulization for the Treatment of Mycobacterium abscessus. Antimicrob Agents Chemother 63.

6. Marchand S, Boisson M, Mehta S, Adier C, Mimoz O, Grégoire N, Couet W. 2018. Biopharmaceutical Characterization of Nebulized Antimicrobial Agents in Rats. 6. Aminoglycosides. Antimicrobial Agents and Chemotherapy AAC.01261-18.

7. Greendyke R, Byrd TF. 2008. Differential antibiotic susceptibility of Mycobacterium abscessus variants in biofilms and macrophages compared to that of planktonic bacteria. Antimicrob Agents Chemother 52:2019–2026.

8. Baeder DY, Yu G, Hozé N, Rolff J, Regoes RR. 2016. Antimicrobial combinations: Bliss independence and Loewe additivity derived from mechanistic multi-hit models. Philos Trans R Soc Lond, B, Biol Sci 371.

9. Ferro BE, Srivastava S, Deshpande D, Sherman CM, Pasipanodya JG, van Soolingen D, Mouton JW, van Ingen J, Gumbo T. 2015. Amikacin Pharmacokinetics/Pharmacodynamics in a Novel Hollow-Fiber Mycobacterium abscessus Disease Model. Antimicrob Agents Chemother 60:1242–1248.

10. Lerat I, Cambau E, Roth Dit Bettoni R, Gaillard J-L, Jarlier V, Truffot C, Veziris N. 2014. In vivo evaluation of antibiotic activity against Mycobacterium abscessus. J Infect Dis 209:905–912.

11. Olivier KN, Shaw PA, Glaser TS, Bhattacharyya D, Fleshner M, Brewer CC, Zalewski CK, Folio LR, Siegelman JR, Shallom S, Park IK, Sampaio EP, Zelazny AM, Holland SM, Prevots DR. 2014. Inhaled amikacin for treatment of refractory pulmonary nontuberculous mycobacterial disease. Ann Am Thorac Soc 11:30–35.

12. Ferro BE, van Ingen J, Wattenberg M, van Soolingen D, Mouton JW. 2015. Time-kill kinetics of antibiotics active against rapidly growing mycobacteria. J Antimicrob Chemother 70:811–817.

13. Rominski A, Schulthess B, Müller DM, Keller PM, Sander P. 2017. Effect of β-lactamase production and β-lactam instability on MIC testing results for Mycobacterium abscessus. J Antimicrob Chemother 72:3070–3078.

14. Schoutrop ELM, Brouwer MAE, Jenniskens JCA, Ferro BE, Mouton JW, Aarnoutse RE, van Ingen J. 2018. The stability of antimycobacterial drugs in media used for drug susceptibility testing. Diagn Microbiol Infect Dis 92:305–308.

15. Ferro BE, Srivastava S, Deshpande D, Pasipanodya JG, van Soolingen D, Mouton JW, van Ingen J, Gumbo T. 2016. Failure of the Amikacin, Cefoxitin, and Clarithromycin Combination Regimen for Treating Pulmonary Mycobacterium abscessus Infection. Antimicrob Agents Chemother 60:6374–6376.

16. Lefebvre A-L, Dubée V, Cortes M, Dorchêne D, Arthur M, Mainardi J-L. 2016. Bactericidal and intracellular activity of β-lactams against Mycobacterium abscessus. J Antimicrob Chemother 71:1556–1563.

17. NCCLS. 2003. Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard. NCCLS document M24-A.

18. Hsieh MH, Yu CM, Yu VL, Chow JW. 1993. Synergy assessed by checkerboard. A critical analysis. Diagn Microbiol Infect Dis 16:343–349.

19. Sueke H, Kaye SB, Neal T, Hall A, Tuft S, Parry CM. 2010. An in vitro investigation of synergy or antagonism between antimicrobial combinations against isolates from bacterial keratitis. Invest Ophthalmol Vis Sci 51:4151–4155.

20. Keizer RJ, van Benten M, Beijnen JH, Schellens JHM, Huitema ADR. 2011. Piraña and PCluster: a modeling environment and cluster infrastructure for NONMEM. Comput Methods Programs Biomed 101:72–79.

21. Beal SL. 2001. Ways to fit a PK model with some data below the quantification limit. J Pharmacokinet Pharmacodyn 28:481–504.

Supplementary data

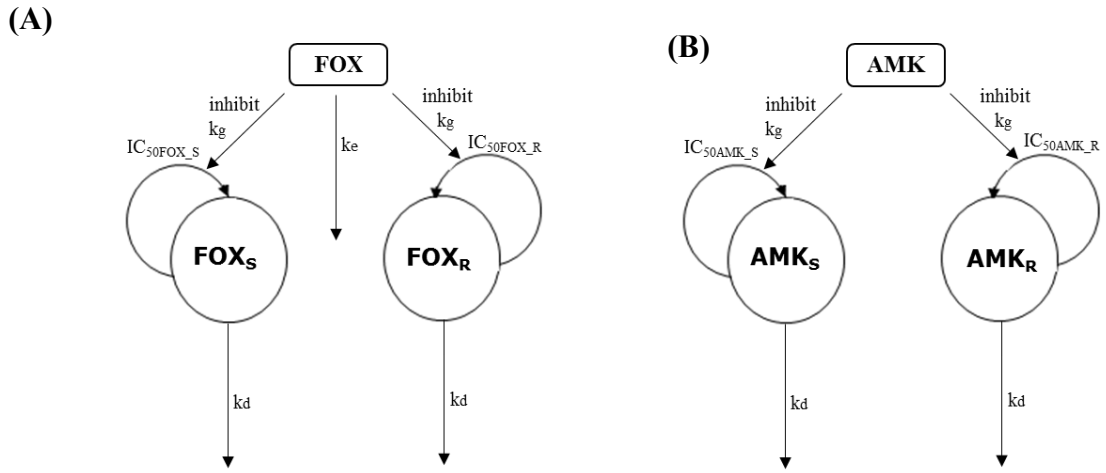

Figure S1: Schematic diagram of the final PK/PD model (A) FOX (B) AMK. Bacteria multiplied with a first-order rate constant (k_g) in the susceptible (*S*) and resistant (*R*) bacterial compartment and all bacteria had natural death-rate (k_d). Cefoxitin followed a with a first-order degradation process (k_e). Antibiotic compartment was driven to the bacterial growth inhibition following an I_{max} model $\left(1 - \left(\frac{I_{max} \times C^{\gamma}}{IC_{50}{}^{\gamma} + C^{\gamma}}\right)\right)$.

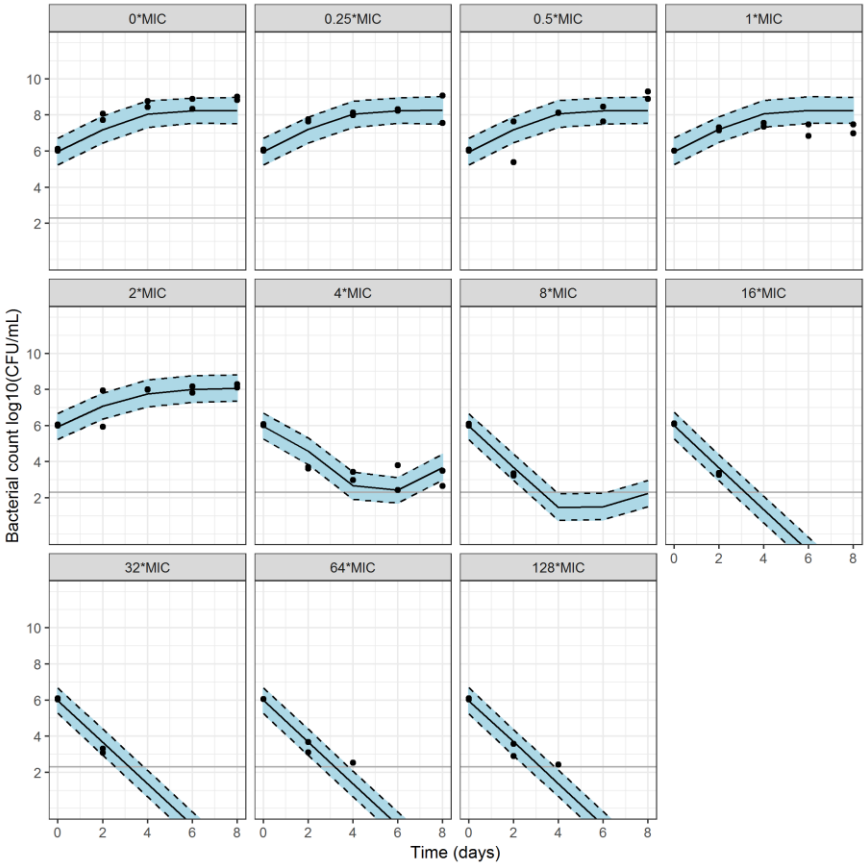

Figure S2: Visual predictive checks (VPCs) for the final PK/PD model of FOX against *M. abscessus CIP104536* with observed bacterial counts (circles), median (black continuous line) and 80% prediction interval (black dotted line) of simulated data. Plots include growth control and experimental data by time-kill kinetics. The indicated concentrations are in multiples of MICs. 50% FOX amount was compensated each 24h to maintain 100% amount of FOX throughout the experiment. Line shows the limit of quantification (200 CFU/mL).

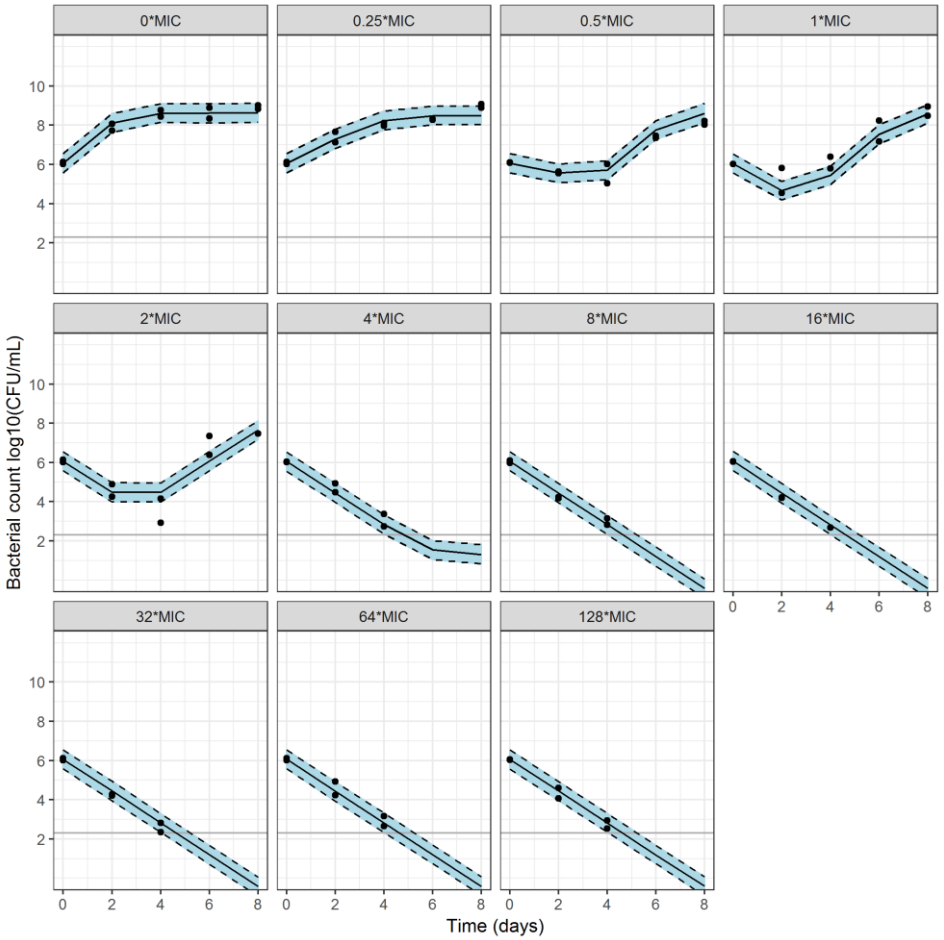

Figure S3: Visual predictive checks (VPCs) for the final PD model of AMK against *M. abscessus* CIP104536 with observed bacterial counts (circles), median (black continuous line) and 80% prediction interval (black dotted line) of simulated data. Plots include growth control and experimental data by time-kill kinetics. The indicated concentrations are in multiples of MICs. Line shows the limit of quantification (200 CFU/mL).

Table S1: PD Parameter estimates for FOX and AMK as monotherapy, derived from the growth inhibition model fitted to time-kill kinetics assay

Parameters (units)	Explanation	Estimations (%RSE)
LGINOC (Log_{10}CFU/mL)	Initial bacterial density	5.98 (1%)
K_g (day^{-1})	Bacterial growth rate constant	4.3 (8%)
B_{max} (Log_{10}CFU/mL)	Bacterial count in stationary phase	8.72 (2%)
K_{id} (day^{-1})	Bacterial death rate constant	1.95 (4%)
I_{max}	Maximum achievable growth inhibition rate constant by FOX and AMK	1 (fixed)
IC_{50FOX_S} (mg/L)	FOX concentration that results in 50% of I_{max} for susceptible subpopulation	18.9 (5%)
IC_{50FOX_R} (mg/L)	FOX concentration that results in 50% of I_{max} for resistant subpopulation	127 (25%)
IC_{50AMK_S} (mg/L)	AMK concentration that results in 50% of I_{max} for susceptible subpopulation	13.2 (7%)
IC_{50AMK_R} (mg/L)	AMK concentration that results in 50% of I_{max} for resistant subpopulation	116 (5%)
$MUTF_{FOX}$	Mutation frequency of bacteria exposed to FOX	-10.9 (13%)
$MUTF_{AMK}$	Mutation frequency of bacteria exposed to AMK	-4.35 (20%)
K_e (day^{-1})	Degradation rate constant for FOX followed by first-order process	0.438 (fixed)
γ_F	Hill factor for growth inhibition due to FOX	4.4 (23%)
γ_A	Hill factor for growth inhibition due to AMK	3.01 (13%)

RSE: Relative Standard Error

Article 4: *In vitro* evaluation of novel bi- or tri-antibiotic combination against clinical isolates of *Mycobacterium abscessus*

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In manuscript

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Résumé en français :

Mycobacterium abscessus est un pathogène émergent, intrinsèquement résistant à de nombreux antimycobactériens. Le traitement recommandé se limite à l'association de l'amikacine (AMK) et de la céfoxitine (FOX) par voie intraveineuse avec la clarithromycine (CLR) par voie orale. Cependant, des études récentes démontrent une résistance intrinsèque à la CLR chez des souches cliniques de *M. abscessus*. De plus, lorsque le traitement standard est inefficace, d'autres antibiotiques tels que les fluoroquinolones, les rifamycines, le linézolide (LZD) ou la clofazimine (CLO) peuvent être ajoutés. Cette étude vise à évaluer l'efficacité *in vitro* de plusieurs combinaisons contre des souches cliniques de *M. abscessus* incluant FOX et AMK pour remplacer l'utilisation de la CLR afin d'éviter le développement de résistances. La concentration minimale inhibitrice (CMI) a été déterminée pour chaque antibiotique. Ensuite, ces antibiotiques ont été étudiés en tri-combinaisons avec la FOX et l'AMK pour comparer leur efficacité avec la souche référence de *M. abscessus* CIP104536, et deux souches cliniques Ma1611 et T28 en utilisant des courbes de bactéricidie. Le souche clinique T28 était résistante à tous les antibiotiques, le CIP104536 et le Ma1611 étaient sensibles ou intermédiaires. Les triple-combinaisons incluant la FOX et l'AMK en présence de LZD, MXF, RIF ou RFB étaient actives contre CIP104536 et Ma1611. Les triple-combinaisons incluant CLO ou CIP étaient également actives contre CIP104536 mais inactives contre Ma1611. Toutes les triple-combinaisons testées étaient inactives contre T28. La souche T28 étant très résistante à l'AMK, l'efficacité de bi-combinaisons incluant la FOX ont été évaluées contre T28. Les bi-combinaisons de FOX avec LZD, RIF et RFB Permettaient d'obtenir une décroissance de l'inoculum initial et ont empêché la repousse observée avec la FOX seule. La bi-combinaison de la FOX avec la RFB était la plus active contre la souche T28. La synergie observée des combinaisons entre FOX et les rifamycines pourrait justifier une optimisation plus poussée du schéma thérapeutique pour le traitement des infections pulmonaires à *M. abscessus*.

Abstract

Mycobacterium abscessus is an emerging pathogen, intrinsically resistance to many antimycobacterial drugs. The recommended treatment is limited to combination of intravenous amikacin (AMK), and cefoxitin (FOX) with oral clarithromycin (CLR). However, recent reports demonstrate intrinsic resistance to CLR in *M. abscessus* clinical isolates. Fluoroquinolones, rifamycins, linezolid (LZD) or clofazimine (CLO) can be added when standard therapy is ineffective. This study aims to evaluate the *in vitro* efficacy of several combinations against clinical isolates of *M. abscessus* including FOX and AMK and replacing CLR to avoid the induced resistance. Minimum inhibitory concentration (MICs) were determined for ciprofloxacin (CIP), moxifloxacin (MXF), rifampicin (RIF), rifabutin (RFB), CLO, LZD and CLR according to CLSI guidelines. Then, these antibiotics were investigated in tri-combinations with FOX plus AMK to compare their efficacy against *M. abscessus* reference strain CIP104536, and two clinical isolates Ma1611 and T28 using time-kill kinetic assays. Efficacy of several bi-combinations was evaluated against T28. Clinical isolate T28 was resistant to all antibiotics, CIP104536 and Ma1611 were susceptible to intermediate against all tested antibiotics. Tri-combinations including FOX plus AMK in presence of LZD, MXF, RIF or RFB were active against CIP104536 and Ma1611. Tri-combinations including CLO or CIP were also active against CIP104536 but inactive against Ma1611. All tested triple combinations were inactive against T28. Since T28 was highly resistant to AMK and FOX alone demonstrated only initial killing followed by regrowth, FOX was used in bi-combinations. Hereafter, bi-combinations of FOX with LZD, RIF and RFB were effective and prevented the regrowth observed with FOX alone. Bi-combination FOX with RFB was the most active against strain T28. Tri-combinations were highly efficient against *M. abscessus* reference strain and intermediate to susceptible clinical isolate Ma1611 but not against multidrug-resistant isolate T28. The synergy between FOX and rifamycins suggests a potent role of this combinations that may warrant further optimization of treatment regimen for the treatment of *M. abscessus* pulmonary infections.

Introduction

Over past few years, *Mycobacterium abscessus*, a rapidly growing mycobacteria, has emerged as an opportunistic pathogen responsible for wide spectrum of infections specially, pulmonary infections in cystic fibrosis (CF) patients, leading to rapid decline in respiratory function (1). *M. abscessus* is naturally resistant to most of the antibiotics including anti-tuberculous agents (2), meaning that there are no effective therapeutic drug regimens to eradicate *M. abscessus* pulmonary infections, as stated by the American Thoracic Society (ATS) (3). The recommended treatment consists of a combination of an oral clarithromycin (CLR) with intravenous (IV) amikacin (AMK) and cefoxitin (FOX), is highly associated with poor prognosis and mortality (4). The induced macrolide resistance in more than 80% cases and inadequate antibiotic concentration after IV administration may explain this treatment failure (5). Since macrolide resistance compromises the treatment efficacy, there is an urgent need to find a replacement and identify better therapeutic options. This issue can be resolved by repurposing existing efficient antimycobacterial agents i.e. FOX and AMK (6–10) by nebulization (NEB) (11, 12). NEB could achieve high lung drug concentration with concomitant low systemic absorption, low serum drug concentration, and consequently reduced toxicity (13). Furthermore, several studies of combinations have shown additive, synergistic or antagonistic effect in different cases against this infection. For example, combinations of clofazimine (CLO) with AMK or CLR (14, 15), AMK with linezolid (LZD) (16), rifampicin (RIF) with carbapenem (17), CLR with LZD or tigecycline (TGC) or vancomycin (16, 18, 19) have shown synergistic activity in various *in vitro* or *in vivo* studies. Despite of showing synergistic activity, these bi-combinations are most of the time associated with the development of resistance and poor outcomes. In such cases, triple combinations could allow to better eradicate *M. abscessus* infections, but the data related triple combinations are quite limited.

