Development of new antibiotic combinations to be administered by dry powder for inhalation to patients with Cystic Fibrosis: microbiological, pharmacological and pharmaceutical studies.

M.Hariri Mustafa, Dipl-Ing(MSc), MRes, PhD student

Molecular and Cellular Pharmacology Unit

SMB Laboratories

Université Catholique de Louvain

Promotor : Pr Françoise Van Bambeke

Co-promotor : Dr Francis Vanderbist

Brussels, Belgium

12/01/2017

Once upon a time, there is a happy place…

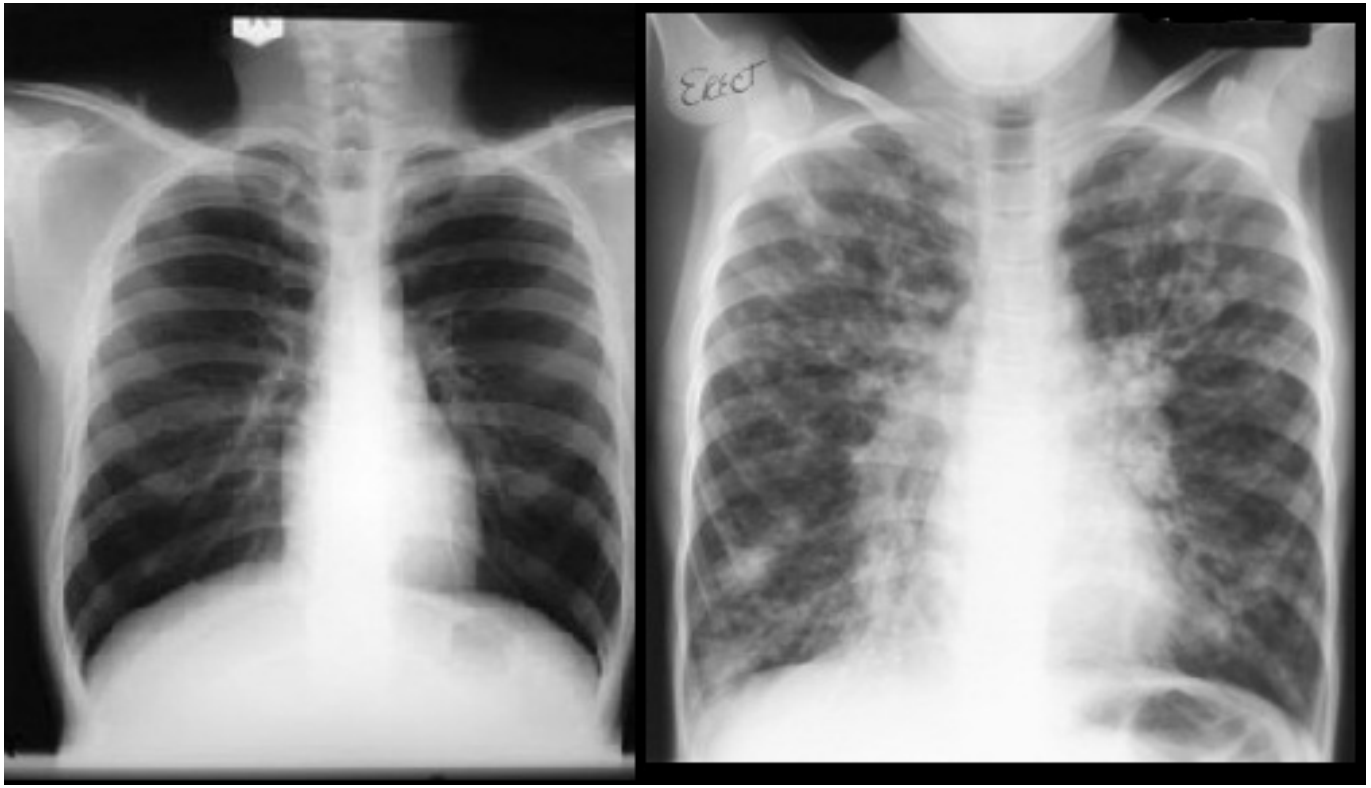
The happy place is open to the outside world…

The dangers come to the happy place…uninvited..

The dangers invade the happy place….

The happy place is the human lung

The happy place is sick with
Cystic Fibrosis



Healthy lung

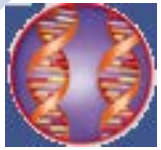
Cystic Fibrosis lung

Cystic Fibrosis: Prevalence

Cystic Fibrosis is the most common lethal genetic disease in Caucasian population with 70,000 people affected worldwide (30,000 in USA and 40,000 in Europe)

- **1/3000 in Europe, North America, and Australia**
- 1/6000 in Middle East (Bahrain)
- 1/7000 in South America (Brazil)
- 1/12 000 in Africa (South Africa)
- 1/350 000 in Asia (Japan)

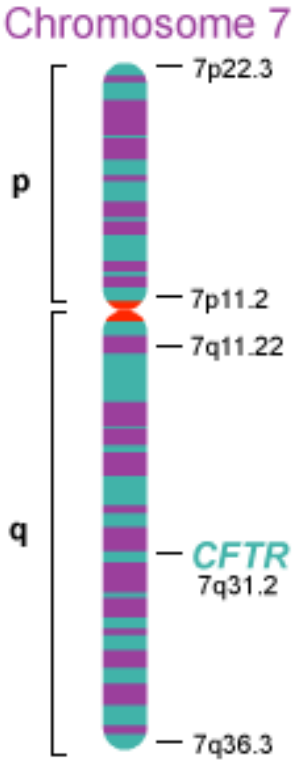
Cystic Fibrosis: Pathophysiology



- CF is caused by mutations in *Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)* gene

***CFTR* gene**

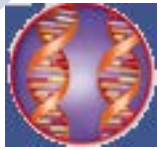
- **located at the q31.2 locus of chromosome 7**
- **230,000 base pairs long**
- **over 2,000 mutations detected**



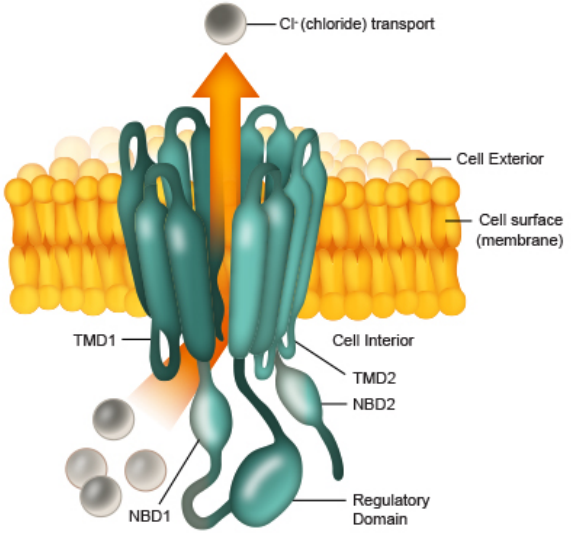
Cystic Fibrosis: Pathophysiology

CFTR protein

- **1,480 amino acids long**
- **transmembrane protein found apical surface of epithelial cells that line the airways, pancreatic ducts, and other tissues**
- **functions as a chloride channel but also regulates other transporters (sodium ions)**

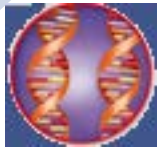


- Mutation in ***CFTR*** gene

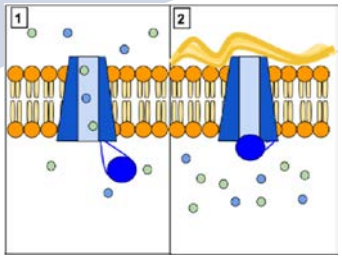


CFTR.info

Cystic Fibrosis: Pathophysiology

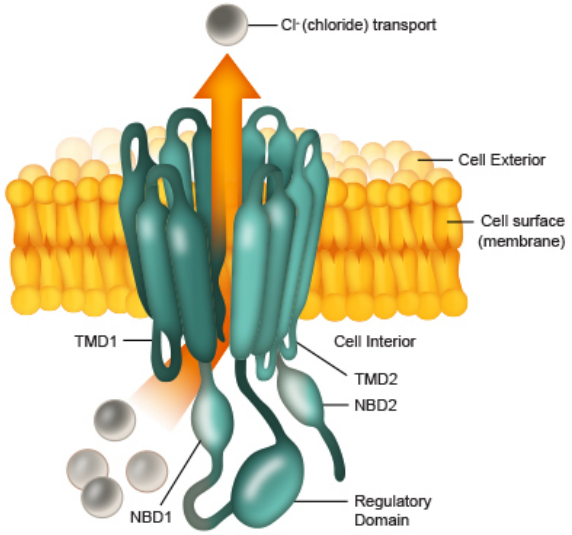


Mutation in ***CFTR*** gene



Defective **CFTR protein** (chloride ion channel) on **epithelial cells membrane**

compromised movement of Cl^-, Na^+, HCO_3^-



CFTR.info

Cystic Fibrosis: Pathophysiology

Skin

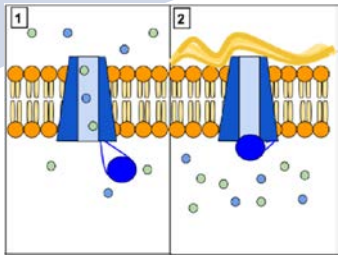
Lung

Liver

Pancreas

Intestine

Genital organs



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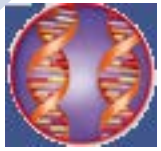

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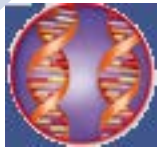

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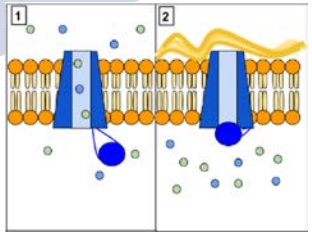


Defective CFTR protein (chloride ion channel)

= compromised movement of Cl^-, Na^+, HCO_3^-

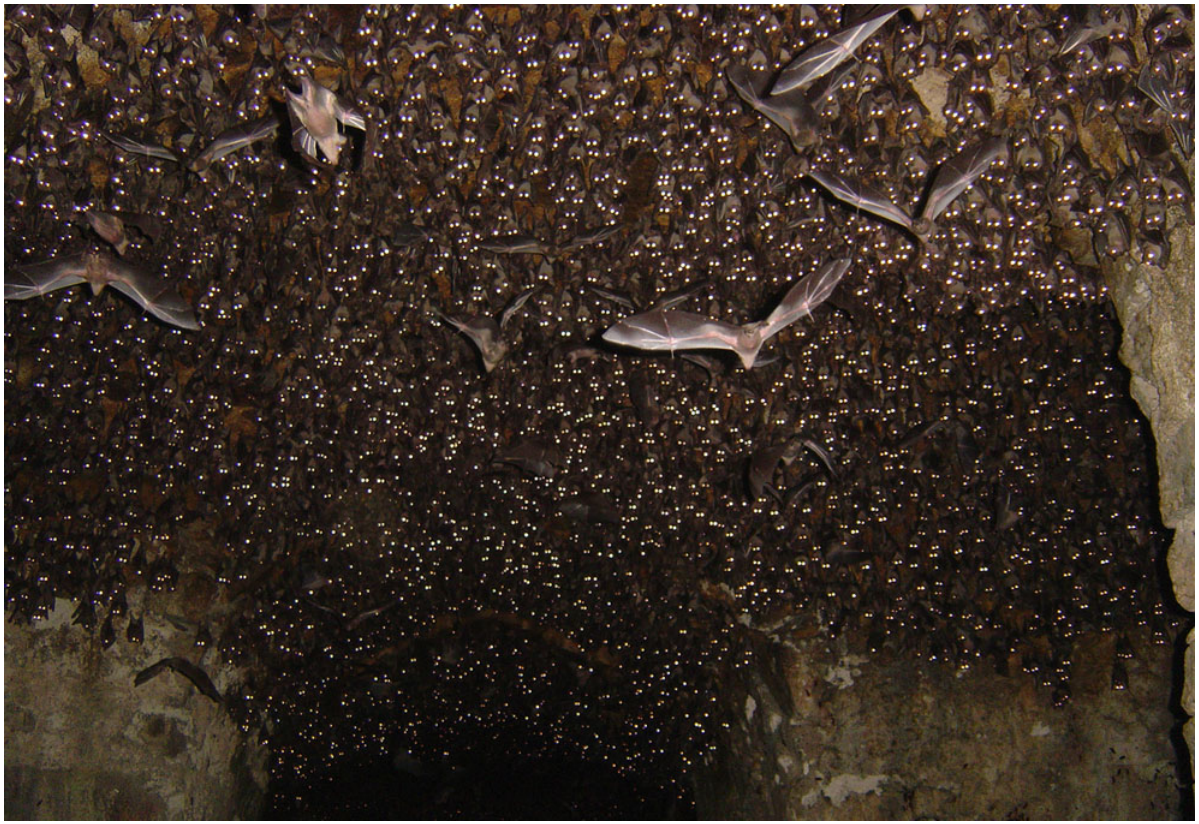
In the **lung**:

- Dehydration
- Mucus thickness
- Impaired mucociliary clearance

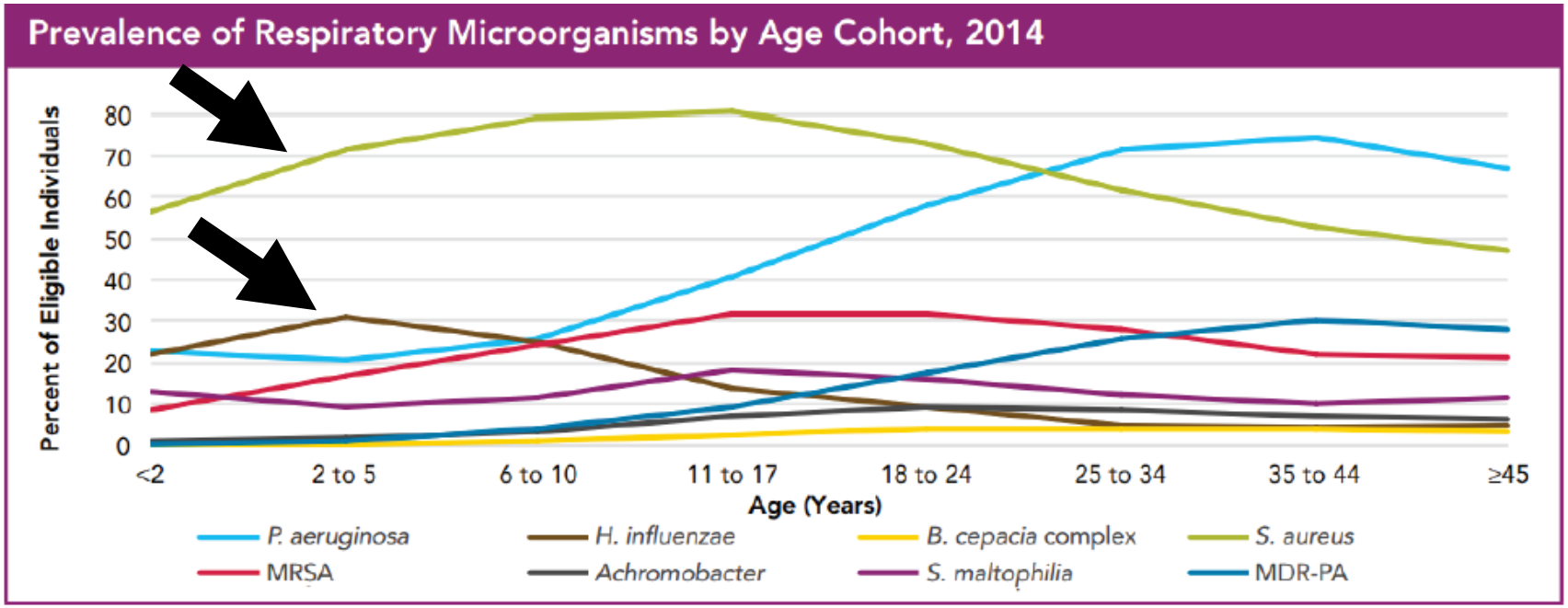
↓

- **Hyper-inflammation**
- Accumulation of **pathogens** and **chronic lung infection**

The dangers is the bacteria infecting the lung of patients with CF



Microorganisms infecting the lung of patients with CF



Cystic Fibrosis Foundation, 2014 Patient Registry Report

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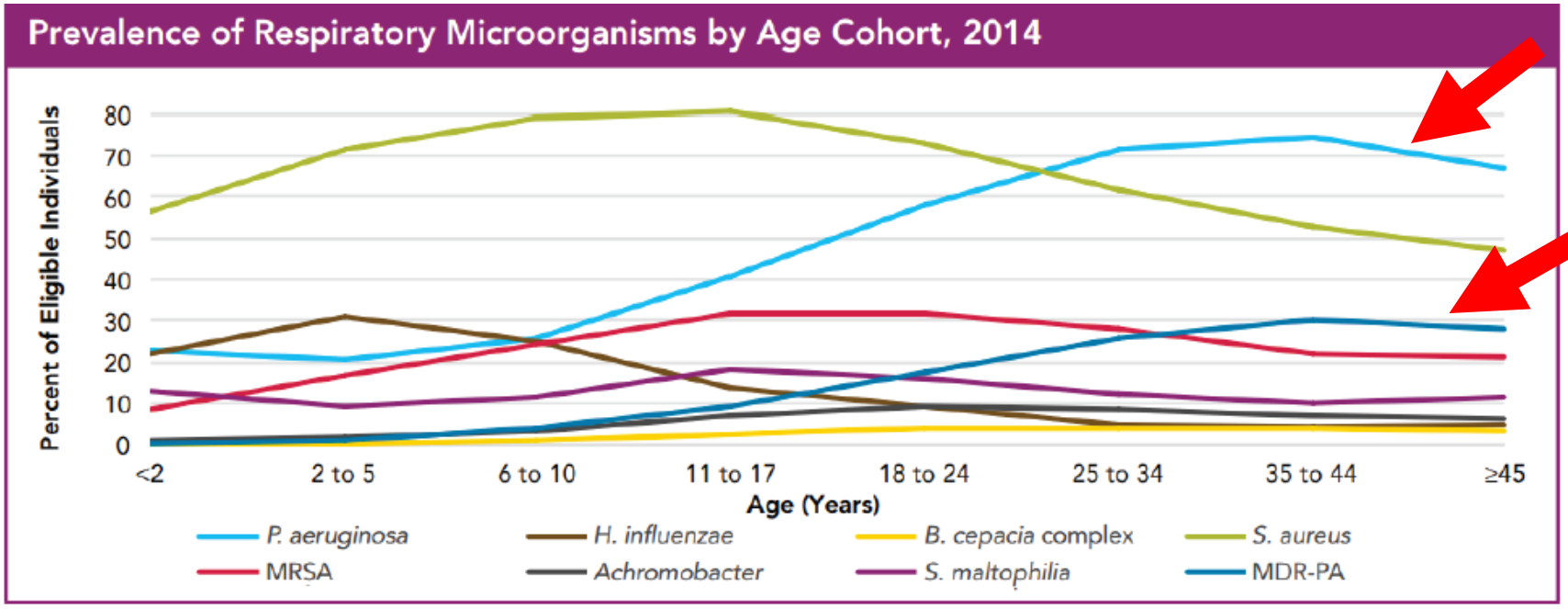


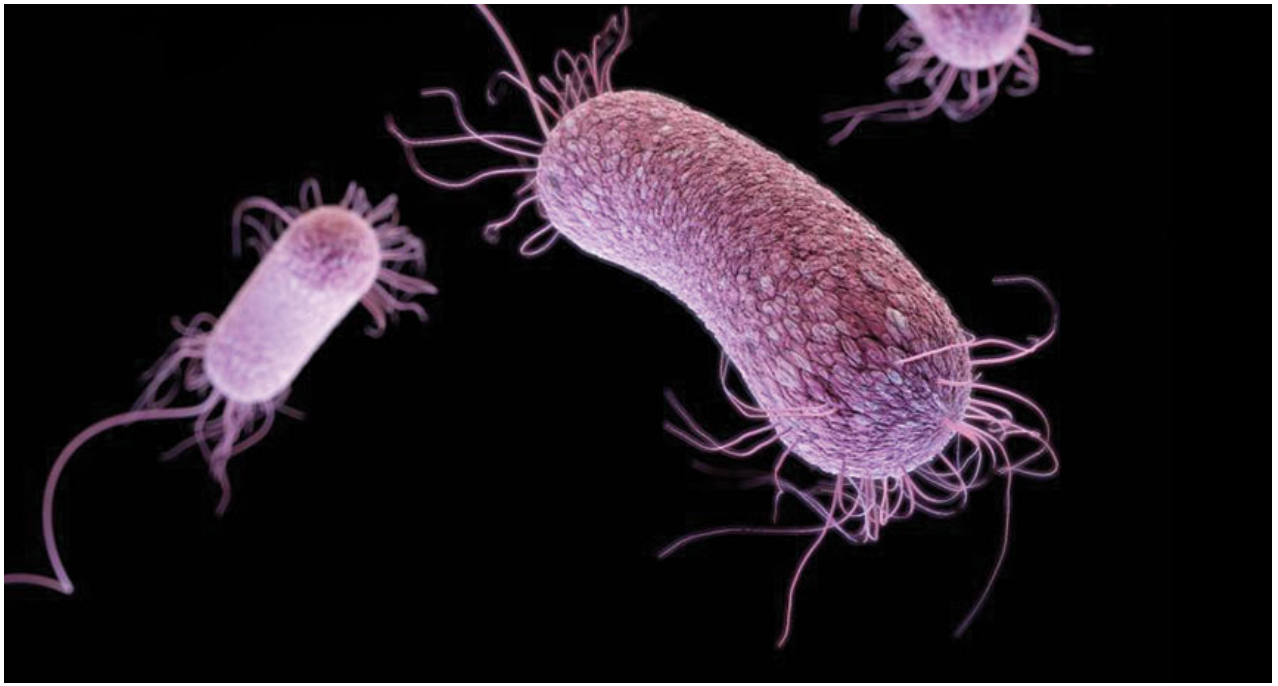
Cystic Fibrosis Foundation, 2014 Patient Registry Report

Pseudomonas aeruginosa **is the most common pathogen in adults patients**

P. aeruginosa: the pathogen

Pseudomonas aeruginosa

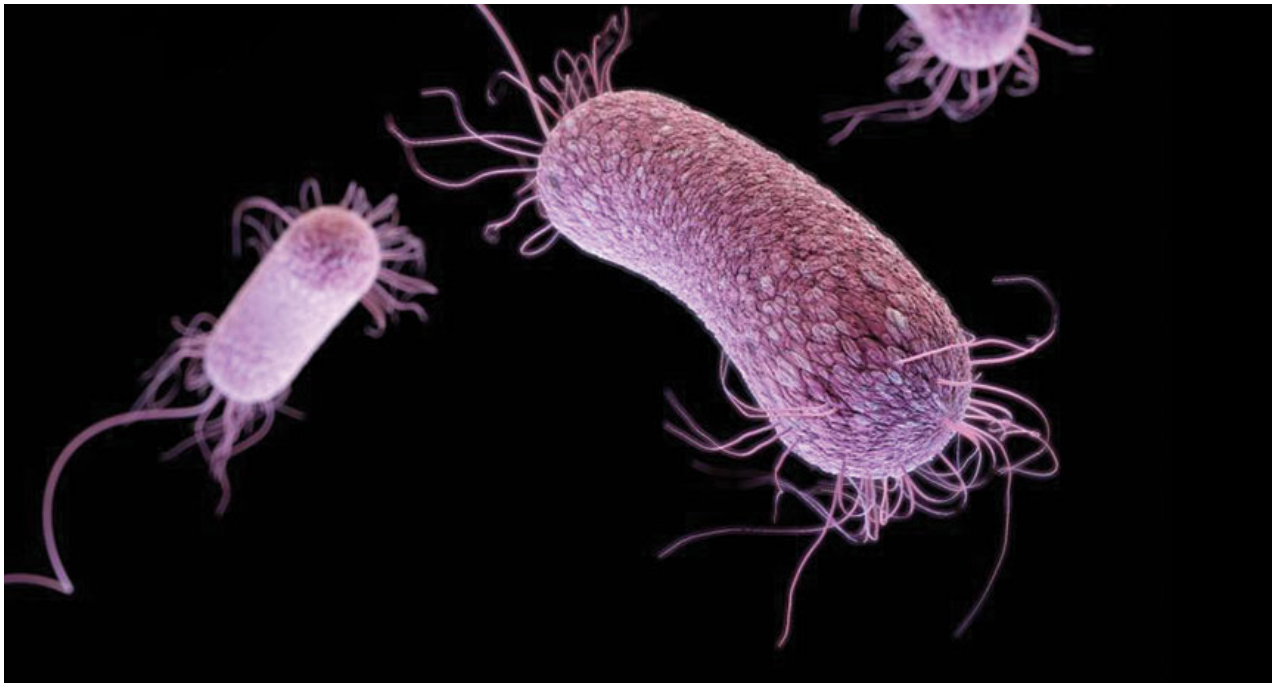
- **Gram-negative bacillus**
- **Opportunistic (immunocompromised patients and CF)**
- **Ubiquitous environmental bacterium**
- **Infects the airway, urinary tract, burns, and wounds, and also causes blood infections**



P. aeruginosa: the pathogen

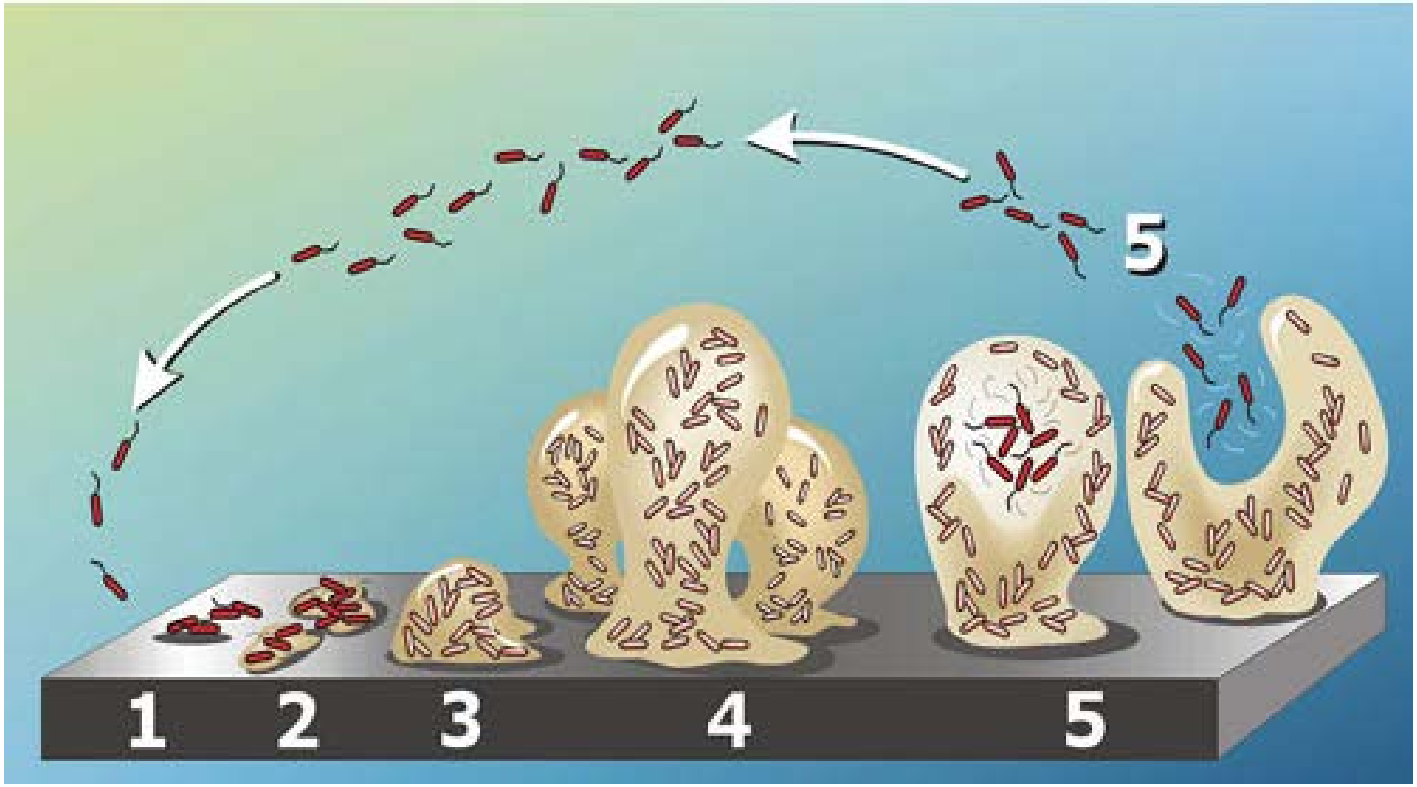
Why *P. aeruginosa* is a problem for patients with CF?

- **Caused chronic lung infection**
- ***P. aeruginosa* is intrinsically resistant and can acquire different resistance mechanism**
- ***P. aeruginosa* forms biofilms**



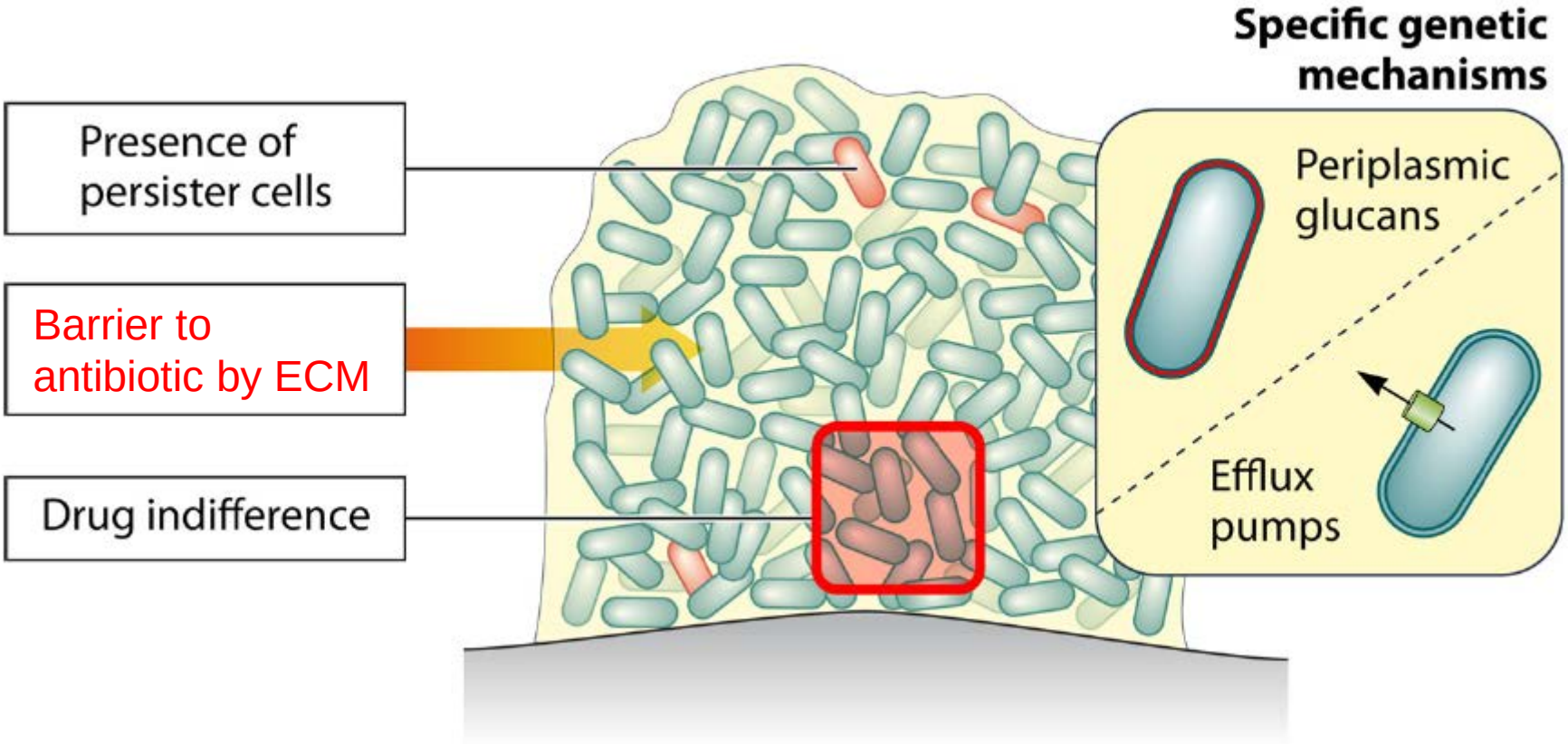
What are biofilms?

= community of bacteria, living together in a self-produced extracellular matrix (ECM) embedded on a surface



Graphic and photos by Peg Dirckx and David Davies © 2003 Center for Biofilm Engineering Montana State University.

Mechanisms of antibiotic tolerance within bacterial biofilm

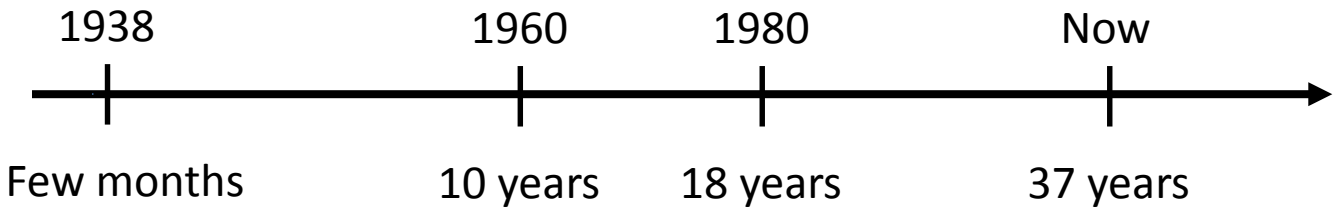


Adapted from David Lebeaux et al, Microbiol Mol Biol Rev. 2014

The happy place needs helps to expulse the dangers ….

Cystic Fibrosis: Life expectancy

Cystic Fibrosis: The evolution of life expectancy of patients



Progress in Pediatrics

CYSTIC FIBROSIS OF THE PANCREAS AND ITS RELATION TO CELIAC DISEASE

A CLINICAL AND PATHOLOGIC STUDY

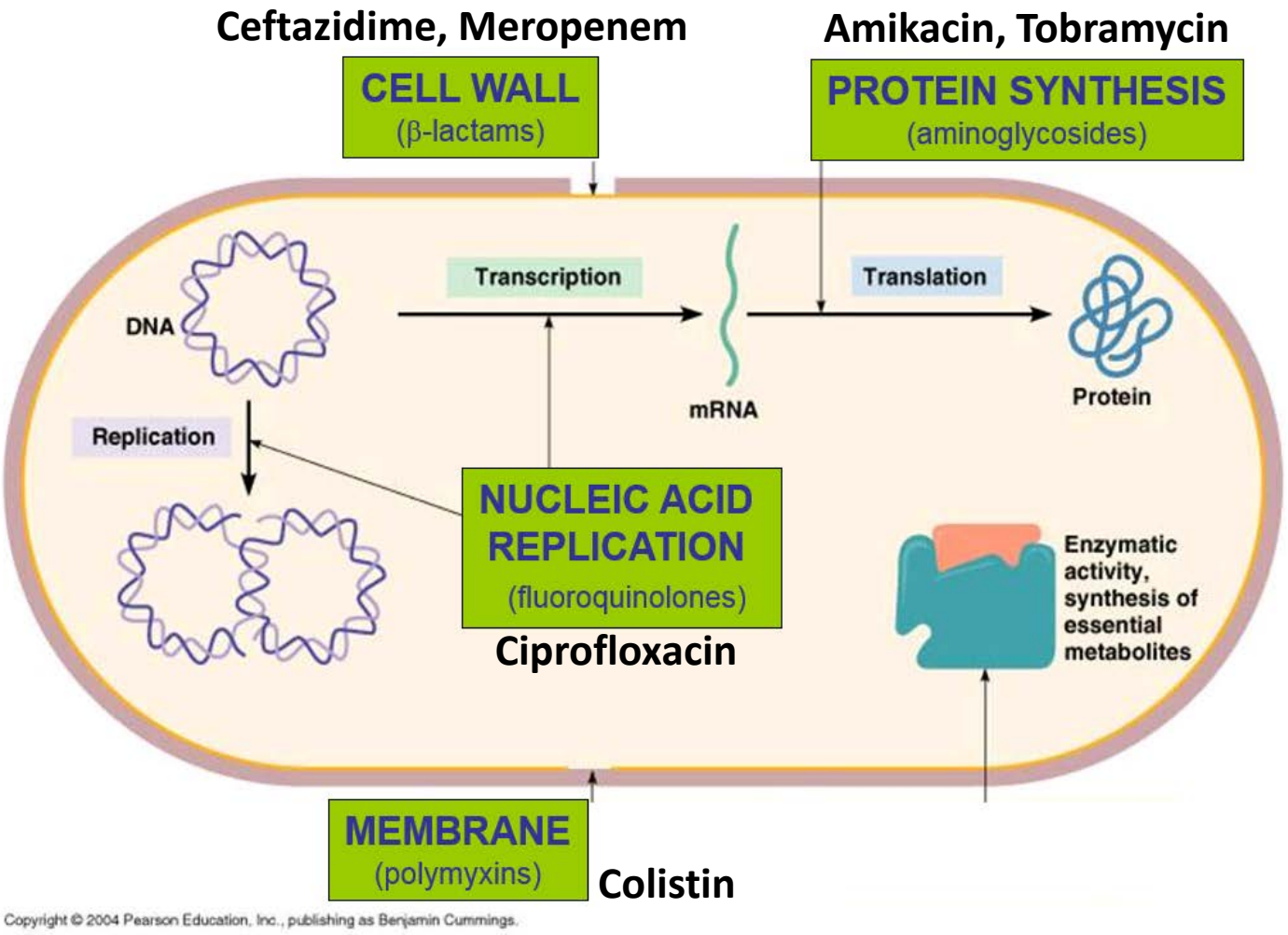
DOROTHY H. ANDERSEN, M.D.
NEW YORK

The pathology and pathologic physiology of celiac disease remain obscure in spite of the many attempts that have been made to understand them. It has become clearer in recent years that celiac disease is a clinical picture "characterized by arrest of growth, a distended abdomen, and attacks of diarrhoea with large, pale, foul-smelling stools" [1] rather than a disease entity and that the underlying pathologic condition may differ in different cases.[2] A tradition exists that pancreatic steatorrhea can be readily differentiated from idiopathic steatorrhea by the low percentage of split fat in the stools associated with the former and the normal percentage characterizing the latter. A careful survey of the literature, however, reveals few cases of either disease in which careful clinical observations have been followed by adequate postmortem examination. The present study was initiated because of the findings in case 44 (XX), in which a patient with celiac disease who had a high percentage of split fat in the stools and who had responded favorably to treatment for celiac disease was found at autopsy to have cystic fibrosis of the pancreas. This demonstrated that the recognized criteria of differentiation between the steatorrhea due to pancreatic insufficiency and that due to other causes are unreliable.

To establish more reliable criteria it is necessary to study cases in which normal acinar tissue has been proved by postmortem examination to be absent or inadequate and to compare them with cases of celiac disease associated with a normal pancreas. The plan of the present investigation has been to collect the cases in which pancreatic insufficiency has been proved by microscopic examination of the pancreas, to

Read at the joint meeting of the American Pediatric Society with the Society for Pediatric Research on May 5, 1938.

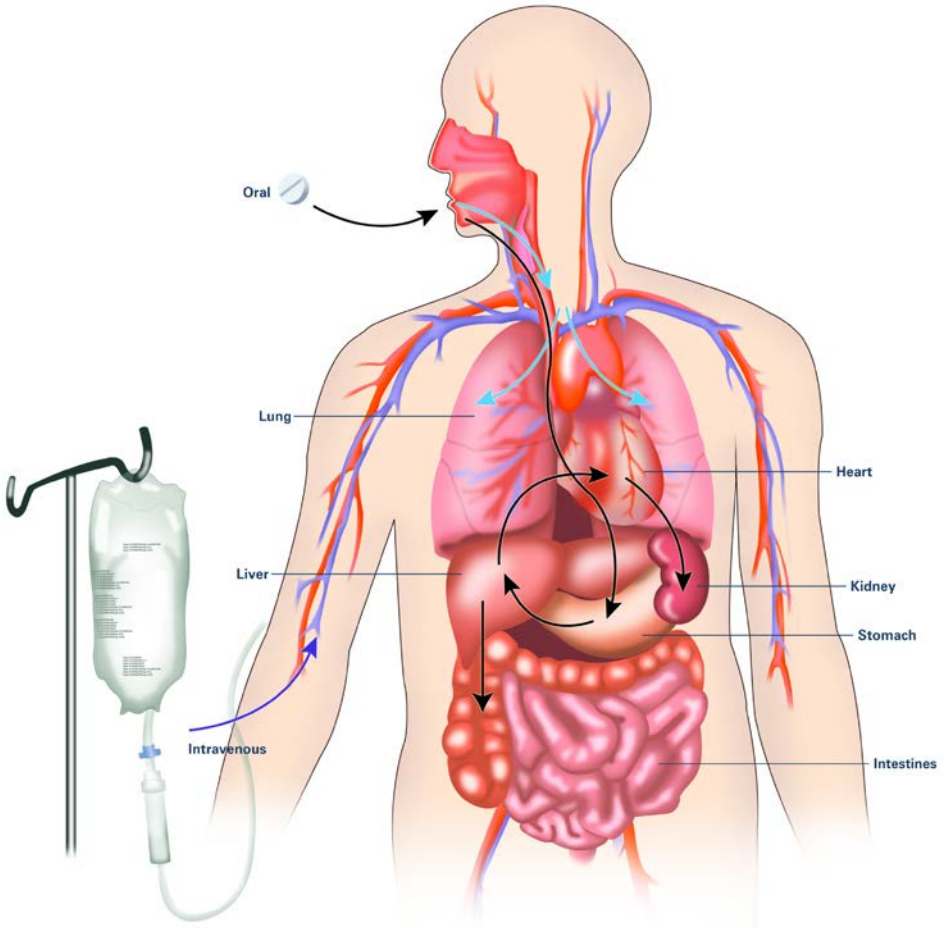
Antibiotic classes used to treat *P. aeruginosa* infection



Antibiotics => The cave explorers to the rescues!

Antibiotic administration route:

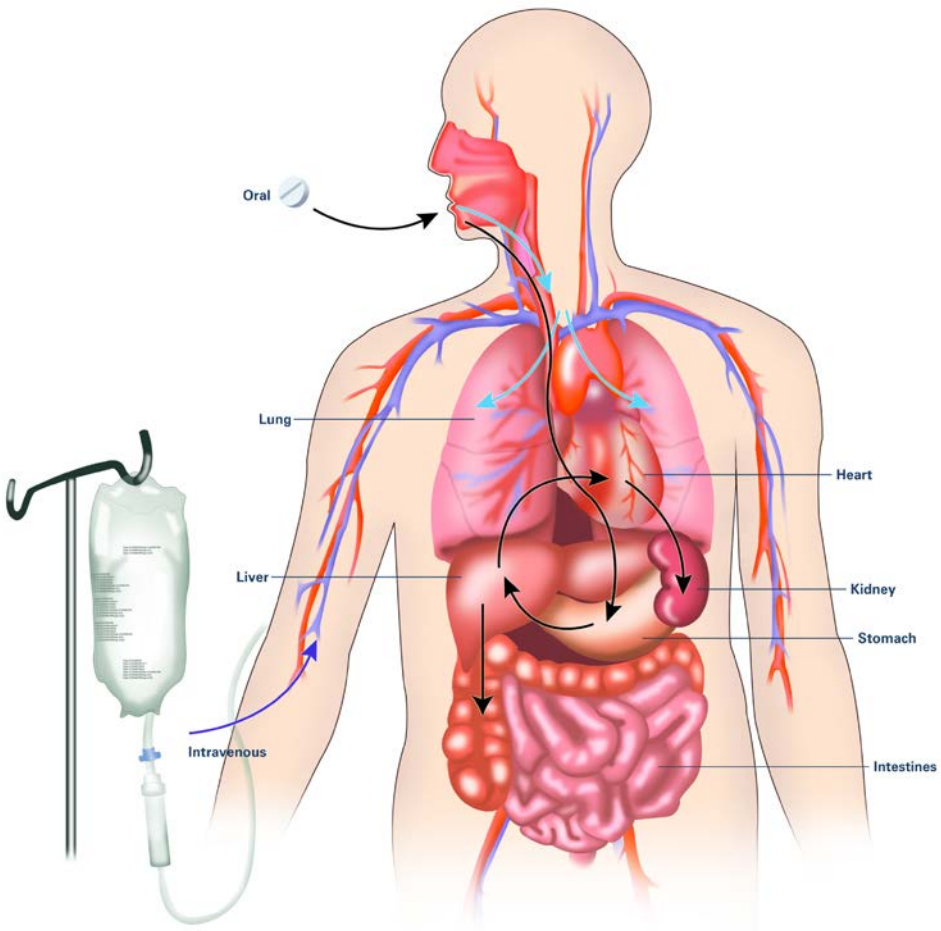
oral and intravenous



National Institute of Health (NCBI)

Antibiotic administration route:

oral and intravenous

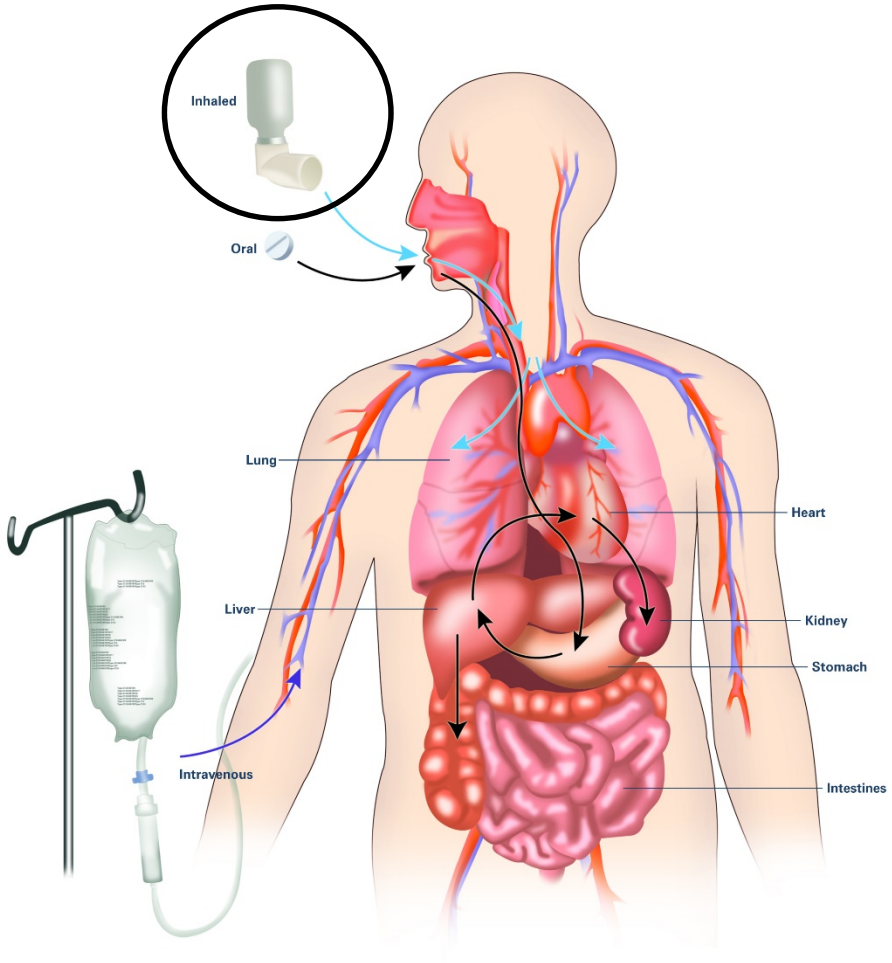


National Institute of Health (NCBI)

But there's a better way for the cave explorers to arrive!

Antibiotic administration route:

pulmonary route (inhalation)

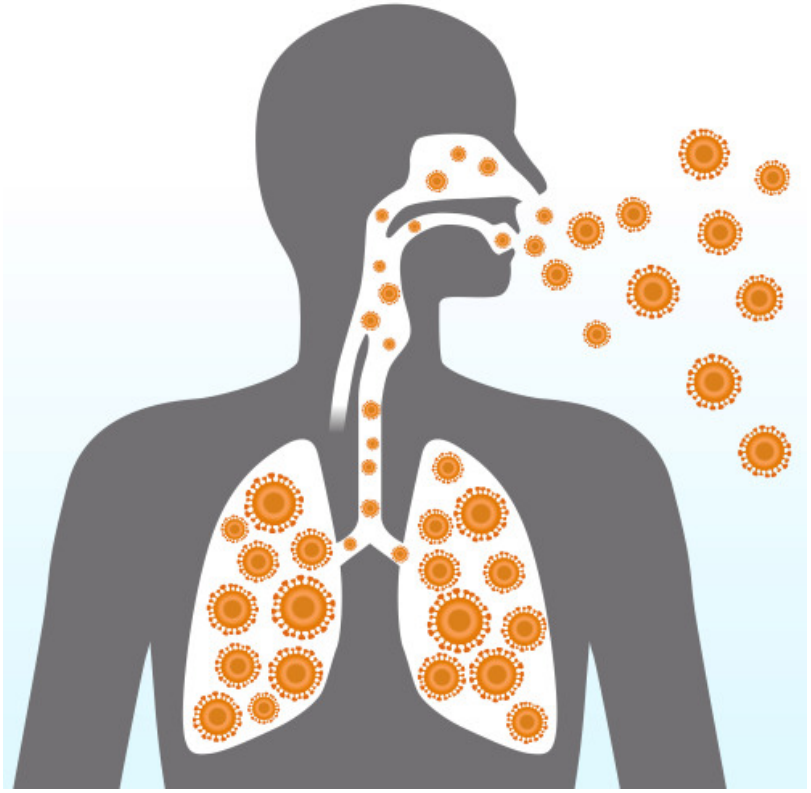


National Institute of Health (NCBI)

Pulmonary Drug Delivery

Local treatment:

- In situ
- Rapid onset of action
- Fewer side effects
- High local concentration



Bronchodilators

Anticholinergics (ipratropium, tiotropium...)

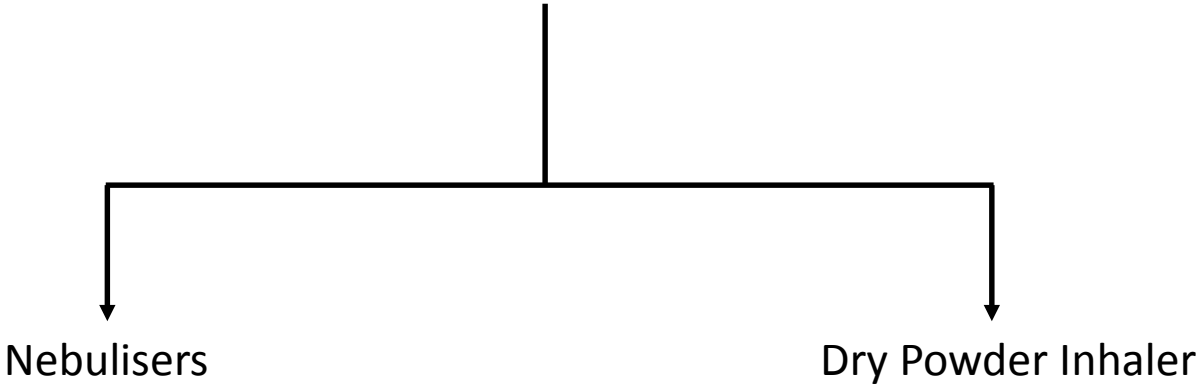
ß$_2$-mimetics (salbutamol, formoterol...)

Corticoids (budesonide, fluticasone...)

Mucolytics (acetylcysteine...)

Antibiotics (tobramycin, colistin...)

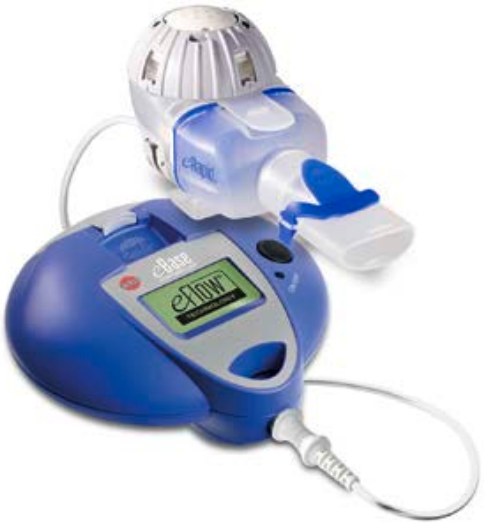
Pulmonary Drug Delivery



Pulmonary Drug Delivery

Nebulisers

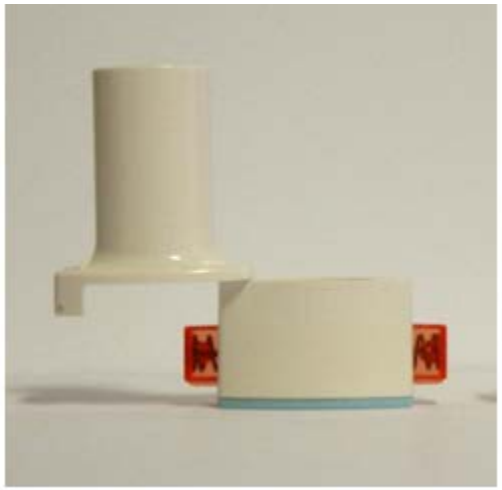
Dry Powder Inhaler



Pulmonary Drug Delivery

Nebulisers

Dry Powder Inhaler



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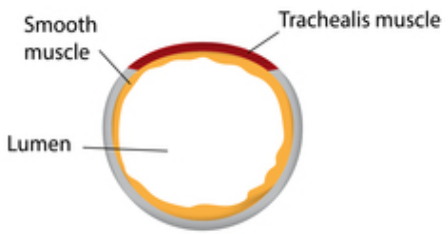
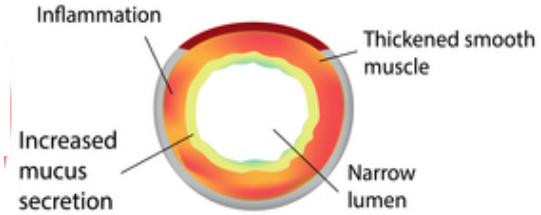
Treatment of pseudomonal infection



Healthy



Cystic Fibrosis

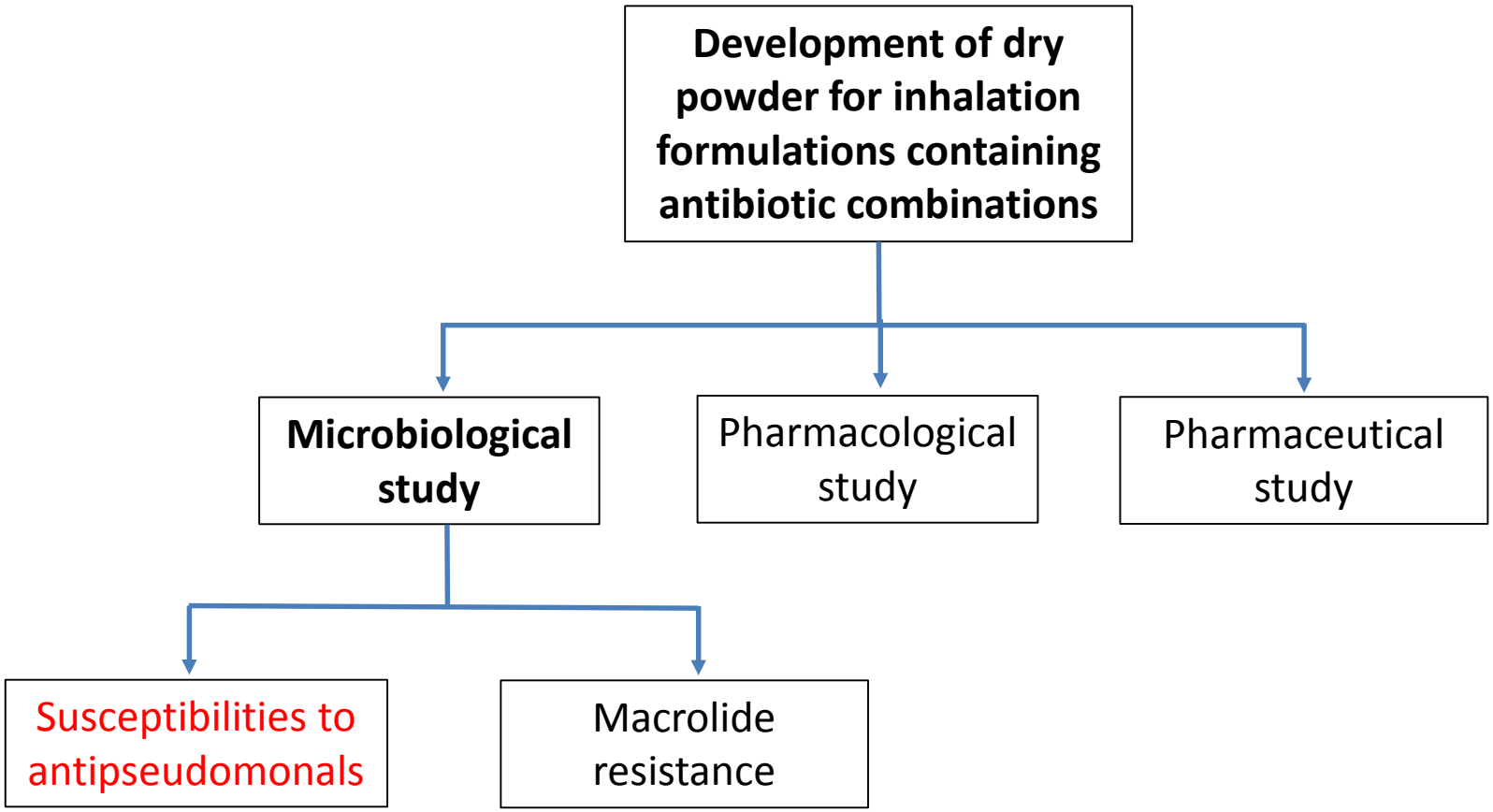
CF lung disease

Hyper-inflammation

Accumulation of **pathogens** and **chronic lung infection**

Combination:

Macrolide and **Antipseudomonal antibiotic**


Development of dry powder for inhalation formulations containing antibiotic combinations
Microbiological study
Pharmacological study
Pharmaceutical study
Susceptibilities to antipseudomonals
Macrolide resistance

Susceptibility to antipseudomonals

TABLE 1 *P. aeruginosa* collection (2006 to 2012)

Country	No. of isolates	No. of patients	Period of sampling
Belgium	44	38	2010
Germany	51	36	2012
United Kingdom	58	46	2006–2009
Total	153	120	

Objective: Characterize the rate of resistance to antipseudomonal antibiotics in a collection of isolates from CF patients in Northern Europe

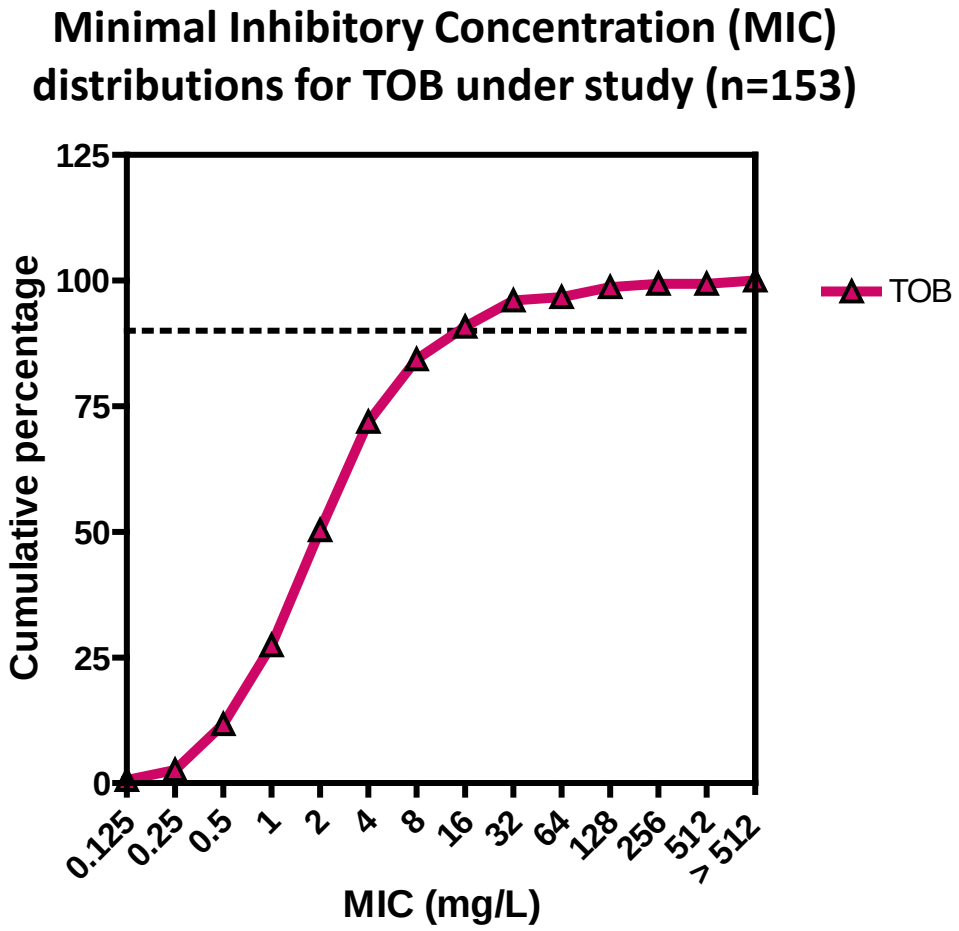
AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Antimicrobial Susceptibility of *Pseudomonas aeruginosa* Isolated from Cystic Fibrosis Patients in Northern Europe

Muhammad-Hariri Mustafa,[a,b] Hussein Chalhoub,[a] Olivier Denis,[c] Ariane Deplano,[c] Anne Vergison,[c]* Hector Rodriguez-Villalobos,[d] Michael M. Tunney,[e] J. Stuart Elborn,[e] Barbara C. Kahl,[f] Hamidou Traore,[b] Francis Vanderbist,[b] Paul M. Tulkens,[a] Françoise Van Bambeke[a]

Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium[a]; SMB Laboratories, Brussels, Belgium[b]; Hôpital Erasme/Hôpital des Enfants Malades, Université libre de Bruxelles, Brussels, Belgium[c]; Departement of Microbiology, Cliniques Universitaires Saint-Luc, Université catholique de Louvain, Brussels, Belgium[d]; The Queen's University of Belfast, Belfast, United Kingdom[e]; University Hospital Münster, Münster, Germany[f]

Susceptibility to antipseudomonals



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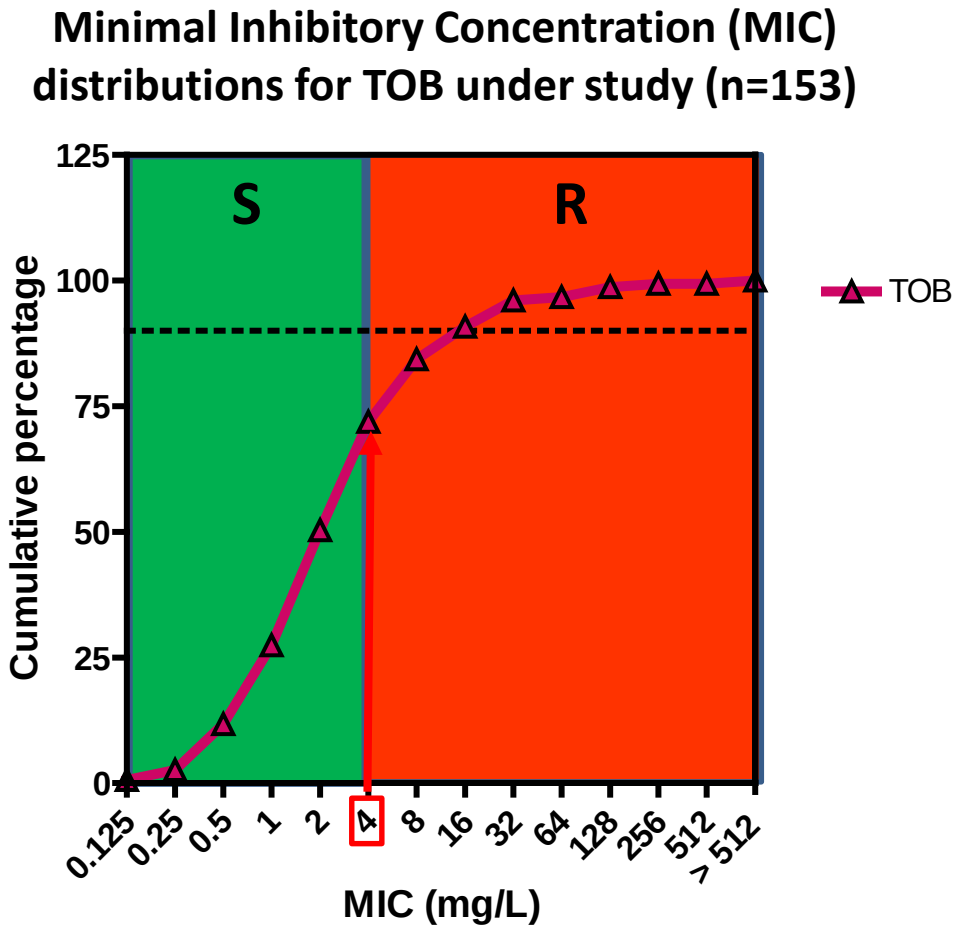

TOB Breakpoint according to EUCAST

Threshold concentration to predict therapeutic success
(in vitro, PK animal and human)

Susceptibility to antipseudomonals

MIC distribution of different antipseudomonals and resistance rates

TIC: Ticarcillin; PIP: Piperacillin; TZP: Piperacillin-tazobactam; CAZ: Ceftazidime; CIP: Ciprofloxacin; AMK: Amikacin; TOB: Tobramycin; MEM: Meropenem; CST: Colistin

	Susceptibility according to:		
	EUCAST[b]		
Antibiotic	% S	% I	% R
TIC	16	NA	84
PIP	24	NA	76
TZP	29	NA	71
CAZ	31	NA	69
CIP	24	20	56
AMK	22	17	61
TOB	72	NA	28
MEM	44	36	20
CST	92	NA	8

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Tobramycin is one of the most active drugs

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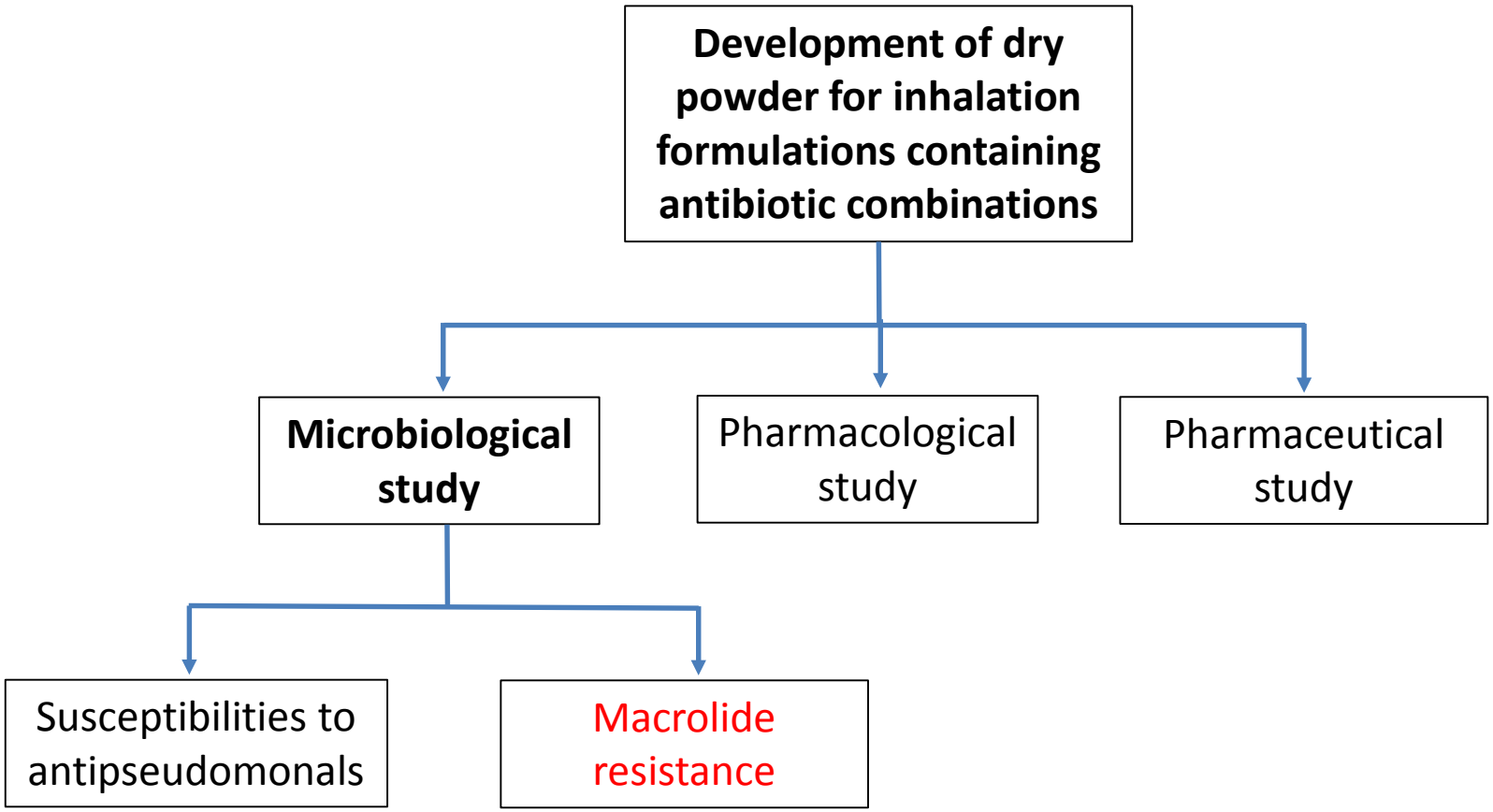
Tobramycin is one of the most active drugs

Multidrug resistant (MDR) isolates = 94/153 isolates => 61%!

