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Présentée par :
Siti Nani Nurbaeti

Études biopharmaceutiques et formulation de chloramphénicol et de thiamphénicol pour le traitement ciblé des infections pulmonaires par voie inhalée

Directeur(s) de Thèse :
Jean-Christophe Olivier, Julien Brillault

Soutenue le 13 décembre 2017 devant le jury

Jury :

Président	**Sandrine Marchand**	Maître de conférences, PH, Université de Poitiers
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Études biopharmaceutiques et formulation du chloramphénicol et du thiamphénicol pour le traitement ciblé des infections pulmonaires par voie inhalée
(Biopharmaceutical studies and formulation of chloramphenicol and thiamphenicol for the treatment of pulmonary infections by inhalation route)

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Directeur de Thèse :

Professeur Jean-Christophe Olivier

Soutenue le 13 décembre 2017

devant la Commission d'Examen

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JURY

Professeur	Frédéric Lagarce	Université d'Angers	Rapporteur
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The research work presented in this thesis was performed under the supervision of Professor Jean-Christophe Olivier and of Doctor Julien Brillault from the INSERM U1070 Pharmacology of Antiinfectious Drugs laboratory, Faculty of Medicine and Pharmacy, University of Poitiers, France.

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Abstract

The rapid emergence of resistant bacteria and the lack of new efficient treatments lead to re-use old forgotten, but still effective, antimicrobials. In particular, chloramphenicol and thiamphenicol have been proposed to treat multidrug-resistant pulmonary bacterial infections. Their direct administration into the lungs as therapeutic aerosols should increase their efficiency and minimize whole body exposure responsible for adverse effects, particularly in the case of prolonged treatments. The purpose of these Ph.D. works was to perform biopharmaceutical studies and to develop an effective aerosol formulation for lung delivery. The membrane permeability of chloramphenicol and thiamphenicol was evaluated in vitro in the Calu-3 bronchial epithelial cell model and pharmacokinetic (PK) studies were carried out in rats after intratracheal and intravenous administration. In vitro membrane permeability of chloramphenicol was high, and intermediate for thiamphenicol. Both compounds were shown to be substrates of membrane efflux transporters. In agreement with these findings, the PK studies showed that the administration route had no impact in the case of chloramphenicol and a moderate one in the case of thiamphenicol. Therefore, in order to prolong lung exposure to chloramphenicol and thiamphenicol, nanoparticle-based formulations with sustained release properties were formulated using the palmitate ester prodrugs of chloramphenicol and thiamphenicol. To ease administration, nanoparticles were included in microsphere-based dry powder for inhalation. These powders showed an optimal content, satisfactory aerodynamic properties and sustained drug release, which make them promising formulations for lung delivery of chloramphenicol and thiamphenicol as aerosols.

Keywords: pulmonary infection, antimicrobial, aerosol formulation, drug permeability, chloramphenicol, thiamphenicol

Résumé

L'émergence rapide de bactéries résistantes et l'absence de nouveaux traitements efficaces ont conduit à réutiliser d'anciens antibiotiques. Le chloramphénicol et le thiamphénicol ont ainsi été proposés pour traiter les infections respiratoires multirésistantes. Leur administration directe dans les poumons sous forme d'aérosols thérapeutiques devrait augmenter leur efficacité et minimiser l'exposition systémique responsable d'effets secondaires, en particulier lors de traitements prolongés. Ce travail de thèse a eu pour objectifs de réaliser des études biopharmaceutiques et de développer des formulations d'aérosols pour la voie pulmonaire. La perméabilité membranaire du chloramphénicol et du thiamphénicol a été évaluée sur le modèle d'épithélium bronchique Calu-3 et les études pharmacocinétiques ont été réalisées chez le rat après administrations intratrachéale et intraveineuse. La perméabilité membranaire in vitro du chloramphénicol s'est révélée élevée, et intermédiaire pour le thiamphénicol. Les deux antibiotiques sont substrats de transporteurs d'efflux. Les études pharmacocinétiques, cohérentes avec les études in vitro, ont montré un impact nul de la voie d'administration dans cas du chloramphénicol et modéré dans le cas du thiamphénicol. Par conséquent, pour prolonger l'exposition pulmonaire à ces antibiotiques, des formulations à libération prolongée de nanoparticules ont été incluses dans des poudres sèches de microsphères pour inhalation. Ces poudres se caractérisent par une teneur optimale, des propriétés aérodynamiques satisfaisantes et un profil de libération prolongée, et sont donc prometteuses pour l'administration pulmonaire de chloramphénicol ou de thiamphénicol sous la forme d'aérosols.

Mots clés: infection pulmonaire, antimicrobien, formulation d'aérosols, perméabilité membranaire aux médicaments, chloramphénicol, thiamphénicol

List of abbreviations

Ap-Bl	Apical to basolateral
Bl-Ap	Basolateral to apical
ABC	ATP binding cassette
ATB	Antibiotics
AUC	Area under the curve
BAL	Bronchoalveolar lavage
BCRP	Breast cancer resistance protein
CF	Cystic fibrosis
CHL	Chloramphenicol
DPI	Dry powder inhalers
ED	Emitted dose
ELF	Epithelial lining fluid
FPD	Fine particle dose
FPF	Fine particle fraction
IHC	Immunohistochemistry
IV	Intravenous
LC-MS/MS	Liquid chromatography-mass spectrometry and liquid chromatography - tandem mass spectrometry
MDR	Multidrug resistance
MIC	Minimum inhibitory concentrations
MMAD	Mass median aerodynamic diameter
MRP2	Multi-drug resistance protein 2
MRPs	Multi-drug resistance proteins

NGI	Next generation pharmaceutical impactor
O/W	Oil in water
OAT	Organic anion transporter
OATP	Organic anion-transporting polypeptide
OCT	Organic cation transport protein
Papp	Apparent permeability coefficient
PEPT	Peptide transporter
PGA	Polyglycolide or poly(glycolic acid)
PK	Pharmacokinetic
P-gp	P-glycoprotein
PLA	Poly(lactic acid)
PLGA	Poly lactic-co-glycolic acid
PVA	Poly(vinyl alcohol)
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
SLC	Solute carrier
THA	Thiamphenicol
W/O/W	Water in oil in water

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CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 Introduction

Respiratory infection is a global health problem and despite advancements in their management severe infections remain difficult to treat (Zhou et al., 2015; File, 2000). Despite the large number of antimicrobials that can be used to treat respiratory infections, the growing occurrence of multidrug microbial resistance jeopardizes the continuous gains in lung infection control. Due to the low antimicrobial resistance for broad-spectrum amphenicol antimicrobials, chloramphenicol and thiamphenicol may constitute a valuable option for the control of resistant infections. They are administered orally, intravenously or intramuscularly (Raymond et al., 2004). Chloramphenicol was originally derived from Streptomyces venezuelae. The use of chloramphenicol is however drastically limited because it induces severe toxicities such as aplastic anemia, bone marrow suppression and grey syndrome (Shukla et al., 2011; Turton et al., 2002). The occurrence of blood dyscrasia is between 1:24 500 to 1:40 800 exposures, and for systemic use the incidence of chloramphenicol-induced aplastic anemia is reported to be 13 times that of the idiopathic form (Wallerstein et al., 1969). In predisposed adult patients, idiosyncratic aplastic anemia occurrence is independent of the chloramphenicol dose. The main metabolite involved in the bone marrow toxicity is nitroso-chloramphenicol, but dehydro-chloramphenicol and, to a lesser extent, dehydro-chloramphenicol base, both produced by enterobacteria of the colon, also showed cytotoxicity and genotoxicity in vitro while

chloramphenicol itself and other intermediates have much less toxic action (Lafarge-Frayssinet et al., 1994). Thiamphenicol is an analogue of chloramphenicol (Drago et al., 2000) and an alternative antimicrobial agent to chloramphenicol (Turton et al., 2000). Without the aromatic nitro group thiamphenicol causes no irreversible aplastic anemia effect and is thus considered to be less toxic than chloramphenicol (Drago et al., 2000). Even though chloramphenicol and thiamphenicol use is rare in developed countries nowadays, they are still widely used in developing country (Ambekar et al., 2000) due to their low price (Wang et al., 2014). Their use might however increase in the future because of high prevalence of antimicrobial resistances to commonly used antibiotics (Wiest et al., 2012). In order to maximize efficacy of chloramphenicol and thiamphenicol in local respiratory tract infections, their targeting into the lungs could be interesting (Zhou et al., 2015). Advantages of drug administration directly into the lungs are high local and low systemic exposure to drugs, with expected reduced systemic toxicity (Fernandes and Vanbever, 2009; Velkov et al., 2015; Weers, 2015), rapid clinical response, and reduced dosing amount (Stigliani et al., 2016). Aerosols of anti-infective drugs are currently used for the maintenance treatment in cystic fibrosis (CF) patients or for the treatment or prevention of a number of additional disease states such as ventilator-associated pneumonia (Hagerman et al., 2006). Furthermore, nebulized antibiotics also can be utilised for non-CF bronchiectasis even though there is no approved product yet (Zhou et al., 2015). Until now only tobramycin, aztreonam and colistin solutions have been approved for nebulization (Zhou et al., 2015; Velkov et al., 2015). So far, there

is no study about chloramphenicol administration into the lungs. The thiamphenicol glycinate ester was evaluated in oncological immunologically compromised patients with respiratory tract infections. Administered into the lungs as aerosols, alone or in association with other antibiotics, thiamphenicol glycinate was effective in more than 95% of patients (Macchi et al., 2011).

The physicochemical properties of the drugs like solubility, lipophilicity, particle size influence their lung penetration (Eixarch et al., 2010). The optimal aerodynamic diameter of the aerosols for lung delivery ranges from 1 to 5 µm (Hagerman et al., 2006). Several side effects probably may occur when drug particles directly contact into the lung such as local irritation, bronchospasm, coughing and wheezing (Zhou et al., 2015; Westerman et al., 2004; Le Brun et al., 2002). Drug formulation for inhaled delivery aims at maintaining drug concentration for the desired length of time, at reducing side effect and at improving efficacy, patient compliance and convenience (Singh et al., 2010). Formulations for inhalation include liquid aerosols or dry powders. In order to modulate release profiles and drug bioavailability, liposome or polymeric micro- or nanoparticle formulations have been proposed (Zhou et al., 2015).

Microsphere or nanosphere formulations are promising options for optimizing lung delivery. Microspheres of the appropriate aerodynamic properties (1 to 5 µm) are generally administered as a dry powder using conventional dry powder inhalers (DPI). DPI offers many advantages over nebulizers, such as portability, quick administration and simple hygienic procedures. Microspheres for lung delivery have been extensively studied and offers interesting features,

such as sustained release profiles (Doan and Olivier, 2009; Gaspar et al., 2016), bioadhesion, macrophage targeting, etc. Due to their small size (<1 µm), nanoparticle cannot be aerosolized as individual particles using conventional DPI. Therefore, they are administered as liquid aerosol using nebulizers or as dry micro-scale aggregates (usually referred to as nano-aggregates, nanocomposite microparticles or Trojan microparticles) using DPI. Micro-scale aggregates are basically composed of nanoparticles and water-soluble additives. Once deposited in the lungs, these aggregates release nanoparticles after dissolution of the water-soluble additives. Interests of nanoparticles are many. Through encapsulation, the binding between the antibiotic molecules and the sputum may be minimized. The small size of the nanoparticles enables them to readily diffuse through the sputum mesh and to reach the deeply embedded bacteria (Suk et al., 2009), resulting in a higher antibacterial efficacy compared to nebulized solutions (Halwani et al., 2009; Meers et al., 2008). The nanoparticles exhibit a longer retention time in the lung compared to microspheres as the lung phagocytic macrophages are less effective in eliminating nanometric particles (Chono et al., 2006). Nanoparticles enhance the diffusion rate to lung tissue and its penetration among the cells because of the optimum of particle size (Kaur et al., 2012; Geys et al., 2006; Gómez-Gaete et al., 2008). They can also promote bioadhesion to pulmonary tissue through appropriate surface properties (Yamamoto et al., 2005).

Both nanospheres and microspheres can modulate drug release kinetic using adequate core and wall polymers. Polymeric matrix of nanospheres and

microspheres using biodegradable compound is widely investigated to control delivery system. Poly(lactide) (PLA) and poly(lactide-co-glycolide) (PLGA) are frequently used as biodegradable polymers for nanoparticles and microparticles (Singh et al., 2010; Kaur et al., 2012), because they are biodegradable and biocompatible (Anderson and Shive, 2012).

The initial step in evolving a new chloramphenicol and thiamphenicol formulation for respiratory infection is to figure out their pharmacokinetic parameters after lung deposition (Stigliani et al., 2016). Correlations between in vitro and in vivo are needed during pharmaceutical development to reduce development time and optimize the formulation (Eixarch et al., 2010). In vitro evaluation of chloramphenicol and thiamphenicol includes their membrane permeability and their transport mechanism into the lung. Calu-3 cells culture is an appropriate broncho-alveolar epithelium model to evaluate the drug transport (Ong et al., 2013) and is used as a primary screening tool for drug candidate in the lung (Stigliani et al., 2016). In order to complete in vitro study, in vivo experiment of chloramphenicol and thiamphenicol in the lung is necessary. In vivo study using animal model as preclinical study is widely used, most predictive, reliable and more practical (Velkov et al., 2015). It is a valuable tool for investigating lung deposition and its bioavailability (Lipworth, 1996). Chloramphenicol and thiamphenicol concentration in epithelial lining fluid (ELF) using the bronchoalveolar lavage (BAL) procedure permits to evaluate antibiotic pharmacokinetics in the lung lumen (Kiem and Schentag, 2008). For proving evidence that lung delivery route is superior to other routes,

it is necessary to compare chloramphenicol and thiamphenicol concentrations in the lung after intratracheal and intravenous administrations in rats (Boisson et al., 2014).

The purpose of this research is to perform a biopharmaceutical characterization of chloramphenicol and thiamphenicol by determination of their apparent membrane permeability coefficient (Papp) and their transport mechanism through Calu-3 lung epithelium model. The second purpose is to evaluate the lung pharmacokinetic properties of chloramphenicol and thiamphenicol in solution after intratracheal and intravenous administrations in rats. Then in vitro and in vivo results will be gathered together to design and optimize chloramphenicol and thiamphenicol innovative dry-powder formulations for inhalation.

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CHAPTER 2

BIBLIOGRAPHY

2. BIBLIOGRAPHY

2.1 Introduction

Respiratory tract infections are caused by bacteria, viruses or fungi (Purushothama and Chien, 1996). They are the second cause of death in children and adults worldwide (Marimón and Navarro-Marí, 2017; Stover and Litwin, 2014). According to the European Lung Foundation and European Respiratory Society 2013 report, in European Union, one of eight deaths is caused by respiratory infection. Each year there are 6 million hospitalizations due to respiratory infections. Respiratory tract infections are classified into upper and lower respiratory tract infection (Purushothama and Chien, 1996). Lower respiratory tract infections such as bronchitis, bronchiolitis and pneumonia (Purushothama and Chien, 1996), are the fifth leading cause of childhood death in the world with the majority in low and middle income countries (Unger and Bogaert, 2017).

Antimicrobial treatment can be delivered by oral, parenteral or inhaled administrations. Systemic administrations of antimicrobials deliver only a small proportion of drug to the lung. To maintain drug levels above efficient concentrations in the lungs, high doses of drug are therefore needed. Such high doses may induce severe adverse effects, while suboptimal dosing may result in antimicrobial resistances (Garonzik et al., 2011; Zhou et al., 2015). Therefore, aerosol administration of antimicrobials into the lung has been proposed to prevent or to treat lung infections (Fernandes and Vanbever,

2009). Pharmacokinetic studies show that high drug concentrations in sputum of the lung can be achieved with the aerosol therapy of colistin methanesulfonate (2 million IU) in cystic fibrosis (CF) patients with low systemic drug exposure (Ratjen et al., 2006). Although aerosol delivery has many advantages, there is a paucity of data on the safety, efficacy and pulmonary pharmacokinetics. Moreover, very few drugs are specifically designed and formulated for pulmonary delivery or under development (Velkov et al., 2015). Until now, there are at least three antibiotics approved for lung infection treatment such as tobramycin, aztreonam, and colistin. Some other antibiotics, such as levofloxacin, fosfomycin, and amikacin are under development (Velkov et al., 2015)

Several factors will influence fate and bioavailability of administered drug via pulmonary delivery such as the nature of the infecting organism, the host (the patient), the physiochemical characteristics of the antibiotic, the lung physiology, formulation factors and the type of inhaler (Kaur et al., 2012, Falagas et al., 2010, Labiris and Dolovich, 2003).

Given the lack of new antimicrobials, old antibiotics (i.e. antibiotics that have been neglected or abandoned in the past because of toxicity issues) have been re-considered for the treatment of infections resistant to commonly used antibiotics and have been shown to be still effective. For example, colistin was recommended by the most recent American Thoracic Society Guidelines as a therapeutic option for the treatment of ventilator-associated pneumonia caused by multiple drug resistance (MDR) Gram-negative organisms (Cassir et al.,

2014). Despite serious side effects such as irreversible aplastic anemia, chloramphenicol is still widely used in developing countries due to its low price. In some developed countries, chloramphenicol is proposed as a second line therapy for the treatment of bacterial infections resistant to modern antibiotics like vancomycin (Norris et al., 1995). It is however not marketed in France. Thiamphenicol, a parent drug devoid of such a severe side effect is available as tablets for oral administration or lyophilized powder for injection in France.

2.2 Amphenicol antibiotics used in human health

Among the amphenicol group of antibiotics, three of them are used in human health: chloramphenicol, thiamphenicol and azidamfenicol. Chloramphenicol and thiamphenicol have been the most studied and are the most used world-wide and will be the focus of this work. Florfenicol, a fluorinated analog of thiamphenicol, is only used in veterinary medicine.

2.2.1 Chloramphenicol

Chloramphenicol, molecular weight 323.13 g/mol, is a nitrobenzene derivative antibiotic. Chloramphenicol succinate ester has been synthesized to increase its aqueous solubility and is used in parenteral forms and chloramphenicol palmitate ester has been synthesized to mask chloramphenicol taste and is used as paediatric suspension.

Chloramphenicol is active against Gram positive or negative bacteria such as

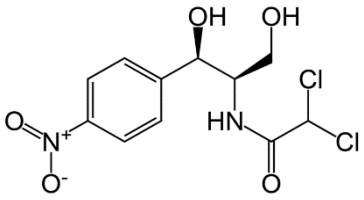
Spirochaetes, Rickettsiae, Mycoplasmas, Tryponemapallidum, Borrelia, Leptospira, Romansky, Pseudomonas Pseudomallei, Actinomyces, Haemophilus influenza, Streptococcus pneumonia, and Neisseria meningitis. It shows no activity against parasites, fungi, mycobacteria and Pseudomonas aeruginosa (Shukla et al., 2011). It is usually used for typhus fever, salmonellosis, acute respiratory infections, meningitis, and conjunctivitis (Ambekar et al., 2000; Turton et al., 2006; Ioannidis and Murdoch, 1957).

Chloramphenicol is mostly bacteriostatic, but possesses bactericidal activity against Haemophilus influenzae, Streptococcus pneumoniae, and Neisseria meningitidis (Feder et al., 1981; Rahal and Simberkoff, 1979). The mechanism of action of chloramphenicol is the inhibition of peptide and protein synthesis by binding to the 50S subunits of the 70S ribosomes (Smith and Weber, 1983).

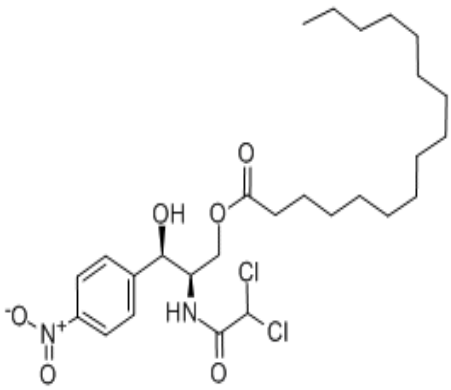
Chloramphenicol can be administered by oral, intravenous, intramuscular, and topical route (Turton et al., 2002; Wiest et al., 2012). Its bioavailability is around 80% after oral administration, and around 70% after its intravenous administration as the prodrug chloramphenicol succinate, because of renal excretion of a fraction of unchanged chloramphenicol succinate (Madhavan and Bagyalakshmi, 2014). Its plasma protein binding is 60% in healthy adults (Ambrose, 1984) and total body clearance is 0.122-0.429 l/kg/h (Sack et al., 1980). The effective serum concentration of chloramphenicol is between 10 and 20 mg/l (Feder et al., 1981; Ambrose, 1984) which is achieved with the dose of 25 mg/kg/day for infants and children and of 50 mg/kg/day for adults (Chaplin, 1986). The dosing can be up to 75-100 mg/kg/day in the case of

meningitis (Feder et al., 1981). Its average half time is 5.1 hours in infants and children (Kauffman et al., 1981), 1.6-3.3 hours in adults (Shukla et al., 2011). It has a good tissue diffusion especially across the blood brain barrier (Wiest et al., 2012) and the volume of distribution is 0.6-1.4 l/kg (Smith and Weber, 1983).

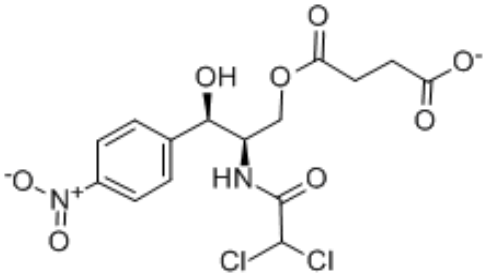
Chloramphenicol induces several side effects such as fatal idiosyncratic aplastic anemia, and bone marrow suppression (Turton et al., 2002; Shukla et al., 2011; Ambekar et al., 2000). The fatal idiosyncratic aplastic anemia effect is dose independent (Wallerstein et al., 1969) and its incidence rates is 1 in 524,000 in the USA, 1.5 per million in France (Madhavan and Bagyalakshmi, 2014). Nitroso-chloramphenicol and hydroxyl-amino-chloramphenicol can be responsible for this effect (Yunis et al., 1980). Due to its serious effects, it is used for serious and potentially fatal infections against which no safer alternative is present (Shukla et al., 2011).



Chloramphenicol



Chloramphenicol Palmitate



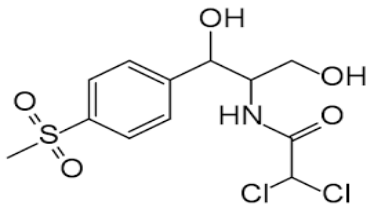
Chloramphenicol Succinate

Figure 1. Chemical structure of chloramphenicol, chloramphenicol palmitate, and chloramphenicol succinate

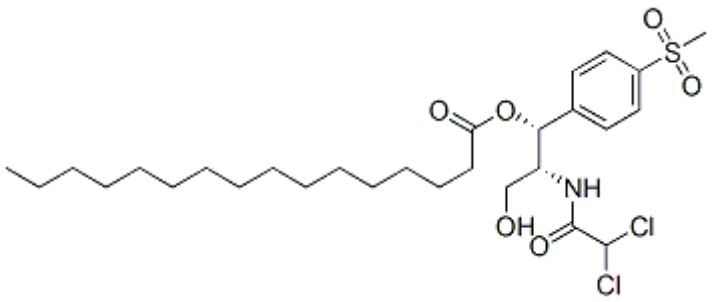
2.2.2 Thiamphenicol

Thiamphenicol, molecular weight 356.22 g/mol, is an analogue of chloramphenicol. A methyl-sulfonyl group substitutes the nitro group of chloramphenicol (Drago et al., 2000). Ester prodrugs are thiamphenicol glycinate and thiamphenicol palmitate. The thiamphenicol glycinate ester, molecular weight 449.70 g/mol, was synthesized to improve its solubility in water and is used in parenteral formulations (Chen et al., 2006). It induces

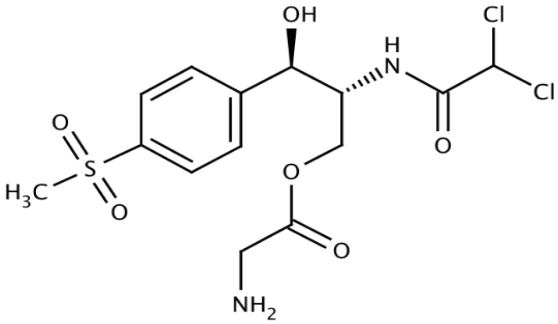
reversible aplastic anemia effect, but, due to the absence of nitro group, it was not reported to be responsible for irreversible aplastic anemia (Turton et al., 2000) and is considered as less toxic than chloramphenicol (Yunis et al., 1980). Its spectrum covers Chlamydia pneumoniae, M. Catarrhalis, H. Influenzae, S. Pneumoniae and S. Pyrogenes (Grassi and De Benedetto, 2002; Serra et al., 2007). It is usually used to treat respiratory infections, bacterial prostatitis, sexually transmitted diseases and urinary tract infections (Chen et al., 2006; Yang et al., 2014). It has bacteriostatic activity with the same mechanism of action as chloramphenicol (Turton et al., 2000). Its absorption is rapid after oral administration and it diffuses rapidly into tissue (Drago et al., 2000). Its half-life in human adults is 3.29 ± 0.40 hours (Wang et al., 2014).



Thiamphenicol



Thiamphenicol Palmitate



Thiamphenicol Glycinate

Figure 2. Chemical structure of thiamphenicol, thiamphenicol palmitate, and thiamphenicol glycinate

2.3 The lungs

For lung administration, the deposited dose and its distribution within the lungs are important factors for therapeutic effectiveness. They are related to the lung physiology in one part and to the physiochemical characteristics of the drug in the other.