Hence, to evaluate the *in vitro* bacterial sterility over time, following the exposure to combined antibiotics, several *in vitro* experiments containing two and three antibiotics in combination were performed on different strains of *M. abscessus.* Then, the activity of bi- or tri-combinations were compared with different *M. abscessus* strains.

Results

Antimicrobial susceptibility testing: The minimal inhibitory concentrations (MIC) determined for each antibiotic has been shown in Table 1. The clinical isolate T28 was fully resistant to almost all antibiotics tested and intermediate for FOX (20). Isolate T28 was highly resistant to AMK with MIC>1024 mg/L alike LZD, RIF and CLR (MIC ≥ 256 mg/L). Both *M. abscessus* CIP104536 and Ma1611 trains demonstrated almost similar profile except CLO and CLR. All antibiotics fall under the breakpoints for susceptible to intermediate, except RIF. CIP104536 was susceptible to CLR (MIC = 1 mg/L) while MIC value of CLR was over the resistant breakpoint for Ma1611 (MIC = 16 mg/L). The clinical breakpoints for RFB and CLO against *M. abscessus* are not determined yet, however MIC value for CLO was higher against Ma1611 (MIC = 16 mg/L) than CIP104536 (MIC = 2 mg/L) and T28 (MIC = 4 mg/L). MIC value for RFB was 8 times higher for T28 (MIC = 16 mg/L) than CIP104536 and Ma1611 (MIC = 2 mg/L). Furthermore, all three strains were resistant to RIF.

Time kill curves of antibiotics alone and in combinations against reference strain: M. abscessus CIP104536 was exposed to several antibiotics including FOX, AMK, CLR, CLO, CIP, MXF, LZD, RIF and RFB, at MICs value that were close to the concentrations achievable in humans (21), as shown on Figure 1A. None of the antibiotic was active alone, showing rapid regrowth from day 2. The time kill curves of triple-combinations containing AMK, FOX and a third molecule against CIP104536 are presented on Figure 1B. All triple combinations were efficient showing rapid bacterial killing to reach detection limit from day 4 for combinations with RIF, RFB, CIP, MXF and CLO, and from day 6 in case of combination with LZD. The combination including CLR was the least active and could not reach detection limit within 8 days.

Time kill curves of triple combinations against clinical isolates: We then compared the activity of these triple combinations against the clinical isolates. All triple combinations were tested using the similar concentration used for reference strain. The activity of the triple combinations was expressed as a change in log CFU at day 4 and day 8 from respective bacterial CFU count at time 0 (Figure 2). First, the bacterial growth in control without antibiotic was lower for multidrug resistant T28 isolate (2-log bacterial growth at day 8) compared to the other strains (around 4-log bacterial growth at day 8). As presented in Figure 1B and Figure 2A, all combinations were effective against reference strain showing bacterial decay up to 4-log (below the limit of detection) at day 8. The effect of triple combinations containing RIF, RFB, LZD,

MXF and CIP against Ma1611 (Figure 2B) was comparable to the reference strain (Figure 2A) but regrowth was observed for triple combinations containing CLR from day 4. The combination with CLO also showed bacterial killing with 2-log CFU decrease against Ma1611, but regrowth was observed at day 8 (Figure 2B). However, none of the triple combinations were active against *M. abscessus* T28 (Figure 2C), showing only bacteriostatic activity for most of the combinations except for the combination with LZD, which achieved 2-log decrease in bacterial density from day 4 with no regrowth observed.

Time kill curves of bi-combinations against M. abscessus multidrug resistant T28 isolate: To find an active combination against T28, we decided to evaluate the combination using comparatively higher concentration of FOX corresponding to its own MIC (i.e. 64 mg/L). Other antibiotics were used at the MICs of reference strain corresponding to human C_{max} (21). For *M. abscessus* T28, all antibiotics tested alone were not active (Figure 3A) except FOX and RFB. Both FOX and RFB have shown initial bacterial killing (2-log CFU decrease, until day 4) followed by regrowth. Hereafter, we evaluated the efficacy of bi-combinations (Figure 3B and 3C). Bi-combination of FOX with RFB was the most active showing complete bacterial killing (CFU count below the limit of detection) after day 4. The combination of FOX with RIF or LZD showed slow decrease in CFU and reached 3 to 4-log decrease in CFU at day 8. Also, the combinations with RFB, RIF and LZD have completely prevented the bacterial regrowth observed with FOX alone. Bi-combinations of FOX with CIP, MXF and CLO have initially shown a bacterial load decrease up to 4-log, but regrowth was observed after day 6 (Figure 3C). The combination with CLR have also shown initial bacterial killing but a rapid regrowth was observed after day 4 and the combination was as active as FOX alone.

Discussion

Starting from the recommended antibiotic combination treatment including AMK, FOX and CLR, we conducted our studies to find a replacement for CLR, as CLR has been reported to be responsible for resistance development and treatment failure in most cases. In present study, the recommended combination was the least active that was consistent with previous *in vitro* studies. Ferro *et al*. (9) reported only a small decrease in bacterial population size when exposed to AMK, FOX or CLR. This combination also failed in hollow fiber system because of observed bacterial regrowth (22).

As mentioned previously (23, 24), FOX is an unstable cephalosporin antibiotic with an *in vitro* degradation half-life of 1.5 days (12). In such cases, Schoutrop *et al.* (24) suggested for daily addition of unstable antibiotic to obtain useful test results, also as already been applied to imipenem. Hence, in this study, 50% FOX amount was added each 24 h in order to compensate the FOX degradation.

In previous studies, Park *et al.* (25) reported moderate activity of CIP and MXF against several *M. abscessus* isolates, but also suggested to use these antibiotics in combination to avoid mutational resistance. Ferro *et al.* (26) observed no activity of MXF in hollow fiber system and also suggested to use MXF only in combination. Maurer *et al.* (27) reported no *in vitro* activity of MXF up to 24h. Consistently, combinations with fluoroquinolones (CIP and MOX) have shown efficient bacterial killing against reference strain and the clinical isolate Ma1611, but were poorly efficient against T28.

As Wallace *et al.* (28) reported excellent potential of LZD against rapid growing mycobacteria, we tested the triple combination with LZD that was efficient against reference strain and Ma1611 and was the most active of the tested triple combinations against T28. But, LZD in combination with AMK, MXF, TGC and FOX were shown rarely synergistic by Zhang *et al.* (16). A possible explanation for this discrepancy could be that we used triple combinations including FOX and AMK, instead of double combinations in the previous study.

Several studies reported CLO as a potent antibiotic for treatment against NTM infection (14, 15). Also in previous study, CLO showed synergistic effect with AMK (14) and prevented bacterial regrowth in presence of AMK against the reference strain (15). Consistently, our tested triple combination with CLO has also shown good activity against reference strain. However, an initial bacterial killing followed by a regrowth was observed in both clinical isolates suggesting limited use of this antibiotic against *M. abscessus* infections.

Anti-tuberculous agents rifamycins are not generally used in clinical practice for the treatment of pulmonary infections caused by *M. abscessus* due to poor *in vitro* activity and development of resistance (29). However, our triple combinations containing RIF and RFB showed bacterial killing against reference strain and Ma1611, and bacteriostatic activity against T28. These results are consistent with a recent study, where RFB exhibited potential *in vitro* activity against the reference strain and CLR-resistant of *M. abscessus* (29). However, the concentration used for RFB was 2 to 3 times higher than achievable peak serum concentration post-oral administration (21), which means that this combination may be unreasonable in clinical setting.

Also, the susceptibility breakpoints for CLO and RFB are not determined yet, but MICs values and achievable peak serum concentration (21) indicates that it might be difficult to use these antibiotics against *M. abscessus* infections. Although TGC showed the best activity and has also shown synergistic effect with AMK (8, 18), we decided to not use TGC, as MIC of TGC for reference strain (30) (MIC = 4 mg/L) was comparatively higher than achievable peak serum concentration (C_{max} = 1.5 mg/L after 0.1g IV).

The clinical isolate *T28* was found to be highly resistant to AMK (MIC>1024 mg/L) due to presence of acquired resistance to aminoglycosides by A1408G mutation of the *rrs* gene encoding rRNA 16S and intermediate to FOX (MIC = 64 mg/L). Consequently, we decided to remove AMK and to evaluate the activity of bi-combinations including FOX (at its corresponding MIC) and a second antibiotic. FOX alone has shown initial bacterial killing followed by regrowth, which were prevented by the combinations with LZD and rifamycins i.e. RIF and RFB.

As explained above, in many cases even though being the antibiotics of choice, FOX and AMK can only be used at high concentration. In addition, in several cases systemic administration of these antibiotics is not tolerable because of toxicity (i.e. ototoxicity and nephrotoxicity by AMK and neutropenia and thrombocytopenia by FOX). Therefore, an approach of replacing systemic administration of FOX (12) and AMK (11) by NEB can be a good option to achieve high lung drug concentration with less systemic side effects.

Furthermore, in case of *M. abscessus* pulmonary infection, bacteria can swiftly grow and survive in extracellular airway mucus as well as intracellularly within macrophages (31). CLR, LZD and RFB accumulate in lung tissues at concentrations above their susceptible MICs values (20). However, CLR and LZD cannot be the first choice of antibiotics because of their associated resistance (2, 16), in contrast to RFB. Indeed, RFB demonstrates intracellular activity in combination against *M. abscessus* within this niche (32). On other hand, AMK acts extracellularly due to its limited permeability in macrophages and FOX penetration within lung tissue is not documented. However, using nebulized antibiotics (AMK and FOX), very high local concentrations can be achieved, also a very small part could penetrate inside cells and reach intracellular forms of *M. abscessus*. Including all these factors, triple combination of nebulized AMK and FOX with an oral RFB (bi-combination in case of AMK and CLR resistance), can be an auspicious treatment option against intracellular *M. abscessus* pulmonary infections.

This experimental study, carried out over 8 days, which is comparatively longer than other reported *in vitro* studies for antibiotics alone or in combination (9, 15, 17, 27, 32–34), has one limitation. The objective of the study was to compare the activity of tri-combinations at Cmax, so antimicrobial activity in bi- or tri-antibiotic combinations at various different concentrations was not investigated. Hence, a follow up study to optimize the most potent combination including RFB could an interesting option, especially against multidrug resistant *M. abscessus* isolates like T28.

In conclusion, time-kill assays revealed that combinations including FOX, AMK and antimicrobials screened from LZD, RIF, RFB, CIP, MXF and CLR may provide an alternative treatment for *M. abscessus* pulmonary infections. The efficient activity of these combinations may warrant a "renaissance" of treatment against *M. abscessus*. The addition of third antibiotic remains controversial as susceptibility to antibiotics depends on the geographical diversity. However, the combination of FOX with RFB, RIF or LZD may act as an effective treatment approach even if *M. abscessus* isolates are resistant to AMK.

Materials and method

Bacterial strain and suspension preparation: Mycobacterium abscessus subspecies abscessus, reference strain CIP104536, was obtained from Institute Pasteur (Paris, France), clinical isolate Ma1611 was isolated from lung expectoration (CHU of Poitiers, France) and T28 was isolated from bronchial aspiration in patients with cystic fibrosis (Lariboisière Hospital, Paris, France). Stock vials were conserved at -80°C. For each experiment, the mycobacterial inoculum was prepared according to CLSI guidelines (20).

Antimicrobial susceptibility testing (AST): MICs were determined by broth microdilution method using Cation-adjusted Mueller–Hinton broth 2 (CAMHB II; Sigma-Aldrich, Saint-Quentin-Fallavier France) supplemented with 10% oleic acid/bovine albumin/dextrose/catalase (OADC) growth supplement (BD, BBL™, Sparks, MD, USA) and 0.5% glycerol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for each antibiotic. The antibiotics were serially diluted to the desired final concentration in a 96-well plate. The bacterial suspension ($1*10^6$ CFU/mL) was added to each well, with the final antibiotic concentrations ranging from 0.125 to 1024 mg/L. A positive control, only with bacterial suspension and without antibiotic was included for each strain. The plates were incubated at 30°C for 3 days (20). MICs were visually determined. All experiments were performed in duplicate.

Time-kill kinetics assay. For single antibiotics: Individual tubes of 20 mL of middlebrook 7H9 broth (BD, BBL™, Sparks, MD, USA) with 10% oleic acid/bovine albumin/dextrose/catalase (OADC) growth supplement (BD, BBL™, Sparks, MD, USA) and 0.5% glycerol containing FOX, AMK, CIP, MXF, LZD, CLO, CLR, RIF and RFB at their MICs values for reference strain CIP104536, were inoculated with the bacterial suspension (~ $1*10^6$ CFU/mL) and incubated at 35° ± 2°C, under shaking conditions (150 rpm) up to 8 days. Bacteria were quantified at 0, 2, 4, 6 and 8 days by plating serial dilutions prepared with sterile phosphate buffer saline (PBS pH 7.4, Gibco™, by life technologies, France) on middlebrook 7H11 agar plates (BD, BBL™, Sparks, MD, USA) with 10% OADC and 0.5% glycerol (20). CFUs were enumerated after 3-5 days of incubation at 35 ± 2°C. The theoretical detection limit was set to 200 CFU/mL i.e. 2.3 $\log_{10}$ CFU/mL. *For antibiotic combination:* Following the same procedure as described above, time-kill assays for triple combinations containing FOX and AMK in presence of 3rd antibiotic were tested against CIP104536, Ma1611 and T28 at concentrations detailed in Table 2. Bi-combinations of FOX with CIP, MXF, LZD, CLO, RFB, RIF and CLR were performed only for T28 isolate using FOX at the T28 MIC concentration.

Antibiotics: The following antibiotics were obtained as microbiological standards from their manufacturers: FOX (Panpharma, Luitré, France), AMK (Acros, Illkirch, France), LZD (Ark Pharma, Neuilly-sur-Seine, France). RIF, RFB, CLR, CIP, MXF and CLO were purchased from Sigma (Saint-Quentin-Fallavier, France). Stock solutions were prepared using appropriate solvents and stored at -80°C.

Curve fittings: Curve fittings were performed with GraphPad Prism (version 7.04) software for Windows (GraphPad Prism Software, San Diego, CA).

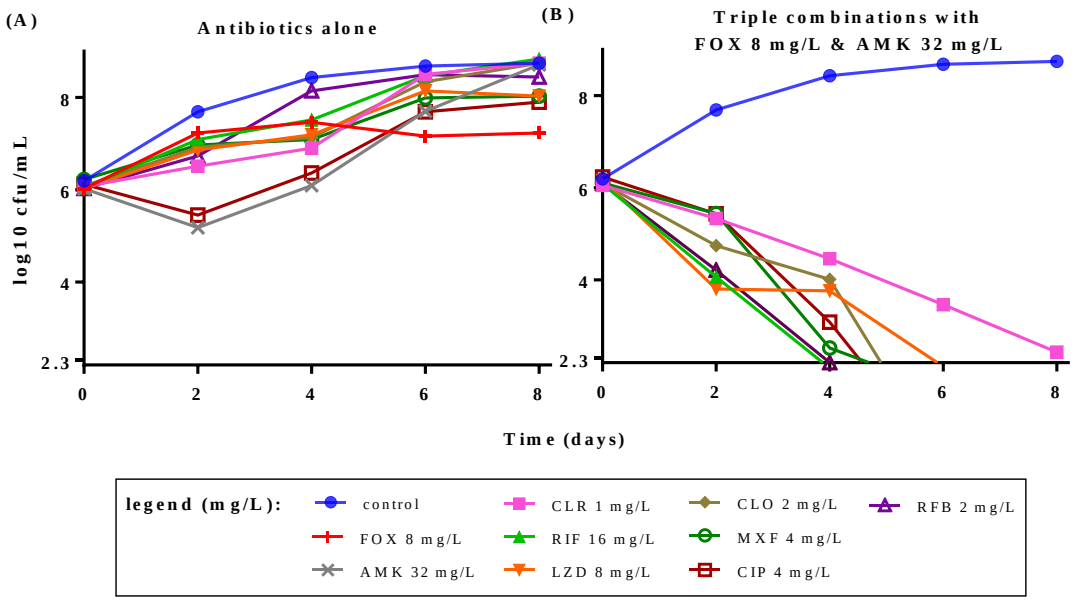

Figure 1: Effect of various combinations on *M. abscessus* CIP 104536. (A) Activity of several screened antibiotics were tested alone and (B) then tested in combination with cefoxitin and amikacin based on the obtained MICs values as shown in Table 1. 50% amount of FOX was compensated each 24h up to 8 days to maintain the FOX amount constant throughout the experiment. CFUs were determined at the interval of 2 days. The limit of quantification is 200 CFU/mL (2.3 in $\log_{10}$).

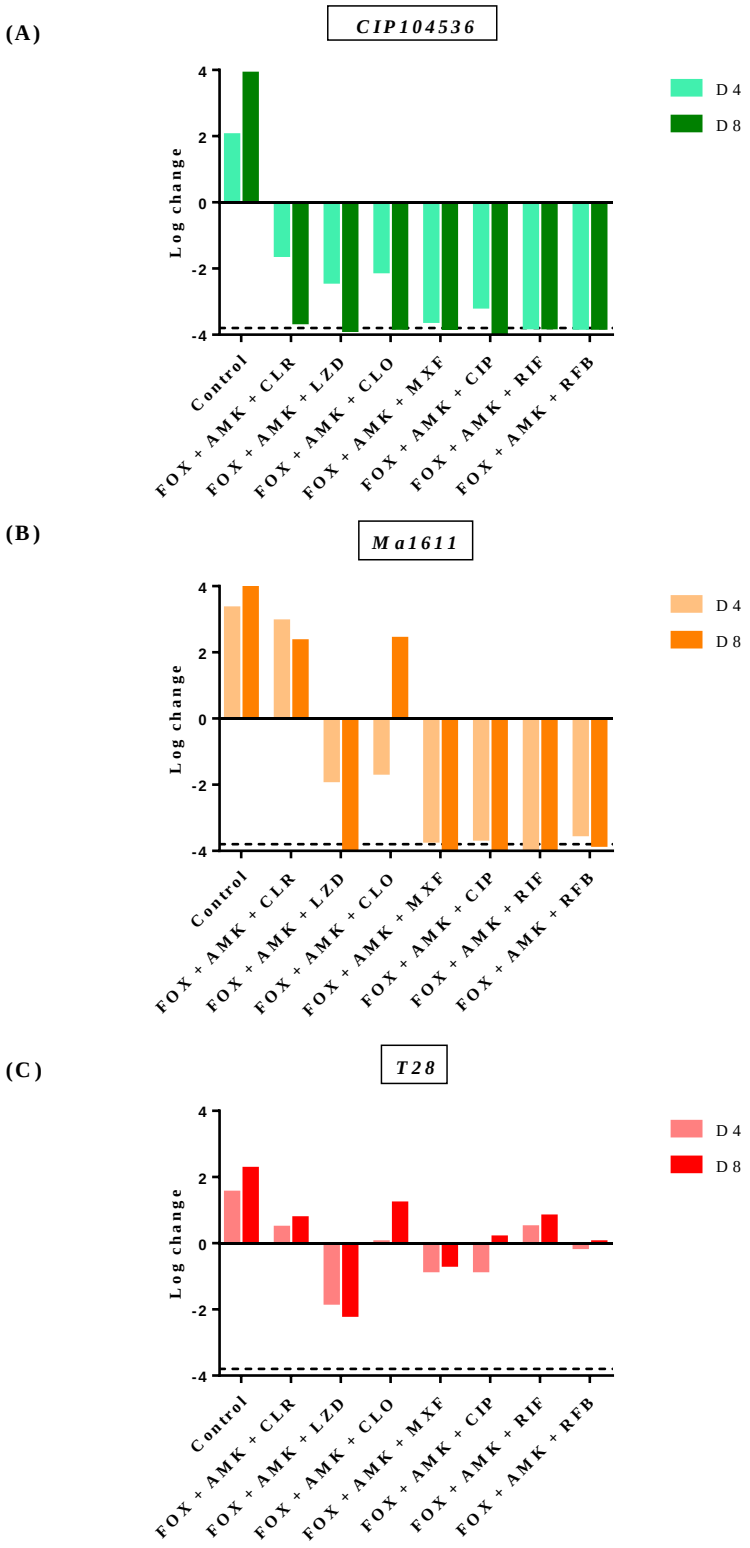

Figure 2: *In vitro* activity of various triple combinations against *M. abscessus* (A) CIP104536 (B) Ma1611 and (C) T28. Concentrations for each antibiotic are as mentioned on Figure 1. Log change was calculated using respective positive control data. For Comparision purpose, results are compared at day 4 and day 8. The dashed line represents the limit of quantification.