Microbiological study - Resistance in CF

Main findings:

- Resistance was highly prevalent in routine *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis in Northern Europe.
- Tobramycin, meropenem and colistin were the most active drugs.
- **Interest in achieving high concentration by pulmonary route.**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Pharmacological study
Pharmaceutical study
Susceptibilities to antipseudomonals
Macrolide resistance

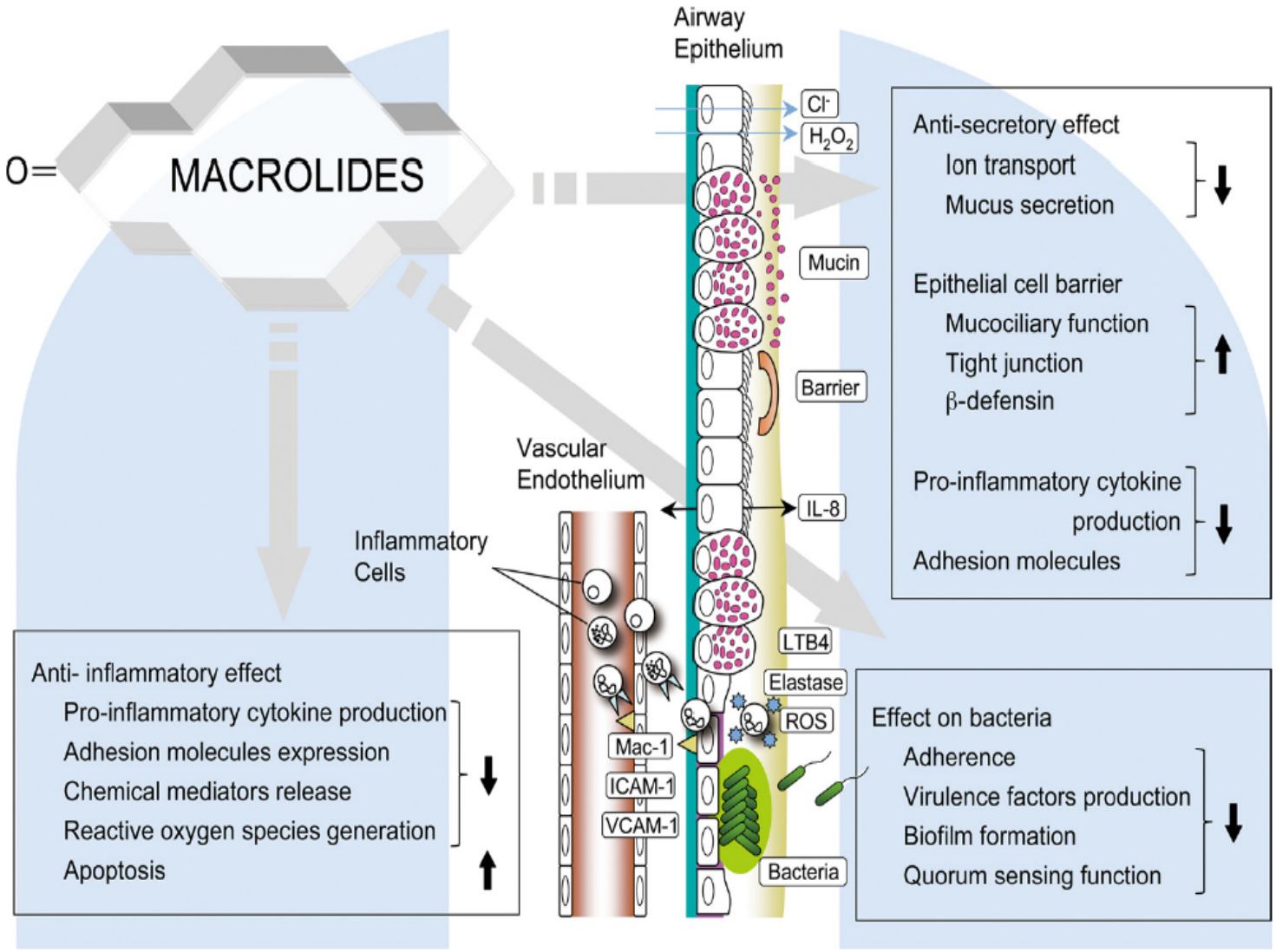
Macrolides and CF

- Not active against *Pseudomonas aeruginosa:*
  - MIC (Azithromycin) = **128 to 512** µg/mL
  - Concentration in the sputum after oral administration: **26.6** µg/mL *(E.B. Wilms et al, 2008)*

- Given to CF patients **via oral route regularly** (70% of patients in USA) for the their **immunomodulatory and anti- inflammatory** effects

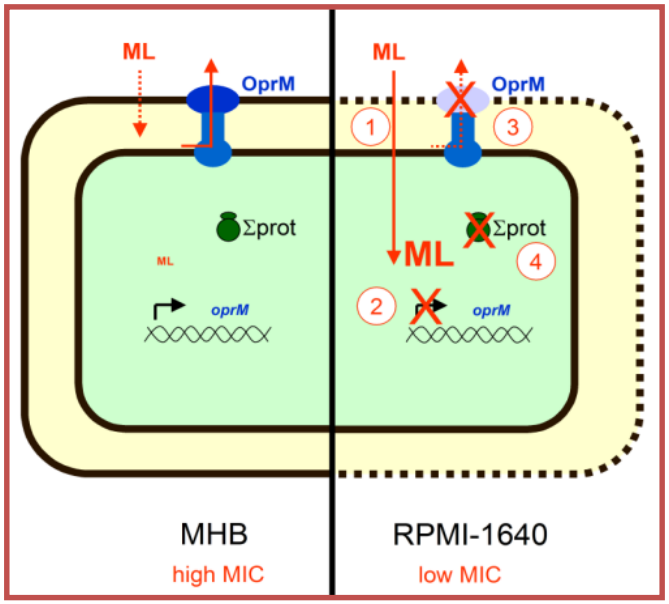
  => improved lung function and reduce exacerbations

Macrolides in CF lung disease



Kanoh et al, Clin Microbiol Rev. 2010

Intrinsic resistance to macrolides

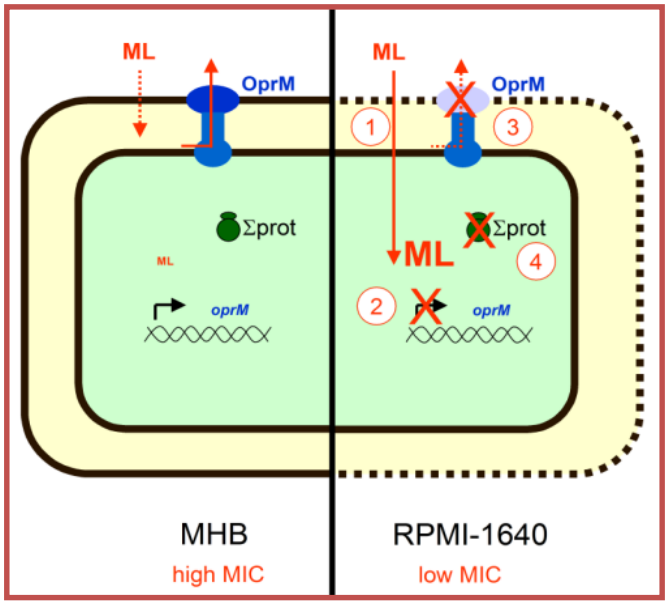


Serum or BAL fluid

Buyck et al. CID (2012)

MIC of PAO1 : 512 µg/mL 32 µg/mL

Intrinsic resistance to macrolides



Buyck et al. CID (2012)

Acquired resistance to macrolides?

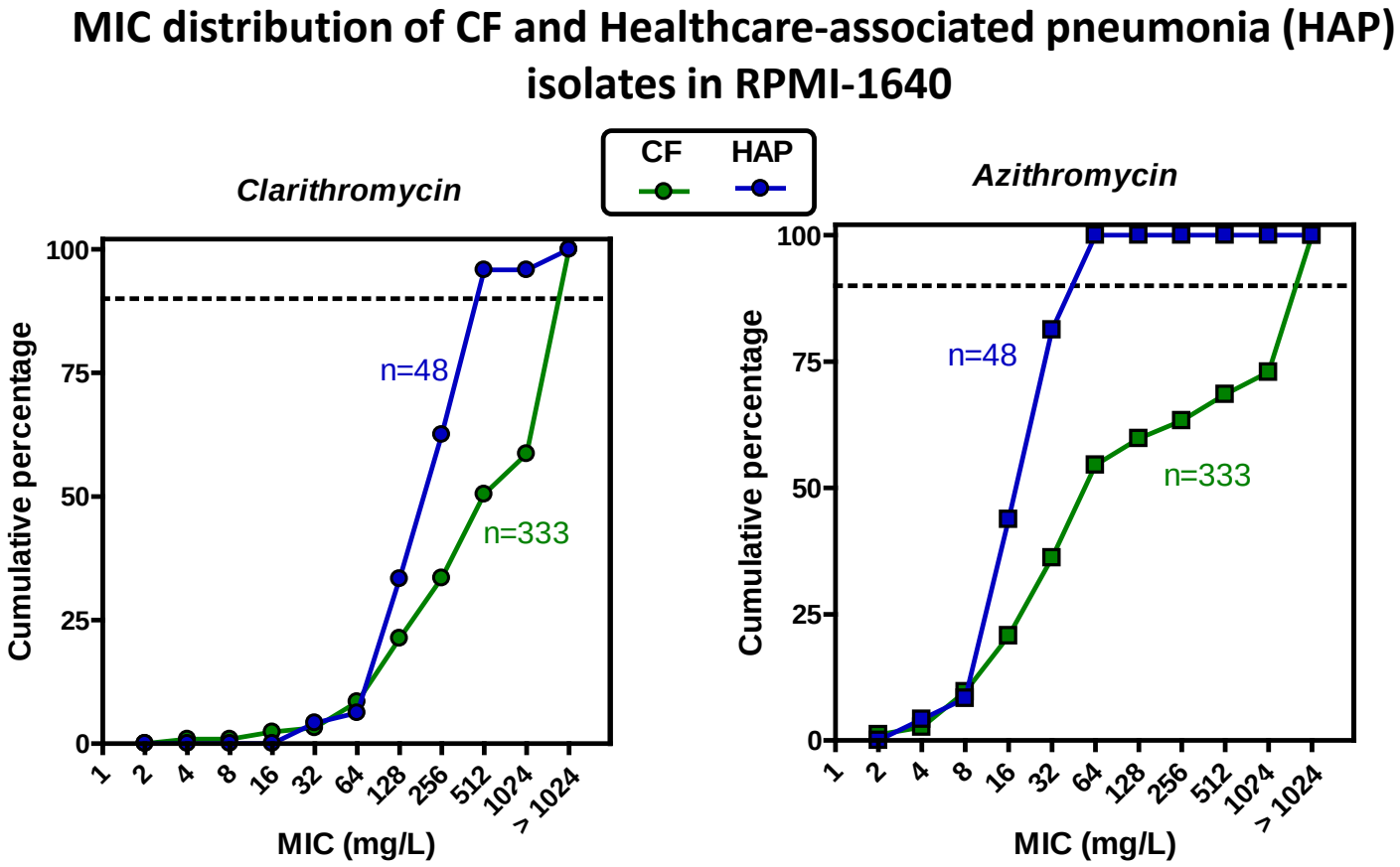
Objective: Study the activity of macrolides and look for resistance mechanisms in CF isolates

EUROPEAN RESPIRATORY *journal*
OFFICIAL SCIENTIFIC JOURNAL OF THE ERS

Acquired resistance to macrolides in *Pseudomonas aeruginosa* from cystic fibrosis patients

Muhammad-Hariri Mustafa[1,2], Shaunak Khandekar[1], Michael M.Tunney[3], J. Stuart Elborn[3], Barbara C. Kahl[4], Olivier Denis[5], Patrick Plésiat[6], Hamidou Traore[2], Paul M. Tulkens[1], Francis Vanderbist[2], Françoise Van Bambeke[1,*]

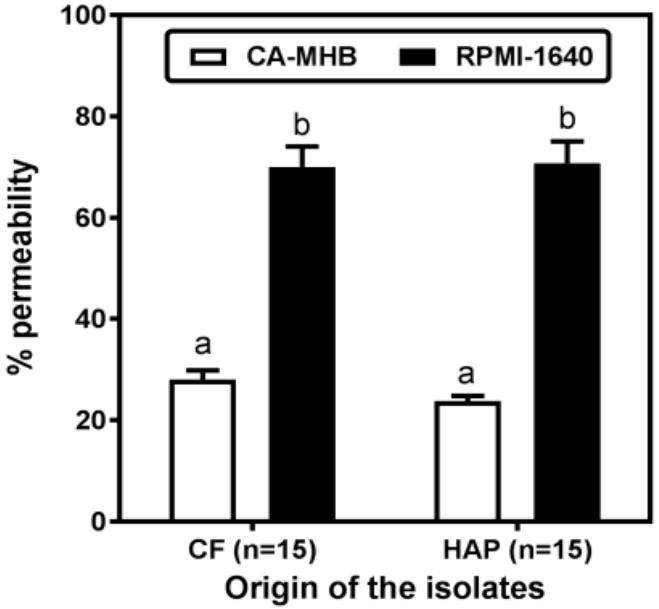
Acquired resistance to macrolides?

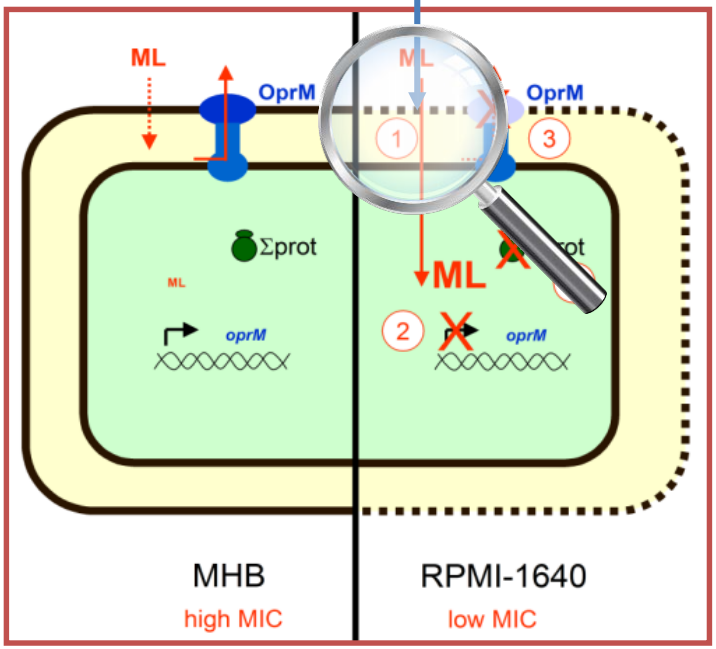


CF isolates are less susceptible than HAP isolates

Acquired resistance to macrolides?

Outer-membrane permeability?



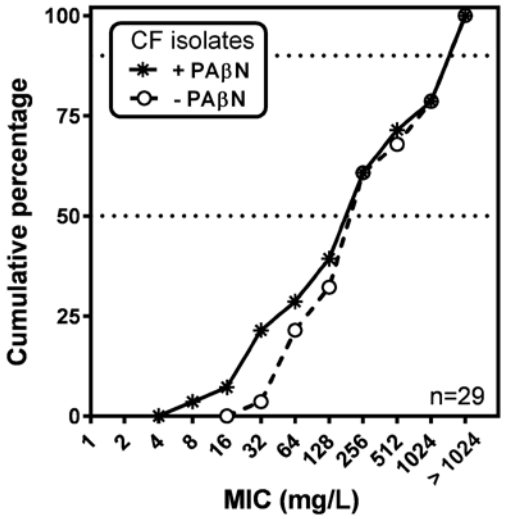


Difference in outer-membrane permeability and is NOT the reason behind high resistance among CF isolates

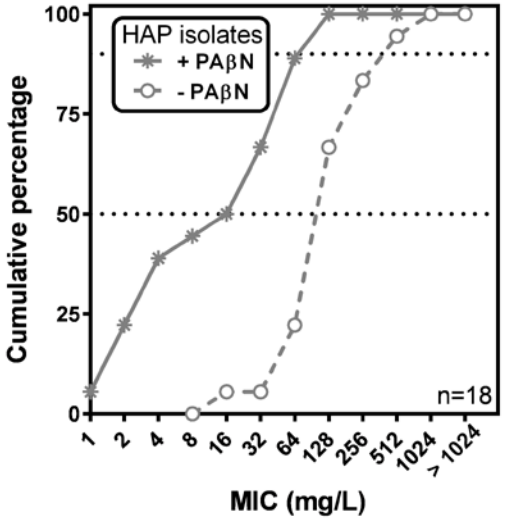
Acquired resistance to macrolides?

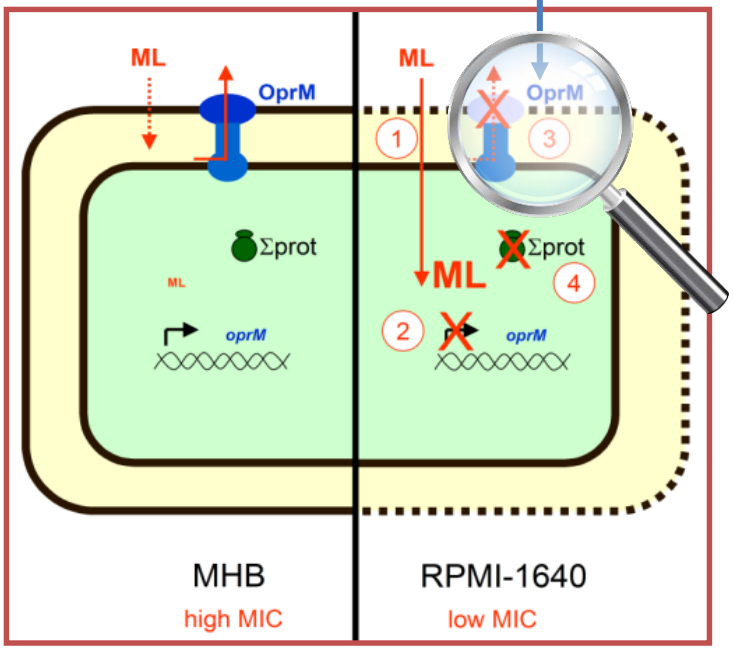
Efflux pumps?

CF isolates



HAP isolates

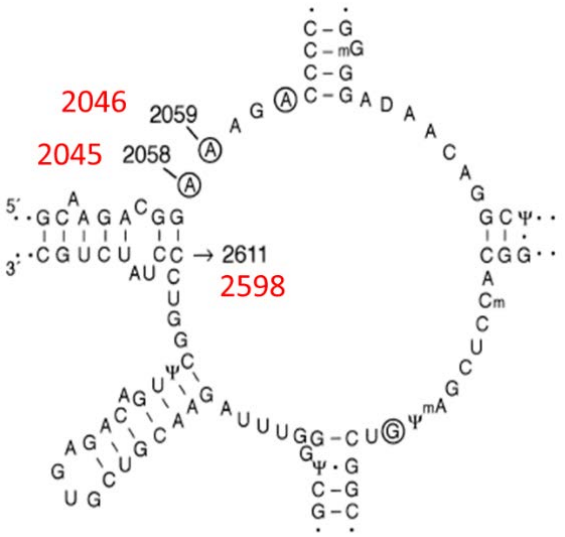


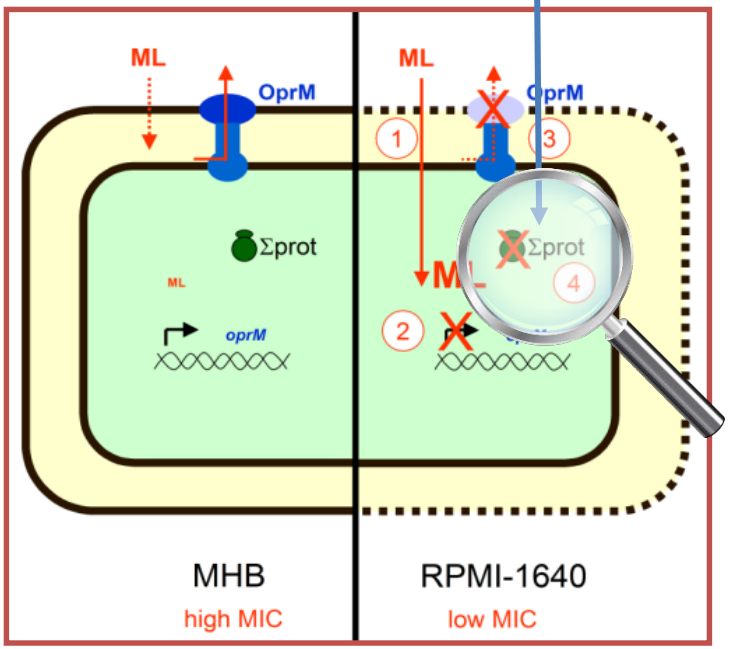


Efflux pump activity is NOT the reason behind high resistance among CF isolates

Acquired resistance to macrolides?

Mutation on macrolide's target!





Secondary-structure models of the peptidyl transferase center in **domain V of 23S rRNA** of *E.coli, Vester B et al. AAC (2012)*

Acquired resistance to macrolides

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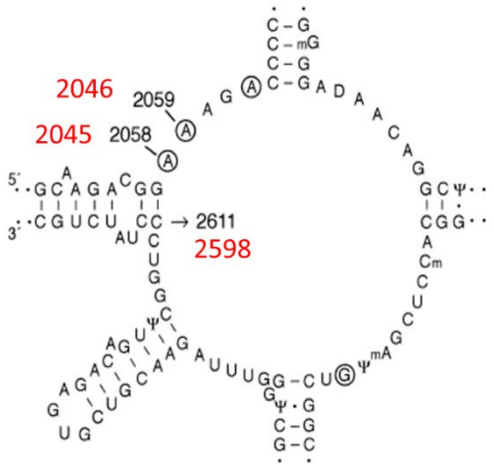
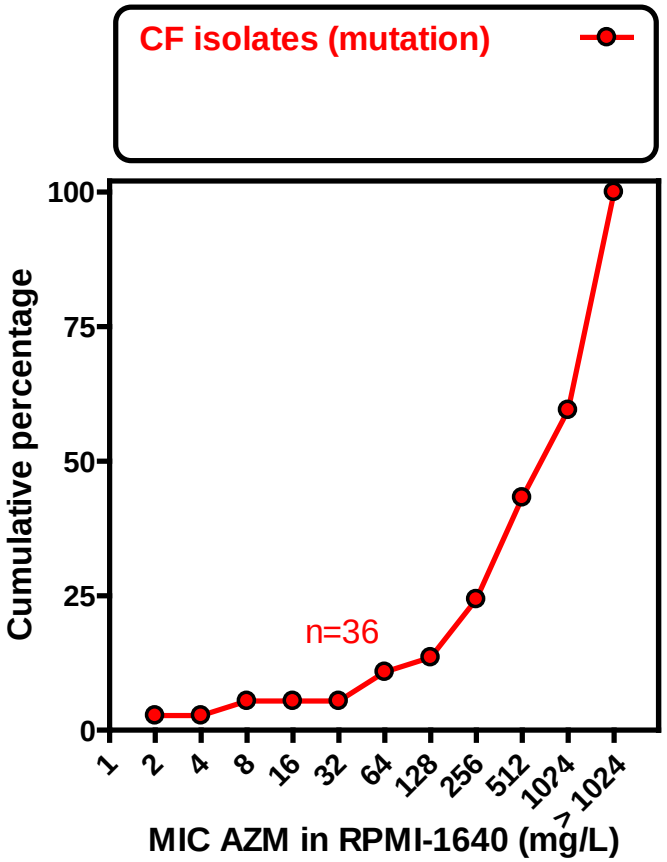


Secondary-structure models of the peptidyl transferase center in **domain V of 23S rRNA** of *E.coli, Vester B et al. AAC (2012)*

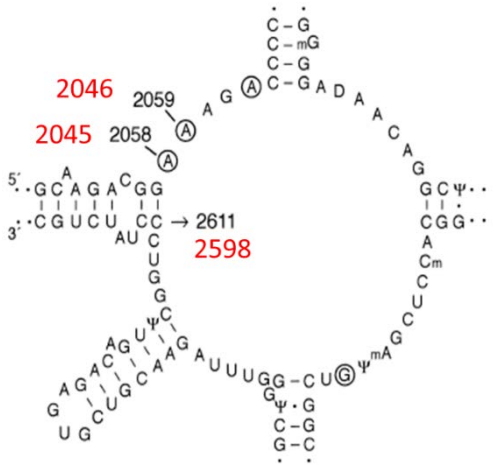
MIC distribution of CF and HAP isolates to AZM in RPMI-1640



- Mutations found in CF isolates

Acquired resistance to macrolides

Mutation on macrolide's target!



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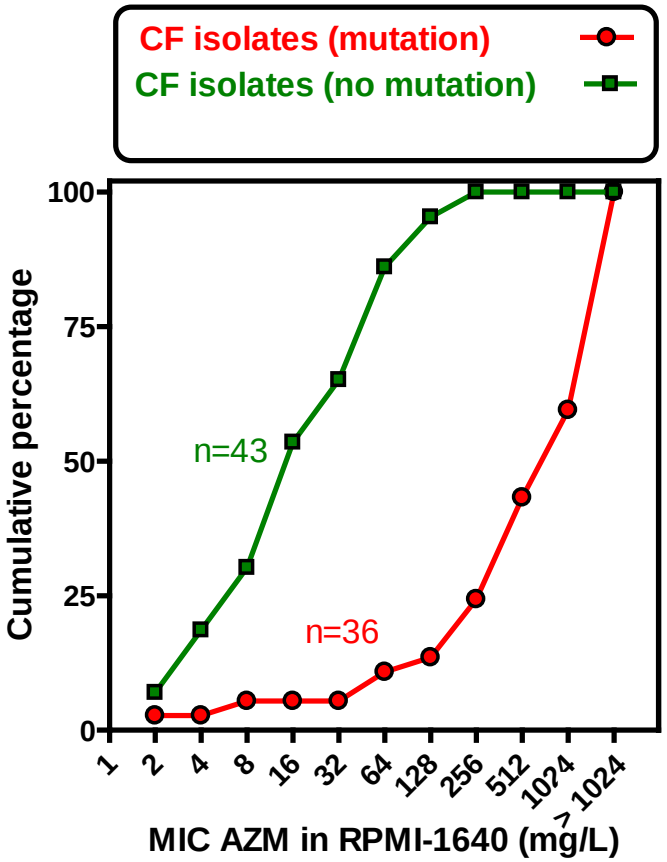

- Mutations found in CF isolates

Acquired resistance to macrolides

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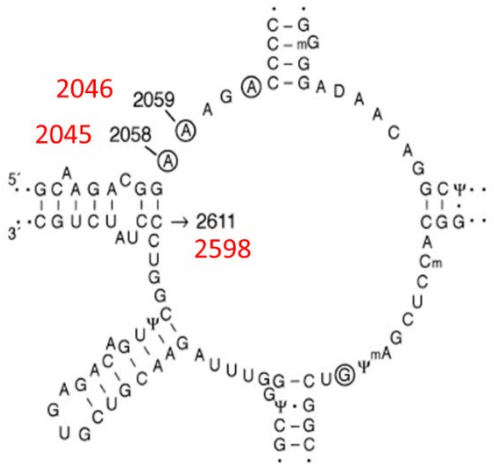

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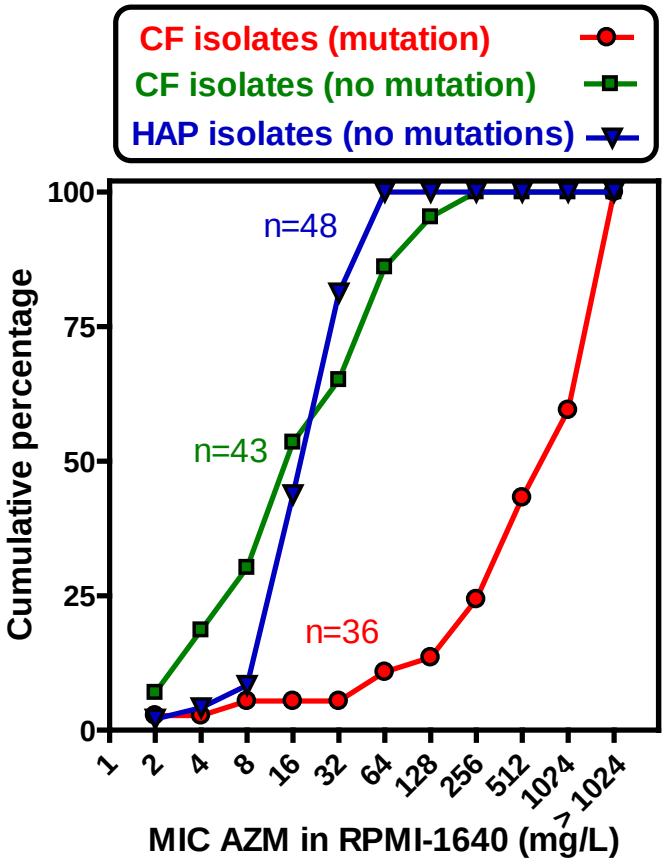
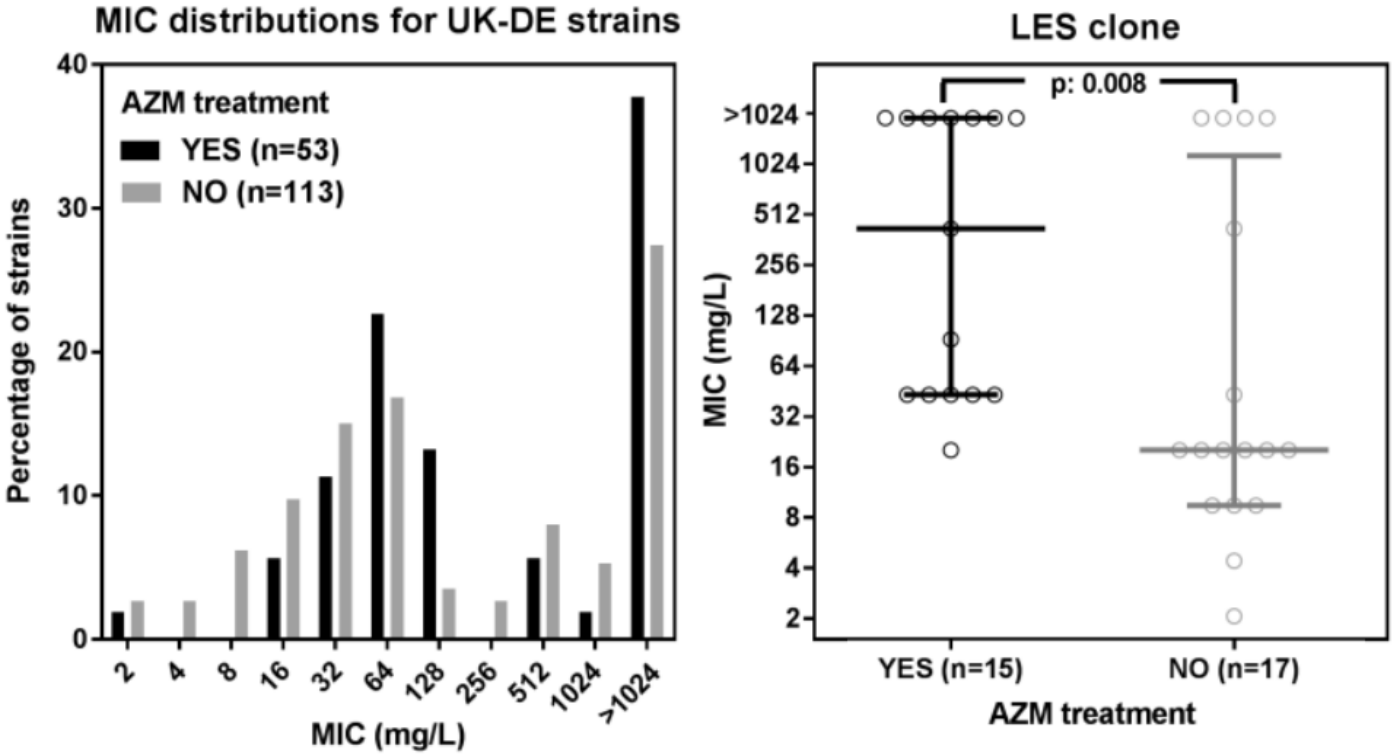


- **Mutations found in CF isolates**
- **No mutation in HAP isolates**

Acquired resistance to macrolides

Clinical association!



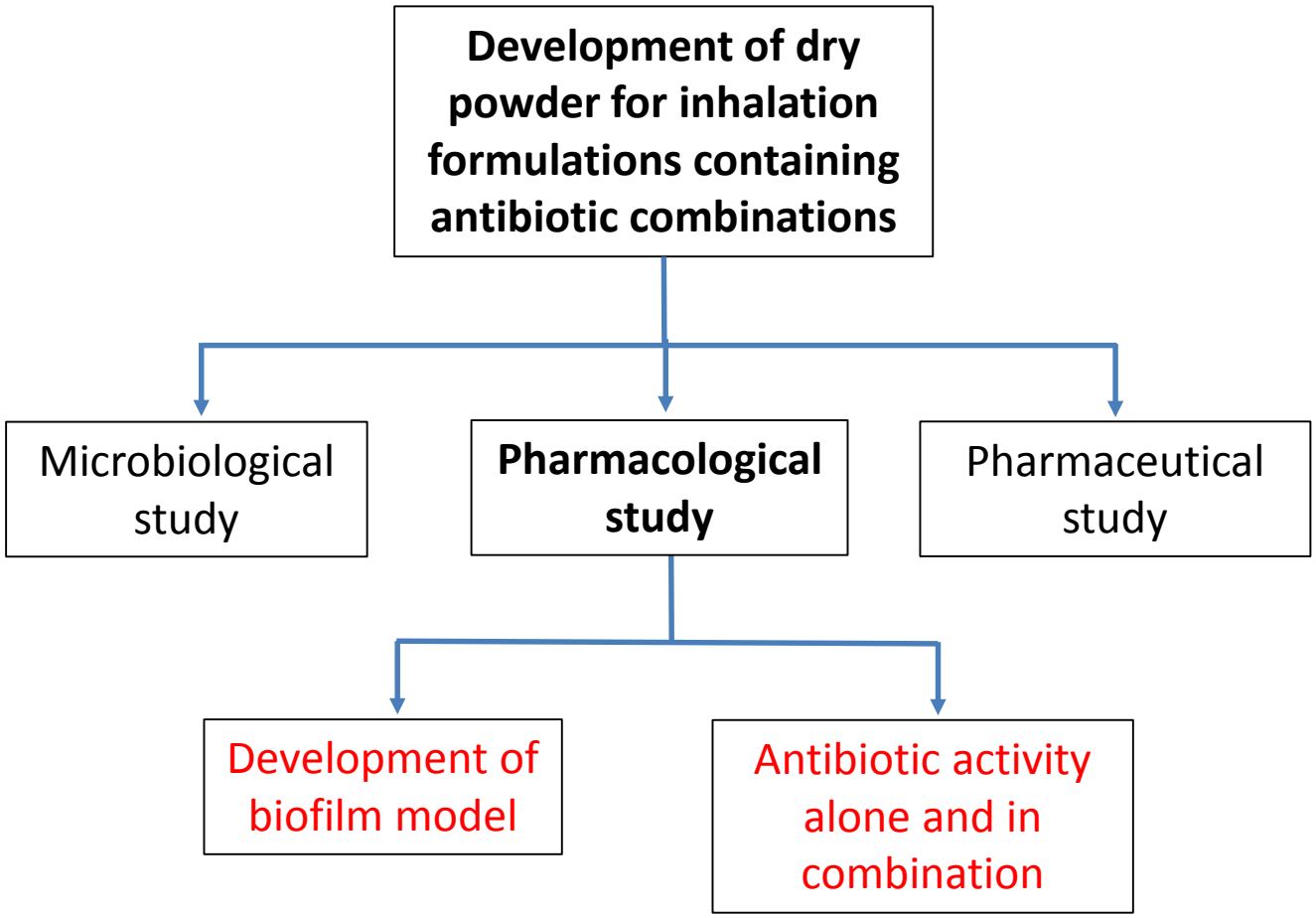
Mutation rate: 39% 12%

- **Isolates are less susceptibles to AZM in YES group**
- **Mutations more frequent in AZM-treated patients from LES**

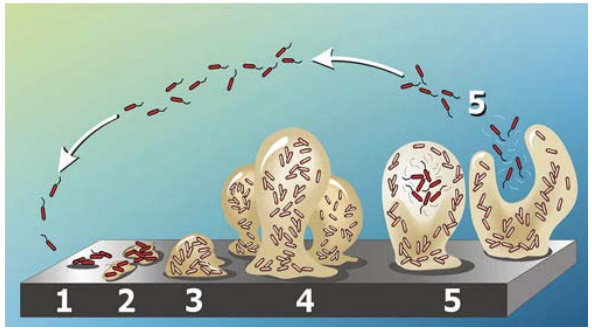
Microbiological study - Resistance in CF

Main findings:

- High resistance to macrolides in patients with CF.
- **Interest in achieving high concentration by pulmonary route.**


Development of dry powder for inhalation formulations containing antibiotic combinations
Microbiological study
Pharmacological study
Pharmaceutical study
Development of biofilm model
Antibiotic activity alone and in combination

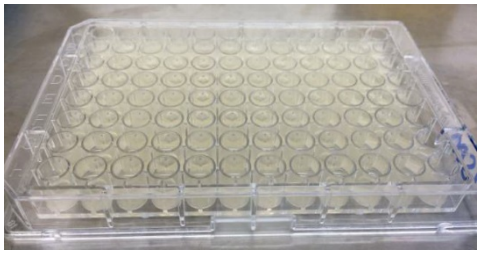
Development of biofilm model



Graphic and photos by Peg Dirckx and David Davies © 2003 Center for Biofilm Engineering Montana State University.

Persistence of chronic infection = biofilm

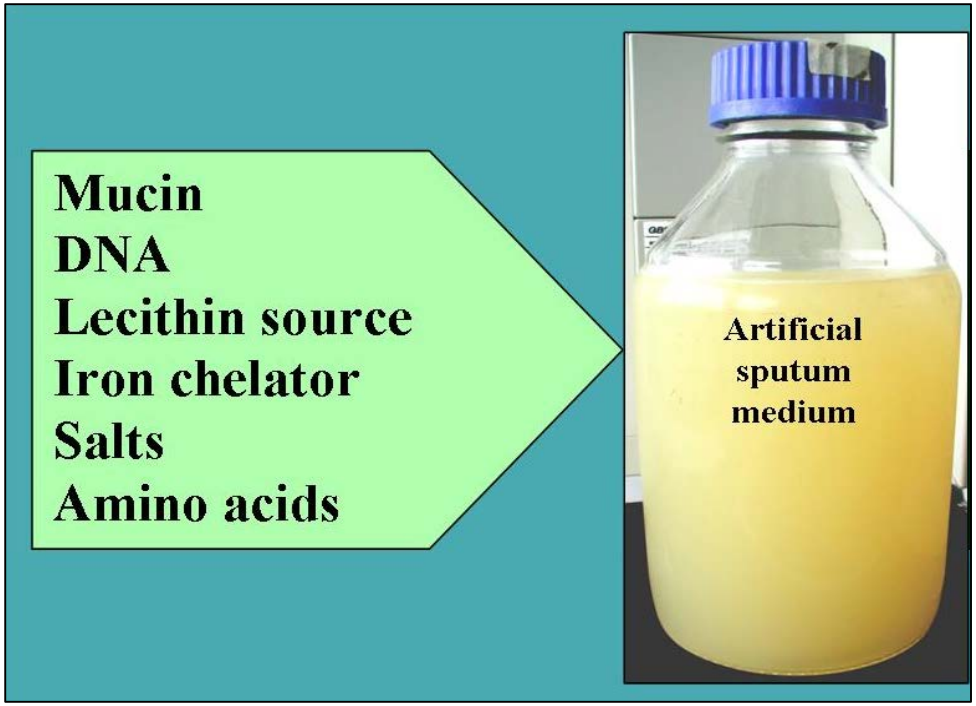
Objective: Establish a biofilm model relevant to CF lung pathophysiology to study antimicrobial activity



Development of biofilm model

Artificial Sputum Medium (ASM)

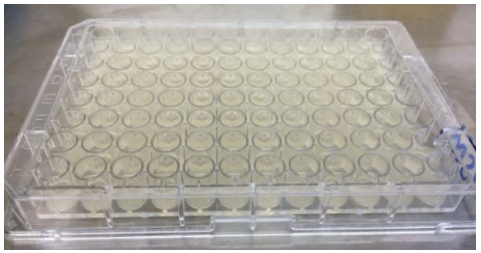
= a medium mimicking the sputum of patients with CF

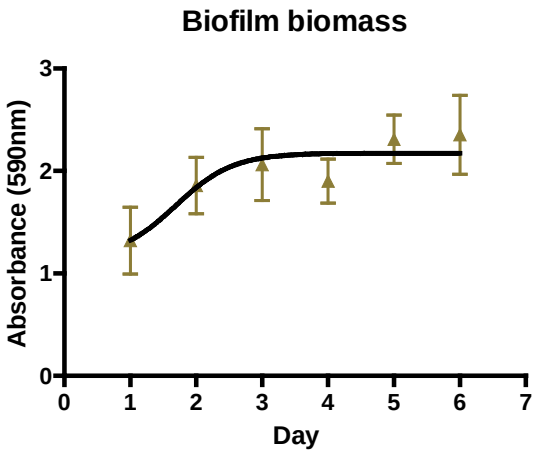


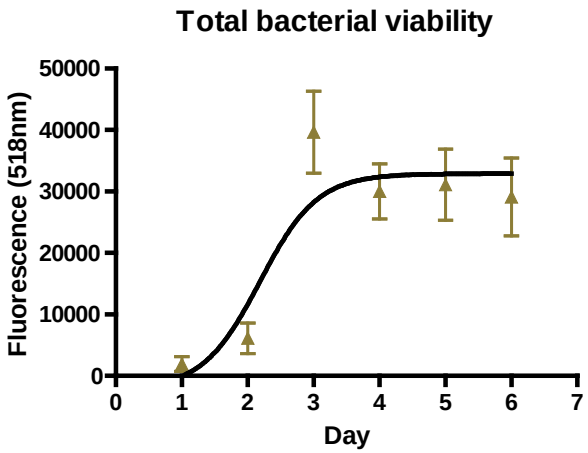
Sriramulu, 2005

Development of biofilm model

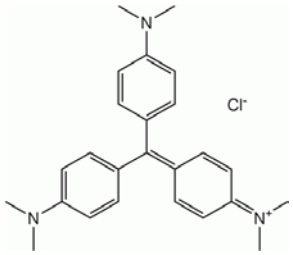
Objective: Establish a biofilm model relevant to CF lung pathophysiology to study antimicrobial activity
=> **Mature, static P. aeruginosa biofilm model in a 96-well plate**

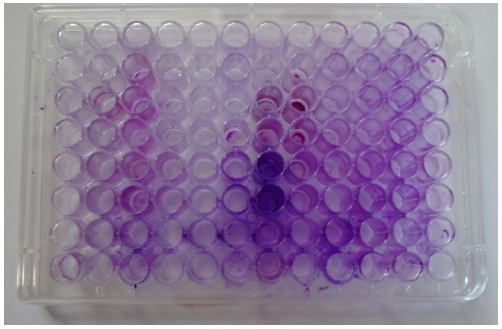




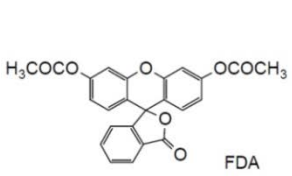


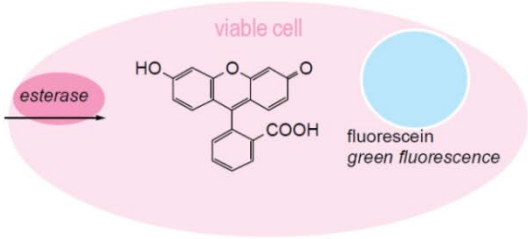
Crystal violet staining





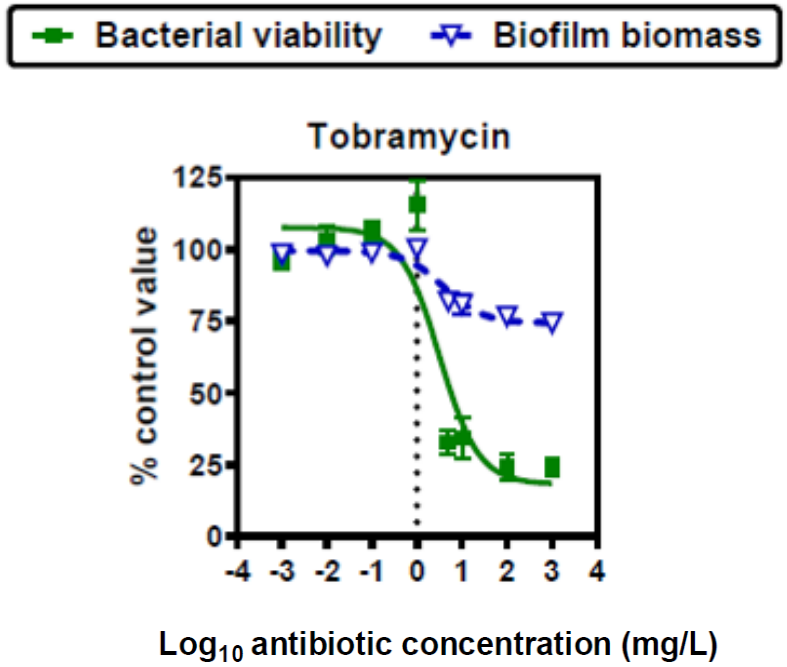
Fluoresceine diacetate assay





Antibiotic activity in biofilm infection

Tobramycin activity on 4-day old mature *P. aeruginosa* PAO1 biofilm after 24-hour of antibiotic exposure



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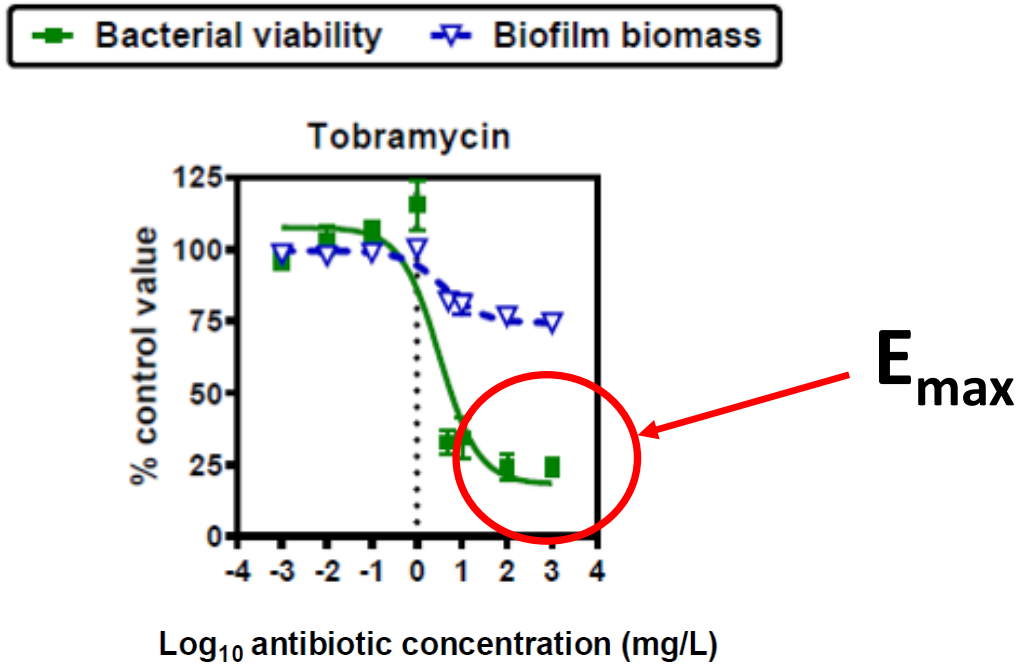
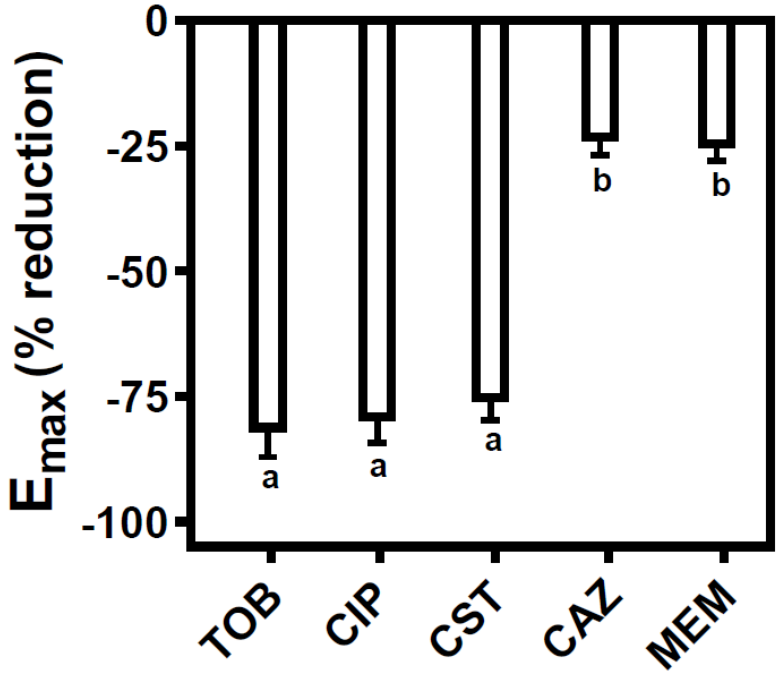


Antibiotic activity in biofilm infection

Activity of different antipseudomonals in biofilm model

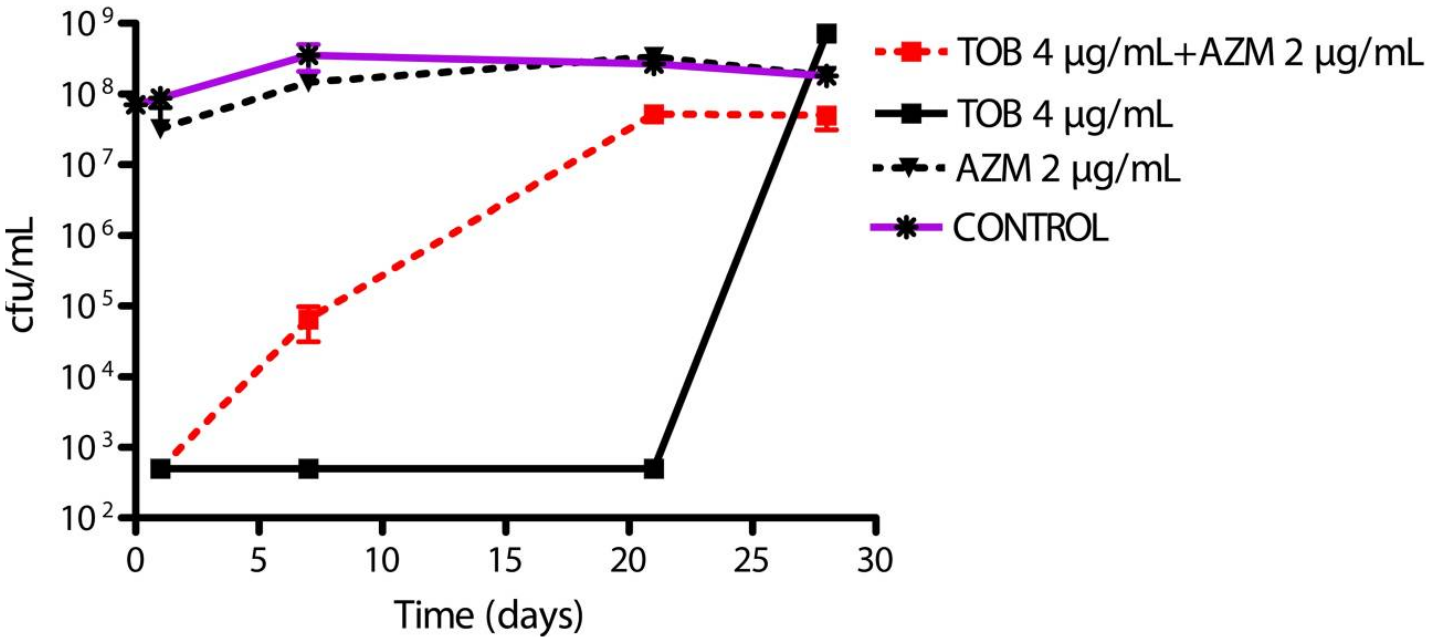
TOB: Tobramycin; CIP: Ciprofloxacin; CST: Colistin; CAZ: Ceftazidime; MEM: Meropenem



Tobramycin is one of the most active drugs

Antibiotic activity in biofilm infection

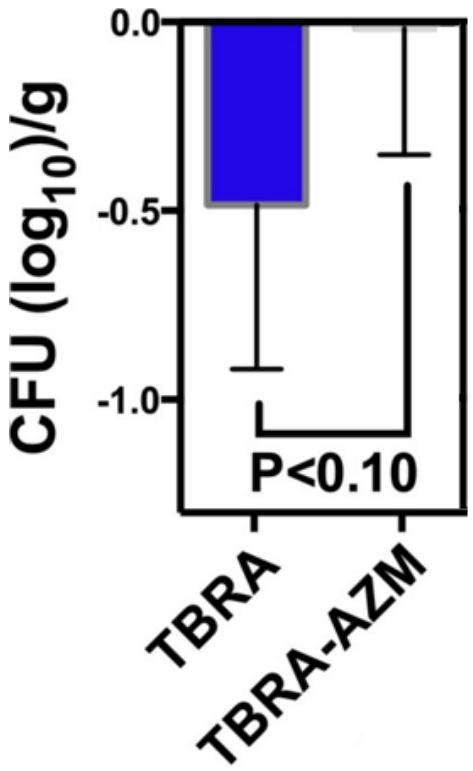
Azithromycin decreased antipseudomonal activity of TOB in biofilm



Tré-hardy et al, Antimicrob Agents Chemother. 2010 Oct;54(10):4409-15.

Antibiotic activity in biofilm infection

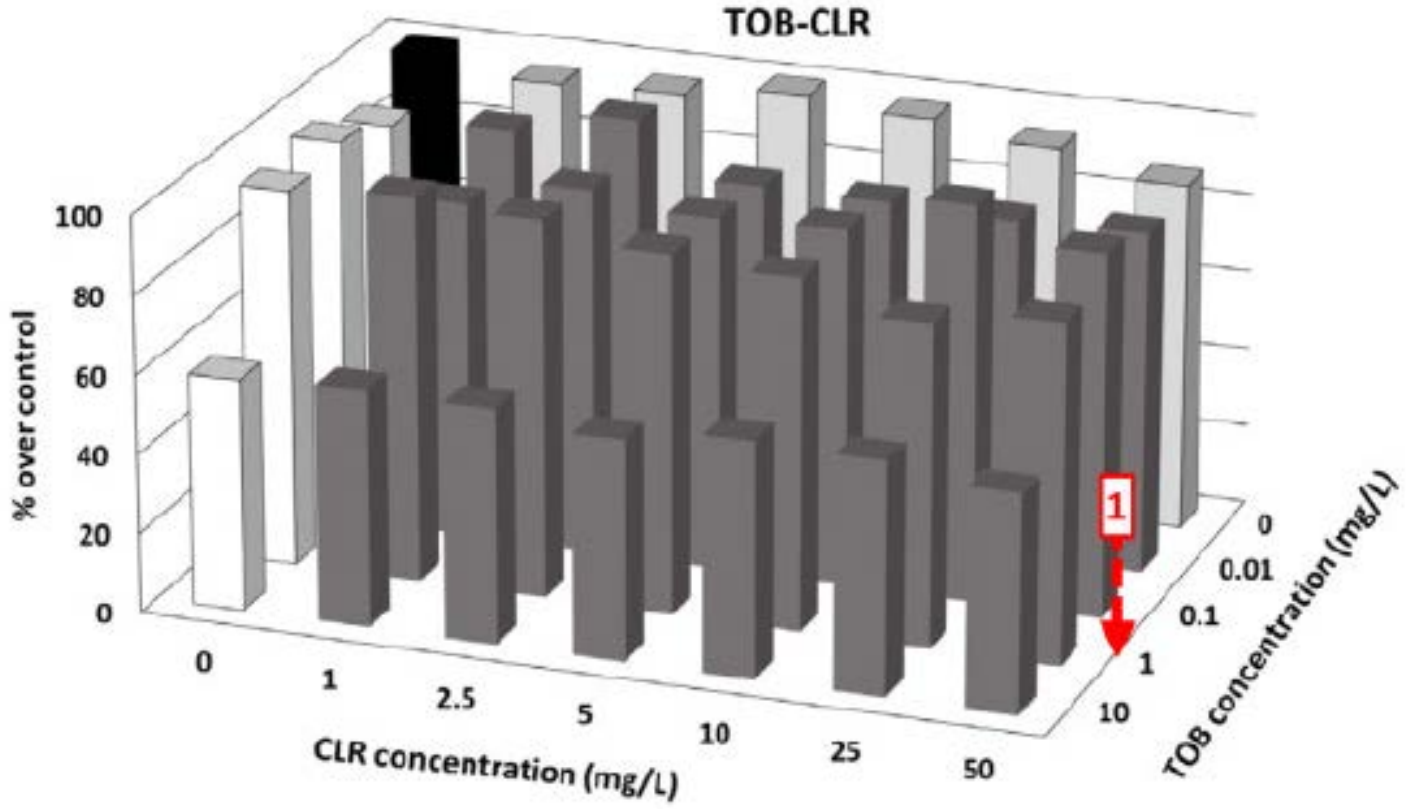
Azithromycin decreased antipseudomonal activity of TOB in patients



Nick et al, Ann Am Thorac Soc. 2014 Mar; 11(3): 342–350.