2.3.1 Structure and physiology of the lungs

Lungs are composed of two functional regions such as airway and alveolar region (Figure 3). The airway region consists of trachea, bronchi and bronchioles. Airway epithelium is composed of epithelial cells – which is the major cell type –, ciliated cells, goblet cells, secretory cells and basal cells (Mortensen et al., 2014) (Figure 4). The mucus is produced by goblet cells within the airway epithelium and by submucosal glands. It consists of 95 % water, 2 % mucin, 1 % salts, 1 % albumin, immunoglobulin, enzymes, less than 1 % lipids (Fernandes and Vanbever, 2009). As protective coating of airways, mucus is involved in the mucociliary or the cough clearance mechanism and trapped particles will end up in the gastrointestinal tract (Olsson et al., 2011).

The alveolar region is composed of the alveolar ducts and the alveolar sacs and represents 95 % of the total surface of the lungs and therefore is of greatest importance when considering drug diffusion.

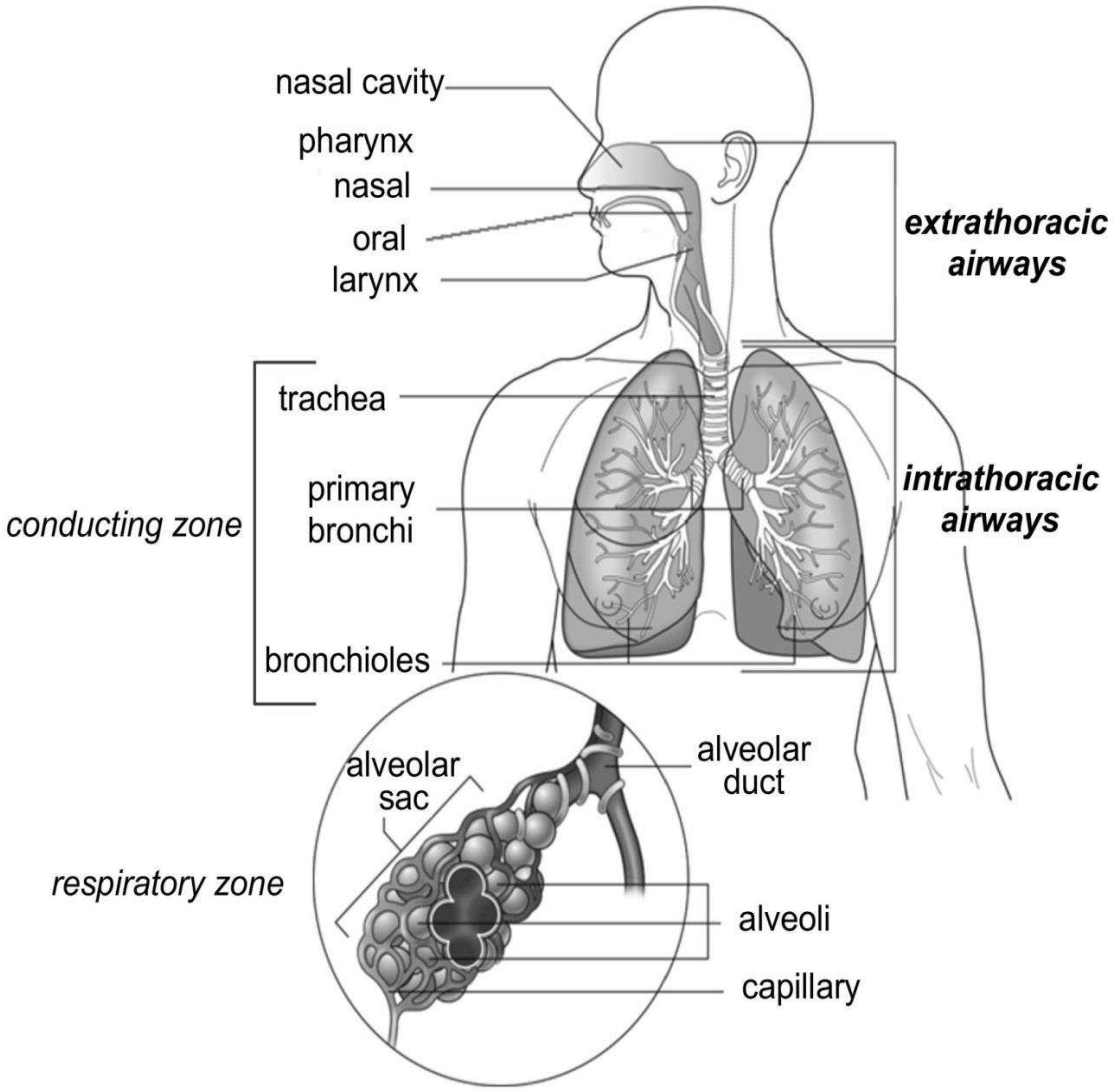


Figure 3. Airways anatomy (Regional aerosol deposition in the human airways) (Koullapis et al., 2017)

The alveolar epithelial cells consist of type I and type II pneumocytes (Fernandes and Vanbever, 2009). Alveolar epithelial type I cells are large squamous cells with diameter 50-100 µm and 0.05 µm thinness (Stone et al., cited Liu et al., 2013). These cells cover > 90% of the alveolar surface and facilitate gas diffusion (Koval and Sidhaye, 2017). They are joined together with tight junction proteins which makes these cells the main paracellular

barrier for drugs or particles.

Alveolar epithelial type II cells are small and cuboidal cells with diameter 7-10 µm (Fehrenbach, 2001). They represent 60% of the cells in the alveolar epithelium but, due to their small size, < 10% of the alveolar surface. The main functions of alveolar epithelial type II cells are to produce surfactant and to serve as progenitors for alveolar epithelial type I cells (Barkauskas et al., 2013; Liu et al., 2013). The lung surfactant is composed of 80% phospholipids, 5-10% neutral lipids and 8-10% protein (Fernandes and Vanbever, 2009). Its role is to decrease surface tension at the pulmonary air-liquid interface (Bernhard, 2016).

Epithelial lining fluid (ELF) is a fluid covering the epithelium in the lung. ELF is considered as a site of lung infections (Kuti and Nicolau, 2015). ELF in the alveolar region comprises proteins, for instance albumin, immunoglobulin G (IgG), secretory IgA, transferrin, and ceruloplasmin (Kim and Malik, 2003) and forms a very thin diphasic layer into which surfactants are inserted.

The alveolar macrophages are found on the alveolar surface. They will quickly engulf particles of sizes between 1.5 and 3.9 µm (El Sherbiny et al., 2015).

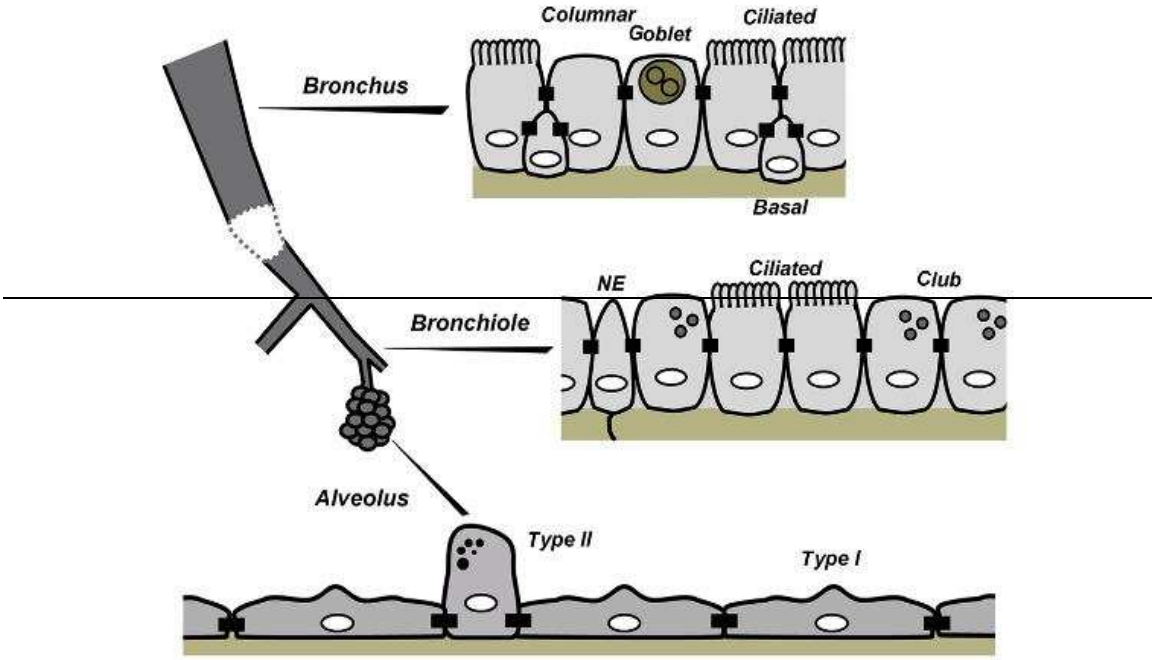


Figure 4. Schematic illustration of epithelial cells in the different regions of the human lung (Koval and Sidhaye, 2017)

2.3.2 Transport mechanisms through the lung epithelium

The main transport mechanisms of molecules across the lung epithelium are passive diffusion and active transport.

2.3.2.1 Passive diffusion

Passive diffusion is driven by a gradient of concentrations (Ware et al., 2004) and is influenced by the physicochemical properties of the molecule such as molecular weight and lipophilicity (Sugano et al., 2010). For instance, better drug diffusion through the intestinal epithelium is expected for drugs with

molecular weight < 500 Da and lipophilicity (log P) in a −0.4 to +5.6 range (Veber et al., 2002). Such information for the lung is not currently available, although the mechanism should be the same.

2.3.2.2 Active transport

The active transport mechanism requires energy and involves membrane transporters (Sugano et al., 2010). Two families of active transporters, the ATP-binding cassette (ABC) and solute-linked carrier (SLC), are present in the lung.

a. ABC transporters use the energy of ATP to transport a large group of various molecules. The most studied transporters are the P-glycoprotein (P-gp), the multidrug resistance associated proteins (MRPs), and the breast cancer resistance protein (BCRP). They function as efflux pumps which transport their substrates from the inside to the outside of cells.
b. SLC transporters do co-transport or secondary transport through the plasma membrane. They function as influx or efflux transporter. They include the organic cation transporter family (OCT), the peptide transporters (PEPT1 and 2), the organic anion transporter family (OAT) and the organic anion transporting polypeptide family (OATP) (Bosquillon, 2010).

In the table 1, the presence of different transporters in the human lung has been reported according to Bosquillon (2010) and Nickel et al. (2016).

Table 1. Transporters in the human lung (adapted from Bosquillon, 2010; Nickel et al., 2016)

Protein		Gene	Expression
ABC transporters	P-gp	ABCB1	- mRNA expressed at low and moderate levels - Apical localization shown by immunohistochemistry (IHC) in bronchial and bronchiolar and alveolar epithelia. While others observed no staining in alveolar epithelia - Protein detected in whole lung and alveolar tissue but absent in bronchial samples - Slightly higher expressed in bronchial mucosa of smokers compared to nonsmokers, although no statistical significance due to high interindividual variations
	MRP1	ABCC1	- Basolateral membrane - Protein detected at high levels expressed in bronchial and bronchiolar epithelial cells, alveolar macrophages, mucus producing cells but absent from pneumocytes
	MRP2	ABCC2	mRNA absent or low level, protein undetectable by LC–MS/MS and in an IHC study, only one out of four antibodies stained positive for MRP2 in the apical membrane of the bronchial and bronchiolar epithelial layers
	MRP3	ABCC3	mRNA expressed at moderate level, protein detected by LC–MS/MS, but undetectable by IHC
	MRP4	ABCC4	mRNA expressed at low-to-moderate level, protein detected by LC–MS/MS
	MRP5	ABCC5	mRNA expressed at moderate level, protein detected by LC–MS/MS
	MRP6	ABCC6	mRNA expressed at moderate level, protein detected by LC–MS/MS
	MRP7	ABCC10	mRNA expressed at moderate-to-high level, protein undetectable by LC–MS/MS
	MRP8	ABCC11	mRNA expressed at moderate level, or absent, protein detected by LC-MS/MS
	BCRP	ABCG2	mRNA expressed at moderate levels. Protein localized to the apical membranes of alveolar epithelial cells with little or no expression in bronchial epithelial cells, mRNA expression detected at low levels by microarray analysis

SLC transporters	OCT1	SLC22A1	mRNA detected at weak to moderate levels by microarray analysis and qRT-PCR. Protein detected at low levels in plasma membranes by LC-MS/MS
	OCT2	SLC22A2	Protein weakly expressed or absent with strong protein expression detected on the apical membranes of ciliated airway cells
	OCT3	SLC22A3	Varying data: mRNA detected at moderate levels in human bronchial tissues and at higher levels in pulmonary parenchyma by microarray analysis. High levels were detected in whole lung tissues and weak expression detected in human airway epithelium
	OCTN1	SCL22A4	mRNA highly expressed in bronchial mucosa, pulmonary parenchyma, central, and peripheral lung samples, protein detected by LC-MS/MS
	OCTN2	SLC22A5	mRNA expression at moderate to high levels, mainly located to the epithelial lining of the trachea
	PEPT1	SLC15A1	mRNA expressed in the 25-50% quartile and expressed weakly in bronchial mucosa, absent in pulmonary parenchyma, protein undetectable by LC-MS/MS
	PEPT2	SLC15A2	- Apical membrane/Cytoplasm for alveolar epithelial type II cells - mRNA expressed at moderate to high level, high protein expression detected by LC-MS/MS, IHC revealed PEPT2 protein to be expressed in tracheal, bronchial, and smaller airway epithelial cells as well as endothelium of small vessels
	OAT1	SLC22A6	mRNA absent protein was undetectable by LC–MS/MS
	OAT2	SLC22A7	Contradictory data regarding mRNA: expressed moderately, or absent, protein detected by LC–MS/MS
	OAT3	SLC22A8	mRNA absent, protein detected by LC–MS/MS
	OAT4	SLC22A11	mRNA absent, protein detected by LC–MS/MS
	URAT1	SLC22A12	Contradictory data regarding mRNA: expressed at low levels or absent
	OAT7	SLC22A9	mRNA absent
	OATP1A2	SLCO1A2	Contradictory data regarding mRNA, expressed at low level or absent, protein detected by LC–MS/MS
	OATP1B1	SLCO1B1	mRNA absent, protein undetectable by LC–MS/MS
	OATP1B3	SLCO1B3	mRNA absent, protein detected by LC–MS/MS

	OATP1C1	SLCO1C1	mRNA absent, protein undetectable by LC–MS/MS
	OATP2A1	SLCO2A1	mRNA expressed at high level, protein detected by LC–MS/MS
	OATP2B1	SLCO2B1	mRNA expressed in the 50–75% quartile, protein detected by LC–MS/MS
	OATP3A1	SLCO3A1	mRNA expressed at moderate-to-high level, protein undetectable by LC–MS/MS
	OATP4A1	SLCO4A1	mRNA expressed at moderate-to-high level, protein undetectable by LC–MS/MS
	OATP4C1	SLCO4C1	mRNA expressed at low-to-moderate level, protein undetectable by LC–MS/MS
	OATP5A1	SLCO5A1	mRNA absent, protein undetectable by LC–MS/MS
	OATP6A1	SLCO6A1	mRNA absent, protein undetectable by LC–MS/MS

2.4 Experimental methods to evaluate drug distribution in the lung

There are several methods to evaluate drug distribution in the lung such as in vivo, ex vivo and in vitro evaluation.

2.4.1 In vivo study

In vivo studies are usually performed in rats. Despite the anatomical differences, rat models predict effectively drug distribution in the human lungs (Ong et al., 2013). Lung distribution is determined by measuring drug concentrations both in the lungs and in the plasma after pulmonary administration or after intravenous administration. Then pharmacokinetic profiles are compared between the two administrations. Drug concentrations can be measured in the lung tissue or in the ELF (Fernandes and Vanbever,

2009). In the lung tissue, concentration measurement may be conducted after lung resection, homogenization and drug extraction using adequate solvents (Fernandes and Vanbever, 2009). Another technique is the microdialyse where a microdialyse probe is inserted into the lung tissues. ELF measurement is performed though bronchoalveolar lavage (BAL) by injecting a normal saline solution into the lungs and then retrieving (Fernandes and Vanbever, 2009). As the advantage, the BAL method is simple to perform both in human and in animals. This method has also disadvantages. First, it is impossible to take ELF samples at several times in the same subject, because the washing process may change the composition of the ELF (Zeitlinger et al., 2005). Second, during the realization of the BAL, cellular lysis of the macrophages may occur and leads to a release of cell content in the ELF. Thus, for drugs that accumulate in the cell, this cell release could bias the results (Kiem and Schentag, 2008).

2.4.2 Ex vivo study

Known as the isolated perfused lung (IPL), ex vivo study is performed to evaluate pulmonary uptake and metabolism of the drug. Rat lungs are isolated and maintained with blood or buffer solution (pH 7.4) at 37℃. The administration of the drug can be done in the isolated lung by intra-tracheal delivery and/or direct injection in the perfusate solution (Fernandes and Vanbever, 2009). It is possible to study drug absorption in the lung without any

interference of other organs. But this method cannot describe the drug absorption process from the airway region of the lung and it requires good surgical skills. However, this method has a short viability time (2-3 hours) for physiological conditions (Fernandes and Vanbever, 2009).

The pros and cons of the different in vivo methods are synthesized in Table 2.

Table 2. Techniques for intrapulmonary concentration determination. Adapted from Dhanani et al. (2010)

	Techniques			
	BAL	Microdialysis	Lung tissue homogenat	Ex vivo
Biological sample	Bronchial and alveolar lining fluid	Interstitium liquid	Total tissue	Isolated perfused lung
Invasiveness	Semi-invasive	Invasive	Invasive	Invasive
Direct measurement of free drug concentration	No	Yes	No	No
Multiple time-based captures of a single subject	No	Yes	No	No
Technical complexities	Low	High	Low	High
Advantages	Sample closer to target site than sputum Easy sampling in mechanically ventilated patients	Possibility of multiple shots of the same subject Evaluation of the free concentration of the drug	Simple technique to realize	Easy sampling of perfusate and lavage fluid
Disadvantages	Potential cell lysis Correction of concentrations by a dilution factor	Invasive technique Authorized technique only in patients undergoing thoracic surgery	Does not allow concentration measurements in the different compartments	Short viability time for controlling physiological conditions Good surgical skills required

2.4.3 In vitro study

In vitro studies involve lung epithelial cells and allow diffusion or transport studies at the cellular and molecular level: transport mechanism, transporter identification, drug-drug interaction and structure-permeability relationship can be evaluated. Cell culture models of the lungs can be obtained from primary cells or from continuous cell lines that are originated from cancerous cells or transformed cells (Ong et al., 2013). Primary cell cultures are more similar to the in vivo condition than continuous cell lines. But the process of purification from the tissues is time consuming, the number of experiments is limited and the results are less reproducible (Ong et al., 2013; Fernandes and Vanbever, 2009; Ehrhardt et al., 2017). Compared to primary cell cultures, continuous cell lines are more consistent and are more suitable for drug screening, despite their cancerous origin. The usual cell lines are: Calu-3, A549, BEAS-2B, 16HBE14o, NCI-H441 and NCI-H292 cells (Fernandes and Vanbever, 2009; Ong et al., 2013). Among these different cell types, Calu-3 is by far the most used cell model for drug transport studies. Acquired from a submucosal adenocarcinoma of a 25-year-old Caucasian male, Calu-3 cells differentiate into polarized monolayer with tight junctions and express the main transporters of the human lungs (Table 3). They are also readily available, easy to culture and robust (Ong et al., Traini, 2013; Bosquillon et al., 2017).

Table 3. Comparison between human lung tissue and human Calu-3 cell expressions of drug transporters (adapted from Nickel et al., 2016)

Transporters	Human lung tissue	Calu-3
P-gp	++	+++
MRP1	+++	+++
MRP2	+	++
MRP3	++	+++
MRP4	++	+
MRP5	++	+++
MRP6	++	+
MRP7	+++	++
MRP8	++	-
BCRP	++	++
OCT1	++	++
OCT2	+	-
OCT3	++	++
OCTN1	+++	++
OCTN2	+++	++
PEPT1	+	++
PEPT2	+++	++
OAT1	-	-
OAT2	+	-
OAT3	+	-
OAT4	+	+
URAT1	+	-
OAT7	-	-
OATP1A2	+	+
OATP1B1	-	+
OATP1B3	+	++
OATP1C1	-	++
OATP2A1	+++	-
OATP2B1	++	++
OATP3A1	+++	++
OATP4A1	+++	+++
OATP4C1	+++	++
OATP5A1	-	+
OATP6A1	-	-

(-) no expression, (+) low expression, (++) intermediate expression, (+++) high expression

Liu et al., 2013 gives summary about cell culture models and their application in drug disposition studies (Table 4).

Table 4. Cell culture models for pulmonary drug disposition studies (adapted from Liu et al., 2013)

Cell culture	Location	Models	Origin	Application
Primary cell culture	Airway epithelium	Epiairway™ system	Normal human derived tracheal/bronchial epithelia	Drug transport study
		Normal human bronchial epithelial cell	Explants of autopsy specimens	Metabolizing enzymes and drug transport study
		Rabbit tracheal epithelial cell monolayers	Trachea from male New Zealand white rabbit	Drug permeability study
	Alveolar epithelium	Human type II alveolar epithelial cells	Specimen of distal portions of normal lung resection	Pulmonary absorption and transport study
		Rat alveolar epithelial cell monolayer	Adult male Sprague-Dawley rat lung	Dextran, beta blocker, amino acids, peptides, protein,nanoparticles transport of mechanism study
		Pig alveolar epithelial cell monolayer	Female 6 months old pig lung	Drug transport study
Cell line	Airway epithelium	16HBE 14o-	Transformation of cultured bronchial-surface epithelial cells from a 1-year- old male heart-lung	Drug transport study

			patient	
		Calu-3	Bronchial submucosal adenocarcinoma in a 25-year-old Caucasian man	Drug transport study; drug metabolism study
		BEAS-2B	Transformation of human bronchial epithelial cells transfected by the adenovirus 12-simian virus 40 hybrid virus	Drug metabolism enzyme expression and activity study
		CFBE41o-	Transformation of bronchial epithelial cells obtained from a CF patient	-
	Alveolar epithelium	A549	Cancerous lung tissue from explanted tumor of a 58-year-old Caucasian male	Drug metabolism and nanoparticle uptake

2.5 Formulation for lung administration

According to Labiris and Dolovich (2003) the formulations for the lung administration must be able:

a. To deliver drugs at the action site, for example by optimizing the particle aerodynamic diameter (Loira-Pastoriza et al., 2014), modifying the surface charge, or through association with ligand (Shah et al., 2017).

b. To modify and to control drug release to extend the residence time of drug in airway or alveolar region and then minimize the risk of adverse effects by decreasing its systemic absorption. Patient compliance should be also increase by reducing dosing frequency (Labiris and Dolovich, 2003).

2.5.1 Particle deposition mechanism, aerodynamic diameter and lung distribution

2.5.1.1 Particle deposition mechanism

The deposition of particles in the respiratory tract is governed by both airway anatomy and the aerodynamics of the inhaled particles. It predominantly results from the combined effect of particle inertia, gravity and Brownian motion. Particles are therefore mainly deposited by inertial impaction to, sedimentation on, and diffusion toward the respiratory tract mucosa (Figure 5).

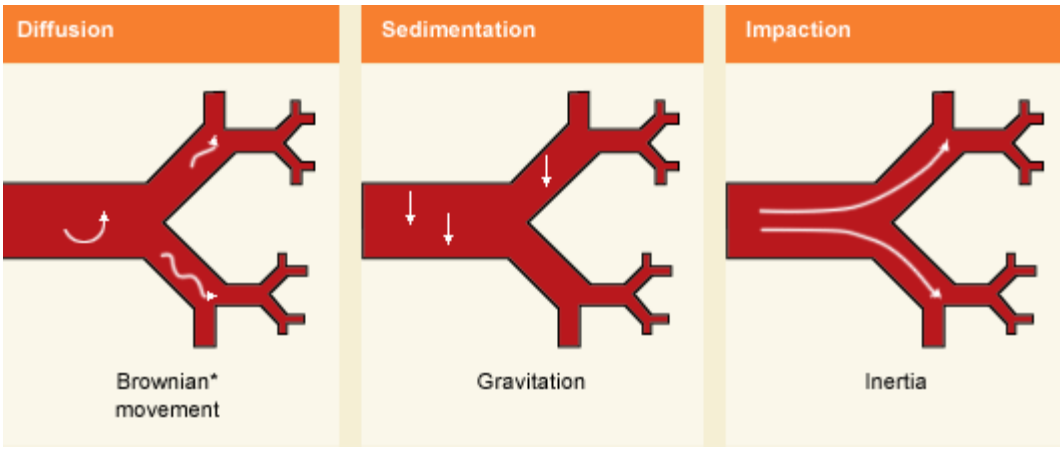


Figure 5. Mechanism of particle deposition in the lung (Vorshaar, 2005)

Impaction occurs predominantly at the airway bifurcations and is influenced by the particle velocity (Smyth and Hickey, 2011). It is the main deposition mechanism in extrathoracic and large conducting airways. It is promoted by the aerosol emission velocity in the case of pressurized metered-dose inhalers or by high respiratory rate.