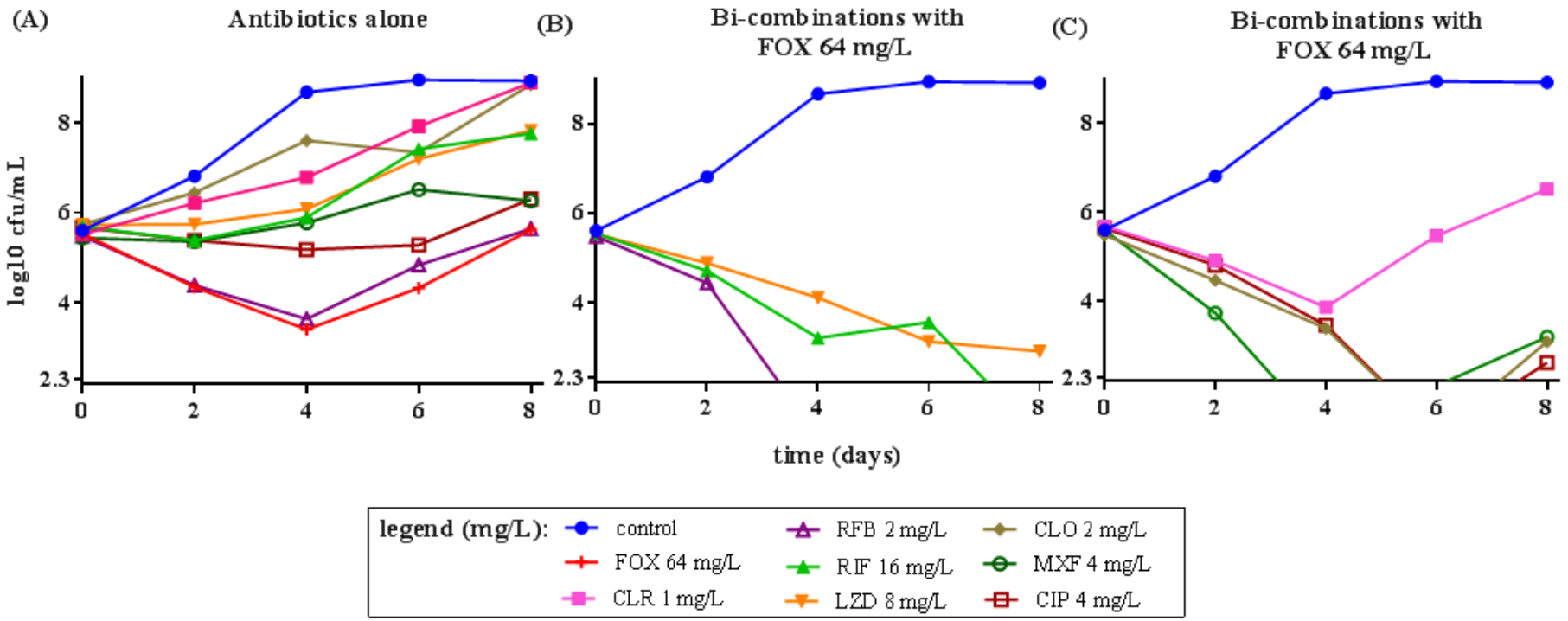

Figure 3: Effect of various combinations on *M. abscessus* T28. (A) Activity of several screened antibiotics alone, (B) cefoxitin in combination with linezolid, rifampicin and rifabutin, and (C) cefoxitin in combination with clarithromycin, clofazimine, moxifloxacin and ciprofloxacin. 50% amount of FOX was compensated each 24h up to 8 days to maintain the FOX amount constant throughout the experiment. CFUs were determined at the interval of 2 days. The dashed line represents the limit of quantification.

Table 1: Susceptibility data for different *M. abscessus* strains test by broth microdilution

Antibiotics	MIC breakpoints			MICs (mg/L)		
	S	I	R	CIP 104536	Ma1611	T28
FOX	<16	32	>128	8	8	64
AMK	<16	32	>64	32	16	>1024
CIP	<1	2	>4	4	4	8
MXF	<1	2	>4	4	4	16
LZD	<8	16	>32	8	8	256
RIF			>1	16	16	256
CLR	<2	4	>8	1	16	>256
CLO	ND			2	16	4
RFB				2	2	16

FOX, cefoxitin ; AMK, amikacin; CIP, ciprofloxacin; MXF, moxifloxacin; LZD, linezolid; CLO, clofazimine; RFB, rifabutin; RIF, rifampicin; CLR, clarithromycin; S, susceptible; I, intermediate; R, resistance; ND, not determined.

Table 2: Antibiotics used for triple combination time-kill assay, containing cefoxitin and amikacin combined with 3rd antibiotic

	3rd antibiotic	**Concentrations used for 3rd antibiotic**
FOX*† + AMK*	+ CIP	4 mg/L
	+ MXF	4 mg/L
	+ LZD	8 mg/L
	+ CLO	2 mg/L
	+ RFB	2 mg/L
	+ RIF	16 mg/L
	+ CLR	1 mg/L

* FOX and AMK concentrations were 8 mg/L and 32 mg/L respectively
† 50% amount of FOX was compensated each 24h up to 8 days to maintain the FOX amount constant throughout the experiment

FOX, cefoxitin; AMK, amikacin; CIP, ciprofloxacin; MXF, moxifloxacin; LZD, linezolid; CLO, clofazimine; RFB, rifabutin; RIF, rifampicin; CLR, clarithromycin

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References

1. Esther CR, Esserman DA, Gilligan P, Kerr A, Noone PG. 2010. Chronic Mycobacterium abscessus infection and lung function decline in cystic fibrosis. J Cyst Fibros 9:117–123.
2. Nessar R, Cambau E, Reyrat JM, Murray A, Gicquel B. 2012. Mycobacterium abscessus: a new antibiotic nightmare. J Antimicrob Chemother 67:810–818.
3. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, Holland SM, Horsburgh R, Huitt G, Iademarco MF, Iseman M, Olivier K, Ruoss S, von Reyn CF, Wallace RJ, Winthrop K, ATS Mycobacterial Diseases Subcommittee, American Thoracic Society, Infectious Disease Society of America. 2007. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med 175:367–416.
4. Oh C-T, Moon C, Park OK, Kwon S-H, Jang J. 2014. Novel drug combination for Mycobacterium abscessus disease therapy identified in a Drosophila infection model. J Antimicrob Chemother 69:1599–1607.
5. Chopra S, Matsuyama K, Hutson C, Madrid P. 2011. Identification of antimicrobial activity among FDA-approved drugs for combating Mycobacterium abscessus and Mycobacterium chelonae. J Antimicrob Chemother 66:1533–1536.
6. Lavollay M, Dubée V, Heym B, Herrmann J-L, Gaillard J-L, Gutmann L, Arthur M, Mainardi J-L. 2014. In vitro activity of cefoxitin and imipenem against Mycobacterium abscessus complex. Clin Microbiol Infect 20:O297-300.
7. Soroka D, Dubée V, Soulier-Escrihuela O, Cuinet G, Hugonnet J-E, Gutmann L, Mainardi J-L, Arthur M. 2014. Characterization of broad-spectrum Mycobacterium abscessus class A β-lactamase. J Antimicrob Chemother 69:691–696.
8. Lerat I, Cambau E, Roth Dit Bettoni R, Gaillard J-L, Jarlier V, Truffot C, Veziris N. 2014. In vivo evaluation of antibiotic activity against Mycobacterium abscessus. J Infect Dis 209:905–912.
9. Ferro BE, van Ingen J, Wattenberg M, van Soolingen D, Mouton JW. 2015. Time-kill kinetics of antibiotics active against rapidly growing mycobacteria. J Antimicrob Chemother 70:811–817.
10. Greendyke R, Byrd TF. 2008. Differential antibiotic susceptibility of Mycobacterium abscessus variants in biofilms and macrophages compared to that of planktonic bacteria. Antimicrob Agents Chemother 52:2019–2026.

11. Marchand S, Boisson M, Mehta S, Adier C, Mimoz O, Grégoire N, Couet W. 2018. Biopharmaceutical Characterization of Nebulized Antimicrobial Agents in Rats. 6. Aminoglycosides. Antimicrobial Agents and Chemotherapy AAC.01261-18.
12. Mehta S, Aranzana-Climent V, Rammaert B, Grégoire N, Marchand S, Couet W, Buyck JM. 2019. Preclinical Pharmacokinetic and Pharmacodynamic Data To Support Cefoxitin Nebulization for the Treatment of Mycobacterium abscessus. Antimicrob Agents Chemother 63.
13. Davis KK, Kao PN, Jacobs SS, Ruoss SJ. 2007. Aerosolized amikacin for treatment of pulmonary Mycobacterium avium infections: an observational case series. BMC Pulm Med 7:2.
14. van Ingen J, Totten SE, Helstrom NK, Heifets LB, Boeree MJ, Daley CL. 2012. In Vitro Synergy between Clofazimine and Amikacin in Treatment of Nontuberculous Mycobacterial Disease. Antimicrob Agents Chemother 56:6324–6327.
15. Ferro BE, Meletiadis J, Wattenberg M, de Jong A, van Soolingen D, Mouton JW, van Ingen J. 2016. Clofazimine Prevents the Regrowth of Mycobacterium abscessus and Mycobacterium avium Type Strains Exposed to Amikacin and Clarithromycin. Antimicrob Agents Chemother 60:1097–1105.
16. Zhang Z, Lu J, Song Y, Pang Y. 2018. In vitro activity between linezolid and other antimicrobial agents against Mycobacterium abscessus complex. Diagn Microbiol Infect Dis 90:31–34.
17. Kaushik A, Makkar N, Pandey P, Parrish N, Singh U, Lamichhane G. 2015. Carbapenems and Rifampin Exhibit Synergy against Mycobacterium tuberculosis and Mycobacterium abscessus. Antimicrob Agents Chemother 59:6561–6567.
18. Huang C-W, Chen J-H, Hu S-T, Huang W-C, Lee Y-C, Huang C-C, Shen G-H. 2013. Synergistic activities of tigecycline with clarithromycin or amikacin against rapidly growing mycobacteria in Taiwan. Int J Antimicrob Agents 41:218–223.
19. Mukherjee D, Wu M-L, Teo JWP, Dick T. 2017. Vancomycin and Clarithromycin Show Synergy against Mycobacterium abscessus In Vitro. Antimicrob Agents Chemother 61.
20. NCCLS. 2003. Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard. NCCLS document M24-A.
21. Bennett J, Dolin R, Blaser M. 2015. Tables of Anti-infective Agent Pharmacology. Principles and Practice of Infectious Diseases, 8th edition.Mandell Infectious Drugs.
22. Ferro BE, Srivastava S, Deshpande D, Pasipanodya JG, van Soolingen D, Mouton JW, van Ingen J, Gumbo T. 2016. Failure of the Amikacin, Cefoxitin, and Clarithromycin

Combination Regimen for Treating Pulmonary Mycobacterium abscessus Infection. Antimicrob Agents Chemother 60:6374–6376.

23. Rominski A, Schulthess B, Müller DM, Keller PM, Sander P. 2017. Effect of β-lactamase production and β-lactam instability on MIC testing results for Mycobacterium abscessus. J Antimicrob Chemother 72:3070–3078.
24. Schoutrop ELM, Brouwer MAE, Jenniskens JCA, Ferro BE, Mouton JW, Aarnoutse RE, van Ingen J. 2018. The stability of antimycobacterial drugs in media used for drug susceptibility testing. Diagn Microbiol Infect Dis 92:305–308.
25. Park S, Kim S, Park EM, Kim H, Kwon OJ, Chang CL, Lew WJ, Park YK, Koh W-J. 2008. In vitro antimicrobial susceptibility of Mycobacterium abscessus in Korea. J Korean Med Sci 23:49–52.
26. Ferro BE, Srivastava S, Deshpande D, Pasipanodya JG, van Soolingen D, Mouton JW, van Ingen J, Gumbo T. 2016. Moxifloxacin's Limited Efficacy in the Hollow-Fiber Model of Mycobacterium abscessus Disease. Antimicrob Agents Chemother 60:3779–3785.
27. Maurer FP, Bruderer VL, Ritter C, Castelberg C, Bloemberg GV, Böttger EC. 2014. Lack of antimicrobial bactericidal activity in Mycobacterium abscessus. Antimicrob Agents Chemother 58:3828–3836.
28. Wallace RJ, Brown-Elliott BA, Ward SC, Crist CJ, Mann LB, Wilson RW. 2001. Activities of linezolid against rapidly growing mycobacteria. Antimicrob Agents Chemother 45:764–767.
29. Aziz DB, Low JL, Wu M-L, Gengenbacher M, Teo JWP, Dartois V, Dick T. 2017. Rifabutin Is Active against Mycobacterium abscessus Complex. Antimicrob Agents Chemother 61.
30. Ferro BE, Srivastava S, Deshpande D, Pasipanodya JG, van Soolingen D, Mouton JW, van Ingen J, Gumbo T. 2016. Tigecycline Is Highly Efficacious against Mycobacterium abscessus Pulmonary Disease. Antimicrob Agents Chemother 60:2895–2900.
31. Wu M-L, Aziz DB, Dartois V, Dick T. 2018. NTM drug discovery: status, gaps and the way forward. Drug Discovery Today 23:1502–1519.
32. Le Run E, Arthur M, Mainardi J-L. 2018. In Vitro and Intracellular Activity of Imipenem Combined with Rifabutin and Avibactam against Mycobacterium abscessus. Antimicrob Agents Chemother 62.

33. Pryjma M, Burian J, Thompson CJ. 2018. Rifabutin Acts in Synergy and Is Bactericidal with Frontline Mycobacterium abscessus Antibiotics Clarithromycin and Tigecycline, Suggesting a Potent Treatment Combination. Antimicrob Agents Chemother 62.
34. Lefebvre A-L, Dubée V, Cortes M, Dorchêne D, Arthur M, Mainardi J-L. 2016. Bactericidal and intracellular activity of β-lactams against Mycobacterium abscessus. J Antimicrob Chemother 71:1556–1563.

Additional experiments

The previous experiments shown in article 4 was an initial screening using several combination, But as the time duration of 8 days may not be sufficient to evaluate the bacterial activity against these rapid growing mycobacteria, experiments with triple combination containing FOX, AMK and MXF were conducted for 21 days against a CLR-resistant *M. abscessus* strain Ma1611.

Here, we searched for the maximum achievable free plasma concentration (free Cmax) for each screened third molecule and from the literature, we could find that the *in vitro* active concentration of moxifloxacin was closer to the free Cmax (Bennett, Dolin, & Blaser, 2015). Then, as the resistance to CLR appears after 14 days, we did experiments up to 21 days to be really sure that the regrowth phase will not occur. Also, the stability of OADC media was confirmed in the safety datasheet of BD based on culture response analysis (BD, 2019).

Materials and Methods

Bacterial strain and suspension preparation: Mycobacterium abscessus subspecies abscessus, clinical isolate Ma1611 was obtained from CHU of Poitiers, France, which was isolated from lung expectoration. Stock vials were conserved at -80°C. For each experiment, the mycobacterial inoculum was prepared according to CLSI guidelines (NCCLS, 2003).

Time-kill kinetics assay: MICs were used from previous experiment as explained in article 4 in this manuscript. Various concentrations to be tested as monotherapy and in combination were selected based on their C_{max} values obtained from Mandell-infectious drugs (Bennett et al., 2015). Various concentrations were tested alone and in combination at multiple of free serum C_{max} as mentioned in Table XII.

For single antibiotics: Individual tubes of 20 mL of middlebrook 7H9 broth (BD, BBL™, Sparks, MD, USA) with 10% oleic acid/bovine albumin/dextrose/catalase (OADC) growth supplement (BD, BBL™, Sparks, MD, USA) and 0.5% glycerol containing FOX, AMK and MXF at various concentrations for clinical isolate Ma1611, were inoculated with the bacterial suspension (~ $1*10^6$ CFU/mL) and incubated at 35° ± 2°C, under shaking conditions (150 rpm) up to 21 days. Bacteria were quantified at 0, 2, 4, 7, 9, 11, 14, 16, 18 and 21 days by plating serial dilutions prepared with sterile phosphate buffer saline (PBS pH 7.4, Gibco™, by life technologies, France) on middlebrook 7H11 agar plates (BD, BBL™, Sparks, MD, USA) with 10% OADC and 0.5% glycerol (NCCLS, 2003). CFUs were enumerated after 3-5 days of

incubation at 35 ± 2°C. The theoretical detection limit was set to 200 CFU/mL i.e. 2.3 $\log_{10}$ CFU/mL.

For antibiotic combination: Following the same procedure as described above, time-kill assays for various bi-combinations and tri-combinations containing FOX, AMK and MXF were tested against the same strain at concentrations detailed in Table XII. As shown in previous study (Mehta *et al.*, 2019), cefoxitin is an unstable β-lactam antibiotic with a half-life of 1.5 days, 35% amount of cefoxitin was added each day throughout the experiment to compensate *in vitro* degradation.

In order to evaluate the existence of resistance subpopulation, several concentrations were plated on antibiotic containing agar plates (AMK 256 mg/L, MXF 32 mg/L and FOX 64 mg/L) at 0, 4, 11, 18 and 21 days.

Antibiotics: The following antibiotics were obtained as microbiological standards from their manufacturers: FOX (Panpharma, Luitré, France), AMK (Acros, Illkirch, France) and MXF (Sigma, Saint-Quentin-Fallavier, France). Stock solutions were prepared using appropriate solvents and stored at -80°C.

Curve fittings: Curve fittings were performed with GraphPad Prism (version 7.04) software for Windows (GraphPad Prism Software, San Diego, CA). In order to evaluate the effect of each antibiotics alone and in combination against Ma1611 at various concentrations, R package mrgsolve (Baron, s. d.) was used to show CFU versus time profiles.