Antibiotic activity in biofilm infection

Activity of combination Tobramycin-Clarithromycin against total bacterial viability



CLR did not interfere with the activity of TOB

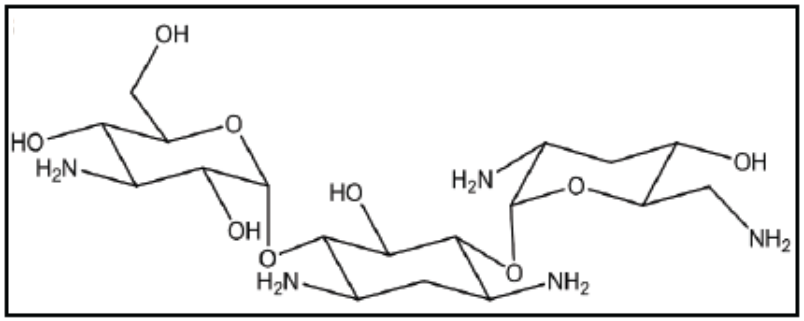
Pharmacological study – Biofilm infection

Main findings:

- Biofilm model relevant to CF pathophysiological condition for simple screening of antibiotic's activity.
- Tobramycin alone as one of the active drugs
- Clarithromycin did not interfere with TOB activity

Development of dry powder for inhalation formulations containing antibiotic combinations

Tobramycin

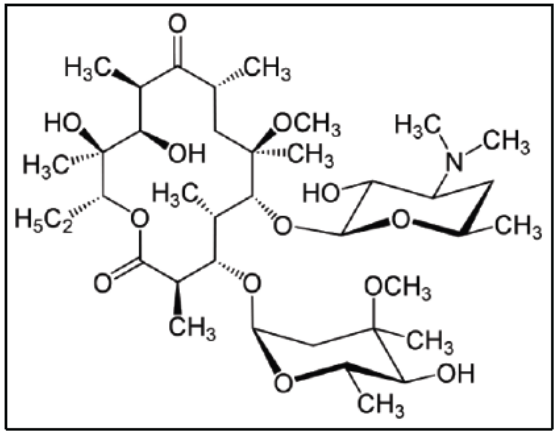


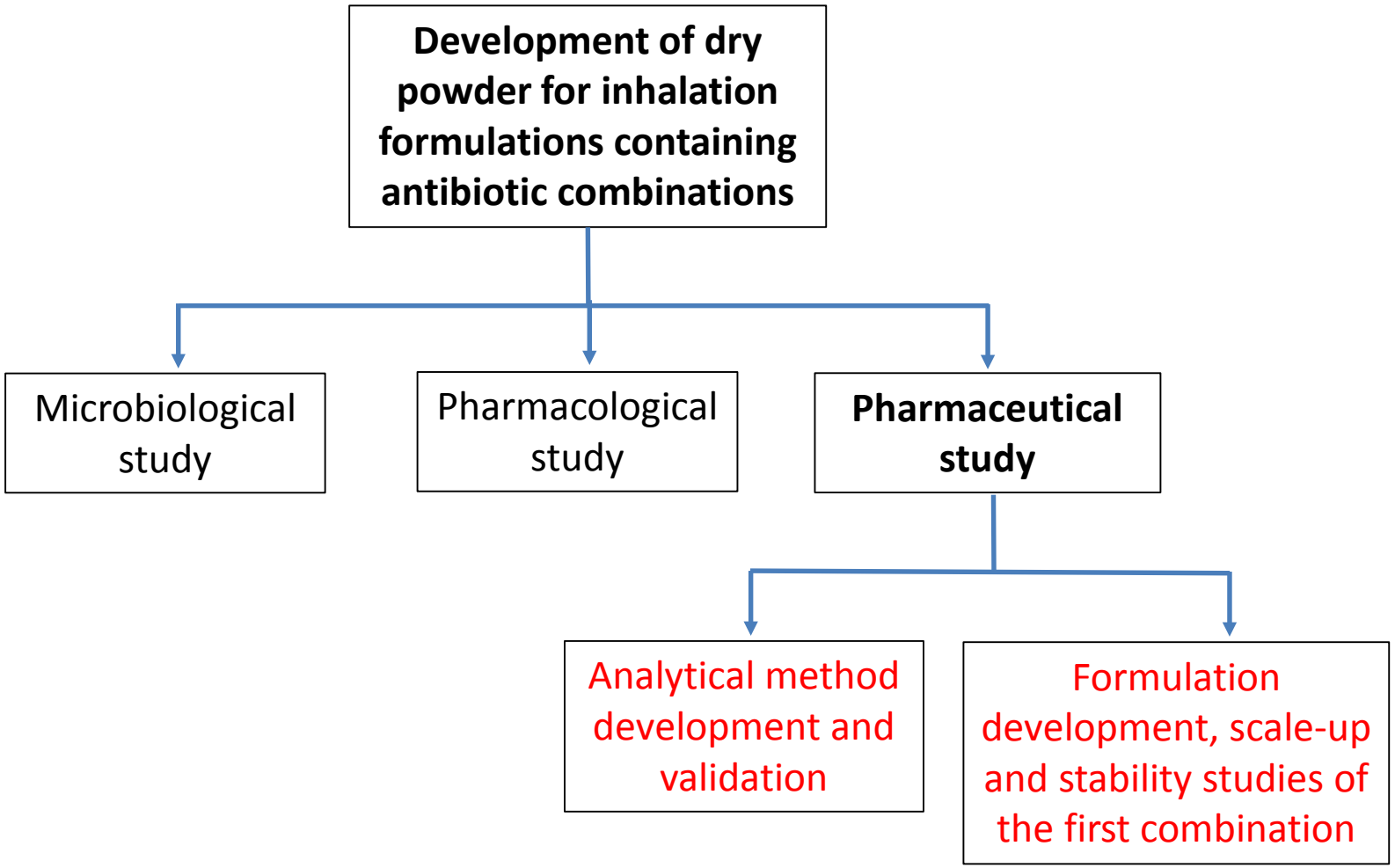
In the market:

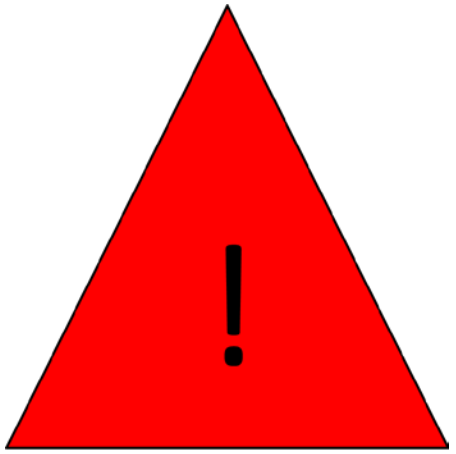
- ***Tobramycin Inhalation Solution***
- ***Tobramycin Inhalation Powder***

+

Clarithromycin




Development of dry powder for inhalation formulations containing antibiotic combinations
Microbiological study
Pharmacological study
Pharmaceutical study
Analytical method development and validation
Formulation development, scale-up and stability studies of the first combination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CONFIDENTIAL
TOP SECRET
CONFIDENTIAL

Process scale-up for production of pilot batches

Laboratory scale

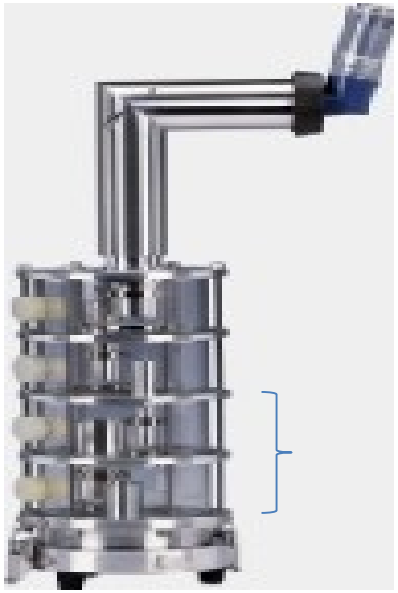
Laboratory planetary mixer

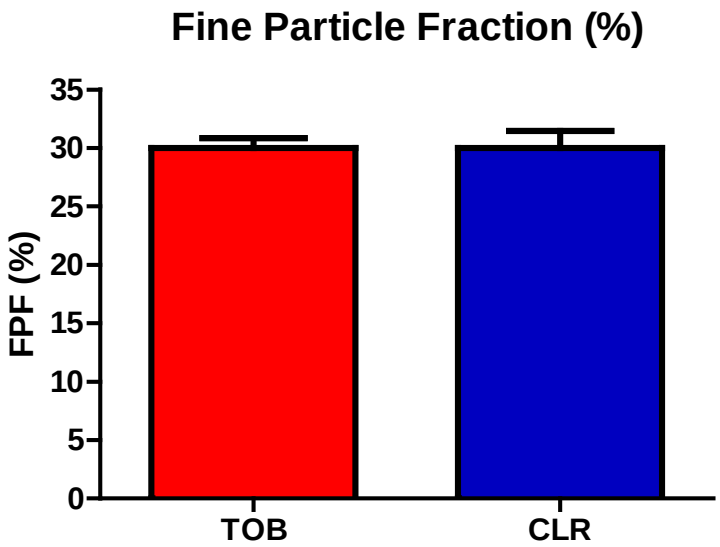
Industrial scale

Industrial planetary mixer

Capsule filling machine

TOB-CLR pulmonary deposition





Stability studies

Packaging:

- High-density polyethylene bottles
- 60 mL
- 2g of dessicant
- 60 capsules/bottle

		Time point (in month)						
		T1	T3	T6	T9	T12	T18	T24
Storage condition (Temperature / Humidity)	Long term (25°C/60% RH)		X	X	X	X	X	X
	Intermediate (30°C/65% RH)		X	X	X	X		
	Accelerated (40°C/75% RH)	X	X	X				

Based on "Stability Testing of New Drug Substances and Products Q1A(R2)" by ICH

Stability studies through 12 months

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	Accelerated (40°C/75% RH)	X	X	X				

Based on "Stability Testing of New Drug Substances and Products Q1A(R2)" by ICH

Pharmaceutical study – TOB-CLR dry powder

Main findings:

- Validated analytical method for detection of TOB-CLR.
- A stable formulation through 12 months at long-term condition.

Summary of the PhD thesis

Main findings:

- Importance of delivering high concentrations of tobramycin and clarithromycin at the infection site because of resistance.
- Stable formulation of the combination for preclinical and clinical development.

Summary of the PhD thesis

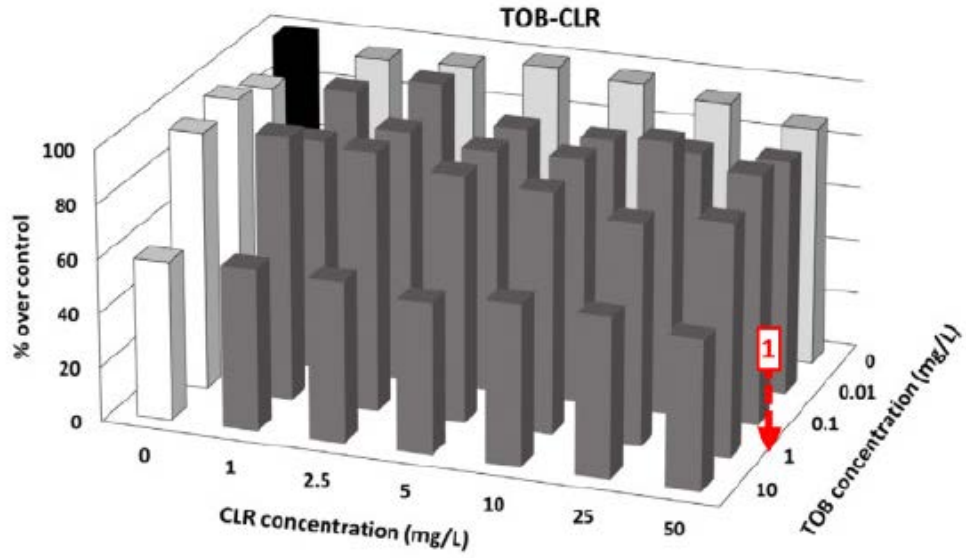
Main findings:

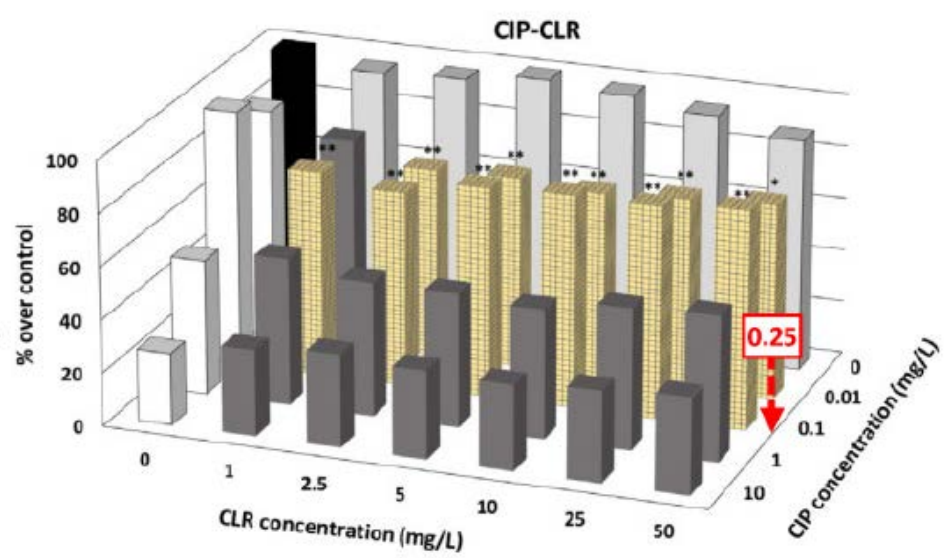
- Importance of delivering high concentrations of tobramycin and clarithromycin at the infection site because of resistance.
- Stable formulation of the combination for preclinical and clinical development.

Perspectives:

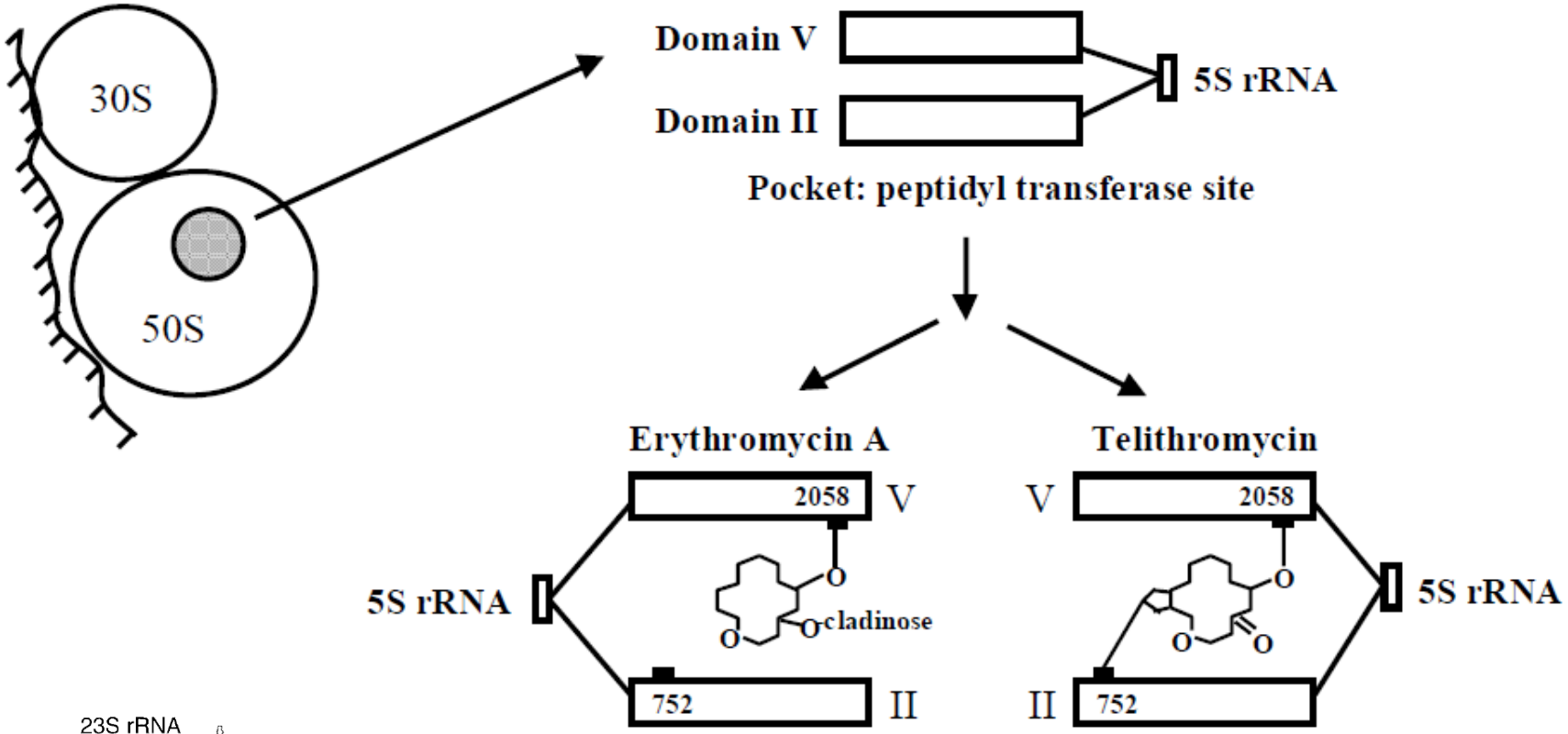
- Developing more pertinent biofilm models (dynamic, co-culture and in vivo) to study a more promising combination

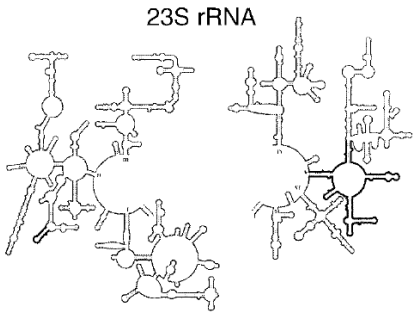
Ciprofloxacin-clarithromycin strong synergism





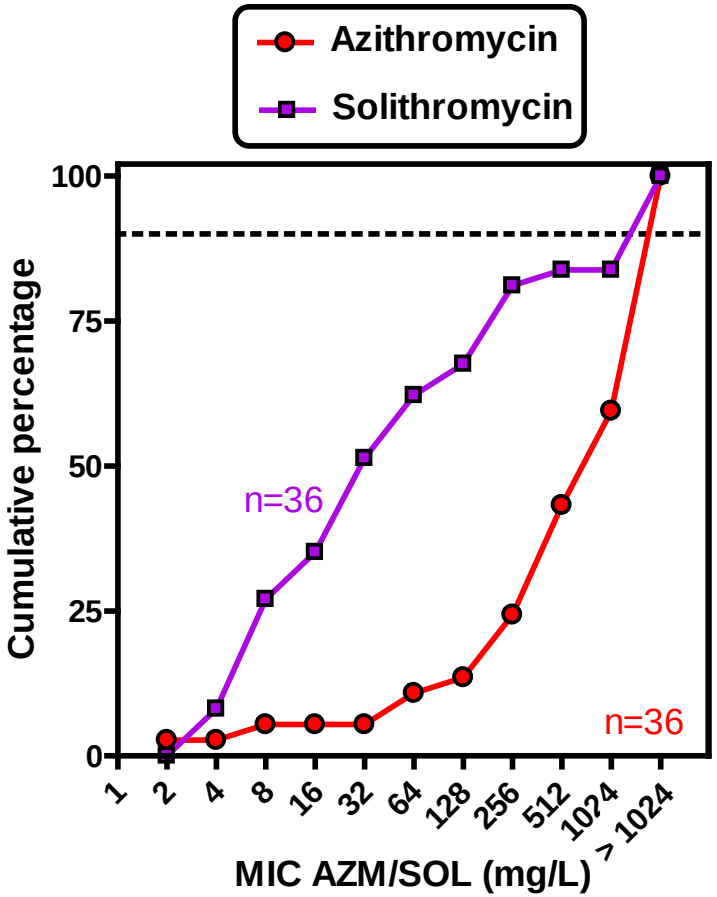
Macrolides vs Ketolides





KETEK (telithromycin) Briefing Document for the FDA

Higher susceptibility of mutated CF isolates to ketolides



Summary of the PhD thesis

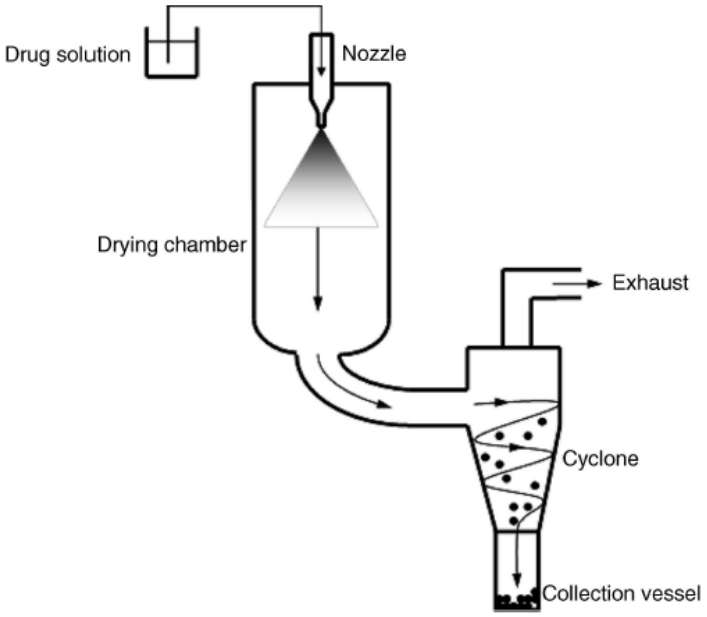
Main findings:

- Importance of delivering high concentrations of tobramycin and clarithromycin at the infection site because of resistance.
- Stable formulation of the combination for preclinical and clinical development.

Perspectives:

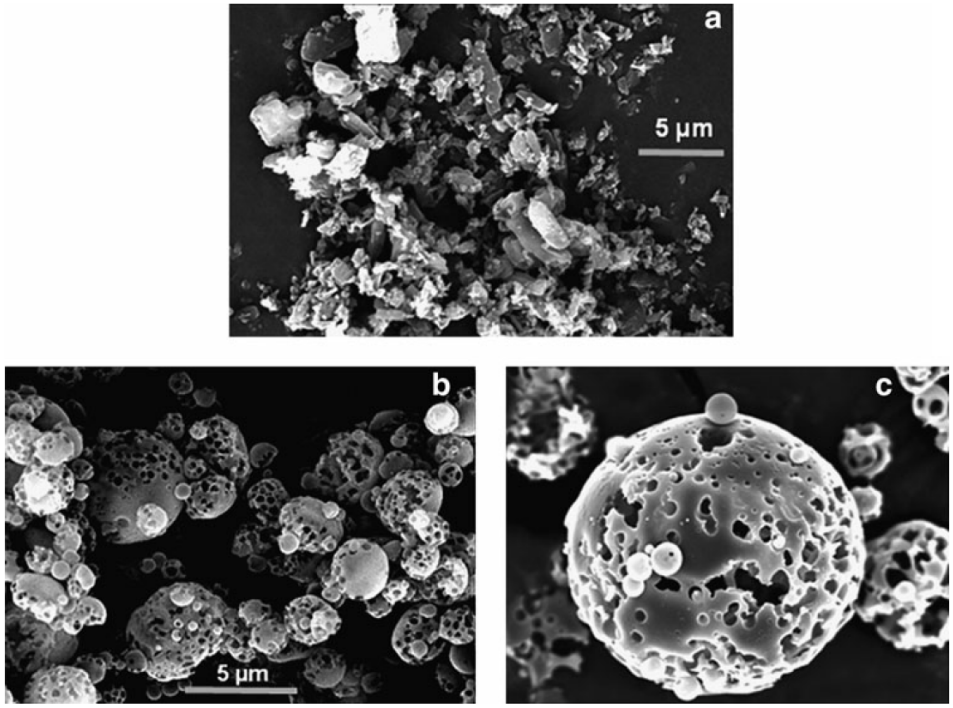
- Developing more pertinent biofilm models (dynamic, co-culture and in vivo) to study a more promising combination
- PK study in mice model for TOB-CLR
- Formulation optimization for higher pulmonary deposition using spray-drying technology

Spray-drying technology



Inhalation Drug Delivery: Techniques and Products: Wiley, 2013

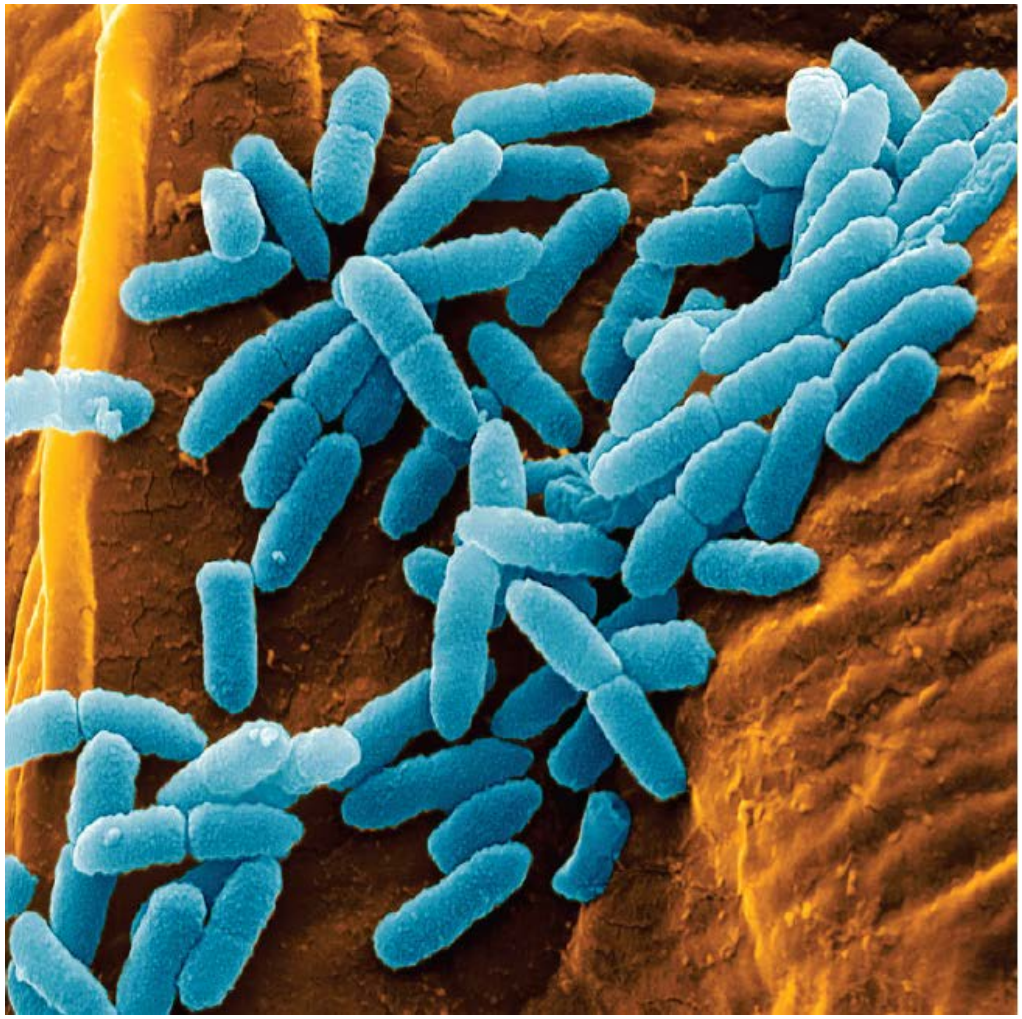
Tobramycin Inhalation Powder (TOBI Podhaler™)
=> FPF around 50%



Geller et al, 2011

The happy place could be happy again?

Thank you for your attention!



Pseudomonas aeruginosa bacteria, coloured scanning electron micrograph (SEM) by Juergen Berger

- Pr Françoise Van Bambeke
- Dr Francis Vanderbist

- Pr Marie-Paul Mingeot
- Pr Paul M. Tulkens

- Pr Véronique Préat
- Pr Rita Vanbever
- Pr Teresinha Léal
- Dr Hector Rodriguez-Villalobos

- Pr William Couet
- Pr Tom Coenye

- Innoviris (The Brussels Institute for Research and Innovation)

- Our collaborators

- FACM team:

- LDRI team

- SMB team: Thami, Laure, Inès, Hamidou, Gaëlle, Danielle, Laurence, Pauline, Alisson, Oussama, Sèverine, Chantal, Fabrice

2016 HAPPY NEW YEAR 2017

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- LDRI team

- SMB team: Thami, Laure, Inès, Hamidou, Gaëlle, Danielle, Laurence, Pauline, Alisson, Oussama, Sèverine, Chantal, Fabrice

- Westley, Ahalieyah, Anushea, Micheline, Eugénie, Amir, Syed Jamal, Dona, Rini, Mekna, OD, Myriam, Hayeon, Nisa, Moïra

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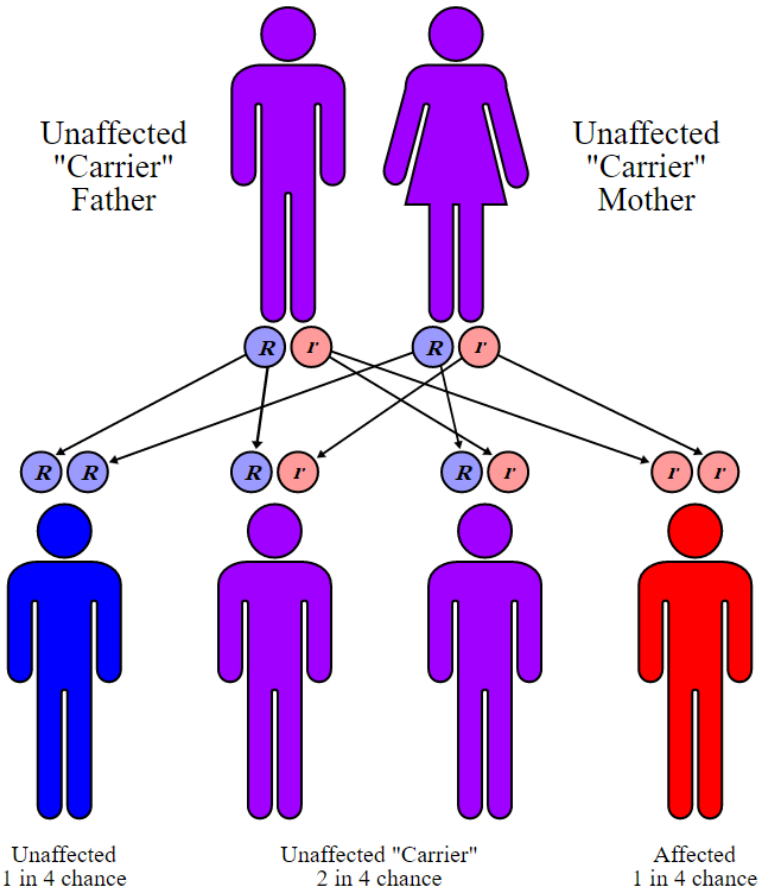
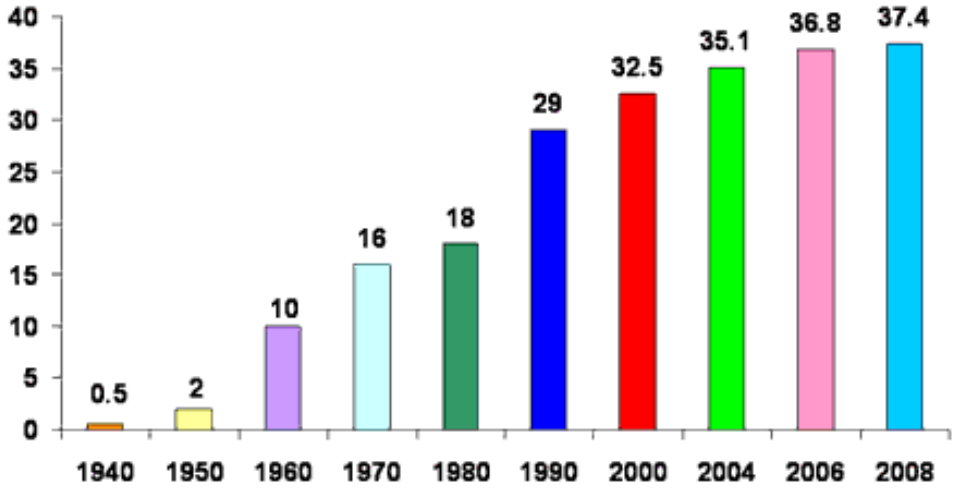
- Ayah
- Kak Nini
- Kak Za
- Kak Ieta
- Nina
- Abang Ridzal
- Abang Kerol
- Abang Shah
- Ierfan
- Lala
- Batrisya
- Iezny
- Daleea
- Iezz
- Jannah
- Eymran
- Izzul
- Maksu
- Faten
- Wafaa
- Quzey

Cystic Fibrosis: Definition

Cystic Fibrosis is an autosomal recessive genetic disease, affecting mainly Caucasian population

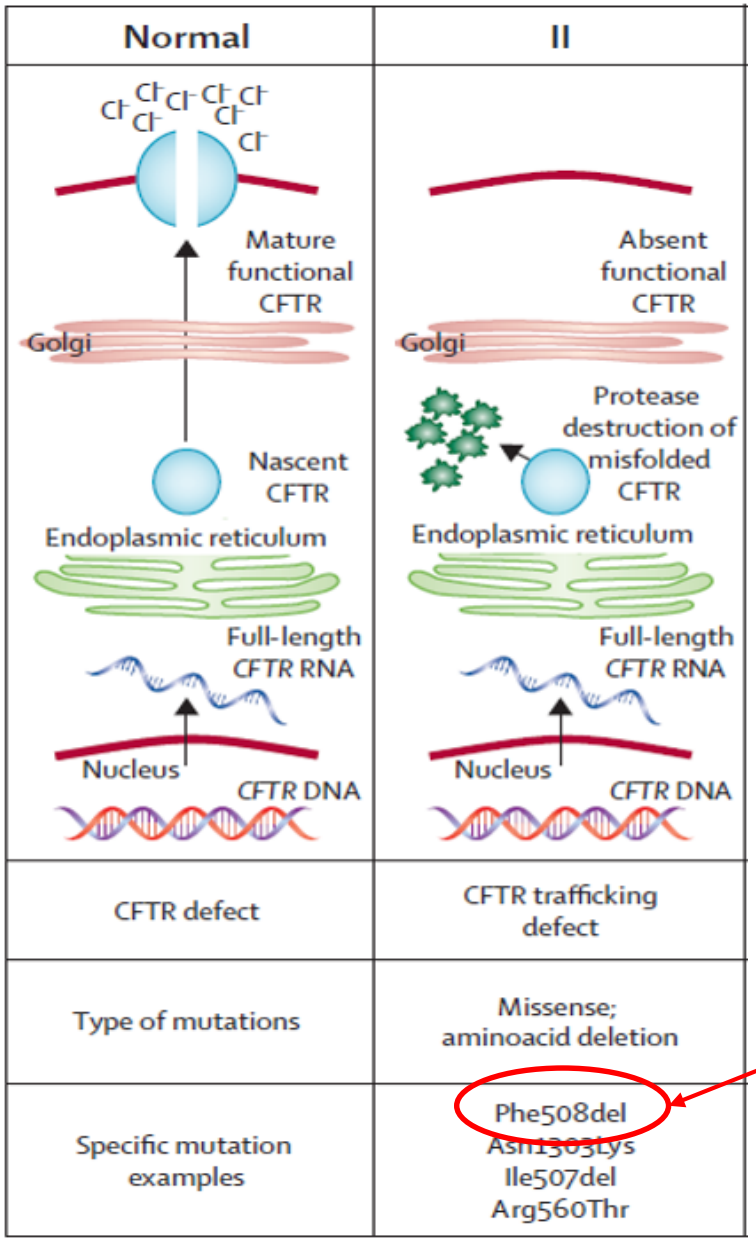


Average Life Expectancy in Cystic Fibrosis
Better Treatment = Improved Survival



Source: Cystic Fibrosis Foundation

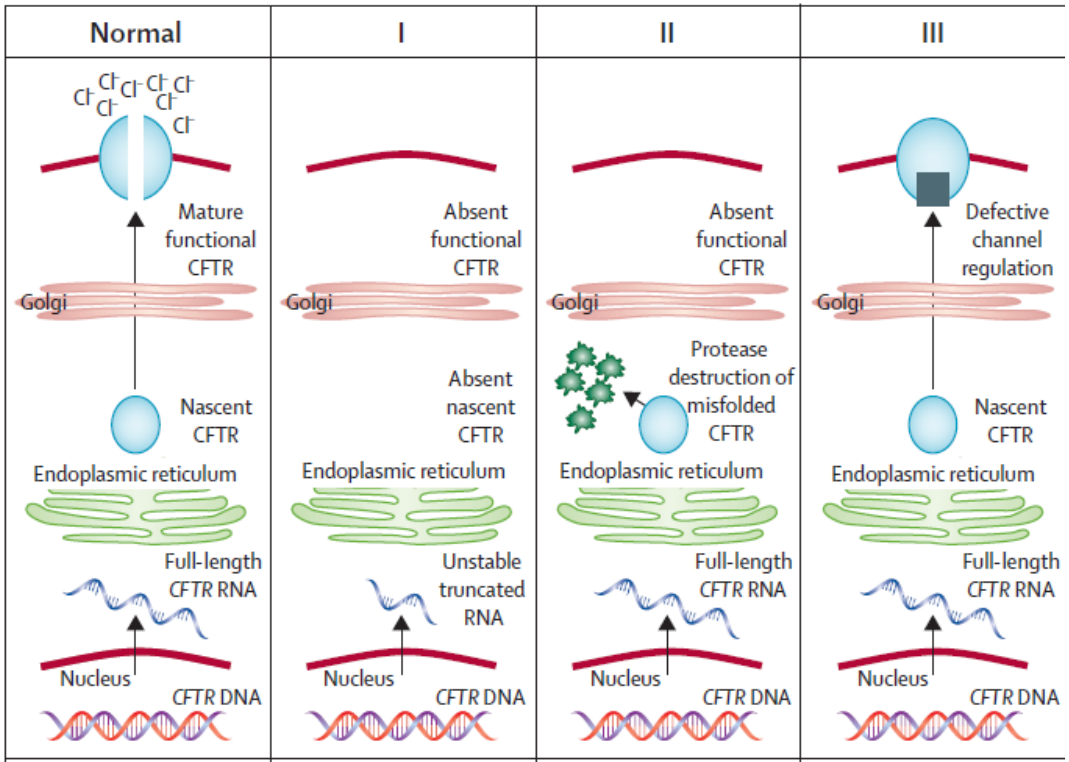
Defective CFTR protein



85-90% of patients with CF

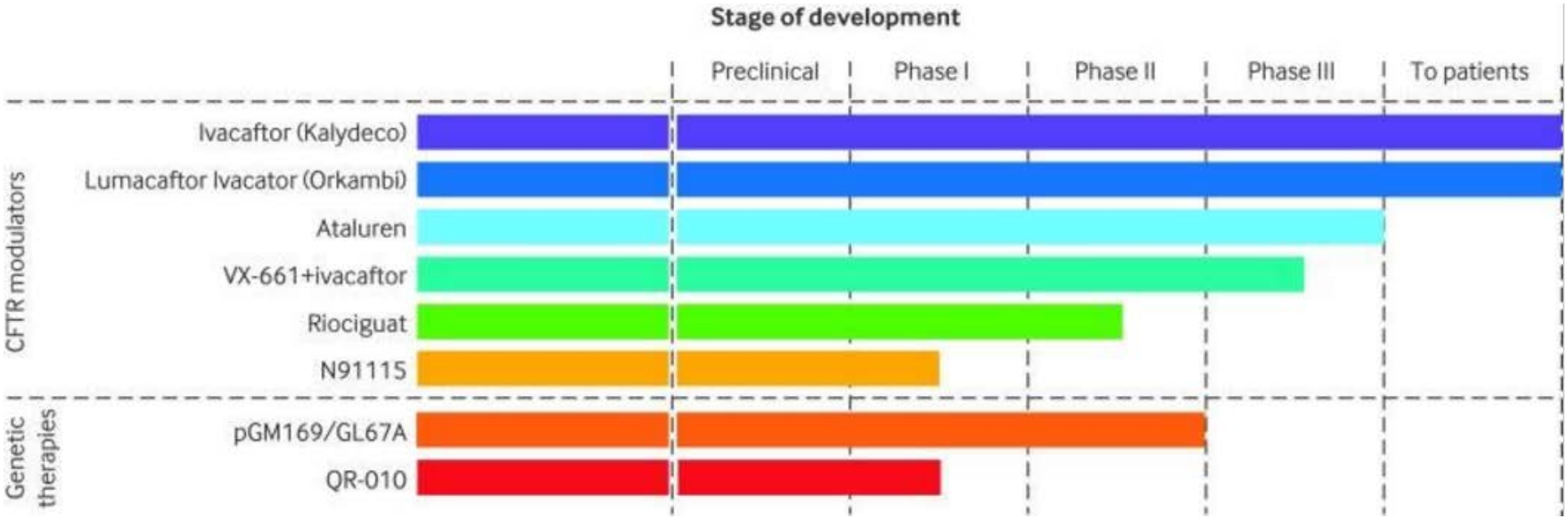
Elborn, 2016

Defective CFTR protein



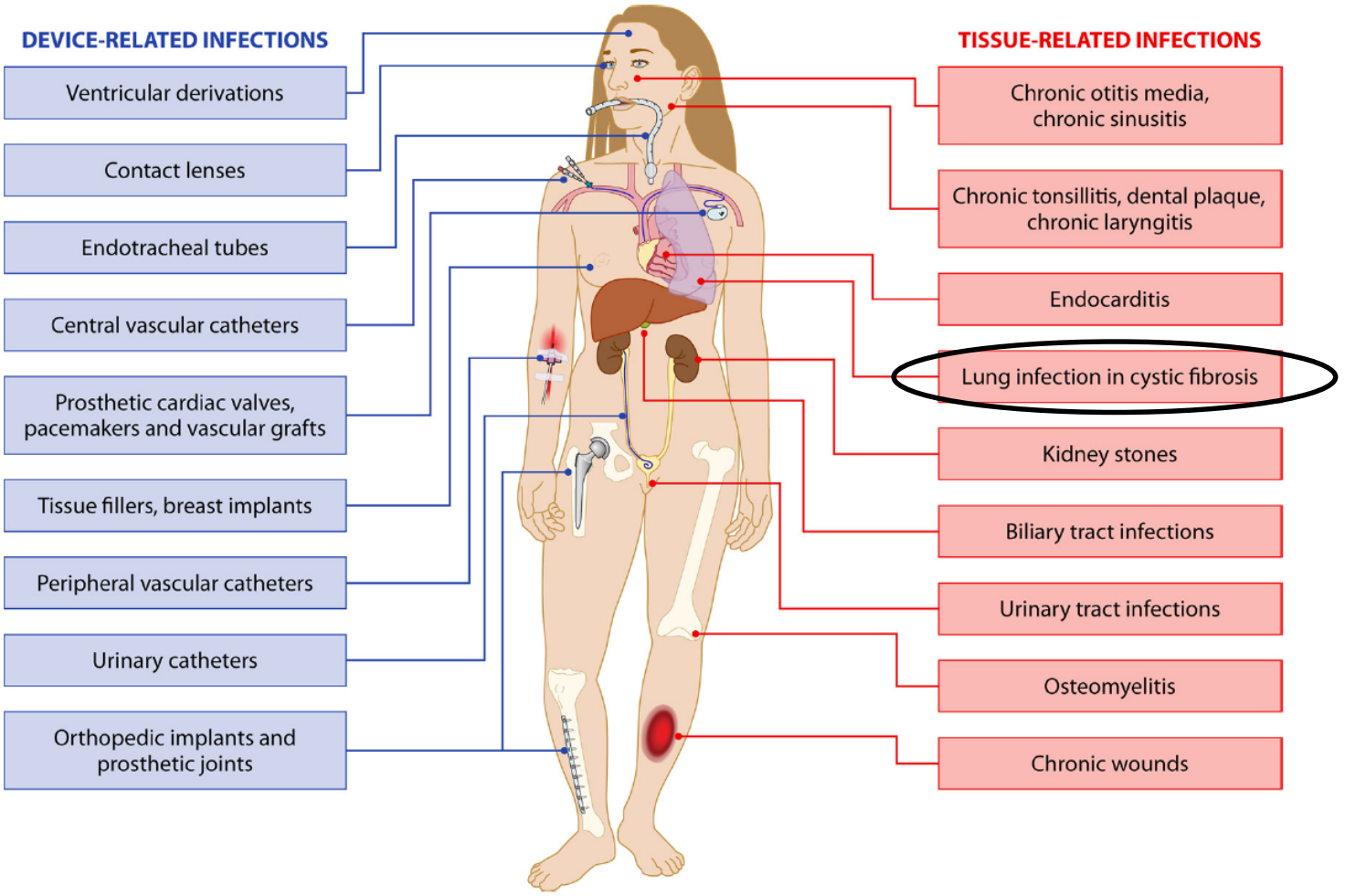
Normal	I	II	III
CFTR defect	No functional CFTR protein	CFTR trafficking defect	Defective channel regulation
Type of mutations	Nonsense; frameshift; canonical splice	Missense; aminoacid deletion	Missense; aminoacid change
Specific mutation examples	Gly542X Trp1282X Arg553X 621+1G→T	Phe508del Asn1303Lys Ile507del Arg560Thr	Gly551Asp Gly178Arg Gly551Ser Ser549Asn

Elborn, 2016



Quon et al, 2016

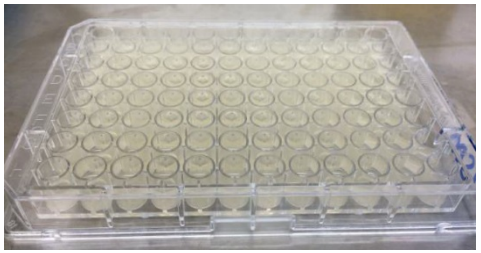
Biofilm-causing diseases

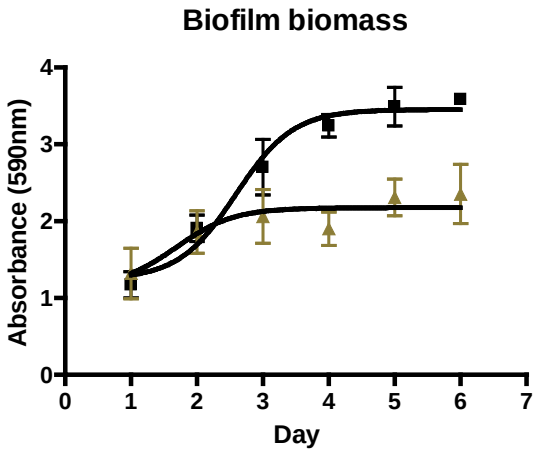


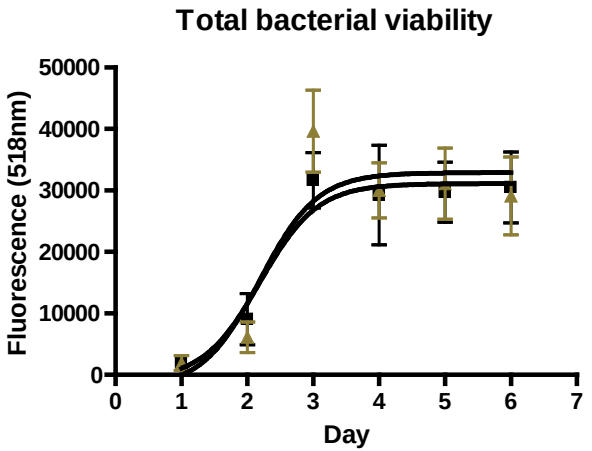
David Lebeaux et al, Microbiology and Molecular Biology Reviews p. 510–543

Development of biofilm model

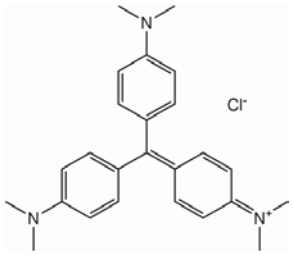
Objective: Establish a biofilm model relevant to CF lung pathophysiology to study antimicrobial activity
=> **Mature, static P. aeruginosa biofilm model in a 96-well plate**

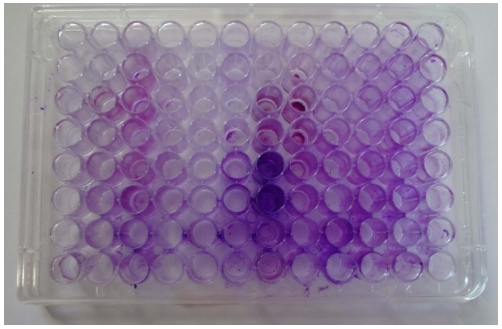




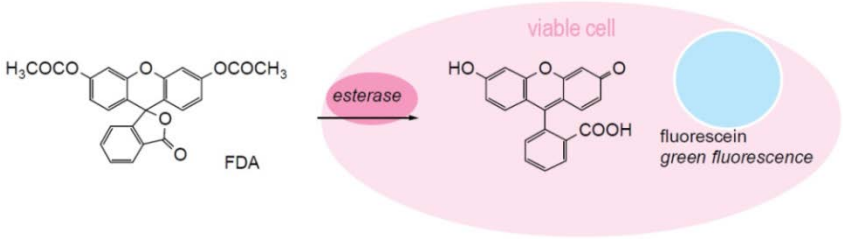


Crystal violet staining



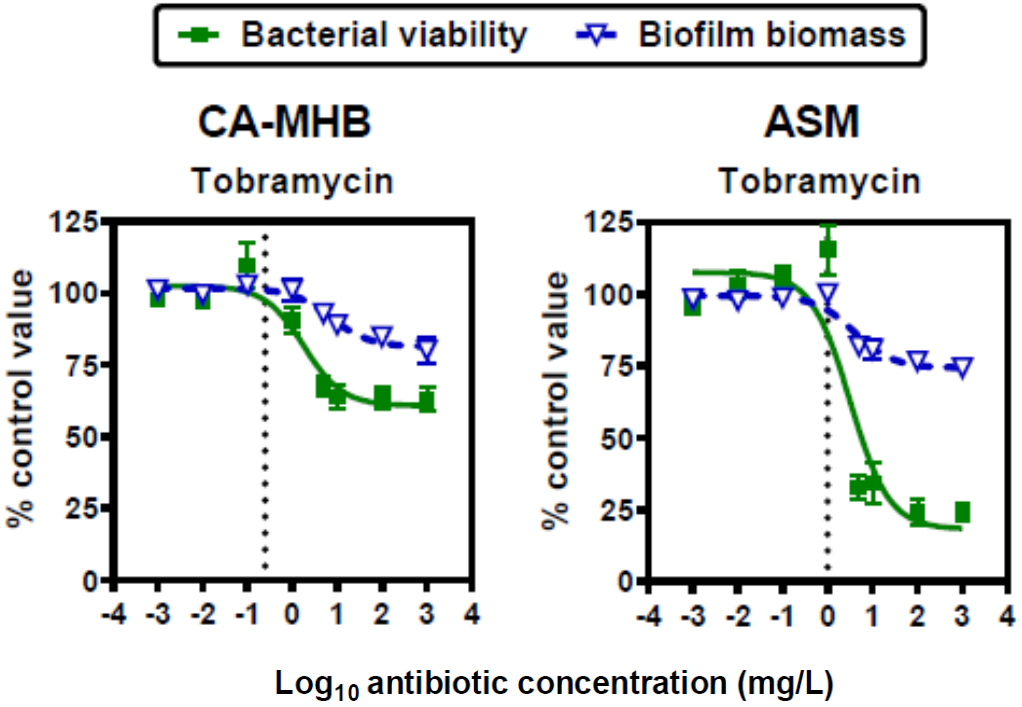


Fluoresceine diacetate assay



Antibiotic activity in biofilm infection

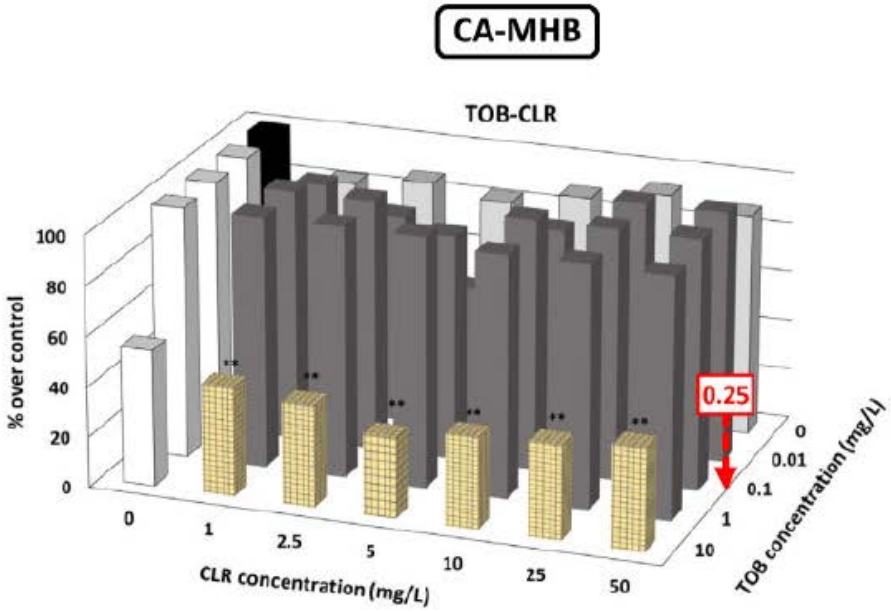
Tobramycin activity on 4-day old mature *P. aeruginosa* PAO1 biofilm after 24-hour of antibiotic exposure

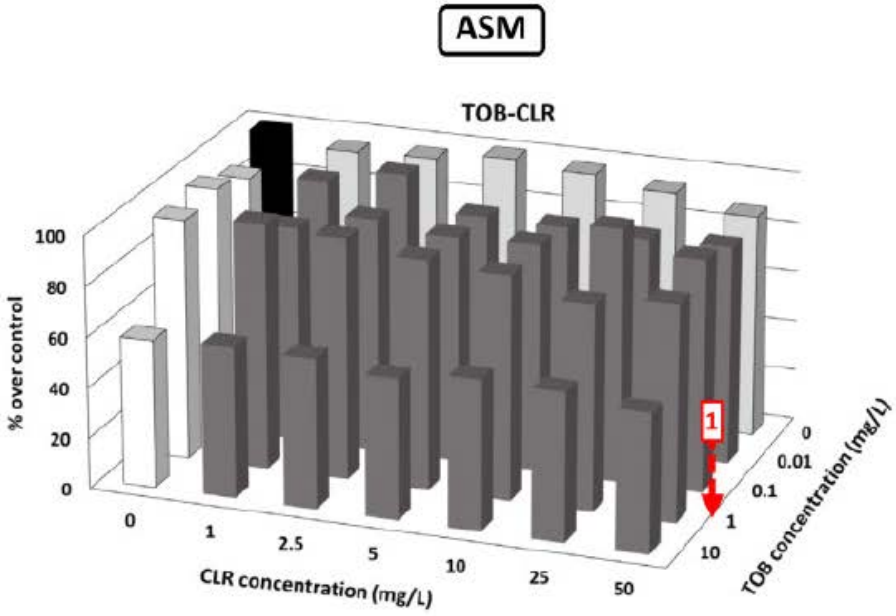


Tobramycin is one of the most active drugs

Antibiotic activity in biofilm infection

Activity of combination Tobramycin-Clarithromycin against total bacterial viability





CLR did not interfere with the activity of TOB

Treatment of pseudomonal infection

Current challenges

- Antibiotics administered by iv route => toxicity
- High resistance => intrinsic and acquired
- Biofilm infection

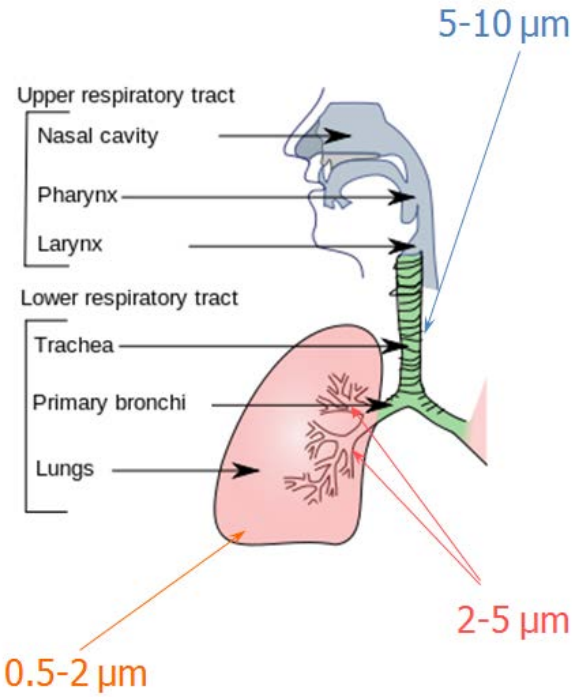
Objective of the PhD thesis

To develop a new dry powder formulation

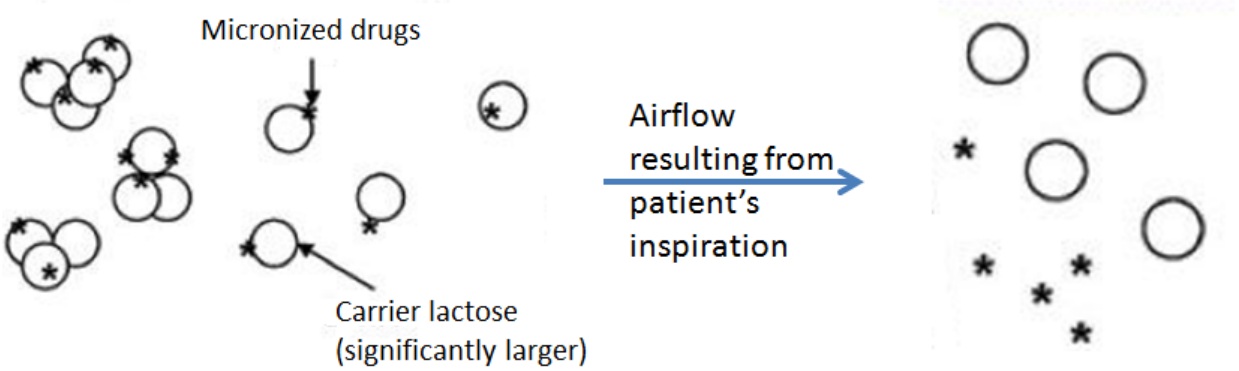
- For inhalation => avoid iv, fewer side effects, high concentration
- Combination => synergy
- Anti-biofilm activity
  => Macrolides

Combination: Macrolides and another class of antibiotics

Formulation strategies:
carrier-based formulation

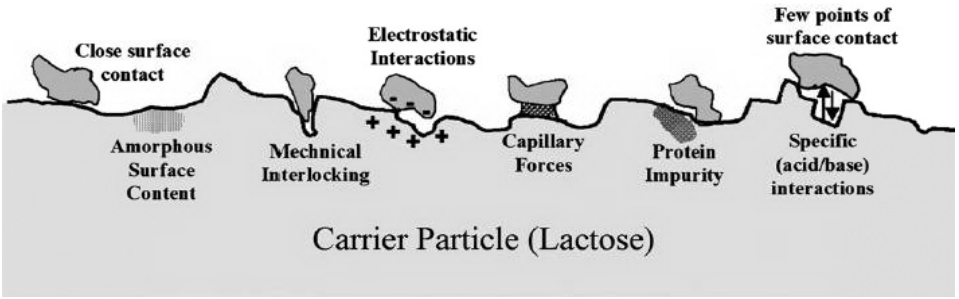


Highly cohesive
- Poor flowability
- Limits dispersibility into aerosol cloud



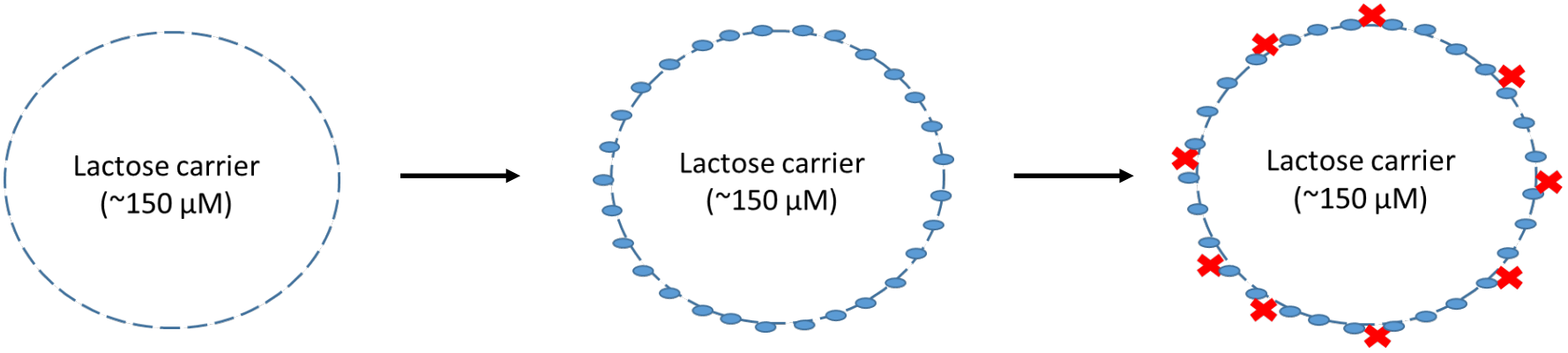
Carrier lactose aids flowability and drug re-dispersion

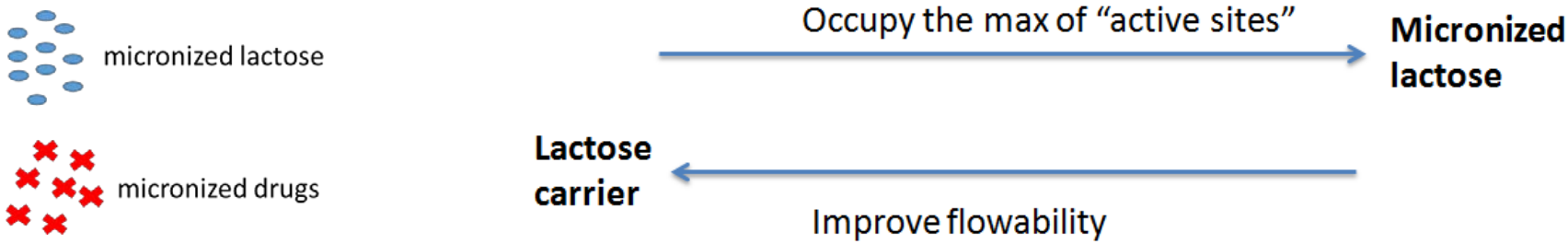
Formulation strategies: micronized lactose as competitor



"Active sites" = cleft/pit = high adhesion potential

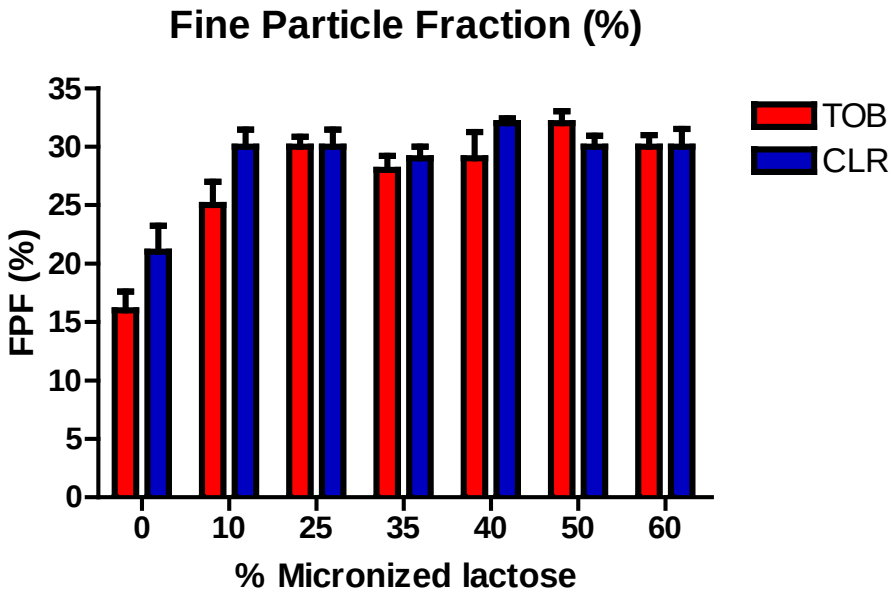
A.J. Hickey et al. (2007)





Lung depositions in function of % micronized lactose

- 20 mg of TOB
- 2 mg of CLR
- 20 mg of Lactose



Lung depositions in function of % micronized lactose

- 20 mg of TOB
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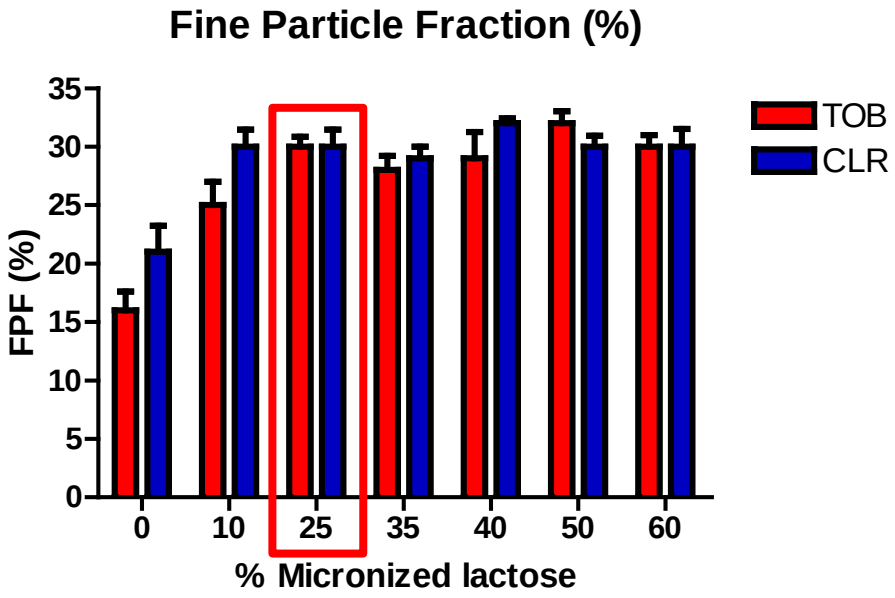
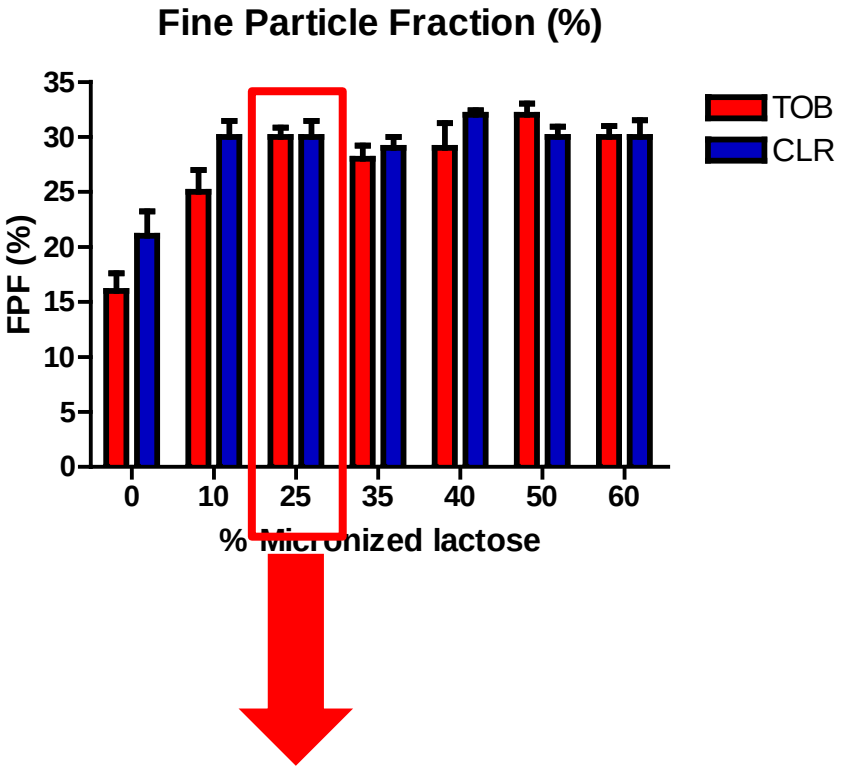


Process scale-up for production of pilot batches

- 20 mg of TOB
- 2 mg of CLR
- 20 mg of Lactose

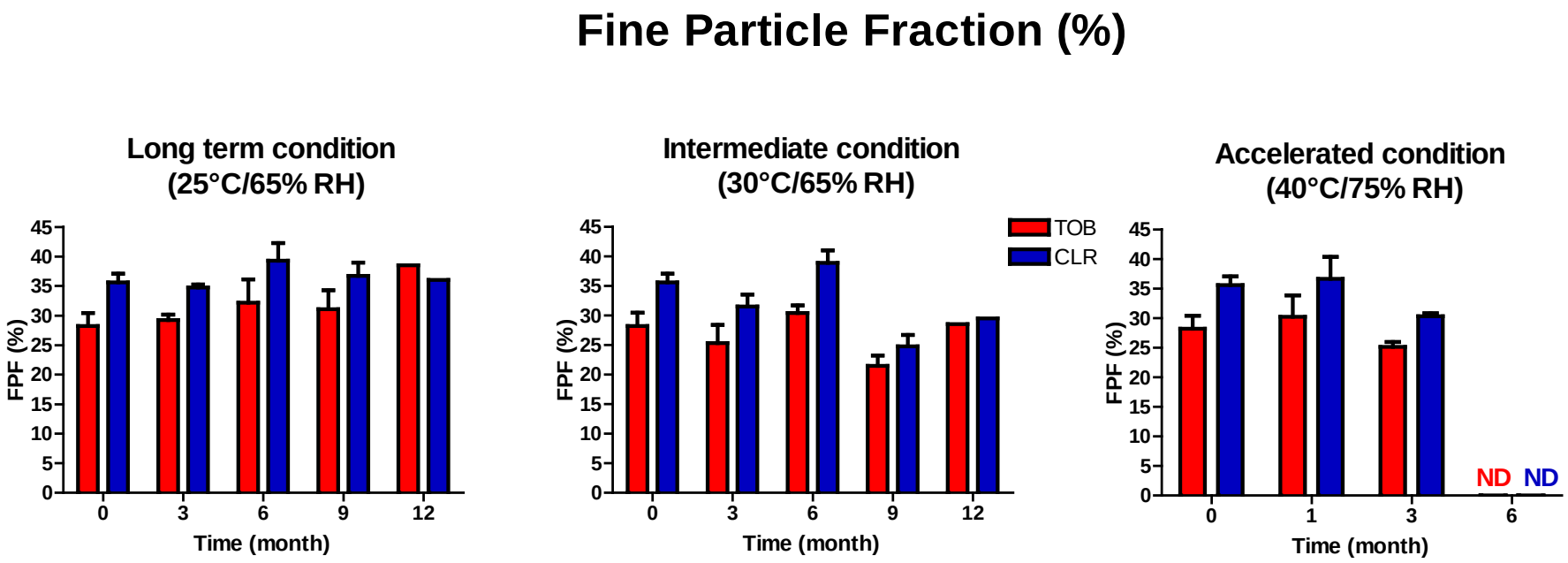


Reproducible capsule filling!