Sedimentation occurs when the velocity of the airflow in the conducting airways cannot counterbalance the effect of gravity and its involvement in the particle deposition is therefore gradually increasing in the deeper areas of the airways where the air flow gradually slows down. Prolonged apnea after aerosol inhalation promotes particle sedimentation.

2.5.1.2 Aerodynamic diameter and particle distribution in the airways

For comparison purpose, particle aerodynamic properties are described by their aerodynamic diameter. The aerodynamic diameter is defined as the diameter of a virtual sphere with a unit density that has the same terminal settling velocity in still air as the particle under consideration. The following equation links the aerodynamic diameter d_{aer} and the geometric diameter d.

$$d_{aer} = d\sqrt{\rho / \rho_o \chi}$$

Where d is the geometric diameter of the particle, ρ the particle density, ρ_o the density of the virtual particle, χ the particle dynamic shape factor denoting deviation of shape from sphericity (Hinds 1999 cited in Loira-Pastoriza et al., 2014)

For lung delivery, since the delivery vehicle of the therapeutic aerosol is the inhaled air flow, the aerodynamic diameter is generally used to predict the potential deposition pattern of the aerosol particles in the airways and lungs (Mortensen et al., 2014).

The mechanism of deposition is dependent on the aerodynamic diameter of the particles (Figure 6):

a. Particles with aerodynamic diameters above 5 µm mainly deposit by inertial impaction in the upper extrathoracic airways and in the large conducting airways (Emami et al., 2009; Høiby, 2011).
b. Particles of aerodynamic diameters ranging from 1 to 5 µm mainly deposit by gravitational sedimentation and inertial impaction in the small conducting airways and the alveolae (Pilcer and Amighi, 2010; Høiby, 2011).
c. Below 1 µm, the fraction of particles that are deposited by gravity tends to be nul and more and more particles are deposited by Brownian diffusion towards the mucosa. The deposited fraction is low, and most particles are exhaled (Emami et al., 2009; Høiby, 2011).

Therefore, particles with aerodynamic diameters ranging from 0.5 to 5 µm are considered to be of interest for drug lung delivery.

Most of the marketed dosage forms for lung delivery generate aerosol particles of density close to one. Therefore, the geometric diameter of aerosol particles that are targeted by developers of inhaled medicines ranges from 0.5 to 5 µm. However there might be some advantages of using low-density particles of “large” geometric diameter (5 to 10 µm), while keeping their aerodynamic diameter within the 0.5 µm to 5 µm range. Particles with geometric diameter larger than 5 µm are more easily aerosolized and are less subjected to macrophage uptake.

Aerodynamic characterization of therapeutic aerosols is usually done using cascade impactors. The next generation pharmaceutical impactor (NGI) consists of seven stages and is possible to operate at any inlet flow rate between 30 and 100 l/min (Marple et al., 2003). Each stage of the impactor consists of a series of nozzles and a collection plate. The particles are deposited in each plate according to their aerodynamic diameter. At the end of the test, particles are removed from each plate using a suitable solvent and then analyzed. Several parameters can be calculated using mathematical programs such as emitted dose (ED, i.e. the total mass of drug emitted from the inhaler), the fine particle dose (FPD, i.e. the mass of drug deposited with aerodynamic diameter smaller than 5 µm), the fine particle fraction (FPF, i.e. the mass fraction of particles with aerodynamic diameter smaller than 5 µm) and the mass median aerodynamic diameter (MMAD, i.e. the median mass of the distribution of airborne particles with respect to the aerodynamic diameter). These parameters are used to predict the deposition patterns of drug particles in the respiratory tract (Moreno-Sastre et al., 2015).

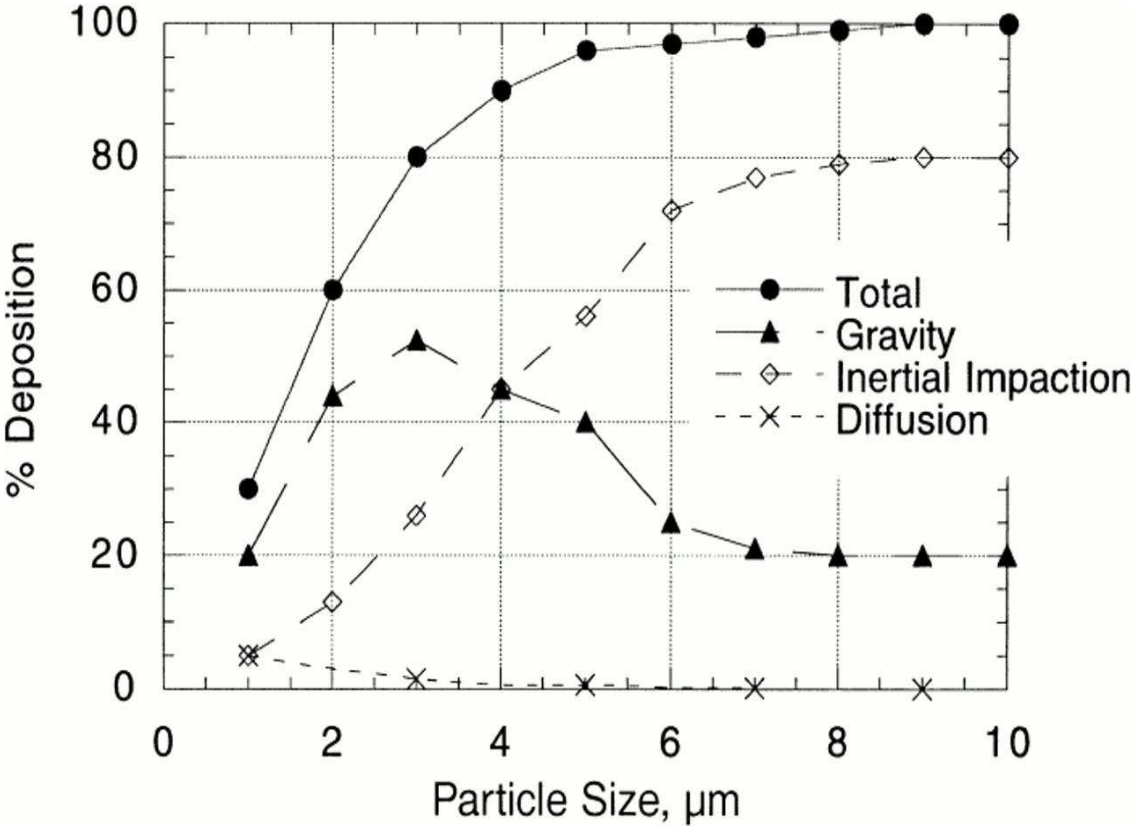


Figure 6. Relative deposition in a human lung model after inhalation of a monodisperse aerosol as a function of aerosol particle aerodynamic diameter, for particles with a mean aerodynamic diameter ranging from 1 to 10 µm. Contributions from the 3 predominant mechanisms of deposition are listed; these include gravitational sedimentation, inertial impaction, and Brownian diffusion (Edwards et al., 1998)

2.5.2 Conventional antimicrobial formulations for lung delivery

To date, marketed formulations of antibiotics for lung aerosol therapy are wet aerosol formulations (solutions for nebulization or powder for solution for

nebulization) or dry powder formulations for inhalation. These are generally basic formulations that may not have optimal properties for efficient therapy.

2.5.2.1 Wet aerosol formulations

Wet aerosols are nebulized solutions or suspensions of antibiotics. During nebulization, antibiotic liquid aerosols are produced mechanically using conventional nebulizers (jet nebulizer, ultrasonic nebulizer), or using more advanced technologies (vibrating mesh nebulizers, or surface acoustic wave microfluidic atomization). Advanced nebulizers generally improve drug delivery efficiencies and patients' compliance (Zhou et al., 2015).

2.5.2.2 Dry powder formulations

Dry powders for inhalation (DPI) have many advantages over solutions, such as long-term physicochemical stability and short time duration of administration (resulting in better patient compliance) (Zhou et al., 2015; Mehta, 2016). For DPI formulations, the micronization process is the basic technology to produce drug powders of the appropriate granulometry for aerosol therapy. Micronized drug powder is often mixed with coarse lactose carriers as an excipient to improve particle dispersion during the aerosolization step. The main inconvenience of a carrier is that for a high dose of drug, which is the case of antibiotics, it increases the powder volume to a level which is incompatible with a lung administration. Therefore, particle engineering is an alternative solution

to produce carrier-free powders with good flowability and good aerosolization properties (Weers, 2015). Spray drying technique is a particle engineering that is used to make inhalable particles such as insulin (Exubera®), tobramycin (TOBI®, podhaler®), or mannitol (Aridol®). As a potential inconvenient, spray drying technique generally produces amorphous forms of drug particles which tend to crystallize into more stable forms with different physicochemical properties, such as dissolution rates. As an innovation to improve lung delivery, porous particles of tobramycin and ciprofloxacin were prepared by spray drying emulsion via the PulmoSphere™ technology which is an emulsion-based spray-drying process that produces light porous particle-based dry-powder formulations, with good flow and aerosolization properties (Geller et al., 2011; Stass et al., 2013; Weers and Tarara, 2014).

2.5.3 Innovative antimicrobial formulations for lung delivery

Since the marketed conventional dosage forms for aerosol therapy only aims at delivering drugs into the lungs without any consideration to the release profiles (they can therefore be considered as immediate release dosage forms), innovative drug delivery systems for lung delivery are needed to optimize release profiles and distribution in the lungs, while fulfilling various issues such as aerosolization properties, stability, biocompatibility, and biodegradation without any adverse effect (Mehta, 2016). NDDS like liposomes, solid lipid nanoparticles, polymeric nanoparticles or polymeric

microspheres have been extensively investigated and may offer several advantages compared to conventional dosage form for inhaled lung delivery (Mehta, 2016) including physical and chemical stability, flow properties, dispersion, tissue distribution, and bioavailability.

2.5.3.1 Liposomal formulations

Liposomes are sphere-shaped vesicles which are composed of one or more phospholipid bilayers (Akbarzadeh et al., 2013). Liposomes can be administered by nebulization of liposome suspension or as dry powders (Loira-Pastoriza et al., 2014). In the respiratory tract, liposomes are engulfed and degraded by the macrophages, then they release the antibiotics, which may be useful in targeting intracellular infections (Zhou et al., 2015). Sustained-release liposome formulations have been produced in order to maintain drug concentrations above their MIC in the respiratory tract, which permits to decrease dosing frequency and to improve patients' compliance (Zhou et al., 2015). Liposomes for lung delivery offer many advantages such as carrier suitability for a lipophilic drug, prevention of local irritation, increased drug potency and reduced toxicity (Desai et al., 2002). Several liposomes of antibiotics have been studied for lung delivery (Table 5). Arikace® is a unique inhaled liposomal formulation that encapsulates aqueous amikacin solution in neutral liposomes (composed of dipalmitoyl-phosphatidylcholine and cholesterol). Once-daily administrations of Arikace® (280 mg or 560 mg) for 28 days demonstrated acute tolerability and safety and efficacy against P aeruginosa infections in cystic fibrosis patients (Clancy et al., 2013). Several

sustained-release liposomal formulations of ciprofloxacin have been investigated. Among them, nebulized ciprofloxacin liposomal formulation (Lipoquin™) and a combination of liposomal and free ciprofloxacin (Pulmaquin™) resulting in rapidly available and slow release ciprofloxacin were shown to be efficacious in cystic fibrosis and bronchitis patients (Zhou et al., 2015).

Table 5. Summary of key liposomal formulations of inhaled antibiotics (adapted from Zhou et al., 2015)

Drug	Formulation	Production method	Major excipient	FPF (%)	Comments
Amikacin	Nebulization (Arikace®)	N/A	N/A	32.5	30-35% loss of entrapped amikacin after nebulization
Ciprofloxacin	Nebulization	Membrane extrusion	Hydrogenated soy phosphatidylcholine, cholesterol	12.5	Drug encapsulation, vesicle size and in vitro release are stable upon nebulization
	Nebulization	Membrane extrusion	Polysorbate 20.04% (w/v), hydrogenated soy phosphatidylcholine, cholesterol	12.5	Faster release rate
	Wet	Thin film method	1,2-Dioleoyloxy-3 trimetylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3 phosphoethanolamine (DOPE), phosphatidyl-	N/A	2-4 times lower MICs against many reference and clinical strains of Pseudomonas aeroginusa, Klebsiella pneumonia, and

			choline (PC), cholesterol		Escheria coli
Colistin	Wet	Dry film method	Dioleoylphosphatidyl choline	N/A	Fast release with 50% of colistin dissolved in 10 min
Isoniazid	Dry powder	Proliposomes by spray drying	1-α-soybean phosphatidylcholine (SPC), cholesterol from lanolin, mannitol	60	Low FPF
Rifapentin	Dry powder	Dry film method	Soya phosphatidylcholine	60	High FPF

N/A: not available

2.5.3.2 Microspheres

Several polymeric sustained-release agents have been investigated such as polyvinyl alcohol (PVA), polylactide or poly(lactic acid) (PLA), polyglycolide or poly(glycolic acid) PGA, and Poly(D, L-lactic-co-glycolic acid) (PLGA). PLGA is commonly used in innovative drug formulations. It shows good physico-chemical and biological properties in biocompatibility, biodegradability, non-toxicity and mechanical strength (Anderson and Shive, 2012), with glass transition temperature (Tg) above of 37℃ (Jain, 2000). Degradation rates depend on the lactic-to-glycolic ratio and on the polymer molecular weight (Jain, 2000). The long-term persistence of PLGA particles in the lungs may trigger pulmonary inflammation and fibrosis (Zhou et al., 2015). Therefore, the choice of the degradation rate is of utmost importance. As foreign particles in

the lung, PLGA particles are expected to be removed either by mucociliary clearance or macrophage (Zhou et al., 2015). Nevertheless, prolonged release times in the lungs have been observed (Doan and Olivier 2009; Gaspar et al., 2016).

There are several preparation methods of drug loaded PLGA of nano/microparticles:

a. Single or multiple emulsion-solvent evaporation permits to prepare nano or microparticles (Julienne et al., 1992) :

    a.1. The single oil-in-water (O/W) emulsion method is suitable for hydrophobic drug. Drug and polymer are both dissolved in the organic solvent (Dichloromethane, chloroform or ethyl acetate). The organic solution is mixed with water phase containing a surfactant (e.g., poly(vinyl alcohol) (PVA)) by a high speed homogenizer or a sonicator to form O/W emulsion. Finally, the organic solvent is removed by extraction or evaporation (Ansary et al., 2014).

    a.2. The multiple oil-in-water (W/O/W) emulsion method is suitable for hydrophilic drug. A water solution of hydrophilic drug is mixed with a polymer solution by a high-speed homogenizer or sonicator to form W/O emulsion. The W/O emulsion is then added to an aqueous phase containing a surfactant. The mixture is then homogenized to obtain a double emulsion with the appropriate

droplet size. Finally, the organic solvent is removed by extraction or evaporation (Ansary et al., 2014).

b. The nanoprecipitation (Fessi et al., 1989) consists of obtaining nanoparticles spontaneously by precipitation and subsequent solidification of the polymer upon fast solvent diffusion. Polymer and drug are dissolved in a water-miscible organic solvent (e.g., acetone, ethanol or methanol). Then, under stirring, the solution is poured into an aqueous solution with surfactant. Finally, the organic solvent is removed by evaporation (Ansary et al., 2014).

Several antibiotics for lung delivery have been formulated: Cheow et al. (2010) investigated the influence of the levofloxacin release profiles from poly (caprolactone) and PLGA nanoparticles on the antibacterial efficacy against E. coli biofilms. The antibiotic release profile had as equal influence on the biofilm eradication rate as the antibiotic dose. Lipid–polymer hybrid nanoparticles of levofloxacin demonstrated improved biofilm affinity and anti-biofilm activity compared to the pure drug (Cheow et al., 2011). Gaspar et al. (2016) showed that intratracheal administrations of levofloxacin-loaded sustained release PLGA microspheres permitted to maintain a high ELF-to-unbound plasma area under the curve (AUC) concentration ratio (around 300) and high concentrations of levofloxacin in ELF over at least 72 hours, confirming previous results obtained with rifampicin (Doan et al., 2013).

Chitosan-modified PLGA nanoparticles loaded with tobramycin released the drug for up to one month and demonstrated good in vitro antimicrobial activities against P. aeruginosa (Ungaro et al., 2012).

Table 6. Summary of key polymeric formulations of inhaled antibiotics (adapted from Zhou et al., 2015)

Drug	Formulation	Production method	Major excipient	FPF (%)
Ciprofloxacin and doxycycline	Dry powder	Co-spray drying	PVA	25.9 for ciprofloxacin and 25.8 for doxycycline
Levofloxacin	Dry powder	Lipid-coated nanoparticles via an emulsification solvent-evaporation method followed by spray drying	PLGA, PVA, phosphatidylcholine, L-leucine	N/A
Tobramycin	Dry powder	Nanoparticle suspension by the emulsion/solvent diffusion method followed by spray drying	PLGA, PVA, chitosan, alginate, lactose	38-52
Isoniazid	Dry powder	Chitosan micropsheres followed by spray drying	Chitosan, tripolyphosphate, lactose, L-leucine	60-70
	Dry powder	Chitosan nanoparticles by ionic gelation method followed by spray drying	Chitosan, tripolyphosphate, lactose, mannitol or maldextrose, L-leucine	7-45 (<5.8 µm), 7.8-11 (<3.3 µm)
Rifampicin	Dry powder	Recrystallization and coating with PLGA/PLA followed	PLGA/PLA	26-45

		by spray coating		
	Dry powder	Amorphous matrix followed by spray drying	PLGA/PLA	23-33
	Dry powder	Poly-(ethylene oxide)-block-distearoyl phosphatidyl-ethanolamine (mPEG-DSPE) nanoparticles followed by lyophilization for rehydration and nebulization	PLGA/PLA mPEG-DSPE w/v	40
	Dry powder	Solvent evaporation of single (w/o) and double emulsion (w/o/w) with premix membrane homogenization followed by freeze-drying	PLGA, PVA	33-70
	Dry powder	Microspheres using single emulsion (o/w) followed by freeze-drying	PLGA, sucrose palmitate	52
	Dry powder	PLGA nanoparticle containing mannitol microspheres followed by four-fluid nozzle spray drying22-32	PLGA, mannitol	35
	Dry powder	Spray drying PLGA-drug solution	PLGA	22-32
	Dry powder	Spray drying PLGA-drug solution	PLA	55-70

N/A: not available

2.6 Conclusion

Lung infections are still major and serious health issues along with the emergence of multidrug microbial resistances. The lack of new antimicrobial discovery leads to the re-use of old antimicrobials such as chloramphenicol and thiamphenicol. The use of chloramphenicol has been abandoned in developed countries due to serious side effects as aplastic anemia, while thiamphenicol has not been reported to cause such fatal adverse effect. However, they are still used in some countries in restricted indications, due to their great tissue penetration profile, broad antimicrobial spectrum and low price. On other hand, the pulmonary administration has several advantages such as rapid clinical response, reduced dosing amount, high local drug concentrations and low systemic exposure to drugs, with expected reduced systemic toxicity. Therefore, the potential for lung administration of chloramphenicol or thiamphenicol should be evaluated as they could be an interesting second-line therapy for lung infections. In order to reevaluate their use for local respiratory infection via pulmonary administration, and to propose an optimum aerosol formulation, the lung diffusion of these drugs has to be evaluated.

First, in vitro experiments will be performed using Calu-3 cells, a well-established model of the lung epithelium for drug transport, with similarity to lung tissue condition such as transporter expression. This first in vitro screening will allow to evaluate the drug permeability through the lung

epithelium, the potential involvement of drug transporter and to predict in vivo lung diffusion.

These in vitro studies will be completed with in vivo experiments in rats which will provide the pharmacokinetic profiles of chloramphenicol and thiamphenicol by measuring their concentration in ELF and in plasma after intra-tracheal and intravenous administrations. Due to the lab expertise, the BAL technique will be the preferred method to measure the drug alveolar content.

Finally, the results of in vitro and in vivo studies will be analyzed to determine the most appropriate aerosol formulation of chloramphenicol or thiamphenicol. The purpose of these formulations will be to maintain drug concentration for the desired length of time, reduce side effects and improve efficacy. The new drug delivery systems like nanosphere or microsphere formulations are promising options for optimizing lung delivery. Both nanospheres and microspheres can modulate drug release kinetic using adequate core and wall polymers. The scheme of this thesis work is reported on Figure 7.

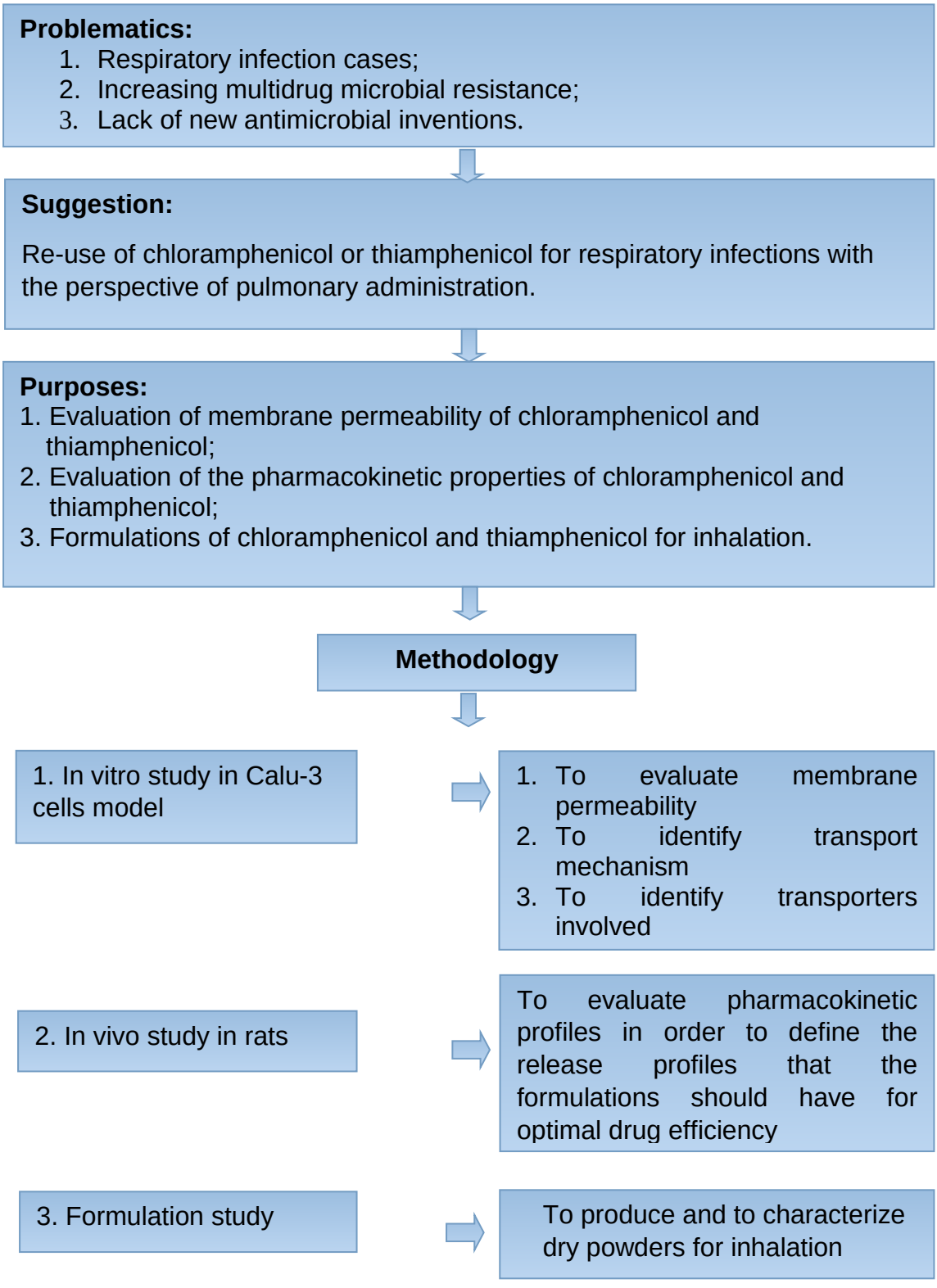


Figure 7. Scheme of research

2.7 References

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CHAPTER 3

EXPERIMENTAL WORK

3.1 In vitro evaluation of lung permeability for chloramphenicol and thiamphenicol using Calu-3 cell model

3. EXPERIMENTAL WORK

The Ph.D. experimental work was divided into three steps. In the first step, basic characteristics such as permeability and efflux transport were evaluated in vitro. Then, pharmacokinetic studies in the blood and ELF compartments were carried out in rats after IV or intratracheal administration. Finally, based on in vitro and in vivo findings, formulations of chloramphenicol and thiamphenicol for the pulmonary route was designed in order to optimize antibiotic treatment efficiencies.

3.1 In vitro evaluation of lung permeability for chloramphenicol and thiamphenicol using Calu-3 cell model

Briefly**,**

Pulmonary administration enables high local concentrations along with limited systemic side-effects, but not all antibiotics (ATB) could be good candidates. In this perspective, diffusion of the ATB chloramphenicol and thiamphenicol through the lung has been evaluated to re-assess their potential for pulmonary administration. The apparent permeability (Papp) was evaluated with the Calu-3 cell model. Influence of drug transporters was assessed with the PSC-833, MK-571, and KO-143 inhibitors. The influence of chloramphenicol and thiamphenicol on the cell uptake of rhodamin123 and fluorescein was also evaluated. Absorptive Papp of chloramphenicol and thiamphenicol were concentration independent with chloramphenicol Papp 4 times higher than that of thiamphenicol. Secretory Papp of chloramphenicol was concentration

independent while it was concentration dependent for thiamphenicol with an efflux ratio of 3.6 for the lowest concentration. The use of inhibitors confirmed that chloramphenicol and thiamphenicol are substrates of efflux transporters, but with a low affinity. In conclusion, the permeability results suggest that the pulmonary route may offer a biopharmaceutical advantage only for thiamphenicol. Due to the influence of drug transporters, a higher concentration in the lung than in the plasma is expected mostly for thiamphenicol, whatever the route of administration.