Table XII: Various concentrations used for monotherapy and triple combinations, containing cefoxitin, amikacin and moxifloxacin against clinical isolate Ma1611

Antibiotics	MICs against Ma1611 (mg/L)	Maximum free serum concentrations (mg/L)			
		1*C_{max}	0.5*C_{max}	0.25*C_{max}	2*C_{max}
FOX†	8	50	25	12.5	100
AMK	32	40	20	10	80
MXF	4	2	1	0.5	4
Several bi-combinations	FOX 100 + AMK 80 FOX 100 + AMK 20 FOX 25 + AMK 80 FOX 100 + MXF 4 FOX 100 + MXF 1 FOX 25 + MXF 4 FOX 25 + MXF 1 AMK 80 + MXF 4 AMK 80 + MXF 1 AMK 20 + MXF 4 AMK 20 + MXF 1	FOX 100 + AMK 40 FOX 50 + AMK 40 FOX 25 + AMK 40 FOX 12.5 + AMK 40 FOX 50 + AMK 80 FOX 50 + AMK 20 FOX 12.5 + AMK 20 FOX 100 + AMK 10 FOX 50 + AMK 10 FOX 25 + AMK 10 FOX 12.5 + AMK 10	FOX 50 + AMK 80 FOX 12.5 + AMK 80 FOX 50 + AMK 40 FOX 12.5 + MXF 2 AMK 40 + MXF 2 FOX 50 + MXF 1 FOX 25 + MXF 2 AMK 20 + MXF 2 AMK 40 + MXF 1	
Several tri-combinations	FOX 100 + AMK 80 + MXF 4 FOX 100 + AMK 80 + MXF 1 FOX 100 + AMK 20 + MXF 4 FOX 100 + AMK 20 + MXF 1 FOX 25 + AMK 80 + MXF 4 FOX 25 + AMK 80 + MXF 1 FOX 25 + AMK 20 + MXF 4 FOX 25 + AMK 20 + MXF 1	FOX 50 + AMK 40 + MXF 2 FOX 50 + AMK 20 + MXF 2 FOX 50 + AMK 20 + MXF 1 FOX 50 + AMK 40 + MXF 1 FOX 25 + AMK 40 + MXF 2 FOX 25 + AMK 20 + MXF 2 FOX 25 + AMK 40 + MXF 1		

† 35% amount of FOX was compensated each 24h up to 21 days to maintain the FOX amount constant throughout the experiment
FOX, cefoxitin; AMK, amikacin; MXF, moxifloxacin

Results

The effect of each antibiotic alone, in combination of two and in combination of three are presented on Figure XIV, XV, XVI and XVII. From Figure XIV, it can be seen that AMK alone was not effective under the concentration below 40 mg/L, but at 80 mg/L AMK showed 2-3 log bacterial killing up to 8 days and then bacteria started regrowing. Which means that AMK was effective even up to 8 days at 2*C_{max} concentrations. The similar phenomenon was observed in case of FOX alone, but FOX alone at 2*C_{max} concentration showed complete bactericidal activity up to 21 days. Several bi-combinations of FOX and AMK showed complete bactericidal activity, which exhibited additive to synergistic effect between these two antibiotics. From Figure XV, it can be seen that MXF alone was not effective at all and the combination with AMK was also not effective against this clinical isolate. From Figure XVI, it

can be seen that MXF in combination with FOX showed complete bactericidal activity at higher concentration by reaching detection limit, however it can be said that this effect was influenced by FOX.

Tri-combinations of FOX, AMK and MXF reached detection limit at several concentration as shown on Figure XVII, but this effect was not superior that bi-combination of AMK and FOX and may even be antagonistic.

Here, it can also be observed that there was the presence of resistance subpopulation to each antibiotic from time 0 as it can be seen on each figure.

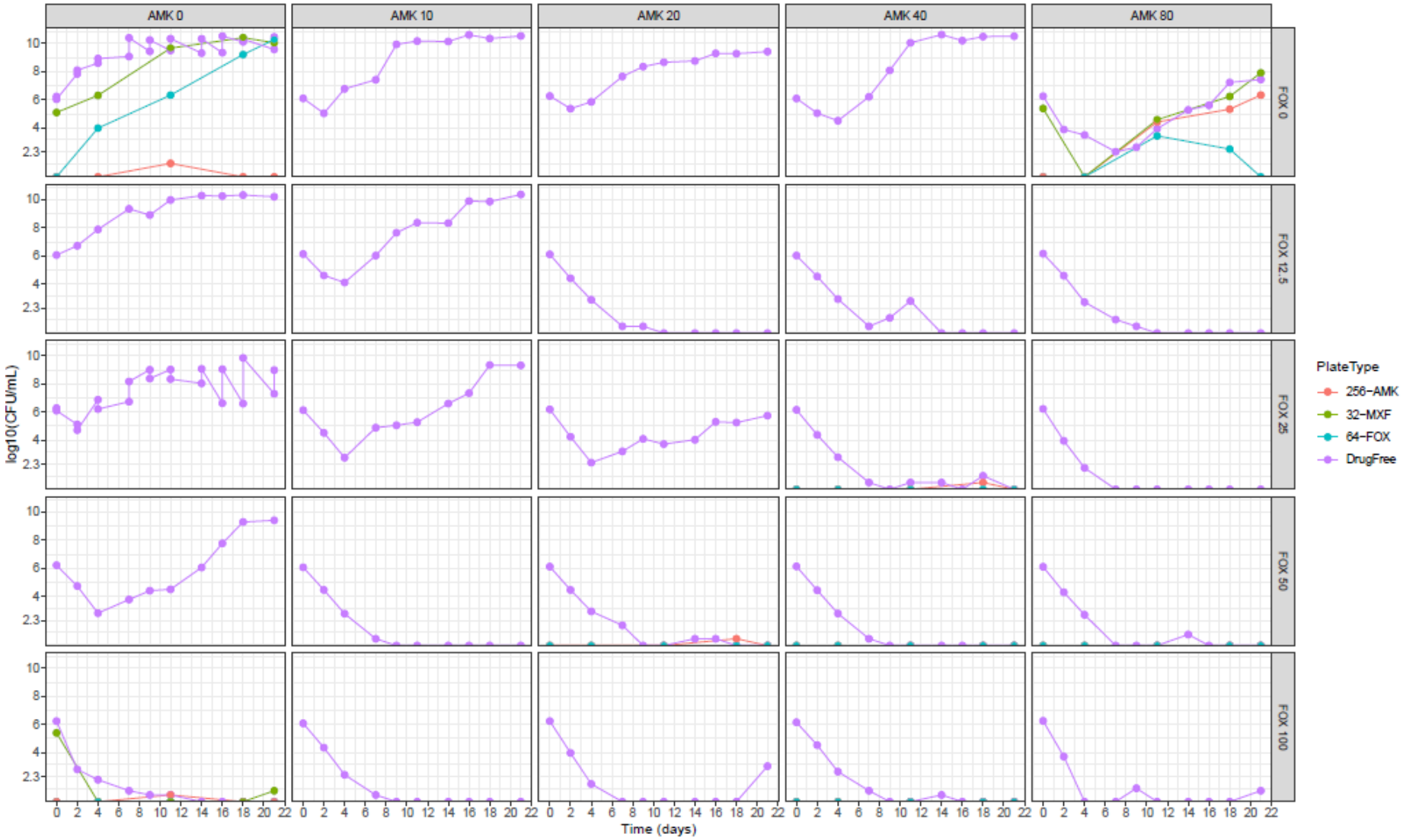

Figure XIV: Effect of cefoxitin and amikacin combination at different concentrations achievable in humans. CFUs were determined at 0, 2, 4, 7, 9, 11, 14, 18 and 21 days.

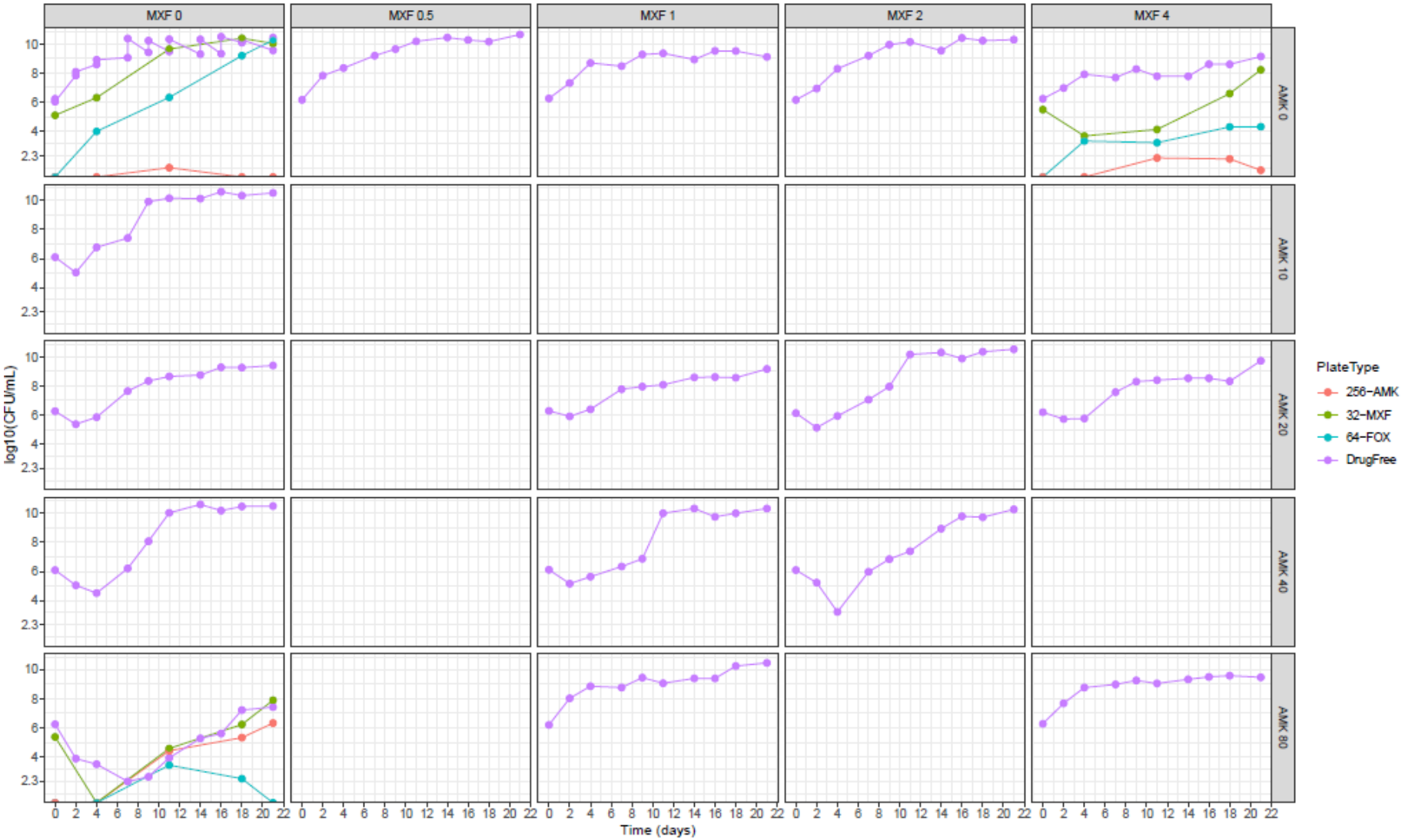

Figure XV: Effect of Moxifloxacin and amikacin combination at different concentrations achievable in humans. CFUs were determined at 0, 2, 4, 7, 9, 11, 14, 18 and 21 days.

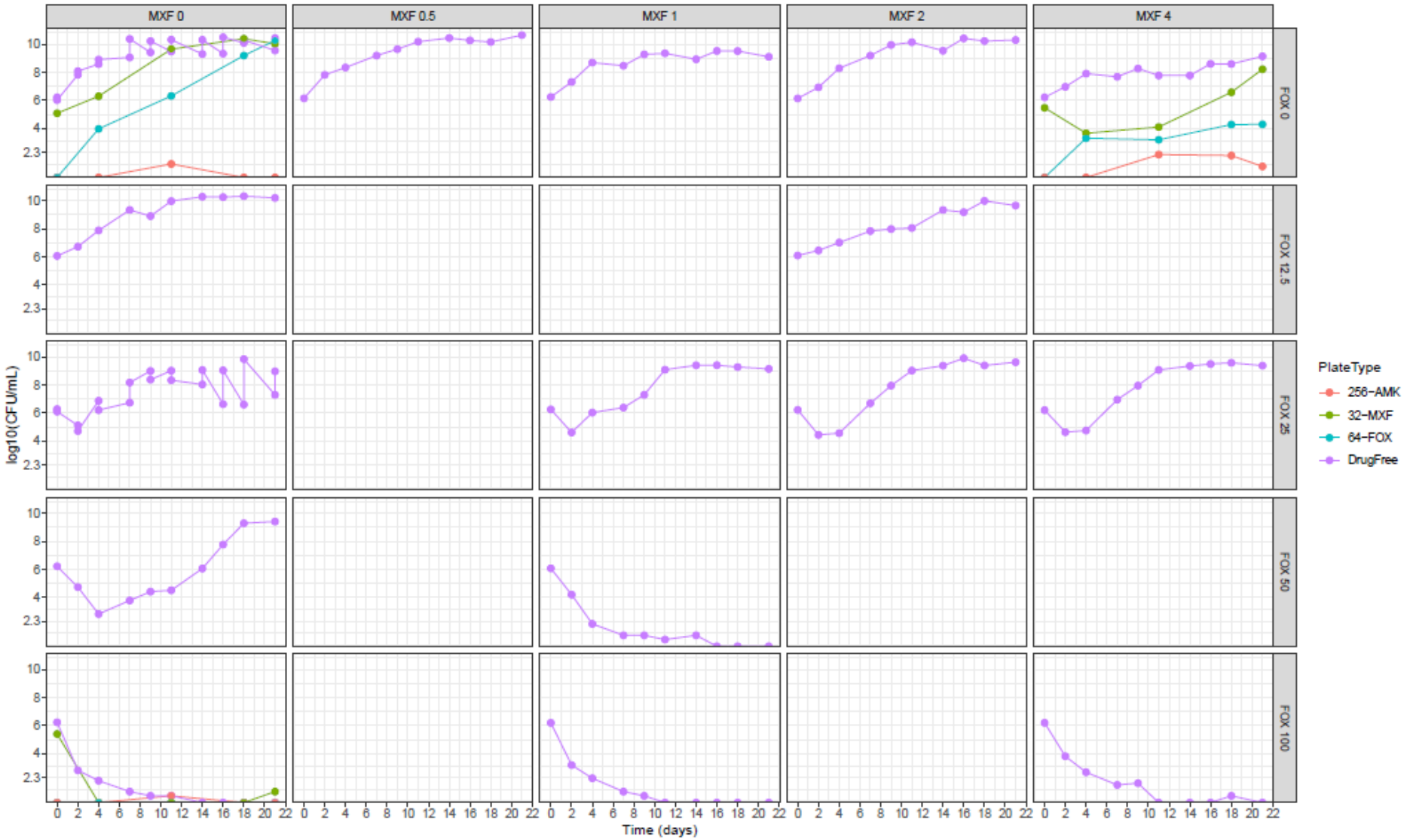

Figure XVI: Effect of cefoxitin and moxifloxacin combination at different concentrations achievable in humans. CFUs were determined at 0, 2, 4, 7, 9, 11, 14, 18 and 21 days.

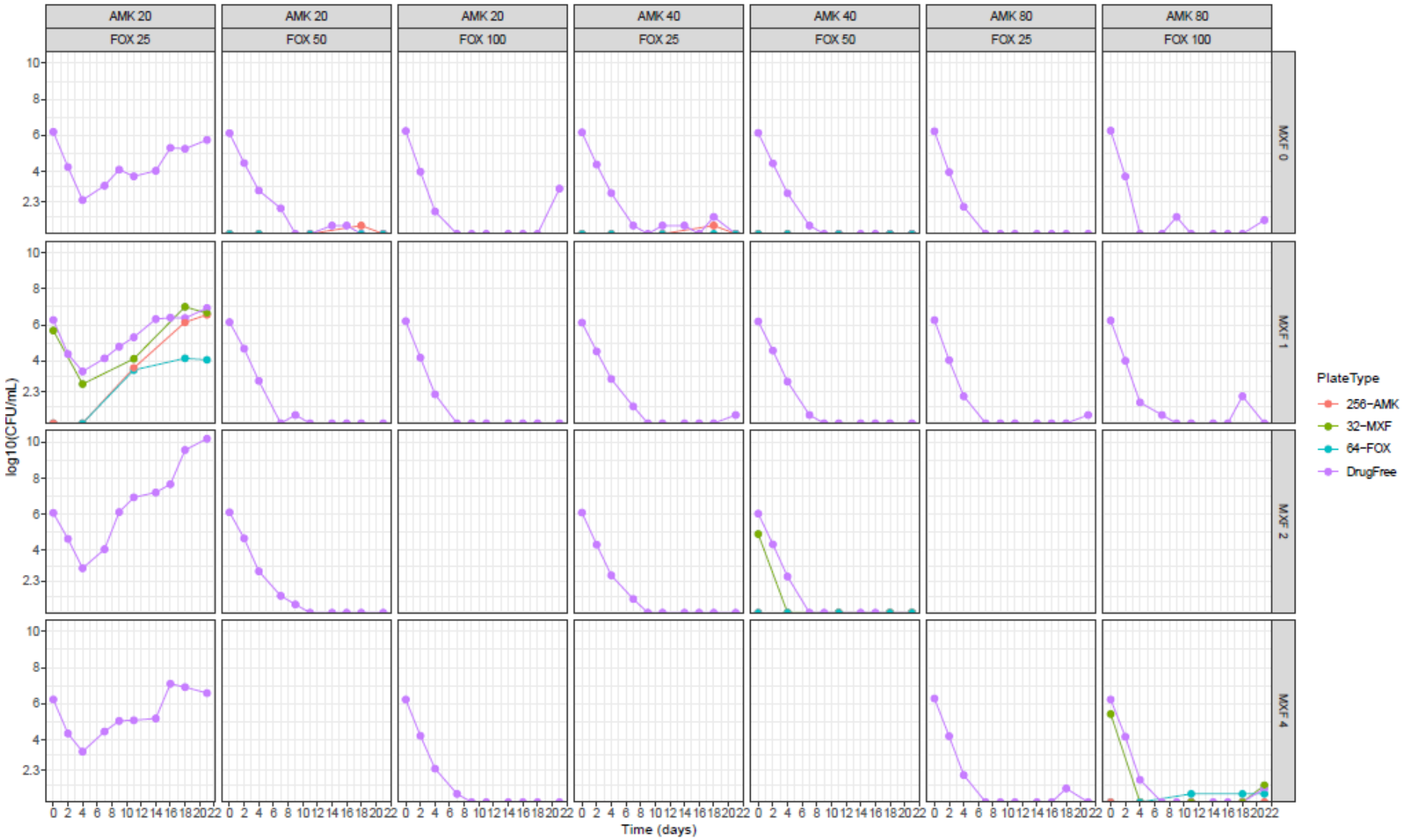

Figure XVII: Effect of cefoxitin, amikacin and moxifloxacin in combination at various concentrations achievable in humans. CFUs were determined at 0, 2, 4, 7, 9, 11, 14, 18 and 21 days.