Planetary mixer Collette

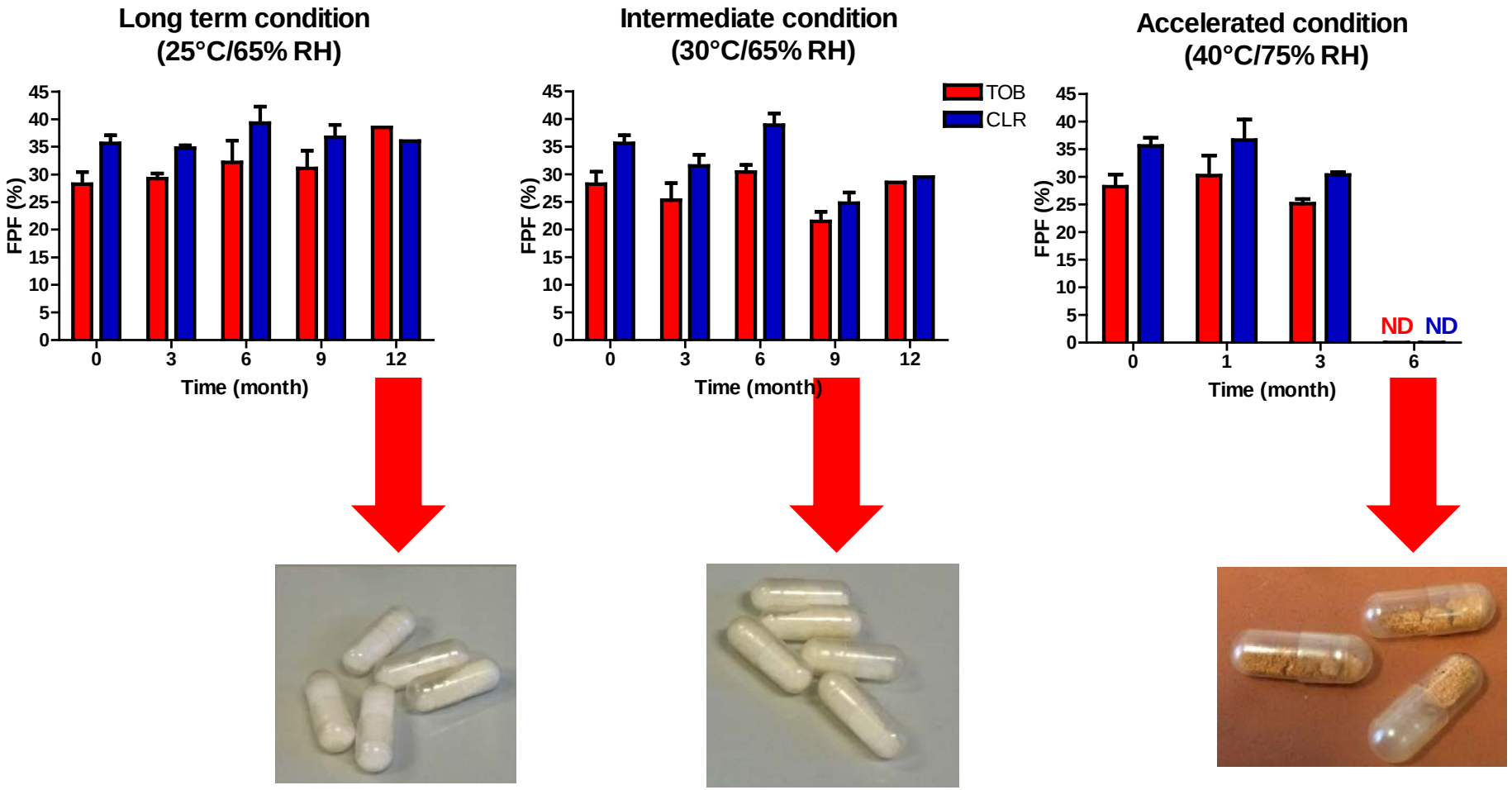
Capsule filling machine MG2 Futura

Stability studies of M25 through 12 months : pulmonary deposition



Stability studies of M25 through 12 months : pulmonary deposition

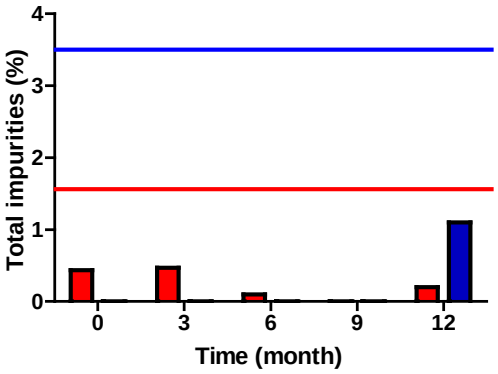
Fine Particle Fraction (%)



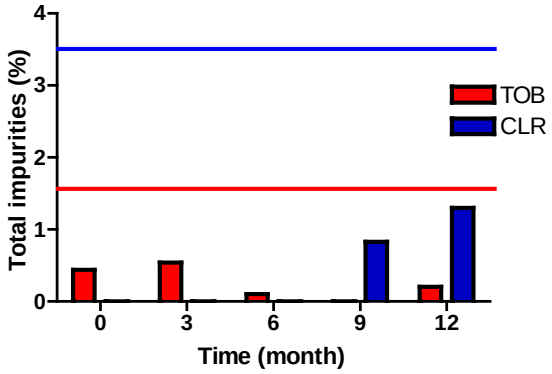
Stability studies of M25 through 12 months : impurities

Total impurities (%)

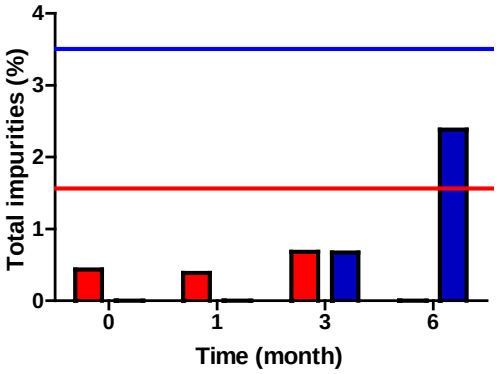
Long term condition (25°C/65%RH)



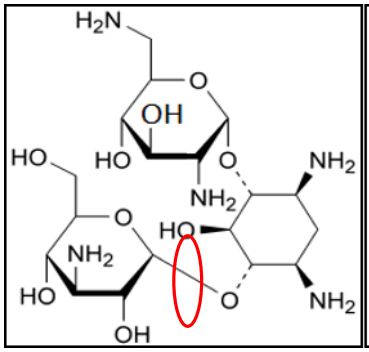
Intermediate condition (30°C/65%RH)



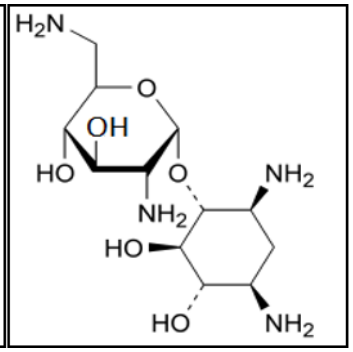
Accelerated condition (40°C/75%RH)



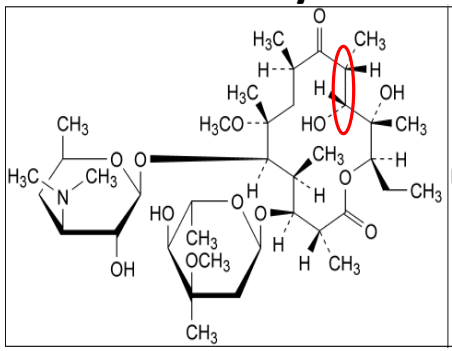
Kanamycin B



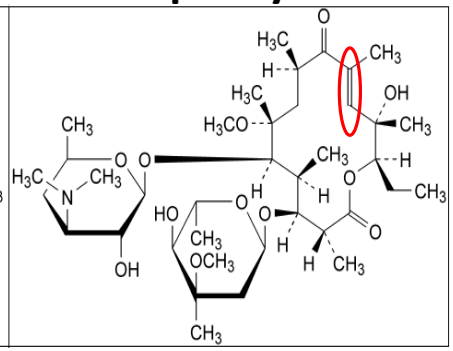
Neamine



Clarithromycin



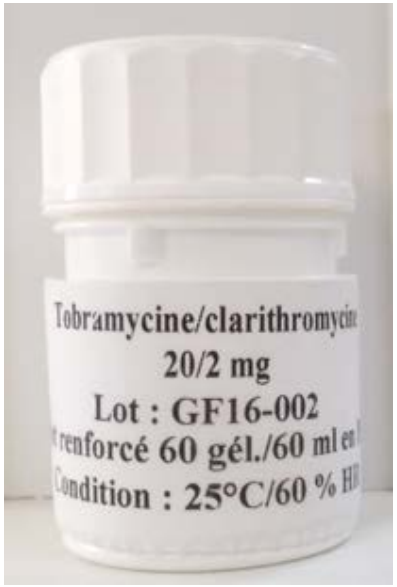
Impurity N



Solution under investigation?

Packaging:

- High-density polyethylene bottles
- 60 mL
- 2g of dessicant
- 60 capsules/bottle

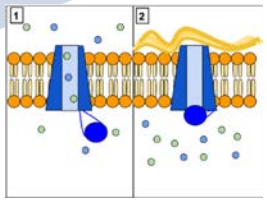


Packaging:

- High-density polyethylene bottles
- 60 mL
- 2g of dessicant
- 60 capsules/bottle
- **Bottles packaged under vacuum into aluminium pockets**

Cystic Fibrosis : Pathophysiology

Mutation in CFTR gene



Defective CFTR protein (chloride ion channel)

= compromised movement of Cl^-, Na^+, HCO_3^-

In the **lung**:

- Dehydration
- Mucus thickness
- Impaired mucociliary clearance

↓

- Hyper-inflammation
- Accumulation of **pathogens** and **chronic lung infection**

↓

S. aureus
H. Influenzae
P. aeruginosa →
S. maltophilia
B. cepacia

- Opportunistic
- 80% in CF patients
- Biofilm,SCV,intra..
- Diverse pathologies

Skin

Lung

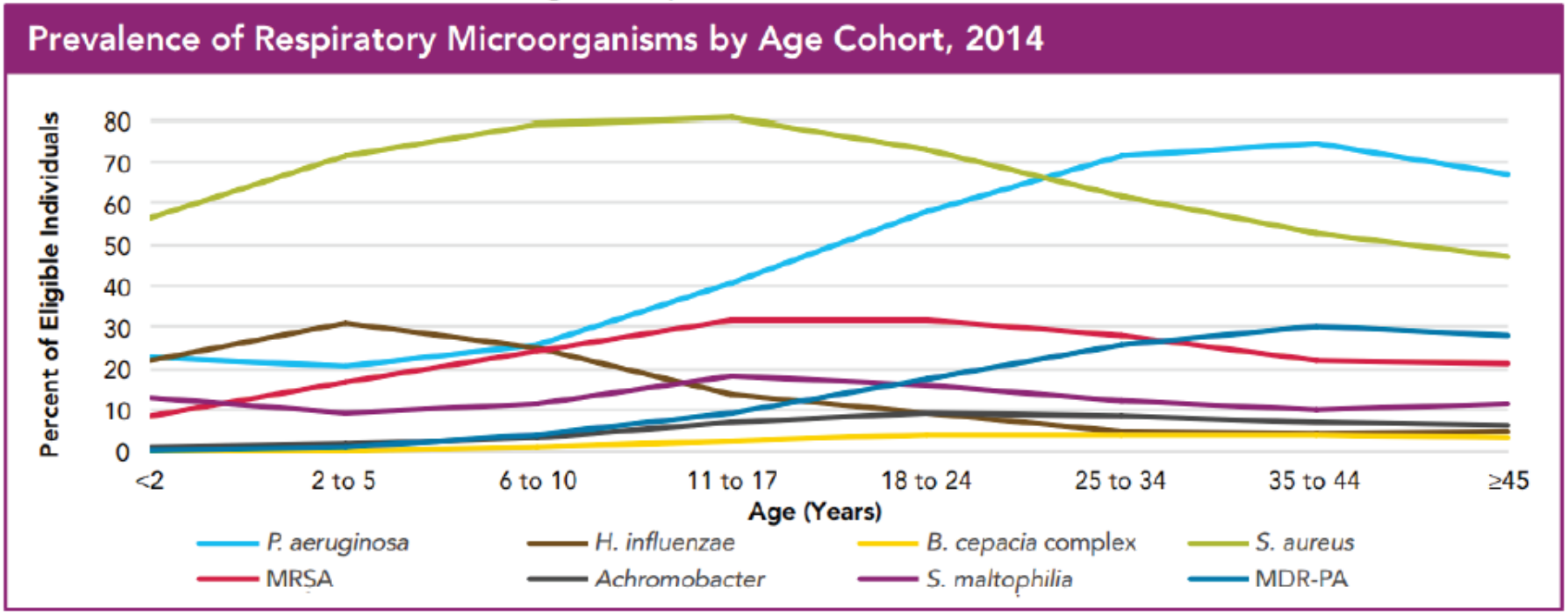
Liver

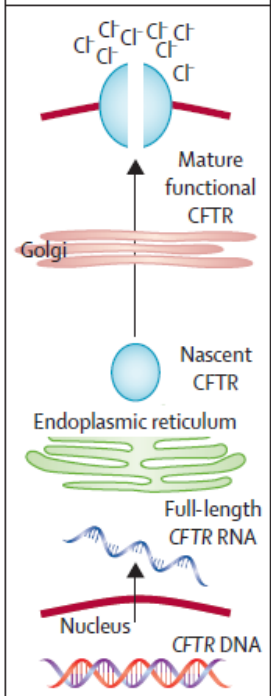
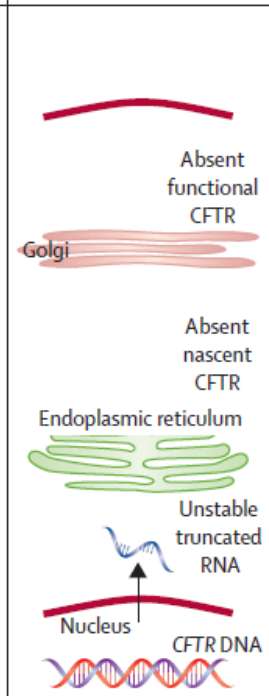
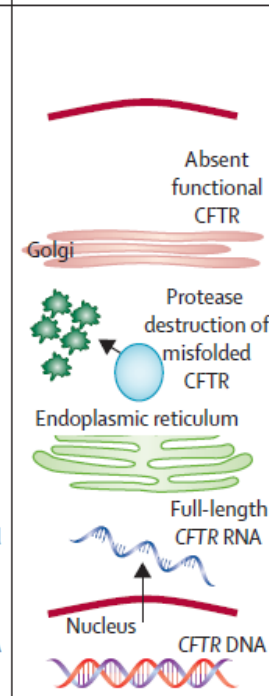
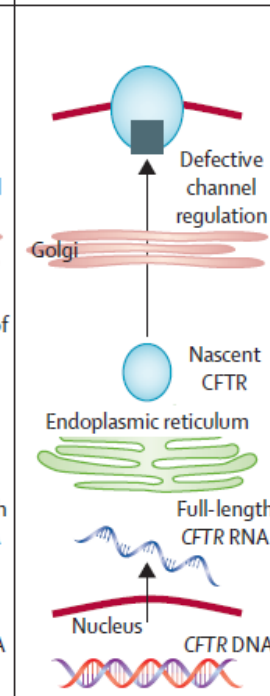
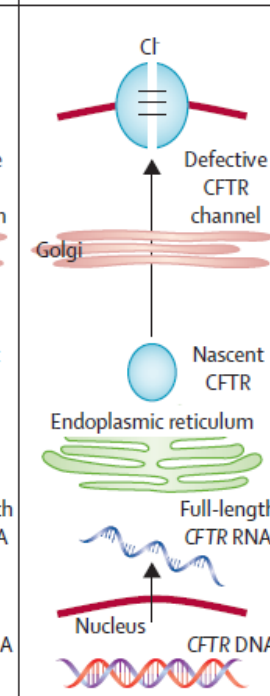
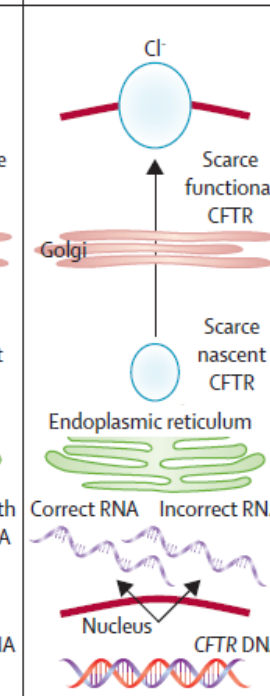
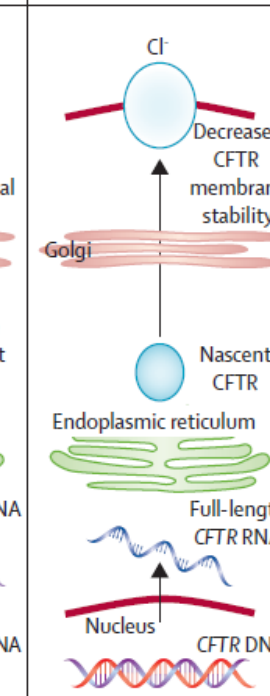
Pancreas

Intestine

Genital organs

Figure 5: Prevalence of different microorganisms infecting the lung of CF patients [3] *(Pseudomonas aeruginosa, Haemophilus influenzae, Burkholderia cepacia complex, Staphylococcus aureus, methicillin-resistant Staphylococcus aureus, Achromobacter xylosoxidans, Stenotrophomonas maltophilia and multidrug-resistant Pseudomonas aeruginosa)*



Normal	I	II	III	IV	V	VI
						
CFTR defect	No functional CFTR protein	CFTR trafficking defect	Defective channel regulation	Decreased channel conductance	Reduced synthesis of CFTR	Decreased CFTR stability
Type of mutations	Nonsense; frameshift; canonical splice	Missense; aminoacid deletion	Missense; aminoacid change	Missense; aminoacid change	Splicing defect; missense	Missense; aminoacid change
Specific mutation examples	Gly542X Trp1282X Arg553X 621+1G→T	Phe508del Asn1303Lys Ile507del Arg560Thr	Gly551Asp Gly178Arg Gly551Ser Ser549Asn	Arg117His Arg347Pro Arg117Cys Arg334Trp	3849+10kbC→T 2789+5G→A 3120+1G→A 5T	4326delTC Gln1412X 4279insA

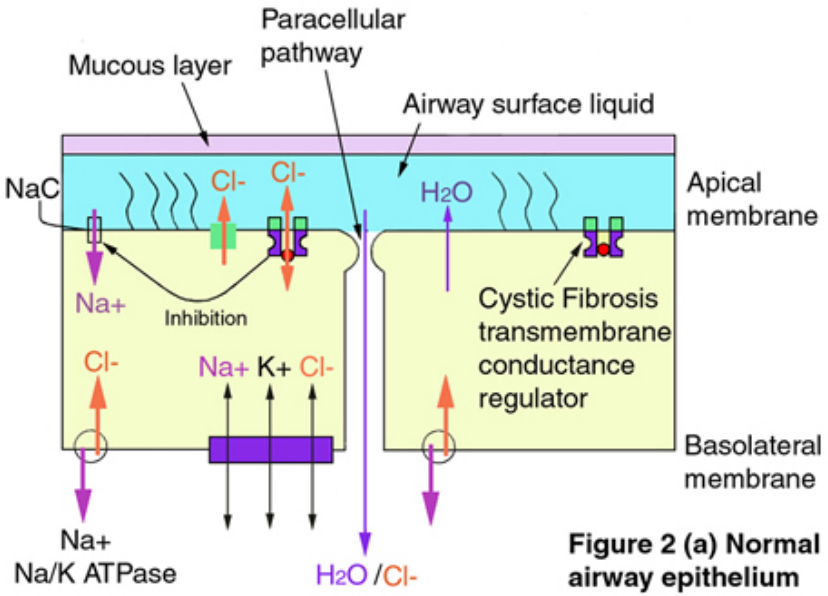


Figure 2 (a) Normal airway epithelium

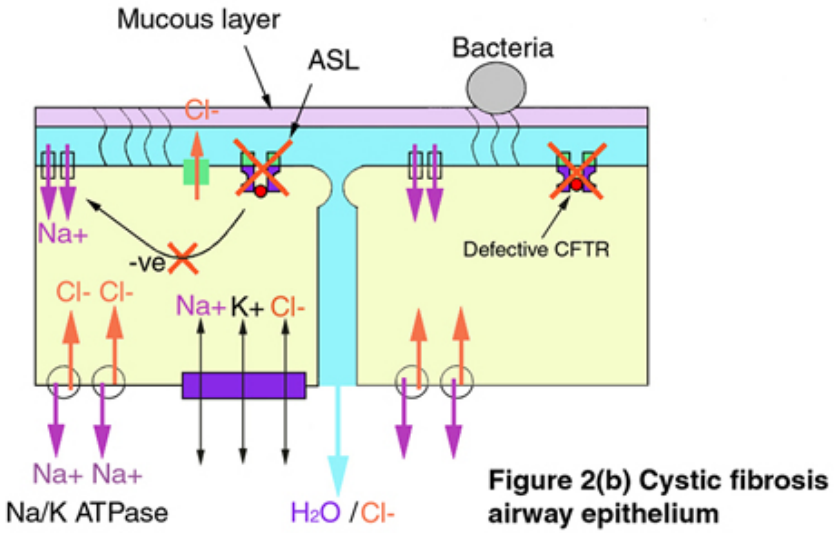
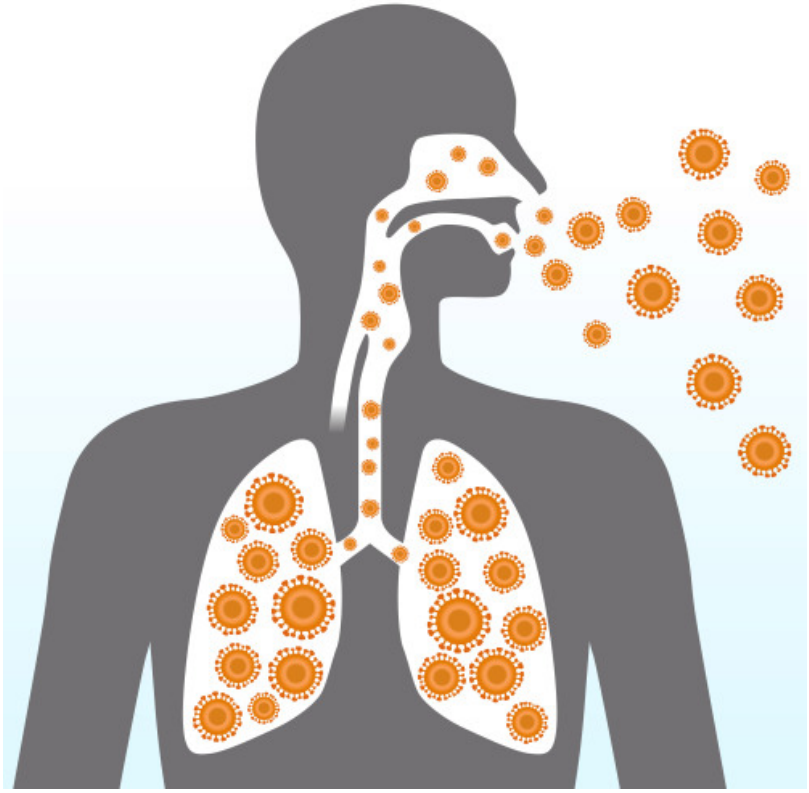


Figure 2(b) Cystic fibrosis airway epithelium

Pulmonary Drug Delivery



1) Local treatment:

- In situ
- Rapid onset of action
- Fewer side effects
- High local concentration

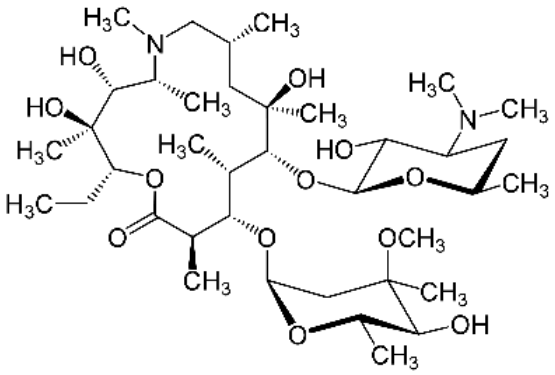
2) Systemic distribution

(drugs including peptides and proteins)

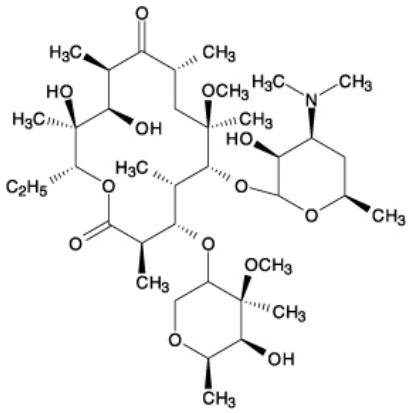
Eg: Insulins

Macrolides and CF

- Macrolides are used to treat infections caused by Gram-positive (e.g., *Streptococcus pneumoniae*) and limited Gram-negative (e.g., *Bordetella pertussis, Haemophilus influenzae*) bacteria.
- Also a substitute for patients with a penicillin allergy
- Not active against *Pseudomonas aeruginosa:*
  - MIC (AZM) = **128 to 512** µg/mL
  - Average concentration of AZM in the sputum of patients receiving high dose therapy (500 mg/day) is **26.6** µg/mL *(E.B. Wilms et al, 2008)*
- Macrolides given to CF patients **via oral route regularly** (70% of patients in USA) for the their immunomodulatory and anti- inflammatory effects => improved lung function and increased body weight



Azithromycin



Clarithromycin

Azithromycin use in Europe (1/2)

Country	Inhaled antibiotics inhaled > 3 months this year number (%)			Oxygen therapy this year number (%)			Macrolides > 3 months this year number (%)		
	Missing/ unknown	No	Yes	Missing/ unknown	No	Yes	Missing/ unknown	No	Yes
Austria	0 (0)	330 (62.03)	202 (37.97)	0 (0)	502 (94.36)	30 (5.64)	3 (0.56)	458 (86.09)	71 (13.35)
Belgium[1]	139 (12.06)	420 (36.43)	594 (51.52)	139 (12.06)	992 (86.04)	22 (1.91)	136 (11.80)	541 (46.92)	476 (41.28)
Czech Republic	24 (4.08)	416 (70.75)	148 (25.17)	24 (4.08)	538 (91.50)	26 (4.42)	24 (4.08)	477 (81.12)	87 (14.80)
Denmark	466 (100)	-	-	466 (100)	-	-	466 (100)	-	-
France[2]	0 (0)	3864 (61.47)	2422 (38.53)	0 (0)	5999 (95.43)	287 (4.57)	0 (0)	3580 (56.95)	2706 (43.05)
Greece	2 (2.04)	30 (30.61)	66 (67.35)	0 (0)	96 (97.96)	2 (2.04)	0 (0)	83 (84.69)	15 (15.31)
Hungary	119 (23.52)	233 (46.05)	154 (30.43)	93 (18.38)	370 (73.12)	43 (8.50)	92 (18.18)	327 (64.62)	87 (17.19)
Ireland	106 (9.97)	558 (52.49)	399 (37.54)	12 (1.13)	1010 (95.01)	41 (3.86)	24 (2.26)	676 (63.59)	363 (34.15)
Israel	7 (1.32)	237 (44.8)	285 (53.88)	9 (1.70)	509 (96.22)	11 (2.08)	8 (1.51)	239 (45.18)	282 (53.31)
Italy	1019 (21.36)	2377 (49.82)	1375 (28.82)	1019 (21.36)	3568 (74.79)	184 (3.86)	1018 (21.34)	2452 (51.39)	1301 (27.27)
Latvia	1 (2.86)	12 (34.29)	22 (62.86)	0 (0)	32 (91.43)	3 (8.57)	1 (2.86)	24 (68.57)	10 (28.57)
Lithuania	0 (0)	13 (100)	0 (0)	0 (0)	12 (92.31)	1 (7.69)	0 (0)	13 (100)	0 (0)
Rep of Macedonia	1 (1.02)	59 (61.20)	38 (38.78)	1 (1.02)	95 (96.94)	2 (2.04)	1 (1.02)	77 (78.57)	20 (20.41)

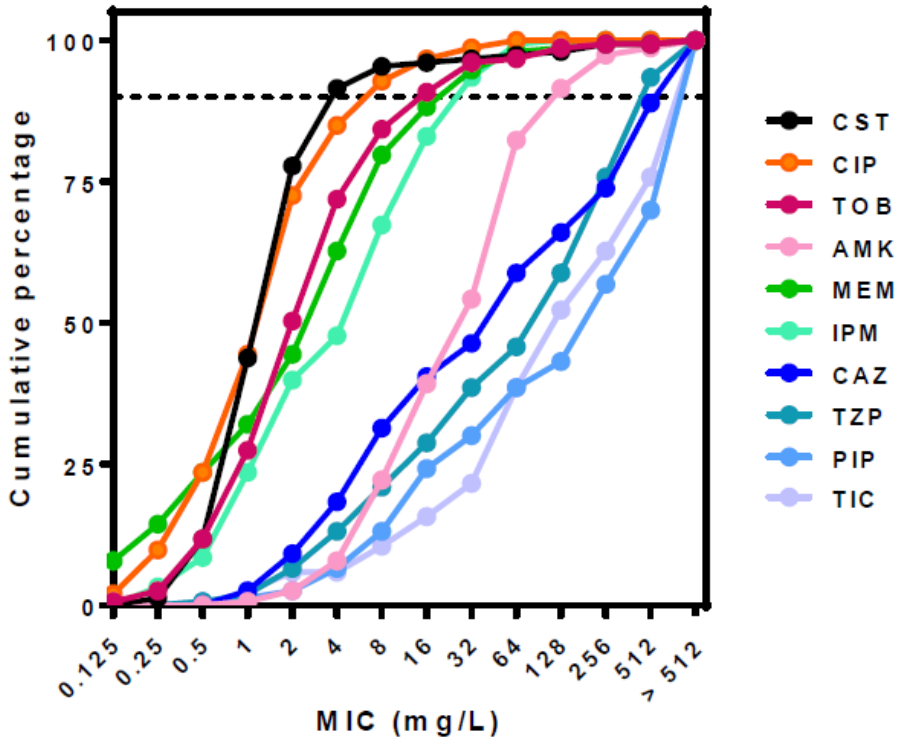
Azithromycin use in Europe (2/2)

Country	Inhaled antibiotics inhaled > 3 months this year number (%)			Oxygen therapy this year number (%)			Macrolides > 3 months this year number (%)		
	Missing/ unknown	No	Yes	Missing/ unknown	No	Yes	Missing/ unknown	No	Yes
Rep of Moldova	0 (0)	29 (47.54)	32 (52.46)	0 (0)	57 (93.44)	4 (6.56)	0 (0)	27 (44.26)	34 (55.74)
The Netherlands	6 (0.45)	782 (58.31)	553 (41.24)	3 (0.22)	1283 (95.67)	55 (4.10)	4 (0.30)	769 (57.35)	568 (42.36)
Portugal	15 (5.86)	115 (44.92)	126 (49.22)	14 (5.47)	228 (89.06)	14 (5.47)	15 (5.86)	145 (56.64)	96 (37.50)
Romania	0 (0)	24 (58.54)	17 (41.46)	0 (0)	41 (100)	0 (0)	0 (0)	38 (92.68)	3 (7.32)
Russian Federation	62 (3.23)	1104 (57.44)	756 (39.33)	64 (3.33)	1756 (91.36)	102 (5.31)	69 (3.59)	1200 (62.43)	653 (33.98)
Serbia	1 (0.65)	100 (64.94)	53 (34.42)	1 (0.65)	146 (94.81)	7 (4.55)	0 (0)	138 (89.61)	16 (10.39)
Slovak Republic	1 (0.67)	70 (46.98)	78 (52.35)	0 (0)	147 (98.66)	2 (1.34)	0 (0)	89 (59.73)	60 (40.27)
Slovenia	1 (1.23)	68 (83.95)	12 (14.81)	0 (0)	79 (97.53)	2 (2.47)	0 (0)	76 (93.83)	5 (6.17)
Spain	31 (2.26)	623 (45.34)	720 (52.40)	28 (2.04)	1301 (94.69)	45 (3.28)	26 (1.89)	824 (59.97)	524 (38.14)
Sweden	30 (4.89)	539 (87.79)	45 (7.33)	24 (3.91)	576 (93.81)	14 (2.28)	23 (3.75)	386 (62.87)	205 (33.39)
Switzerland	6 (0.91)	406 (61.33)	250 (37.76)	4 (0.60)	633 (95.62)	25 (3.78)	4 (0.60)	460 (69.49)	198 (29.91)
Ukraine	3 (2.86)	65 (61.90)	37 (35.24)	1 (0.95)	99 (94.29)	5 (4.76)	3 (2.86)	7 (6.67)	95 (90.48)
United Kingdom[3]	10 (0.11)	4081 (45.09)	4959 (54.80)	83 (0.92)	8358 (92.35)	609 (6.73)	1 (0.01)	5194 (57.39)	3855 (42.60)

Antipseudomonals susceptibility studies

CST: Colistin; CIP: Ciprofloxacin TOB: Tobramycin; AMK: Amikacin; MEM: Meropenem; IPM: Imipenem; CAZ: Ceftazidime; TZP: Piperacillin-tazobactam; PIP: Piperacillin; TIC: Ticarcillin

MIC distributions for the antibiotics under study (n=153)



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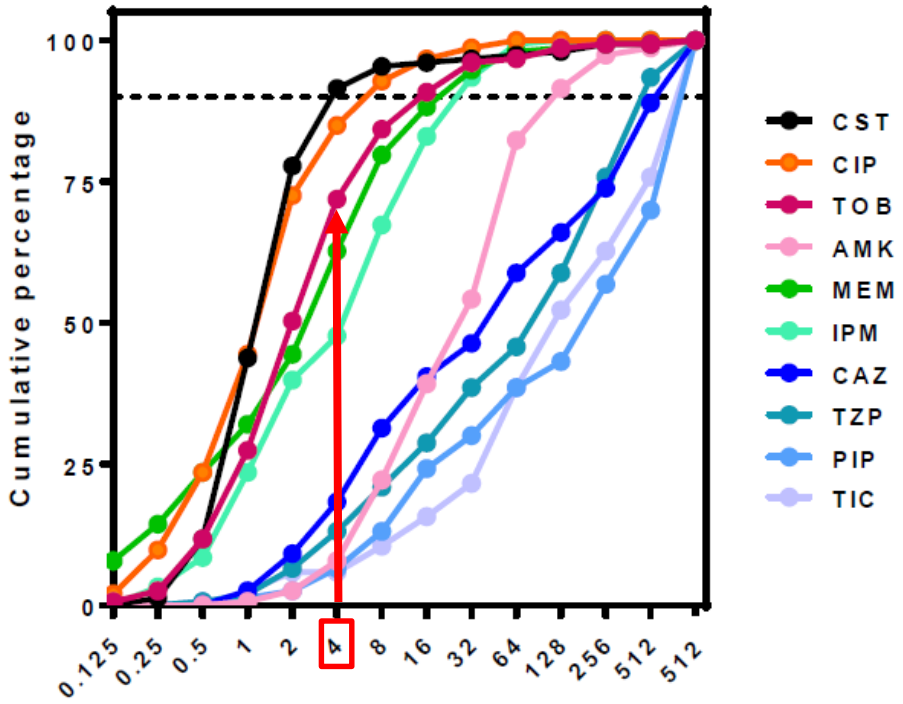
MIC distributions for the antibiotics under study (n=153)



TOB Breakpoint according to EUCAST

Antipseudomonals susceptibility studies

TABLE 2 MIC distributions for antipseudomonal antibiotics and corresponding percent susceptibility according to EUCAST or CLSI breakpoints[a]

	MIC (mg/liter)				Susceptibility according to: EUCAST[b]			CLSI[c]		
Antibiotic	Min	Max	50%	90%	% S	% I	% R	% S	% I	% R
TIC	1	>512	128	>512	16	NA	84	16	23	61
PIP	0.5	>512	256	>512	24	NA	76	24	15	61
TZP	0.5	>512	128	512	29	NA	71	29	17	54
CAZ	1	>512	64	512	31	NA	69	31	10	59
IPM	0.25	128	4	32	48	19	33	48	19	33
MEM	0.032	256	2	16	44	36	20	63	17	20
AMK	1	>512	32	128	22	17	61	39	15	46
TOB	0.064	>512	2	16	72	NA	28	72	12	16
CIP	0.064	64	1	8	24	20	56	44	29	27
CST	0.25	>512	1	4	92	NA	8	78	14	8

Multidrug resistant (MDR) isolates = 94 isolates => 61%!

Antipseudomonals susceptibility studies

TABLE 1 *P. aeruginosa* collection (2006 to 2012)

Country	No. of isolates	No. of patients	Period of sampling
Belgium	44	38	2010
Germany	51	36	2012
United Kingdom	58	46	2006–2009
Total	153	120	

Multidrug resistant (MDR) isolates = 94 isolates => 61%!

56 isolates (resistant to at least 4 classes) were genotyped (PFGE)

TABLE 4 Distribution of pulsotypes among the MDR *P. aeruginosa* clinical isolates

Country	No. of MDR strains	No. of pulsotypes		No. of strains in epidemic pulsotype								
		Sporadic	Epidemic	CA[a]	CK	CM	CD	H	WW	YI	CJ	YY
Belgium	10	3	4	0	0	2	2	0	2	0	0	1
Germany	22	11	5	0	2	0	0	3	0	2	2	2
United Kingdom	24	5	2	18	0	0	0	0	0	0	0	1

[a] CA pulsotype corresponds to the LES epidemic clone pulsotype.

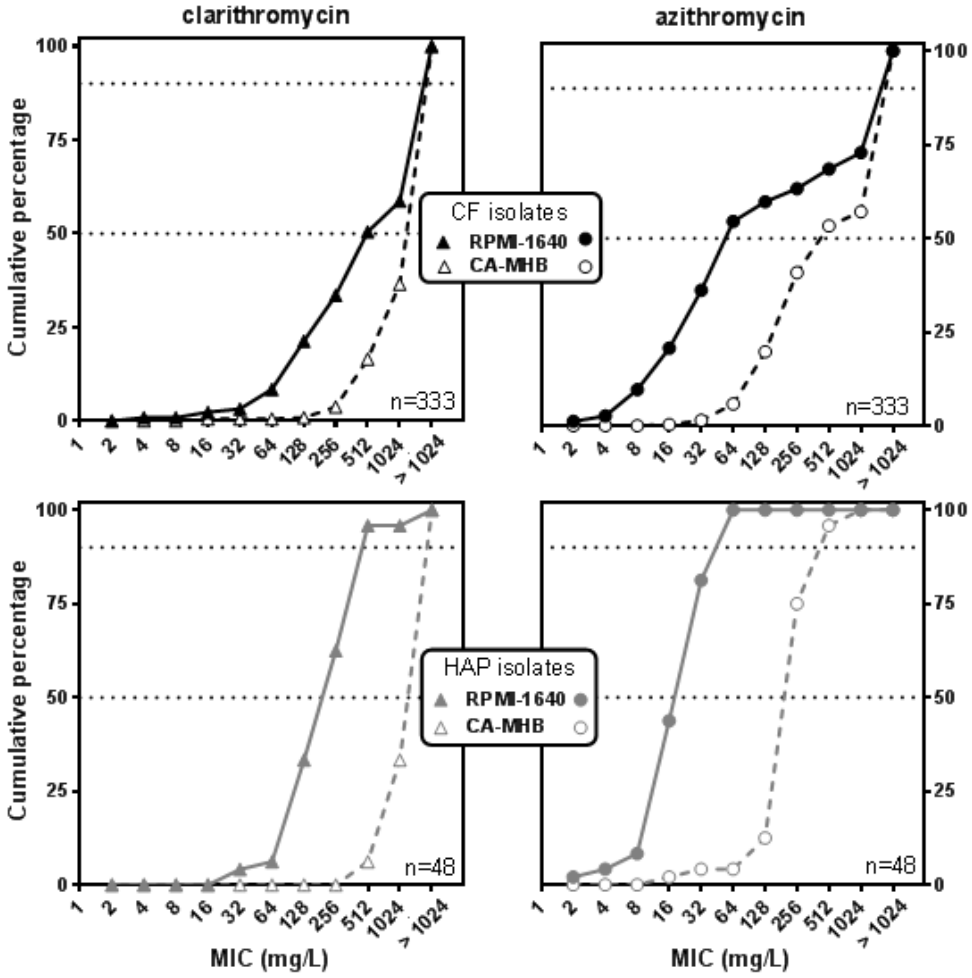
A **high genetic diversity** was observed, with 19 sporadic pulsotypes and 9 epidemic pulsotypes

Macrolide resistance

Fig 1: MIC distributions of clarithromycin (left) and azithromycin (right) in RPMI-1640 (closed symbols) and CA-MHB (open symbols) for CF (top) and HAP (bottom) isolates.
The Table below the graphs shows MIC_{50} and MIC_{90} values in both media. The number of isolates included in the study is indicated in each panel.

CF

Healthcare-associated pneumonia (HAP)



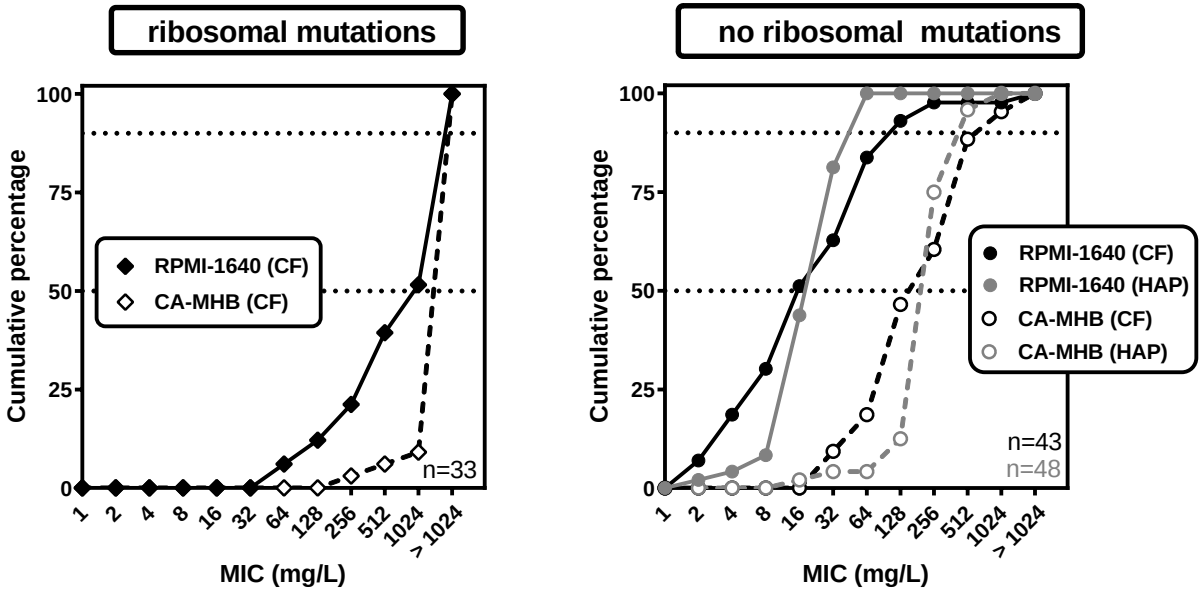
origin	medium	clarithromycin		azithromycin	
		MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
CF	CA-MHB	> 1024	> 1024	512	>1024
	RPMI-1640	512	>1024	64	>1024
HAP	CA-MHB	>1024	>1024	256	512
	RPMI-1640	256	512	32	64

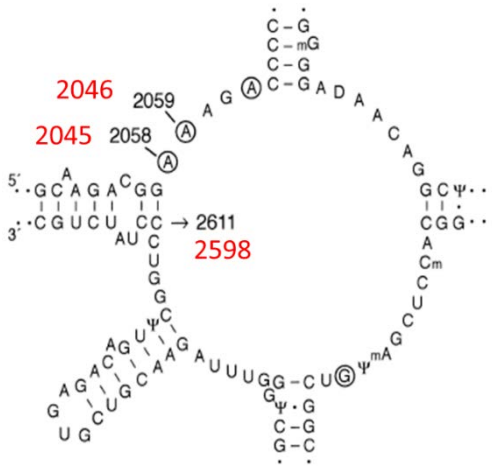
CF isolates are less susceptible than HAP isolates

Macrolide resistance

Mutation on macrolide's target!

Fig 5: MIC distributions of azithromycin (left) and solithromycin (right) in CA-MHB (open symbols) and RPMI-1640 (closed symbols) for CF and HAP isolates presenting mutations (top) or not (bottom) in domain V of 23 S rRNA.
The Table below the graphs shows MIC_{50} and MIC_{90} values in both media for mutated (YES) or non-mutated (NO) isolates. The number of isolates included in the study is indicated in each panel.





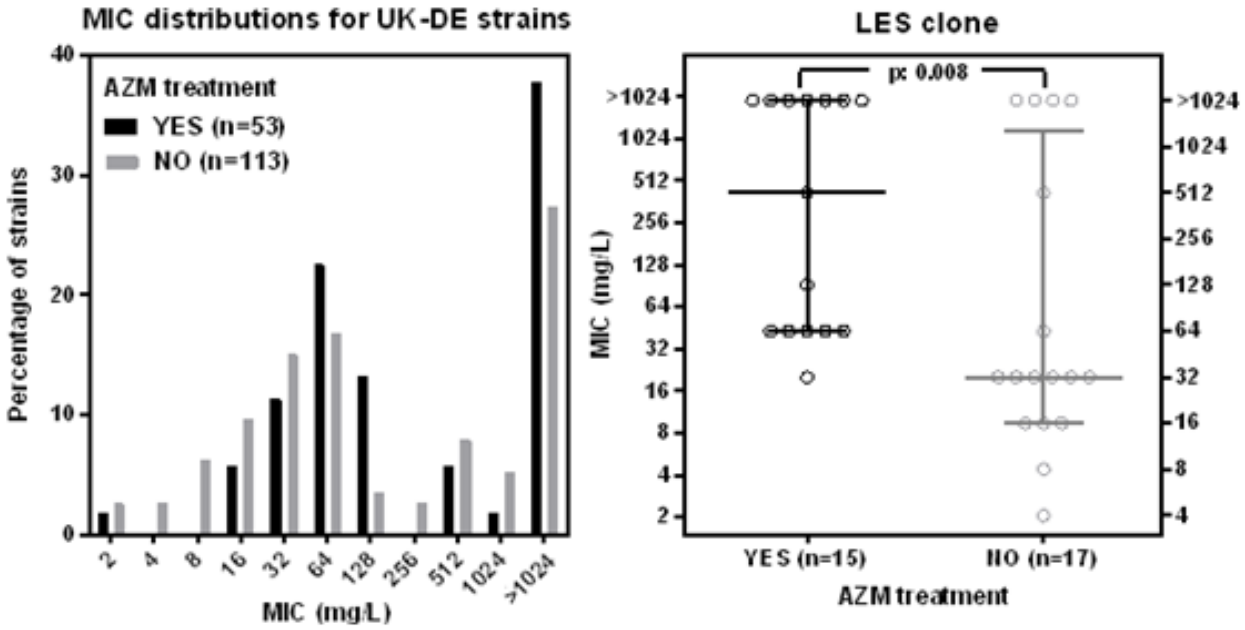
Secondary-structure models of the peptidyl transferase center in **domain V of 23S rRNA** of *E.coli, Vester B et al. AAC (2012)*

origin	ribosomal mutations	medium	azithromycin	
			MIC_{50}	MIC_{90}
CF	YES	CA-MHB	1024	>1024
		RPMI-1640	1024	>1024
	NO	CA-MHB	256	1024
		RPMI-1640	16	128
HAP	NO	CA-MHB	256	512
		RPMI-1640	32	64

Mutations only found in CF isolates

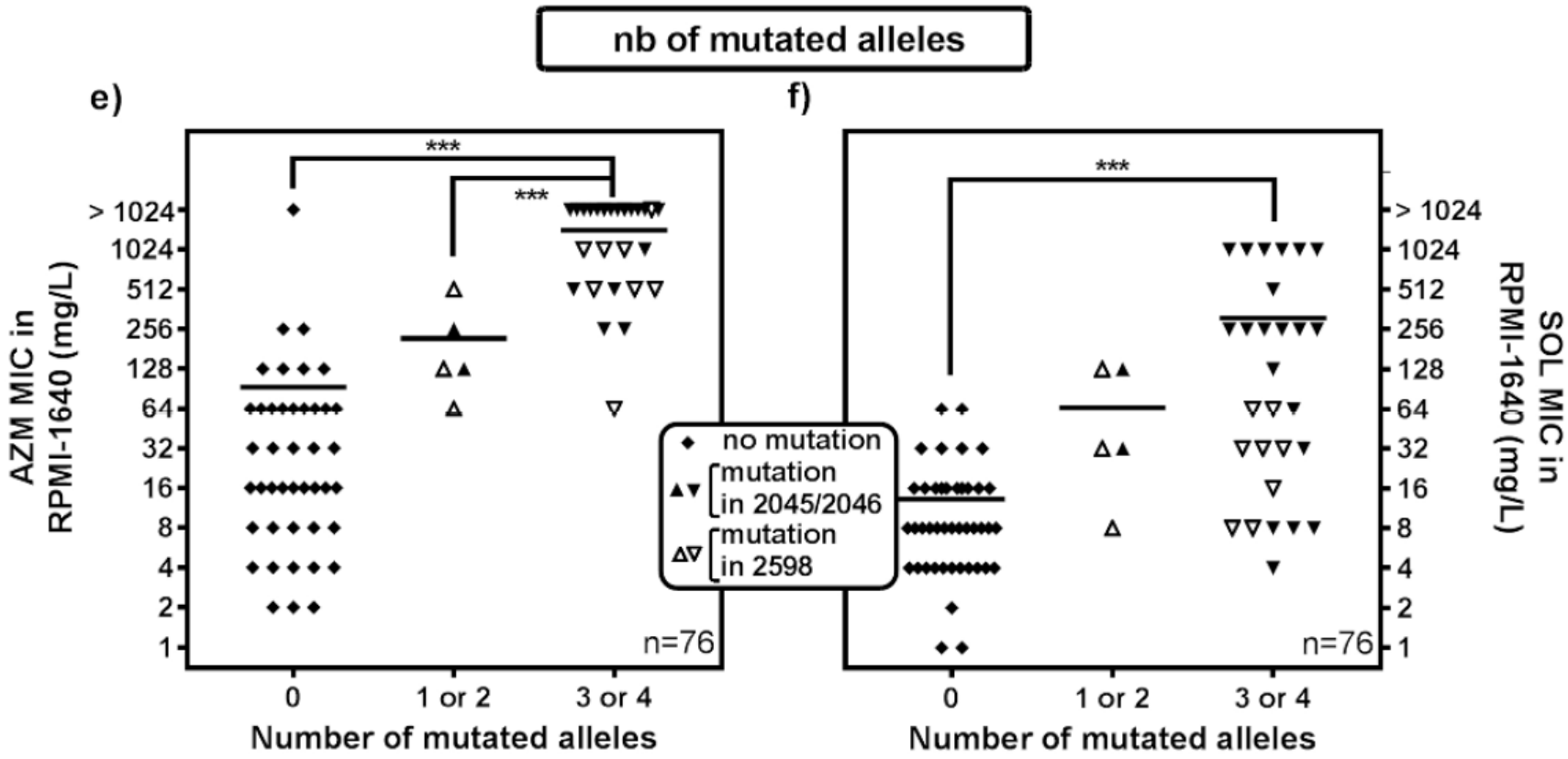
Acquired resistance to macrolides?

Clinical association!

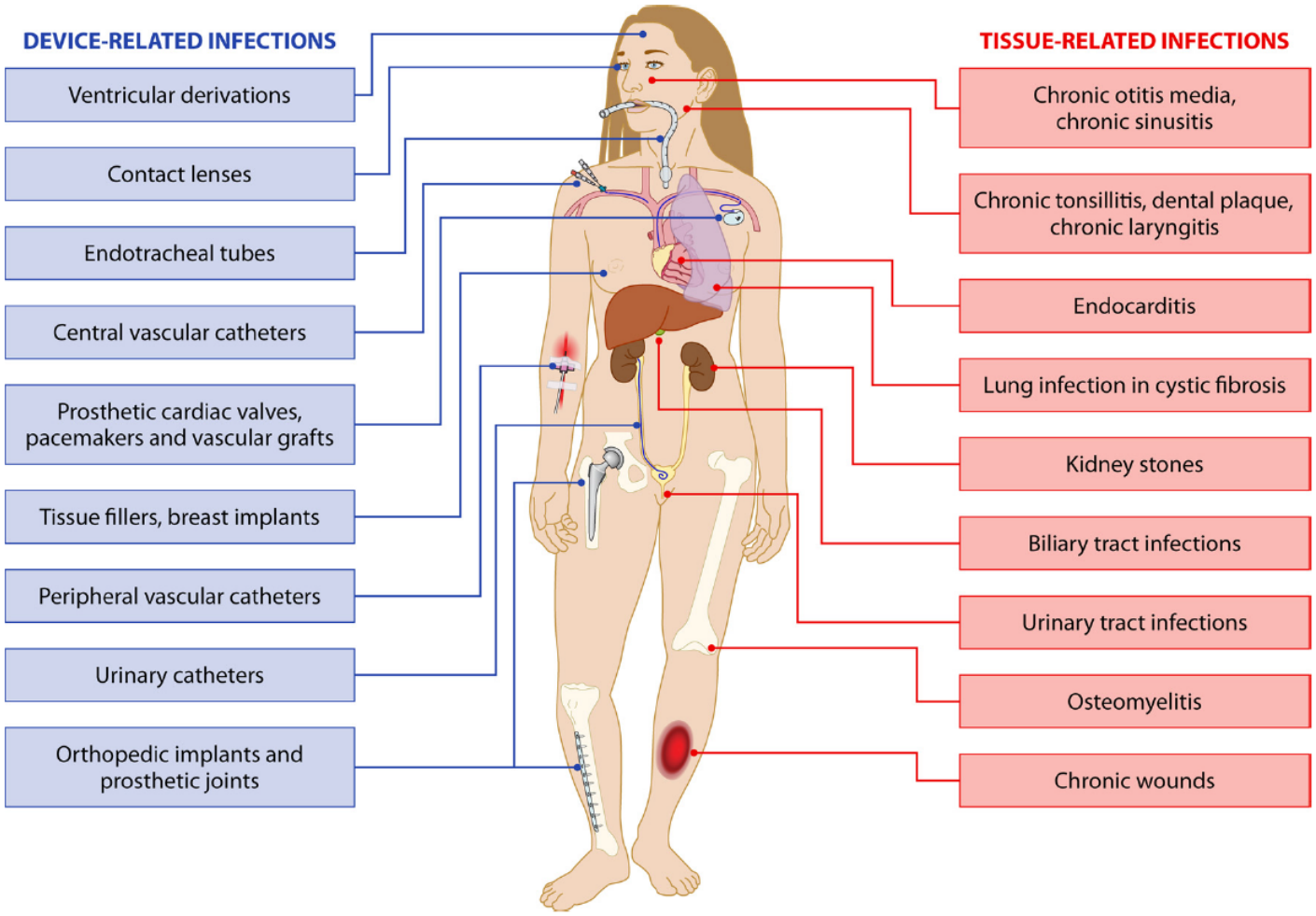


Mutation rate: 39% 12%

- **Isolates are less susceptibles to AZM in YES group**
- **Mutations more frequent in AZM-treated patients from LES**

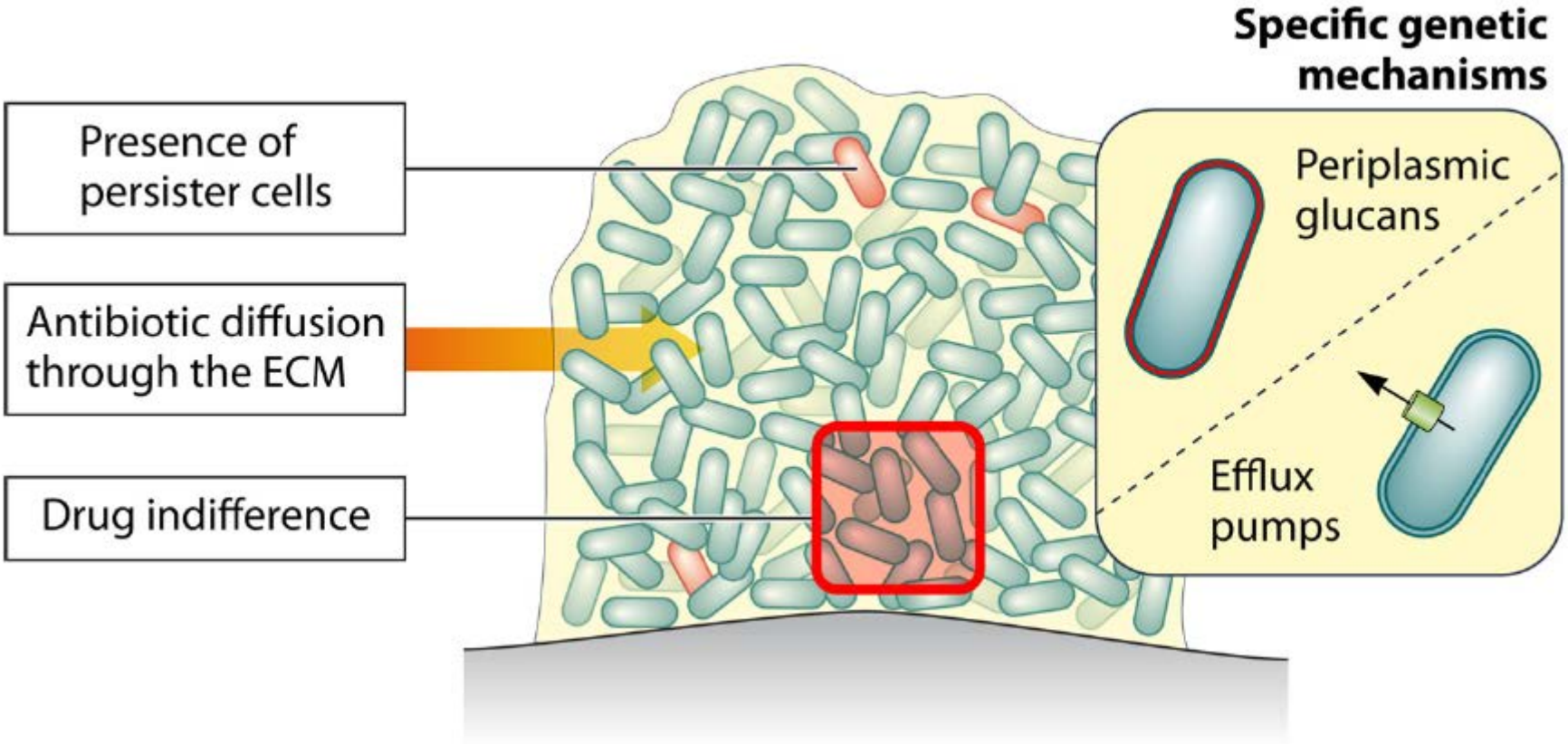

nb of mutated alleles
e)
f)
***
***
***
> 1024
1024
512
256
128
64
32
16
8
4
2
1
AZM MIC in RPMI-1640 (mg/L)
SOL MIC in RPMI-1640 (mg/L)
no mutation
mutation in 2045/2046
mutation in 2598
n=76
n=76
0
1 or 2
3 or 4
Number of mutated alleles
Number of mutated alleles

Mechanisms of antibiotic tolerance within bacterial biofilm



David Lebeaux et al, Microbiology and Molecular Biology Reviews p. 510–543

Mechanisms of antibiotic tolerance within bacterial biofilm



David Lebeaux et al, Microbiology and Molecular Biology Reviews p. 510–543

Table 3: Inhaled antibiotics for the treatment of pseudomonal infection in CF patients in Europe

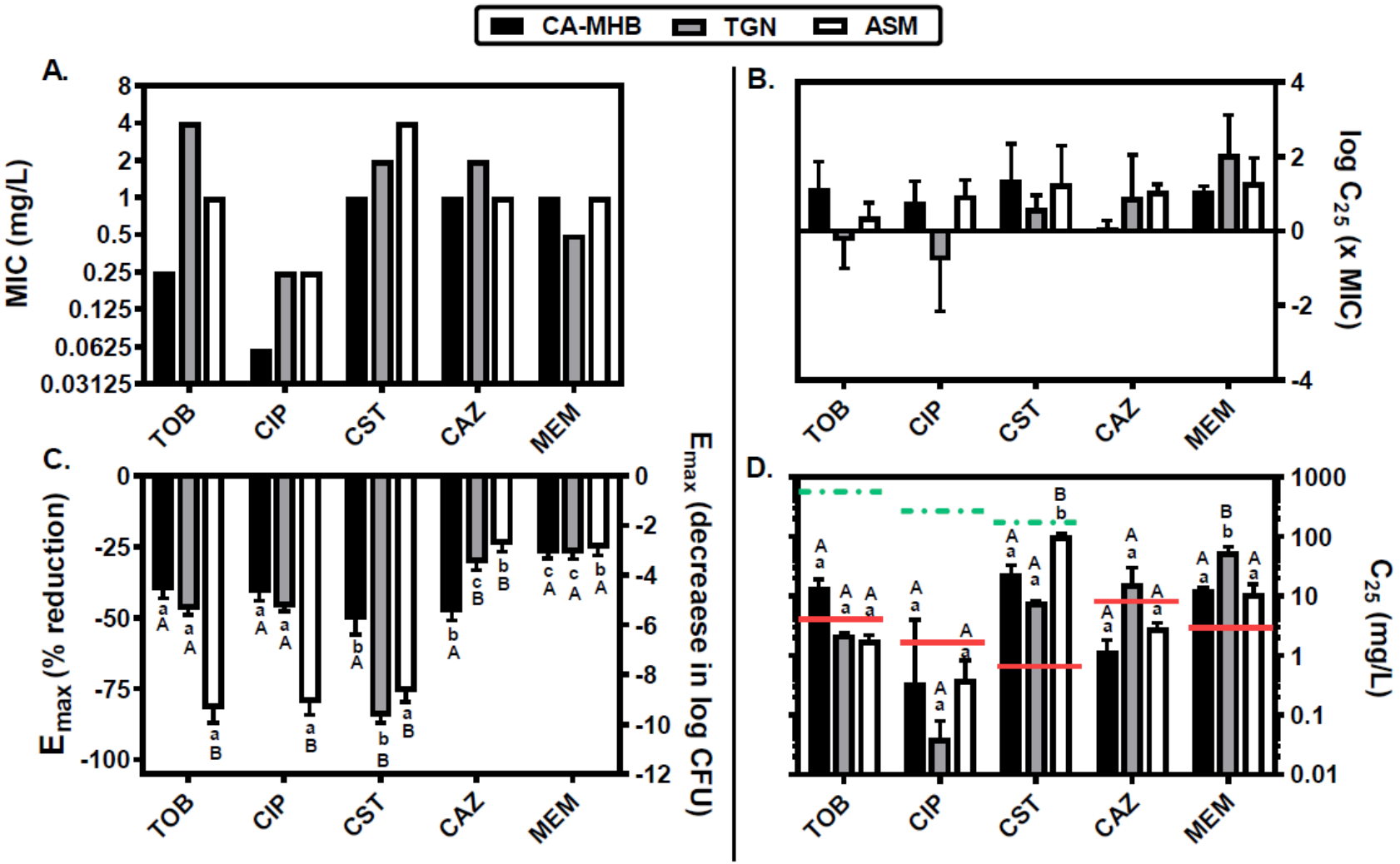
Antibiotic	Formulation	Trade name	Dosage	Sputum concentration (in µg/g of sputum)*
Tobramycin	Solution	TOBI	300 mg/5 mL Twice daily	737
	Inhalation powder	TIP	112 mg twice daily	1047
Aztreonam	Solution	Cayston	75 mg three times daily	726
Levofloxacin	Solution	Aeroquin	240 mg twice daily	4690
Colistin	Inhalation powder	Colobreathe	1 662 500 IU twice daily	202

*based on European Medicine Agency Summary of Product Characteristics

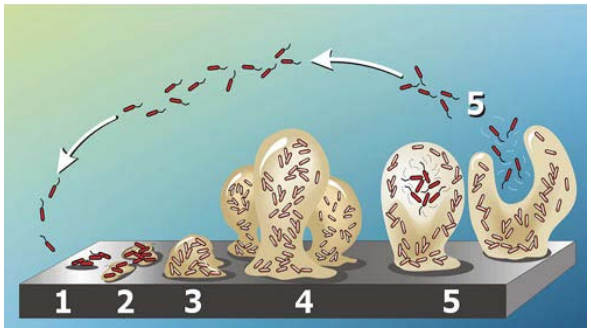
	iv on CF patients*		
	dose	Cmax sputum (µg/mL)	Reference
Tobra	8mg/kg	3.88	(1)
Cipro	200 mg	1.6	(3)
Colistin	400 mg colistin methanesulfonate	0.72	(2)
Cefta	3g	9.5	(1)
Meropenem*	500 mg	~1	(4)

*not CF patients (respiratory infection, particularly bronchopneumonia)

(1) https://www.ncbi.nlm.nih.gov/pubmed/17902147
(2) https://www.ncbi.nlm.nih.gov/pubmed/24550334
(3) https://www.ncbi.nlm.nih.gov/pmc/articles/PMC284211/
(4) https://www.ncbi.nlm.nih.gov/pubmed/9126702


CA-MHB
TGN
ASM
A.
MIC (mg/L)
8
4
2
1
0.5
0.25
0.125
0.0625
0.03125
TOB
CIP
CST
CAZ
MEM
B.
log C25 (x MIC)
4
2
0
-2
-4
C.
Emax (% reduction)
0
-25
-50
-75
-100
Emax (decreaese in log CFU)
0
-2
-4
-6
-8
-10
-12
D.
C25 (mg/L)
1000
100
10
1
0.1
0.01

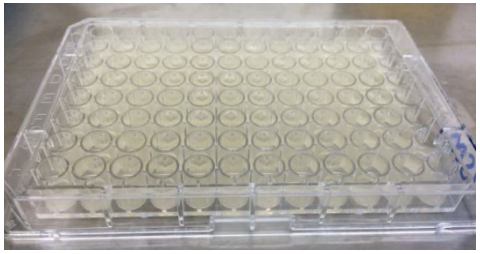
Development of biofilm model

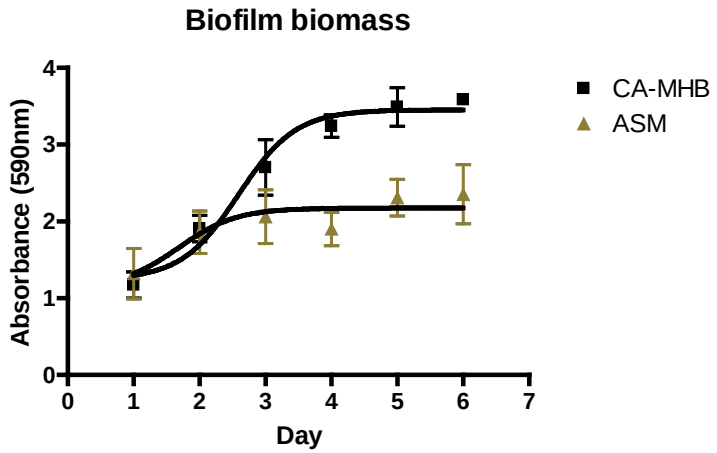


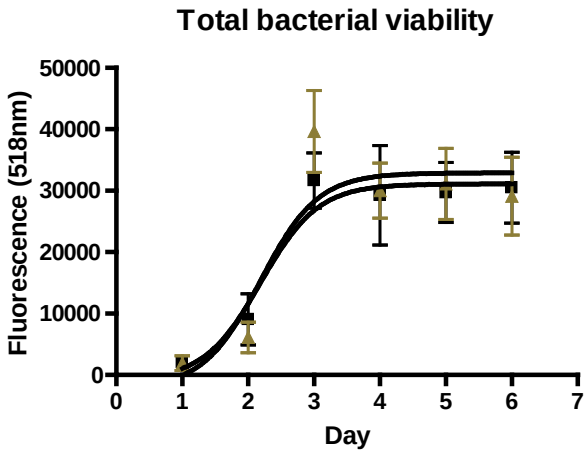
Graphic and photos by Peg Dirckx and David Davies © 2003 Center for Biofilm Engineering Montana State University.