Résumé développé en français:

Evaluation de la perméabilité pulmonaire du chloramphénicol et du thiamphénicol à l'aide d'un modèle Calu-3 d'épithélium pulmonaire.

Le but de ce travail est d'évaluer in vitro la perméabilité et le transport du chloramphénicol ou du thiamphénicol à travers le modèle d'épithélium bronchique Calu-3. Le modèle Calu-3 est un modèle bien connu et largement utilisé pour l'étude du transport de médicaments, où l'expression des principaux transporteurs de médicaments, tels que la P-gp, les MRPs et la BCRP a été démontrée. Dans ce modèle, les cellules sont cultivées sur des inserts Transwell® et dans les conditions air-liquide (Figure 8), où la face basale des cellules baigne dans le milieu de culture et la face apicale est exposée à l'air afin de mimer les conditions physiologiques de l'épithélium pulmonaire. Les études de transport ont été menées dans deux directions: apicale-basolatérale (Ap-Bl) et basolatérale-apicale (Bl-Ap). Ces études ont

été réalisées avec différentes concentrations de chloramphénicol ou thiamphénicol, à différents temps et en présence ou en absence d'inhibiteurs de pompes d'efflux. La quantité de chloramphénicol ou de thiamphénicol passée à travers les cellules Calu-3 a été analysée par LC-MS/MS.

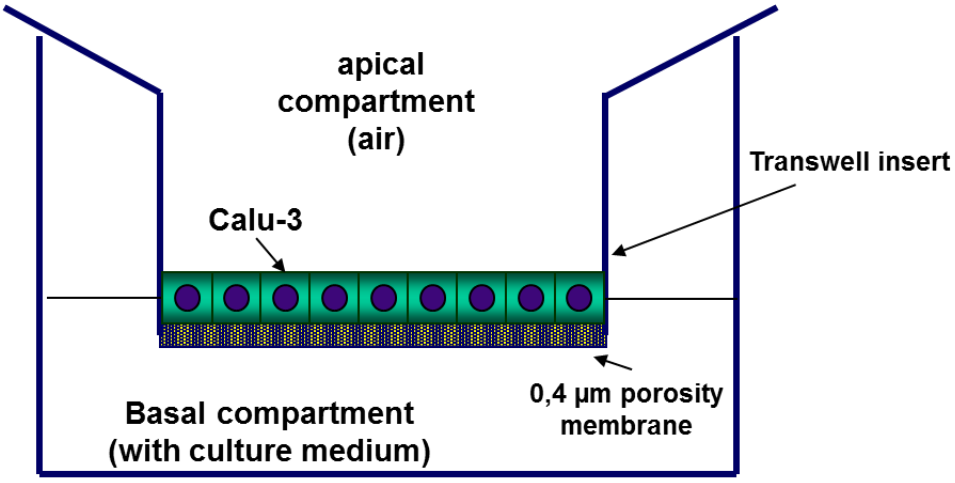

Figure 8. Culture des cellules en condition air-liquide. Les cellules Calu-3 sont ensemencées dans des inserts de marque Transwell® dont la surface est composée de polystyrène traité pour la culture cellulaire (diamètre 12 mm, porosité 0.4 µm). Elles adhèrent et forment un épithélium étanche couvrant toute la surface. Seule la partie basale des cellules est en contact avec le milieu de culture. La partie apicale est exposée à l'air afin de reproduire les conditions de l'épithélium pulmonaire in vivo.

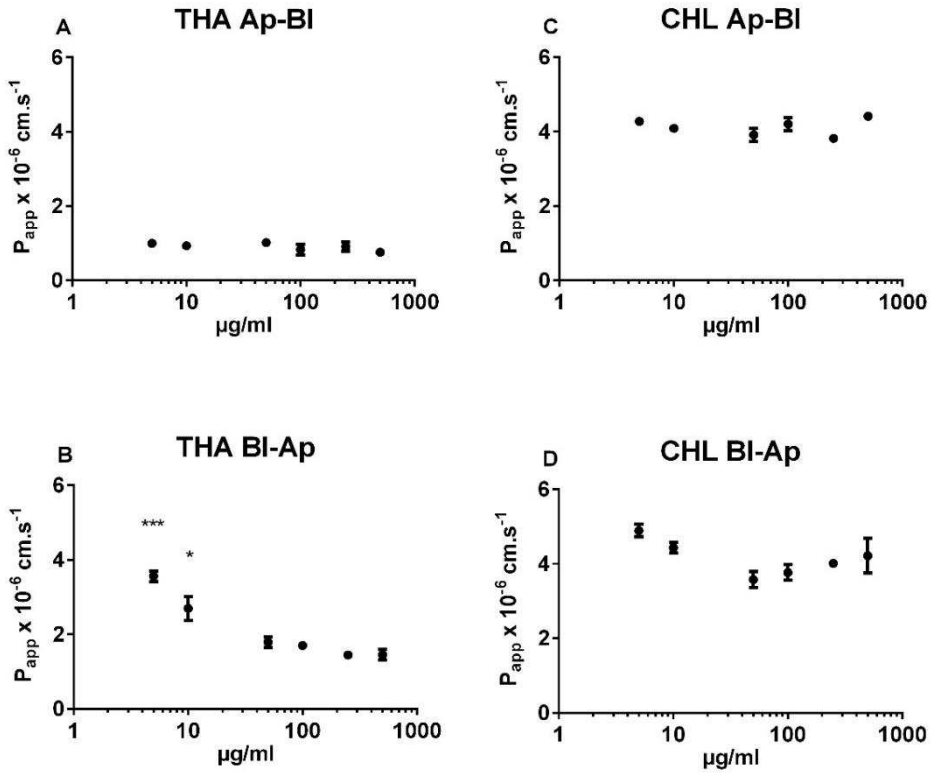

Figure 9. Perméabilité versus concentration du thiamphénicol ou du chloramphénicol dans les directions Ap-Bl et Bl-Ap à travers les cellules Calu-3. Moyenne ± erreur standard (n = 3). * P<0.05, ***P<0.001

Les résultats présentés sur la Figure 9 montrent deux éléments importants. Premièrement, les deux molécules ont des perméabilités différentes: celle du thiamphénicol (Papp ≈ $1x10^{-6}$ cm/s) est 4 fois inférieure à celle du chloramphénicol (Papp ≈ $4x10^{-6}$ cm/s) dans le sens Ap-Bl. Ceci s'explique par une plus grande lipophilicité du chloramphénicol par rapport au thiamphénicol (LogP = 1.15 pour chloramphénicol et 0.33 pour thiamphénicol, d'après le

logiciel ALOGPS 2.1). Deuxièmement, les résultats montrent que la perméabilité du thiamphénicol est dépendante de la concentration, associée à un phénomène de saturation dans la direction Bl-Ap à partir de 100 µg/ml environ (Figure 9B). Ce qui suggère l'effet d'un transporteur actif. Dans le cas du chloramphénicol, cet effet est moins important, avec des résultats non statistiquement significatifs (Figure 9D). L'effet d'un transport actif pour le chloramphénicol est peut-être observable à des concentrations plus faibles. Cependant, les limites de la méthode analytique ne permettent pas de mesurer la perméabilité pour des concentrations inférieures à 5 µg/ml.

Afin de mieux caractériser les transporteurs impliqués, l'épithélium de cellules Calu-3 ont été co-incubées avec des inhibiteurs spécifiques, tels que le PSC-833 (P-gp), MK-571 (MRP1) et KO-143 (BCRP), pendant les études de transport du chloramphénicol ou du thiamphénicol.

D'après la Figure 10 le PSC-833 diminue significativement la perméabilité Bl-Ap du thiamphénicol et du chloramphénicol, ce qui confirme le rôle de transporteurs tels que la P-gp. Cependant, dans le cas du thiamphénicol, les autres inhibiteurs ont aussi un effet sur la perméabilité, ce qui suggère l'implication des MRP1 et BCRP. Des études complémentaires d'uptake, utilisant des substrats spécifiques, telles que la rhodamine123 (P-gp) et la fluorescéine (MRP1), n'ont pas permis de clarifier les rôles respectifs des différents transporteurs.

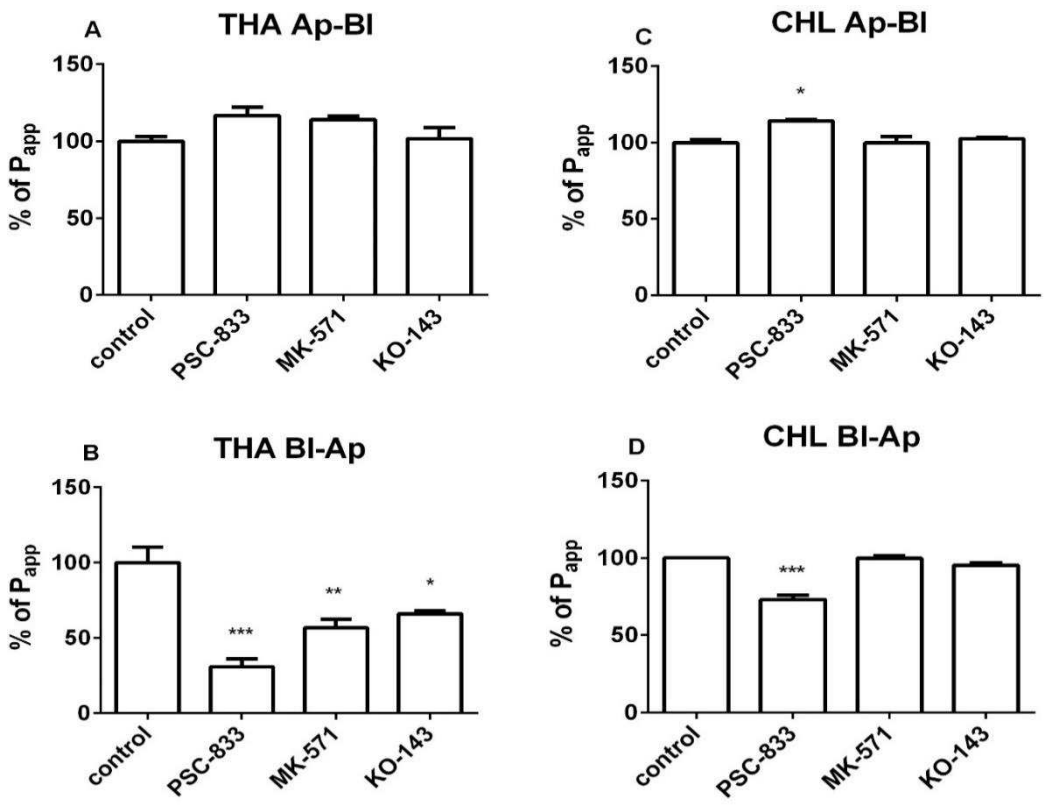

Figure 10. Papp du thiamphénicol ou du chloramphénicol dans les directions Ap-Bl et Bl-Ap à travers les cellules Calu-3, en absence ou en présence 3 µM PSC-833, MK-571 ou KO-143. Moyenne ± erreur standard (n = 3). * P<0.05, ***P<0.001

En conclusion, les études de transport ont démontré que la perméabilité du chloramphénicol était de type élevé, comparable à celle des fluoroquinolones telles que la moxifloxacine. Dans ce cas, une diffusion rapide du

chloramphénicol dans les poumons est attendue, quelle que soit la voie d'administration. Ainsi aucun avantage de l'administration par la voie pulmonaire n'est attendu par rapport à une voie IV ou orale. Dans le cas du thiamphénicol, les valeurs de perméabilité sont de type intermédiaire, proche de celle de la ciprofloxacine ou de la norfloxacine, ce qui peut conférer à l'administration pulmonaire un avantage par rapport aux voie IV ou orale. Même si l'identité des pompes d'efflux reste à découvrir, le rôle de ces transporteurs a été démontré. Ainsi, pour le chloramphénicol et le thiamphénicol, des concentrations plus élevées dans le compartiment pulmonaire que dans le compartiment sanguin sont attendues in vivo.

Article number 1

ACTIVE MEDIATED TRANSPORT OF CHLORAMPHENICOL AND THIAMPHENICOL IN A CALU-3 LUNG EPITHELIAL CELL MODEL

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Keywords: pulmonary delivery/absorption, cell culture, ABC transporters, permeability

ABSTRACT

Pulmonary administration enables high local concentrations along with limited systemic side-effects, but not all ATB could be good candidates. In this perspective, diffusion of the ATB chloramphenicol (CHL) and thiamphenicol (THA) through the lung has been evaluated to re-assess their potential for pulmonary administration. The apparent permeability (Papp) was evaluated

with the Calu-3 cell model. Influence of drug transporters was assessed with the PSC-833, MK-571, and KO-143 inhibitors. The influence of CHL and THA on the cell uptake of rhodamin123 and fluorescein was also evaluated. Absorptive Papp of CHL and THA were concentration independent with CHL Papp 4 times higher than that of THA. Secretory Papp of CHL was concentration independent while it was concentration dependent for THA with an efflux ratio of 3.6 for the lowest concentration. The use of inhibitors confirmed that CHL and THA are substrates of efflux transporters, but with a low affinity. In conclusion, the permeability results suggest that the pulmonary route may offer a biopharmaceutical advantage only for THA. Due to the influence of drug transporters, a higher concentration in the lung than in the plasma is expected mostly for THA, whatever the route of administration.

INTRODUCTION

The rapid emergence of resistant bacteria is a complex and major threat, endangering the efficacy of antibiotics (ATB). The development of new ATB is urgently needed but has been almost abandoned by the industry due to reduced economic incentives. Therefore, old forgotten ATB constitute a valuable alternative to eradicate emerging bacteria becoming resistant to most of the currently used ATB [1]. The use of such ATB had been limited mainly because of lower tolerability, compared to the new generations of ATB. However, by choosing the best dosing regimen and/or route of administration,

side effects could be limited, and a new therapeutic interest could be given to these old drugs. This is particularly obvious in the case of lung infectious diseases where the pulmonary route of administration could give a real advantage for the ATB treatments where high local concentrations could be achieved. Sub-optimal exposure, that favors the development of resistances, could be then avoided along with a limited systemic concentration and decreased side effects. In this perspective, the re-use of CHL has been gaining interest for the treatment of lung infectious diseases. CHL is an old broad-spectrum ATB discovered in 1947 and effective against many Gram-positive and Gram-negative bacteria [2]. Its activity is high against methicillin-resistant Staphylococcus aureus, fair against Klebsiella pneumoniae or Streptococcus pneumonia and poor against Acinetobacter baumannii or Pseudomonas aeruginosa [3,4].Due to its effectiveness, availability and low price, it is still frequently used in the developing world. Regarding the toxicity, dose related, and reversible bone marrow suppression is the most common. Another toxic effect, rare but fatal, is aplastic anemia which occurs in 1 in 25 000 - 40 000 patients [5]. Due to these adverse effects, CHL is rather recommended for second-line treatments. THA is a methane-sulfonate derivative of CHL, with a comparable antimicrobial spectrum and activity. It has been widely used in Europe and Japan, especially for respiratory tract infections [6]. THA has been also associated with reversible bone marrow suppression but never with fatal aplastic anemia. With the lack of new ATB, phenicol drugs should be re-evaluated, especially in the case of lung infections where the pulmonary route of administration could lead to high local concentrations and limited adverse

effects. However, the pulmonary route is to be chosen only for drugs with physico-chemical properties that favor a longer residence time and/or a higher concentration in the lung than in in the plasma compartment. The main factor that affects the drug absorption from lung to plasma is the epithelial cell permeability. Several in vivo studies have shown a higher concentration of ATB in the lung compartment after pulmonary administration for drugs with a low membrane permeability such as colistin or aztreonam [7-9]. For higher permeability drugs such as fluoroquinolones, in vivo studies have demonstrated that their concentrations equilibrated quickly between the lung and plasma compartments whatever the route of administration, suggesting a limited advantage of the pulmonary route [10]. A second factor that affects drug concentrations in the lung is the presence of drug transporters at the broncho-alveolar epithelium. Indeed, several ABC transporters (P-gp, MRPs and BCRP) have been reported to be expressed in lung tissue and ATB approved by the FDA for inhalation, such as fluoroquinolones or aztreonam, have been shown to be substrate of such drug transporters [11]. If ATB are substrates of efflux transporters, different concentrations are expected in the lung compartment and in the plasma. As evidences on the drug transporter presence in the lung are raising, a better understanding of their contribution in the drug distribution is needed [12]. In this regard, information about permeability and efflux transport for CHL and THA do not exist to date in the literature, with the last studies dating mostly from the early 80's. Knowledge about ATB membrane permeability is necessary in order to choose the best route of administration in the case of lung infection treatment or to design a suitable

formulation for the pulmonary administration. The objective of this study was to assess the permeability, drug uptake and efflux transport of CHL and THA. The in vitro Calu-3 cell model was chosen to perform these experiments as it is a well-established model for drug transport, where expression of the main drug transporters, such as P-gp, MRPs and BCRP have been demonstrated [11,13].

MATERIALS AND METHODS

Chemicals

THA (98% pure), CHL (99% pure), dimethyl sulfoxide (DMSO), MK-571, KO-143, Triton X-100, sodium fluorescein and rhodamin123 were purchased from Sigma-Aldrich. PSC-833 was kindly supplied by Novartis (Basel, Switzerland). Hanks' balanced salt solution (HBSS) and phosphate-buffered saline (PBS) pH 7.4, sodium bicarbonate, Dulbecco's modified Eagle's medium (DMEM)-F12, fetal bovine serum (FBS), HEPES buffer, were supplied from PAN Biotech GmbH (Aidenbach, Germany). Transwell® clear polyester membranes with a 1.12-cm^2 area and a pore size of 0.4 µm were obtained from Corning Costar (NY). Nunclon Delta Surface 96-well plates were supplied from Thermo Fisher Scientific (Roskilde, Denmark). All other reagents were of analytical grade.

Calu-3 cell culture

The Calu-3 cells were purchased from the American Type Culture Collection (ATCC® HTB55™, Rockville, MD). The cells between passages 41 and 60 were cultured in DMEM-F12 medium with 2.2 g/l bicarbonate and supplemented with L-glutamine (2 mM), 10% (vol/vol) FBS. The cells were seeded at a density of 15 x10^4 cells/well into Transwell® inserts (12 well plates, 12 mm diameter inserts, 0.4 µm pore size, tissue culture treated, polyester membrane) with a volume of 0.5 ml medium in the apical (Ap) compartment and of 1 ml in the basolateral (Bl) compartment. Cells were then cultured under air-interface conditions at 37℃ in air with 5% CO_2 and 90-95% relative humidity, for 15 days before experiment with the renewal of the basal compartment with 1.5 ml of medium every other day.

Time effect

Solutions of 10 and 500 µg/ml of CHL or THA were prepared in transport medium (TM: HBSS supplemented with 10 mM HEPES buffer). The flux of drug through the Calu-3 cell monolayer was evaluated in apical-to-basolateral (Ap-Bl) and basolateral-to-apical (Bl-Ap) directions. On the day of the experiment, inserts with cells were first washed 3 times 10 min with TM in both compartments. Then the acceptor compartment was filled with TM (1.5 ml for Bl compartment or 0.5 ml for Ap compartment). Solution of CHL or THA was added in the donor compartment (1.5 ml for Bl compartment or 0.5 ml for Ap compartment) and the cells returned to the incubator. At 60, 120 and 180 min,

150µl from the acceptor compartment was sampled and replaced with the same volume of TM. The samples were stored at -80℃ until analysis. In order to check for the integrity of the Calu-3 cell barrier, the transport of fluorescein (FLU) was carried out at the end of the experiments in Ap-Bl direction. The Calu-3 cells were rinsed once with TM in both compartments and the cells were incubated with TM in the Bl compartment and 10 µg/ml of sodium FLU in TM in the Ap compartment. Samples were collected after 60 min in the incubator and the concentration of FLU was evaluated with a fluorescent plate reader (TECAN Infinite 200 pro, Männedorf, Switzerland) and the apparent permeability (Papp) value for FLU was calculated. A threshold Papp value of 0.7×10^{-6} $cm \cdot s^{-1}$ was retained for the tight junction integrity rejection parameter for all experiments. This corresponds to the transfer of <0.5% of the initial amount in the apical compartment (Brillault et al., 2010).

Concentration effect

The experiments were conducted as described above with the following modifications: transport experiments were evaluated with 5, 10, 50, 100, 250, and 500 µg/ml concentrations for CHL and THA. Only one sample was collected after 60 min since time experiments demonstrated linearity over 180 min and samples were stored at -80℃ until analysis. The Papp (cm/s) was calculated using Eq. (1) where Q (µg) is the amount of drug in the acceptor compartment after a time Δt (s), S is the insert membrane surface (1.12 cm^2) and [Co] is the initial drug concentration in the donor compartment (µg/ml).

$$Papp = \frac{Q}{[Co].\Delta t.S}$$ Eq. (1)

Inhibition studies

Stock solutions of 300 µM PSC-833, MK-571 and KO-143 were prepared in DMSO and stored at -20℃. Cells were first equilibrated and rinsed two times with TM for 10 min each and then rinsed a third time with TM in presence of inhibitors in both compartments. Transport experiments were then realized as described above with 10 µg/ml of CHL, THA, FLU or rhodamin123 (RHO) in the donor compartment and in the presence of 3 µM inhibitors in both compartments. Control experiments without inhibitors were done in the TM with 1% DMSO. Cells were incubated for 60 min and samples were collected in the acceptor compartment. The samples were stored at -80℃ until analysis.

Uptake experiments

The uptake of FLU or RHO in Calu-3 cells was evaluated in presence of inhibitors, CHL or THA. The cells were seeded in 96-well plates at a density of 2 x10^4 per well and incubated with culture medium for 5 days. On the day of the experiment, the cells were first washed once with TM for 10 min and then incubated with 100µL of 10µg/ml FLU or RHO in presence of PSC-833, MK-571, KO-143, CHL or THA. The concentrations were 8 serial dilutions starting from 700 µM for CHL and THA and starting from 3 µM for PSC-833, MK-571 and KO-143. Preliminary experiments showed that an equilibrium in the uptake of FLU or RHO was reached after 90 min (data not shown). After this incubation time, the medium was aspirated, and the cells were rinsed six times

rapidly with ice-cold PBS. The cells were then lysed with 100µl of 1% triton in PBS for 30 min. The amount of FLU and RHO was determined by fluorescence (490/572 and 425/540 nm, respectively) with the TECAN plate reader. For control, cells were incubated with 10 µg/ml of FLU or RHO in TM with 1% DMSO. Data were analysed with the following equation (eqn 2).

$$E(X) = Emax - \frac{(Emax - E0)}{1+10^{(logIC50 - X)}} \qquad \text{Eq. (2)}$$

where E(X) is the uptake of FLU or RHO at a concentration X, [X] is the log of the inhibitor concentration, E_{max} is the maximum uptake of FLU or RHO by the cells, E_0 is the uptake of FLU or RHO in the absence of inhibitors and was normalized at 100%, IC50 is the inhibitor concentration at which E([IC50])= (E_{max} – E_0)/2 .

Analytical assays

Analysis of CHL and THA were conducted by LC-MS/MS using an Alliance 2695 system (Waters, Saint-Quentin En Yvelines, France) coupled with a Quattro Micro API mass spectrometer (Waters, Saint-Quentin En Yvelines, France) and Masslynx version 4 software. CHL and THA chromatographic separations were performed with an X bridge C18 column (5.0 µm, 150 x 2.1 mm ID, Waters, St-Quentin en Yvelines, France). The mobile phase consisted of water and acetonitrile (60:40, v/v) with 0.1% formic acid using isocratic elution. The flow rate was 0.2 ml/min and the injection volume was between 30-50 µl. The run time analysis was 5 min and divert valve was used to divert the eluent to waste from 0 to 1.5 min, to MS from 4.5 to 5.0 min. For CHL

determination, the negative mode was used with Multiple-Reactions-Monitoring (MRM) in the mass analyzers. The MRM transitions were m/z 321/152.1 for CHL and 326/157.1 for internal standard (CHL deuterium 5). CHL serial calibration standards ranged from 10 to 5000 ng/ml and QC sample concentrations were 25, 100, 250, 500, 3750 ng/ml. For THA determination, the positive mode was used with Multiple-Reactions-Monitoring (MRM) in the mass analyzers. The MRM transitions were m/z 356.1/338 for THA and 323.2/305 for internal standard (CHL). THA serial calibration standards were ranged from 5 to 1000 ng/ml and QC sample concentrations were 20, 50, 100, 750 ng/ml.