DISCUSSION/PERSPECTIVE

Within this thesis, three major issues related to the *M. abscessus* pulmonary infections have been raised:

(1) The "gold standard therapy" against these infections, consisting clarithromycin in combination amikacin and cefoxitin or imipenem, is often associated with high treatment failure or poor outcomes mainly because of intravenous administration of cefoxitin and amikacin and intrinsic clarithromycin resistance. In such cases, use of nebulization of low permeability compounds (Gontijo, Grégoire, *et al.,* 2014; Marchand *et al.,* 2018; Yapa *et al.,* 2014) such as cefoxitin and amikacin may improve the treatment outcomes by allowing high concentrations at target site and limiting the systemic toxicity (Lee *et al.,* 2015; Mougari *et al.,* 2016; Olivier *et al.,* 2017; van Ingen *et al.,* 2013).

(2) The combination of cefoxitin and amikacin may improve the treatment outcomes; particularly when used in nebulization. However, the resistance due to clarithromycin is still a challenge and being recognized as a rising threat. In such cases, the replacement of clarithromycin with another efficient antibiotic combined with cefoxitin and amikacin should be considered to avoid emergence of resistance.

(3) β-lactams are known to have limited *in vitro* stability (Rominski *et al.,* 2017). Here, the PK/PD type modeling approach not only help to incorporate *in vitro* unstability of cefoxitin but also help in efficacy data interpretation using single antibiotic or in combination.

Cefoxitin and amikacin represent two backbones in the treatment of *M. abscessus* pulmonary infections, but the treatment for long duration (3-4 months to few years in case of *M. abscessus* infection) with these drugs given as intravenous administration lead to poor compliance for patients and systemic toxicity (David E. Griffith *et al.,* 2007). As discussed above, the administration via pulmonary route may be interesting for optimizing their efficiency, improving compliance and controlling systemic toxicity. For amikacin, which is a molecule with a PK/PD index as Cmax/MIC, to reach very high amount inside the lung may improve directly the efficiency. On the other hand, for an antibiotic showing a PK/PD index as T>MIC profile like cefoxitin, only one nebulized dose per day (or per week) might be sufficient instead of 2 or 3 IV administration per day improving thus the compliance for patients and ease of treatment in order to avoid the emergence of resistance.

To know their efficacy via pulmonary route, it is necessary to study their pharmacokinetics *in vivo*. In our laboratory, the series of experiments for biopharmaceutical optimization have been conducted for several molecules in order to determine preliminary biopharmaceutical classification of antibiotics for pulmonary delivery (Galindo Bedor *et al.*, 2016; Gontijo, Grégoire, *et al.*, 2014; Marchand *et al.*, 2018, 2015; Marchand, Grégoire, *et al.*, 2016; Yapa *et al.*, 2014). First two studies presented in this thesis present the interest of using intratracheal route of administration for amikacin and cefoxitin. Amikacin suspension for inhalation (Arikayce®) is already commercially available but no trace of cefoxitin being used as nebulized form. Even if our results are very preliminary data about the use of cefoxitin as nebulization, it is of note that from all the antibiotics evaluated in the laboratory, it was a molecule with the highest Targeting advantage (>1000), which makes it interesting to further evaluate the potential impact of the nebulization of cefoxitin on the *M. abscessus* pulmonary infections. Moreover, as for these infections a single agent is generally not recommended, development of inhaled formulation of cefoxitin and then use it in combination with existing amikacin inhaled formulation could be a nice companion in order to avoid emergence of resistance and improve efficacy. Here, other low permeability molecules like imipenem may also be good candidate for nebulization, but it needs to be analyzed. β-lactams are described to have limited *in vitro* stability (Rominski *et al.*, 2017) which is generally a neglected problem during *in vitro* static (antibiotic concentration) experiments with bacteria (replicates within hours) other than mycobacteria (takes days to weeks to replicate).

The evaluation and consideration of degradation of such molecules would avoid misinterpretation of MIC or time kill curves data as it has been raised as an important issue in the literature (Rominski *et al.*, 2017). To overcome this problem, we have developed a PK/PD type modeling approach as discussed in Article 2 to describe the time-kill data, including two subpopulations (FOX-susceptible and FOX-resistant), *in vitro* degradation and an I_{max} type growth inhibition model with Hill's coefficient. The development of these models was based on the *in vitro* experiments carried out against *M. abscessus* reference strain. The maximum information was extracted using these models to understand the pharmacodynamics of these antibiotics. Especially, using these models we could consider the *in vitro* degradation of cefoxitin over time and thus to estimate more accurately the emergence of cefoxitin resistance against these bacteria. Our model was able to describe our data and to go through this problem of antibiotic degradation showing that to kill cefoxitin resistance subpopulation, comparatively high concentration is required, which cannot be achieved using routine systemic administration

which is still in favor of nebulization as an effective alternative. As mentioned in one published work (Schoutrop *et al.,* 2018) that support administration may be a useful option in such case, an approach of 50% cefoxitin amount compensation each 24 h to support the degradation, throughout the *in vitro* experiment was adopted to resolve this problem.

The study of the PK and PD characteristics of cefoxitin and amikacin, and the clinical practice of treating *M. abscessus* infections using multidrug regimens led us to evaluate the efficacy of these two molecules in combination against *M. abscessus*. The purpose of the third and fourth studies were to evaluate *in vitro* efficacy of antibiotics alone and/or in combination and to develop semi-mechanistic PK/PD models to analyze these data.

The model describing the combined effect of cefoxitin and amikacin allowed us to verify the effect of combination against this mycobacterial strain. The combined effect of cefoxitin and amikacin was analyzed using Bliss independence model and Loewe additivity model (Foucquier & Guedj, 2015; Rao *et al.,* 2018). The final growth inhibition I_{max} model, which fit the individual data well for both antibiotics, determined that the joint activity of these combinations was better than could be achieved by either antibiotic alone and most of the combinations were additive to synergistic.

We then proposed to use cefoxitin and amikacin via nebulization in combination with a third molecule to replace macrolides leading to resistance during treatment. We preferentially chose oral antibiotics for ease of administration including linezolid, ciprofloxacin, moxifloxacin, rifampicin, rifabutin or clofazimine. So, despite of being good candidate against these bacteria, we didn't use tigecycline in this study as it is given by only IV administration. Antibiotic combination of low permeable molecules like nebulized cefoxitin and/or amikacin, which may kill extracellular mycobacteria but might be inefficient against intracellular bacteria which can replicate and survive inside macrophages (Wu, Aziz, Dartois, & Dick, 2018). The reason of being backbone of clarithromycin for this treatment is its capacity to accumulate inside macrophages and act intracellularly. To associate these molecules with one high permeable molecule like fluoroquinolones, rifamycins (preferentially rifabutin), linezolid or macrolides, which may accumulate inside macrophages may improve the killing of intracellular bacteria and lead to efficient therapy.

Here, despite of their properties to accumulate in lung tissues at concentrations above their susceptible MICs values (NCCLS, 2003), clarithromycin and linezolid cannot be the first choice of antibiotics because of their associated resistance (Nessar *et al.*, 2012; Zhang, Lu, Song, &

Pang, 2018), in contrast to rifabutin. Low achievable plasma concentration of antibiotics except moxifloxacin render all screened antibiotics in this study, poor candidates for novel treatment regimens. However, plasma concentrations for rifabutin still need to be evaluated. Also, rifabutin is a good candidate with its intracellular activity in combination against *M. abscessus* within this niche (Le Run *et al.*, 2018). Including all these factors, triple combination of nebulized amikacin and cefoxitin with an oral fluoroquinolone or rifamycin, can be an auspicious treatment option against intracellular *M. abscessus* pulmonary infections.

But these studies have several limitations:

- The real issue is a long period of experiments. As it is known that mycobacteria grow slowly than other bacteria like *Pseudomonas, E.coli*, etc., it necessitates prolonged clinical treatment (over some months). Also, the resistance due to clarithromycin appears after 14 days, which requires *in vitro* experiments at least >14 days in order to obtain unbiased and appropriate results. All these reasons lead to a long duration commitment with heavy workload (Ruth *et al.*, 2019). We have already started performing several experiments in combination up to 21 days of experiments with daily dose addition of cefoxitin in order to compensate its degradation over time.
- In addition to quantifying individual or combined effects, PK/PD modeling of antibiotics can improve the understanding of antibiotic effect at various concentration, the concentrations need to kill mycobacterial subpopulations at target site, their mechanism of actions in order to extrapolate *in vitro* results to develop clinical studies and improve the treatment options. In order to build a much-constrained mechanistic model, there is also a need for information like results obtained from case studies, study of resistance mechanisms from a genetic point of view including gene expression, characteristics of mycobacterial species, etc. Here, we can include microbiological technics like MUTF, PAPs to determine subpopulations and sequencing and qPCR analysis to identify mechanistic informations in order to improve PK/PD models. In this direction, we have already started several combination experiments including PAPs analysis.
- The use of very standardized media to "enhance" *in vitro* growth of this bacteria is also one limitation as performing *in vitro* experiments in rich and standardized media (e.g. Middlebrook 7H9 broth supplemented with cations for MIC determinations), are not representative of human infection sites, which may influence the results *in vitro* and in clinical practice like it has been showed for other bacterial species (Buyck *et al.*, 2012).

Performing the experiments in a culture medium closer to the *in vivo* infection medium may improve the quality of our predictions like artificial sputum medium, which can mimic the bacterial growth during CF infections, possibly with the formation of biofilms (Miranda-CasoLuengo, Staunton, Dinan, Lohan, & Loftus, 2016).

- Another limitation is that simple agar counting does not reveal all physiological states of bacteria. In general, we are just able to distinguish between susceptibility and resistance but additional physiological states like tolerance, persistence, etc. could also play a role in antibiotic resistance and in recurrence of infection. Techniques such as the oCelloScope (Fredborg *et al.*, 2013) and/or flow cytometry (Brown *et al.*, 2019) could bring other informations like shape (for the oCelloScope) and size (for flow cytometry) or persisters and viable but non-cultivable cells that we cannot detect using classical microbiology methods based on the growth of bacteria on agar plate. The use of all these techniques will allow us to better identify and categorize the different bacterial subpopulations present in our experiments which probably play a role in resistance to antimicrobials.

In vitro experiments showed in this manuscript revealed the efficiency of combined regimens against *M. abscessus* reference strain as well as clinical isolates including clarithomycin resistance to multidrug resistance. The efficient activity of these combinations may warrant a "renaissance" of treatment against *M. abscessus*. But as the *in vitro* static conditions determined here doesn't represent the dynamic condition which can be evaluated by means of hollow fiber analysis or *in vivo* experiments, we cannot extrapolate these data into clinical practice. Also, the information considered in *in vivo* experiments or clinical setting like daily intake of drugs and their pharmacokinetics and target site of mycobacterial infection create a different scenario. Thus, the next step should be to continue these studies using dynamic PK/PD models and mechanism-based mathematical models using intracellular infection data, hollow fiber experiments may be mixed with intracellular models, *in vivo* experiments with chronic lung infection and preclinical assessment to evaluate the effect of multidrug regimens and then clinical implementation. The use of nebulized drugs leads to high concentration but maybe not at the right site of action (intracellular) which requires a development of such antibiotic in formulation may be as liposome or nanoparticles like commercially available Amikacin suspension for inhalation (Arikayce®). Different formulation in order to optimize drug release at target site and coformulation might lead to new effective treatment modalities.

In recent years, PK/PD modelling has gained importance, particularly for drug safety agencies that actively promote its use. From now on, the challenge of extrapolating the results obtained

in vitro into an *in vivo* context must be met. To do this, the integration of mechanistic information into our models seems to be a good way forward and the work presented in this thesis is a first step in this direction. Different sustained release formulations may be an option to think about. Also, the experiments to evaluate the better combination activity should be considered. Many experimental techniques like PAPs, intracellular model, biofilm model etc. can be the medium to better evaluate the combination activity and understand the mechanisms involved in antibiotic treatment. These different models may help to design more efficient treatment regimens.

ANNEXES

A. Trail experiments

Based on literature review and their ability of being given as nebulized antibiotic, we decided cefoxitin and amikacin as backbones for the treatment of this infection. In addition to this, we have tested MICs of several antibiotic against reference strain and few clinical isolates and results are presented in Table XIII. *Mycobacterium abscessus subspecies abscessus,* reference strain CIP104536, was obtained from Institute Pasteur (Paris, France), clinical isolates Ma1611, Ma1507, Ma1703 and Ma1111 were isolated from lung expectoration (CHU of Poitiers, France) and T28 was isolated from bronchial aspiration in patients with cystic fibrosis (Lariboisière Hospital, Paris, France). Stock vials were conserved at -80°C. For each experiment, the mycobacterial inoculum was prepared according to CLSI guidelines. MICs were determined by broth microdilution method using Cation-adjusted Mueller–Hinton broth 2 (CAMHB II; Sigma-Aldrich, Saint-Quentin-Fallavier France) for each antibiotic. The antibiotics were serially diluted to the desired final concentration in a 96-well plate. The bacterial suspension ($1*10^6$ CFU/mL) was added to each well, with the final antibiotic concentrations ranging from 0.125 to 1024 mg/L. A positive control, only with bacterial suspension and without antibiotic was included for each strain. The plates were incubated at 30°C for 3 days. MICs were visually determined. All experiments were performed in duplicate.

Based on the MIC results, it can be seen that all clinical isolates showed almost similar activity as reference strain except clinical isolate T28. Rifampicin, linezolid, clofazimine, clarithromycin, ciprofloxacin, moxifloxacin and tigecycline have been chosen to perform time-kill kinetics analysis. Then to check the activity of each effective antibiotic alone, time-kill kinetics experiments were performed with multiples of MICs (0.25*MIC, 0.5*MIC, 1*MIC and 4*MIC) up to 8 days, as per the procedure explained in the articles above. The results obtained for cefoxitin and amikacin are mentioned in article 2 and 3, other results are shown here.

Table XIII: Antimicrobial susceptibility testing for several antibiotics against *M. abscessus* reference strain and few clinical isolates

Antibiotics	MICs (mg/L)					
	CIP 104536	Ma1507	Ma1611	Ma1703	Ma1111	T28
Ciprofloxacin	4	16	4	8	8	8
Moxifloxacin	4	16	4	8	8	16
Linezolid	8	4	8	8	2	256
Meropenem	8	4	16	16	16	64
Rifabutin	2	2	2	2	2	16
Clofazimine	2	0.5	16	16	16	4
Rifampicin	16	16	16	16	16	256
Ethambutol	>256	>256	>256	>256	>256	64
Tobramycin	16	16	256	128	>256	>256
Colistin	256	>256	>256	>256	>256	>256
Polymyxin B	128	>256	>256	>256	>256	>256
Imipenem	0.125	0.25	0.5	0.5	2	64
Dapson	>256	>256	>256	>256	>256	>256
Cefoxitin	8	8	8	8	8	64
Amikacin	32	16	16	16	32	>1024
Cefuroxime	4	4	4	4	4	>256
Tigecycline	4	>256	>256	>256	>256	>256
Clarithromycin (D3)	1	2	16	32	32	>256
Farnesol (Nano-capsules)	>256	>256	>256	>256	>256	>256
Geraniol (Nano-capsules)	>256	>256	>256	>256	>256	>256

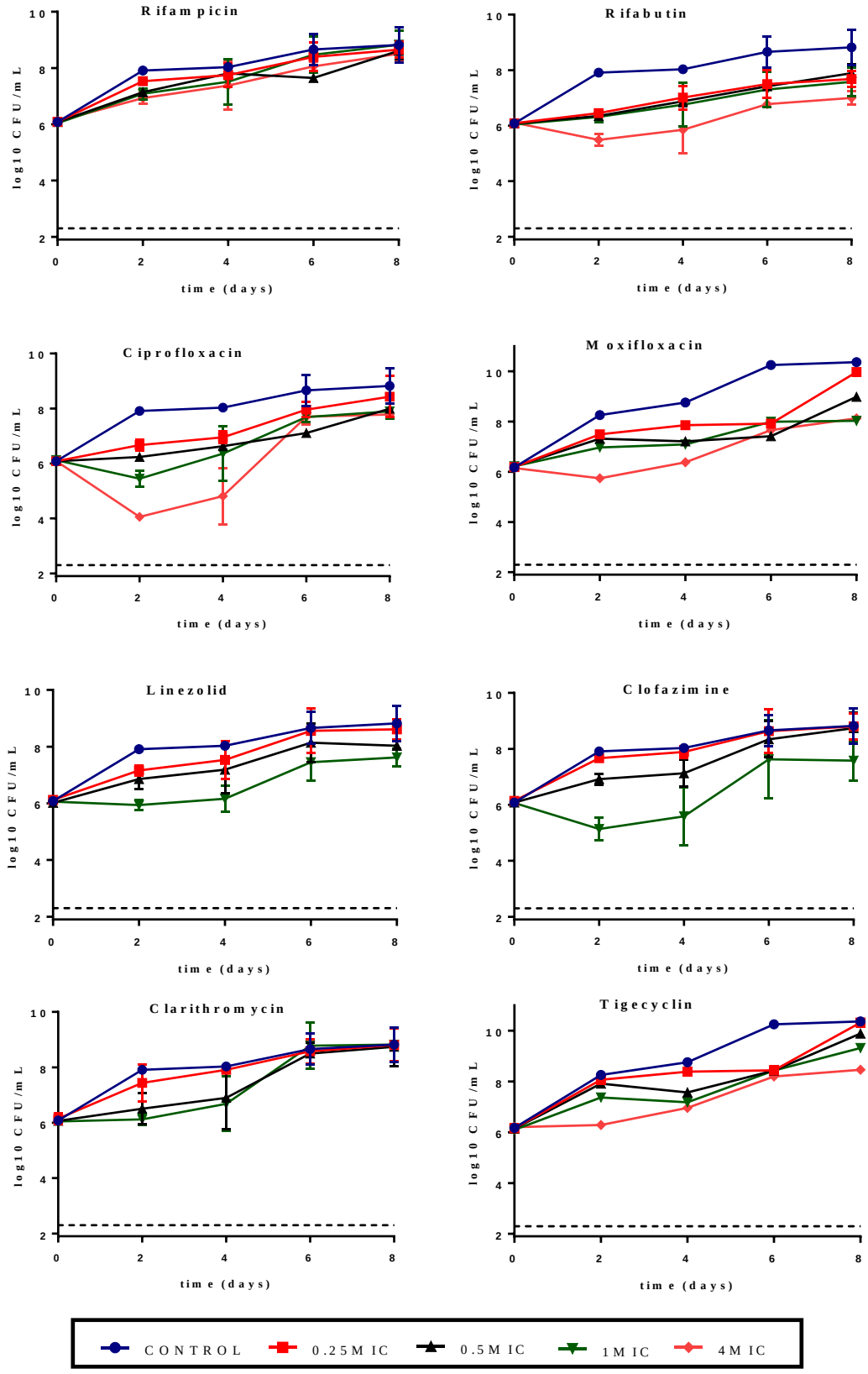

Figure XVIII: Time-kill kinetics experiments of several antibiotics alone at 0.25*MIC, 0.5*MIC, 1*MIC and 2*MIC of each antibiotic against *M. abscessus* CIP104536

Not a single antibiotic was effective against these bacteria. Then to check the activity of these antibiotic in combination with cefoxitin or amikacin, several bi-combinations were tested against reference strain CIP104536 using checkerboard analysis and E-test method.