Persistence of chronic infection = biofilm

Objective: Establish a biofilm model relevant to CF lung pathophysiology to study antimicrobial activity
=> **Mature, static P. aeruginosa biofilm model in a 96-well plate**



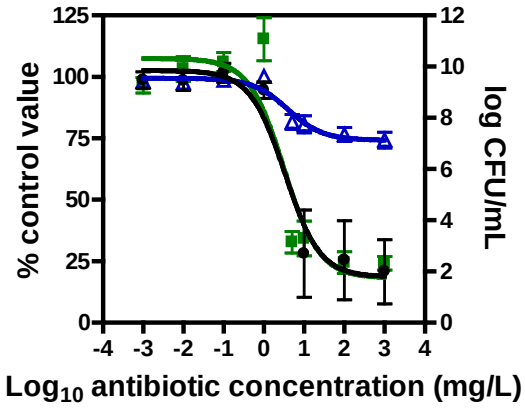




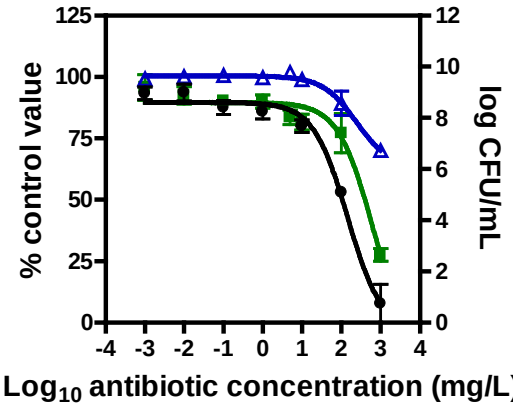
Biofilm model and antibiotic activity

Antibiotic activity on 4-day old mature P. aeruginosa PAO1 biofilm in ASM after 24-hour of antibiotic exposure

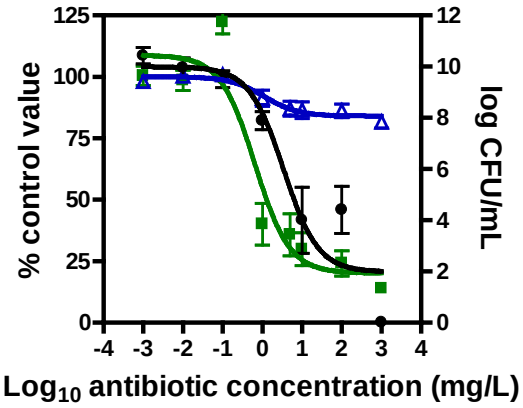
Tobramycin



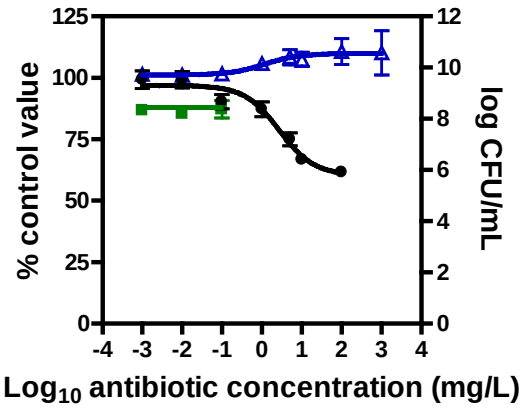
Colistin



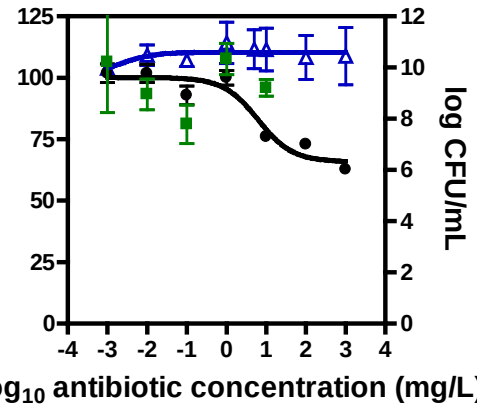
Ciprofloxacin



Ceftazidime

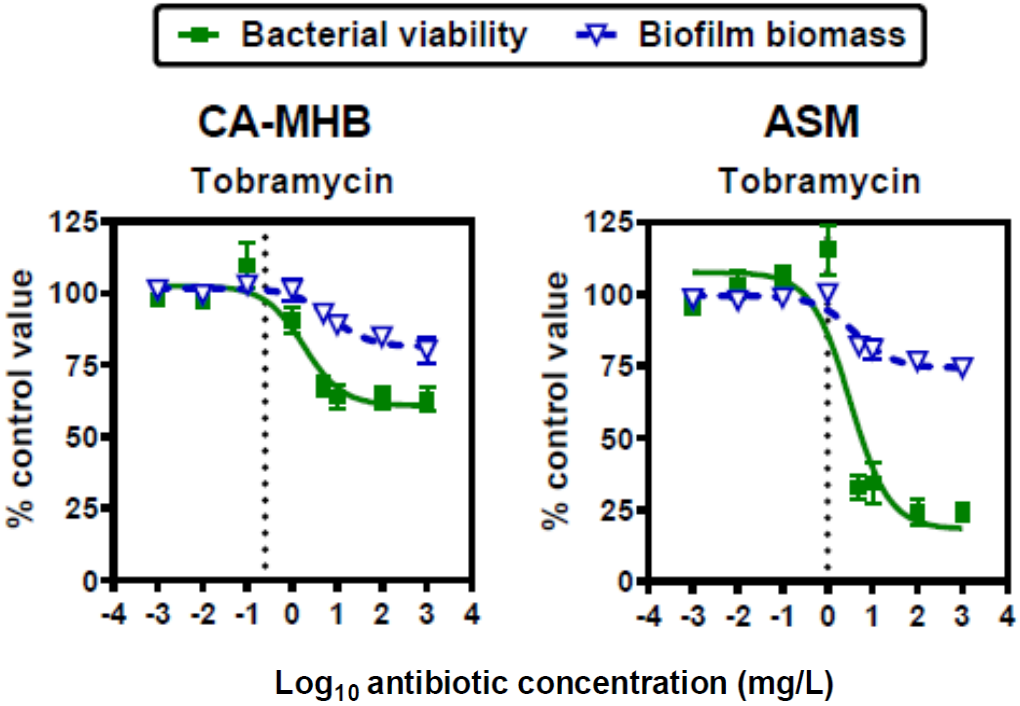


Meropenem



Antibiotic activity in biofilm infection

Tobramycin activity on 4-day old mature *P. aeruginosa* PAO1 biofilm after 24-hour of antibiotic exposure



Tobramycin is one of the most active drugs

Biofilm model and antibiotic activity

Objective: To study whether CLR can increase antipseudomonal activity of different drugs in biofilm model

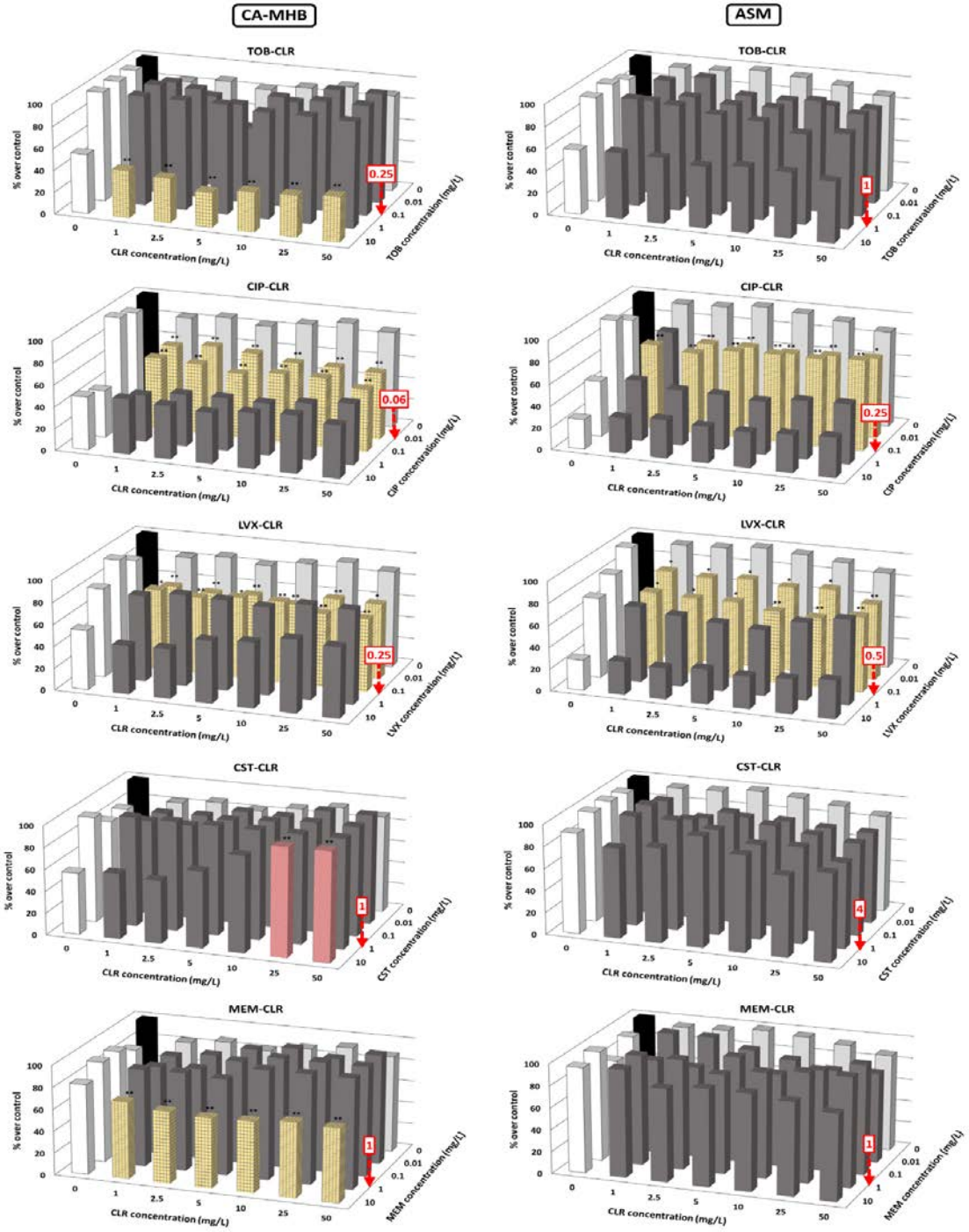
Antipseudomonal drugs

- Tobramycin — Aminoglycoside
- Meropenem — β-lactam
- Colistin — Polymyxin
- Ciprofloxacin
- Levofloxacin — Fluoroquinolones

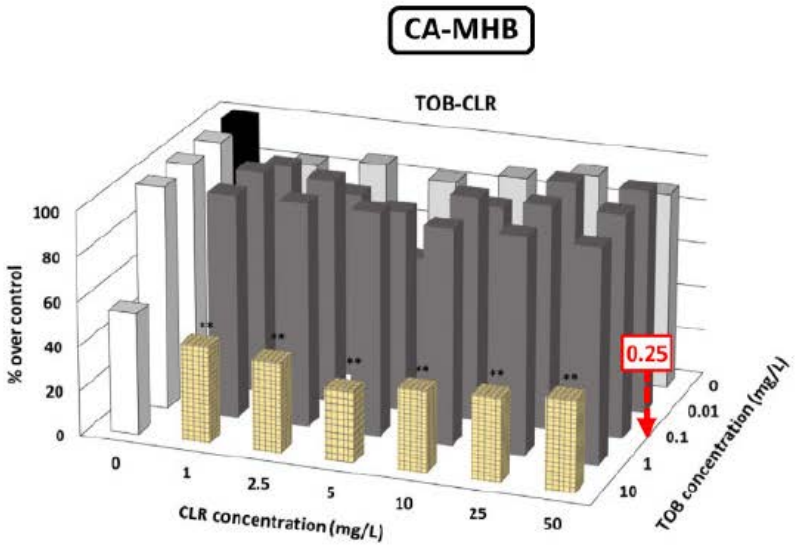
+

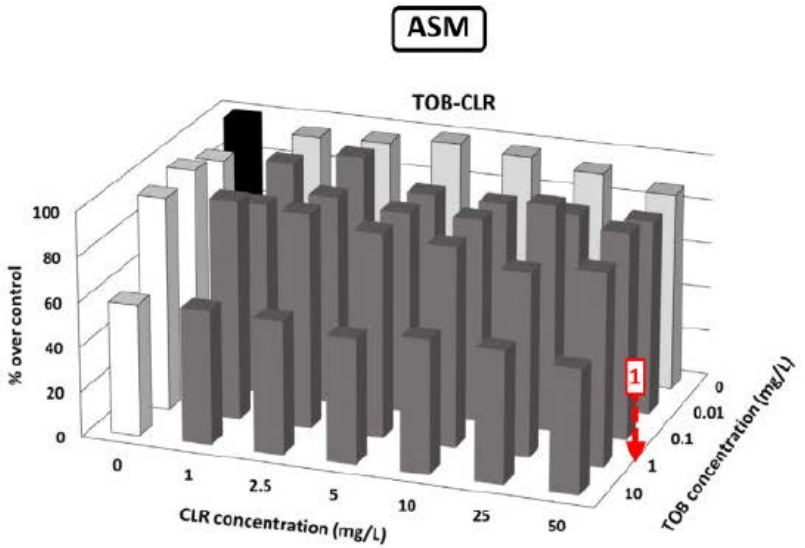
Anti-inflammatory agent

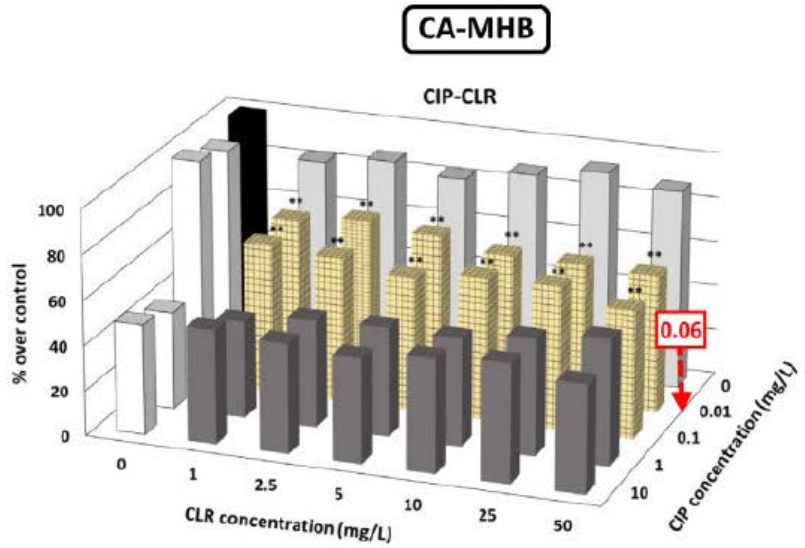
Clarithromycin (Macrolide)

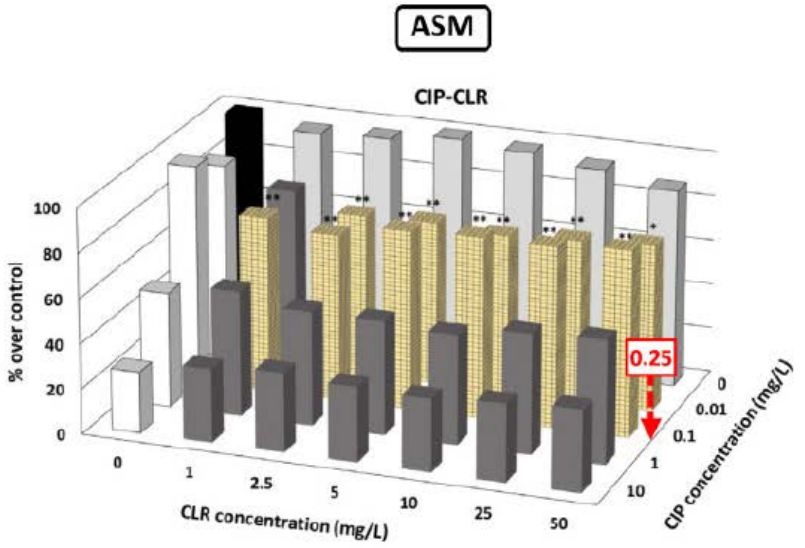

CA-MHB
ASM
TOB-CLR
CIP-CLR
LVX-CLR
CST-CLR
MEM-CLR
% over control
CLR concentration (mg/L)
TOB concentration (mg/L)
CIP concentration (mg/L)
LVX concentration (mg/L)
CST concentration (mg/L)
MEM concentration (mg/L)
0.25
1
0.06
0.25
0.25
0.5
1
4
1

Ciprofloxacin-clarithromycin strong synergism in both media



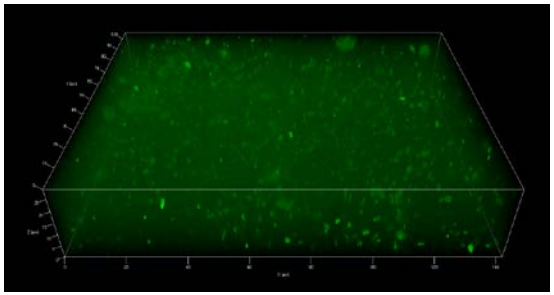




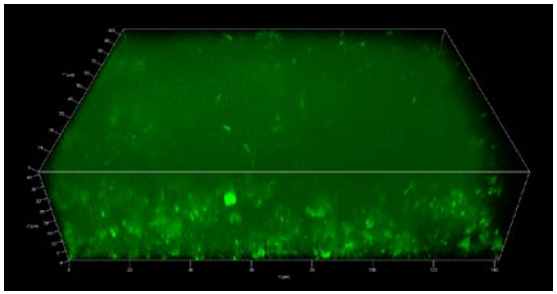


Biofilm model and antibiotic activity

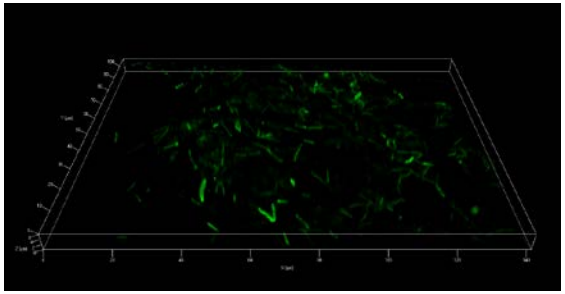
2) Assessing biofilm thickness using confocal microscopy



Control

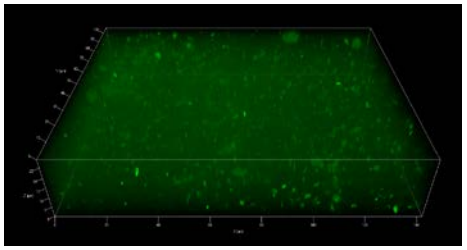


Ciprofloxacin



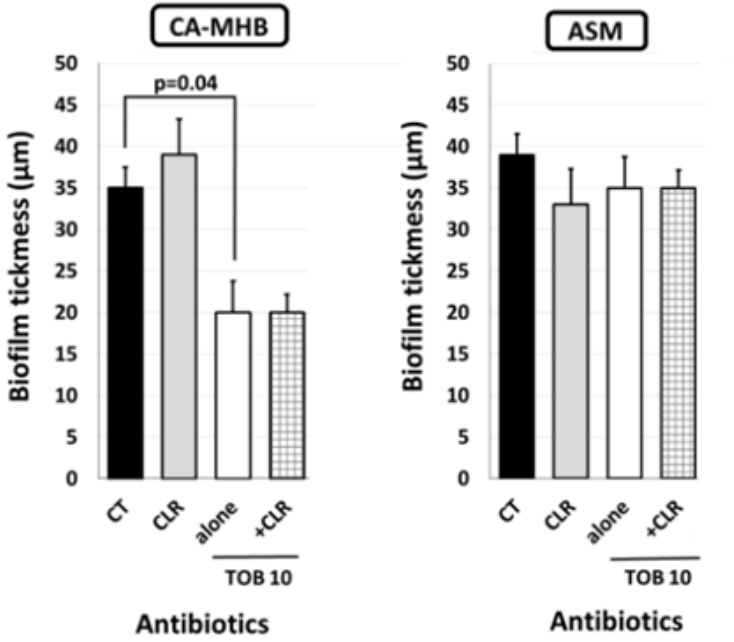
Ciprofloxacin + Clarithromycin

Biofilm model and antibiotic activity



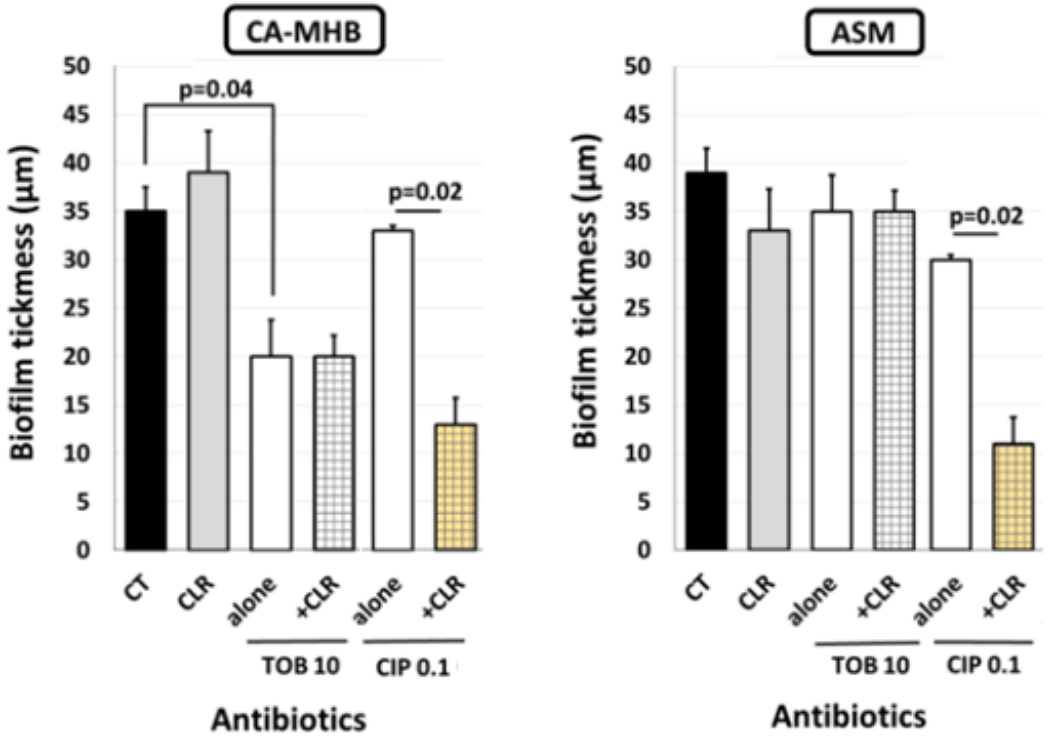
Activity of combination Tobramycin-Clarithromycin

2) Reduction in biofilm thickness using confocal microscopy

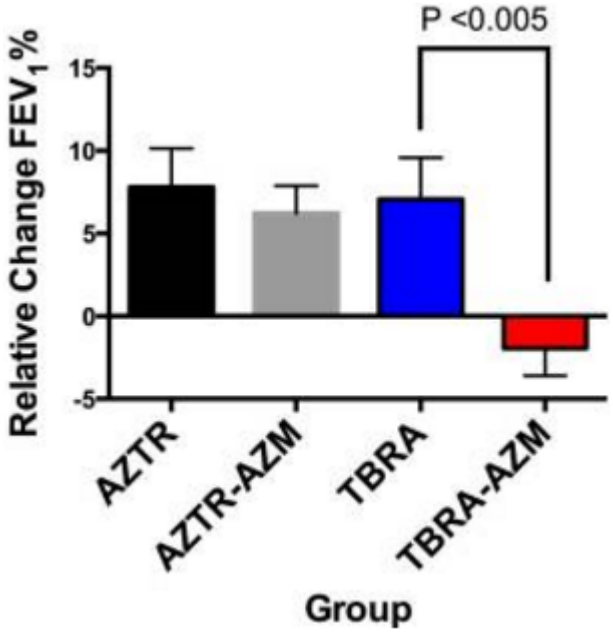


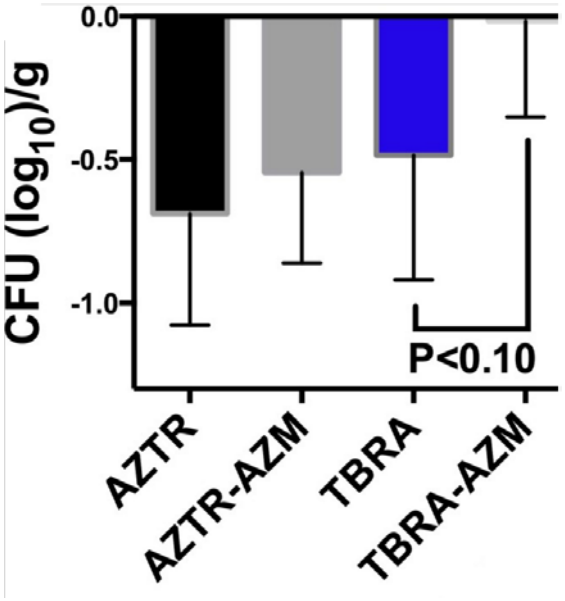
TOB alone is active in reducing biofilm thickness in CA-MHB

Ciprofloxacin-clarithromycin strong synergism in both media



Antagonism Tobramycin-Azithromycin





Nick et al, Ann Am Thorac Soc. 2014 Mar; 11(3): 342–350.

Main studies

Microbiology, Pharmacology : University	**Pharmaceutics, Formulation : SMB**
1) Antibiotic susceptibility surveillance 2) Macrolide resistance 3) *In vitro* biofilm model 4) Antibiotic combinations in biofilm model	1) Analytical method development and validation 2) Inhaled formulation development

2) Macrolide resistance (under review)

Outer-membrane permeability?

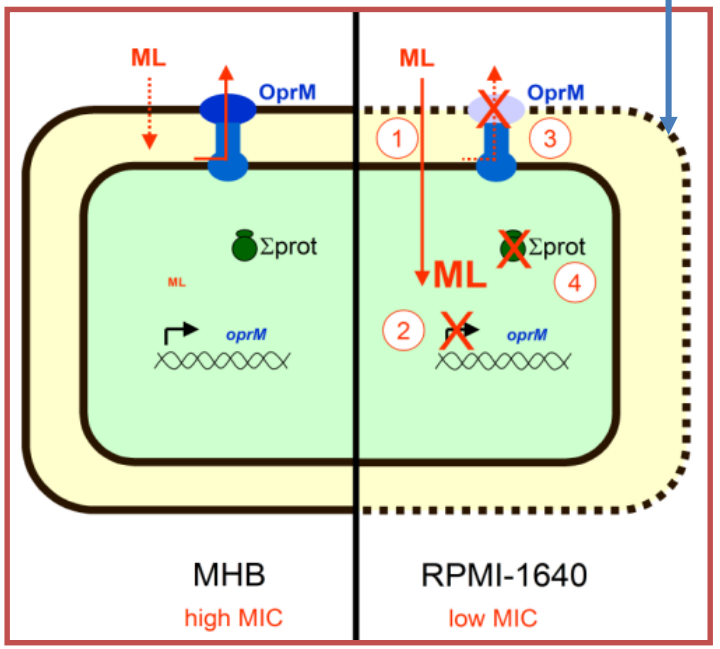
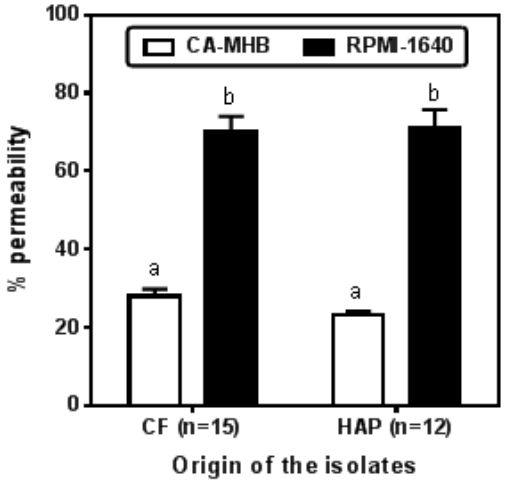


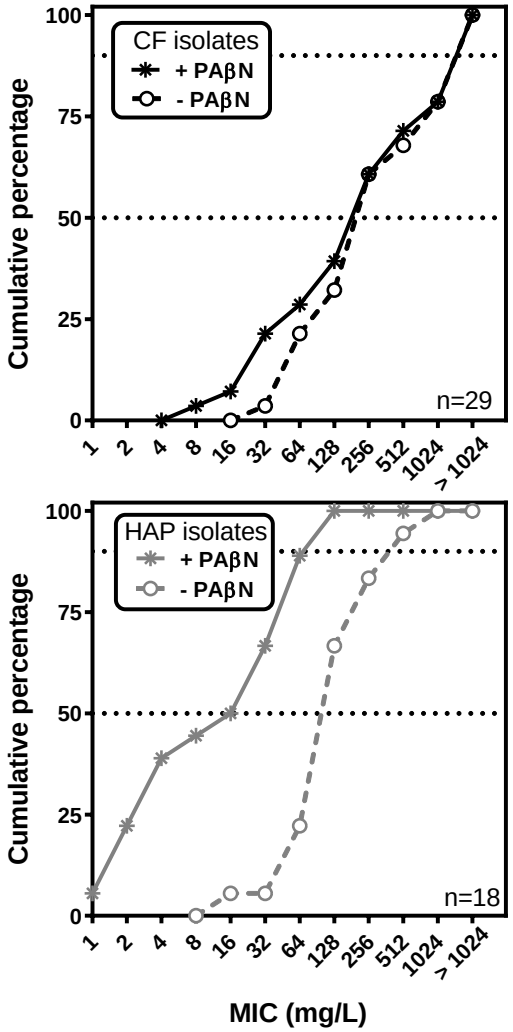
Fig 3: Outer-membrane permeability of CF and HAP isolates in CA-MHB and RPMI-1640. Bacteria were incubated for 4 hours in cation-adjusted Muller-Hinton broth (CA-MHB; open bars) or RPMI-1640 medium (closed bars) and then with 25 µM 1-N-phenylnaphthylamine. Data are expressed as a percentage of the maximal value recorded in the presence of 3% Triton and are the mean ± SD of the values recorded for the different isolates studied. Statistical analysis: 2-ways ANOVA with Tukey multiple comparison test: data with different letters are significantly different from one another. The number of isolates included in the study is indicated on the X axis.



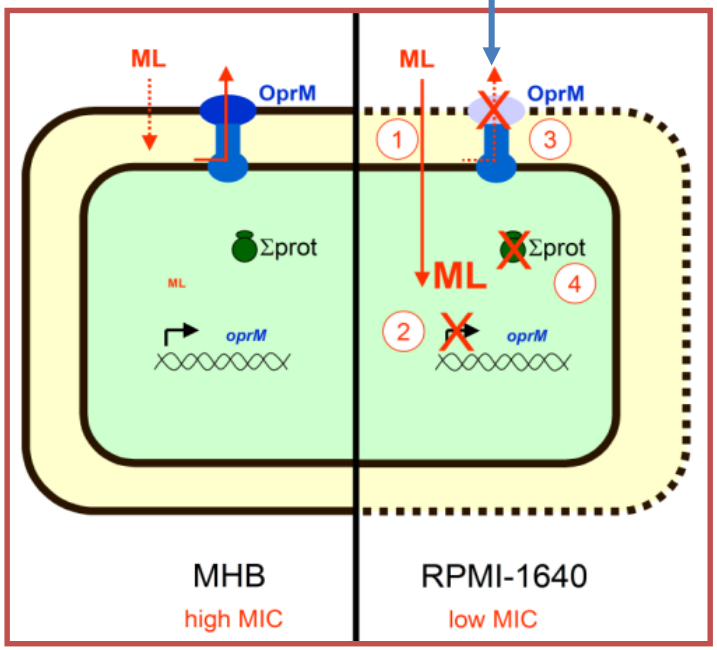
Difference in outer-membrane permeability and is NOT the reason behind high resistance among CF isolates

2) Macrolide resistance (under review)

Efflux pumps?



strains	medium	azithromycin	
		MIC_{50}	MIC_{90}
CF	-PAβN	256	> 1024
	+PAβN	256	>1024
HAP	-PAβN	128	>1024
	+PAβN	16	64

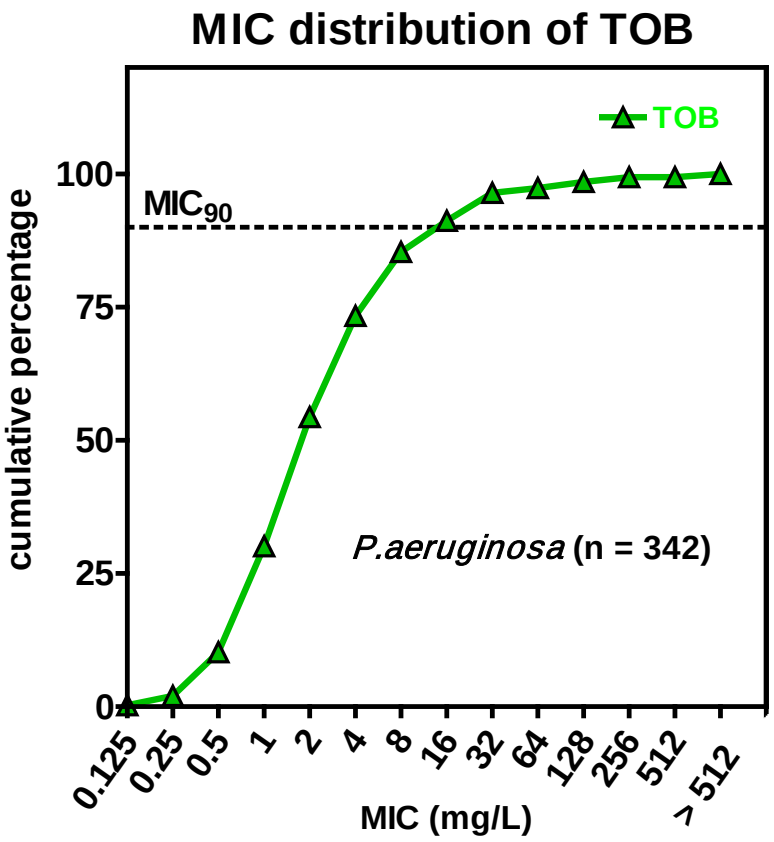


Efflux pump activity is NOT the reason behind high resistance among CF isolates

General programme

	Microbiology, Pharmacology : University	**Pharmaceutics, Formulation : SMB**
3rd year	• Macrolide resistance • Study of other combinations on *in vitro* biofilm model	• Optimization of the formulation and its process • Process scale-up for production of pilot batches • Stability study of the first formulation
4th year	• Macrolide resistance • Study of other combinations on *in vitro* biofilm model • Study of mode of action of the new combination : effect of macrolides	• Continuation of the stability study of the first combination • Packaging optimization • Industrial scale-up for pilot manufacturing of the first combination • Development of the new combination (analytical method and formulation)

Activity of antipseudomonal agents



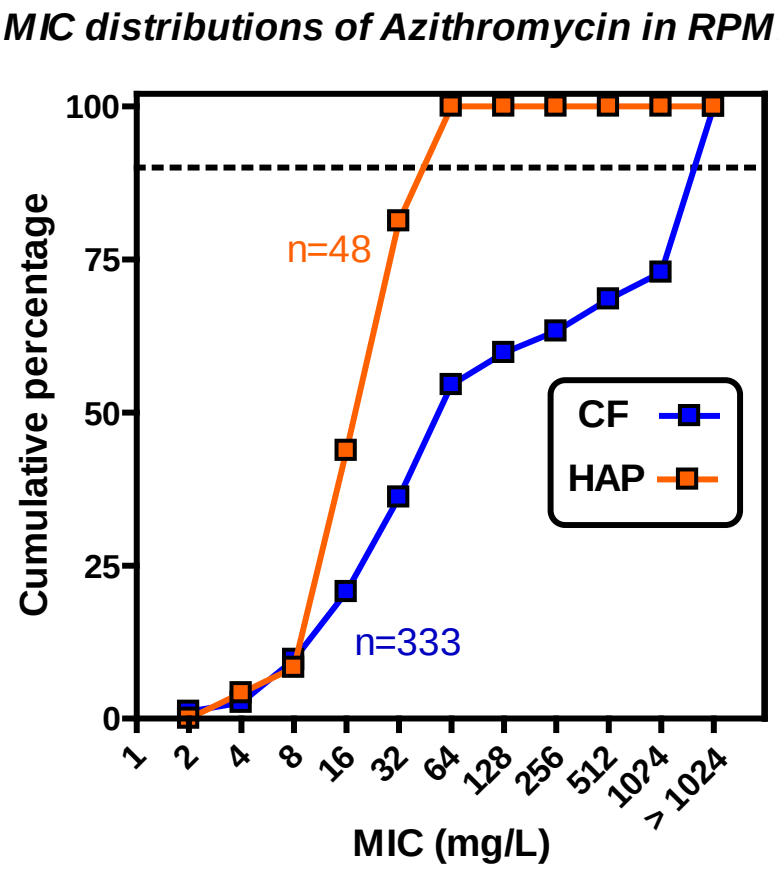
Drug	EUCAST	MIC_{90} (mg/L)	% S	% I-R
Colistin	S≤ 4; R>4	4	93	7
Tobramycin	S≤ 4; R>4	16	73	26
Amikacin	S≤ 8; R>16	128	25	75
Meropenem	S≤ 2; R>8	16	48	52
Ceftazidime	S≤ 8; R>8	512	36	64
Piperacillin-tazobactam	S≤ 16; R>16	512	31	69
Ciprofloxacin	S≤0.5; R>1	8	32	68

(According to EUCAST breakpoints)

⇒ **Resistance high**

⇒ **Best options : Meropenem, Tobramycin, Colistin**

Activity of macrolides :
CF *vs* Healthcare associated pneumonia (HAP) isolates



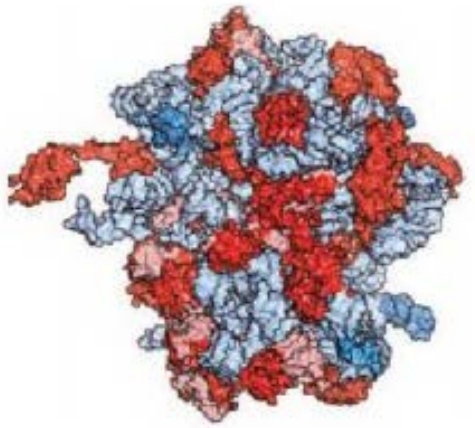
=> CF strains showed more resistance!

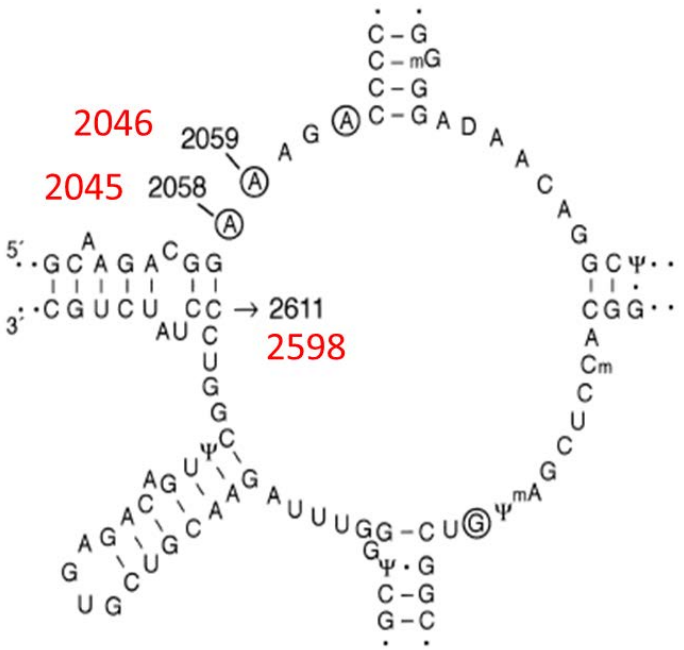
Searching for the main mechanism of resistance to macrolides in CF vs HAP isolates

Mechanism	CF isolates	HAP isolates
Outer-membrane permeability	=	
Activity of efflux pumps eliminating macrolides	=	
Modification of macrolide site of action: **nucleotide mutation in 23S subunit** of the bacterial ribosome	+++	-

Sequencing Domain V of 23S ribosome: Presence of mutations in CF isolates

- 79 CF isolates and 48 HAP isolates
- **6 different mutations were found in 46% of CF isolates**:
  - A2045G
  - A2045T
  - A2046G
  - A2046T
  - C2598G
  - C2598T
- **No mutation among HAP isolates**.



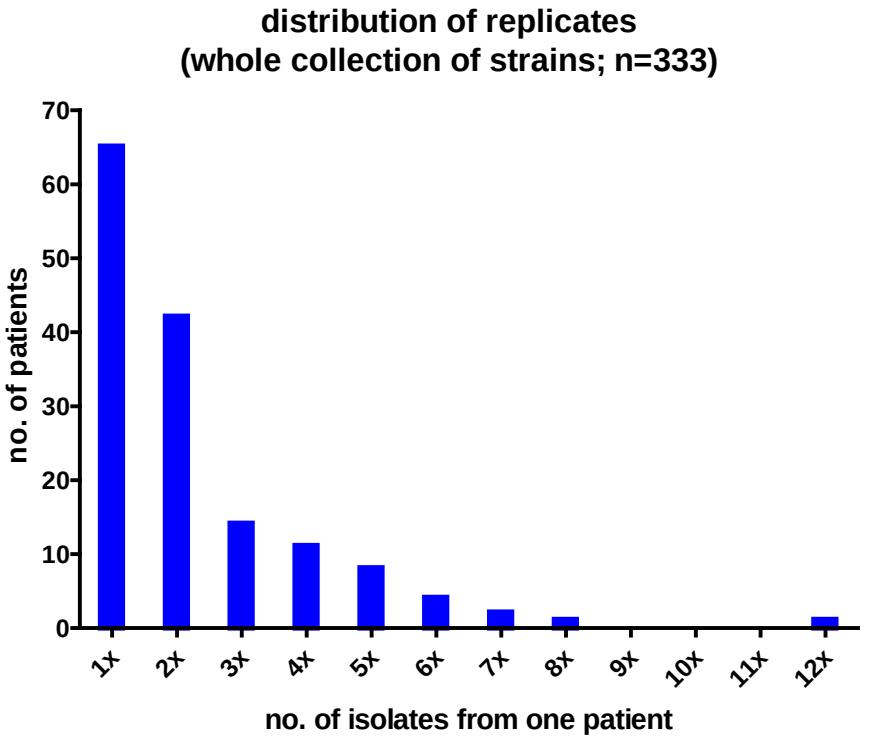


Secondary-structure models of the peptidyl transferase center in domain V of 23S rRNA of *E.coli, Vester B et al. AAC (2012)*

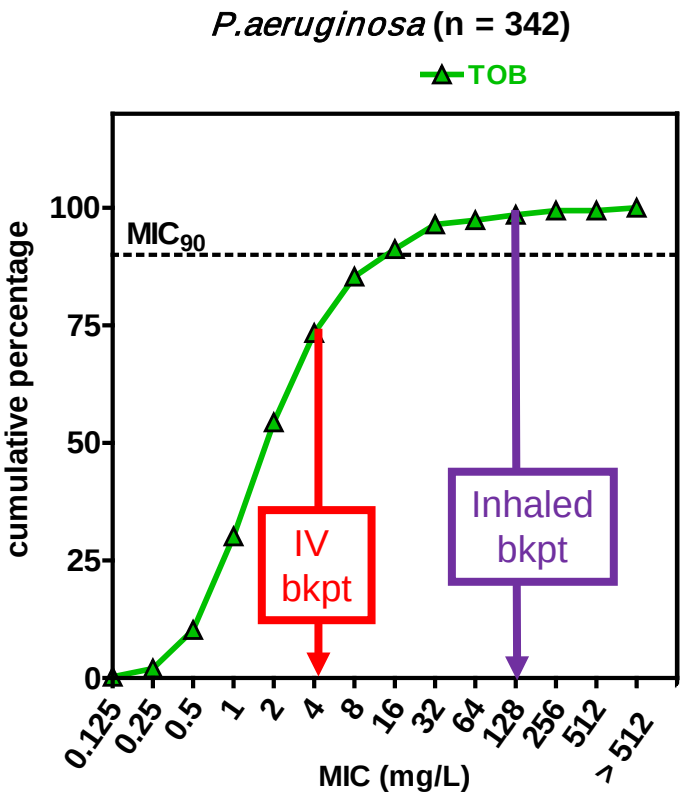
Origin of the strains

342 clinical isolates

- 99 : Dr M. Tunney, The Queen's University of Belfast, UK.
- 91 : Drs A. Vergison / O. Denis from the "Hôpital Erasme", Brussels
- 81 : Dr P. Plésiat, Hôpital Jean Minjoz, Besançon, France
- 71 : Dr B. Kahl, University Hospital Münster, Münster, Germany
- Quality control strain ***P. aeruginosa ATCC 27853***



Tobramycin by inhalation

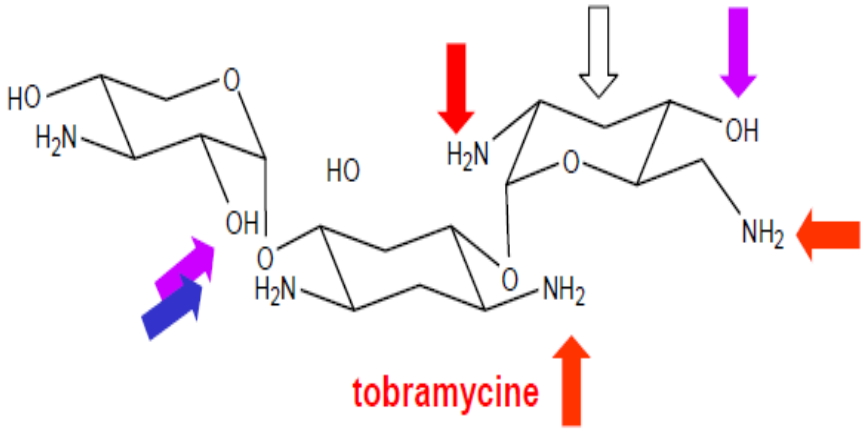


Admin. route	Dose	peak	PD Bkpt (mg/L) [C_{max}/MIC > 8]
IV	10 mg/kg	20-30 mg/L (serum)	30/8 ~ 4
Inhalation (TOBI® podhaler)	112 mg	1050 µg/g ~1050 µg/mL (lung)	1200/8 ~ 128

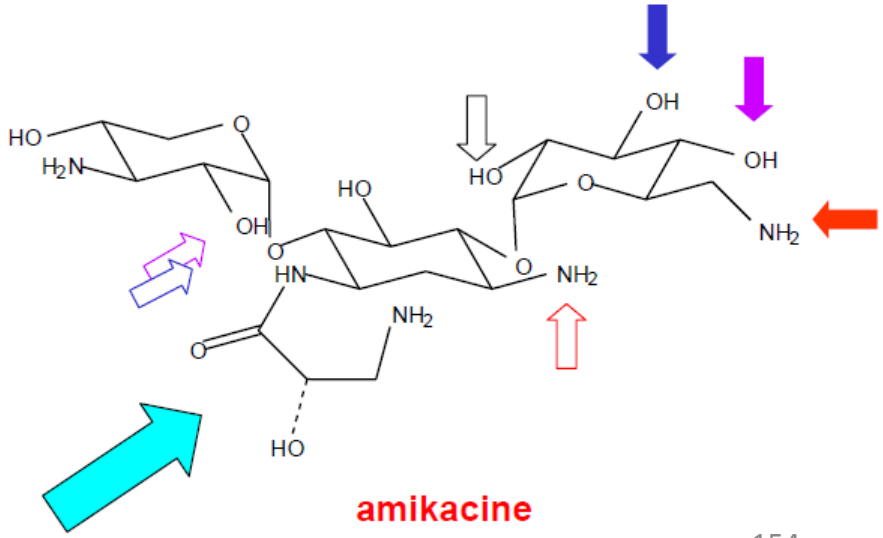
MIC (mg/L)	% R « IV » bkpt	% R « inhaled » bkpt
MIC_{50} = 4 MIC_{90} = 16	26%	1.5%
	S : ≤ 4 R : > 4	S : ≤ 128 R : > 128

Pourquoi?

- Amikacine a plus d'affinité pour MexX par rapport à la tobramycine ? *(JC Hurley et al , Mechanism of amikacin resistance in Pseudomonas aeruginosa isolates from patients with cystic fibrosis, 1995)*
- Activité de l'enzyme 3'phosphotransférase ?



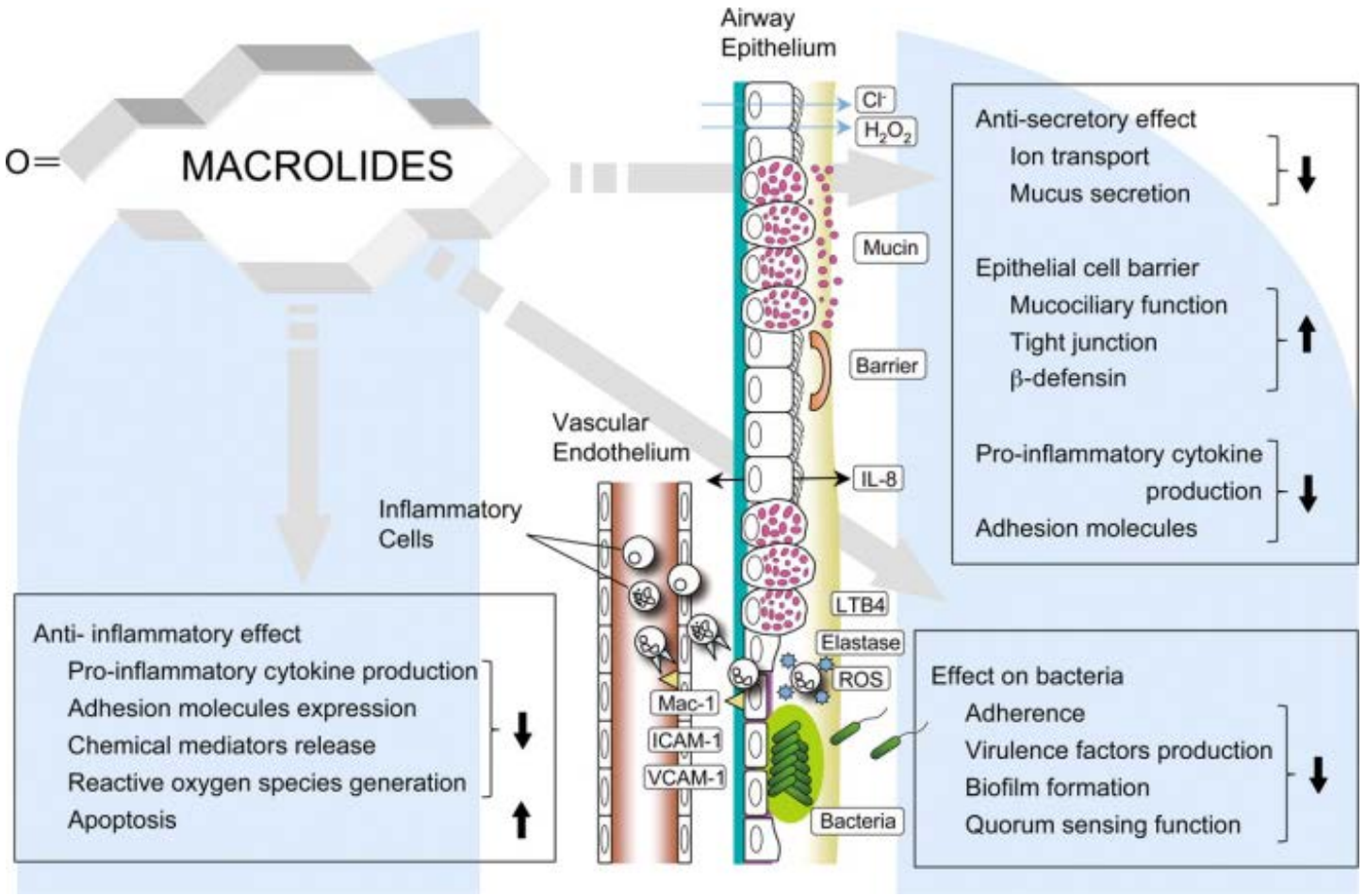
tobramycine



amikacine

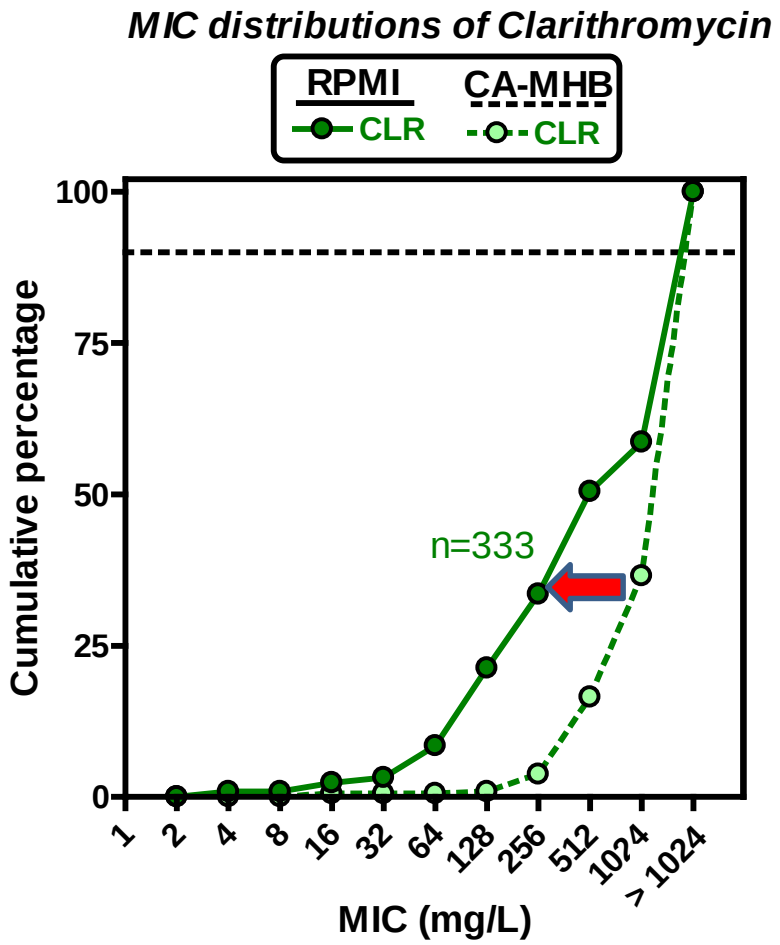
And what about macrolides ?