Data Analysis

The statistical evaluation of the data was performed using GraphPad Prism, version 6.01, for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com

RESULTS

Time effect. The amount of CHL or THA in the acceptor compartment of the Calu-3 model increased linearly over time and up to 180 min suggesting an absence of toxicity or saturation phenomenon during this time window (Fig. 1). For CHL, the flux rate was similar in both directions and at both concentrations. For THA, the Bl-Ap flux rate was 3 times higher than in the Ap-Bl direction for

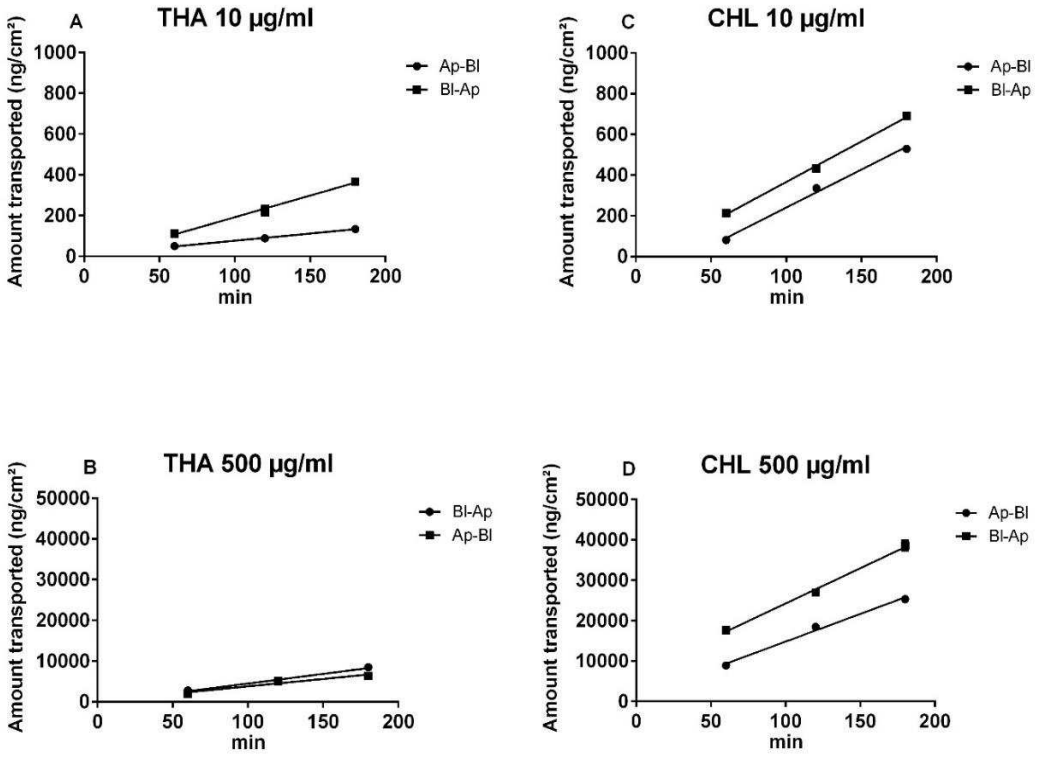

Figure 1. Amounts of CHL or THA transported across Calu-3 cell monolayers over time. The concentration in the donor compartment was 10 µg/ml (A,C) or 500 µg/ml (B,D). Data are expressed as means ± SEM (n = 3).

the 10 µg/ml concentration (2.12 ± 0.20 and 0.70 ± 0.04 ng/cm^2/min) and the flux rates were similar in both directions for the 500 µg/ml. These results suggested an asymmetrical transport of THA for the lowest concentrations.

Concentration effect. Transport experiments were performed at different concentrations and the Papp of the cell monolayer for CHL or THA was evaluated. In the Ap-Bl direction, Papp of CHL was independent of the concentration (Fig. 2) with Papp values close to 4 x 10^{-6} cm/s. In the Bl-Ap direction for CHL, no significant effect of the concentration was observed, and the Papp values were also close to 4 x 10^{-6} cm/s (Table 1). For THA, in the Ap-Bl direction Papp was independent of the concentration (Fig. 2) and Papp values were close to 1 x 10^{-6} cm/s (Table 1). In the Bl-Ap direction, the Papp for THA was concentration dependent with a Papp 2.5 times higher for the lowest concentration than for the highest (Fig 2). Efflux ratio between Ap-Bl and Bl-Ap directions were around 1 for CHL and between 1.8 and 3.6 for THA (Table 1).

Inhibition studies. In the Ap-Bl direction, the presence of the inhibitors did not affect the Papp for CHL or THA, except for CHL where PSC-833 had a limited (increased to 114 % of its initial value) but statistically significant effect (Fig. 3 A, C). In the Bl-Ap direction, for CHL, only PSC-833 had a significant effect by decreasing the Papp down to 73% of its initial value. For THA, in the Bl-Ap direction, Papp values were decreased significantly down to 31%, 57% and 66% of the control in the presence of PSC-833, MK-571 and KO-143, respectively (Fig. 3 B, D). As a comparison, Papp for FLU and RHO were

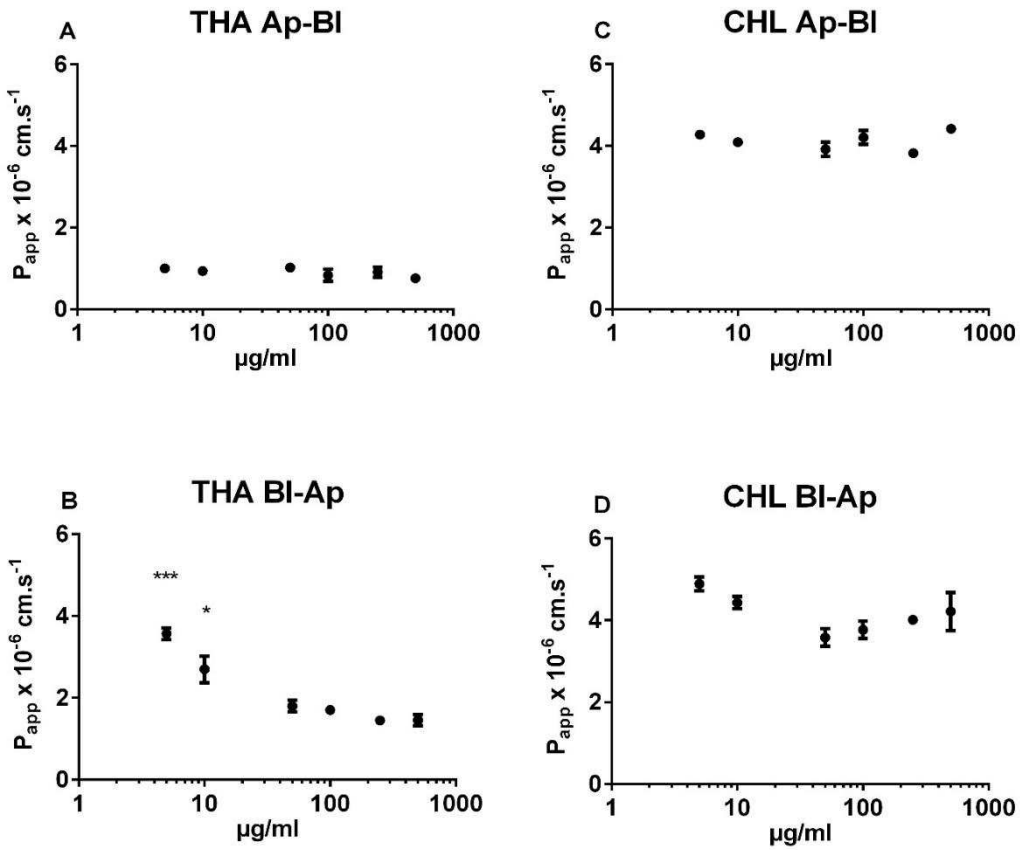

Figure 2. THA or CHL Papp versus concentration in the absorptive (Ap-Bl) or secretory (Bl-Ap) directions across Calu-3 cell monolayers. Data are expressed as means ± SEM (n = 3). One-way ANOVA and Bonferroni's post hoc test were used to evaluate the effect of each concentration compared to the highest 500 µg/ml concentration (* $P<0.05$, ***$P<0.001$).

determined in absence or presence of inhibitors. For the control conditions, efflux ratios of FLU and RHO were 0.7 and 2.1, respectively (Table 2). In the presence of inhibitors, only PSC-833 had a significant effect on Bl-Ap RHO Papp. No significant differences were obtained for FLU Papp in presence of inhibitors.

Uptake experiments. FLU uptake by the Calu-3 cells was increased in the presence of PSC-833 or MK-571 and not KO-143. Estimated IC50 were 0.08 and 0.25 µM for PSC-833 and MK-571, respectively. IC50 for CHL and THA were estimated at 46.85 and 74.71 µM, respectively. E_{max} of the inhibitors on FLU uptake was estimated at 125% for PSC-833, MK-571, CHL and 128% for THA (Fig. 4 A, C). RHO uptake was increased up to 241% in presence of 3 µM PSC-833 and was not affected by MK-571 or KO-143. Because PSC-833 had toxic effects at concentrations >3 µM, E_{max} and IC50 values could not be estimated. RHO uptake was not affected by the presence of CHL or THA even at concentrations up to 700 µM (Fig. 4 B, D). CHL or THA concentrations > 700 µM leaded to cell toxicity (i.e. cell toxicity was assumed since with these concentrations the cells detached from the bottom of the well during the washing step of the uptake protocol and fluorescence values were not interpretable).

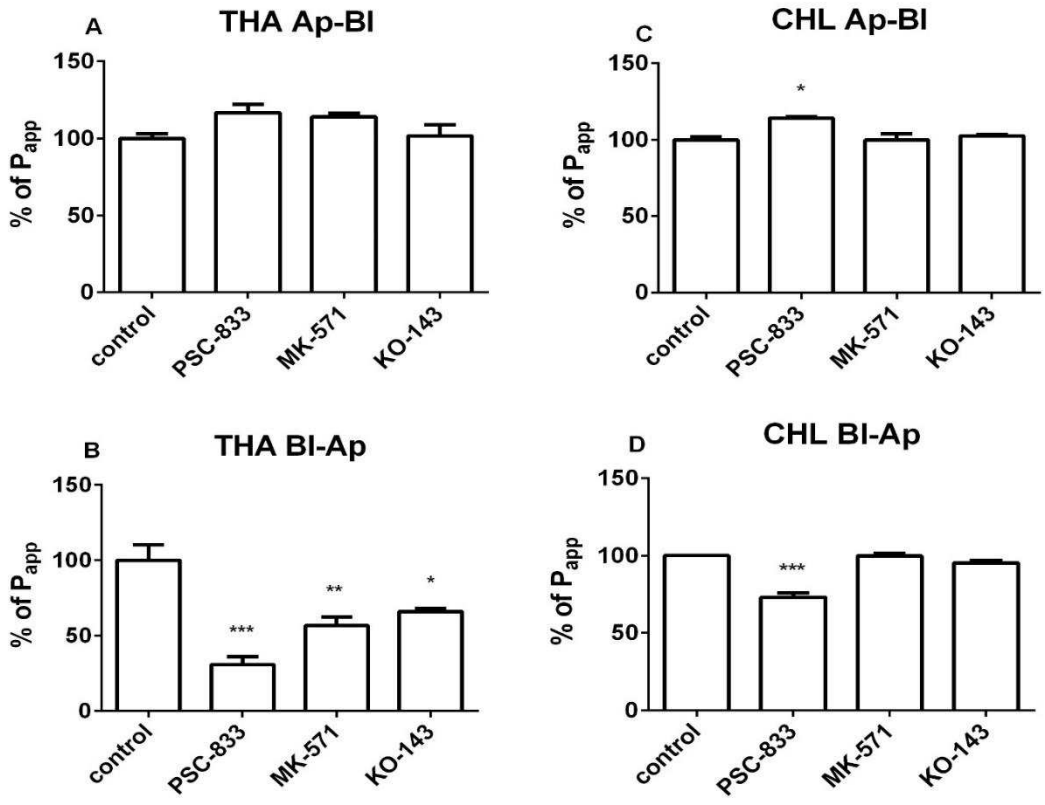

Figure 3. Absorptive (Ap-Bl) or secretory (Bl-Ap) Papp of THA or CHL across Calu-3 cell monolayers in the absence (control) or presence of 3 µM PSC-833, MK-571 or KO-143. The concentration of THA or CHL in the donor compartment was 10 µg/ml. Data are expressed as a percentage of control Papp and as means ± SEM (n = 3). One-way ANOVA and Bonferroni's post hoc test were used to compare drug treatments to control (* P<0.05, ** P<0.01, ***P<0.001).

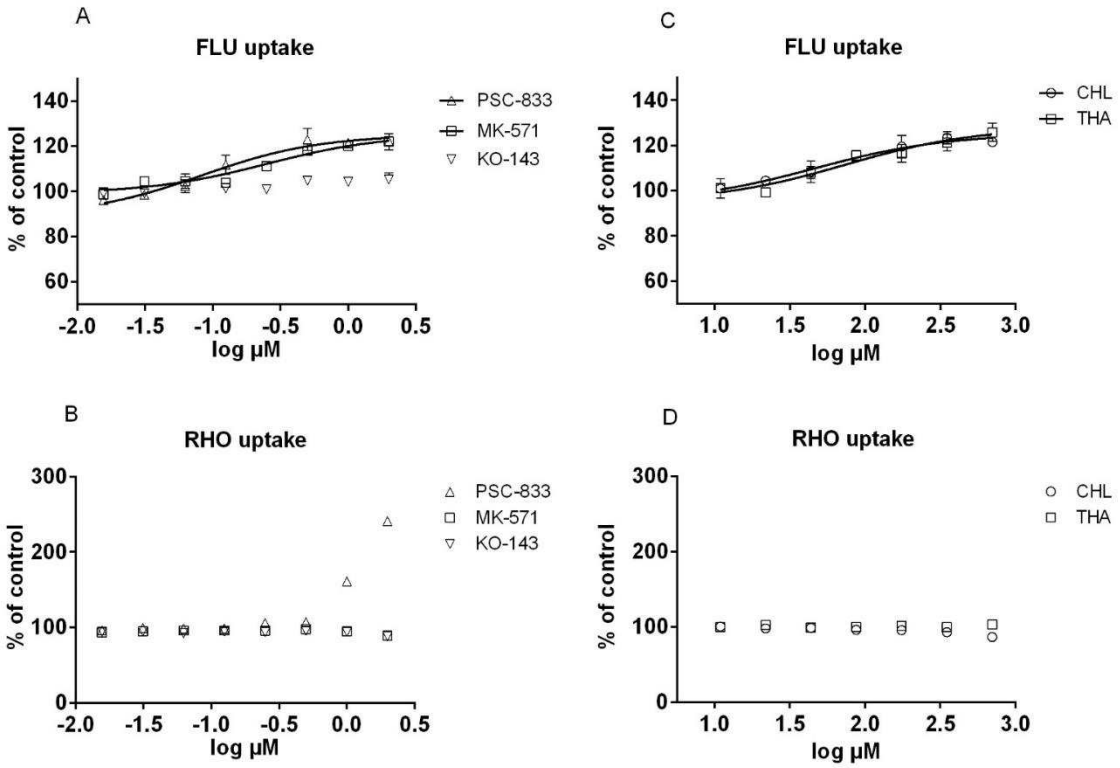

Figure 4. Uptake of 10 µg/ml FLU or RHO in the Calu-3 cells in presence of PSC-833, MK-571, KO-143 (A,B), THA or CHL (C, D). Data are expressed as a percentage of control (uptake in absence of inhibitors) and as means ± SEM (n = 6 of two different experiments).

DISCUSSION

The flux and permeability studies indicated that Papp for CHL was close to 4 x 10^{-6} cm/s in both directions (Table 1), which can be considered as a high Papp value. This is in accordance with its high lipophilicity reflected by its high Log P value (for instance, ALOGPS 2.1 program evaluated the log P of CHL at 1.15 [14]). Indeed, higher lipophilicity is generally associated with a faster cell membrane diffusion and then cell permeability. When Papp values are compared across ATB using the same Calu-3 model, CHL Papp compares with these of high permeability ATB and in particular fluoroquinolones such as moxifloxacin (MXF) or levofloxacin (LVX) (2.4 and 5 x 10^{-6} cm/s) [15]. Within the tested concentration range, no significant effect of concentration was observed in both directions and the efflux ratios were close to 1 (Table 1). Altogether these results suggest that CHL permeability is not dependent of efflux transporters. However, global permeability for a drug through the cells is the resultant of two components with opposite actions: efflux transport and diffusion. CHL may have a low affinity for the efflux transporters, along with a high lipophilicity and then rapid diffusion. Effect of the transporters on the Papp would be then mostly observed at low concentrations. Unfortunately, concentrations <5 µg/ml could not be tested in this experiment due to the limits of the analytical method. In an attempt to indirectly characterize the efflux transporters that may be involved, inhibitors have been used during transport experiments. For CHL, only PSC-833 had an effect on Papp, that appeared statistically significant although moderate, and suggesting that CHL is

substrate of the P-gp. However, PSC-833 is a potent inhibitor of the P-gp but is also known to have a moderate inhibitory effect on MRP1 [16]. For the uptake experiments, FLU and RHO were chosen as specific substrates for the MRP1 and P-gp efflux transporters, respectively [17]. In parallel, transport experiments were determined with FLU and RHO in absence or presence of inhibitors (Table 2). For RHO, uptake experiments (Fig. 4B) and transport experiments (Table 2) clearly show the active transport of RHO through the cell and the specific inhibitory effect of PSC-833. For FLU, the cell uptake was affected by the presence of both MK-571 and PSC-833, suggesting a lack of specificity of FLU as a substrate of MRP1 (Fig 4A). This effect was moderate (125%) when compared to the RHO uptake inhibition (241%) and may explain in part the absence of effect of the inhibitors on FLU Papp (Table 2). Altogether, these data may suggest that MRP1 is not the sole actor in the transport of FLU through the Calu-3 cells. CHL had an inhibitory effect on FLU uptake but to a lesser extent than for MK-571 or PSC-833. Indeed, IC50 for CHL were about 200 times higher than that for MK-571. CHL had no effect on the P-gp dependent uptake of RHO, suggesting that CHL is not substrate of P-gp or with a very low affinity compared to RHO. Finally, the uptake experiments failed to confirm the identity of the CHL efflux transporter.

Regarding THA, Papp in the Ap-Bl direction was close to 1 x 10^{-6} cm/s (Table 1). This lower Papp than that of CHL is in accordance with its lower lipophilicity than for CHL (as reflected by ALOGPS predicted log P = 0.33) and close to the Papp of other fluoroquinolones with relatively lower Papp, such as

ciprofloxacin (CIP) or norfloxacin (NOR) (0.6 and 0.7 x 10^{-6} cm/s, respectively), which can be considered as intermediate permeabilities [15]. Indeed, these Papp values are lower than the one of high permeability MXF and LVX, but still higher than the one of low permeability ATB such as colistin, aztreonam or tobramycin, for which Papp value are ranging between 0.04 - 0.07 x 10^{-6} cm/s [9,18,19]. In the secretory Bl-Ap direction, permeability for THA was concentration dependent, suggesting an efflux transport of THA. Due to the limit of quantification of the analytical method, lower concentrations of THA could not be tested, and parameters characteristic of a Michaelis-Menten model (Vm and Km) could not be estimated to characterize this efflux transport. However, a significant effect of transporters was demonstrated from 5 to 50 µg/ml. This corresponds probably to the therapeutic range, since in human studies, plasma Cmax of THA reached 5 and 27 µg/ml in healthy volunteers after oral and IV administration of 500 mg, respectively [20,21]. Thus, an efflux transport effect should be expected in patient lungs after IV or oral administration, and higher concentrations of THA in the lung compartment than in the plasma should be expected, whatever the route of administration. For example, in previous rat pharmacokinetics studies, MXF concentrations were always almost 10x higher in the lung compartment than in the plasma, due to the influence of drug transporters [10]. Although the extent of this difference for THA has to be determined in vivo, this is of importance for the use in lung infection since the higher local concentration would lead to better efficacy. In an attempt to characterize the efflux transporters, inhibitors have been used during THA transport experiments. Presence of such inhibitors decreased the secretory Bl-

Ap Papp, with PSC-833 having the most important effect (Fig 3B). The MRP-1 and BCRP inhibitors, namely MK-571 and KO-143, also significantly lowered the THA Papp but to a lesser extent. THA had an inhibitory effect on the FLU uptake. Yet, the IC50 of THA was about 300 times higher than that of MK-571, suggesting that THA is not as potent inhibitor as MK-571. Moreover, THA had no effect on the P-gp dependent transport of RHO, suggesting THA is not substrate of P-gp or with a very low affinity compared to RHO. As for CHL, the uptake experiments failed to confirm the identity of the THA efflux transporter.

As a conclusion, important differences have been shown regarding the Papp of CHL and THA, where CHL Papp was 4 times higher than that of THA. CHL is a high permeability compound while THA should be considered as an intermediate permeability compound when compared with fluoroquinolones. Regarding the efflux transport, the influence of efflux transporters in the Papp of CHL and THA has been confirmed mainly for THA. Unfortunately, the identity of these transporters is still not clear. Regarding the route of administration, no advantages of the pulmonary route should be expected for CHL, compared to the IV or oral route. On the other hand, the pulmonary administration of THA, considering its intermediate permeability properties, may be an advantage compared to IV or oral. In vivo experiments are currently under completion to further document the potential of THA for this route.

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TABLE 1. Efflux ratios and Papp for CHL or THA at different concentrations in the Calu-3 cell model.

		Papp 10^{-6} cm/s		
	µg/ml	Ap-Bl	Bl-Ap	ER
CHL	5	4.27 ± 0.05	4.89 ± 0.17	1.1
	50	3.91 ± 0.17	3.58 ± 0.22	0.9
	500	4.41 ± 0.08	4.22 ± 0.46	1.0
THA	5	0.99 ± 0.03	3.57 ± 0.14	3.6
	50	1.02 ± 0.10	1.80 ± 0.14	1.8
	500	0.76 ± 0.08	1.45 ± 0.14	1.9

ER: Bl-Ap to Ap-Bl Papp ratios. Data are expressed as means ± SEM (n = 3).

TABLE 2. Efflux ratios and Papp for FLU or RHO 10 µg/ml in presence of 3µM inhibitors.

		Papp 10^{-6} cm/s		
		Ap-Bl	Bl-Ap	ER
FLU	control	0.26 ± 0.01	0.17 ± 0.01	0.7
	PSC-833	0.28 ± 0.02	0.17 ± 0.01	0.6
	MK-571	0.28 ± 0.01	0.17 ± 0.01	0.6
	KO-143	0.28 ± 0.01	0.18 ± 0.01	0.6
RHO	control	2.52 ± 0.14	5.19 ± 0.08	2.1
	PSC-833	2.20 ± 0.02	3.21 ± 0.26***	1.5
	MK-571	2.52 ± 0.11	5.23 ± 0.13	2.4
	KO-143	2.55 ± 0.15	5.35 ± 0.23	2.1

ER: Bl-Ap to Ap-Bl Papp ratios. Data are expressed as means ± SEM (n = 3). Two-way ANOVA and Bonferroni's post hoc test were used to compare drug treatments to control for each direction (***P<0.001).

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CHAPTER 3

EXPERIMENTAL WORK

3.2 In vivo evaluation of lung permeability for chloramphenicol and thiamphenicol in rats

3.2 In vivo evaluation of lung permeability for chloramphenicol and thiamphenicol in rats

Briefly,

The aim of this second study was to determine pulmonary epithelial lining fluid pharmacokinetics of chloramphenicol and thiamphenicol. After intravenous bolus and intra-tracheal nebulization in rats (3 $mg.kg^{-1}$), blood was collected and broncho alveolar lavages were performed. No effect of route of administration was observed for chloramphenicol, whereas epithelial lining fluid over plasma area under the curve ratio was 467 times higher after nebulization than after intravenous administration for thiamphenicol.

Résumé développé en français:

Evaluation de la perméabilité pulmonaire du chloramphénicol et du thiamphénicol in vivo chez le rat.

Le but de ces travaux est d'évaluer la pharmacocinétique du chloramphénicol ou du thiamphénicol dans le liquide épithélial pulmonaire (ELF) et dans le plasma après administration intra-trachéale et intraveineuse (IV) chez des rats mâles Sprague-Dawley (250-350 g). L'administration IV de chloramphénicol ou thiamphénicol a été réalisée à 1 mg/ml dans du sérum physiologique (0.9 % NaCl). L'injection de 1 ml a été réalisée dans l'artère caudale en bolus. Dans le cas de l'administration intra-trachéale, pour des raisons de solubilité, 10 mg de chloramphénicol ou de thiamphénicol ont été dissous dans 1 ml de sérum

physiologique en présence de 60 mg d'hydroxypropyl-β-cyclodextrine. 100 µl de cette solution a ensuite été nébulisée avec le microsprayer 1A-1B (Penn-Century, Wyndmoor, USA) dans la trachée. Les échantillons de sang ont été prélevés dans le cœur. Les échantillons d'ELF ont été récupérés après lavage broncho-alvéolaire (LBA) : pour cela, 1 ml de sérum physiologique est administré dans la trachée puis immédiatement ré-aspiré. La dilution de l'ELF apportée par la solution de lavage est corrigée par dosage de l'urée. Les LBA et les prélèvements de sang ont été effectués à 0,25, 0,5, 1, 2, 3 h pour le chloramphénicol et à 0,25, 0,5, 1, 3, 4 h pour le thiamphénicol. Ensuite, les concentrations de chloramphénicol ou de thiamphénicol ont été déterminées par LC-MS/MS. La liaison aux protéines plasmatiques a été évaluée par ultra-filtration (UF) (Centrifree, Millipore, Molsheim, France) avec du plasma de rat surchargé avec 0.1 et 1 µg/ml de chloramphénicol ou thiamphénicol, respectivement. La fraction libre ainsi évaluée in vitro est de 42.7 ± 0.5 % pour le chloramphénicol et de 90.9 ± 5.1 % pour le thiamphénicol.