Table XIV*:* Fractional Inhibitory concentrations indices of antibiotic in combination

Antibiotic combinations	MIC of single agent (mg/L)	MIC in combinations (mg/L)	FICi
Cefoxitin and amikacin	8/32	4/16	1
Cefoxitin and rifampicin	8/16	8/8	1.5
Cefoxitin and rifabutin	8/2	4/1	1
Cefoxitin and ciprofloxacin	8/4	4/8	2.5
Cefoxitin and moxifloxacin	8/4	4/4	1.5
Cefoxitin and linezolid	8/8	4/4	1
Cefoxitin and clofazimine	8/2	8/2	2
Cefoxitin and clarithromycin	8/1	8/2	3
Amikacin and rifampicin	32/16	16/8	1
Amikacin and rifabutin	32/2	32/2	2
Amikacin and ciprofloxacin	32/4	16/4	1.5
Amikacin and moxifloxacin	32/4	16/2	1
Amikacin and linezolid	32/8	32/4	1.5
Amikacin and clofazimine	32/2	16/1	1
Amikacin and clarithromycin	32/1	32/1	2

After repeated for twice, it was difficult to interpret the results from checkerboard analysis. No synergy or antagonism was detected in most of the combinations. Furthermore, the combination of cefoxitin and amikacin was tested using time-kill kinetics analysis as explained in article 3 and then several tri-combinations were tested against reference strain and selected clinical isolates.

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REFERENCES

Aguilar-Ayala, D. A., Cnockaert, M., André, E., Andries, K., Gonzalez-Y-Merchand, J. A., Vandamme, P., … Martin, A. (2017). In vitro activity of bedaquiline against rapidly growing nontuberculous mycobacteria. *Journal of Medical Microbiology*, *66*(8), 1140-1143. https://doi.org/10.1099/jmm.0.000537

Amidon, G. L., Lennernäs, H., Shah, V. P., & Crison, J. R. (1995). A theoretical basis for a biopharmaceutic drug classification : The correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharmaceutical Research*, *12*(3), 413-420.

Anders, K. L., Nguyen, H. L., Nguyen, N. M., Van Thuy, N. T., Hong Van, N. T., Hieu, N. T., … Simmons, C. P. (2015). Epidemiology and virology of acute respiratory infections during the first year of life : A birth cohort study in Vietnam. *The Pediatric Infectious Disease Journal*, *34*(4), 361-370. https://doi.org/10.1097/INF.0000000000000643

Aziz, Dinah B., Teo, J. W. P., Dartois, V., & Dick, T. (2018). Teicoplanin—Tigecycline Combination Shows Synergy Against Mycobacterium abscessus. *Frontiers in Microbiology*, *9*, 932. https://doi.org/10.3389/fmicb.2018.00932

Aziz, Dinah Binte, Low, J. L., Wu, M.-L., Gengenbacher, M., Teo, J. W. P., Dartois, V., & Dick, T. (2017). Rifabutin Is Active against Mycobacterium abscessus Complex. *Antimicrobial Agents and Chemotherapy*, *61*(6). https://doi.org/10.1128/AAC.00155-17

Banaschewski, B., & Hofmann, T. (2019). Inhaled Antibiotics for Mycobacterial Lung Disease. *Pharmaceutics*, *11*(7). https://doi.org/10.3390/pharmaceutics11070352

Barger, A., Fuhst, C., & Wiedemann, B. (2003). Pharmacological indices in antibiotic therapy. *The Journal of Antimicrobial Chemotherapy*, *52*(6), 893-898. https://doi.org/10.1093/jac/dkg482

Baron, K. T. (s. d.). *mrgsolve : Simulate from ODE-Based Population PK/PD and Systems Pharmacology Models*. Consulté à l'adresse https://github.com/metrumresearchgroup/mrgsolve

BD. (2019). *Middlebrook 7H10 Agar • Middlebrook and Cohn 7H10 Agar • Middlebrook OADC Enrichment*. https://doi.org/Difco™ & BBL™ Manual, 2nd Edition

Bennett, J., Dolin, R., & Blaser, M. (2015). Tables of Anti-infective Agent Pharmacology. Principles and Practice of Infectious Diseases, 8th edition. In *Mandell Infectious Drugs*: *Vol. Chapter 54*.

Bermudez, L., Blanchard, J., Hauck, L., & Gonda, I. (2015). Treatment of Mycobacterium avium subsp hominissuis (MAH) Lung Infection with Liposome-Encapsulated Ciprofloxacin Resulted in Significant Decrease in Bacterial Load in the Lung | D108. DIAGNOSIS AND MANAGEMENT OF NONTUBERCULOUS MYCOBACTERIA INFECTIONS. Consulté 6 septembre 2018, à l'adresse https://www.atsjournals.org/doi/pdf/10.1164/ajrccm-conference.2015.191.1_MeetingAbstracts.A6293

Bernut, A., Le Moigne, V., Lesne, T., Lutfalla, G., Herrmann, J.-L., & Kremer, L. (2014). In vivo assessment of drug efficacy against Mycobacterium abscessus using the embryonic zebrafish test system. *Antimicrobial Agents and Chemotherapy*, *58*(7), 4054-4063. https://doi.org/10.1128/AAC.00142-14

Bliss, C. I. (1939). THE TOXICITY OF POISONS APPLIED JOINTLY1. *Annals of Applied Biology*, *26*(3), 585-615. https://doi.org/10.1111/j.1744-7348.1939.tb06990.x

Boisson, M., Mimoz, O., Hadzic, M., Marchand, S., Adier, C., Couet, W., & Grégoire, N. (2018). Pharmacokinetics of intravenous and nebulized gentamicin in critically ill patients. *The Journal of Antimicrobial Chemotherapy*. https://doi.org/10.1093/jac/dky239

Bosquillon, C., Madlova, M., Patel, N., Clear, N., & Forbes, B. (2017). A Comparison of Drug Transport in Pulmonary Absorption Models : Isolated Perfused rat Lungs, Respiratory Epithelial Cell Lines and Primary Cell Culture. *Pharmaceutical Research*, *34*(12), 2532-2540. https://doi.org/10.1007/s11095-017-2251-y

Breen, R. a. M., Smith, C. J., Bettinson, H., Dart, S., Bannister, B., Johnson, M. A., & Lipman, M. C. I. (2004). Paradoxical reactions during tuberculosis treatment in patients with and without HIV co-infection. *Thorax*, *59*(8), 704-707. https://doi.org/10.1136/thx.2003.019224

Brown, M. R., Hands, C. L., Coello-Garcia, T., Sani, B. S., Ott, A. I. G., Smith, S. J., & Davenport, R. J. (2019). A flow cytometry method for bacterial quantification and biomass estimates in activated sludge. *Journal of Microbiological Methods*, *160*, 73-83. https://doi.org/10.1016/j.mimet.2019.03.022

Brown-Elliott, B. A., Nash, K. A., & Wallace, R. J. (2012). Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria. *Clinical Microbiology Reviews*, *25*(3), 545-582. https://doi.org/10.1128/CMR.05030-11

Brown-Elliott, B. A., & Wallace, R. J. (2017). In Vitro Susceptibility Testing of Tedizolid against Nontuberculous Mycobacteria. *Journal of Clinical Microbiology*, *55*(6), 1747-1754. https://doi.org/10.1128/JCM.00274-17

Brown-Elliott, B. A., & Wallace, R. J. (2019). In Vitro Susceptibility Testing of Bedaquiline against Mycobacterium abscessus Complex. *Antimicrobial Agents and Chemotherapy*, *63*(2), e01919-18. https://doi.org/10.1128/AAC.01919-18

Bryant, J. M., Grogono, D. M., Rodriguez-Rincon, D., Everall, I., Brown, K. P., Moreno, P., … Floto, R. A. (2016). Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium. *Science (New York, N.Y.)*, *354*(6313), 751-757. https://doi.org/10.1126/science.aaf8156

Buyck, J. M., Plésiat, P., Traore, H., Vanderbist, F., Tulkens, P. M., & Van Bambeke, F. (2012). Increased Susceptibility of Pseudomonas aeruginosa to Macrolides and Ketolides in Eukaryotic Cell Culture Media and Biological Fluids Due to Decreased Expression of oprM and Increased Outer-Membrane Permeability. *Clinical Infectious Diseases*, *55*(4), 534-542. https://doi.org/10.1093/cid/cis473

Camargos, P., Le Bourgeois, M., Revillon, Y., Tatsuo, E., Sermet-Gaudelus, I., Scheinmann, P., & de Blic, J. (2008). Lung resection in cystic fibrosis : A survival analysis. *Pediatric Pulmonology*, *43*(1), 72-76. https://doi.org/10.1002/ppul.20742

CF foundation, B. (2017). About Cystic Fibrosis. Consulté 20 août 2018, à l'adresse /What-is-CF/About-Cystic-Fibrosis/

CF foundation Patient Registry. (2016). *2016 Patient Registry Annual Data Report*. 94.

Chalermskulrat, W., Sood, N., Neuringer, I. P., Hecker, T. M., Chang, L., Rivera, M. P., … Aris, R. M. (2006). Non-tuberculous mycobacteria in end stage cystic fibrosis : Implications for lung transplantation. *Thorax*, *61*(6), 507-513. https://doi.org/10.1136/thx.2005.049247

Chiaradia, L., Lefebvre, C., Parra, J., Marcoux, J., Burlet-Schiltz, O., Etienne, G., … Daffé, M. (2017). Dissecting the mycobacterial cell envelope and defining the composition of the native mycomembrane. *Scientific Reports*, *7*(1), 12807. https://doi.org/10.1038/s41598-017-12718-4

Choi, G.-E., Min, K.-N., Won, C.-J., Jeon, K., Shin, S. J., & Koh, W.-J. (2012). Activities of moxifloxacin in combination with macrolides against clinical isolates of Mycobacterium abscessus and Mycobacterium massiliense. *Antimicrobial Agents and Chemotherapy*, *56*(7), 3549-3555. https://doi.org/10.1128/AAC.00685-12

Cremades, R., Santos, A., Rodríguez, J. C., Garcia-Pachón, E., Ruiz, M., & Royo, G. (2009). Mycobacterium abscessus from respiratory isolates : Activities of drug combinations. *Journal of Infection and Chemotherapy: Official Journal of the Japan Society of Chemotherapy*, *15*(1), 46-48. https://doi.org/10.1007/s10156-008-0651-y

Czaja, C. A., Levin, A., Moridani, M., Krank, J. L., Curran-Everett, D., & Anderson, P. L. (2014). Cefoxitin Continuous Infusion for Lung Infection Caused by the Mycobacterium abscessus Group. *Antimicrobial Agents and Chemotherapy*, *58*(6), 3570-3571. https://doi.org/10.1128/AAC.02763-14

Dailloux, M., Abalain, M. L., Laurain, C., Lebrun, L., Loos-Ayav, C., Lozniewski, A., … French Mycobacteria Study Group. (2006). Respiratory infections associated with nontuberculous mycobacteria in non-HIV patients. *The European Respiratory Journal*, *28*(6), 1211-1215. https://doi.org/10.1183/09031936.00063806

Dedrick, R. M., Guerrero-Bustamante, C. A., Garlena, R. A., Russell, D. A., Ford, K., Harris, K., … Spencer, H. (2019). Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant Mycobacterium abscessus. *Nature Medicine*, *25*(5), 730-733. https://doi.org/10.1038/s41591-019-0437-z

Derendorf, H., & Meibohm, B. (1999). Modeling of Pharmacokinetic/Pharmacodynamic (PK/PD) Relationships : Concepts and Perspectives. *Pharmaceutical Research*, *16*(2), 176-185. https://doi.org/10.1023/A:1011907920641

Dhanani, J., Roberts, J. A., Chew, M., Lipman, J., Boots, R. J., Paterson, D. L., & Fraser, J. F. (2010). Antimicrobial chemotherapy and lung microdialysis : A review. *International Journal of Antimicrobial Agents*, *36*(6), 491-500. https://doi.org/10.1016/j.ijantimicag.2010.08.013

Duan, H., Chu, N., Wang, Q., Wang, J., Huang, H., & Liang, Q. (2013). [Mycobacterium abscessus group lung disease : Case reports and review of the literature]. *Zhonghua Jie He He Hu Xi Za Zhi = Zhonghua Jiehe He Huxi Zazhi = Chinese Journal of Tuberculosis and Respiratory Diseases*, *36*(9), 671-674.

Dubée, V., Bernut, A., Cortes, M., Lesne, T., Dorchene, D., Lefebvre, A.-L., … Arthur, M. (2015). β-Lactamase inhibition by avibactam in Mycobacterium abscessus. *The Journal of Antimicrobial Chemotherapy*, *70*(4), 1051-1058. https://doi.org/10.1093/jac/dku510

Dubée, V., Soroka, D., Cortes, M., Lefebvre, A.-L., Gutmann, L., Hugonnet, J.-E., … Mainardi, J.-L. (2015). Impact of β-lactamase inhibition on the activity of ceftaroline against Mycobacterium tuberculosis and Mycobacterium abscessus. *Antimicrobial Agents and Chemotherapy*, *59*(5), 2938-2941. https://doi.org/10.1128/AAC.05080-14

Eixarch, H., Haltner-Ukomadu, E., Beisswenger, C., & Bock, U. (2010). Drug Delivery to the Lung : Permeability and Physicochemical Characteristics of Drugs as the Basis for a Pulmonary Biopharmaceutical Classification System (pBCS). *Journal of Epithelial Biology & Pharmacology*, *3*(1). Consulté à l'adresse https://benthamopen.com/ABSTRACT/JEBP-3-1

Falkinham, J. O. (2002). Nontuberculous mycobacteria in the environment. *Clinics in Chest Medicine*, *23*(3), 529-551.

Falkinham, J. O. (2010). Impact of human activities on the ecology of nontuberculous mycobacteria. *Future Microbiology*, *5*(6), 951-960. https://doi.org/10.2217/fmb.10.53

Falkinham, J. O., Iseman, M. D., de Haas, P., & van Soolingen, D. (2008). Mycobacterium avium in a shower linked to pulmonary disease. *Journal of Water and Health*, *6*(2), 209-213. https://doi.org/10.2166/wh.2008.032

Fernandes, C. A., & Vanbever, R. (2009a). Preclinical models for pulmonary drug delivery. *Expert Opinion on Drug Delivery*, *6*(11), 1231-1245. https://doi.org/10.1517/17425240903241788

Fernandes, C. A., & Vanbever, R. (2009b). Preclinical models for pulmonary drug delivery. *Expert Opinion on Drug Delivery*, *6*(11), 1231-1245. https://doi.org/10.1517/17425240903241788

Ferro, B. E., Meletiadis, J., Wattenberg, M., de Jong, A., van Soolingen, D., Mouton, J. W., & van Ingen, J. (2016). Clofazimine Prevents the Regrowth of Mycobacterium abscessus and Mycobacterium avium Type Strains Exposed to Amikacin and Clarithromycin. *Antimicrobial Agents and Chemotherapy*, *60*(2), 1097-1105. https://doi.org/10.1128/AAC.02615-15

Ferro, B. E., Srivastava, S., Deshpande, D., Pasipanodya, J. G., van Soolingen, D., Mouton, J. W., … Gumbo, T. (2016a). Failure of the Amikacin, Cefoxitin, and Clarithromycin Combination Regimen for Treating Pulmonary Mycobacterium abscessus Infection. *Antimicrobial Agents and Chemotherapy*, *60*(10), 6374-6376. https://doi.org/10.1128/AAC.00990-16

Ferro, B. E., Srivastava, S., Deshpande, D., Pasipanodya, J. G., van Soolingen, D., Mouton, J. W., … Gumbo, T. (2016b). Moxifloxacin's Limited Efficacy in the Hollow-Fiber Model of Mycobacterium abscessus Disease. *Antimicrobial Agents and Chemotherapy*, *60*(6), 3779-3785. https://doi.org/10.1128/AAC.02821-15

Ferro, B. E., Srivastava, S., Deshpande, D., Pasipanodya, J. G., van Soolingen, D., Mouton, J. W., … Gumbo, T. (2016c). Tigecycline Is Highly Efficacious against Mycobacterium

abscessus Pulmonary Disease. *Antimicrobial Agents and Chemotherapy*, *60*(5), 2895-2900. https://doi.org/10.1128/AAC.03112-15

Ferro, B. E., Srivastava, S., Deshpande, D., Sherman, C. M., Pasipanodya, J. G., van Soolingen, D., … Gumbo, T. (2015). Amikacin Pharmacokinetics/Pharmacodynamics in a Novel Hollow-Fiber Mycobacterium abscessus Disease Model. *Antimicrobial Agents and Chemotherapy*, *60*(3), 1242-1248. https://doi.org/10.1128/AAC.02282-15

Ferro, B. E., van Ingen, J., Wattenberg, M., van Soolingen, D., & Mouton, J. W. (2015). Time-kill kinetics of antibiotics active against rapidly growing mycobacteria. *The Journal of Antimicrobial Chemotherapy*, *70*(3), 811-817. https://doi.org/10.1093/jac/dku431

Floto, R. A., Olivier, K. N., Saiman, L., Daley, C. L., Herrmann, J.-L., Nick, J. A., … Haworth, C. S. (2016). US Cystic Fibrosis Foundation and European Cystic Fibrosis Society consensus recommendations for the management of non-tuberculous mycobacteria in individuals with cystic fibrosis : Executive summary. *Thorax*, *71*(1), 88-90. https://doi.org/10.1136/thoraxjnl-2015-207983

Foucquier, J., & Guedj, M. (2015). Analysis of drug combinations : Current methodological landscape. *Pharmacology Research & Perspectives*, *3*(3). https://doi.org/10.1002/prp2.149

Fredborg, M., Andersen, K. R., Jørgensen, E., Droce, A., Olesen, T., Jensen, B. B., … Sondergaard, T. E. (2013). Real-Time Optical Antimicrobial Susceptibility Testing. *Journal of Clinical Microbiology*, *51*(7), 2047-2053. https://doi.org/10.1128/JCM.00440-13

Fujita, K., Ito, Y., Hirai, T., Kubo, T., Togashi, K., Ichiyama, S., & Mishima, M. (2014). Prevalence and risk factors for chronic co-infection in pulmonary Mycobacterium avium complex disease. *BMJ Open Respiratory Research*, *1*(1), e000050. https://doi.org/10.1136/bmjresp-2014-000050

Galindo Bedor, D. C., Marchand, S., Lamarche, I., Laroche, J., Pereira de Santana, D., & Couet, W. (2016). Biopharmaceutical Characterization of Nebulized Antimicrobial Agents in Rats : 5. Oseltamivir Carboxylate. *Antimicrobial Agents and Chemotherapy*, *60*(8), 5085-5087. https://doi.org/10.1128/AAC.00909-16

Ganapathy, U. S., Dartois, V., & Dick, T. (2019). Repositioning rifamycins for Mycobacterium abscessus lung disease. *Expert Opinion on Drug Discovery*, *0*(0), 1-12. https://doi.org/10.1080/17460441.2019.1629414

Gilljam, M., Scherstén, H., Silverborn, M., Jönsson, B., & Ericsson Hollsing, A. (2010). Lung transplantation in patients with cystic fibrosis and Mycobacterium abscessus infection.