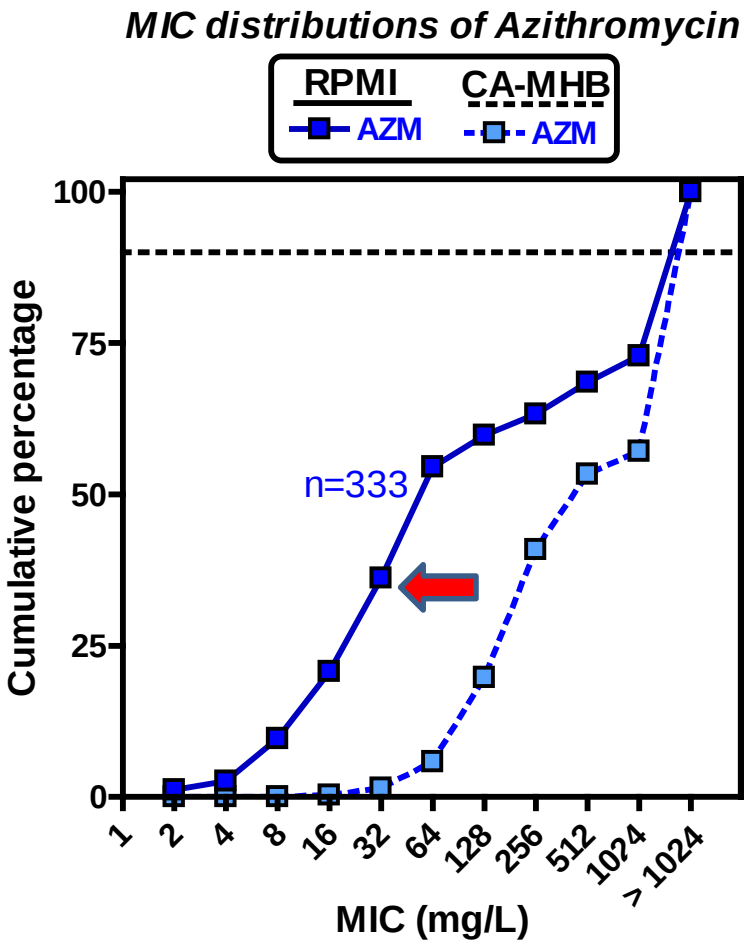
Azithromycin is widely and successfully used in Cystic Fibrosis patients



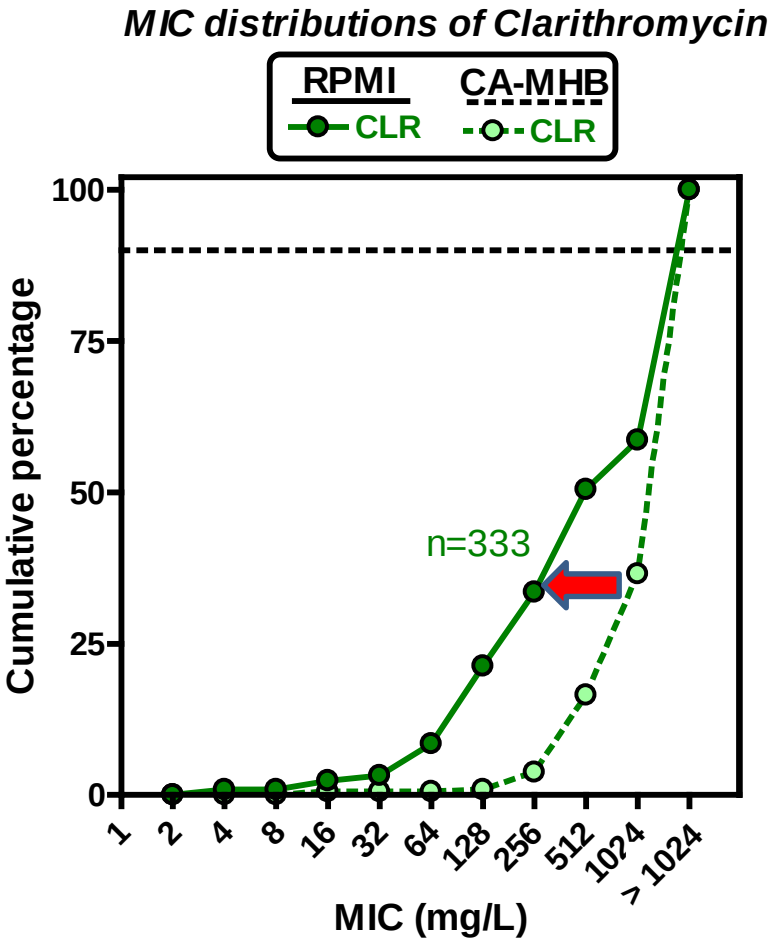
Kanoh & Rubin; Clin. Microbiol. Rev. (2010) 23:590-615

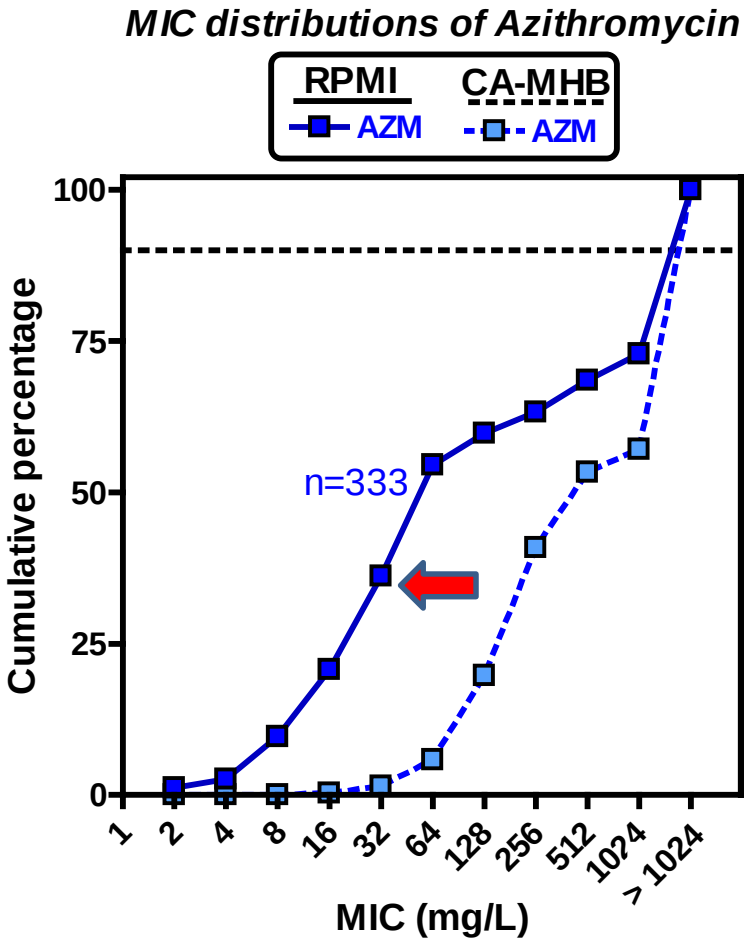
Activity of macrolides : influence of medium





Activity of macrolides : influence of medium



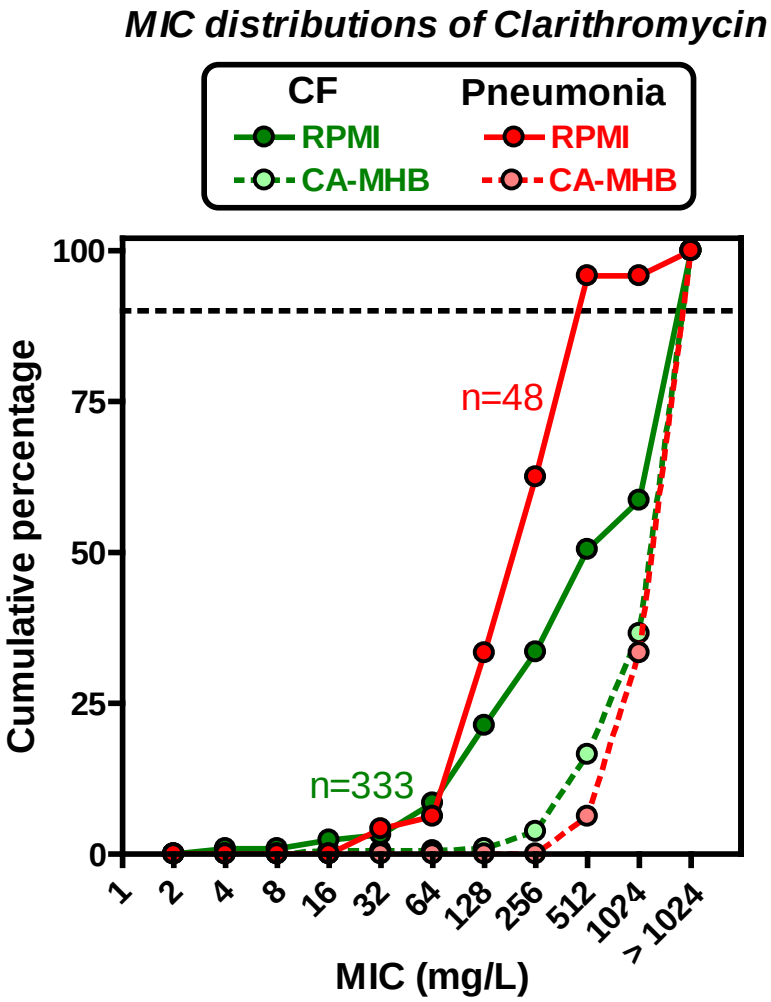


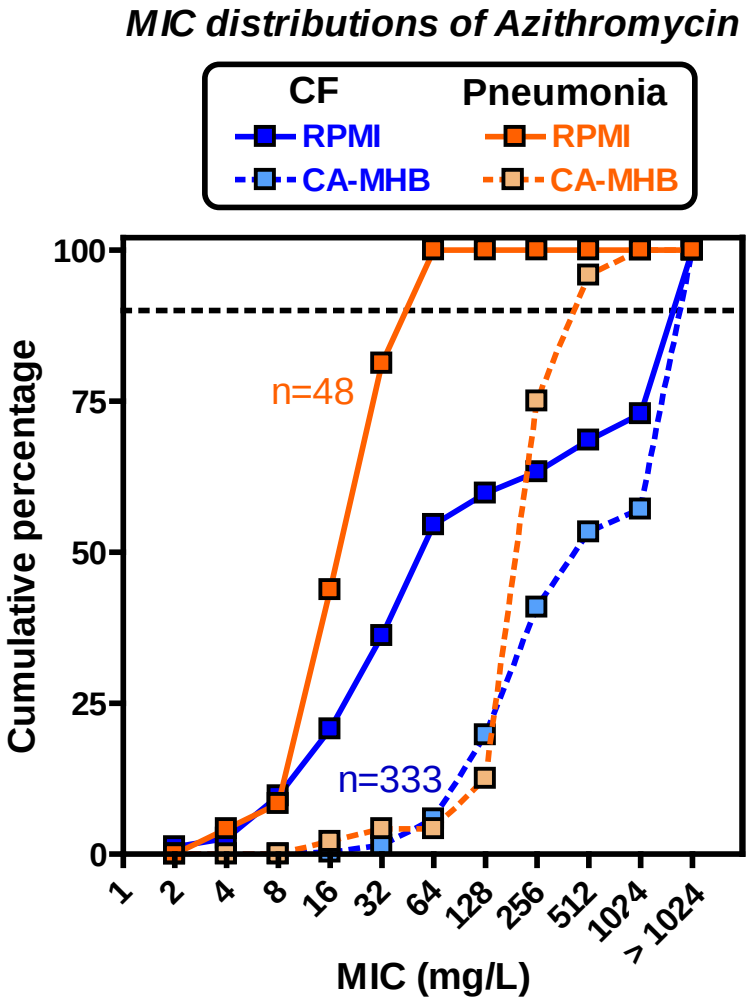
Strain PAO1 : (Buyck et al. CID (2012))

- **MIC CLR/AZM in MHB = 512/128 mg/L**
- **MIC CLR/AZM in RPMI 1640 = 128/32 mg/L**

=> CF strains showed more resistance

Activity of macrolides : CF vs Healthcare associated pneumonia (HAP) isolates

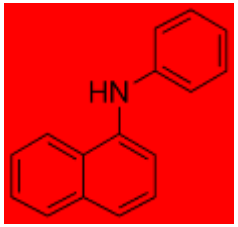


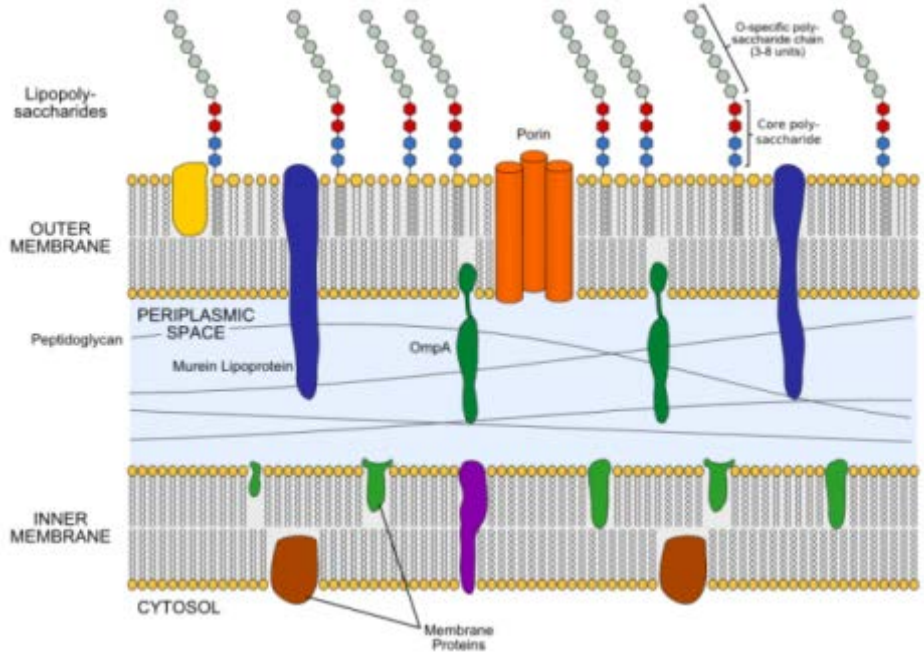


=> CF strains showed more resistance!

2nd part : Resistance mechanism (Outer-membrane permeability?)

NPN (N-phenylnaphtalene-1-amine)





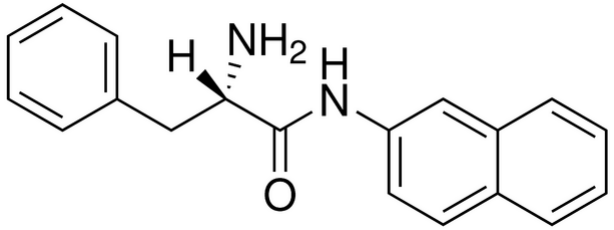
Fluorescence

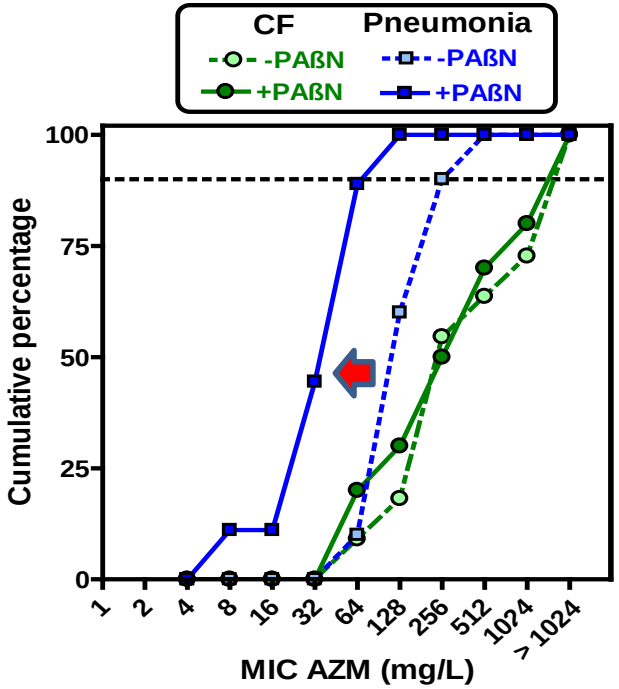
Outer-membrane permeability ↗ => Fluorescence ↗

2nd part : Resistance mechanism (Efflux pumps?)

Macrolides → *MexAB-OprM* (constitutive)
→ *MexCD-OprJ* (inducible)
→ *MexXY-OprM* (constitutive)

L-Phenylalanine β-naphthylamide (PAβN) : inhibitor of efflux pumps



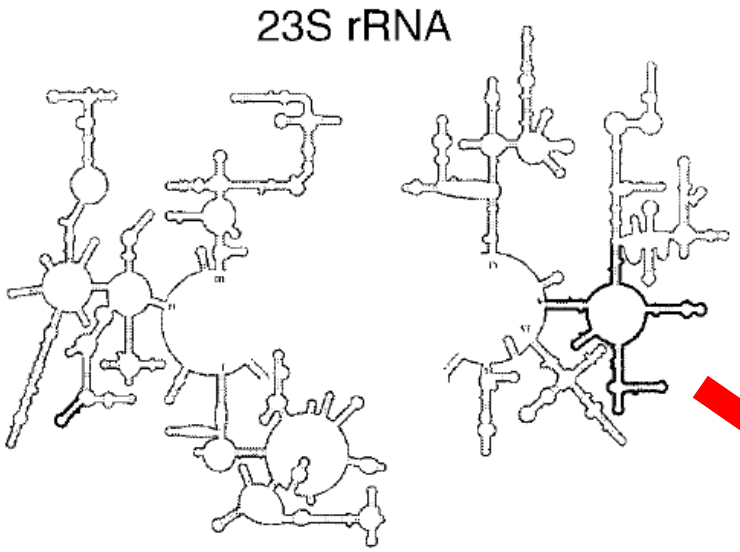


PN strains : regain of activity (2-fold dilution) in the presence of PAβN

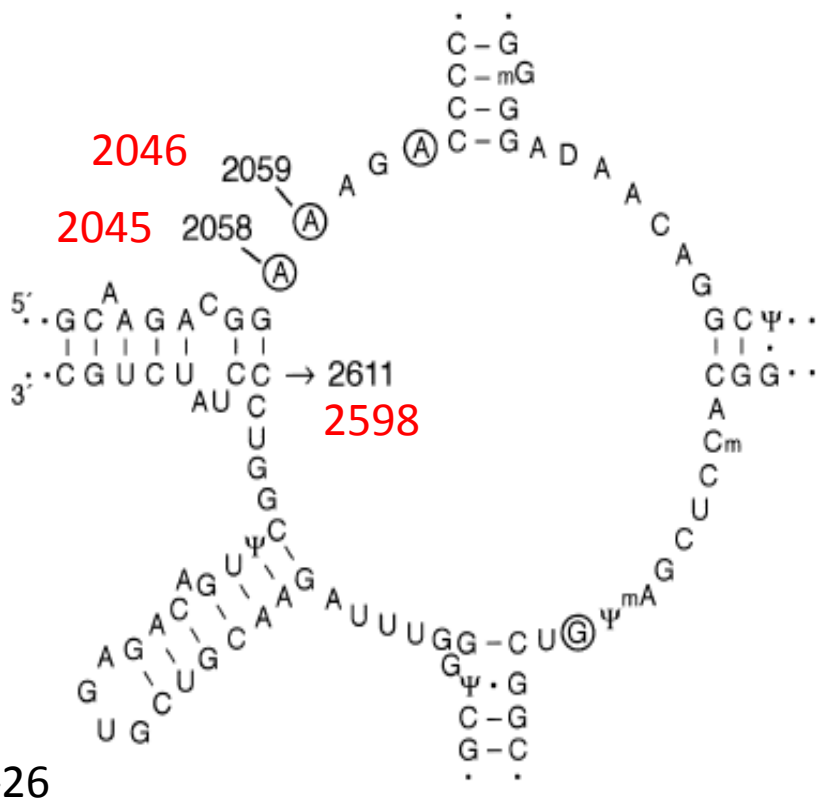
Cf strains : no regain of activity (presence of another resistance mechanism!)

→ **Ribosomal mutation?**

Secondary-structure model of the peptidyl transferase center in domain V of 23S rRNA of *E.coli*



In red : corresponding position in P.aeruginosa

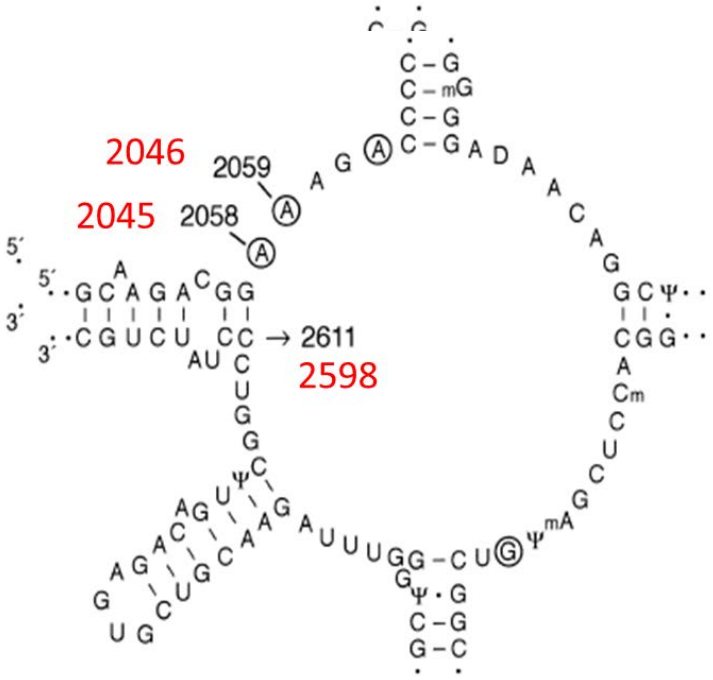


Gutell *et al*, Microbiol Rev. 1994 Mar;58(1):10-26

Assessing the effect of ribosomal mutation on reference strain PAO1

6 different mutations to study:

- A2045G
- A2045T
- A2046G
- A2046T
- C2598G
- C2598T

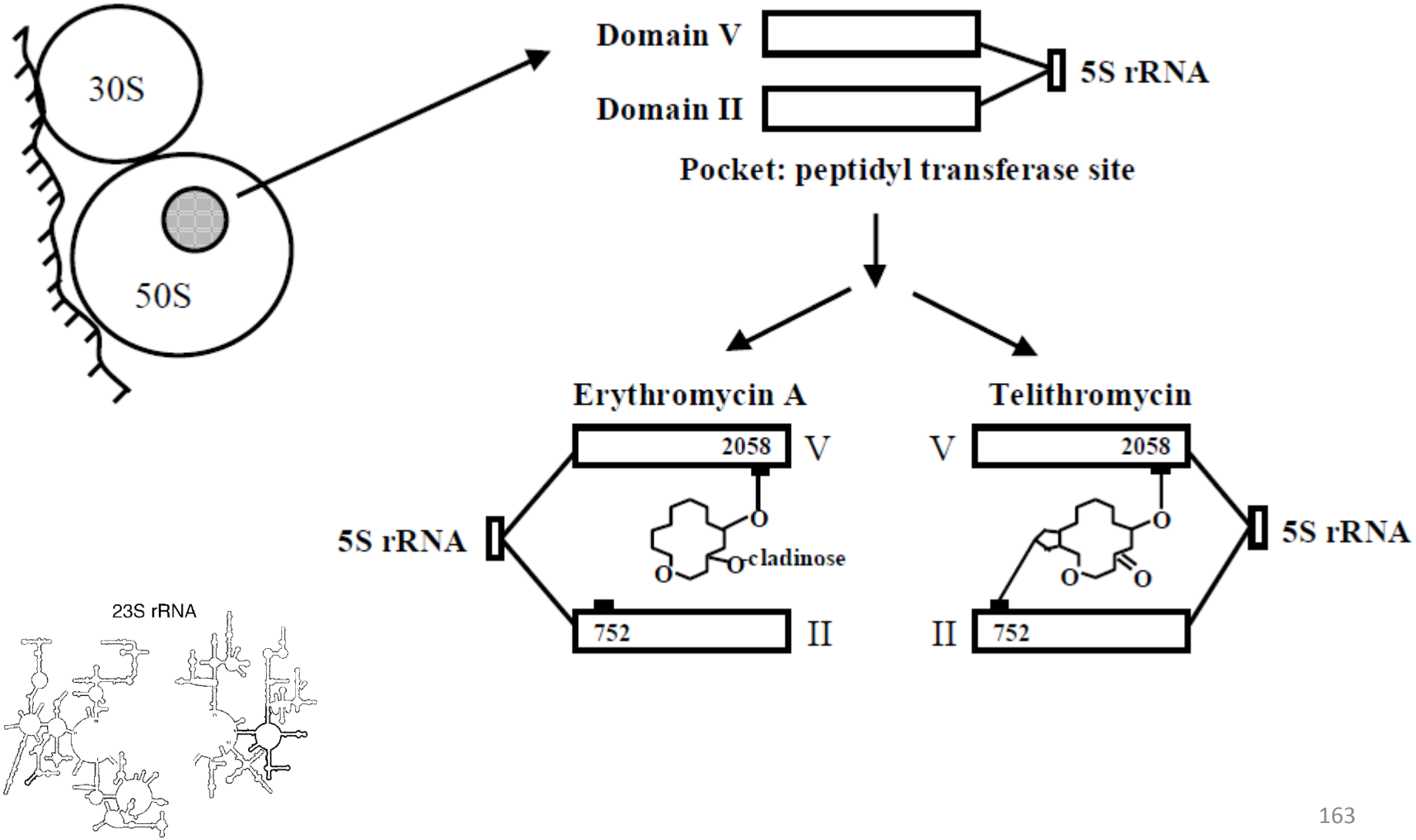


⇒ **Cloning** process

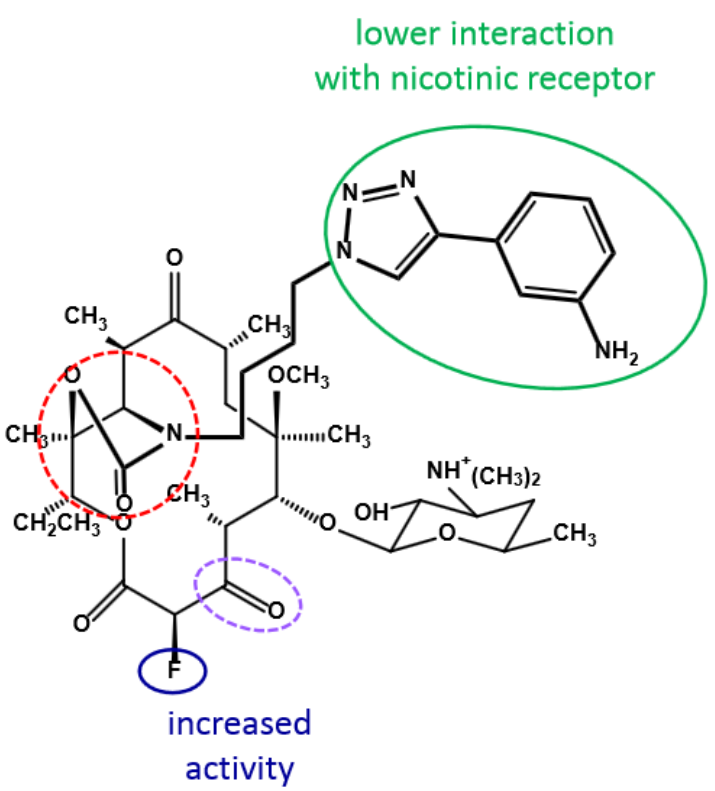
⇒ Transformation of plasmid containing wild-type or mutated rRNA operon into PAO1

⇒ MIC determination

Macrolides vs Ketolides

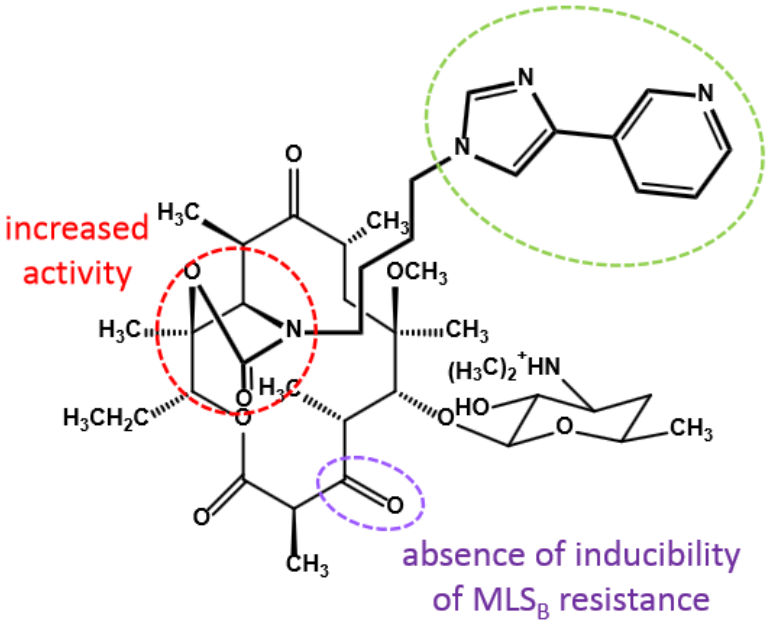


Solithromycin vs Telithromycin



solithromycin

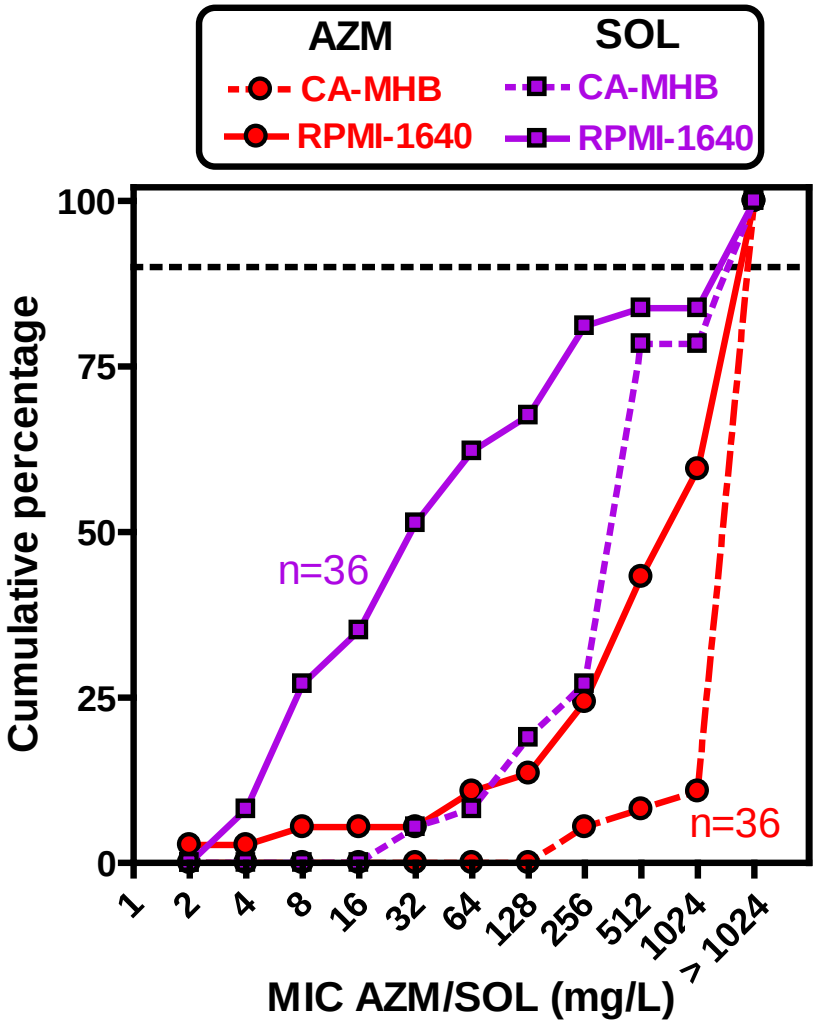
- binding to ribosomal domain II
- poor recognition by pneumococci efflux pumps



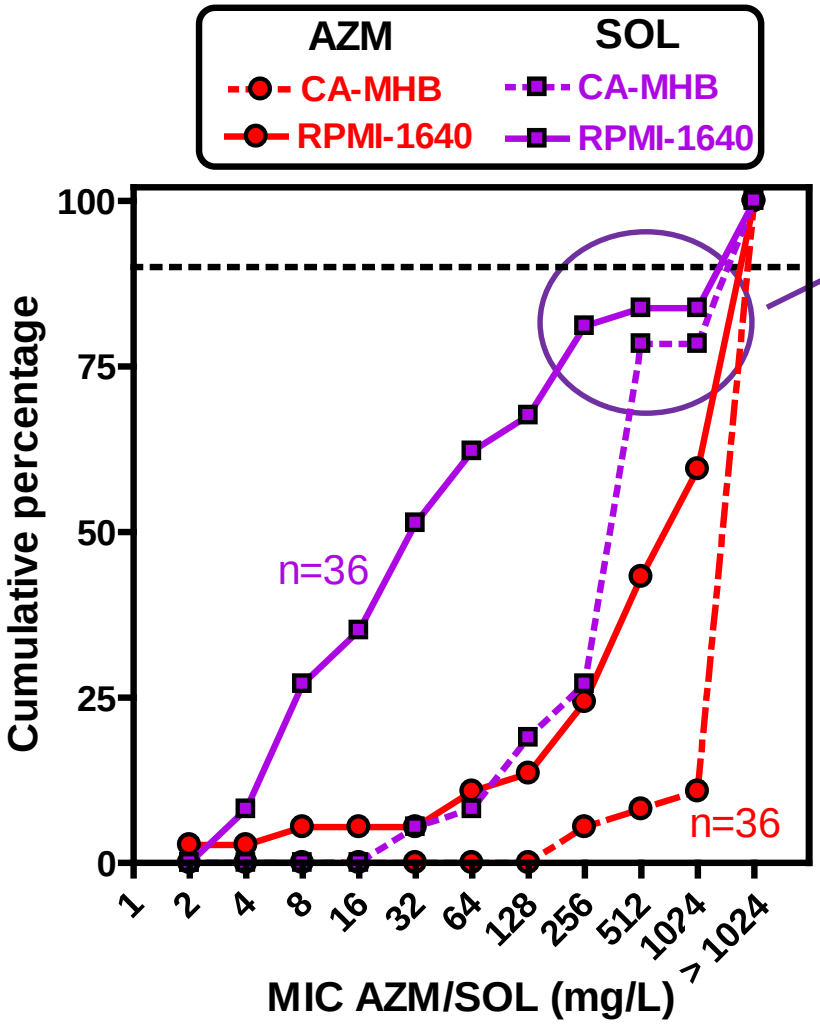
telithromycin

Adapted from Van Bambeke, Ann. Med (2014) 46:512-29

Higher susceptibility of mutated CF strain to ketolides



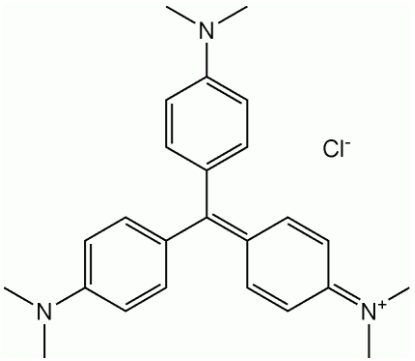
Some have high MICs even for ketolides…

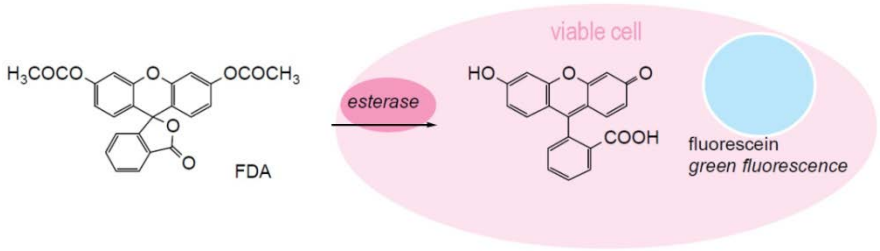


No mutation on Domain II (ketolide's binding site) !

Static biofilm model in 96-well plate

- Change of medium every day to stimulate the production of biofilm
  - ⇒ Accelerate the formation process
  - ⇒ More stability in term of change in medium
  - ⇒ Approaching dynamic biofilm model (continuous flow of medium)
- Antibiotic treatment on day 4 for mature biofilm model
- Antibiotic treatment for 24 hours
- Quantification of biofilm with Crystal violet staining (for biofilm biomass) and with fluoresceine diacetate enzymatic assay (for total bacterial viability)

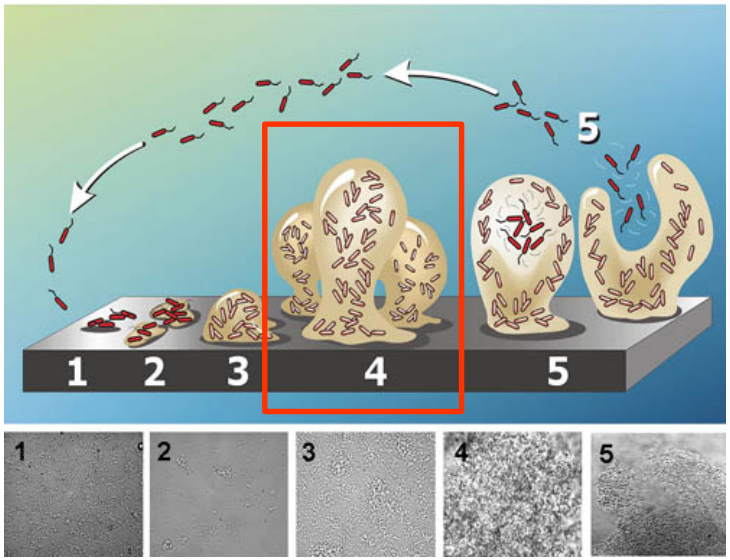


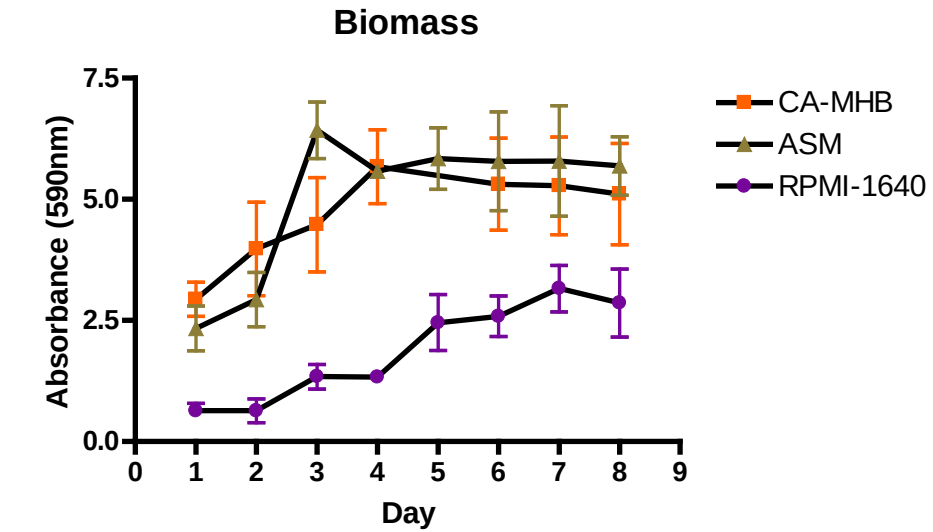


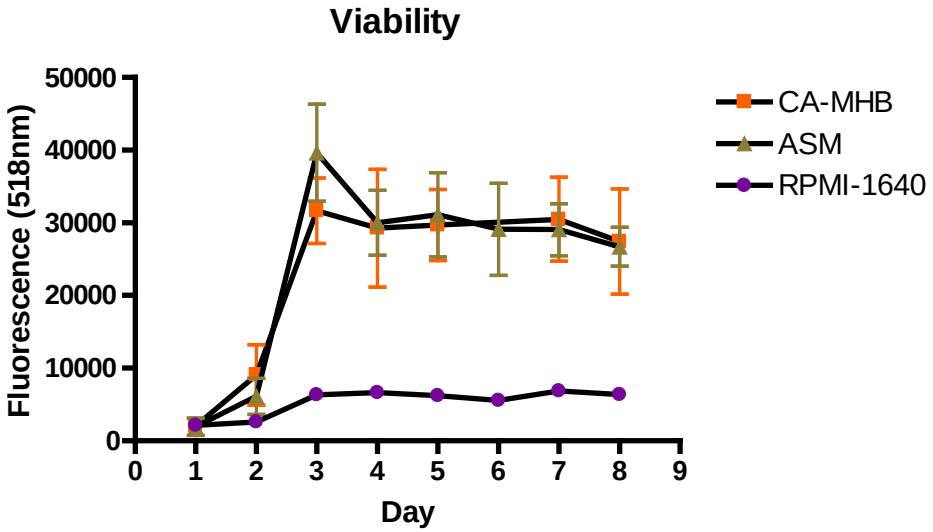
Crystal violet positively charged molecule will bind negatively charged surface molecules and to polysaccharides in the extracellular matrix

Bacteria will convert non-fluorescent fluorescein diacetate into highly fluorescent fluorescein by non-specific esterases

Biofilm development of PAO1 in three media

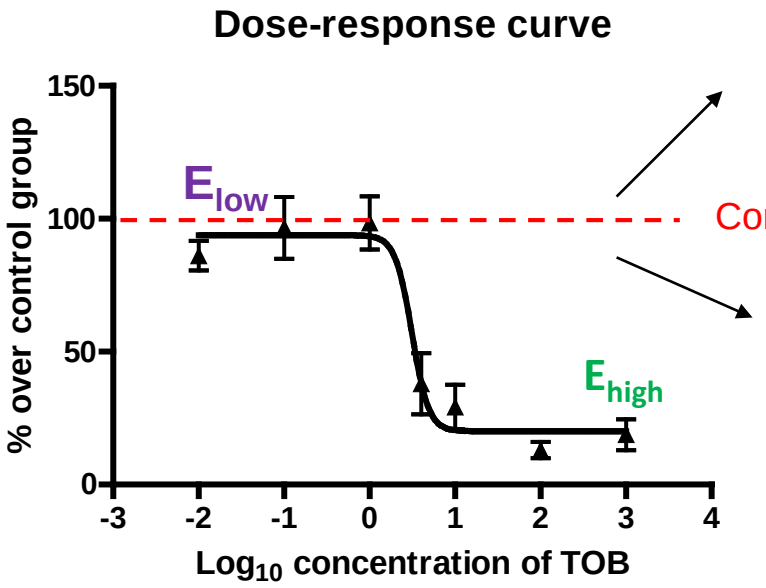






Parameters to determine AB's efficacy

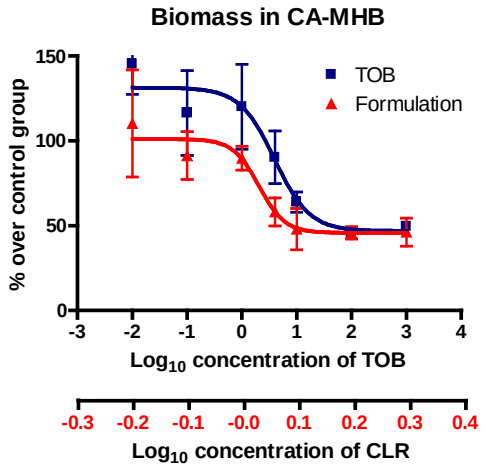
- E_{low} : Difference in biomass or viability at the lowest concentration tested (top plateau) compared to control group (in %)
- E_{high} : Difference in biomass or viability at the highest concentration tested (bottom plateau) compared to control group (in %)

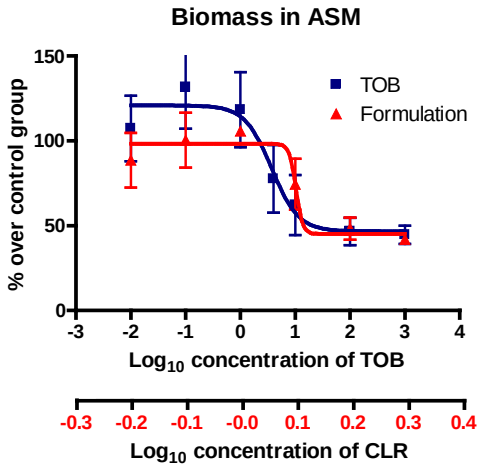


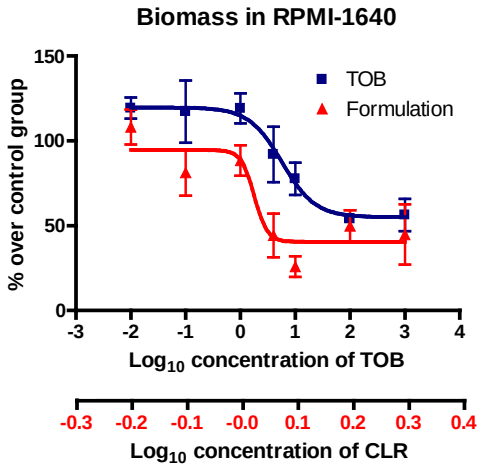
- Positive value: stimulation of growth/biomass as compared to controls
- Negative value: loss of growth/biomass as compared to controls

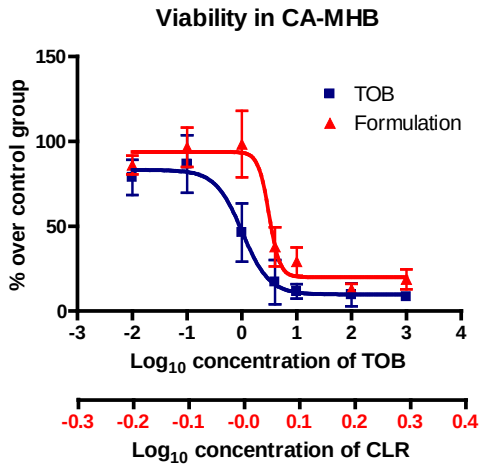
⇒ Identical E_{high} for TOB and TOB/CLR with around 50% reduction of biomass and more than 75% reduction in viability

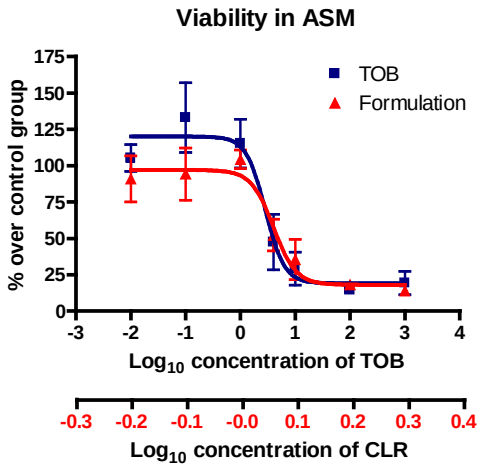
Dose-response curves for TOB alone and the formulation against mature biofilm of PAO1

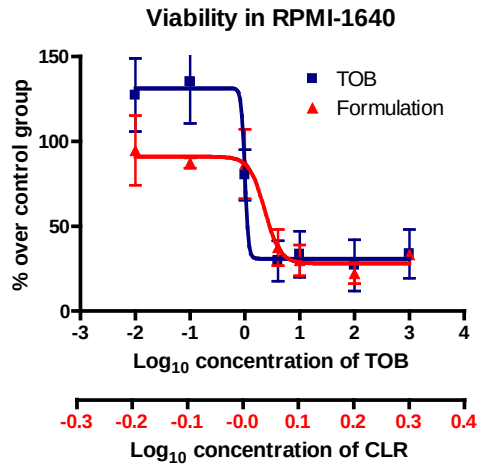












Parameters to determine AB's efficacy

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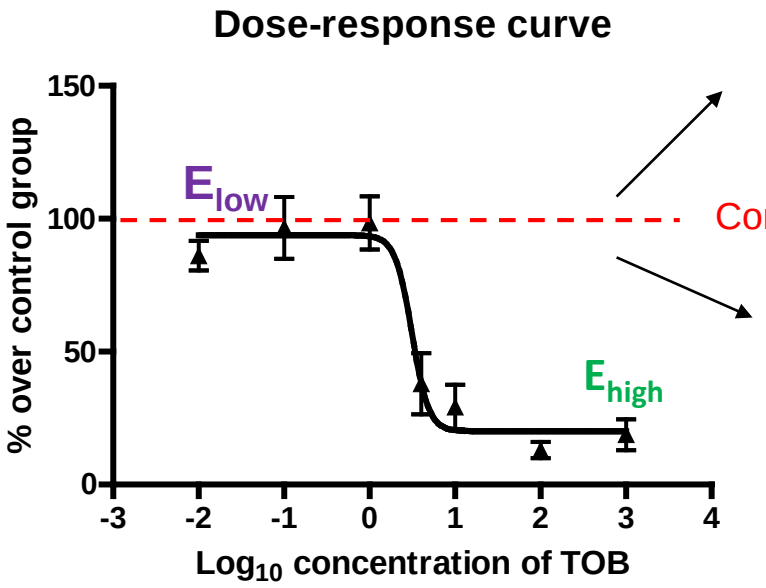
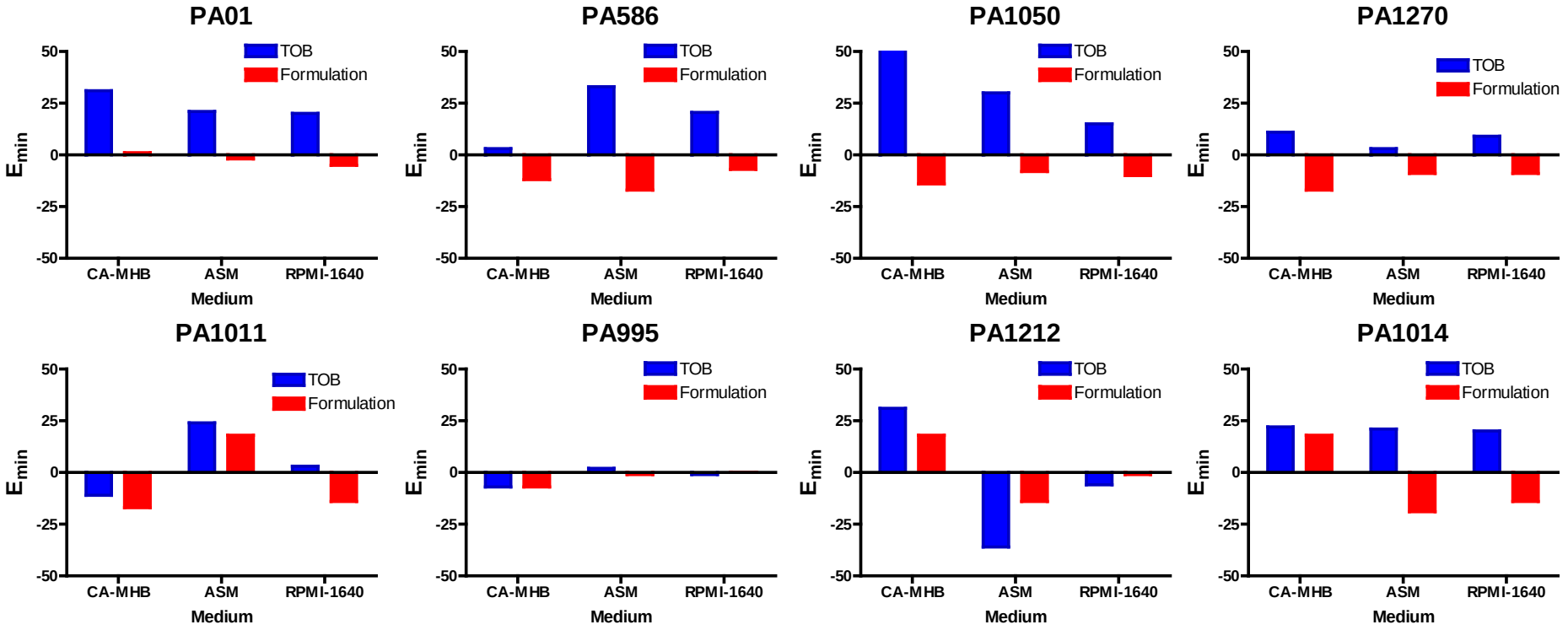
- E_{high} : Difference in biomass or viability at the highest concentration tested (bottom plateau) compared to control group (in %)



- Positive value: stimulation of growth/biomass as compared to controls
- Negative value: loss of growth/biomass as compared to controls

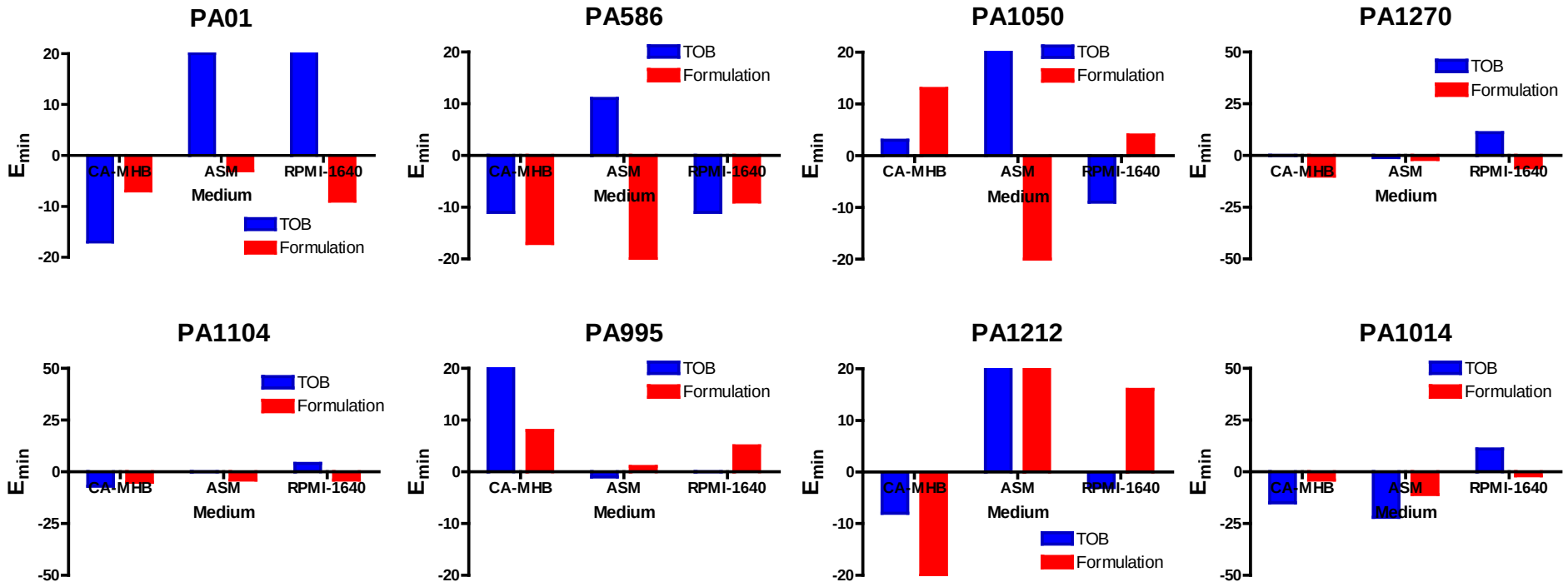
⇒ Identical E_{high} for TOB and TOB/CLR with around 50% reduction of biomass and more than 75% reduction in viability

Biofilm Biomass : E_{min}

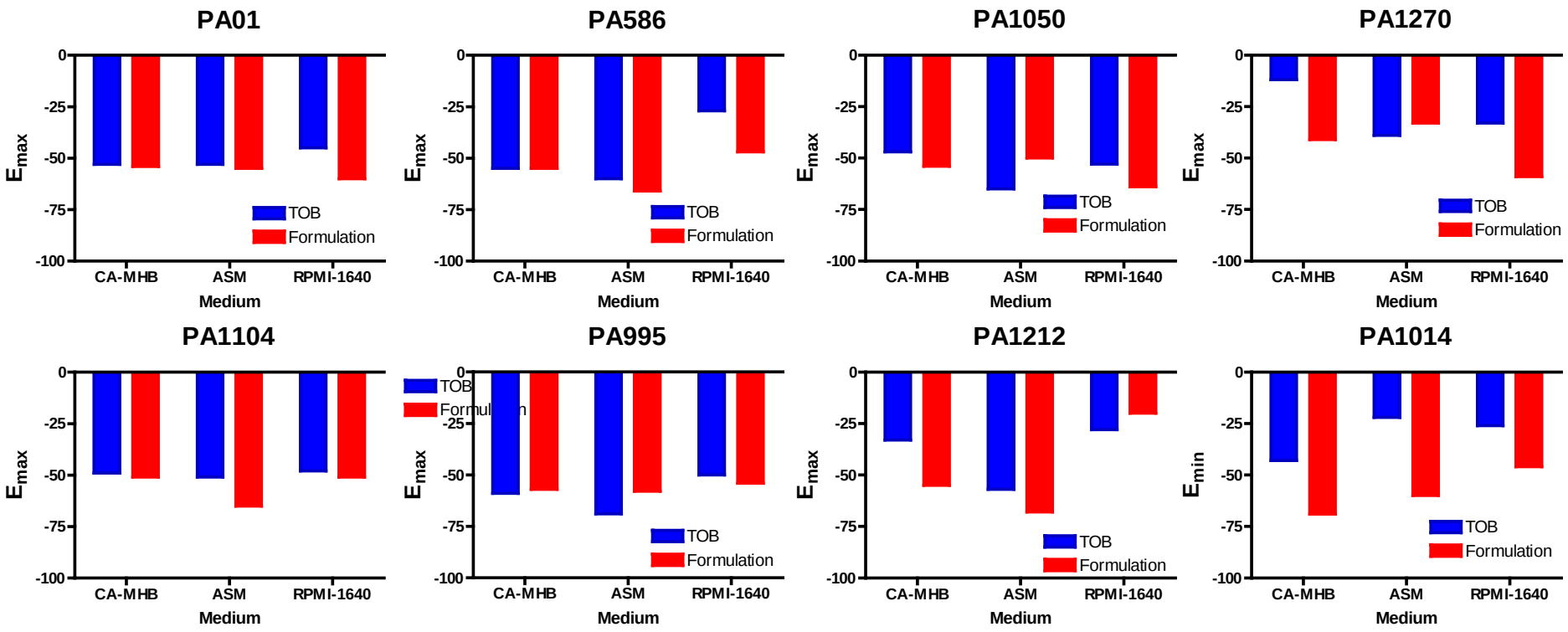


- Synergy observed at E_{min} for biomass, but not systematic for viability
- Same effect at E_{max}

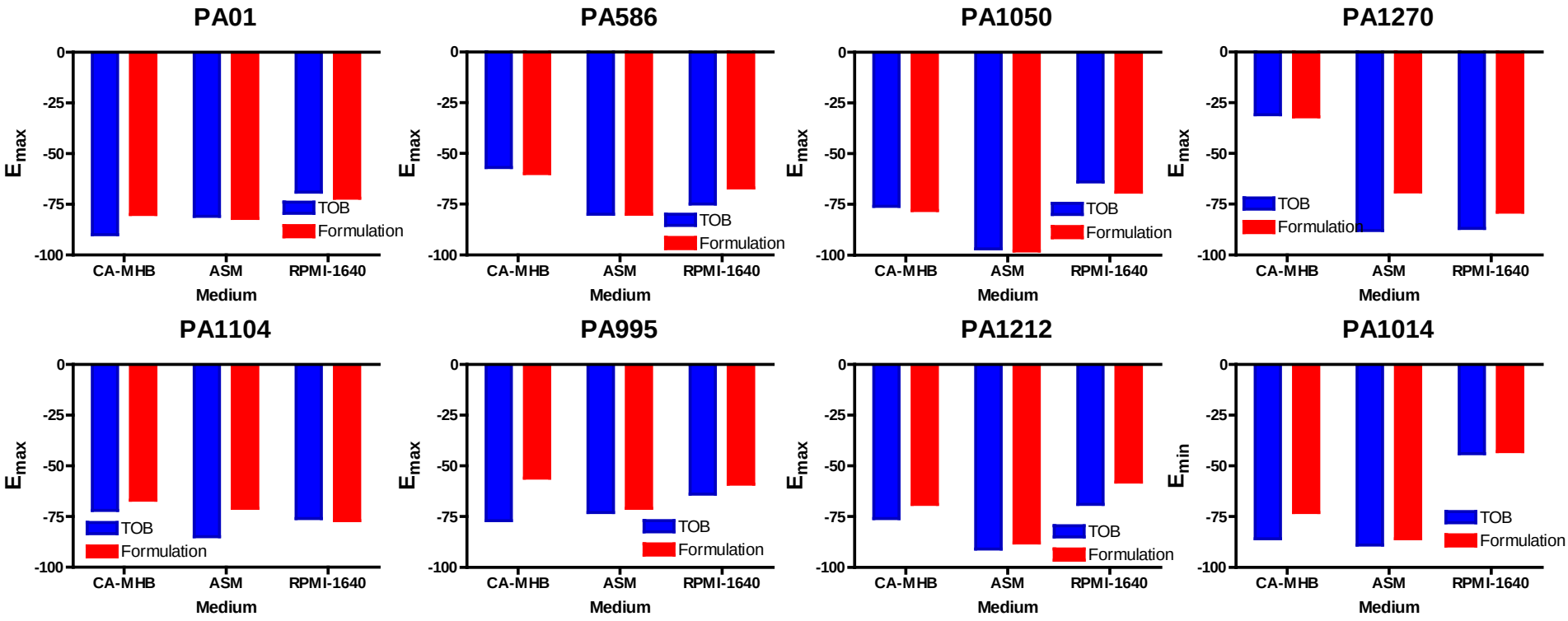
Bacterial Viability: E_{min}



Biofilm Biomass : E_{max}



Bacterial Viability: E_{max}



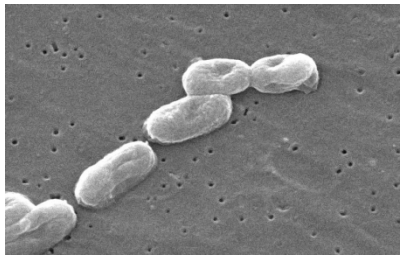
CF Strains		MIC (mg/L) in CA-MHB			MIC (mg/L) in RPMI 1640	
Strain	**Origin**	**TOB**	**CLR**	**AZM**	**CLR**	**AZM**
PA01	Ref. strain	2-4	**>1024**	256	256	32
PA586	Italy	4	**1024**	512	256	32
PA1050	Germany	4	256	64	16	2
PA1270	Belgium	1	16	16	8	4
PA1104	UK	**128**	**>1024**	**>1024**	**>1024**	**>1024**
PA995	France	**256**	**1024**	256	128	16
PA1212	Belgium	0.25	**>1024**	**>1024**	**>1024**	**>1024**
PA1014	Germany	2	**>1024**	**>1024**	**>1024**	**>1024**

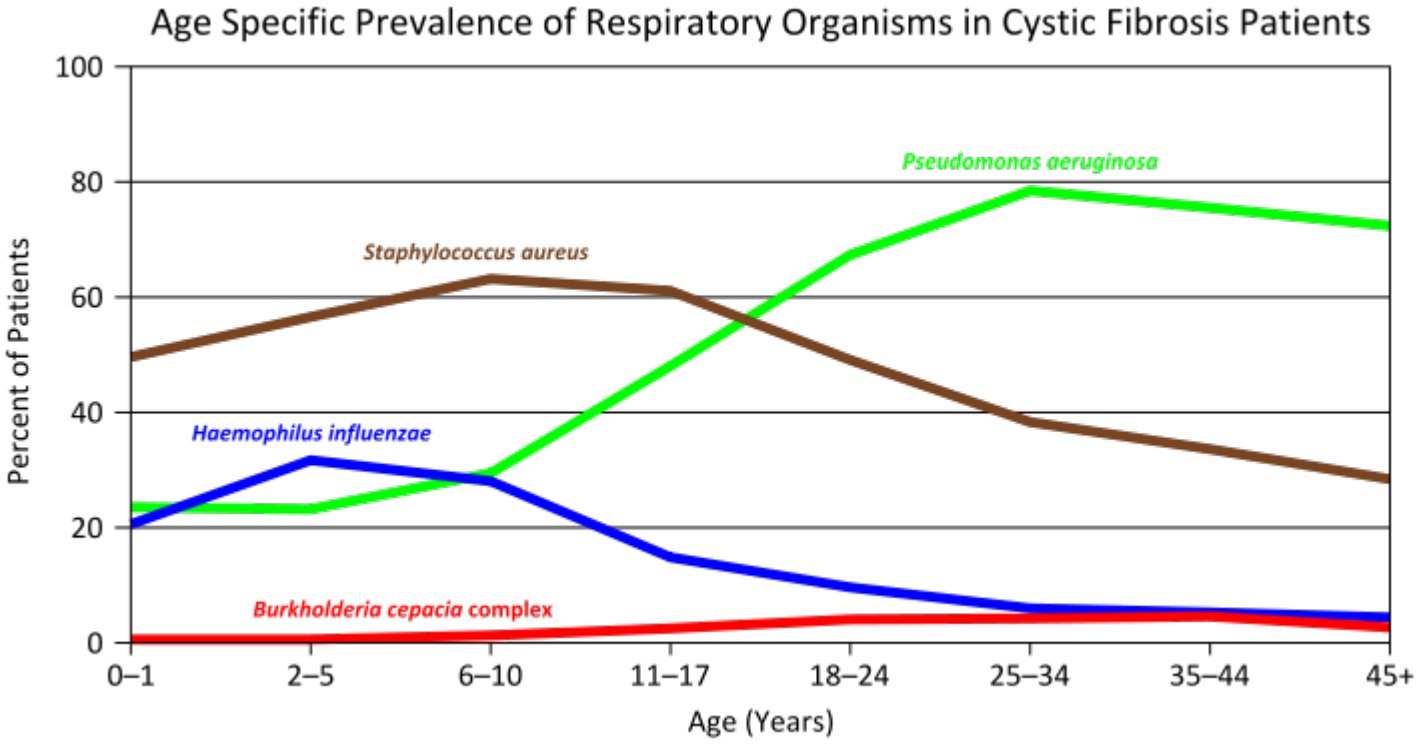
		C_{50} (mg/L) vs biofilm biomass						C_{50} (mg/L) vs bacterial viability					
CF Strains		CA-MHB		ASM		RPMI-1640		CA-MHB		ASM		RPMI-1640	
Strain	Origin	TOB	F	TOB	F	TOB	F	TOB	F	TOB	F	TOB	F
PA01	Ref. strain	43.4	7.96	21.1	14.3	NA	2.74	0.91	3.40	3.95	4.83	1.11	2.81
PA586	Italy	11.3	11.5	12.8	10.1	NA	NA	11.6	60.7	4.69	10.6	12.6	23.0
PA1050	Germany	NA	8.31	6.89	NA	4.98	3.64	5.69	4.21	1.42	1.09	6.28	3.83
PA1270	Belgium	NA	NA	NA	NA	NA	4.04	NA	NA	17.4	10.7	0.86	1.15
PA1104	UK	NA	36.6	NA	146	NA	17.2	9.48	9.53	8.02	10.2	12.3	12.5
PA995	France	719	11.3	68.7	20.2	319	7.96	189	205	9.79	10.6	6.04	13.7
PA1212	Belgium	NA	1.86	10.1	15.2	NA	NA	1.54	10.4	3.99	4.68	4.54	3.70
PA1014	Germany	NA	1.14	NA	30.0	NA	NA	1.30	1.01	3.39	3.47	NA	NA

NA: Antibiotic treatment could not eradicate 50% of biofilm biomass/bacterial viability over control.
For the formulation, the concentrations indicated in the table correspond to those of TOB.
Concentration for CLR is ten times less than these concentrations indicated

Burkholderia cepacia complex (*BCC*)

- Gram-negative bacteria
- Opportunist
- Infect minority of CF patients





Data is from the 2009 Patient Registry Report, issued by the Cystic Fibrosis Foundation.

MIC of Aminoglycosides and Macrolides in CA-MHB and RPMI-1640 for 10 *BCC* strains

CF Strains			MIC (mg/L) in CA-MHB			
Strain	**Origin**	**Species**	**AMK**	**TOB**	**CLR**	**AZM**
PA1092	Germany	*B. multivorans*	128	32	64	64
PA1093	Germany	*B. cenocepacia*	256	128	128	64
PA1094	Germany	*B. multivorans*	128	64	128	64
PA1095	Germany	*B. multivorans*	256	128	>1024	>1024
PA1096	Germany	*B. gladioli*	32	8	64	32
PA1097	Germany	*B. multivorans*	512	256	64	64
PA1098	Germany	*B. cenocepacia*	128	64	256	64
PA1099	Germany	*B. multivorans*	128	64	256	128
PA1100	Germany	*B. cepacia*	64	32	>1024	>1024
PA1101	Germany	*B. cepacia*	>512	>512	>1024	>1024

Burkholderia cepacia complex (*BCC*)

Low activity of macrolides and aminoglycosides in CA-MHB

→ Causes ?

- **efflux pump eliminating aminoglycosides and macrolides are constitutively expressed: AmrAB-OprA**
  (Moore et al, Antimicrob Agents Chemother. (1999) 43:465-70).

- **low number of phosphate and carboxylate groups in the lipopolysaccharide**
  ⇨ resistance to cationic antibiotics (aminoglycosides, colistin)
  (Cox et al, Mol Microbiol. (1991) 5:641-6).