Les résultats pharmacocinétiques ont montré que le chloramphénicol diffusait rapidement dans l'ELF après administration IV avec une concentration maximale de 3,2 ± 1,3 µg/ml à 0,25 h, puis diminuait en parallèle avec le profil plasmatique (Figure 11a), ce qui est typique des antibiotiques à forte perméabilité comme les fluoroquinolones. De plus, les concentrations dans l'ELF sont toujours supérieures à celles dans le plasma, suggérant l'intervention de transporteurs d'efflux. En effet, le ratio d'AUC ELF/plasma après administration IV est de 7.9. Enfin, l'évolution des concentrations

plasmatiques et ELF ont les mêmes profils que ce soit après administration IV ou intra-trachéale. Ce qui montre l'absence d'effet de la voie d'administration (Figure 11a et b). Dans le cas de thiamphénicol, les concentrations dans l'ELF augmentent plus progressivement après administration IV que pour le chloramphénicol (Figure 11c), ce qui suggère une plus faible perméabilité membranaire du thiamphénicol par rapport au chloramphénicol, comme observé précédemment in vitro (article 1). Par contre, le ratio d'AUC ELF/plasma après administration IV est de seulement 1.4, suggérant une faible contribution des transporteurs d'efflux. Comme déjà observé avec des antibiotiques de faible perméabilité, un effet de la voie d'administration est observé. En effet le ratio d'AUC ELF/plasma est plus élevé (659) après administration intra-trachéale qu'après administration IV (1.4). Cependant, les concentrations de thiamphénicol dans l'ELF ne sont supérieures à celles dans le plasma que pendant la première heure après administration (Figure 11d), tandis que pour d'autres antibiotiques à faible perméabilité (Aztreonam, tobramicyn, colistin), cet effet durait pendant toute la durée de l'étude (4 h) (Marchand et al., 2015 and 2016 ; Gontijo et al., 2014). La perméabilité du thiamphénicol peut ainsi être considérée comme intermédiaire entre celles des composés à forte et à faible perméabilité. En conclusion, dans le cas du traitement des infections pulmonaires, ces études pharmacocinétiques montrent que l'administration intra-trachéale, en comparaison de la voie IV, offrent un avantage biopharmaceutique modéré pour le thiamphénicol et pas pour le chloramphénicol.

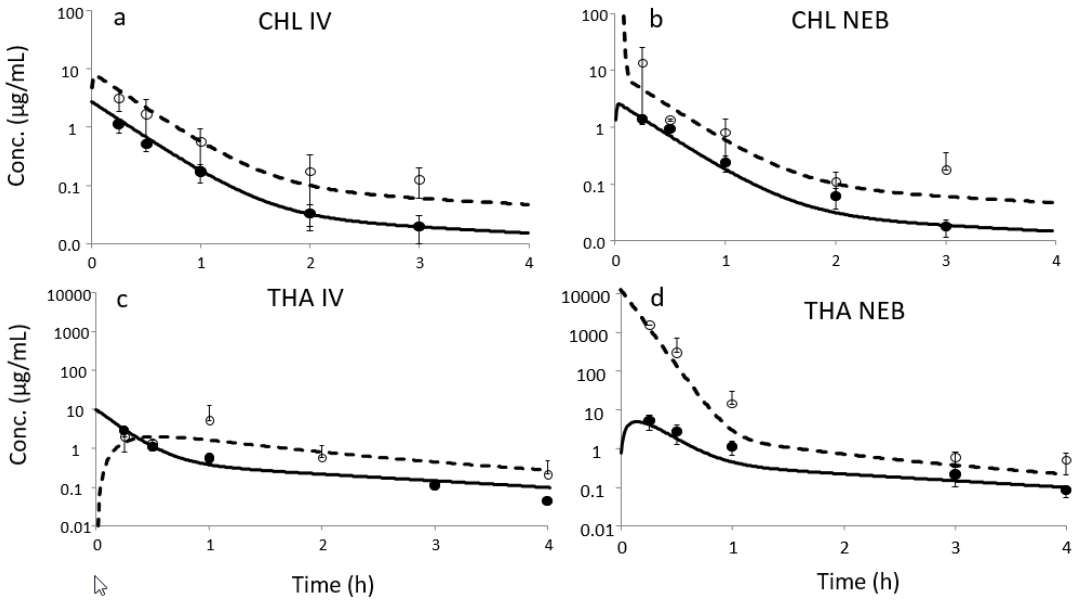

Figure 11. Profils pharmacocinétiques pour du chloramphénicol et du thiamphénicol (3 mg.kg-1) dans le plasma total (ligne pleine) et dans l'ELF (ligne pointillée) après IV (Figures 11a and 11c) et administration intra-trachéale (NEB) (Figures 11b and 11d). Les données sont représentées comme moyennes et écarts-types.

Article number 2

BIOPHARMACEUTICAL CHARACTERIZATION OF NEBULIZED ANTIMICROBIAL AGENTS IN RATS: 6. CHLORAMPHENICOL AND THIAMPHENICOL

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Running Title: Biopharmaceutical characterization of phenicols

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ABSTRACT

The aim of this study was to determine pulmonary epithelial lining fluid pharmacokinetics of chloramphenicol and thiamphenicol. After intravenous bolus and intra-tracheal nebulization in rats (3 $mg.kg^{-1}$), blood was collected and broncho alveolar lavages were performed. No effect of route of administration was observed for chloramphenicol, whereas epithelial lining fluid over plasma area under the curve ratio was 467 times higher after nebulization than after intravenous administration for thiamphenicol.

SHORT COMMUNICATION

Nebulization may improve the treatment of pulmonary infections by increasing antibiotic concentrations at the infection site and reducing systemic exposure and therefore toxicity, but not all antibiotics may be good candidates for nebulization. We have recently started a series of experiments in controlled and standardized experimental conditions in order to allow comparisons between compounds. This first set of experiments has demonstrated that antibiotics with low membrane permeability such as colistin (1), tobramycin (2) or aztreonam (3) present suitable characteristics for nebulization, in agreement with clinical practice. By contrast fluoroquinolones present higher membrane permeability and are therefore rapidly absorbed after lung delivery at least in healthy rats (4). Yet in this situation, appropriate formulation may solve the issue (5). The objective of this new study was to complete this initial series of

experiments, testing other antibiotics with various physico-chemical properties and therefore permeability characteristics. Chloramphenicol (CHL) and thiamphenicol (THA) were considered because their permeability could be intermediate between low and high membrane permeability but also because after being virtually forgotten for many years, they are still frequently active against multidrug resistant (MDR) bugs responsible for pulmonary infections (6). Therefore, reducing the systemic exposure of phenicol antibiotics would be of great potential benefit considering their systemic toxicity (7).

Solutions for nebulization (NEB) were prepared by mixing 10 mg of CHL or THA powder (Sigma-Aldrich, Saint Quentin Fallavier, France) with 60 mg of hydroxypropyl-β-cyclodextrin, (Kleptose®, Rocquette, Vecquemont France) (8). The mixture was dissolved into 1 mL of NaCl 0.9% under vortex for 1 min to obtain a final concentration of 10 $mg.mL^{-1}$ and then ultrasonicated (Advantage-Lab, Schilde, Belgium) over 30 min at room temperature before administration. Animal experiments were conducted in compliance with EC Directive 2010/63/EU and registered by the French Ministry of Higher Education and Research (n° 2015042116017243). Male Sprague Dawley (250 to 350 g) from Charles River Laboratories (Saint Germain Nuelles, France) were divided into 4 groups (n=23-30 per group). The two first groups received an IV bolus of CHL or THA at a dose of 3 $mg.kg^{-1}$ via the tail artery (solutions of 1 $mg.mL^{-1}$ in NaCl 0.9% and volume of injection closed to 1mL). The two others received the same dose via intra-tracheal NEB of 100µL of the10 $mg.mL^{-1}$ solution using microsprayer 1A-1B (Penn-Century, Wyndmoor, USA)

(9). Broncho alveolar lavages (BAL) and blood sampling were performed at 0.25 0.5, 1, 2, 3h post administration for CHL and at 0.25, 0.5, 1, 3 and 4h for THA. Epithelial lining fluid (ELF) antibiotic concentrations were derived from measured BAL concentrations after correction by urea (9). Plasma protein binding was estimated by ultra-filtration (UF) (Centrifree?, Millipore, Molsheim, France) using rat plasma spiked with CHL or THA at concentrations respectively equal to 0.1 µg.mL^{-1} and 1 µg.mL^{-1} (n=2) and after testing that no non-specific drug adsorption on membranes was observed. Biological samples of both molecules were analyzed by LC-MS/MS using an Alliance Waters 2695 HPLC system module coupled with a QUATTRO MICRO API mass spectrometer (Saint-Quentin-en-Yvelines, France). A mobile phase (water/ acetonitrile, 60/40, v/v with 0.1% formic acid) was delivered at 0.18 mL.min^{-1} in an XBridge BEH300 C18 column (5.0 µm, 150 x 2.1 mm ID, Waters, St-Quentin en Yvelines, France). Negative and positive modes were respectively used for CHL and THA for mass spectrometer with Multiple-Reactions-Monitoring (MRM) in the mass analyzers. The MRM transitions were m/z 321/152.1 for CHL and 326/157.1 for its internal standard (CHL-d5) and were m/z 356/308 and 356/338 for THA and 323/305 for its internal standard (CHL). Limits of quantification were respectively estimated at 1 ng.mL^{-1} in BAL for both molecules and at 5 and 10 ng.mL^{-1} in plasma for CHL and THA. The between-day variability was evaluated at three concentrations in each media and precision and bias were always less than 15% (n=4-9). Urea concentrations in plasma and BAL were measured as previously described (9).

The S-ADAPT software (10) was used for simultaneous analysis of unbound plasma and ELF concentrations versus time for each compound. CHL data were fitted with a two-compartment model for plasma concentrations with linear elimination from central compartment. One compartment was used to describe ELF concentrations and was connected to the central compartment by distribution clearance and by the addition of an efflux clearance from central to ELF compartment. For THA, two compartments were used to describe profiles in plasma with distribution clearance between compartments and two compartments with distinct volumes to describe lung with first order distribution constants between compartments. Only unbound drugs were assumed to distribute into lung compartments and systemic bioavailability after nebulization was fixed at its maximum value (100%) (2). Areas under unbound plasma concentrations and ELF concentrations versus time-curves from 0 to infinity ($AUC_{u,plasma}$, AUC_{ELF}) were calculated from the model (Berkeley Madonna, version 8.3.18, University of California).

In vitro unbound fractions were estimated at 42.7 ± 0.5 % for CHL in accordance with literature (11) and at 90.9 ± 5.1 % for THA. After IV administration of CHL, distribution within ELF was rapid with a maximum concentration (3.2 ± 1.3 $\mu g.mL^{-1}$) observed at 0.25 h after administration, and then ELF concentrations profile decreased in parallel with plasma profile (Fig. 1a), as typically observed with highly permeable antibiotics such as fluoroquinolones (4). Noticeably also, ELF concentrations were always higher than corresponding unbound plasma concentrations with an ELF over plasma

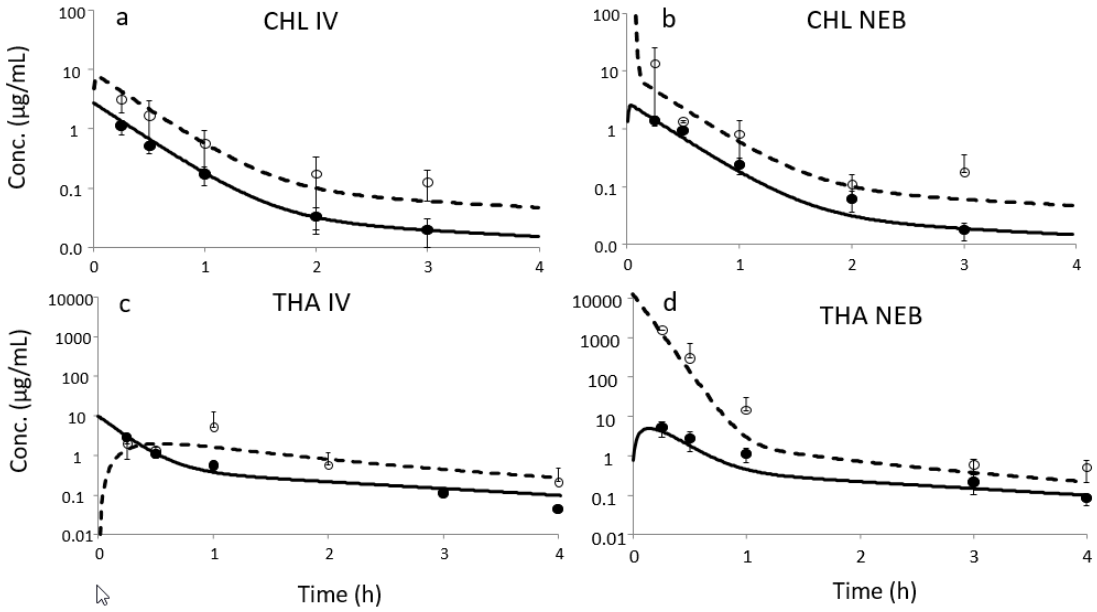

FIG. 1. Predicted concentration-time profiles of CHL and THA (3 mg.kg^{-1}) in total plasma (solid line) and in ELF (dashed line) from simultaneous PK modeling after IV (Fig 1a and 1c) and NEB administrations (Fig 1b and 1d). Closed and open symbols represent respectively mean ± SD of experimental concentrations in total plasma and in ELF.

unbound AUC ratio higher than unity ($AUC_{ELF,IV,}/AUC_{u,plasma,\ IV=}$ 7.9) suggesting that CHL is substrate of an efflux transporter, as previously observed with moxifloxacin (4). Lastly, CHL ELF and plasma concentrations profiles presented the same general shape after IV and NEB, demonstrating a lack of effect of the route of administration (Fig. 1a and 1b), which is another characteristics of highly permeable compounds (4). THA data look quite different with ELF concentrations increasing more progressively after IV administration as previously observed with low membrane permeability antibiotics (2, 3). The ELF over unbound plasma AUC ratio after IV ($AUC_{ELF,IV}$ / $AUC_{u,plasma,IV}$) was close to one (1.4) suggesting modest if any active efflux. As already seen with low permeability antibiotics, an effect of the route of administration on ELF PK of THA was observed, with higher ELF concentrations after NEB than after IV administration (Fig 1c, 1d) corresponding to an AUC_{ELF} 513 fold higher after NEB than after IV administration. This effect of the route of administration may be further evidenced by the higher ELF over unbound plasma AUC ratio after NEB ($AUC_{ELF,NEB}/AUC_{u,plasma,NEB}$ = 659) than after IV administration ($AUC_{ELF,IV}$ / $AUC_{u,plasma,IV}$ = 1.4). However with previously investigated low permeability antibiotics (colistin, tobramycin and aztreonam), after NEB ELF concentrations were much higher than plasma concentrations for the whole study duration (4h) and both decayed in parallel, whereas for THA, only concentrations measured during the first hour post nebulization were much higher in ELF than in plasma. These data suggest that THA permeability at the broncho-alveolar barrier should be intermediate between that of high and low permeability

compounds, which is presently investigated in in vitro experiments. Yet permeability is not the only issue and dissolution characteristics may also determine drug pharmacokinetics after nebulization. Therefore, it may not be excluded that solubilization of CHL and THA with hydroxypropyl-β-cyclodextrin may have had an impact on their apparent permeability characteristics.

These results demonstrated that THA nebulization offered a biopharmaceutical advantage compared to IV administration for the treatment of pulmonary infections, which was not observed for CHL.

ACKNOWLEDGEMENTS

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CHAPTER 3

EXPERIMENTAL WORK

3.3 Sustained release dry powder formulations of chloramphenicol or thiamphenicol prodrugs for lung delivery as aerosols

3.3 Sustained release dry powder formulations of chloramphenicol or thiamphenicol prodrugs for lung delivery as aerosols

Briefly,

The purpose of this study was to design inhalable sustained-release nanoparticle-in-microspheres for the lung delivery of chloramphenicol or thiamphenicol as aerosols, in order to optimize therapeutic efficiency against pulmonary bacterial infections. The palmitate ester prodrugs of the two antibiotics were used as they were expected to be efficiently entrapped within PLGA nanoparticles or to form pure prodrug nanoparticles. Prodrug-loaded PLGA nanoparticles or pure prodrug nanoparticles were prepared using the emulsion-solvent evaporation method. Dry microsphere powders for inhalation were then produced by spray-drying the nanoparticle suspensions supplemented with lactose as a bulking agent and leucin as a dispersing enhancer. The obtained microspheres appeared to be shriveled by scanning electron microscopy, with no presence of crystalline structures. Drug loading was satisfactory (14 to 34 % (m/m)) and the aerodynamic properties determined with a Next Generation Impactor were appropriate for lung delivery, with MMAD values close to 3µm. The in vitro release profiles showed that sustained released was achieved with these formulation, with a longer release time for the powders prepared from the pure prodrug nanoparticles (14 days at least) than from the prodrug-loaded PLGA nanoparticles (8 to 10 days).

Keywords: chloramphenicol, thiamphenicol, aerosol, lung delivery, antimicrobial, prodrug, palmitate, PLGA.

Résumé développé en français:

Formulations à libération prolongée de promédicaments de chloramphénicol ou thiamphénicol pour administration pulmonaire sous forme d'aérosols

En accord avec les études de perméabilité réalisées in vitro sur le modèle d'épithélium broncho-pulmonaire Calu-3, les études pharmacocinétiques réalisées chez le rat ont montré que l'administration intra-trachéale de chloramphénicol ou de thiamphénicol chez le rat n'avait pas ou peu d'impact significatif sur l'exposition pulmonaire par rapport à l'administration intraveineuse (iv). Par conséquent, afin de maintenir une concentration pulmonaire en chloramphénicol ou en thiamphénicol efficace sur une période de temps prolongée, tout en minimisant l'exposition systémique, il est nécessaire de concevoir des formulations à libération prolongée administrées par voie inhalée et déposées dans les poumons afin de libérer localement au plus près du site d'infection les antibiotiques. En effet, des études antérieures ont montré que des formulations à base de microsphères de PLGA administrées par voie intratrachéale permettaient de maintenir pendant plusieurs jours des concentrations de rifampicine ou de lévofloxacine élevées dans le fluide épithélial pulmonaire, alors même que les concentrations

plasmatiques, par conséquent l'exposition systémique à ces antibiotiques, étaient beaucoup plus faibles (Gaspar et al., 2016, Doan et al., 2013).

Au cours d'études préliminaires, la production de microsphères de PLGA de chloramphénicol ou de thiamphénicol basée sur la méthode d'évaporation de solvant conduisait à une teneur insuffisante en antibiotique (de 1 à 5% (w/w). Gómez-Gaete et al. (2008) ont montré que l'utilisation d'un promédicament, l'ester acétate de dexaméthasone, a permis d'augmenter la teneur des nanoparticules par rapport à la dexaméthasone. Ainsi, dans ce travail, les esters palmitate de chloramphénicol ou de thiamphénicol ont été formulés dans des nanoparticules avec ou sans poly (D, L-lactide-co-glycolide) (PLGA) (1:1) et produites par la méthode d'évaporation de solvant en émulsion. Les nanoparticules ont ensuite été agrégées par spray-drying en présence de lactose, excipient de charge, et de leucine, agent de dispersion, afin d'obtenir une poudre de microsphères aux propriétés aérodynamiques adéquates pour l'administration pulmonaire sous la forme d'aérosols. Les nanoparticules produites et les microsphères ont été caractérisées et leurs propriétés aérodynamiques et de libération in vitro ont été évaluées.

Les nanoparticules de PLGA chargées en palmitate de chloramphénicol ou en palmitate de thiamphénicol possèdent une teneur en antibiotique satisfaisante et le rendement d'encapsulation est de l'ordre de 100%. Leur diamètre moyen est de 160 nm environ. Les nanoparticules à base de promédicament pures ont été préparées dans les mêmes conditions et leur diamètre moyen est d'environ 170 nm. La microscopie électronique à balayage (MEB) effectuée

sur les microsphères a montré que les microsphères étaient d'aspect et de dimension homogènes et que leur surface était ridée. Toutes les poudres ont montré des propriétés aérodynamiques satisfaisantes avec des valeurs MMAD proches de 3 µm et des valeurs FPF et ED élevées. Les études de libération in vitro ont montré que les formulations permettaient une libération prolongée de palmitate de chloramphénicol ou de palmitate de thiamphénicol sur 8 jours à plus de 14 jours, avec une cinétique de libération plus rapide avec les poudres préparées à partir de nanoparticules de PLGA qu'avec celles préparées avec des promédicament pures.

En conclusion, des formulations en poudre sèche de palmitate de chloramphénicol ou de palmitate de thiamphénicol avec des profils de libération prolongée et des propriétés aérodynamiques appropriées pour l'administration pulmonaire sous forme d'aérosol ont été obtenues avec succès grâce à la technologie de production par incorporation de nanoparticules dans des microsphères.

Article number 3

Sustained release dry powder formulations of chloramphenicol or thiamphenicol prodrugs for lung delivery as aerosols

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Abstract

The purpose of this study was to design inhalable sustained-release nanoparticle-in-microspheres for the lung delivery of chloramphenicol or thiamphenicol as aerosols, in order to optimize therapeutic efficiency against pulmonary bacterial infections. The palmitate ester prodrugs of the two antibiotics were used as they were expected to be efficiently entrapped within PLGA nanoparticles or to form pure prodrug nanoparticles. Prodrug-loaded PLGA nanoparticles or pure prodrug nanoparticles were prepared using the emulsion-solvent evaporation method. Dry microsphere powders for inhalation were then produced by spray-drying the nanoparticle suspensions supplemented with lactose as a bulking agent and leucin as a dispersing

enhancer. The obtained microspheres appeared to be shriveled by scanning electron microscopy, with no presence of crystalline structures. Drug loading was satisfactory (14 to 34 % (m/m)) and the aerodynamic properties determined with a Next Generation Impactor were appropriate for lung delivery, with MMAD values close to 3µm. The in vitro release profiles showed that sustained released was achieved with these formulation, with a longer release time for the powders prepared from the pure prodrug nanoparticles (14 days at least) than from the prodrug-loaded PLGA nanoparticles (8 to 10 days).

Keywords: chloramphenicol, thiamphenicol, aerosol, lung delivery, antimicrobial, prodrug, palmitate, PLGA.

1. Introduction

Chloramphenicol (CHL) and thiamphenicol (THA) are antibiotics of the amphenicol class that possess similar broad spectra of activity against Gram positive and Gram negative bacteria (Eliakim-Raz et al. 2015, Serra et al. 2007). Commonly used in the past due to its broad spectrum and its good diffusion into tissues, chloramphenicol was reported to cause rare, irreversible and fatal idiosyncratic aplastic anaemia, which was attributed to the nitroso-chloramphenicol metabolite responsible for DNA damage (Dinos et al. 2017, Ferrari 1984). Possessing a methyl sulfonyl group in place of the nitro group thiamphenicol was not associated with such a lateral effect (Yunis 1984, Ferrari 1984). Both chloramphenicol and thiamphenicol are however

responsible for reversible dose-dependent bone marrow suppression in the case of prolonged treatments (more than 7 days) (Ferrari 1984, Eliakim-Raz et al. 2015). In the advanced countries, they have therefore been replaced in clinical practice with less toxic antibiotics, and their use is recommended as the second line treatments of life-threatening infections not responding to other antibiotics (Eliakim-Raze et al. 2015). Chloramphenicol is inexpensive, and is still widely used as topical preparations, e.g. in eye drops for the prevention and treatment of superficial eye infections, or as oral tablets or capsules or pediatric suspension in low-income countries (Lam et al. 2002). Though potentially useful for the treatment of life-threatening infections resistant to other antibiotics, chloramphenicol and thiamphenicol are not available world-wide and are considered as “forgotten” antibiotics in advanced countries (Pulcini et al. 2017). In particular, CHL and THA have been proposed to treat multidrug-resistant pulmonary bacterial infections. Their direct administration into the lungs as therapeutic aerosols should be considered to increase treatment efficiency and minimize whole body exposure responsible for adverse effects, particularly in the case of prolonged treatments. Thiamphenicol, in the form of its glycinate ester prodrug, was administered as an aerosol in oncological patients with respiratory infections and was effective in more than 95% of the patients (Macchi et al. 2011). Due to their lipophilicity both CHL and THA were shown to have a high permeability in in vitro studies on a Calu-3 cell line broncho-alvelolar model (Nurbaeti et al. submitted), in consistency with the rapid absorption through the intestinal epithelium reported in human PK studies after oral administration (Ferrari 1984, Ambrose 1984).

The CHL or THA intratracheal administration in rats had a little or not significant impact on lung exposure compared to IV administration (Nurbaeti et al. submitted). It is therefore relevant to formulate them in sustained release systems for lung delivery as aerosol. In previous works, we showed that antibiotics-loaded PLGA microspheres with sustained-release properties permitted to dramatically increase the concentration of antibiotics in the pulmonary epithelial lining fluid over a prolonged time period (more than 72 h), compared to an intratracheally nebulized solution or an IV infusion (Gaspar et al. 2016, Doan et al. 2013). In preliminary experiments, the formulation of PLGA microspheres with chloramphenicol or thiamphenicol and their preparation using the solvent evaporation method resulted in insufficient drug loading (from 1 to 5% (w/w)) (data not shown). Previous studies showed that using hydrophobic prodrugs dramatically increased the nanoparticle drug content compared to the parent drug and prolong the release time (Gómez-Gaete et al. 2008, Han et al. 2015). Lipophilic palmitate esters of chloramphenicol or of thiamphenicol are available. Chloramphenicol palmitate (CHLP) was synthetized in the past as a tasteless prodrug for oral administration in children. Different from chloramphenicol it is poorly absorbed, but it is quickly and almost completely hydrolyzed into the parent drug by esterases in the small intestine, which results in a chloramphenicol bioavailability similar to oral dosage forms of chloramphenicol (Ambrose, 1984). CHLP is marketed as a pediatric oral suspension in some countries (India, etc.). In the present work, lipophilic palmitate esters of chloramphenicol or of thiamphenicol were therefore investigated and formulated as dry powders

for inhalation based on nanoparticle aggregation through the spray-drying of a nanoparticle aqueous suspension. Formulations based on nanoparticles made from the pure prodrugs were also investigated. Once released in the lungs, ester prodrugs are expected to be hydrolyzed by lung lipase into active drugs (Camps et al. 1991).