Journal of Cystic Fibrosis: Official Journal of the European Cystic Fibrosis Society, *9*(4), 272-276. https://doi.org/10.1016/j.jcf.2010.03.008

Gontijo, A. V. L., Brillault, J., Grégoire, N., Lamarche, I., Gobin, P., Couet, W., & Marchand, S. (2014). Biopharmaceutical Characterization of Nebulized Antimicrobial Agents in Rats : 1. Ciprofloxacin, Moxifloxacin, and Grepafloxacin. *Antimicrobial Agents and Chemotherapy*, *58*(7), 3942-3949. https://doi.org/10.1128/AAC.02818-14

Gontijo, A. V. L., Grégoire, N., Lamarche, I., Gobin, P., Couet, W., & Marchand, S. (2014). Biopharmaceutical Characterization of Nebulized Antimicrobial Agents in Rats : 2. Colistin. *Antimicrobial Agents and Chemotherapy*, *58*(7), 3950-3956. https://doi.org/10.1128/AAC.02819-14

Greendyke, R., & Byrd, T. F. (2008). Differential antibiotic susceptibility of Mycobacterium abscessus variants in biofilms and macrophages compared to that of planktonic bacteria. *Antimicrobial Agents and Chemotherapy*, *52*(6), 2019-2026. https://doi.org/10.1128/AAC.00986-07

Griffith, D. E., & Wallace, R. J. (1996). New developments in the treatment of nontuberculous mycobacterial (NTM) disease. *Seminars in Respiratory Infections*, *11*(4), 301-310.

Griffith, David E., Aksamit, T., Brown-Elliott, B. A., Catanzaro, A., Daley, C., Gordin, F., … Infectious Disease Society of America. (2007). An official ATS/IDSA statement : Diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *American Journal of Respiratory and Critical Care Medicine*, *175*(4), 367-416. https://doi.org/10.1164/rccm.200604-571ST

Hallstrand, T. S., Ochs, H. D., Zhu, Q., & Liles, W. C. (2004). Inhaled IFN-gamma for persistent nontuberculous mycobacterial pulmonary disease due to functional IFN-gamma deficiency. *The European Respiratory Journal*, *24*(3), 367-370. https://doi.org/10.1183/09031936.04.00036704

Hastedt, J. E., Bäckman, P., Clark, A. R., Doub, W., Hickey, A., Hochhaus, G., … Weers, J. G. (2016). Scope and relevance of a pulmonary biopharmaceutical classification system AAPS/FDA/USP Workshop March 16-17th, 2015 in Baltimore, MD. *AAPS Open*, *2*(1), 1. https://doi.org/10.1186/s41120-015-0002-x

Haverkamp, M. H., van Wengen, A., de Visser, A. W., van Kralingen, K. W., van Dissel, J. T., & van de Vosse, E. (2012). Pulmonary Mycobacterium abscessus : A canary in the cystic fibrosis coalmine. *The Journal of Infection*, *64*(6), 609-612. https://doi.org/10.1016/j.jinf.2012.02.010

Haworth, C. S., Banks, J., Capstick, T., Fisher, A. J., Gorsuch, T., Laurenson, I. F., … Floto, R. A. (2017). British Thoracic Society guidelines for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). *Thorax*, *72*(Suppl 2), ii1-ii64. https://doi.org/10.1136/thoraxjnl-2017-210927

Hoefsloot, W., van Ingen, J., Andrejak, C., Angeby, K., Bauriaud, R., Bemer, P., … Nontuberculous Mycobacteria Network European Trials Group. (2013). The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples : An NTM-NET collaborative study. *The European Respiratory Journal*, *42*(6), 1604-1613. https://doi.org/10.1183/09031936.00149212

Hsieh, M. H., Yu, C. M., Yu, V. L., & Chow, J. W. (1993). Synergy assessed by checkerboard. A critical analysis. *Diagnostic Microbiology and Infectious Disease*, *16*(4), 343-349.

Huang, C.-W., Chen, J.-H., Hu, S.-T., Huang, W.-C., Lee, Y.-C., Huang, C.-C., & Shen, G.-H. (2013). Synergistic activities of tigecycline with clarithromycin or amikacin against rapidly growing mycobacteria in Taiwan. *International Journal of Antimicrobial Agents*, *41*(3), 218-223. https://doi.org/10.1016/j.ijantimicag.2012.10.021

Jacobs, M., Grégoire, N., Couet, W., & Bulitta, J. B. (2016). Distinguishing Antimicrobial Models with Different Resistance Mechanisms via Population Pharmacodynamic Modeling. *PLoS Computational Biology*, *12*(3), e1004782. https://doi.org/10.1371/journal.pcbi.1004782

Jeon, K., Kwon, O. J., Lee, N. Y., Kim, B.-J., Kook, Y.-H., Lee, S.-H., … Koh, W.-J. (2009). Antibiotic Treatment of Mycobacterium abscessus Lung Disease. *American Journal of Respiratory and Critical Care Medicine*, *180*(9), 896-902. https://doi.org/10.1164/rccm.200905-0704OC

Johnson, M. M., & Odell, J. A. (2014). Nontuberculous mycobacterial pulmonary infections. *Journal of Thoracic Disease*, *6*(3), 210-220. https://doi.org/10.3978/j.issn.2072-1439.2013.12.24

Jönsson, B. E., Gilljam, M., Lindblad, A., Ridell, M., Wold, A. E., & Welinder-Olsson, C. (2007). Molecular epidemiology of Mycobacterium abscessus, with focus on cystic fibrosis. *Journal of Clinical Microbiology*, *45*(5), 1497-1504. https://doi.org/10.1128/JCM.02592-06

Jordan, P. W., Stanley, T., Donnelly, F. M., Elborn, J. S., McClurg, R. B., Millar, B. C., … Moore, J. E. (2007). Atypical mycobacterial infection in patients with cystic fibrosis : Update on clinical microbiology methods. *Letters in Applied Microbiology*, *44*(5), 459-466. https://doi.org/10.1111/j.1472-765X.2007.02130.x

Kai, R., Annegret, H., Sandra, B., Nadine, R., Sebastian, S., & Franz-Christoph, B. (2014). Characterization of Rough and Smooth Morphotypes of Mycobacterium abscessus Isolates from Clinical Specimens. Consulté à l'adresse J Clin Microbiol. 52(1): 244–250.

Kaushik, A., Makkar, N., Pandey, P., Parrish, N., Singh, U., & Lamichhane, G. (2015). Carbapenems and Rifampin Exhibit Synergy against Mycobacterium tuberculosis and Mycobacterium abscessus. *Antimicrobial Agents and Chemotherapy*, *59*(10), 6561-6567. https://doi.org/10.1128/AAC.01158-15

Kazwala, R. R., Kusiluka, L. J. M., Sinclair, K., Sharp, J. M., & Daborn, C. J. (2006). The molecular epidemiology of Mycobacterium bovis infections in Tanzania. *Veterinary Microbiology*, *112*(2-4), 201-210. https://doi.org/10.1016/j.vetmic.2005.11.026

Kim, C.-J., Kim, N.-H., Song, K.-H., Choe, P. G., Kim, E. S., Park, S. W., … Oh, M. (2013). Differentiating rapid- and slow-growing mycobacteria by difference in time to growth detection in liquid media. *Diagnostic Microbiology and Infectious Disease*, *75*(1), 73-76. https://doi.org/10.1016/j.diagmicrobio.2012.09.019

Koh, W.-J., Stout, J. E., & Yew, W.-W. (2014). Advances in the management of pulmonary disease due to Mycobacterium abscessus complex. *The International Journal of Tuberculosis and Lung Disease: The Official Journal of the International Union Against Tuberculosis and Lung Disease*, *18*(10), 1141-1148. https://doi.org/10.5588/ijtld.14.0134

Koh, Won-Jung, Hong, G., Kim, K., Ahn, S., & Han, J. (2012). Pulmonary sequestration infected with nontuberculous mycobacteria : A report of two cases and literature review. *Asian Pacific Journal of Tropical Medicine*, *5*(11), 917-919. https://doi.org/10.1016/S1995-7645(12)60172-2

Koh, Won-Jung, Jeon, K., Lee, N. Y., Kim, B.-J., Kook, Y.-H., Lee, S.-H., … Kwon, O. J. (2011). Clinical significance of differentiation of Mycobacterium massiliense from Mycobacterium abscessus. *American Journal of Respiratory and Critical Care Medicine*, *183*(3), 405-410. https://doi.org/10.1164/rccm.201003-0395OC

Kuti, J. L., & Nicolau, D. P. (2015). Presence of infection influences the epithelial lining fluid penetration of oral levofloxacin in adult patients. *International Journal of Antimicrobial Agents*, *45*(5), 512-518. https://doi.org/10.1016/j.ijantimicag.2014.12.028

Laencina, L. (2018). *Avantages génomiques conférés à Mycobacterium abscessus pour une existence intracellulaire*. 263.

Lavollay, M., Dubée, V., Heym, B., Herrmann, J.-L., Gaillard, J.-L., Gutmann, L., … Mainardi, J.-L. (2014). In vitro activity of cefoxitin and imipenem against Mycobacterium abscessus complex. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *20*(5), O297-300. https://doi.org/10.1111/1469-0691.12405

Le Run, E., Arthur, M., & Mainardi, J.-L. (2018). In Vitro and Intracellular Activity of Imipenem Combined with Rifabutin and Avibactam against Mycobacterium abscessus. *Antimicrobial Agents and Chemotherapy*, *62*(8). https://doi.org/10.1128/AAC.00623-18

Lee, M.-R., Sheng, W.-H., Hung, C.-C., Yu, C.-J., Lee, L.-N., & Hsueh, P.-R. (2015). Mycobacterium abscessus Complex Infections in Humans. *Emerging Infectious Diseases*, *21*(9), 1638-1646. https://doi.org/10.3201/2109.141634

Lefebvre, A.-L., Dubée, V., Cortes, M., Dorchêne, D., Arthur, M., & Mainardi, J.-L. (2016). Bactericidal and intracellular activity of β-lactams against Mycobacterium abscessus. *The Journal of Antimicrobial Chemotherapy*, *71*(6), 1556-1563. https://doi.org/10.1093/jac/dkw022

Lerat, I., Cambau, E., Roth Dit Bettoni, R., Gaillard, J.-L., Jarlier, V., Truffot, C., & Veziris, N. (2014). In vivo evaluation of antibiotic activity against Mycobacterium abscessus. *The Journal of Infectious Diseases*, *209*(6), 905-912. https://doi.org/10.1093/infdis/jit614

Lindman, M., & Dick, T. (2019). Bedaquiline Eliminates Bactericidal Activity of β-Lactams against Mycobacterium abscessus. *Antimicrobial Agents and Chemotherapy*, *63*(8), e00827-19. https://doi.org/10.1128/AAC.00827-19

Loewe, S. (1953). The problem of synergism and antagonism of combined drugs. *Arzneimittel-Forschung*, *3*(6), 285-290.

Lyons, M. A., & Lenaerts, A. J. (2015). Computational pharmacokinetics/pharmacodynamics of rifampin in a mouse tuberculosis infection model. *Journal of pharmacokinetics and pharmacodynamics*, *42*(4), 375-389. https://doi.org/10.1007/s10928-015-9419-z

Marchand, S., Boisson, M., Mehta, S., Adier, C., Mimoz, O., Grégoire, N., & Couet, W. (2018). Biopharmaceutical Characterization of Nebulized Antimicrobial Agents in Rats. 6. Aminoglycosides. *Antimicrobial Agents and Chemotherapy*, AAC.01261-18. https://doi.org/10.1128/AAC.01261-18

Marchand, S., Chauzy, A., Dahyot-Fizelier, C., & Couet, W. (2016). Microdialysis as a way to measure antibiotics concentration in tissues. *Pharmacological Research*, *111*, 201-207. https://doi.org/10.1016/j.phrs.2016.06.001

Marchand, S., Grégoire, N., Brillault, J., Lamarche, I., Gobin, P., & Couet, W. (2015). Biopharmaceutical Characterization of Nebulized Antimicrobial Agents in Rats : 3. Tobramycin. *Antimicrobial Agents and Chemotherapy*, *59*(10), 6646-6647. https://doi.org/10.1128/AAC.01647-15

Marchand, S., Grégoire, N., Brillault, J., Lamarche, I., Gobin, P., & Couet, W. (2016). Biopharmaceutical Characterization of Nebulized Antimicrobial Agents in Rats. 4. Aztreonam. *Antimicrobial Agents and Chemotherapy*, *60*(5), 3196-3198. https://doi.org/10.1128/AAC.00165-16

Martiniano, S. L., Nick, J. A., & Daley, C. L. (2016). Nontuberculous Mycobacterial Infections in Cystic Fibrosis. *Clinics in Chest Medicine*, *37*(1), 83-96. https://doi.org/10.1016/j.ccm.2015.11.001

Maselli, D. J., Keyt, H., & Restrepo, M. I. (2017). Inhaled Antibiotic Therapy in Chronic Respiratory Diseases. *International Journal of Molecular Sciences*, *18*(5). https://doi.org/10.3390/ijms18051062

Maurer, F. P., Bruderer, V. L., Ritter, C., Castelberg, C., Bloemberg, G. V., & Böttger, E. C. (2014). Lack of antimicrobial bactericidal activity in Mycobacterium abscessus. *Antimicrobial Agents and Chemotherapy*, *58*(7), 3828-3836. https://doi.org/10.1128/AAC.02448-14

Mdegela, R. H., Kusiluka, L. J. M., Kapaga, A. M., Karimuribo, E. D., Turuka, F. M., Bundala, A., … Kambarage, D. M. (2004). Prevalence and Determinants of Mastitis and Milk-borne Zoonoses in Smallholder Dairy Farming Sector in Kibaha and Morogoro Districts in Eastern Tanzania. *Journal of Veterinary Medicine, Series B*, *51*(3), 123-128. https://doi.org/10.1111/j.1439-0450.2004.00735.x

Medjahed, H., Gaillard, J.-L., & Reyrat, J.-M. (2010). Mycobacterium abscessus : A new player in the mycobacterial field. *Trends in Microbiology*, *18*(3), 117-123. https://doi.org/10.1016/j.tim.2009.12.007

Mehta, S., Aranzana-Climent, V., Rammaert, B., Grégoire, N., Marchand, S., Couet, W., & Buyck, J. M. (2019). Preclinical Pharmacokinetic and Pharmacodynamic Data To Support Cefoxitin Nebulization for the Treatment of Mycobacterium abscessus. *Antimicrobial Agents and Chemotherapy*, *63*(7). https://doi.org/10.1128/AAC.02651-18

Millar, B. C., & Moore, J. E. (2019). Antimycobacterial strategies to evade antimicrobial resistance in the nontuberculous mycobacteria. *International Journal of Mycobacteriology*, *8*(1), 7-21. https://doi.org/10.4103/ijmy.ijmy_153_18

Miranda-CasoLuengo, A. A., Staunton, P. M., Dinan, A. M., Lohan, A. J., & Loftus, B. J. (2016). Functional characterization of the Mycobacterium abscessus genome coupled with condition specific transcriptomics reveals conserved molecular strategies for host adaptation and persistence. *BMC Genomics*, *17*(1), 553. https://doi.org/10.1186/s12864-016-2868-y

Misra, A., Hickey, A. J., Rossi, C., Borchard, G., Terada, H., Makino, K., … Colombo, P. (2011). Inhaled drug therapy for treatment of tuberculosis. *Tuberculosis (Edinburgh, Scotland)*, *91*(1), 71-81. https://doi.org/10.1016/j.tube.2010.08.009

Miyasaka, T., Kunishima, H., Komatsu, M., Tamai, K., Mitsutake, K., Kanemitsu, K., … Kaku, M. (2007). In vitro efficacy of imipenem in combination with six antimicrobial agents against Mycobacterium abscessus. *International Journal of Antimicrobial Agents*, *30*(3), 255-258. https://doi.org/10.1016/j.ijantimicag.2007.05.003

Morimoto, K., Hasegawa, N., Izumi, K., Namkoong, H., Uchimura, K., Yoshiyama, T., … Mitarai, S. (2017). A Laboratory-based Analysis of Nontuberculous Mycobacterial Lung Disease in Japan from 2012 to 2013. *Annals of the American Thoracic Society*, *14*(1), 49-56. https://doi.org/10.1513/AnnalsATS.201607-573OC

Mougari, F., Guglielmetti, L., Raskine, L., Sermet-Gaudelus, I., Veziris, N., & Cambau, E. (2016). Infections caused by Mycobacterium abscessus : Epidemiology, diagnostic tools and treatment. *Expert Review of Anti-Infective Therapy*, *14*(12), 1139-1154. https://doi.org/10.1080/14787210.2016.1238304

Mouton, J. W., & Vinks, A. A. (2005). Pharmacokinetic/pharmacodynamic modelling of antibacterials in vitro and in vivo using bacterial growth and kill kinetics : The minimum inhibitory concentration versus stationary concentration. *Clinical Pharmacokinetics*, *44*(2), 201-210. https://doi.org/10.2165/00003088-200544020-00005

Mukherjee, D., Wu, M.-L., Teo, J. W. P., & Dick, T. (2017). Vancomycin and Clarithromycin Show Synergy against Mycobacterium abscessus In Vitro. *Antimicrobial Agents and Chemotherapy*, *61*(12). https://doi.org/10.1128/AAC.01298-17

Muwonge, A., Kankya, C., Godfroid, J., Djonne, B., Opuda-Asibo, J., Biffa, D., … Skjerve, E. (2010). Prevalence and associated risk factors of mycobacterial infections in slaughter pigs from Mubende district in Uganda. *Tropical Animal Health and Production*, *42*(5), 905-913. https://doi.org/10.1007/s11250-009-9506-5

Nash, K. A., Brown-Elliott, B. A., & Wallace, R. J. (2009). A novel gene, erm(41), confers inducible macrolide resistance to clinical isolates of Mycobacterium abscessus but is absent from Mycobacterium chelonae. *Antimicrobial Agents and Chemotherapy*, *53*(4), 1367-1376. https://doi.org/10.1128/AAC.01275-08

NCCLS. (2003). *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard. NCCLS document M24-A.*

Nessar, R., Cambau, E., Reyrat, J. M., Murray, A., & Gicquel, B. (2012). Mycobacterium abscessus : A new antibiotic nightmare. *The Journal of Antimicrobial Chemotherapy*, *67*(4), 810-818. https://doi.org/10.1093/jac/dkr578

Nielsen, E. I., Cars, O., & Friberg, L. E. (2011). Pharmacokinetic/pharmacodynamic (PK/PD) indices of antibiotics predicted by a semimechanistic PKPD model : A step toward model-based dose optimization. *Antimicrobial Agents and Chemotherapy*, *55*(10), 4619-4630. https://doi.org/10.1128/AAC.00182-11

Nielsen, E. I., & Friberg, L. E. (2013). Pharmacokinetic-Pharmacodynamic Modeling of Antibacterial Drugs. *Pharmacological Reviews*, *65*(3), 1053-1090. https://doi.org/10.1124/pr.111.005769

Obregón-Henao, A., Arnett, K. A., Henao-Tamayo, M., Massoudi, L., Creissen, E., Andries, K., … Ordway, D. J. (2015). Susceptibility of Mycobacterium abscessus to antimycobacterial drugs in preclinical models. *Antimicrobial Agents and Chemotherapy*, *59*(11), 6904-6912. https://doi.org/10.1128/AAC.00459-15

Oh, C.-T., Moon, C., Park, O. K., Kwon, S.-H., & Jang, J. (2014). Novel drug combination for Mycobacterium abscessus disease therapy identified in a Drosophila infection model. *The Journal of Antimicrobial Chemotherapy*, *69*(6), 1599-1607. https://doi.org/10.1093/jac/dku024

Olivier, K. N., Griffith, D. E., Eagle, G., McGinnis, J. P., Micioni, L., Liu, K., … Wallace, R. J. (2017). Randomized Trial of Liposomal Amikacin for Inhalation in Nontuberculous Mycobacterial Lung Disease. *American Journal of Respiratory and Critical Care Medicine*, *195*(6), 814-823. https://doi.org/10.1164/rccm.201604-0700OC