MIC of Aminoglycosides and Macrolides in CA-MHB and RPMI-1640 for 10 *BCC* strains

CF Strains			MIC (mg/L) in CA-MHB				MIC (mg/L) in RPMI 1640			
Strain	**Origin**	**Species**	**AMK**	**TOB**	**CLR**	**AZM**	**AMK**	**TOB**	**CLR**	**AZM**
PA1092	Germany	*B. multivorans*	128	32	64	64	32	4	16	2
PA1093	Germany	*B. cenocepacia*	256	128	128	64	32	32	32	2
PA1094	Germany	*B. multivorans*	128	64	128	64	16	8	32	4
PA1095	Germany	*B. multivorans*	256	128	>1024	>1024	8	32	>1024	>1024
PA1096	Germany	*B. gladioli*	32	8	64	32		1	8	2
PA1097	Germany	*B. multivorans*	512	256	64	64	No growth			
PA1098	Germany	*B. cenocepacia*	128	64	256	64		16	32	4
PA1099	Germany	*B. multivorans*	128	64	256	128		16	64	4
PA1100	Germany	*B. cepacia*	64	32	>1024	>1024		4-8	>1024	64
PA1101	Germany	*B. cepacia*	>512	>512	>1024	>1024		256	>1024	16

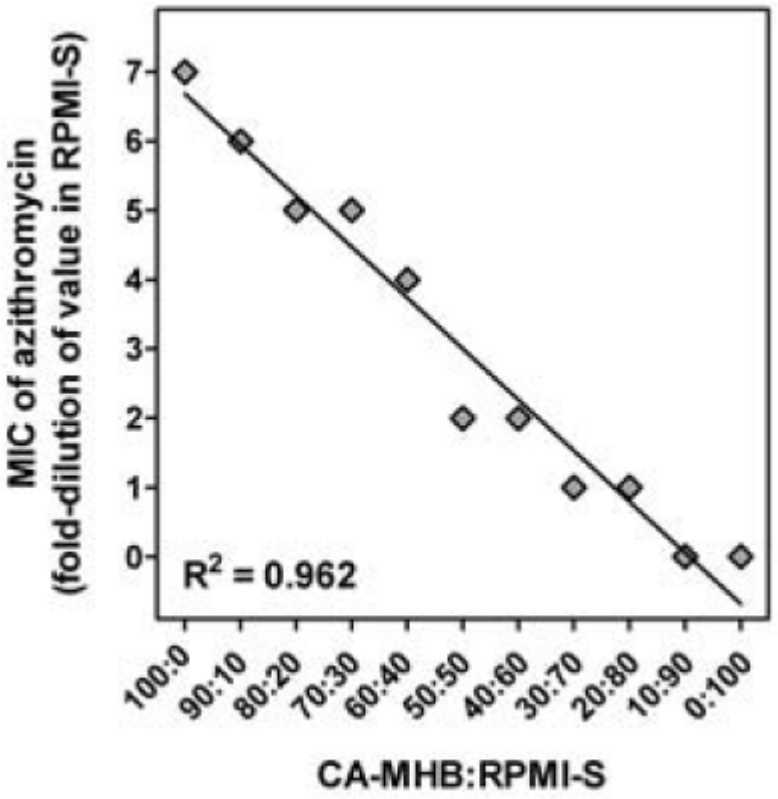


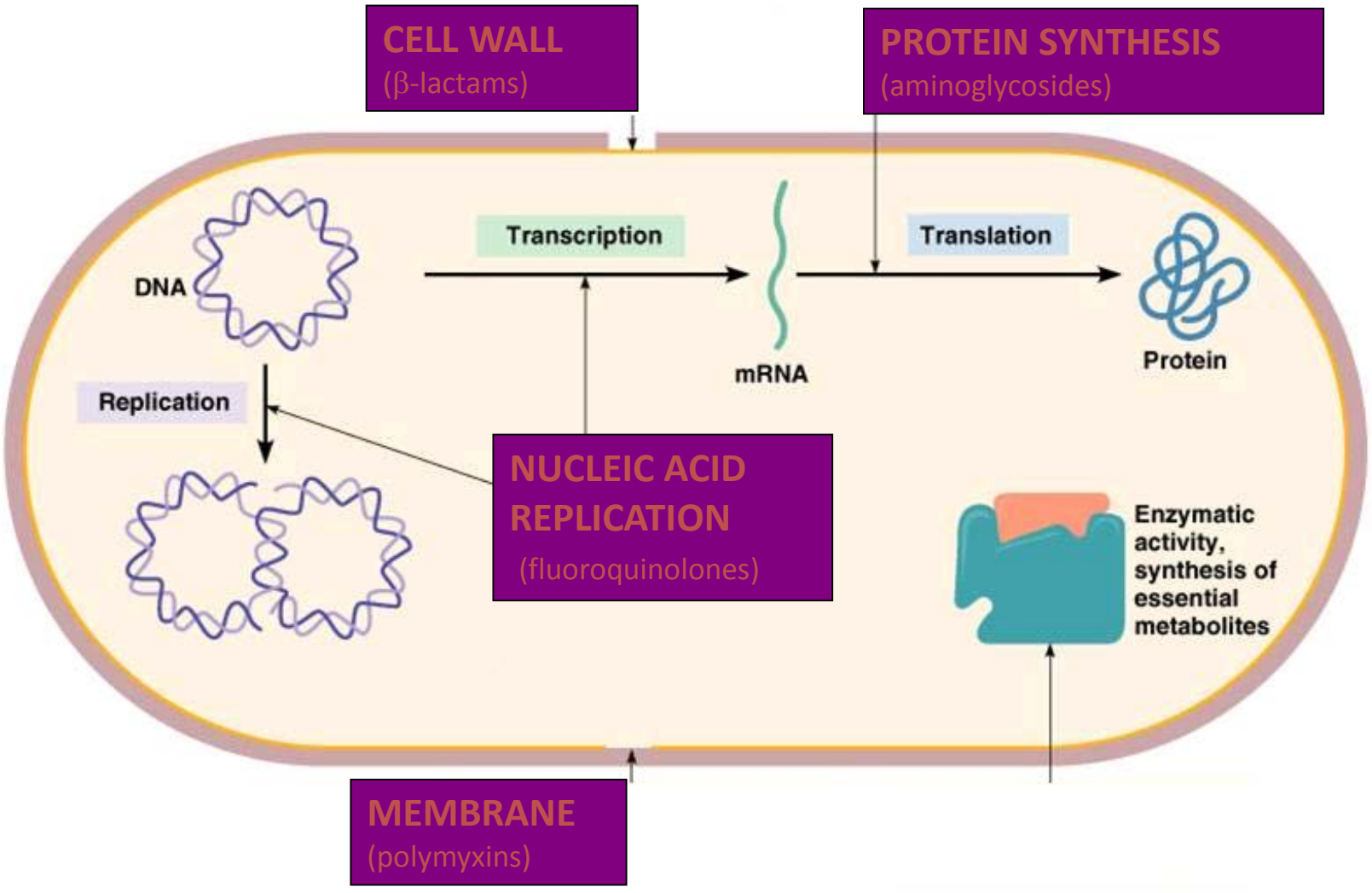
Table S1. MIC of azithromycin against *P. aeruginosa* PAO1 as measured in CA-MHB supplemented with specific constituents of RPMI medium.

RPMI constituent added to CA-MHB[a]	concentration	MIC (mg/L)
none	-	128
D-glucose	2000 mg/L	64
$Ca(NO3)_2$	0.424 mg/L	128
$MgSO_4$	0.407 mg/L	128
KCl	5.33 mg/L	128
NaCl	103.45 mg/L	128
thiamine hydrochloride	0.00297 mg/L	128
niacinamide	0.0082 mg/L	128
pyridoxine	0.00485 mg/L	128
non essential amino acids	1 %	256
salt mixture [$Ca(NO3)_2$, $MgSO_4$, KCl, NaCl]	same as above [b]	128
vitamin mixture [thiamine, niacinamide, pyridoxine]	same as above [b]	64
phenol red	0.0133 mg/L	128

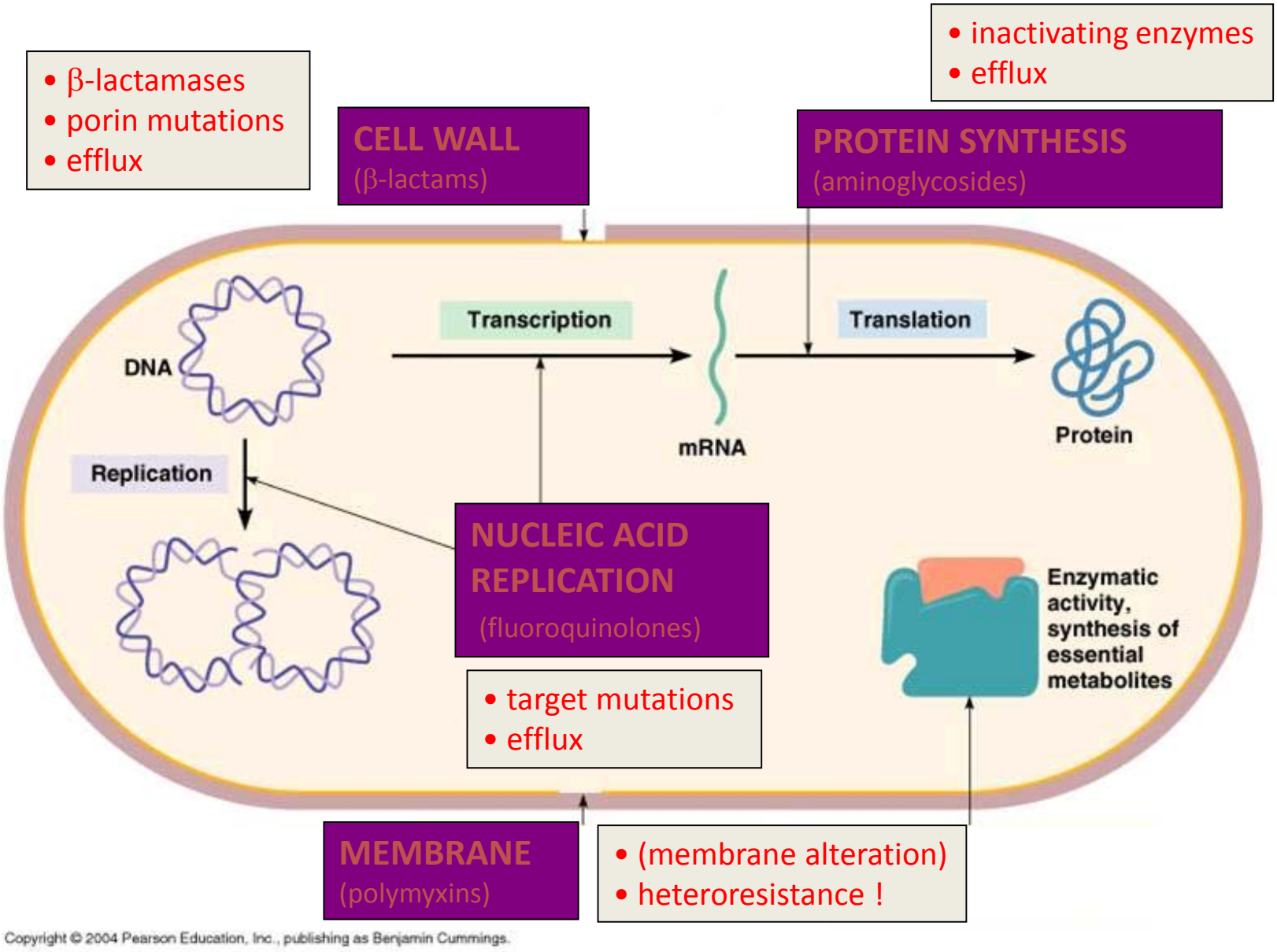
[a] each constituent was added to CA-MHB (for mixtures, in the order indicated in the Table), and the supplemented medium carefully mixed before being used for MIC determination.

[b] with respect to individual constituents

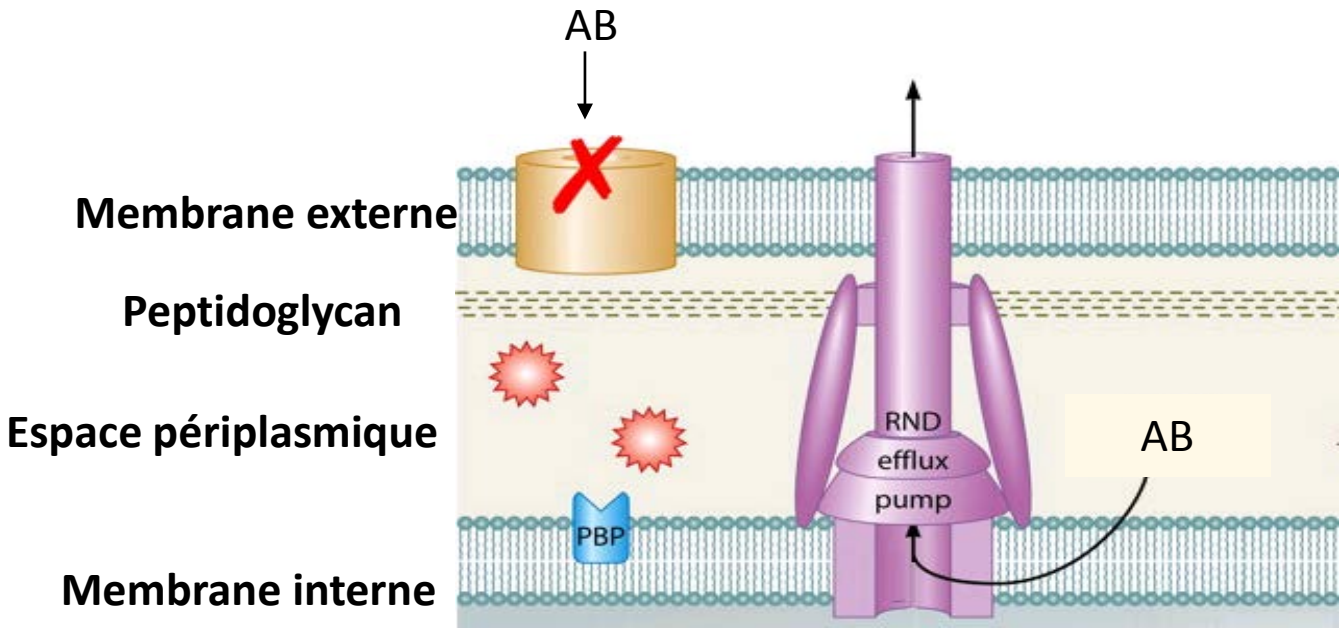
Our current armamentorium



Our current armamentorium: resistance !



Enveloppe de *Pseudomonas aeruginosa*



Faible perméabilité de l'enveloppe : porines peu perméables

effl

Rem: Les MLS sont substrtas de toutes les pompes, pas seulement MEXCD

Adapté de Lister et al. Clin. Microbiol. Rev. 2009; 22: 582-610

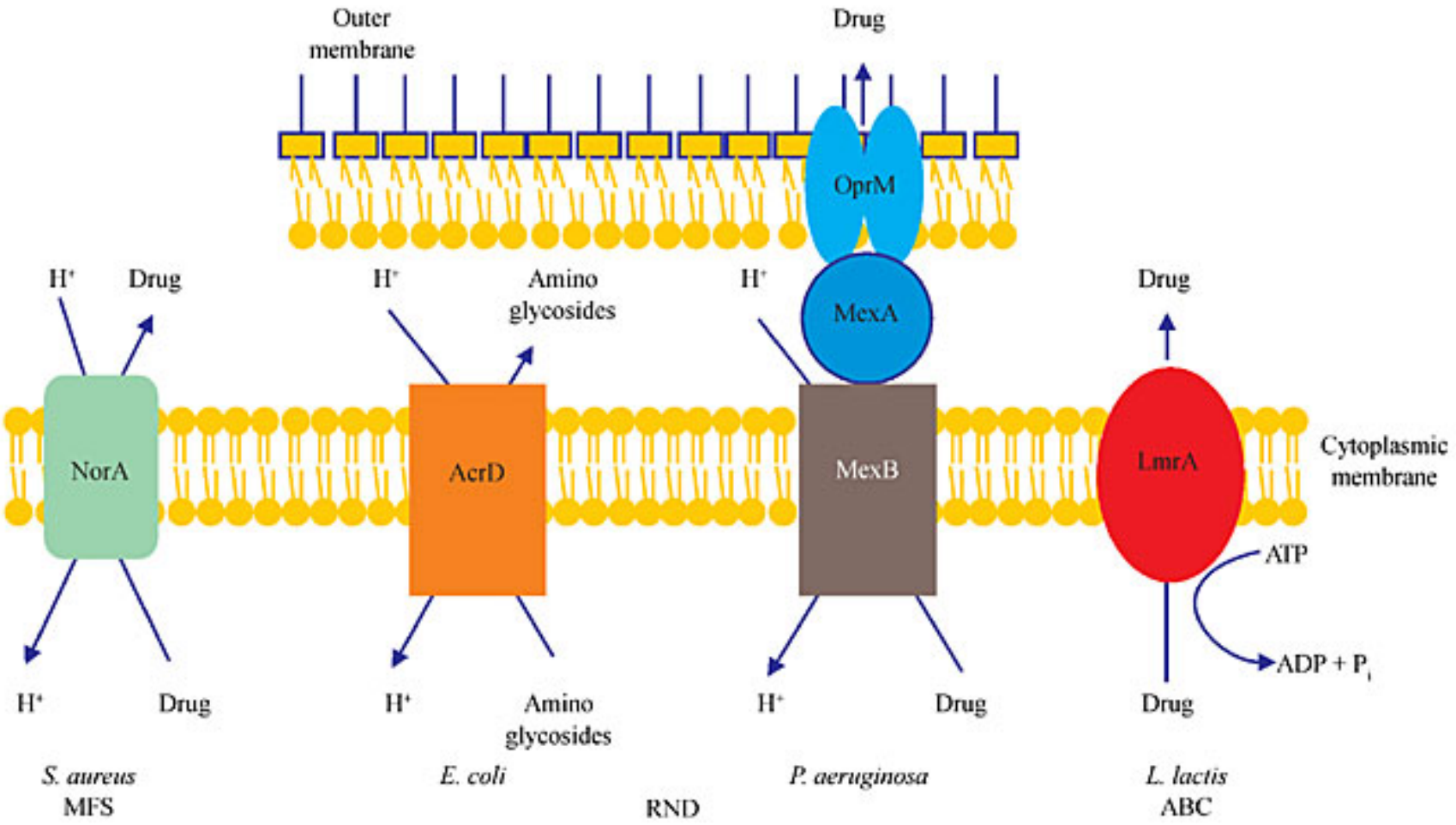
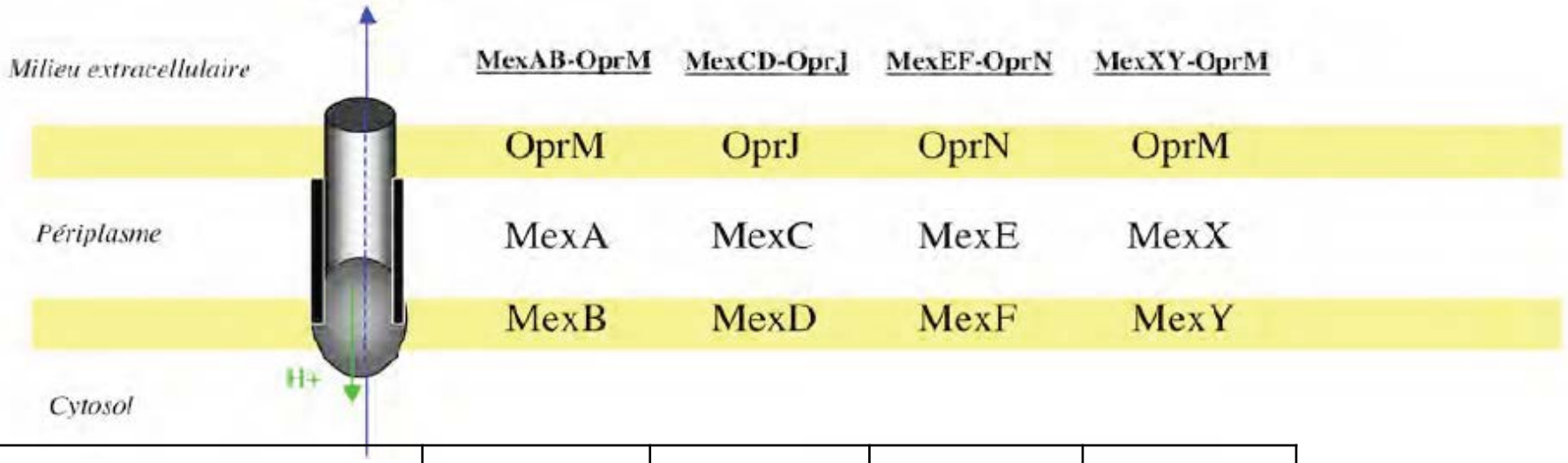


Figure 1. Schematic illustration of the main types of bacterial drug efflux pumps shown in *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Lactobacillus lactis*. Illustrated are NorA, a member of the major facilitator superfamily (MFS); AcrD and MexAB-OprM, two members of the resistance-nodulation-division (RND) family and LmrA, a member of the ATP-binding cassette (ABC) family. All systems extrude drugs in an energy-dependent manner, either by using proton motif force or ATP. The two other types of efflux systems found in bacteria, multidrug and toxic compound extrusion (MATE), and small multidrug resistance (SMR), look structurally similar to the MFS but are designated as distinct families, based on phylogenetic diversity (MATE) or size (SMR).

Pompes à efflux



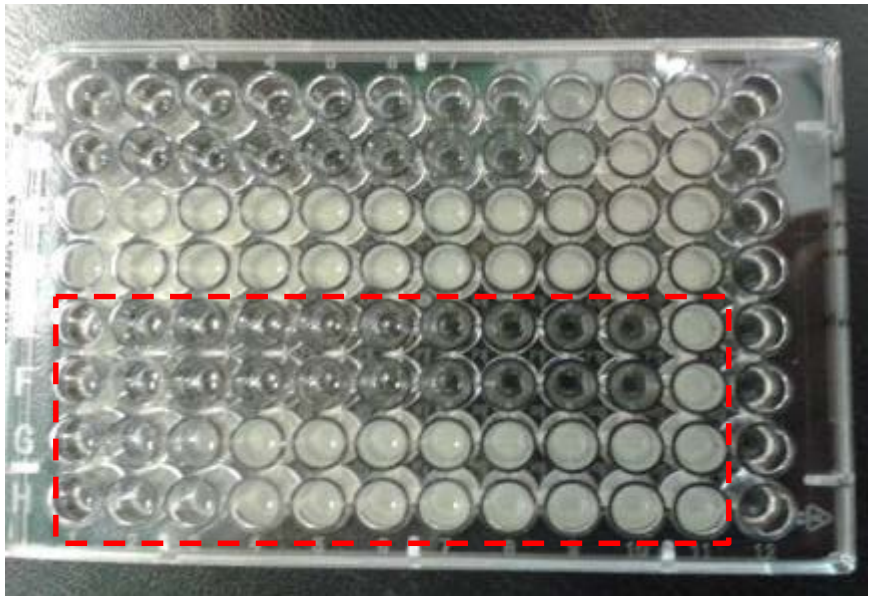
B-lactames	**XX**	**X**		**X**
Rifampicine	X			
Fluoroquinolones	X	X	X	X
Chloramphenicol	X	X	X	X
Tetracycline	X	X	X	X
Macrolides	X	X		X
Aminoglycosides				X

Figure adapté de l'Université Paris Descartes, thèse de doctorat présentée et soutenue publiquement par Gilles PHAN (2008).
Tableau adapté de *Van Bambeke et al.* J Antimicrob Chemother. 2003 May;51(5):1055-65.

Characterisation of the strains

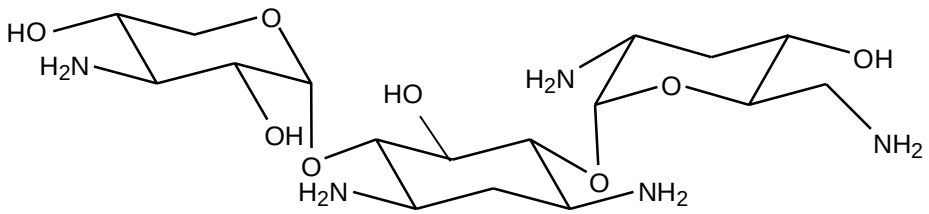
- Measure of **Minimal Inhibitory Concentrations (MIC)** : the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation
- by *microdilution* in **cation-adjusted Mueller-Hinton (CA-MHB)** broth and/or in **RPMI 1640**
- ATCC 27853 was used as quality control strain and susceptibility was assessed according to EUCAST and CLSI breakpoints.

  (Starting inoculum: approx. $5*10^5$ CFU/ml; 24h incubation @ 37° C)



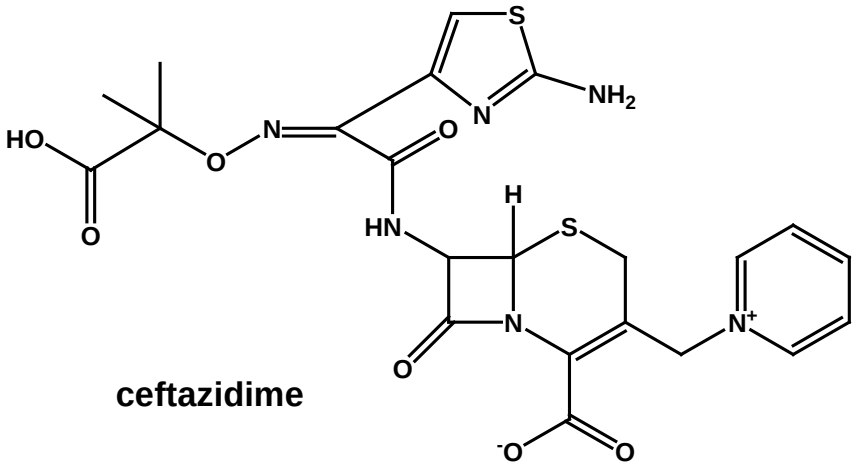
Antipseudomonal antibiotics

- Aminoglycosides

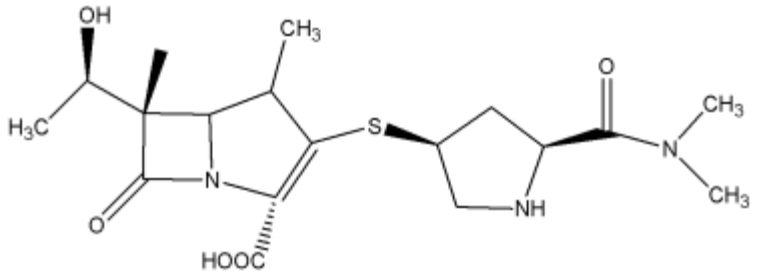


tobramycine

- β-lactams



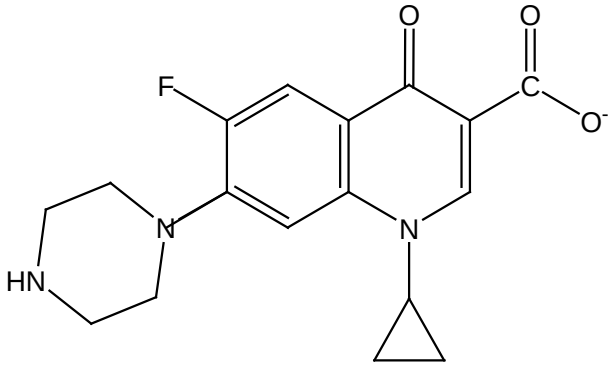
ceftazidime



meropeneme

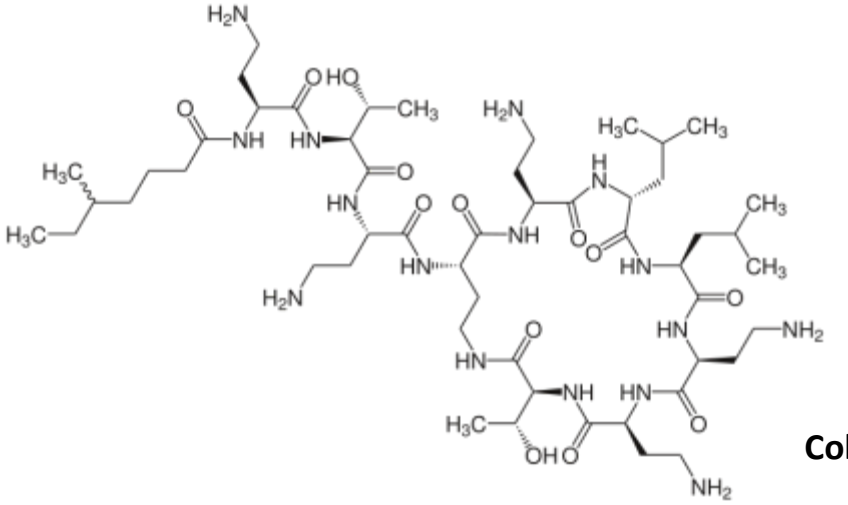
Antipseudomonal antibiotics

- Fluoroquinolones



ciprofloxacin

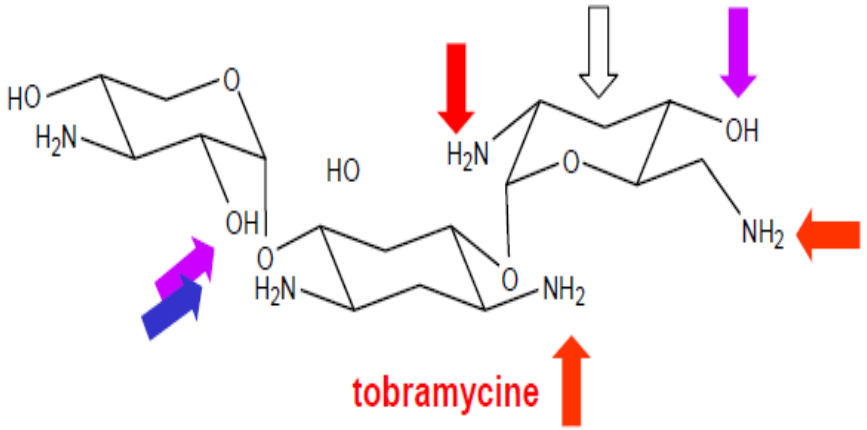
- Polymyxines

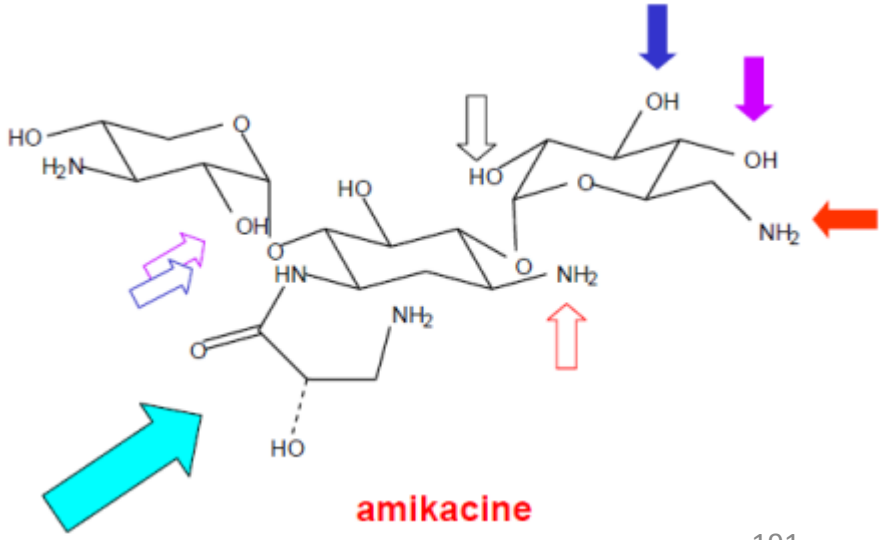


Colistin

Pourquoi?

- Amikacine a plus d'affinité pour MexX par rapport à la tobramycine ? *(JC Hurley et al , Mechanism of amikacin resistance in Pseudomonas aeruginosa isolates from patients with cystic fibrosis, 1995)*
- Activité de l'enzyme 3'phosphotransférase ?





Burkholderia strains : MICs in both media

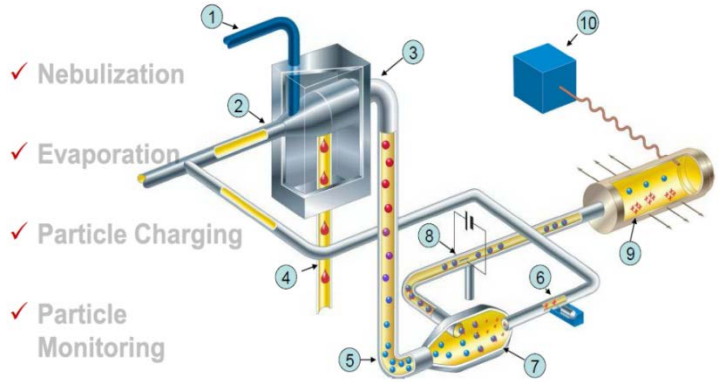
CF Strains			MIC (mg/L) in CA-MHB				MIC (mg/L) in RPMI 1640			
Strain	**Origin**	**Species**	**AMK**	**TOB**	**CLR**	**AZM**	**AMK**	**TOB**	**CLR**	**AZM**
PA1092	Germany	*B. multivorans*	128	32	64	64	32	4	16	2
PA1093	Germany	*B. cenocepacia*	256	128	128	64	32	32	32	2
PA1094	Germany	*B. multivorans*	128	64	128	64	16	8	32	4
PA1095	Germany	*B. multivorans*	256	128	>1024	>1024	8	32	>1024	>1024
PA1096	Germany	*B. gladioli*	32	8	64	32		1	8	2
PA1097	Germany	*B. multivorans*	512	256	64	64	No growth			
PA1098	Germany	*B. cenocepacia*	128	64	256	64		16	32	4
PA1099	Germany	*B. multivorans*	128	64	256	128		16	64	4
PA1100	Germany	*B. cepacia*	64	32	>1024	>1024		4-8	>1024	64
PA1101	Germany	*B. cepacia*	>512	>512	>1024	>1024		256	>1024	16

Comparison of HPLC Detectors

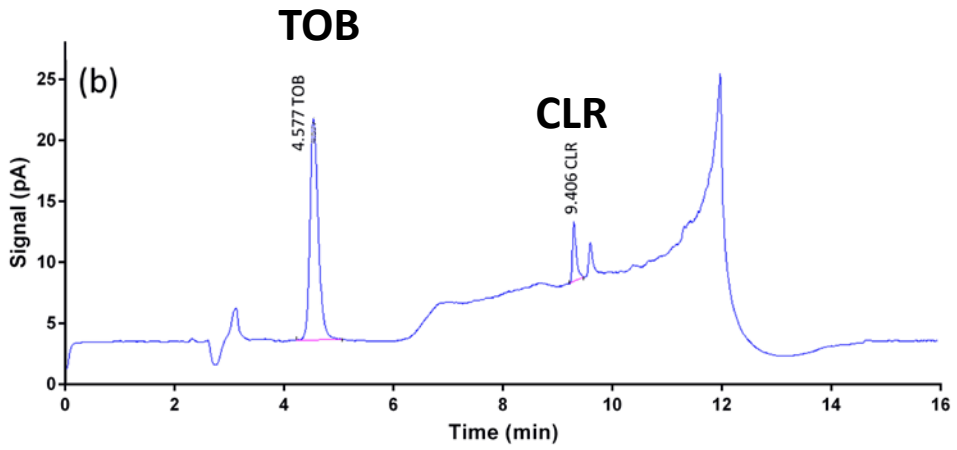
HPLC Detector Type	RI	ELSD	Corona CAD	UV	MS	FL	EC
Range of Applications	Universal	Universal	Universal	Selective	"Universal"*	Very Selective	Very Selective
Minimal Detectable Quantity	µg	High ng	Low ng	Low ng	pg	pg	fg - pg
Linear Range / Dinamic Range	10^3	10^3	10^4	10^4	$10^3 \cdot 10^4$	$10^3 \cdot 10^4$	10^5

* Not all compounds will undergo ionization on the MS detector

Analytical method development and validation for the first combination TOB-CLR



HPLC-Charged aerosol detector

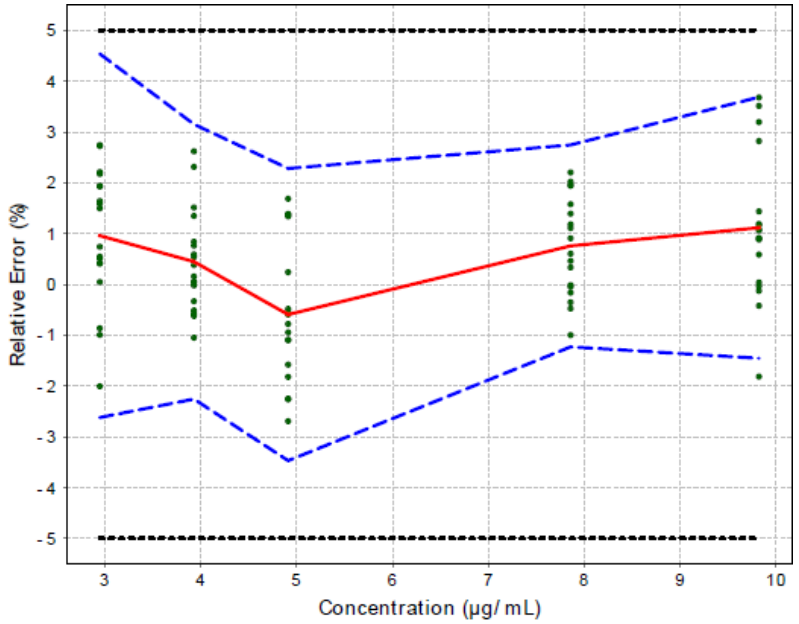


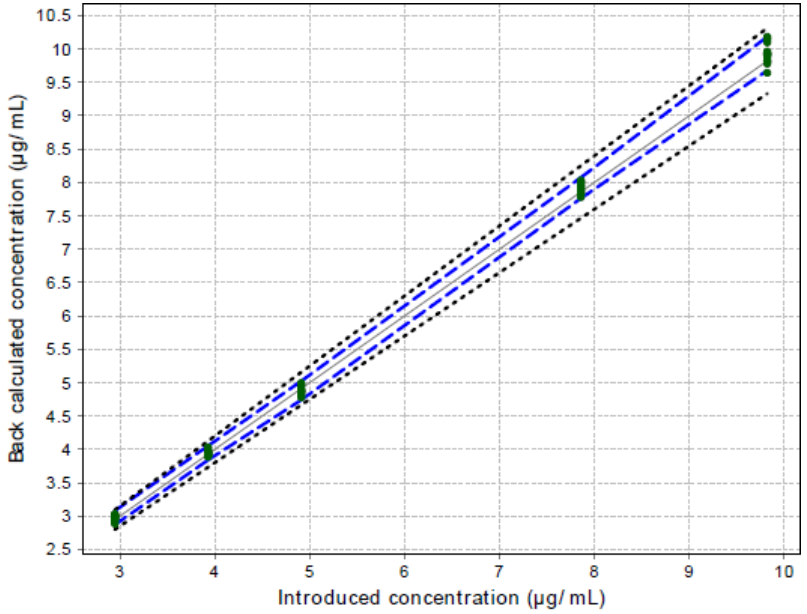
Change of phase (gradient elution)

Accuracy

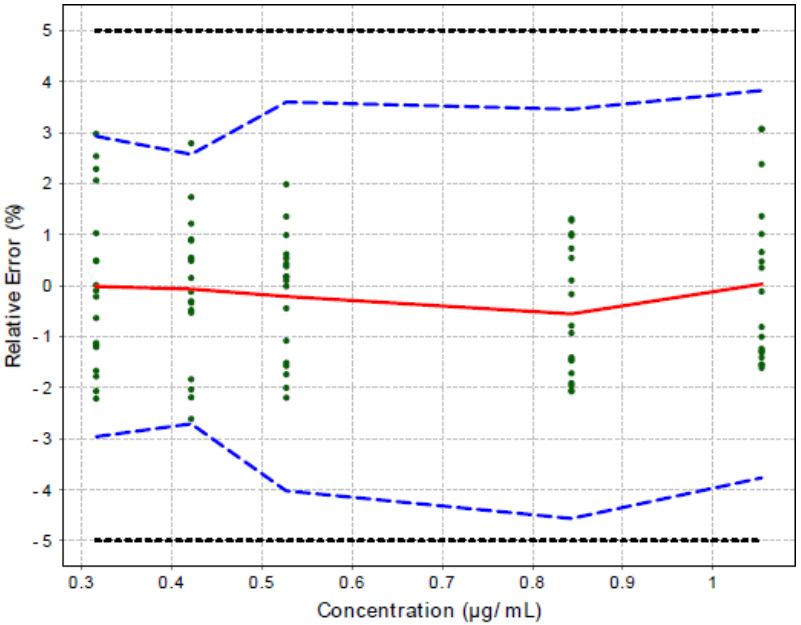
Linearity

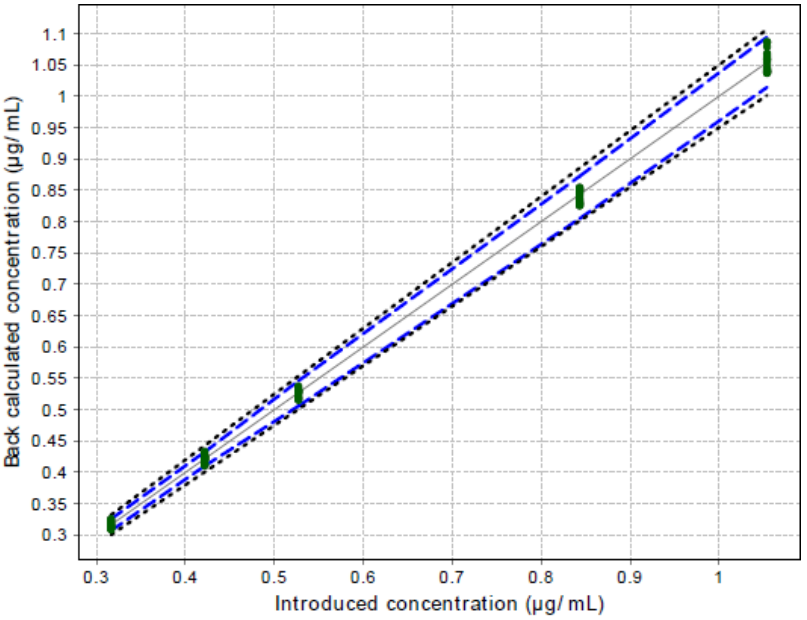
TOB





CLR





Method development : main challenges

- Problems with previous method developed with mass spectrometer
  - High variability
  - Matrix effect (lactose)
- Problems with the nature of the ABs:
  - TOB : polar, lack chromophore group
  - CLR : hydrophobic

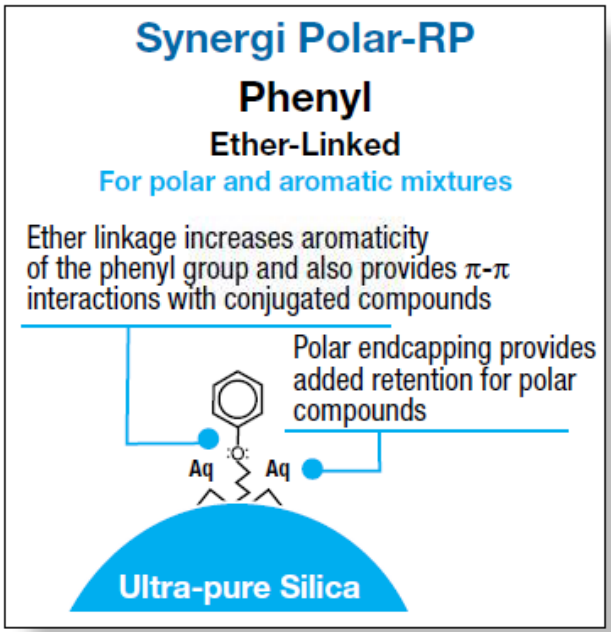
=> Method using Corona charged aerosol detector

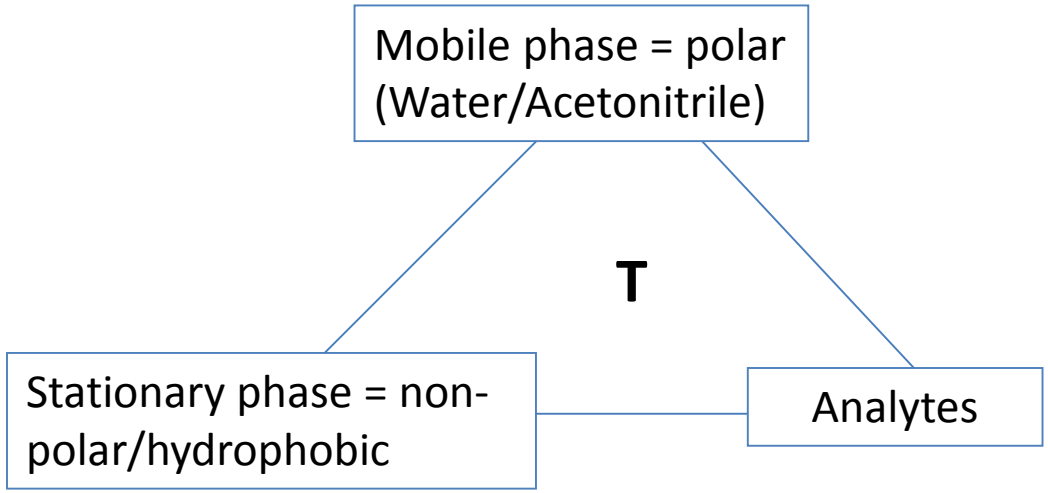
What is Corona CAD?

A novel, mass sensitive, **“universal”** detector for the routine determination of **any non-volatile and many semi-volatile** chemical species

Method principles

- High Performance Liquid Chromatography (Agilent 1100)
- In reversed-phase mode : Synergi 4µm Polar-RP 80 Å, LC 150x4.6mm column (from Phenomenex).

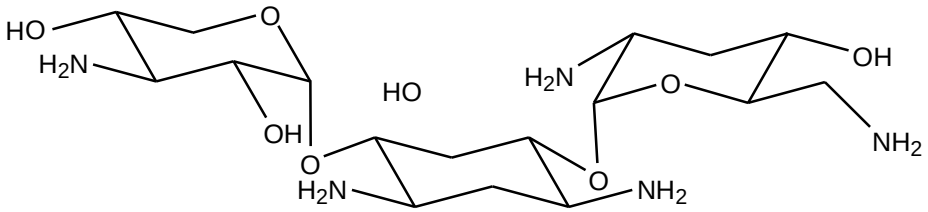




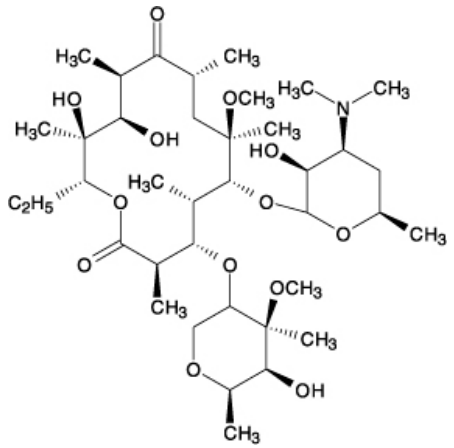
- Detection : Corona Charged Aerosol Detector (CAD)

Why Corona CAD?

- No UV (Tobramycin lack of chromophore)



Tobramycine

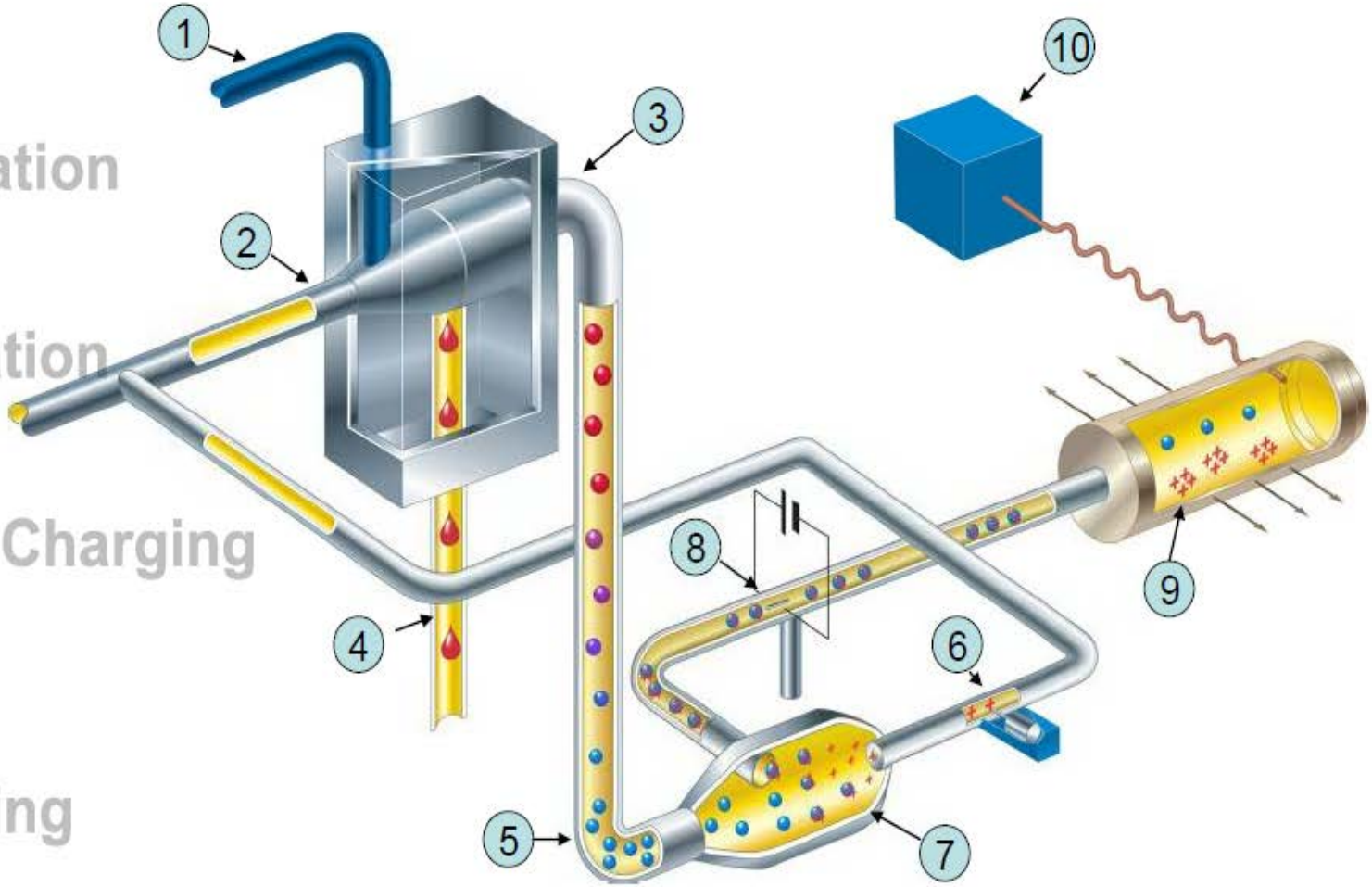


Clarithromycin

- No MS!
  - high variability
  - complex
  - matrix effect (Lactose)

How does it work? : Corona CAD Principles

- ✓ Nebulization
- ✓ Evaporation
- ✓ Particle Charging
- ✓ Particle Monitoring



Chromatographic conditions

- Stationary phase : Synergi 4µm Polar-RP 80 Å
- Mobile phase :
  - Purified water (miliQ) + 0.1% Pentafluoropropionic anhydride acid (PFPA) as a counter ion
  - Acetonitrile (ACN) LC/MS grade + 0.1% PFPA
- Gradient elution :

Time (min)	ACN + 0.1% PFPA	Water + 0.1% PFPA
0	20	80
2.5	20	80
9.5	90	10
10	20	80
16	20	80

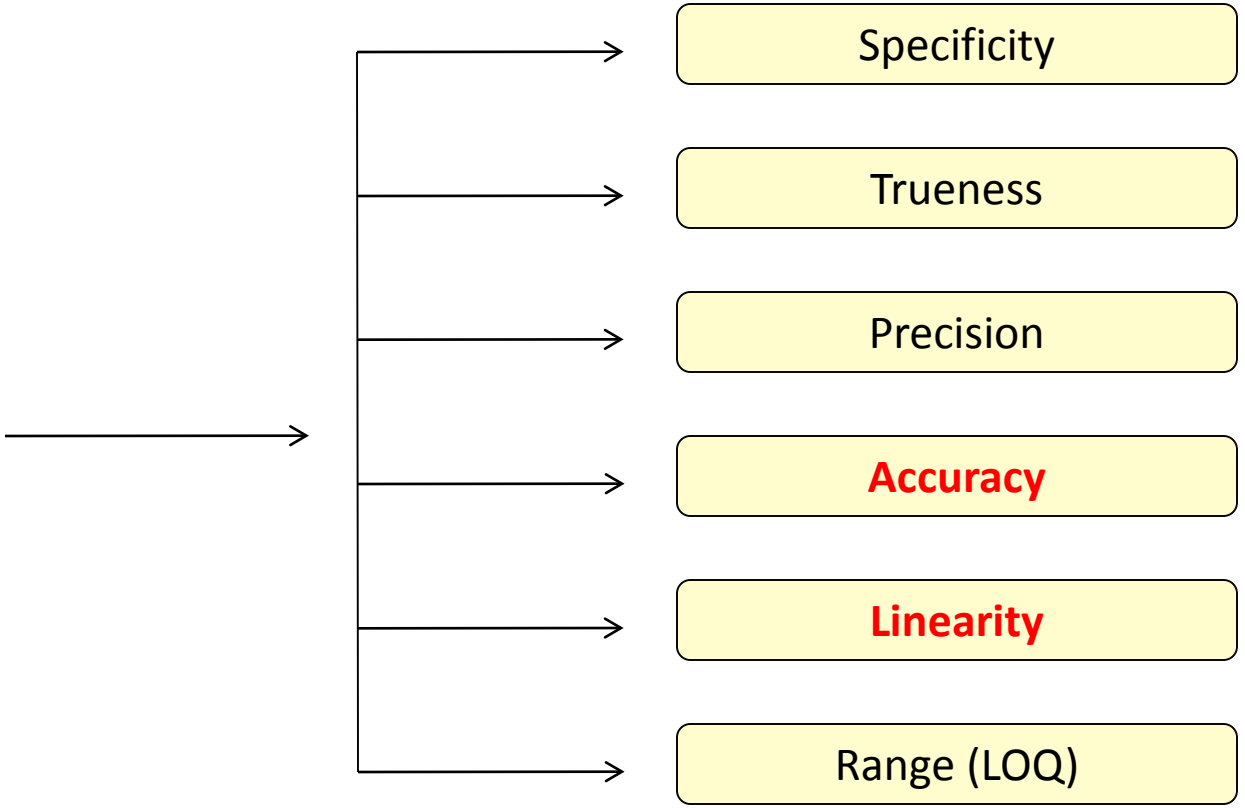
Specificity

= the ability of an analytical method to unequivocally assess the analyte in the presence of components which may be expected to be present

Method validation

Method Validation

= the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use

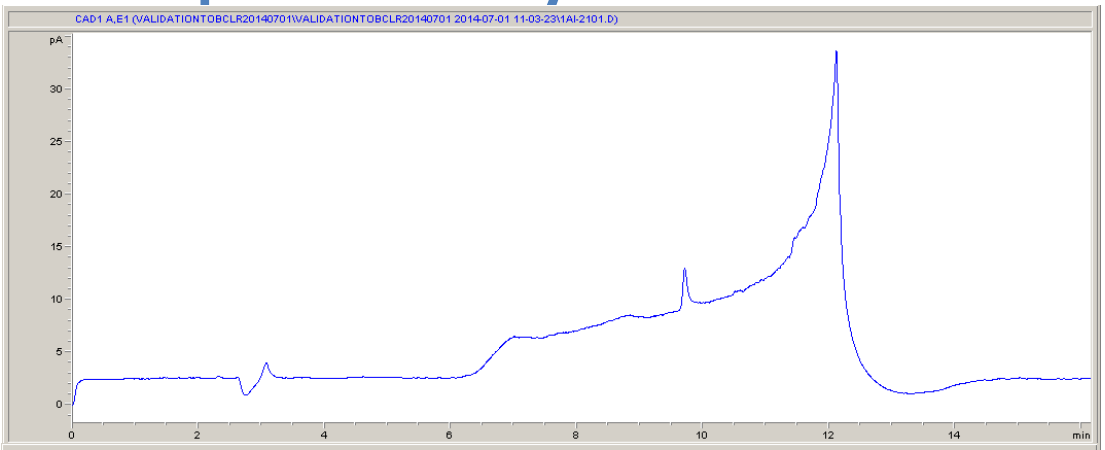


Acceptance limit set at ±5 %
(analysis for pharmaceutical drug development)

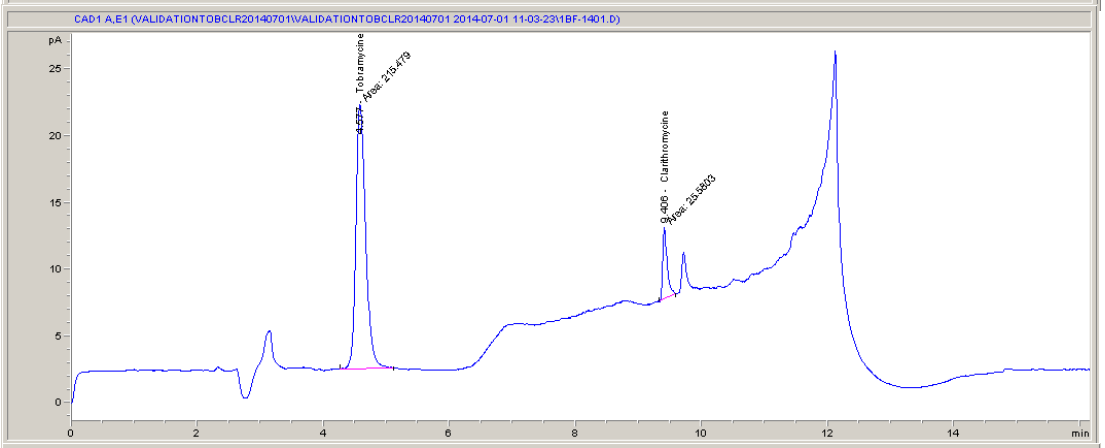
M. Feinberg, B. Boulanger, W. Dewé, Ph. HubertAnal. Bioanal. Chem., 380 (2004), pp. 502–514

Specificity

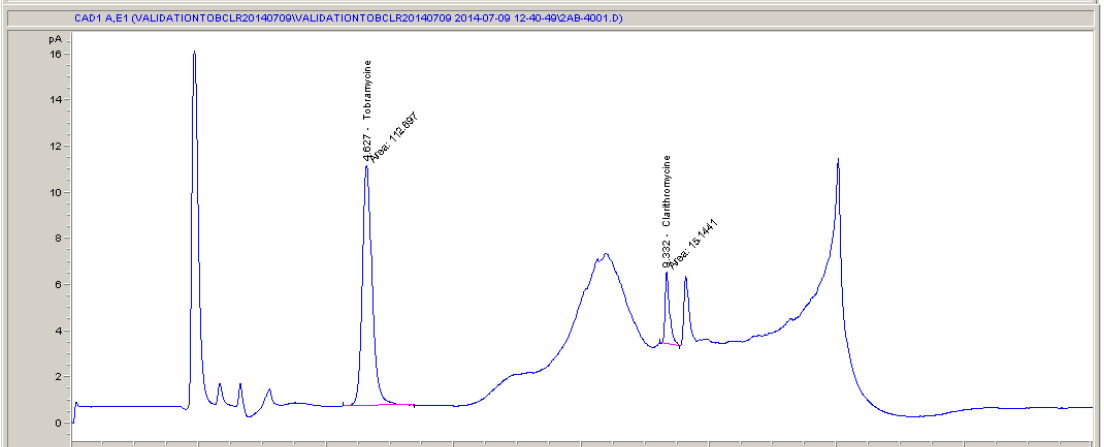
Blank sample



Sample containing only TOB and CLR



Sample containing TOB and CLR and the matrix (lactose and HPMC capsule)



Response function

= choosing the validated calibration model for the analyte

=> Quadratic regression for Corona CAD

TOB

Model	Lower and upper limits of quantitation (LOQ) (µg/mL)	Dosing Range Index
Quadratic Regression	[2.946 , 9.820]	1.000

CLR

Model	Lower and upper limits of quantitation (LOQ) (µg/mL)	Dosing Range Index
Linear Regression	[0.3162 , 1.054]	1.0000
Quadratic Regression	[0.3162 , 1.054]	1.0000

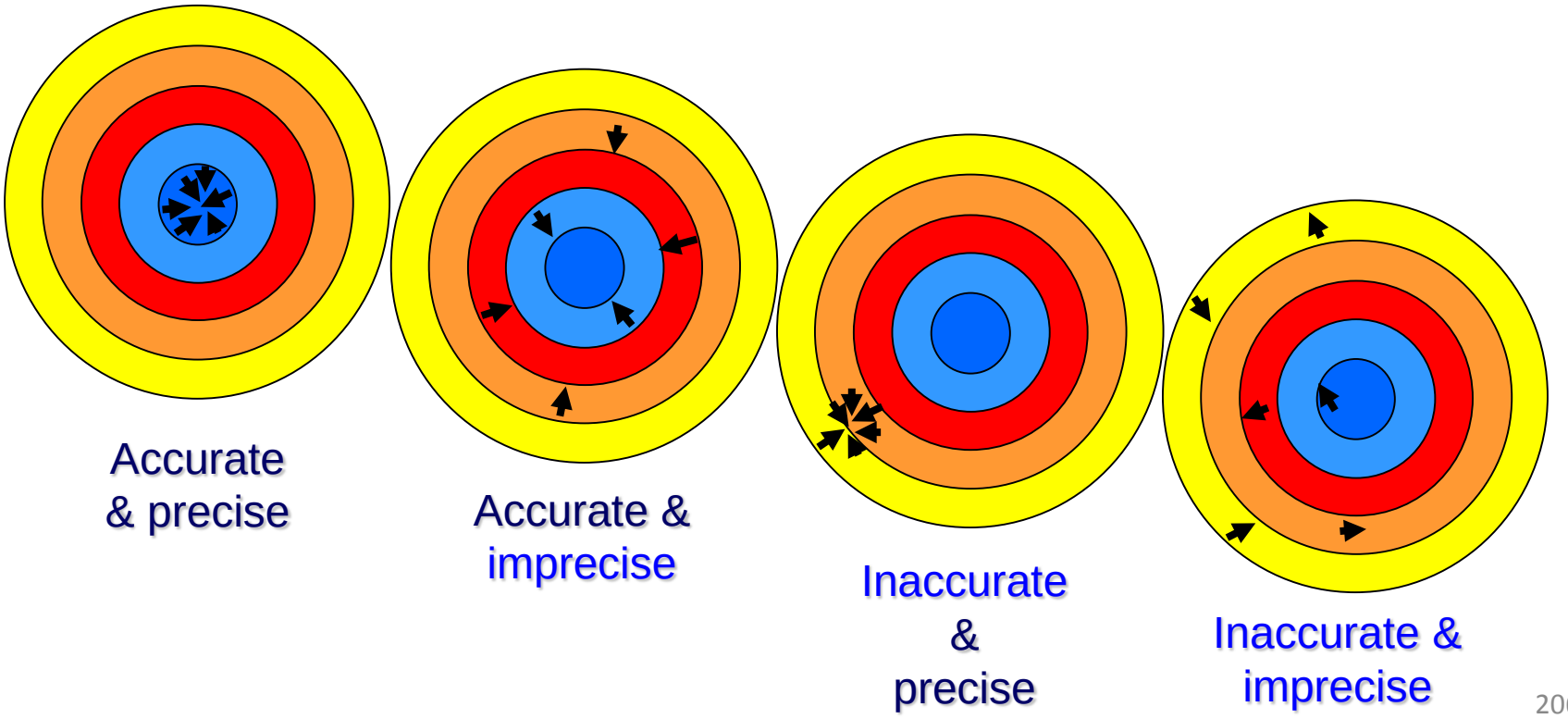
Accuracy

= the closeness of agreement between the test result and the accepted reference value, namely the conventionally true value

The closeness of agreement observed is the resultant of the sum of the systematic and random errors (the total error linked to the result).

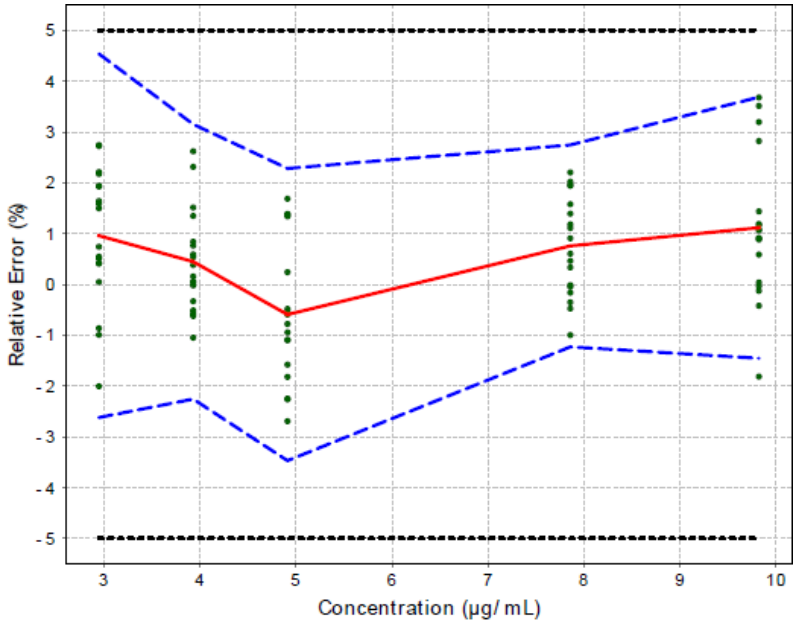
=> Accuracy = Trueness + Precision

(Acceptance limit set at ±5 %)

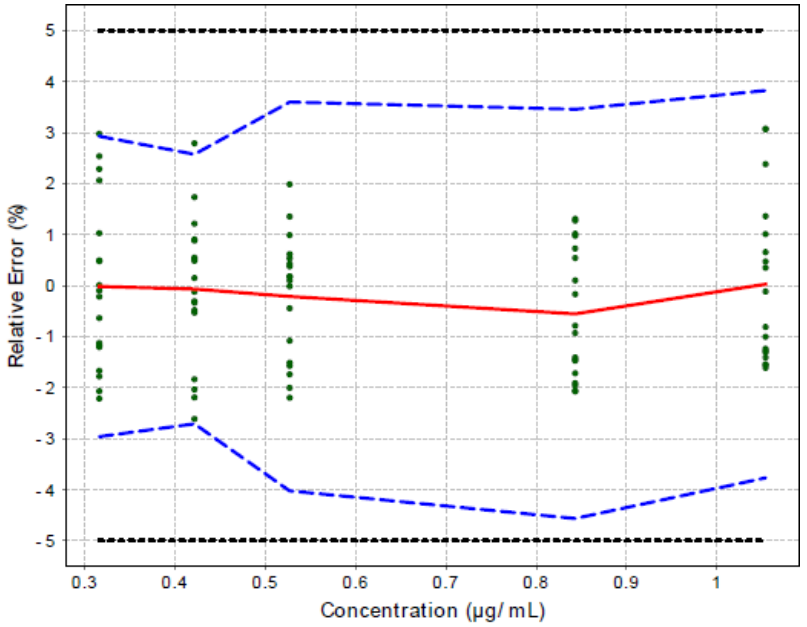


Accuracy

Accuracy profile for TOB



Accuracy profile for CLR



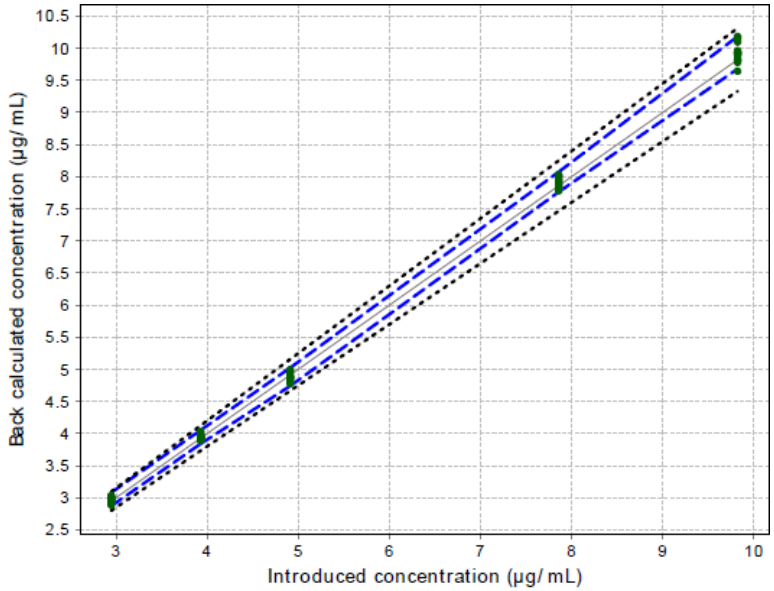
Linearity

= the ability of an analytical method to obtain results directly proportional to the concentration (quantity) of the analyte in the sample.