2. Materials and methods

2.1. Materials

Chloramphenicol palmitate was purchased from Chem-Impex International, Inc. (Wood Dale, IL, USA). Thiamphenicol palmitate was obtained from Abcam. Resomer® RG 502 H (PLGA 50:50, acid terminated), alpha-lactose monohydrate (purity, 99%), L-leucine and lipase from porcine pancreas were obtained from Sigma. Rhodoviol 4/125 (polyvinylalcohol, degree of hydrolysis of 88%) was purchased from Prolabo (France). Purified water was produced with a milli DI™ Millipore system and HPLC quality grade water used for HPLC analysis was purchased from Carlo Erba. Hank's Balanced Salt Solution (HBSS) was obtained from PAN Biotech GmbH (Aidenbach, Germany). All other chemicals were of analytical grade or equivalent.

2.2. Methods

2.2.1. Nanoparticle preparation

Nanoparticles were prepared by an emulsion - solvent evaporation method. For prodrug-loaded PLGA nanoparticles, a solution of PLGA (100 mg) and

prodrug (100 mg) in 20 mL ethyl acetate (EA) was vortex mixed with 80 ml of a solution W of polyvinylalcohol (0.5% w/v) saturated with EA (4.68 mL), then subjected to ultrasonication on ice and under magnetic stirring for 6 min using a probe sonicator (Branson Sonifier 450) set at 20% maximum power. In the case of prodrug nanoparticles, 200 mg prodrug were dissolved in EA and the procedure was the same. EA was evaporated off under vacuum at 30℃ duri ng 1 hour using a rotary evaporator. Volume was adjusted to 80 mL with water and the suspension was filtered on a Fischerbrand glass microfibres filter (2.7 µm porosity). The mean diameter of the volume distribution (D_v) of the nanoparticles was determined in purified water by laser light diffraction (Microtrac® X100 particle size analyzer).

For yield (%) and prodrug nanoparticle content determination, an aliquot of 4 mL of the nanoparticle suspension was collected and nanoparticles were washed twice with water through two cycles of centrifugation (11,000 rpm, 30 min, Eppendorf centrifuge 5804R (14,610g)). After the last centrifugation, pellets were dissolved in 3 mL EA and dried at 45℃ under a nitrogen f low. The dry weight was measured and 3 mL EA was added. Then 1.5 mL was collected and centrifuged (14,000 rpm, 30 min, Eppendorf centrifuge 5418R (16,900 g)) for the prodrug spectrophotometric determinations.

The nanoparticle suspension was supplemented with leucine (2 mg/mL final concentration) and centrifuged (11,000 rpm, 30 min, Eppendorf centrifuge 5804R (14,610g)). The supernatant was discarded and the pellet was re-dispersed in 20 mL of a solution of leucine (2 mg/mL) and lactose (8 mg/mL),

which corresponded to a nanoparticle-to-additive weight ratios ranging from 0.60 to 0.85. Then the suspension was spray dried.

2.2.2. Spray-drying

The nanoparticle suspension supplemented with leucine and lactose (2 and 8 mg/mL respectively) were spray dried using a Büchi® Mini Spray Dryer B-290 (Switzerland) set up in blowing mode and equipped with a 0.7 mm nozzle. Settings were: 5% pump rate for the feed, air flow rate set at maximum level and aspiration rate of 100 %, with the pneumatic automatic nozzle cleaning operated 3 to 5 times per min. The inlet and outlet temperatures were 81-83℃ and 52-54℃, respectively. The obtained powders were then characterized according to their size, aerodynamic properties, drug content, morphology and release profile.

2.3. Formulation characterization

Scanning electron microscopy (SEM)

Microsphere powders were dispersed on double-sided adhesive carbon tapes that were fixed on copper stubs and sputter coated with a platinum film. SEM images were taken using a Teneo VolumeScope Electron microscope (FEI Thermo Scientific). The images were obtained from the collection of secondary electrons under a voltage of 20 kV and 11 mm of working distance.

Aerodynamic diameter determination

The aerodynamic diameter was determined using a Next Generation Impactor (NGI, Copley Ltd., Nottingham, UK), equipped with a TPK 2000 critical flow controller and a HCP5 vacuum pump (Copley HCP5, Nottingham, UK) as previously decribed (Gaspar et al. 2015). For each measurement, a size-three hard gelatin capsule was filled with 25 ± 1 mg of powder, inserted in a dry-powder inhaler (DPI) Handihaler® (Boehringer-Ingelheim, Germany) and pierced according to the DPI user's manual. The DPI was tightly connected to the NGI induction port via a silicone adapter. The airflow rate was set at 41 ± 5% L/min in order to produce a pressure drop of 4 kPa through the inhaler. The test duration time was set in order to draw 4L of air through the inhaler. The powder discharged from the capsule and deposited in the induction port, all the stages and the filter was collected with EA for the prodrug spectrophotometric determination. Powder deposited in the DPI and in the adapter was collected with ethanol, evaporated off at 45℃ under a nitrogen flow and t he dry residue was dissolved in EA.

The emitted dose, i.e. the mass of prodrug deposited in the induction port, the stages and the MOC, was expressed as the percentage (ED%) of the prodrug mass contained in the capsule. The percent cumulative mass fractions of prodrug deposited in MOC and stages 1 to 7 were plotted versus the log aerodynamic diameters. The mass median aerodynamic diameter (MMAD) was calculated by linear interpolation using the equation of the linear interpolant that links the curve points immediately below and above 50%

deposition. The fine particle fraction (FPF) (i.e. the fraction of the prodrug within the particles of 1-to-5 µm aerodynamic diameter) was expressed as the percentage of the ED.

Differential Thermal Analysis (DTA) and Thermal Gravimetric Analysis (TGA)

DTA and TGA were carried out on a SDT Q600 Instrument (TA Instruments, USA). Samples were placed on platinum pans and studies were carried out over a temperature range from 30℃ to 350℃ at a 10℃/min heating rate a nd under a 100 mL/min air flow rate.

In vitro release studies

For in vitro release studies under sink conditions, powders (50 mg) were dispersed in 10 mL of HBSS, pH 7.4, supplemented with 400µL of a 1 mg/mL porcine lipase solution in HBSS, and incubated at 37 ℃ under ma gnetic stirring (300 rpm) (Gaspar et al., 2016). At pre-determined time points, aliquots were collected over 2 weeks and centrifuged at 14,000 rpm for 10 min (Eppendorf centrifuge 5418R, 16,900). Then, supernatants were collected and CHL and THA were determined by HPLC. Lipase (400µL) was added every day to the release medium to compensate for loss of enzyme activity.

Prodrug spectrophotometric determination

The nanoparticle or spray-dried powder prodrug content (weight%), i.e. the amount of prodrug (mg) per 100 mg of dry weight (including entrapped

prodrug), was determined by spectrophotometry at 273 nm using a Varian Cary 50 UV-Visible spectrophotometer after dissolution in EA using a prodrug calibration curve (3 – 60 µg/mL concentration range for CHLP and 100 – 1000 µg/mL for THAP).

Thiamphenicol and chloramphenicol HPLC determination

The chromatographic system consisted of Waters 717 plus autosampler, a Hitachi L-7110 pump and a Eurosep 785 A spectrophotometric detector (Applied Biosystem). For CHL determination, it was equipped with a C18 X Terra HPLC column (5 µm, 2.1x150 mm) with a Gemini C18 (4x2 mm) Phenomenex precolumn. The mobile phase was run at a 0.2 mL/min flow rate and was composed of a 75:25 (v/v) acetonitrile: water mixture supplemented with 0.1% (v/v) formic acid and 0.2% (v/v) PIC B7. The injection volume was 50 µL and the run time was 9 min. CHL was detected by spectrophotometry at 275 nm. The calibration curve was constructed by linear regression of the peak areas versus the added concentrations (0.125 - 10 µg/mL in HBSS (pH 7.4)) with a weighting factor of $1/x^2$ (R^2 = 0.999). Appropriate quality controls (QCs) were also included to monitor the performance of the method.

For THA determination, it was equipped with a C18 X Terra HPLC column (5 µm, 3.9x150 mm) with a Gemini C18 (4x2 mm) Phenomenex precolumn. The mobile phase was run at a 0.4 mL/min flow rate and was composed of an 85:15 (v/v) acetonitrile: water mixture supplemented with 0.1% (v/v) formic acid and 0.2% (v/v) PIC B7. The injection volume was 50 µL and the run time was

18 min. THA was detected by spectrophotometry at 225 nm. The calibration curve was constructed by linear regression of the peak areas versus the added concentrations (0.5 - 40 µg/mL in HBSS (pH 7.4)) with a weighting factor of $1/x^2$ (R^2 = 0.999). Appropriate quality control solutions were also included to monitor the performance of the method.

3. Results and discussion

3.1. Nanoparticle preparation

A low molecular weight PLGA polymer with a rapid degradation rate (Gaspar et al., 2016; Díez and Tros de Ilarduya, 2006) was used for the preparation of PLGA nanoparticles in order to avoid polymer accumulation within the lung upon repeated dosing. The visual inspection of the suspensions of nanoparticles made with or without PLGA using a photonic microscope did not show any prodrug crystal-like precipitates (not shown), as present in the commercial prodrug powder (Fig. 1). In the case of dexamethasone acetate (ester), Gómez-Gaete et al. (2008) observed that the acetate prodrug was partially incorporated within PLGA nanoparticles and the unentrapped fraction precipitated as typical crystal needles.

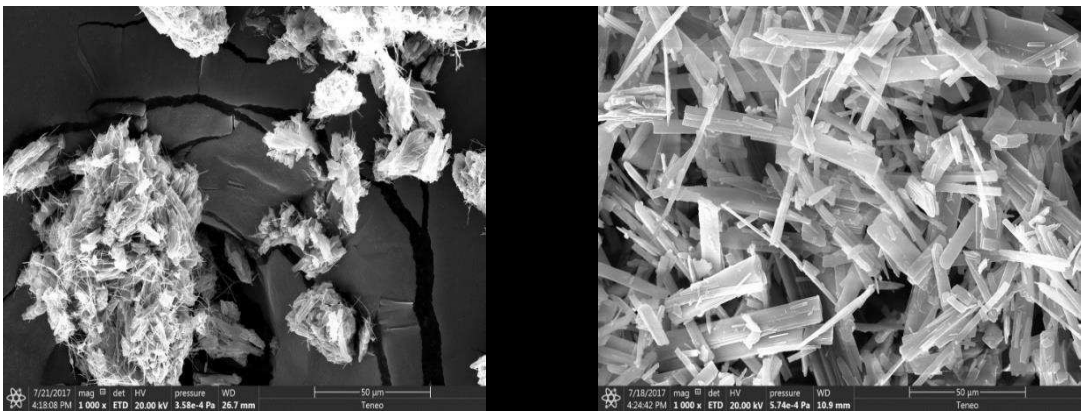

Fig. 1: SEM of the commercial CHLP (left) and THAP (right) powders

Studying the effect of the chain length of the carboxylate ligands (acetate to decanoate) of a platinum derivative, Johnstone and Lippard (2013) showed a good correlation between the lipophilicity and the entrapment efficiency within PLGA nanoparticles, with the highest efficiency for the decanoate derivative. Palmitate ester prodrugs are usually entrapped with high efficiency in PLGA nanoparticles (Debotton et al., 2008; Tangsumranjit et al., 2006). In the case of prodrug-loaded PLGA nanoparticles, the prodrug content in PLGA nanoparticles ranged from 50 to 60% (m/m), i.e. in accordance with a loading efficiency of 100%, which indicated that the lipophilic prodrugs had a high affinity for the polymer (Table 1). It is interesting to note that using the same procedure as for preparing prodrug-loaded PLGA nanoparticles, nanoparticles were successfully prepared from pure CHLP or THAP prodrugs, without the formation of crystalline precipitates, as observed by visual inspection of the preparations under a photonic microscope (not shown). As expected, the prodrug contents were close to 100% in the case of nanoparticles made of

pure prodrug (Table 1). Prodrug-loaded PLGA nanoparticles and pure prodrug nanoparticles had virtually similar size, with a narrow size distribution (span value below 1) (Table 1). THAP-based nanoparticles were slightly larger than CHLP-based ones.

Table 1: Characterization of nanoparticles (mean±SD, n = 8-9)

Nanoparticle type	Dv (nm)	Span	Prodrug content (mg/100 mg)	Yield (%)
CHLP nanoparticles	159±6	0.54±0.07	82±11	86±5
CHLP-loaded PLGA nanoparticles	160±4	0.64±0.09	50±13	63±18
THAP nanoparticles	177±6	0.79±0.07	91±22	84±9
THAP-loaded PLGA nanoparticles	178±12	0.77±0.04	60±14	70±7

3.2. Dry microsphere powders

Dry microsphere powders were obtained by spray-drying the nanoparticle aqueous suspensions supplemented with lactose and leucine. The spray-drying production process produces monodisperse microparticles with high encapsulation efficiency and drug loading (Gómez-Gaete et al. 2008). Lactose was used as a bulking agent and leucine as a dispersibility enhancer in order to obtain powders with appropriate aerosolisation properties for inhaled delivery (Seville et al., 2007). At the x2,500 magnification the dry powders

appeared to be made of microspheres with diameter below 5 µm and were similar whatever the prodrug or whatever the use of PLGA to prepare the nanoparticles (Fig. 2 and 3). As expected, no crystalline structure of CHLP or THAP like the crystals of the commercial prodrug powder (Fig. 1) was observed, since powders generated by spray-drying are predominately amorphous in nature (Corrigan 1995). At the x10,000 and x40,000 magnifications, the microsphere surface appear smooth and it is not possible to distinguish nanoparticles like in the case of previously described Trojan particles (Gómez-Gaete et al., 2008). The use of a different bulking agent and of a PLGA polymer with a higher molecular weight than in the present study (with a higher glass transition temperature) may explain the different morphology of the final product. In addition the microspheres appeared to be shriveled like raisin. This typical shape was attributed to the collapse of the microsphere structure during the spray-drying process and was reported to be promoted by leucine (Seville et al., 2007, Yang et al. 2015). Particles with a high degree of surface corrugation were shown to possess better aerosolisation properties due to reduced contact area (Chew et al., 2005; Adi et al.,2008). Prodrug contents are presented in Table 2. As expected, the prodrug content is higher when the spray-dried powder is obtained from nanoparticles made of pure prodrug, than with the prodrug-loaded PLGA nanoparticle suspension.

Table 2: Characterization of nanoparticles (mean±SD, n = 3-4)

Dry microsphere powder made with:	Prodrug content (%, m/m)	ED (%)	FPF (%) (1-5µm)	MMAD (µm)
CHLP nanoparticles	30.2±1.0	75±9	33±10	3.1±0.2
CHLP-loaded PLGA nanoparticles	13.9±3.0	79±8	27±13	3.3±0.5
THAP nanoparticles	34.5±4.8	91±8	47±9	2.9±0.2
THAP-loaded PLGA nanoparticles	21.0±0.7	82±19	36±10	2.8±0.3

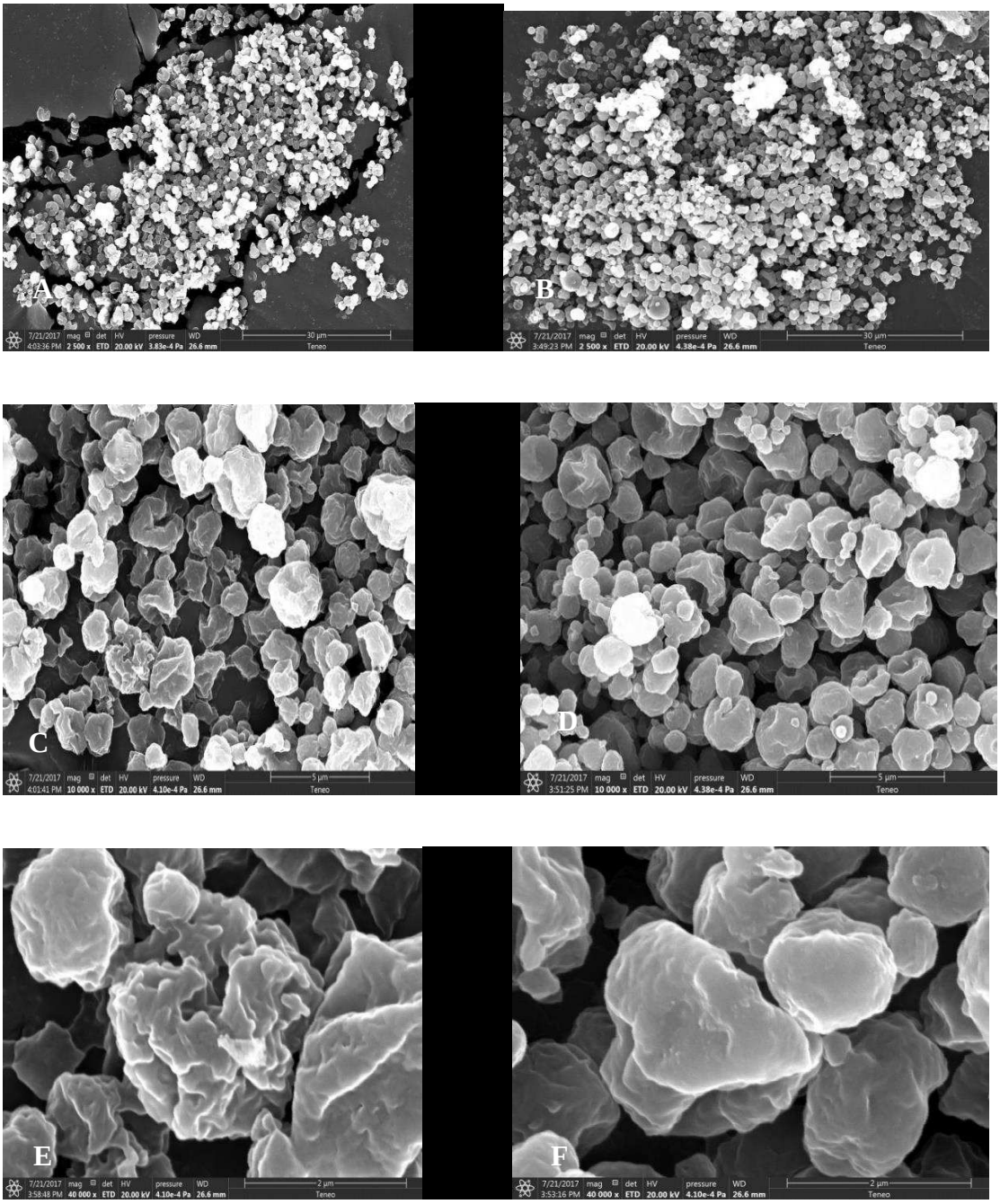

Figure 2: SEM of microsphere powders prepared with CHLP nanoparticles (left, i.e. A, C and E) or CHLP-loaded PLGA nanoparticles (right, i.e. B, D and E)

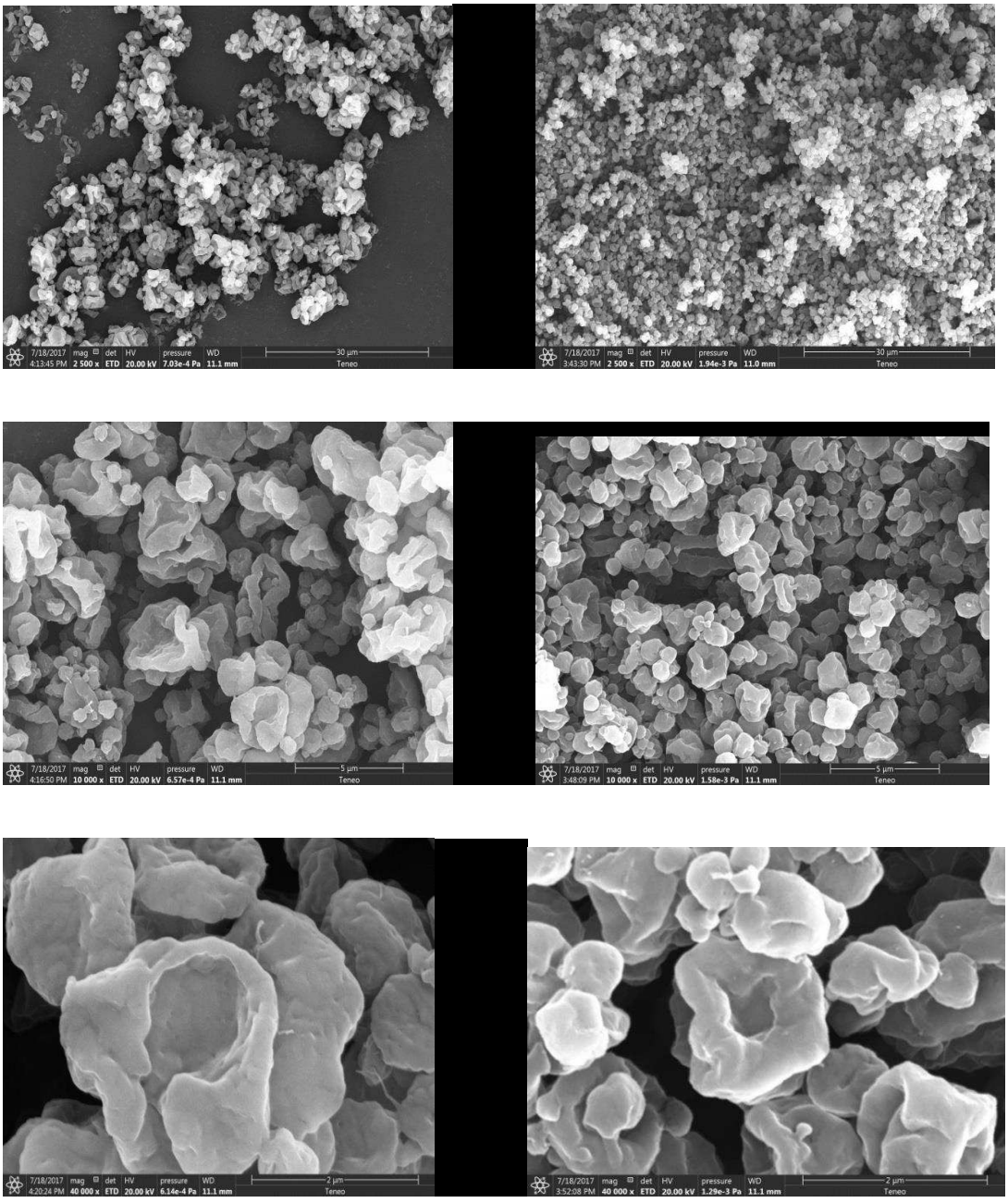

Figure 3: SEM of microsphere powders prepared with THAP nanoparticles (left, i.e. A, C and E) or THAP-loaded PLGA nanoparticles (right, i.e. B, D and F)

3.3. DTA-TGA analysis

DTA-TGA analysis was conducted to analyze the interaction between chloramphenicol or thiamphenicol with PLGA, leucin and lactose. Fig. 4A and 4B show endothermic peaks at 153.64℃ and 167.06℃ correspon ding to the melting points of CHL and THA respectively (Aiassa et al. 2015, and compendial values). Degradation occurs with an onset at 190℃ as shown b y the exothermic peaks and the gradual weight loss. The prodrug CHLP exists as three described polymorphs: one stable form A and two metastable forms B and C (Kaneniwa and Otsuka, 1985), with different solubility and oral bioavailability. Forms B and C are suitable for therapeutic use, but form A is considered as biologically inactive, which was attributed to a different susceptibility to pancreatic lipase in the upper intestinal tract. Forms A and B have respective melting points at 90.3 or 92℃ and 86.7 ℃ respectively (Kaneniwa and Otsuka, 1985; Gamberini et al. 2006). The endothermic peak at 88.31℃ on the CHLP thermogram (Fig. 4A) may correspond to form B or a mix of forms A and B, since the metastable form B tends to transform into the stable form A (Gamberini et al. 2006). The thermogram of THAP (Fig. 4B) shows an endothermic peak at 111.27℃ which was attributed to the melting point of THAP. In both cases, taking account of the small size of the endothermic peaks of CHLP or of THAP it is likely that most prodrugs are in the amorphous or dissolved state in the microspheres. For both prodrugs, degradation occurred with an onset 190℃ (Fig. 4A and 4B), as deduced from the exothermic peaks and from the weight loss (Fig. 4C and 4D).

For the four microsphere powders, the weight loss of 2 to 5 % observed over 40 to 70℃ (Fig. 4C and 4D) was due to the evaporation of residual w ater (Raula et al. 2012). A further weight loss with an onset at around 140℃ was simultaneous with an endothermic event (over 145 to 190℃) and was attributed to the sublimation of leucine (Lähde et al. 2009). Since all the components were reported to degrade at temperature above 200℃, the exothermic event with an onset at 190-200℃ (Fig. 4A and 4B) which is simultaneous with an important weight loss (Fig. 4C and 4D) was attributed to the thermal degradation of the MS components. MS powders made with CHLP nanoparticles or CHLP-loaded PLGA nanoparticles had roughly similar thermograms (Fig. 4A) with an endothermic peak at 85.84℃ attributed to the melting of the CHLP form B. MS powders made with THAP nanoparticles or THAP-loaded PLGA nanoparticles (with our without PLGA) had also similar thermograms (Fig. Y C&D). The endothermic peaks at 108-109℃ were attributed to THAP melting.