Olivier, K. N., Shaw, P. A., Glaser, T. S., Bhattacharyya, D., Fleshner, M., Brewer, C. C., … Prevots, D. R. (2014). Inhaled amikacin for treatment of refractory pulmonary nontuberculous mycobacterial disease. *Annals of the American Thoracic Society*, *11*(1), 30-35. https://doi.org/10.1513/AnnalsATS.201307-231OC

Olivier, K. N., Weber, D. J., Wallace, R. J., Faiz, A. R., Lee, J.-H., Zhang, Y., … Nontuberculous Mycobacteria in Cystic Fibrosis Study Group. (2003). Nontuberculous

mycobacteria. I : Multicenter prevalence study in cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, *167*(6), 828-834. https://doi.org/10.1164/rccm.200207-678OC

Parte, A. C. (2018). LPSN – List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on. *International Journal of Systematic and Evolutionary Microbiology*, *68*(6), 1825-1829. https://doi.org/10.1099/ijsem.0.002786

Petrini, B. (2006). Non-tuberculous mycobacterial infections. *Scandinavian Journal of Infectious Diseases*, *38*(4), 246-255. https://doi.org/10.1080/00365540500444652

Pham, D.-D., Fattal, E., & Tsapis, N. (2015). Pulmonary drug delivery systems for tuberculosis treatment. *International Journal of Pharmaceutics*, *478*(2), 517-529. https://doi.org/10.1016/j.ijpharm.2014.12.009

Piersimoni, C., & Scarparo, C. (2009). Extrapulmonary Infections Associated with Nontuberculous Mycobacteria in Immunocompetent Persons. *Emerging Infectious Diseases*, *15*(9), 1351-1358. https://doi.org/10.3201/eid1509.081259

Pryjma, M., Burian, J., & Thompson, C. J. (2018). Rifabutin Acts in Synergy and Is Bactericidal with Frontline Mycobacterium abscessus Antibiotics Clarithromycin and Tigecycline, Suggesting a Potent Treatment Combination. *Antimicrobial Agents and Chemotherapy*, *62*(8). https://doi.org/10.1128/AAC.00283-18

Qvist, T., Gilljam, M., Jönsson, B., Taylor-Robinson, D., Jensen-Fangel, S., Wang, M., … Scandinavian Cystic Fibrosis Study Consortium (SCFSC). (2015). Epidemiology of nontuberculous mycobacteria among patients with cystic fibrosis in Scandinavia. *Journal of Cystic Fibrosis: Official Journal of the European Cystic Fibrosis Society*, *14*(1), 46-52. https://doi.org/10.1016/j.jcf.2014.08.002

Rao, G. G., Li, J., Garonzik, S. M., Nation, R. L., & Forrest, A. (2018). Assessment and modelling of antibacterial combination regimens. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *24*(7), 689-696. https://doi.org/10.1016/j.cmi.2017.12.004

Ripoll, F., Pasek, S., Schenowitz, C., Dossat, C., Barbe, V., Rottman, M., … Gaillard, J.-L. (2009). Non mycobacterial virulence genes in the genome of the emerging pathogen Mycobacterium abscessus. *PloS One*, *4*(6), e5660. https://doi.org/10.1371/journal.pone.0005660

Rominski, A., Schulthess, B., Müller, D. M., Keller, P. M., & Sander, P. (2017). Effect of β-lactamase production and β-lactam instability on MIC testing results for Mycobacterium

abscessus. *The Journal of Antimicrobial Chemotherapy*, *72*(11), 3070-3078. https://doi.org/10.1093/jac/dkx284

Rose, S. J., Neville, M. E., Gupta, R., & Bermudez, L. E. (2014). Delivery of aerosolized liposomal amikacin as a novel approach for the treatment of nontuberculous mycobacteria in an experimental model of pulmonary infection. *PloS One*, *9*(9), e108703. https://doi.org/10.1371/journal.pone.0108703

Rottman, M., Catherinot, E., Hochedez, P., Emile, J.-F., Casanova, J.-L., Gaillard, J.-L., & Soudais, C. (2007). Importance of T cells, gamma interferon, and tumor necrosis factor in immune control of the rapid grower Mycobacterium abscessus in C57BL/6 mice. *Infection and Immunity*, *75*(12), 5898-5907. https://doi.org/10.1128/IAI.00014-07

Roux, A., Hermann, J., Gaillard, J., & Rottman, M. (2010). Mycobacterium abscessus, pathogène émergent dans la mucoviscidose—ScienceDirect. Consulté 5 septembre 2018, à l'adresse https://www.sciencedirect.com/science/article/pii/S0923253210000074

Roux, A.-L., Catherinot, E., Soismier, N., Heym, B., Bellis, G., Lemonnier, L., … OMA group. (2015). Comparing Mycobacterium massiliense and Mycobacterium abscessus lung infections in cystic fibrosis patients. *Journal of Cystic Fibrosis: Official Journal of the European Cystic Fibrosis Society*, *14*(1), 63-69. https://doi.org/10.1016/j.jcf.2014.07.004

Roux, A.-L., Viljoen, A., Bah, A., Simeone, R., Bernut, A., Laencina, L., … Herrmann, J.-L. (2016). The distinct fate of smooth and rough Mycobacterium abscessus variants inside macrophages. *Open Biology*, *6*(11). https://doi.org/10.1098/rsob.160185

Ruth, M. M., van Rossum, M., Koeken, V. A. C. M., Pennings, L. J., Svensson, E. M., Ruesen, C., … van Ingen, J. (2019). Auranofin Activity Exposes Thioredoxin Reductase as a Viable Drug Target in Mycobacterium abscessus. *Antimicrobial Agents and Chemotherapy*, *63*(9). https://doi.org/10.1128/AAC.00449-19

Sanguinetti, M., Ardito, F., Fiscarelli, E., La Sorda, M., D'Argenio, P., Ricciotti, G., & Fadda, G. (2001). Fatal pulmonary infection due to multidrug-resistant Mycobacterium abscessus in a patient with cystic fibrosis. *Journal of Clinical Microbiology*, *39*(2), 816-819. https://doi.org/10.1128/JCM.39.2.816-819.2001

Schoutrop, E. L. M., Brouwer, M. A. E., Jenniskens, J. C. A., Ferro, B. E., Mouton, J. W., Aarnoutse, R. E., & van Ingen, J. (2018). The stability of antimycobacterial drugs in media used for drug susceptibility testing. *Diagnostic Microbiology and Infectious Disease*, *92*(4), 305-308. https://doi.org/10.1016/j.diagmicrobio.2018.06.015

Sfeir, M., Walsh, M., Rosa, R., Aragon, L., Liu, S. Y., Cleary, T., … Abbo, L. M. (2018). Mycobacterium abscessus Complex Infections : A Retrospective Cohort Study. *Open Forum Infectious Diseases*, *5*(2). https://doi.org/10.1093/ofid/ofy022

Simons, S., van Ingen, J., Hsueh, P.-R., Van Hung, N., Dekhuijzen, P. N. R., Boeree, M. J., & van Soolingen, D. (2011). Nontuberculous Mycobacteria in Respiratory Tract Infections, Eastern Asia. *Emerging Infectious Diseases*, *17*(3), 343-349. https://doi.org/10.3201/eid1703100604

Singh, S., Bouzinbi, N., Chaturvedi, V., Godreuil, S., & Kremer, L. (2014). In vitro evaluation of a new drug combination against clinical isolates belonging to the Mycobacterium abscessus complex. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *20*(12), O1124-1127. https://doi.org/10.1111/1469-0691.12780

Skolnik, K., Kirkpatrick, G., & Quon, B. S. (2016). Nontuberculous Mycobacteria in Cystic Fibrosis. *Current Treatment Options in Infectious Diseases*, *8*(4), 259-274. https://doi.org/10.1007/s40506-016-0092-6

Sopirala, M. M., Mangino, J. E., Gebreyes, W. A., Biller, B., Bannerman, T., Balada-Llasat, J.-M., & Pancholi, P. (2010). Synergy Testing by Etest, Microdilution Checkerboard, and Time-Kill Methods for Pan-Drug-Resistant Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy*, *54*(11), 4678-4683. https://doi.org/10.1128/AAC.00497-10

Soroka, D., Dubée, V., Soulier-Escrihuela, O., Cuinet, G., Hugonnet, J.-E., Gutmann, L., … Arthur, M. (2014). Characterization of broad-spectrum Mycobacterium abscessus class A β-lactamase. *The Journal of Antimicrobial Chemotherapy*, *69*(3), 691-696. https://doi.org/10.1093/jac/dkt410

Stout, J. E., Koh, W.-J., & Yew, W. W. (2016). Update on pulmonary disease due to non-tuberculous mycobacteria. *International Journal of Infectious Diseases: IJID: Official Publication of the International Society for Infectious Diseases*, *45*, 123-134. https://doi.org/10.1016/j.ijid.2016.03.006

Stover, C. S., & Litwin, C. M. (2014). The Epidemiology of Upper Respiratory Infections at a Tertiary Care Center : Prevalence, Seasonality, and Clinical Symptoms [Research article]. https://doi.org/10.1155/2014/469393

Sugino, K., Kobayashi, M., Iwata, M., Gocho, K., Kaburaki, K., Muramatsu, Y., … Homma, S. (2009). Successful treatment with pneumonectomy for pulmonary Mycobacterium abscessus infection. *Internal Medicine (Tokyo, Japan)*, *48*(6), 459-463.

Tissot, A., Thomas, M. F., Corris, P. A., & Brodlie, M. (2018). NonTuberculous Mycobacteria infection and lung transplantation in cystic fibrosis : A worldwide survey of clinical practice. *BMC Pulmonary Medicine*, *18*(1), 86. https://doi.org/10.1186/s12890-018-0635-3

Tortoli, E., Kohl, T. A., Brown-Elliott, B. A., Trovato, A., Leão, S. C., Garcia, M. J., … Cirillo, D. M. (2016). Emended description of Mycobacterium abscessus, Mycobacterium abscessus subsp. Abscessus and Mycobacteriumabscessus subsp. Bolletii and designation of Mycobacteriumabscessus subsp. Massiliense comb. Nov. *International Journal of Systematic and Evolutionary Microbiology*, *66*(11), 4471-4479. https://doi.org/10.1099/ijsem.0.001376

Tronde, A., Krondahl, E., von Euler-Chelpin, H., Brunmark, P., Bengtsson, U. H., Ekström, G., & Lennernäs, H. (2002). High airway-to-blood transport of an opioid tetrapeptide in the isolated rat lung after aerosol delivery. *Peptides*, *23*(3), 469-478.

van Ingen, J., Boeree, M. J., van Soolingen, D., & Mouton, J. W. (2012). Resistance mechanisms and drug susceptibility testing of nontuberculous mycobacteria. *Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, *15*(3), 149-161. https://doi.org/10.1016/j.drup.2012.04.001

van Ingen, J., Ferro, B. E., Hoefsloot, W., Boeree, M. J., & van Soolingen, D. (2013). Drug treatment of pulmonary nontuberculous mycobacterial disease in HIV-negative patients : The evidence. *Expert Review of Anti-Infective Therapy*, *11*(10), 1065-1077. https://doi.org/10.1586/14787210.2013.830413

van Ingen, J., Wagner, D., Gallagher, J., Morimoto, K., Lange, C., Haworth, C. S., … NTM-NET. (2017). Poor adherence to management guidelines in nontuberculous mycobacterial pulmonary diseases. *The European Respiratory Journal*, *49*(2). https://doi.org/10.1183/13993003.01855-2016

Vankeerberghen, A., Cuppens, H., & Cassiman, J.-J. (2002). The cystic fibrosis transmembrane conductance regulator : An intriguing protein with pleiotropic functions. *Journal of Cystic Fibrosis: Official Journal of the European Cystic Fibrosis Society*, *1*(1), 13-29.

Vesenbeckh, S., Schönfeld, N., Roth, A., Bettermann, G., Krieger, D., Bauer, T. T., … Mauch, H. (2017). Bedaquiline as a potential agent in the treatment of Mycobacterium abscessus infections. *European Respiratory Journal*, *49*(5), 1700083. https://doi.org/10.1183/13993003.00083-2017

Veyrier, F., Pletzer, D., Turenne, C., & Behr, M. A. (2009). Phylogenetic detection of horizontal gene transfer during the step-wise genesis of Mycobacterium tuberculosis. *BMC Evolutionary Biology*, *9*, 196. https://doi.org/10.1186/1471-2148-9-196

Wallace, R. J., Dukart, G., Brown-Elliott, B. A., Griffith, D. E., Scerpella, E. G., & Marshall, B. (2014). Clinical experience in 52 patients with tigecycline-containing regimens for salvage treatment of Mycobacterium abscessus and Mycobacterium chelonae infections. *The Journal of Antimicrobial Chemotherapy*, *69*(7), 1945-1953. https://doi.org/10.1093/jac/dku062

World Health Organisation. (2017). *Global tuberculosis report 2017*.

Wu, M.-L., Aziz, D. B., Dartois, V., & Dick, T. (2018). NTM drug discovery : Status, gaps and the way forward. *Drug Discovery Today*, *23*(8), 1502-1519. https://doi.org/10.1016/j.drudis.2018.04.001

Yapa, S., Li, J., Patel, K., Wilson, J. W., Dooley, M. J., George, J., … McIntosh, M. P. (2014). Pulmonary and systemic pharmacokinetics of inhaled and intravenous colistin methanesulfonate in cystic fibrosis patients : Targeting advantage of inhalational administration. *Antimicrobial Agents and Chemotherapy*, *58*(5), 2570-2579. https://doi.org/10.1128/AAC.01705-13

Zhang, Z., Lu, J., Song, Y., & Pang, Y. (2018). In vitro activity between linezolid and other antimicrobial agents against Mycobacterium abscessus complex. *Diagnostic Microbiology and Infectious Disease*, *90*(1), 31-34. https://doi.org/10.1016/j.diagmicrobio.2017.09.013

Abstract

Mycobacterium abscessus is rapidly growing non-tuberculous mycobacteria responsible for difficult-to-treat pulmonary infections in humans. Current recommended treatment is associated with high treatment failure and emergence of resistance to most of the antibiotics. Also, with only a few new antibiotic drugs active against multidrug-resistant bacteria approved every year, it is important to optimize the use of already existing antibiotics using biopharmaceutical approach like Pharmacokinetic/pharmacodynamic (PK/PD). In pulmonary infections, direct administration of low permeability drugs such as cefoxitin (FOX) and amikacin (AMK) into lungs as therapeutic aerosols should increase their efficiency and minimize whole body exposure responsible for adverse effects, particularly in the case of prolonged treatments. Moreover, the use of antibiotics in combination may reduce the risk of resistance. Several points have been addressed in this thesis:

1. Biopharmaceutical studies of AMK and FOX: It was shown that after nebulization of AMK and FOX, pulmonary concentrations were almost 1000-fold higher than after intravenous administration for both antibiotics, making them a good candidate for nebulization.
2. Pharmacokinetic/pharmacodynamic (PK/PD) study of cefoxitin: a semi-mechanistic PK/PD model was developed from *in vitro* time kill-kinetics assay data, enabling identification of concentration-effect relationships for two bacterial sub-populations while taking into account the unstability degradation of cefoxitin.
3. PK/PD study of bi-combination: Using a mechanism-based mathematical model and data obtained from time kill-kinetics study, it was shown that the combined effect of AMK and FOX was additive to synergistic at different concentration.
4. Bi-or tri-combinations: several tri-combinations including AMK, FOX and a 3rd antibiotic (including clarithromycin, linezolid, clofazimine, ciprofloxacin, moxifloxacin, rifampicin and rifabutin) were tested against reference strain, clarithromycin resistance-clinical isolate (Ma1611) and multidrug-resistance-clinical isolate (T28). All tri-combinations were active against reference strain. Similar observation was made with Ma1611 except combination with clofazimine and clarithromycin. Any combination was active against T28. Bi-combinations with highest concentrations of FOX and rifamycins were effective against T28. The synergy between FOX and fluoroquinolones or rifamycins suggests a potent role of these combinations that may warrant further optimization of treatment regimen for the treatment of *M. abscessus* pulmonary infections.
5. Tri-combination including AMK, FOX and moxifloxacin (MXF) up to 21 days against clarithromycin-resistance clinical isolate has shown no importance of using MXF as tri-combination was not more effective than the bi-combination of AMK and FOX.

Keywords: *Mycobacterium abscessus*, cefoxitin, amikacin, PK/PD modeling, *in vitro* combination, antibiotic resistance

Résumé

Mycobacterium abscessus est une mycobactérie non-tuberculeuse responsable d'infections pulmonaires difficiles à traiter en clinique. Le traitement actuellement recommandé est associé à un taux d'échec élevé et à l'émergence de résistances à la plupart des antibiotiques. Dans le contexte actuel, avec un faible nombre de nouveaux antibiotiques mis sur le marché, il est important de d'optimiser l'utilisation des antibiotiques à notre disposition par des approches pharmacocinétique/pharmacodynamiques (PK/PD). Dans les infections pulmonaires, l'administration directe de médicaments à faible perméabilité tels que la céfoxitine (FOX) et l'amikacine (AMK) dans les poumons sous forme d'aérosols devrait accroître leur efficacité au site d'infection tout en diminuant la toxicité systémique responsable des effets indésirables, particulièrement dans le cas des traitements prolongés. De plus, l'utilisation d'antibiotiques en combinaison pourrait réduire le risque de développement de résistance. Plusieurs points ont été abordés dans cette thèse :

1. Études biopharmaceutiques sur AMK et FOX : Il a été démontré qu'après la nébulisation d'AMK et de FOX, les concentrations pulmonaires étaient presque 1000 fois plus élevées qu'après l'administration intraveineuse des deux antibiotiques, ce qui en fait de bons candidats pour la nébulisation.
2. Étude PK/PD de céfoxitine : un modèle PK/PD semi-mécanique a été mis au point à partir de données pharmacocinétiques *in vitro*, permettant d'identifier les relations concentration-effet pour deux sous-populations de bactéries tout en tenant compte de la dégradation de la céfoxitine.
3. Étude pharmacocinétique et pharmacodynamique de la bi-combinaison : à l'aide d'un modèle mathématique basé sur un mécanisme et de données obtenues à partir données *in vitro*, il a été démontré que l'effet combiné d'AMK et de FOX était additif et/ou synergique selon les différentes concentrations.
4. Bi- ou tri-combinaisons : plusieurs tri-combinaisons incluant AMK, FOX et un 3ème antibiotique (incluant clarithromycine, linézolide, clofazimine, ciprofloxacine, moxifloxacine, rifampicine et rifabutine) ont été testés contre une souche de référence, un isolat clinique résistant à la clarithromycine (Ma1611) et un isolat clinique multirésistant (T28). Toutes les tri-combinaisons étaient actives contre la souche de référence. Les combinaisons étaient également actives sur la souche Ma1611 sauf pour les associations avec la clofazimine et la clarithromycine. Aucune combinaison n'était active contre T28. Les bi-combinaisons avec de plus fortes concentrations de FOX et les fluoroquinolones ou les rifamycines étaient efficaces contre T28. Cela suggère une optimisation du schéma thérapeutique pour le traitement des infections pulmonaires à *M. abscessus.*
5. La tri-combinaison comprenant AMK, FOX et moxifloxacine (MXF) jusqu'à 21 jours contre un isolat clinique résistant à la clarithromycine Ma1611 n'a montré aucun effet supplémentaire de l'utilisation de MXF car la tri-combinaison n'était pas plus efficace que la bi-combinaison d'AMK et FOX.

Mots-clés : *Mycobacterium abscessus*, céfoxitine, amikacine, modélisation PK/PD, *in vitro* combinaison, antibiorésistance.