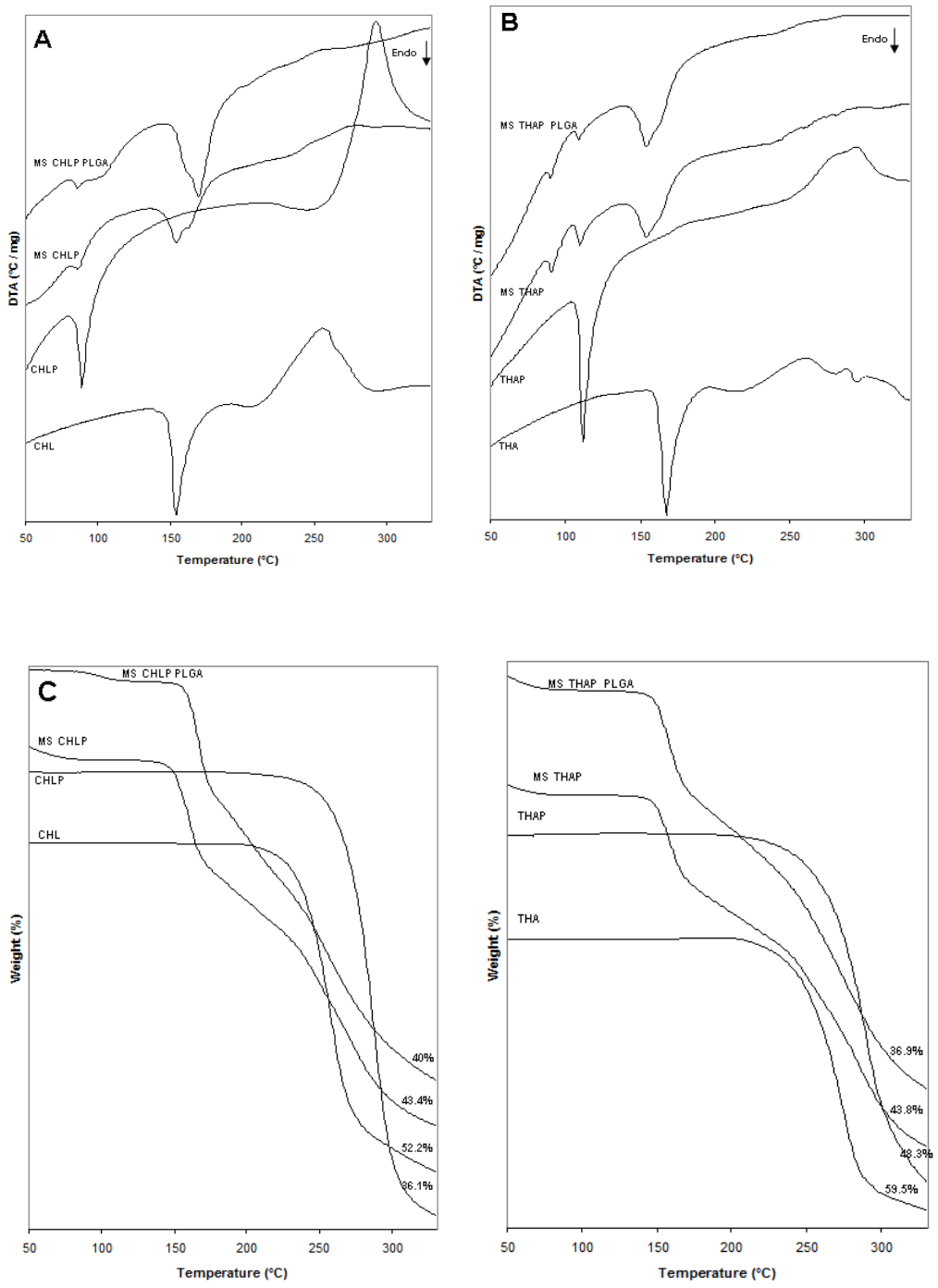

Fig. 4: DTA (A & B) and TGA (C & D) thermograms of raw materials (CHL, CHLP, THA and THAP) and of dry microsphere powders made with CHLP or THAP nanoparticles (MS CHLP or MS THAP) or with CHLP- or THAP-loaded PLGA nanoparticles (MS PLGA CHLP or MS PLGA THAP). Total weight loss (%) is indicated on TGA curves.

3.4. Aerodynamic properties

All the formulations showed a high dispersibility with high ED% values (above 70%) indicating that the microsphere powder was efficiently emitted from the DPI (Table 2). MMAD values were around 3µm, suggesting that the MS successfully de-aggregated during the aerosolisation process within the DPI. FPF (%) were very satisfactory in the range of values reported for marketed formulations (15-30%) for pulmonary administration (Smith and Parry-Billings, 2003), indicating that the MS obtained in the present work should be efficient to deliver CHLP and THAP to the lungs.

3.5. Prodrug release studies

Release prodrug studies were carried out at 37℃ in HBSS buffer, pH 7.4. In order to maintain sink conditions, porcine lipase was added to the release medium, in order to convert the poorly soluble prodrugs released in the release medium into the more soluble drugs, which were eventually determined by HPLC. For all the four microsphere powder formulations tested, release profiles were rather linear and showed a sustained release of the prodrugs (Fig 5).

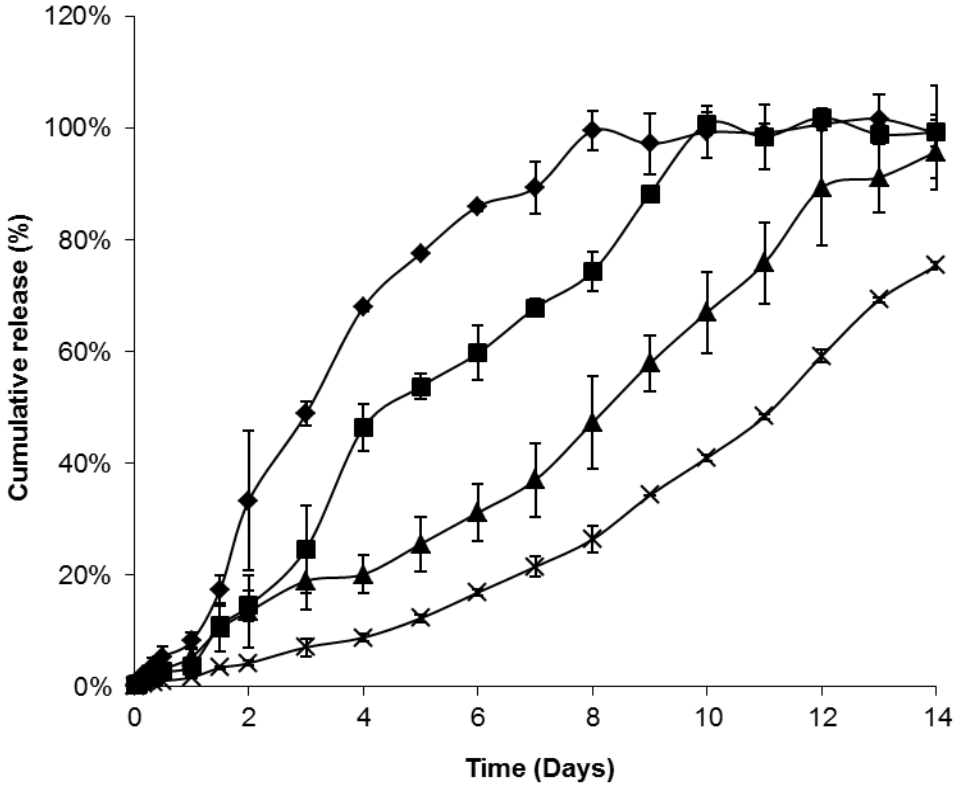

Figure 5: In vitro prodrug release profiles in HBSS medium, 37℃, for dry microsphere powders made with CHLP nanoparticles (×), with THAP nanoparticles (■), with CHLP-loaded PLGA nanoparticles (▲) or with THAP-loaded PLGA nanoparticles (◆).

THAP release was completed within 8 or 10 days for the microspheres made with THAP-loaded PLGA nanoparticles or with THAP nanoparticles, respectively. In the case of CHLP, release was longer with 14 days or even more than 14 days, for the microspheres made with CHLP-loaded PLGA nanoparticles or with CHLP nanoparticles, respectively. Therefore, release rates were clearly higher with the THAP prodrug than with the CHLP prodrug.

This was attributed to the higher water solubility of the THA moiety than the solubility of the CHL moiety, leading to a higher dissolution rate in the release medium. Release rates were also higher with the microsphere powders made with prodrug-loaded PLGA nanoparticles than with those made with pure prodrug nanoparticles. In the case of prodrug-loaded PLGA nanoparticles, prodrugs are dispersed within the PLGA matrix that gradually degrades. Such degradation is likely to produce pores within the nanoparticles that permit the entry of water and increase the exchange surface area with the release medium.

For both prodrugs, whatever the formulation, the releases were much more slowly than in previously reported results obtained with dexamethasone acetate-loaded PLGA nanoparticles (Gómez-Gaete et al., 2008). This can be explained by a higher affinity of the CHLP or THAP prodrugs towards the PLGA polymer, resulting in a more slowly drug release.

It is difficult to estimate whether such relatively slow sustained release profiles of the prodrugs and their hydrolysis rate into the active drugs within the lungs will be appropriate to reach efficient CHL or THA concentrations in the lung areas. Previous PK studies with sustained release rifampicin or levofloxacin-loaded PLGA microspheres showed that such delivery systems permitted to reach high epithelial lining fluid (ELF) to blood plasma drug concentration ratios due to the local release in the small ELF volume. In addition, little is known about the esterase activity within the lungs (Camps et al. 1991).

Therefore PK studies will be necessary to further investigate the therapeutic potential of the present formulations.

4. Conclusion

Dry powder formulations of CHLP or THAP with sustained release profiles and appropriate aerodynamic properties for lung delivery as aerosols were successfully prepared with the nanoparticle-within-microsphere spray-drying production technology. Further PK investigations are needed to evaluate the potential efficiency of such innovative CHLP or THAP drug delivery systems.

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CHAPTER 4

GENERAL DISCUSSION

4. GENERAL DISCUSSION

4.1 In vitro and in vivo studies

Chloramphenicol and thiamphenicol are old antibiotics with therapeutic potential against lung infections resistant to commonly used antibiotics. Their administration via the pulmonary route may be interesting for optimizing their efficiency and for controlling their systemic toxicity. In order to design an appropriate drug formulation for lung delivery as aerosol, it is necessary to study their lung permeability in vitro and their pharmacokinetics in vivo.

In vitro study was performed using Calu-3 cells, while in vivo study was performed in rats. The result of in vitro study showed that Papp of chloramphenicol through Calu-3 cell monolayer was higher than Papp of thiamphenicol. This is correlated to the higher lipophilicity of chloramphenicol compared to thiamphenicol. Bur et al., 2010 and Stigliani et al., 2016 reported that there is a relationship between cell membrane diffusion and cell permeability. With an apparent permeability value of 4 x 10^{-6} cm/s chloramphenicol can be considered as a high permeability compound. Its apparent permeability value is indeed similar to the ones of high-permeability fluoroquinolones such as moxifloxacin and levofloxacin with Papp 2.4 x 10^{-6} cm/s and 5 x 10^{-6} cm/s, respectively. On the other hand, thiamphenicol is categorized as an intermediate permeability compounds with Papp ≈ 1 x 10^{-6} cm/s in Ap-Bl. It is almost similar to the permeability of ciprofloxacin or norfloxacin with Papp 0.6 and 0.7 x 10^{-6} cm/s (Brillault et al., 2010). These in

vitro results were confirmed by in vivo pharmacokinetics which demonstrated that the pharmacokinetic profiles of chloramphenicol in ELF and plasma were similar to certain fluoroquinolones known as highly permeable antibiotics (Gontijo et al., 2014). The pharmacokinetic profile of thiamphenicol was similar with low membrane permeability antibiotics such as tobramycin and aztreonam (Marchand et al., 2015, 2016). At this point, both in vitro and in vivo studies suggested the lack of advantages of the lung administration route over IV or oral administration routes for chloramphenicol. Indeed, due to the high permeability, chloramphenicol concentrations will equilibrate quickly in both lung and plasma compartments, whatever the route of administration. For thiamphenicol, in link to its intermediate permeability, the pulmonary route may be an advantage at least at early times post administration, since the ELF concentrations were shown to be higher than in the plasma. This may however have a very limited effect on the thiamphenicol antibiotic efficiency.

The role of the transporters in the chloramphenicol and thiamphenicol lung diffusion has been evaluated by in vitro and in vivo experiments. The in vitro experiments showed that permeability of chloramphenicol and thiamphenicol was influenced by the inhibitor PSC-833, suggesting they are substrates of efflux pumps such as P-gp. However, the methods used in this study failed to ascertain the identity of the transporter(s). Specific inhibitors or fluorescent substrates have proven their utility in such objectives in other studies but they may not be sufficient when the tested drug has low affinity and/or specificity to one transporter. This may be the case for chloramphenicol and thiamphenicol,

which modified slightly or not the cell uptake of rhodamin123 and fluorescein, suggesting a low affinity to the transporters. Moreover, for thiamphenicol, several specific inhibitors influenced the permeability, suggesting the role of several transporters at the same time and a low specificity. In this particular case, more adapted tools should be used such as respective transporter-expressing systems. For instance, the canine renal epithelial MDCK cell line forms confluent monolayers suitable for drug transport studies. They have been also transfected with the P-gp human gene (MDR1). Chloramphenicol and thiamphenicol transport studies could be done with wild-type MDCK and MDR1-MDCK, and the comparison between the two permeability studies would give information on the role of P-gp. However, this model has also some flaws since the wild-type MDCK cells express endogenous canine P-gp leading to a potential bias (Kuteykin-Teplyakov et al., 2010). Since Calu-3 cells already express human drug transporters such as P-gp, another promising approach would be to design a new Calu-3 cell line where the MDR1 gene had been inactivated with the CRISPR-Cas9 tool. This methodology could also apply to the other transporters MRP1 and BCRP. Clarifying the identity of the transporters for chloramphenicol and thiamphenicol will help to prevent drug-drug interaction in a future usage in patients.

The role of the transporters was confirmed in vivo for both chloramphenicol and thiamphenicol. However, important differences occurred between in vitro and in vivo data. In vitro studies showed an impact of the transporters mainly for thiamphenicol and in a limited extend for chloramphenicol. On the contrary,

in vivo data showed a clear role of transporters for chloramphenicol and a very limited one for thiamphenicol. Indeed, the AUC ratio ELF/plasma after IV was 7.9 for chloramphenicol and only 1.4 for thiamphenicol. There are several explanations for this discrepancy. First, Calu-3 cells are from human origin and the nature and expression level of transporters may be different between the human and rat species. Second, the Calu-3 cells may be a too simplified model of the lung that does not take into account the diverse anatomical and physiological diversity of the entire lung. Finally, the Calu-3 cells, due to their airway epithelium nature, may not fully mimic the alveolar epithelium of the lung where most of the diffusion/transport occurs. These limits of the Calu-3 model may explain the differences in the importance of the role of transporters. The use of a different cell line such as broncho-alveolar NCI-H441 cell line or rat alveolar primary cells could help to better understand these differences.

4.2 Formulation study

Since both in vitro and in vivo results showed that the intratracheal administration had no advantage for chloramphenicol and a limited advantage for thiamphenicol compared to the systemic IV route of administration, formulations with sustained release properties may be a promising strategy to reach high pulmonary concentrations for a prolonged time period, while providing a reduced systemic exposure to the two antibiotics. The challenges of the formulations are to produce:

a. Powders for inhalation, i.e. powders with aerodynamic diameter from 1 to 5 µm.

b. With sustained release properties in order to maintain high concentrations of antibiotics in the ELF

c. With a high antibiotic content, in order to minimize the administration of materials into the lungs

In preliminary feasibility formulation studies (not shown), the production of chloramphenicol- or thiamphenicol-loaded PLGA microspheres using the solvent evaporation method resulted in insufficient drug loading (from 1 to 5% (w/w)) (data not shown). Previous studies showed that using hydrophobic prodrugs dramatically increased the nanoparticle drug content compared to the parent drug and prolong the release time (Gómez-Gaete et al., 2008; Han et al., 2015). Therefore, the use of lipophilic palmitate esters of chloramphenicol or thiamphenicol was investigated to produce sustained release powder for aerosol antibiotherapy. For that purpose, chloramphenicol palmitate or thiamphenicol palmitate were formulated as nanoparticles, which were then aggregated into microspheres by spray-drying. Nanoparticles were produced from pure prodrugs or in the presence of PLGA in order to evaluate the impact of the polymer on the final product properties.

Nanoparticle preparation and properties

The O/W emulsion evaporation method was used to produce nanoparticle loaded with chloramphenicol palmitate or thiamphenicol palmitate. This method is suitable to produce carrier systems with high drug content in the case of poorly water-soluble drugs (Westesen et al., 1997). Chloramphenicol palmitate or thiamphenicol palmitate and PLGA in 1:1 weight ratio or the prodrugs alone were dissolved in ethyl acetate (Song et al., 2006). A low molecular weight PLGA polymer with a rapid degradation rate (Gaspar et al., 2016; Díez and Tros de Ilarduya, 2006) was used for the preparation of PLGA nanoparticles in order to avoid polymer accumulation within the lung upon repeated dosing. The organic phase was then emulsified in a solution of PVA at a concentration of 0.5%.

For both prodrugs, the suspensions of nanoparticles did not show any prodrug crystal-like precipitates. In the case of dexamethasone acetate (ester), Gómez-Gaete et al. (2008) observed that the acetate prodrug was partially incorporated within PLGA nanoparticles and the unentrapped fraction precipitated as typical crystal needles.

Nanoparticles had mean particle diameter (Dv) less than 200 nm with a satisfactory span value (i.e. below 1). Considering each prodrug, PLGA had no impact on the nanoparticle size. Chloramphenicol palmitate-loaded nanoparticles were slightly smaller than thiamphenicol palmitate-loaded nanoparticles.

In the case of prodrug-loaded PLGA nanoparticles the entrapment efficiency was around 100%. This proves that palmitate esters of chloramphenicol or thiamphenicol had a good affinity for the PLGA polymer and are suitable to optimize their drug loading in nanoparticles formulation, in agreement with previous works. Studying the effect of the chain length of the carboxylate ligands (acetate to decanoate) of a platinum derivative, Johnstone and Lippard (2013) showed a good correlation between the lipophilicity and the entrapment efficiency within PLGA nanoparticles, with the highest efficiency obtained with the decanoate derivative. Palmitate ester prodrugs are usually entrapped with high efficiency in PLGA nanoparticles (Debotton et al., 2008; Tangsumranjit et al., 2006). It is interesting to note that using the same procedure as for preparing prodrug-loaded PLGA nanoparticles, nanoparticles were successfully prepared from pure chloramphenicol palmitate or thiamphenicol palmitate prodrugs, without the formation of crystalline precipitates, as observed by visual inspection of the preparations under a photonic microscope.

Microsphere preparation and properties

In order to prepare microspheres by spray-drying, the aqueous suspensions of nanoparticles were supplemented with lactose as a bulking agent and with leucin as a dispersing agent. The spray-drying method is widely used for drug encapsulation and produces monodisperse microspheres with high encapsulation efficiency and high drug loading (Gómez-Gaete et al., 2008). In the present work, all the dry powder formulations had a similar aspect under

scanning electron microscopy (SEM) which clearly shows discrete microsphere structures. The commercial powder, chloramphenicol palmitate and thiamphenicol palmitate appeared as crystalline, which was not the case in all prodrug dry powder formulations. This indicated that with this formulation, the drug was successfully entrapped, probably mostly in an amorphous and/or dissolved state. The microsphere surface appeared corrugated, but it was not possible to distinguish individual nanoparticles like in the case of previously described Trojan particles (Gómez-Gaete et al., 2008). This might be caused by the use of a different bulking agent and/or the melting and merging of the nanoparticles exposed to high temperatures during the spray-drying process. This shriveled shape of the microspheres was attributed to the collapse of the microsphere structure during the spray-drying process and was reported to be promoted by leucine (Seville et al., 2007; Yang et al., 2015). Particles with a high degree of surface corrugation were shown to possess better aerosolisation properties due to reduced contact area (Chew et al., 2005; Adi et al., 2008).

DTA-TGA analysis performed to analyze the interaction between chloramphenicol or thiamphenicol prodrugs with PLGA, leucin and lactose showed that CHLP and THAP are likely to be mostly in the amorphous or dissolved state in the microspheres.

All the powder formulations had MMAD appropriate to lung delivery as aerosol, as determined with the NGI. All the formulations showed a high dispersibility with high ED% values (above 70%) indicating that the microsphere powder

was efficiently emitted from the DPI. Fine particle fraction (FPF) of this formulation was satisfactory and was higher than values reported for some marketed formulations (15-30%) (Smith and Parry-Billings, 2003). These formulations may be efficient in delivering chloramphenicol palmitate and thiamphenicol palmitate to the lungs. As expected, the prodrug content was higher when the spray-dried powder was obtained from nanoparticles made of the pure prodrug, than from the prodrug-loaded PLGA nanoparticle suspension.

In release studies, all the formulations do not show any burst effects, which was attributed to the low solubility of the prodrugs. Prodrugs were released over 8 to 14 days depending on the formulation and on the prodrug. Release rate was faster in the case of microspheres of thiamphenicol palmitate than in the case of microspheres of chloramphenicol palmitate. This was attributed to the higher water solubility of the thiamphenicol moiety than the solubility of the chloramphenicol moiety, leading to a higher dissolution rate in the release medium. Moreover, release rates from microsphere powders prepared with PLGA nanoparticles were higher than from those made with pure prodrug nanoparticles. In the case of prodrug-loaded PLGA nanoparticles, prodrugs are dispersed within the PLGA matrix that gradually degrades. Such degradation is likely to produce pores within the nanoparticles that permit the entry of water and increase the exchange surface area with the release medium.

For both prodrugs, whatever the formulation, the release rates were much more slowly than in previously reported results obtained with dexamethasone

acetate-loaded PLGA nanoparticles (Gómez-Gaete et al., 2008). This can be explained by a higher affinity of the chloramphenicol palmitate or thiamphenicol palmitate prodrugs towards the PLGA polymer, resulting in a more slowly drug release.

It is difficult to estimate whether such relatively slow sustained release profiles of the prodrugs and their hydrolysis rate into the active drugs within the lungs will be appropriate to reach efficient chloramphenicol or thiamphenicol concentrations in the lung areas. Previous PK studies with sustained release rifampicin or levofloxacin-loaded PLGA microspheres showed that such delivery systems permitted to reach high epithelial lining fluid (ELF) to blood plasma drug concentration ratios due to the local release in the small ELF volume. In addition, little is known about the esterase activity within the lungs (Camps et al., 1991). Therefore, PK studies will be necessary to further investigate the therapeutic potential of the present formulations.

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CHAPTER 5

CONCLUSION AND PERSPECTIVES

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According to the biopharmaceutical investigations performed in vitro and in vivo, due to its high permeability, chloramphenicol had no advantage to be administered directly into the lungs compared to a systemic (intravenous) administration. Therefore, developing an immediate drug release formulation (solution or drug powder) of chloramphenicol for the pulmonary route would be useless to increase lung exposure and to diminish systemic exposure compared to the oral or the intravenous route of administration. Due to its intermediate permeability and to efflux transports inhaled delivery of thiamphenicol using an immediate release formulation may offer a little advantage compared to systemic routes. Therefore, sustained-release powders for inhalation were formulated in order to maintain high concentrations of chloramphenicol or thiamphenicol in the lungs, while permitting low systemic exposure responsible for toxicity. For technical and formulation reasons, palmitate prodrugs of chloramphenicol or thiamphenicol were used to design innovative microsphere-based sustained release powder for inhalation. Release studies performed in vitro showed that prodrugs were released over 8 to 14 days, depending on the formulation and the prodrug. Further studies are therefore needed to be carried out to evaluate the potential of the designed formulations:

a. It is necessary to evaluate the activity of esterases in the lungs, since the formulations are made with ester prodrugs which are converted into drugs

through ester hydrolysis. Therefore, the formulation efficiency is highly dependent on the pulmonary esterase activity.

b. Pharmacokinetic studies need to be performed in healthy rats in order to investigate the adequate dose of formulated powder to maintain efficient drug concentrations in the ELF, while minimizing the systemic exposure responsible for adverse effects. The amount of powder that can be administered is limited, and reformulation may be necessary to adapt the release profile to obtain efficient ELF chloramphenicol or thiamphenicol concentrations.

c. Pharmacokinetic studies in infected rats shall be necessary to evaluate the effect of pathologic conditions on the release profiles of the formulations.

d. Meanwhile, in vitro pharmacodynamic studies shall be performed to demonstrate the antimicrobial activity of the sustained-release chloramphenicol and thiamphenicol powders for inhalation.

e. Toxicity studies shall also be performed in order to demonstrate that low systemic exposure that is expected with the innovative dry powders for inhalation suppresses or at least mitigates chloramphenicol or thiamphenicol toxicity, in particular the reversible dose-dependent bone marrow suppression that are reported in the case of prolonged treatments (more than 7 days).