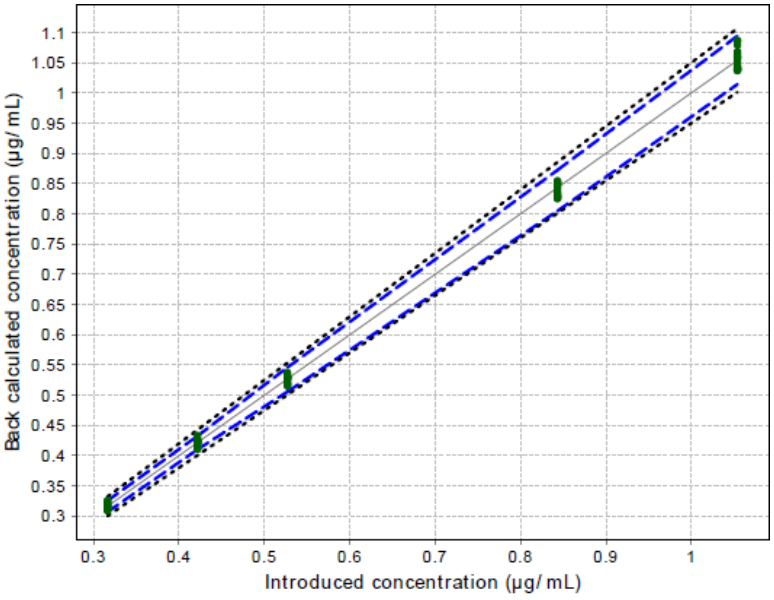
A linear regression model is fitted on the back-calculated concentrations as a function of the introduced concentrations

Linearity

Linearity graph for TOB



Linearity graph for CLR



Range - Limit of quantitation (LOQ)

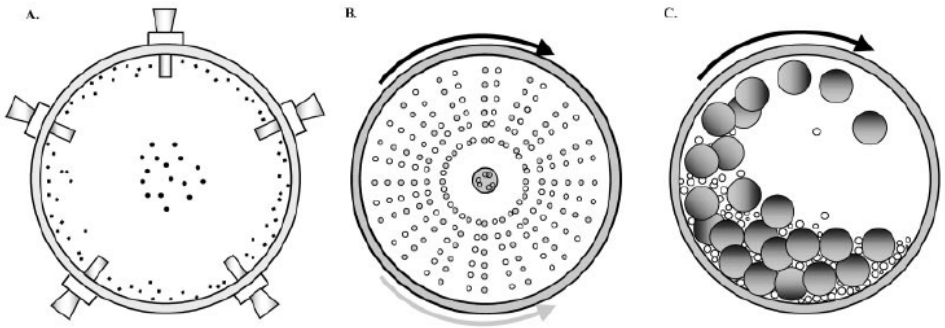
= the range between the lower and upper concentration limits (including these limits) for which it has been demonstrated that the analytical procedure has a suitable level of accuracy (trueness + precision) and linearity.

For TOB : 2.9 µg/mL to 9.8 µg/mL
For CLR : 0.32 µg/mL to 1.05 µg/mL

=> Method validated for these ranges of concentration for TOB and CLR!

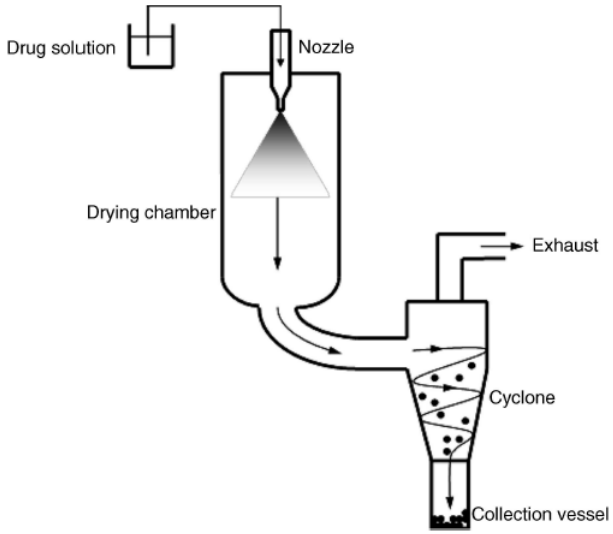
Micronized drug particles

Top-down process
= Breaking solid materials into smaller pieces
Eg: Milling

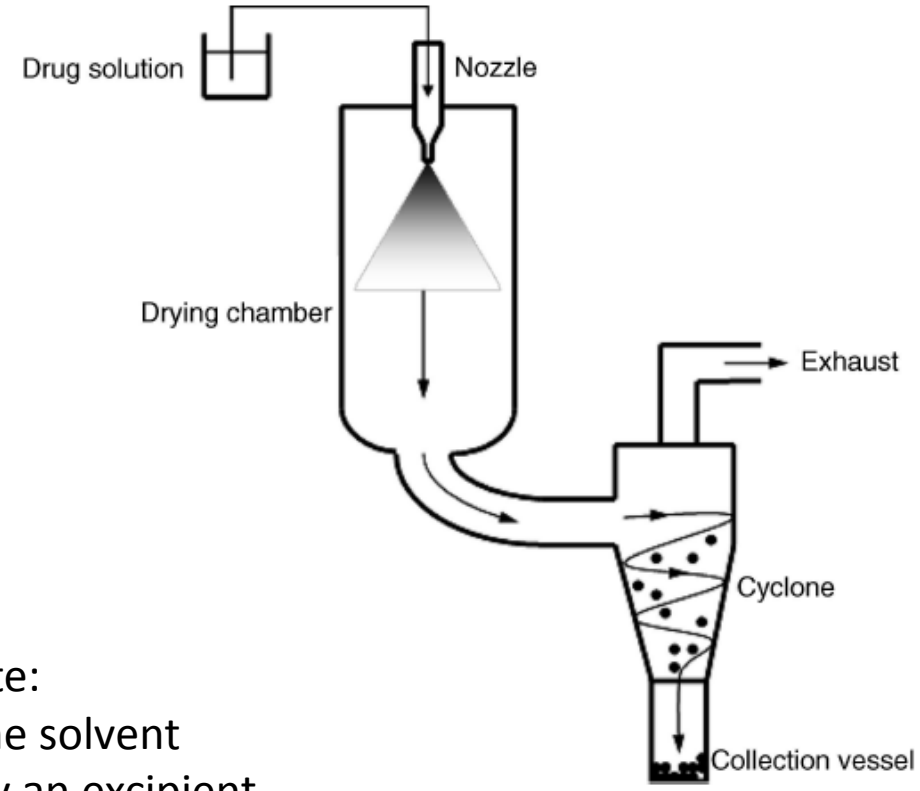


A: Jet mill. B: Pin mill. C: Ball mill.

Bottom-up process
= Particle size increase
Eg: Spray-drying



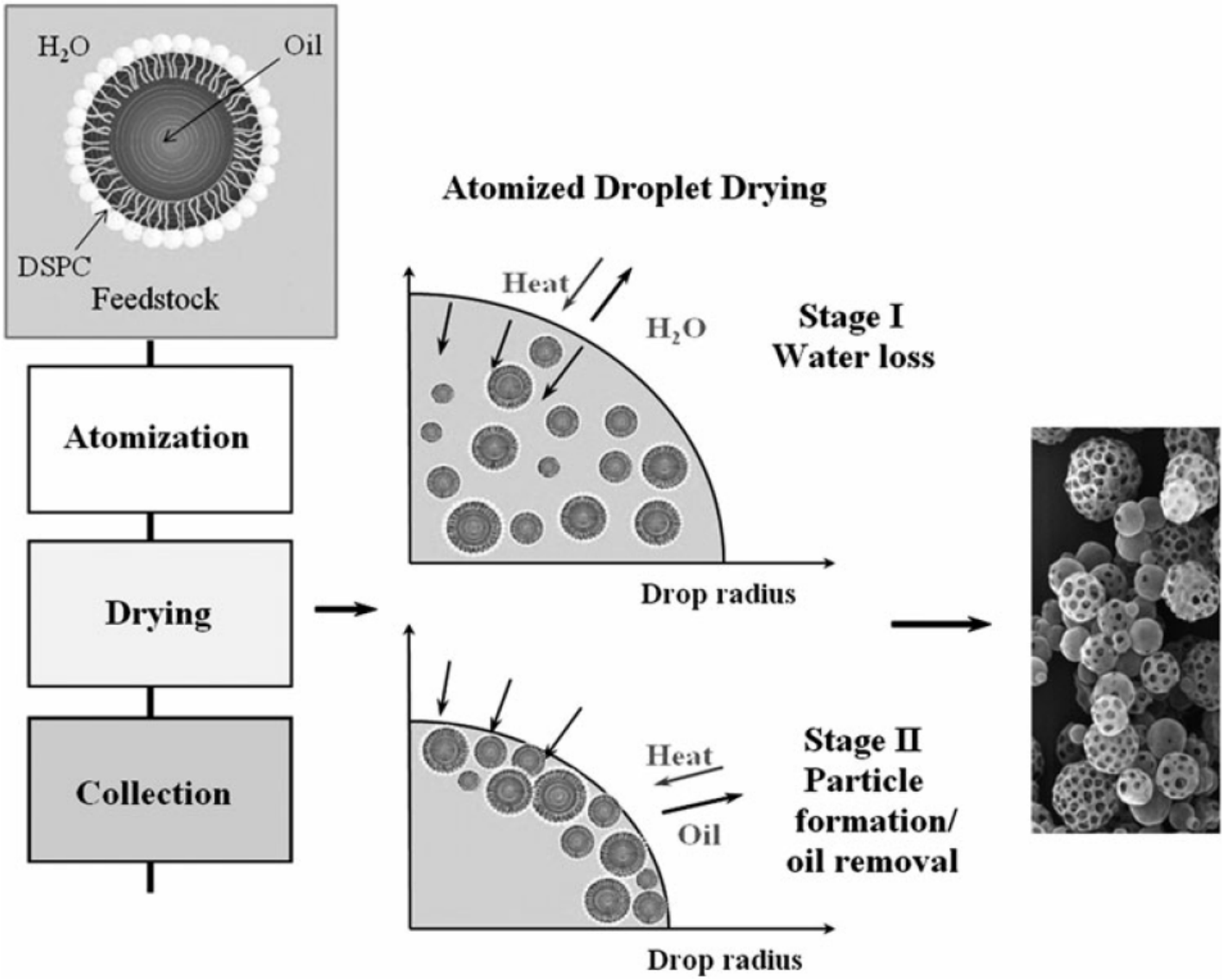
Spray-drying process



Parameters to manipulate:

- the composition of the solvent
- coating of particles by an excipient
- solute concentration
- solution feed rate
- gas feed rate
- drying rate
- Viscosity of the liquid feed
- relative humidity

Tobramycin inhalation powder: TOBI



Tobramycin inhalation powder: TOBI

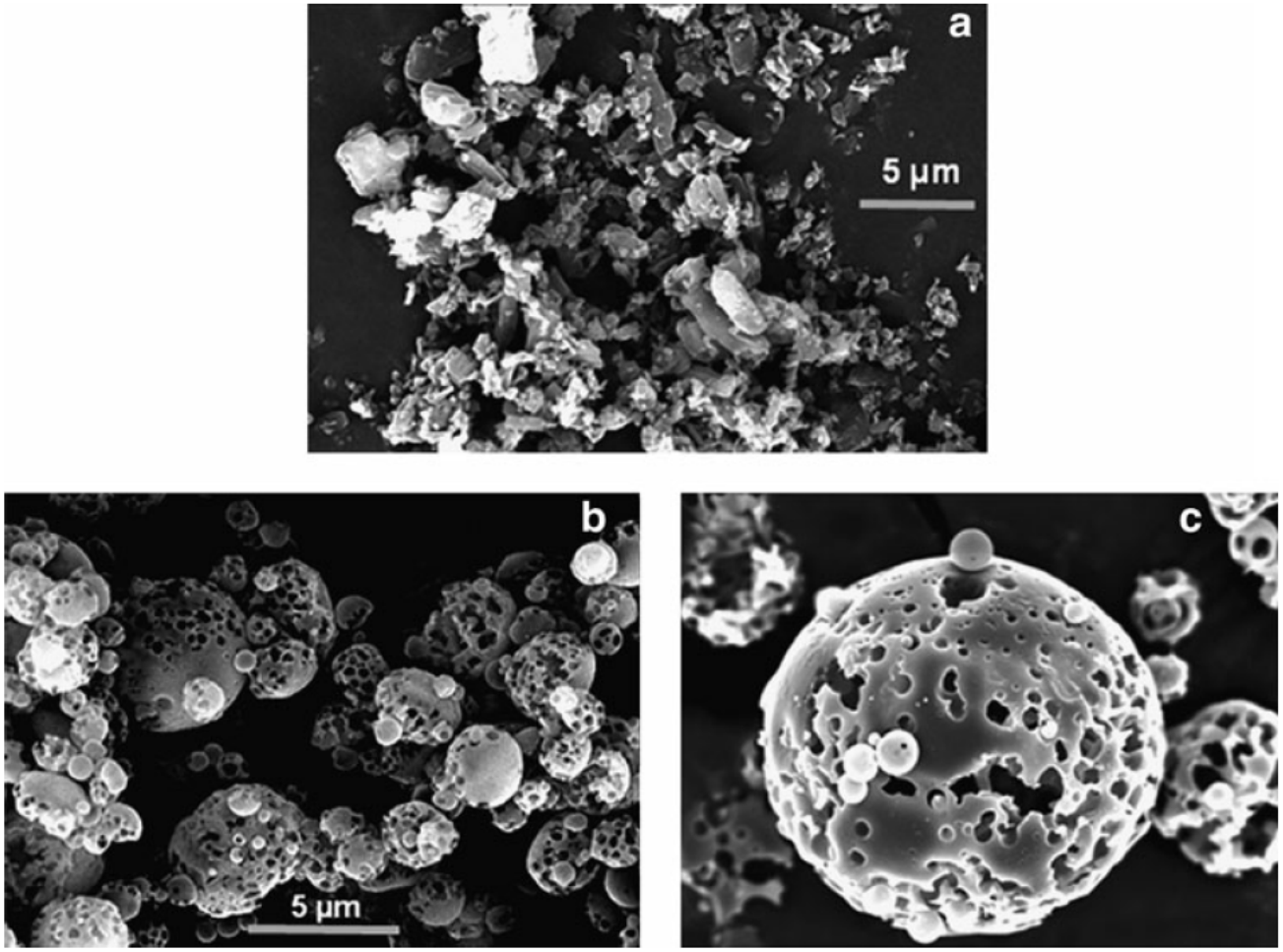
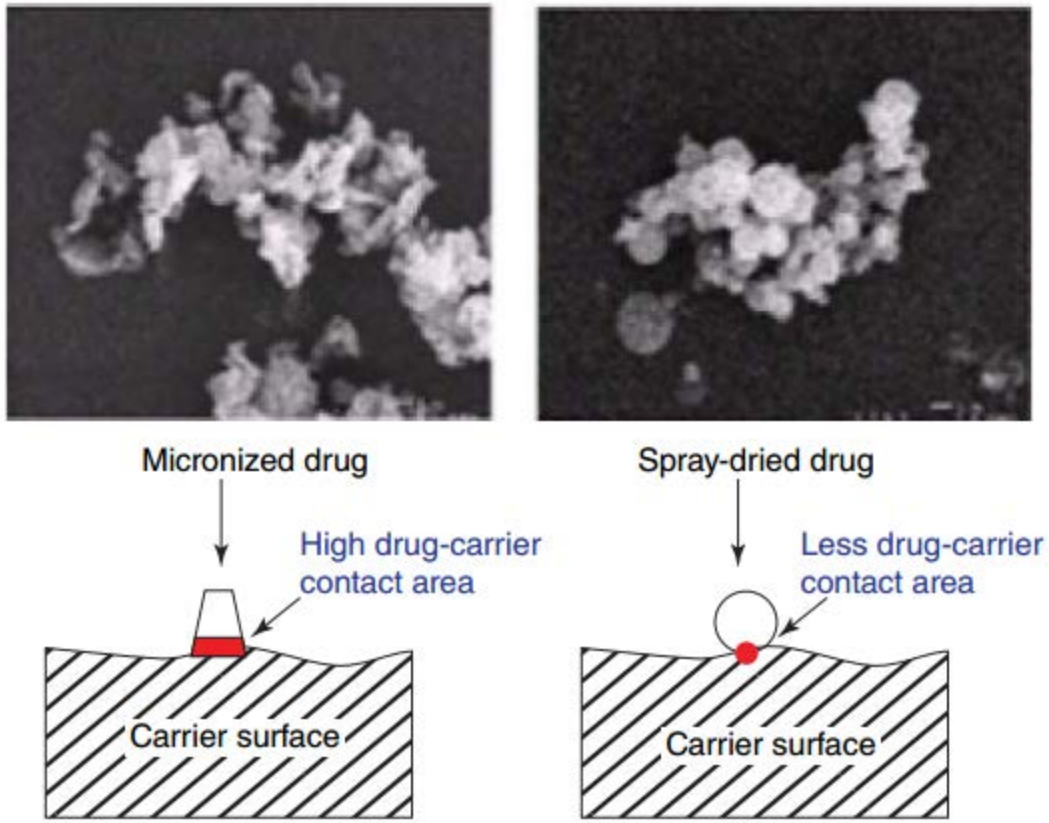


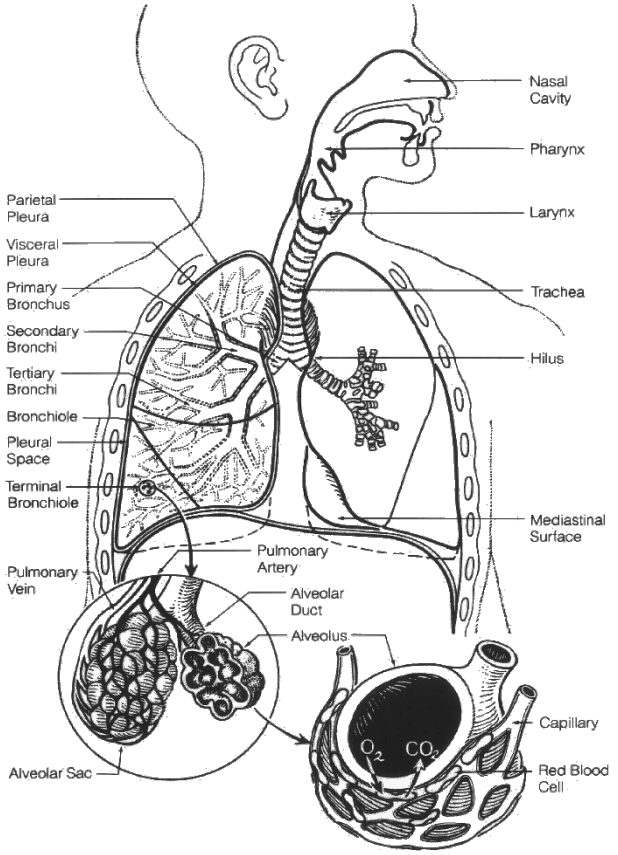
Figure 67 : Spray dryer MOBILE MINOR™ from GEA, Germany

Schematic representation of drug–carrier interactions of a micronized drug and a spray-dried drug



Louey et al. Pharmaceutical Research, 21(7), 1200–1206.

Pulmonary drug delivery



Local treatment:

- In situ
- Rapid onset of action
- Side effects

Systemic distribution

(Peptides and Proteins)

Bronchodilators

Anticholinergics (ipratropium, tiotropium...)

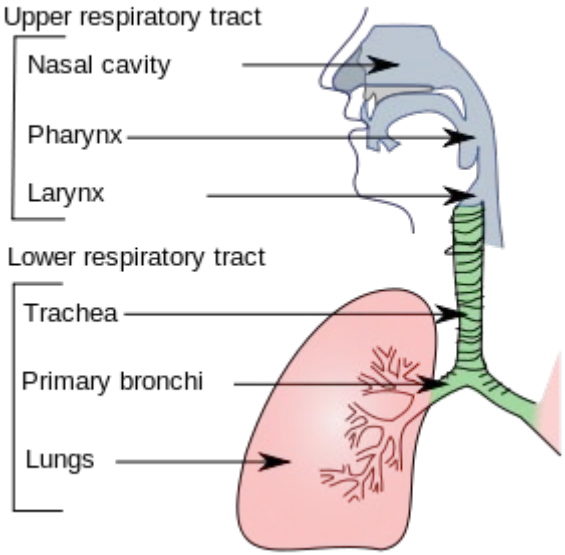
ß$_2$-mimétics (salbutamol, formoterol...)

Corticoids (budesonide, fluticasone...)

Mucolytics (acetylcysteine...)

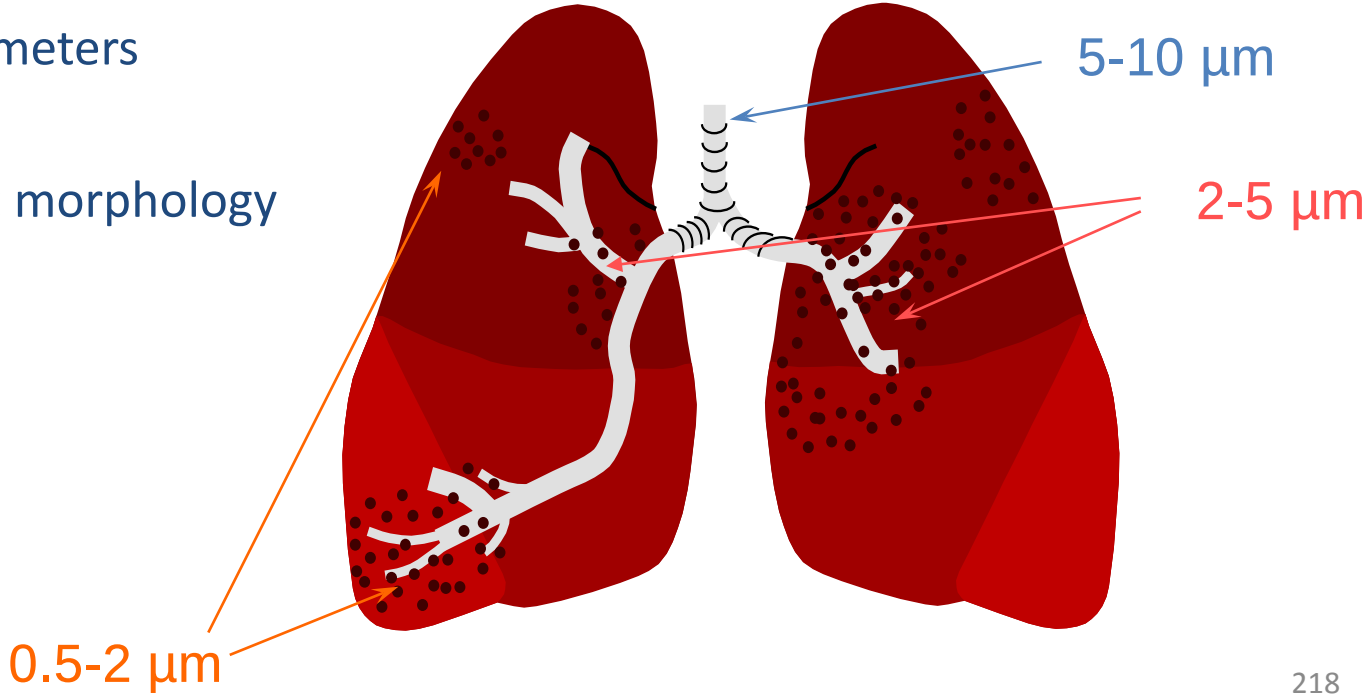
Antibiotics (tobramycin, gentamycin...)

Pulmonary Deposition



Parameters influencing the deposition of inhaled particles:

- Particle size (aerodynamic diameter)
- Ventilatory parameters
- Respiratory tract morphology



Pulmonary Deposition

Main Deposition mechanisms of inhaled particles:

- Inertial impaction

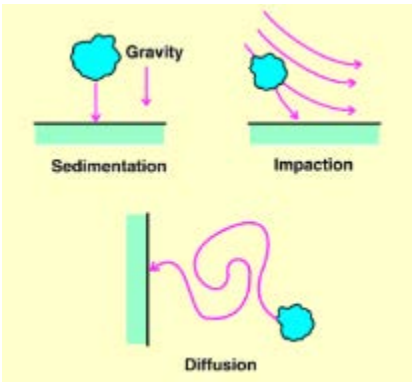
Larges conducting airways – big particles (>10 µm)

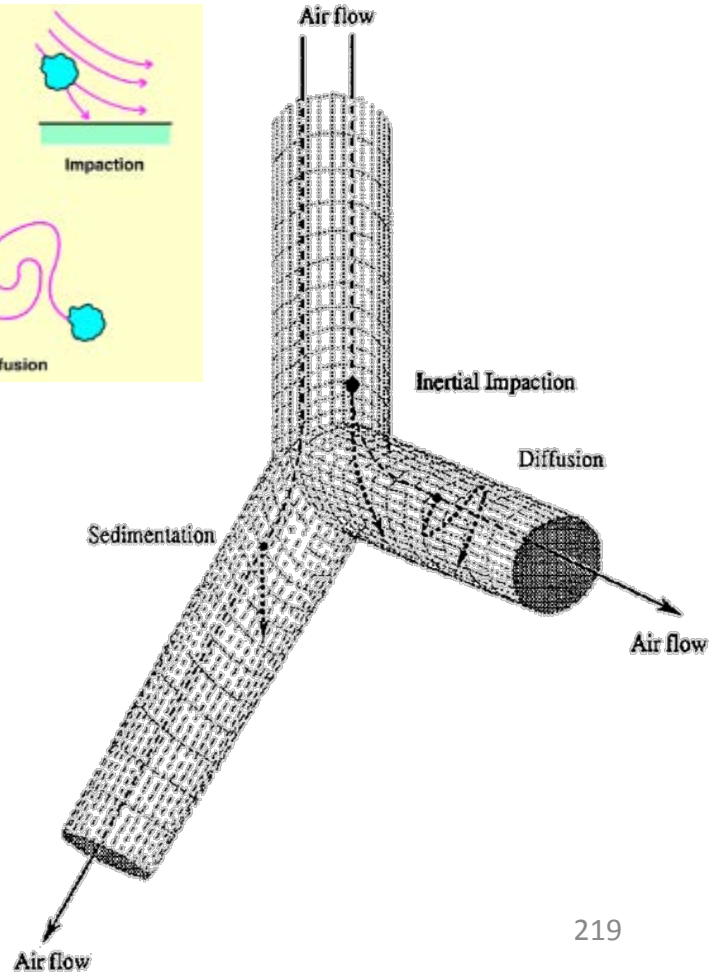
- Gravitational sedimentation

Small conducting airways – small particles (0.5-5 µm)

- Diffusion

Brownian motion in alveoli – submicrometer-sized particles (< 0.5 µm)





Pulmonary drug delivery

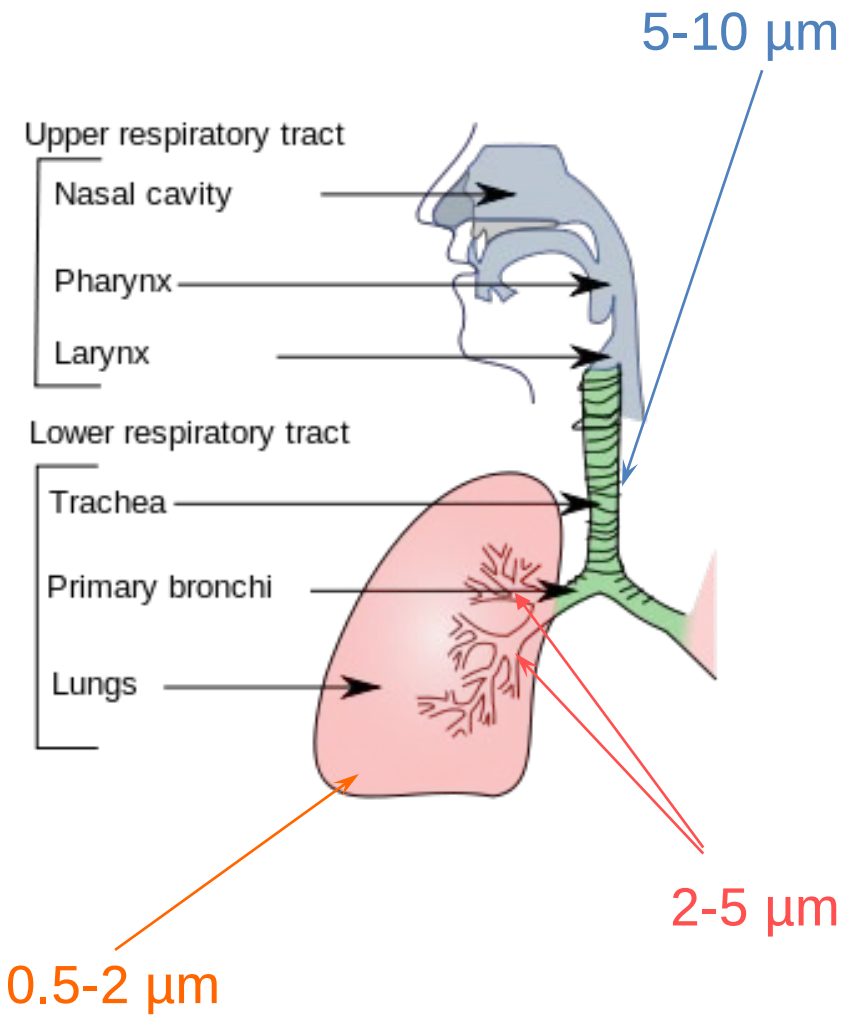
Local treatment:

- In situ
- Rapid onset of action
- Side effects ↘

Pulmonary deposition

Parameters influencing the deposition of inhaled particles:

- **Particle size (aerodynamic diameter)**
- Ventilatory parameters
- Respiratory tract morphology



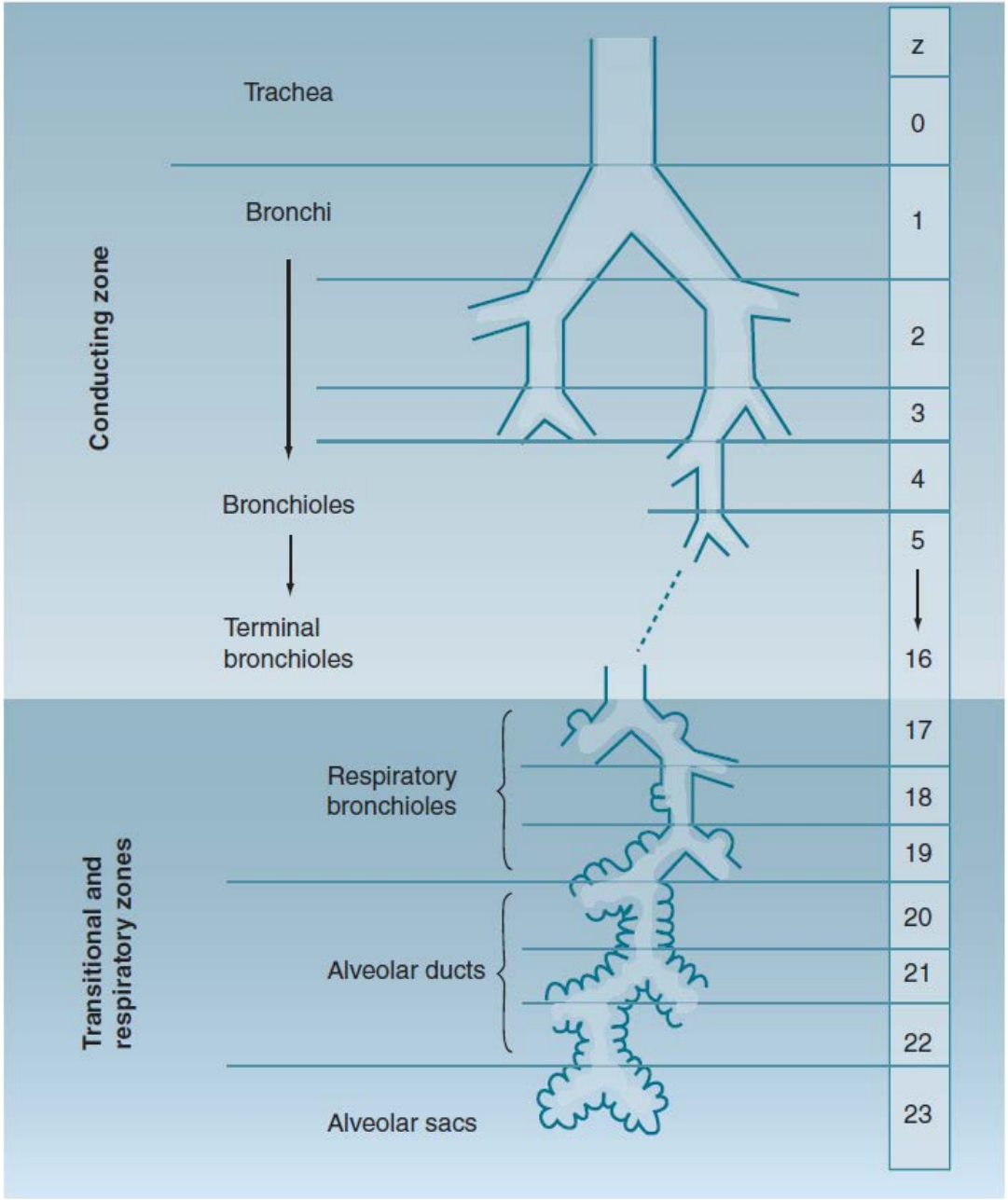


Figure 7. The conductive and respiratory zones of the lungs.

Formulation development : Objectives

- Develop dry powder formulations of TOB, CLA and Lactose (ratio of **10 : 1 : 10**)
- In a HPMC (Hydroxypropyl methylcellulose) capsule size 3 :

Dose 1
- 20 mg of TOB
- 2 mg of CLA
- 20 mg of Lactose

Dose 2
- 30 mg of TOB
- 3 mg of CLA
- 30 mg of Lactose

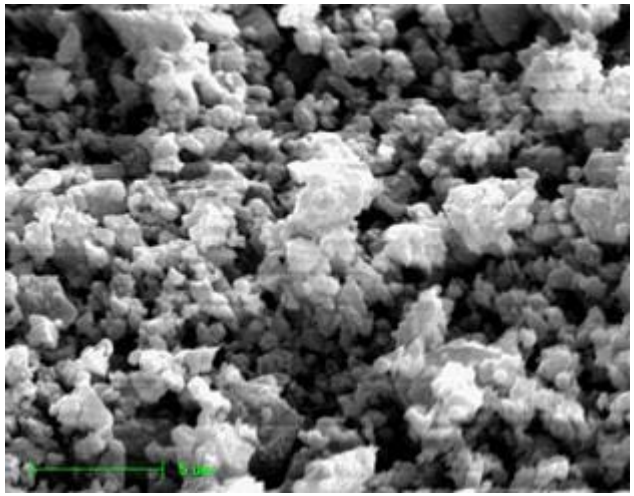
- To be administered by AXAHALER® 100 L/min

Formulation principles : main challenges

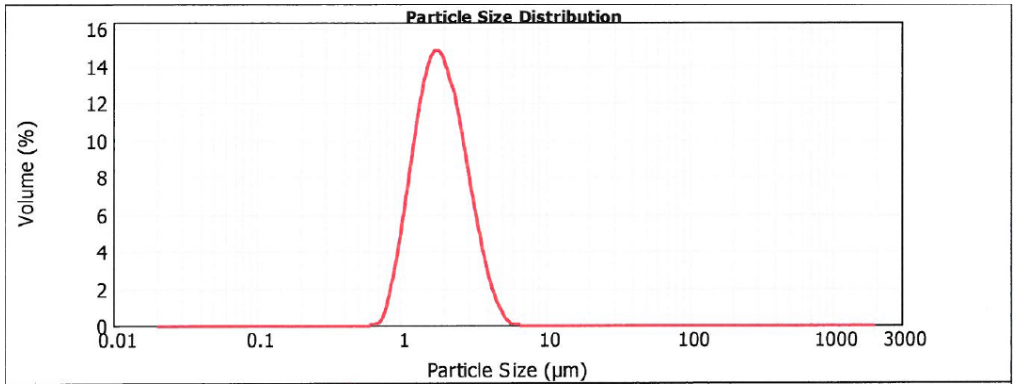
Micronized powders of TOB and CLR => cohesive nature!

- Poor flowability
- Limits dispersibility into aerosol cloud

=> Poor product performance!

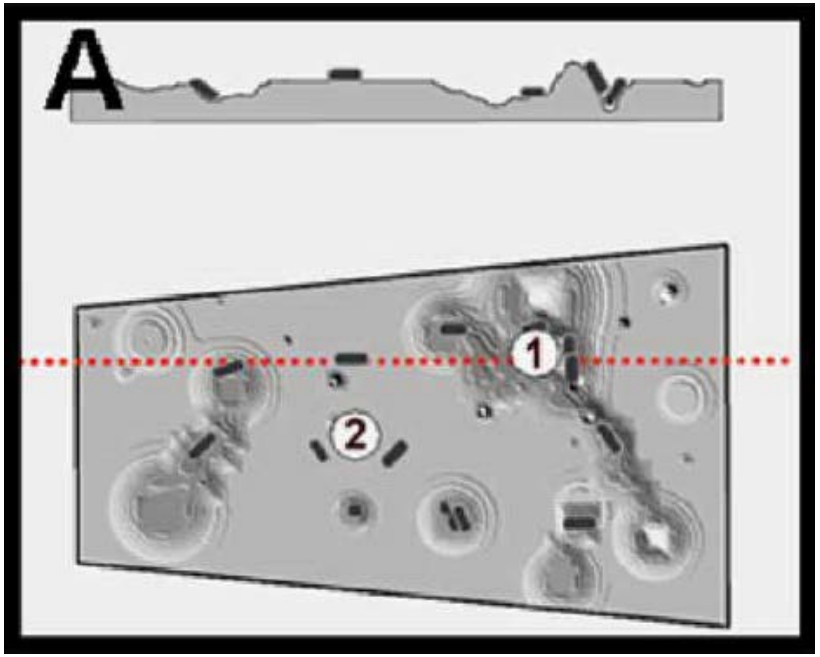


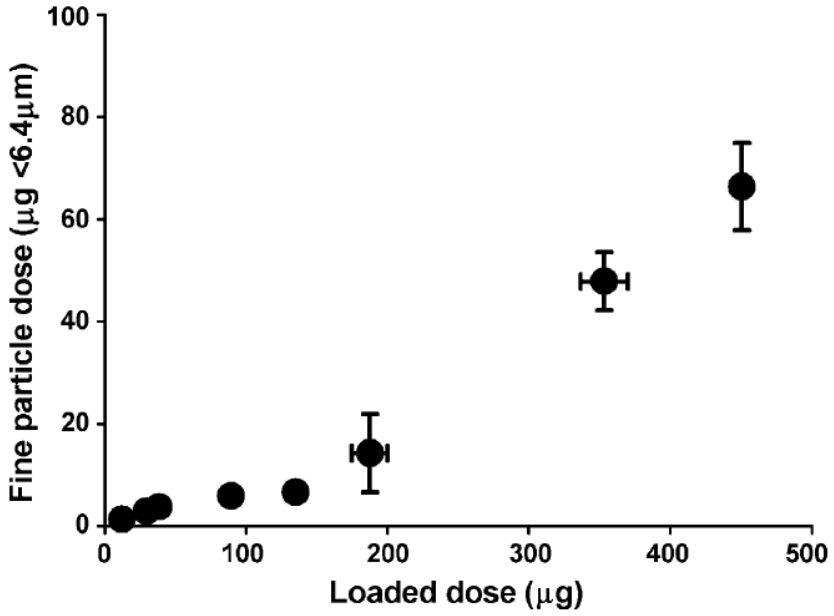
tobramycine micronisée 5000x



Formulation development : Why micronized lactose?

- Active sites = cleft/pit = high adhesion potential
  => drug particles adhered to these areas will thus be more difficult to remove

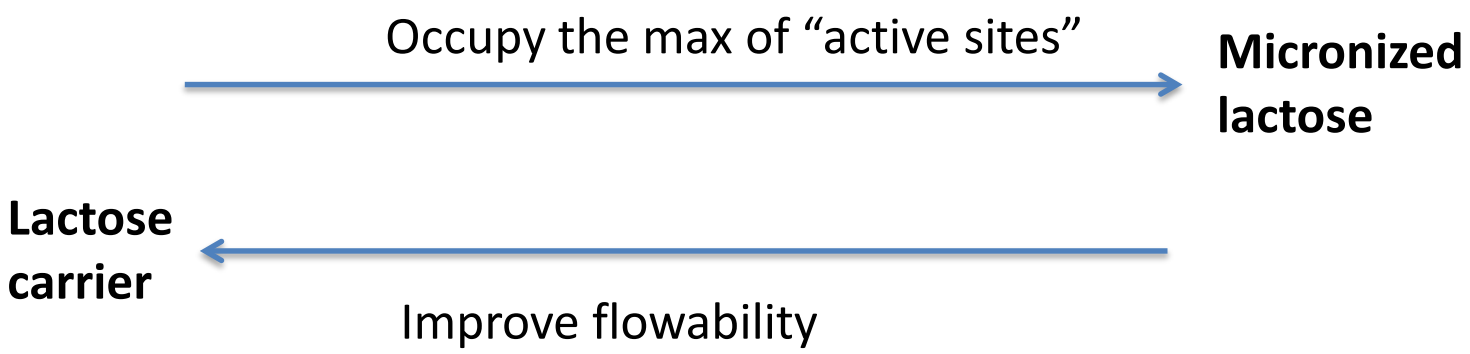




P.M. Young et al. The influence of dose on the performance of dry powder inhalation systems, Int. J. Pharm. 296 (2005) 26–33.

Formulation development :
Use of force control agent

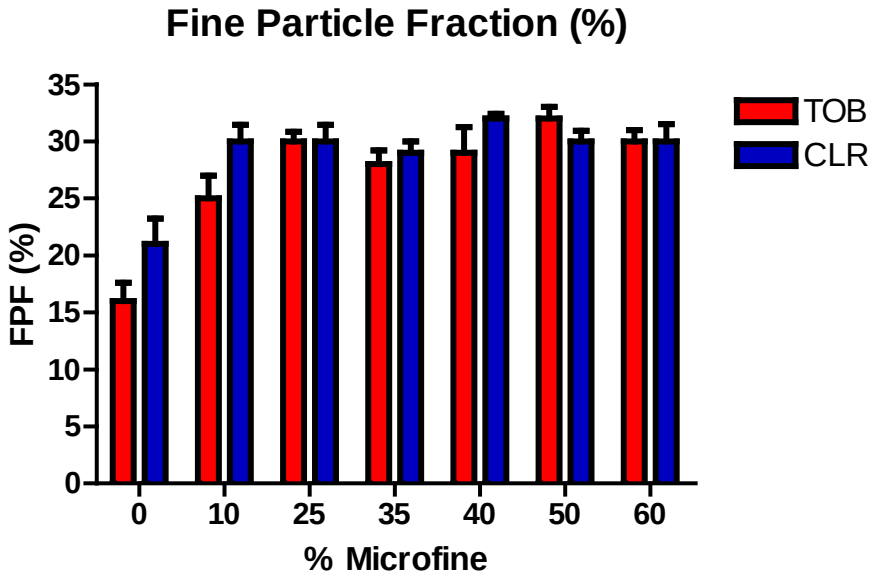
- Enhance aerosol performance using a force control agent (micronized lactose) => pre-treatment of lactose carrier with the agent => **optimal ratio of lactose carrier and micronized lactose**



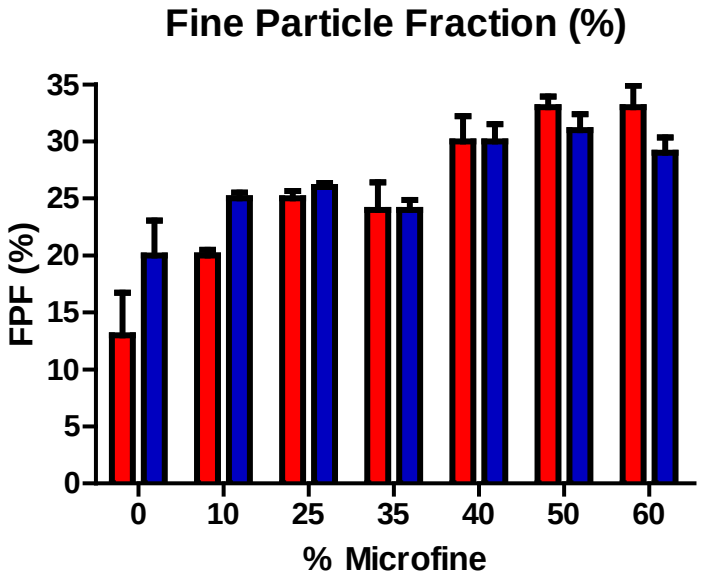
Formulation development :
Results

Dose 1
- 20 mg of TOB
- 2 mg of CLA
- 20 mg of Lactose

Dose 2
- 30 mg of TOB
- 3 mg of CLA
- 30 mg of Lactose



Hausner ratio/ Flow character	1.11 Excellent	1.12 Good	1.12 Good	1.13 Good	1.16 Good	1.17 Good



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Formulation development :
Results

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- 20 mg of TOB
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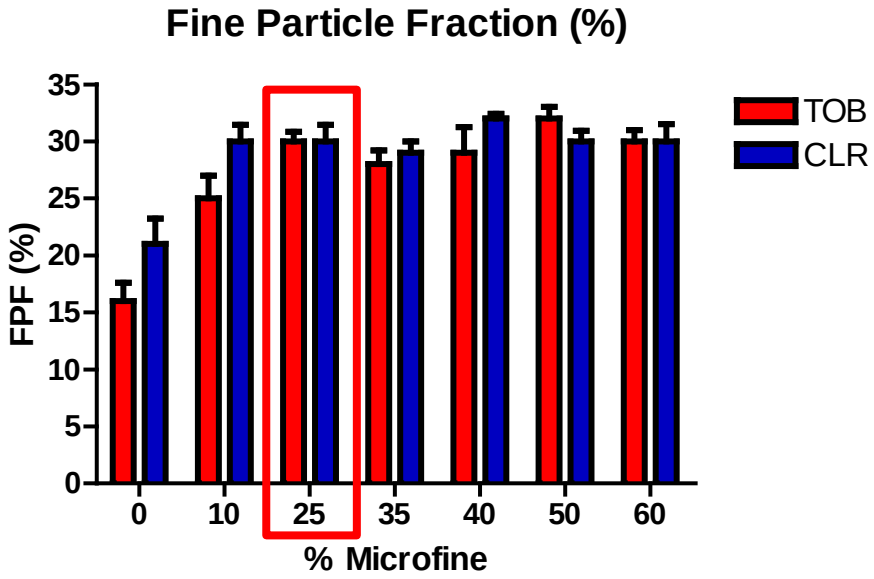
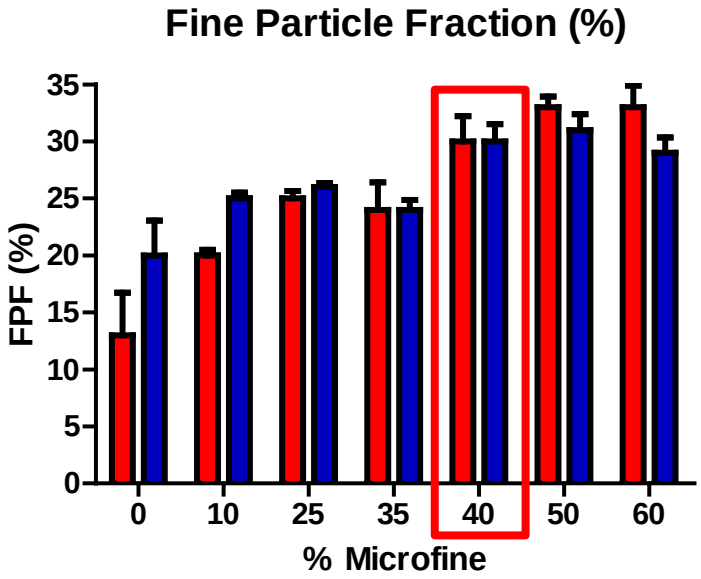
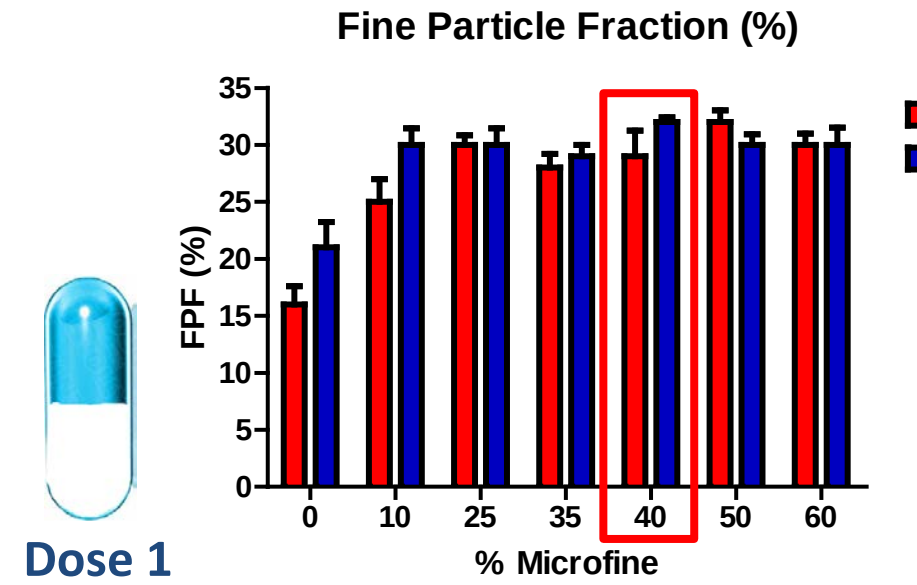


Hausner ratio/ Flow character	1.11 Excellent	1.12 Good	1.12 Good	1.13 Good	1.16 Good	1.17 Good

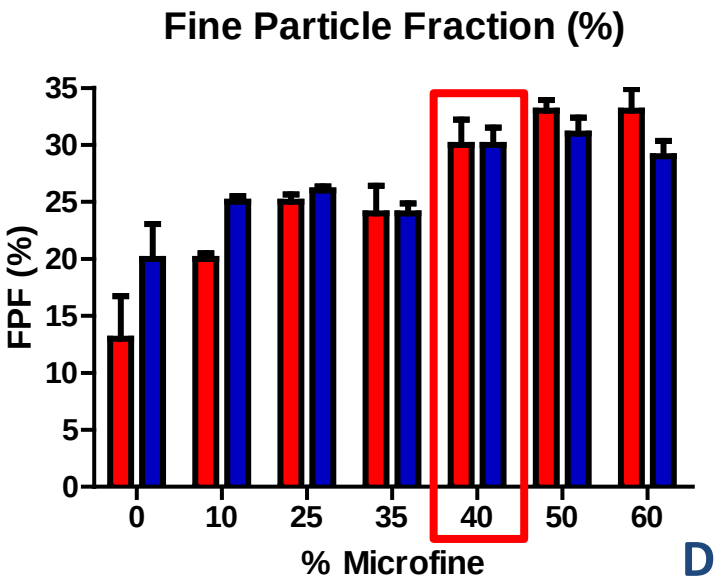


1.11 Excellent	1.12 Good	1.12 Good	1.13 Good	1.16 Good	1.17 Good

Process scale-up for production of pilot batches



Dose 1

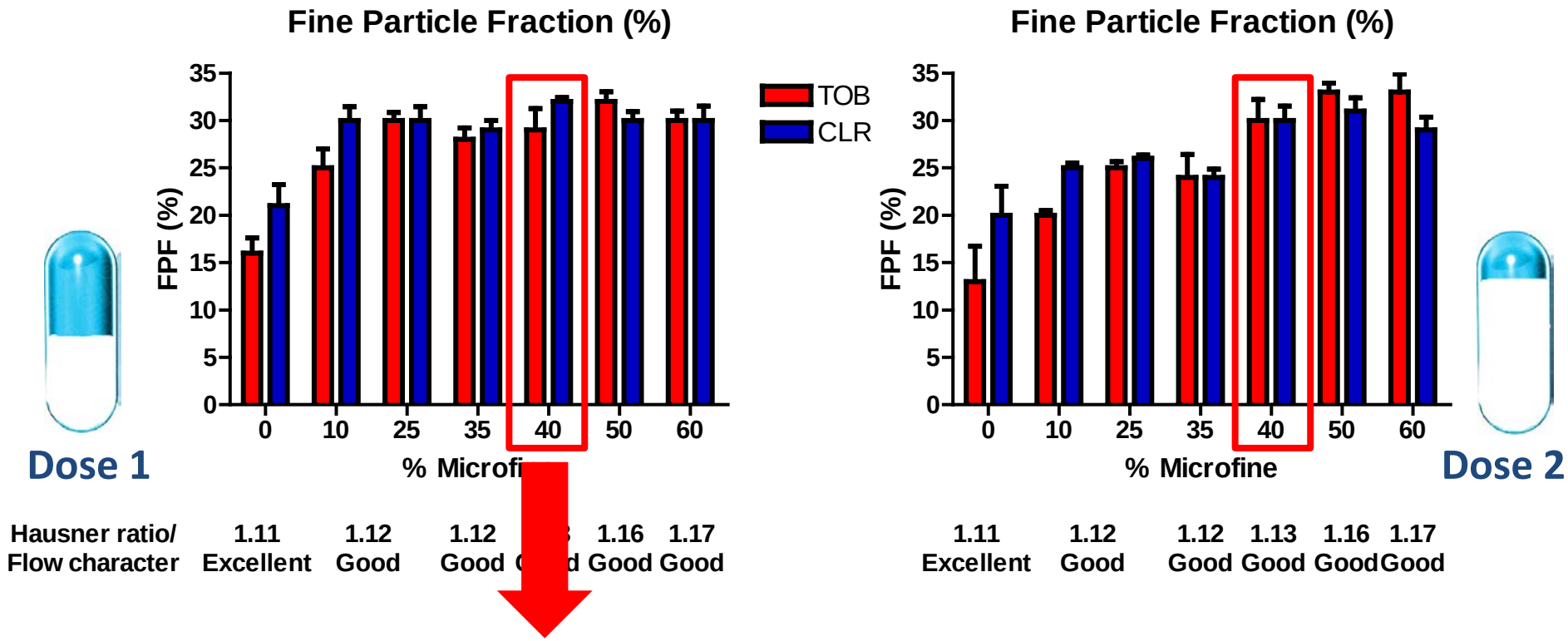


Dose 2

Hausner ratio/ Flow character	1.11 Excellent	1.12 Good	1.12 Good	1.13 Good	1.16 Good	1.17 Good

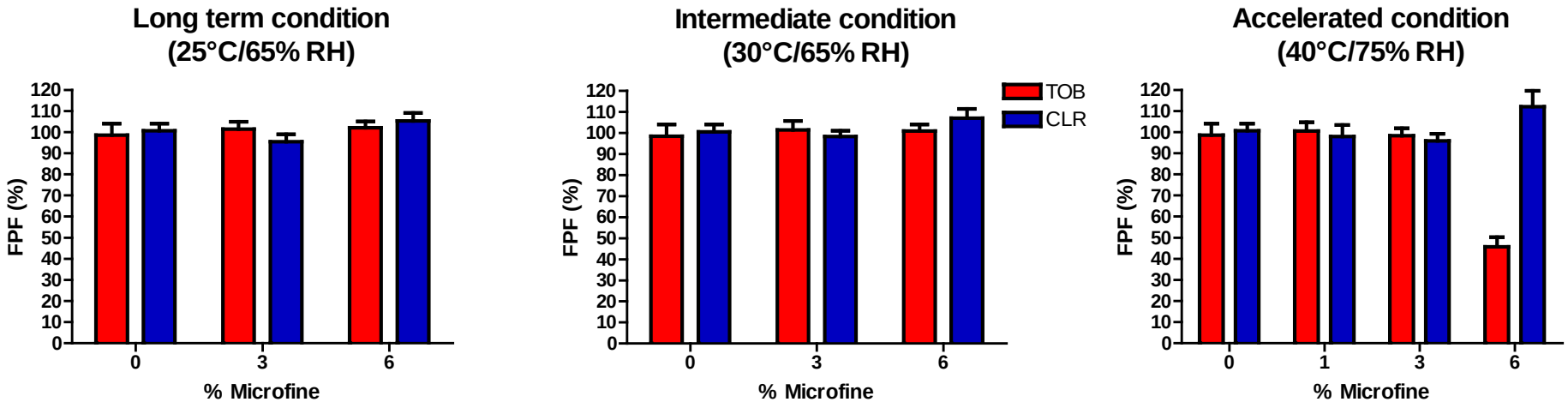
1.11 Excellent	1.12 Good	1.12 Good	1.13 Good	1.16 Good	1.17 Good

Process scale-up for production of pilot batches



Stability studies of M25 through 6 months : drug quantification

Drug quantification (% over theoretical value)



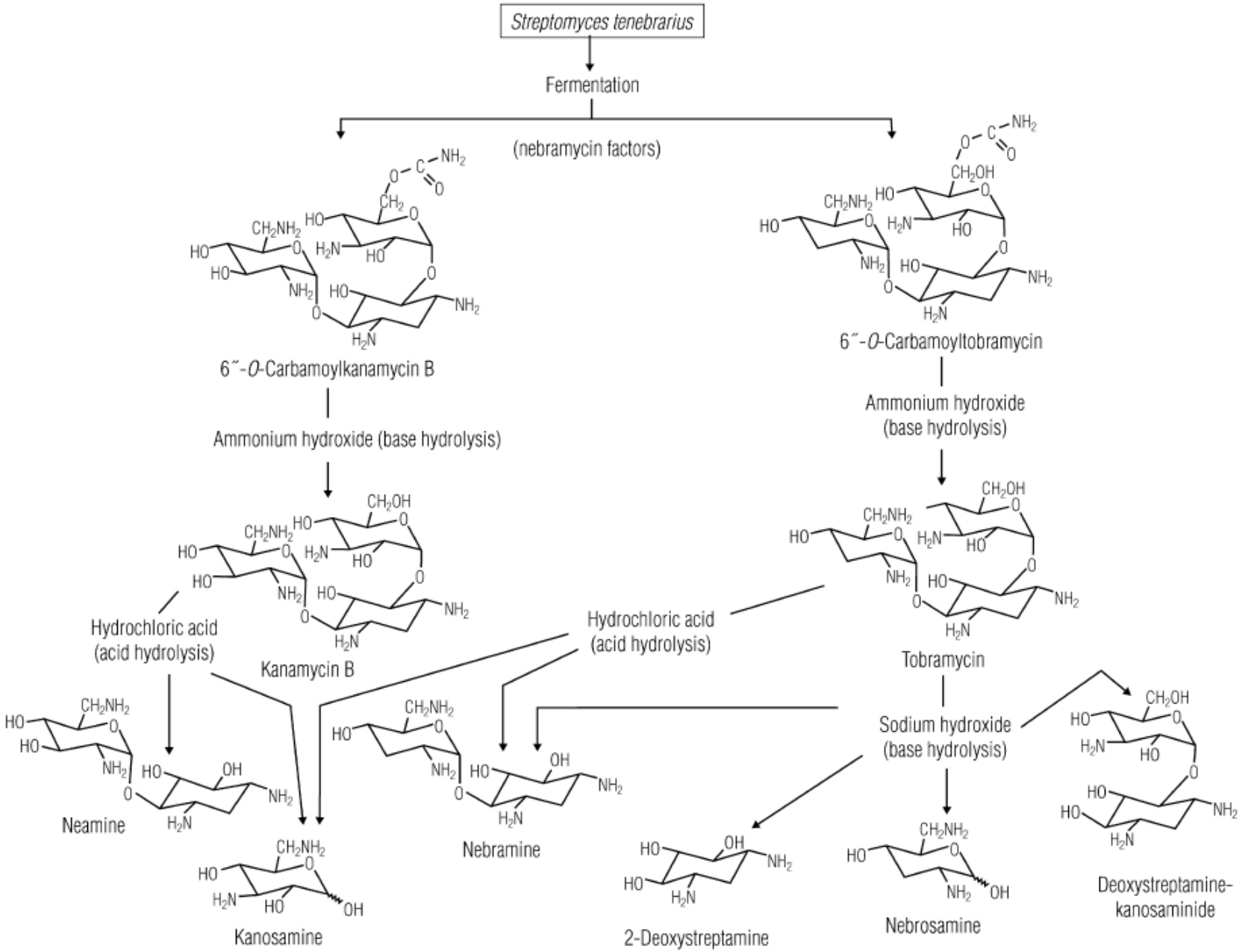


Fig. 1. The chemical structures of tobramycin, process intermediates, and degradation products.

Delivery Devices

- Nebulizers
- Metered Dose Inhalers (MDI)
- Dry Powder Inhalers (DPI)
    - Combine powder technology with device design in order to disperse dry particles as an aerosol in the patient's inspiratory airflow
    - Unit-Dose or Multi-Dose Devices
    - Four basic features:

        A dose-metering mechanism

        An aerolization mechanism

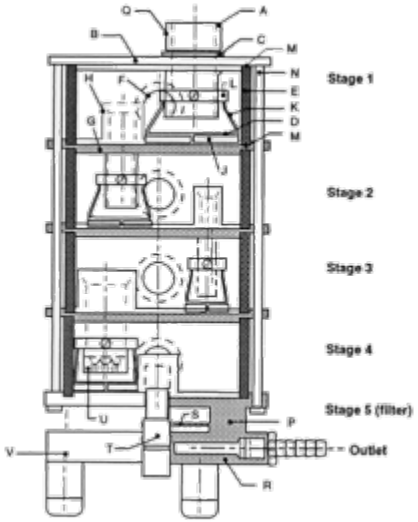
        A deaggregation mechanism

        An adaptator to direct the aerosol into the patient's mouth

Impactors

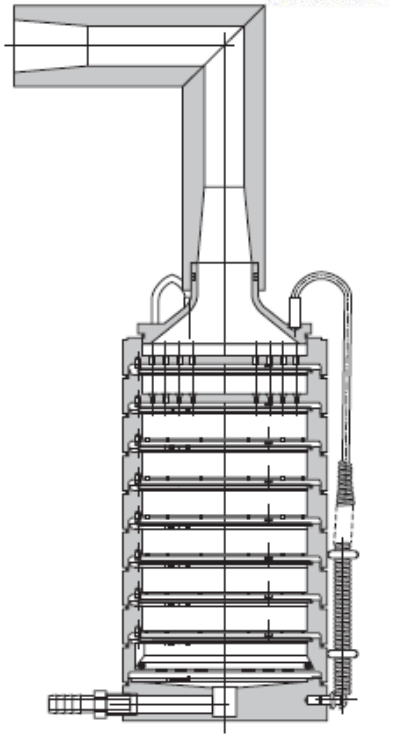
Ph. E : *This test is used to determine the fine particle characteristics of the aerosol clouds generated by preparations for inhalation.*

- Multi-Stage Liquid Impinger (MLI)



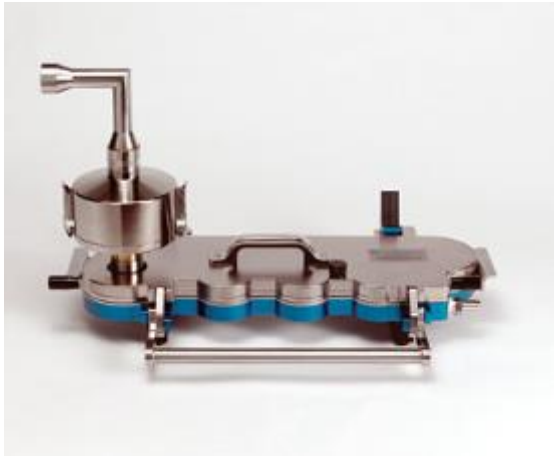
Impactors

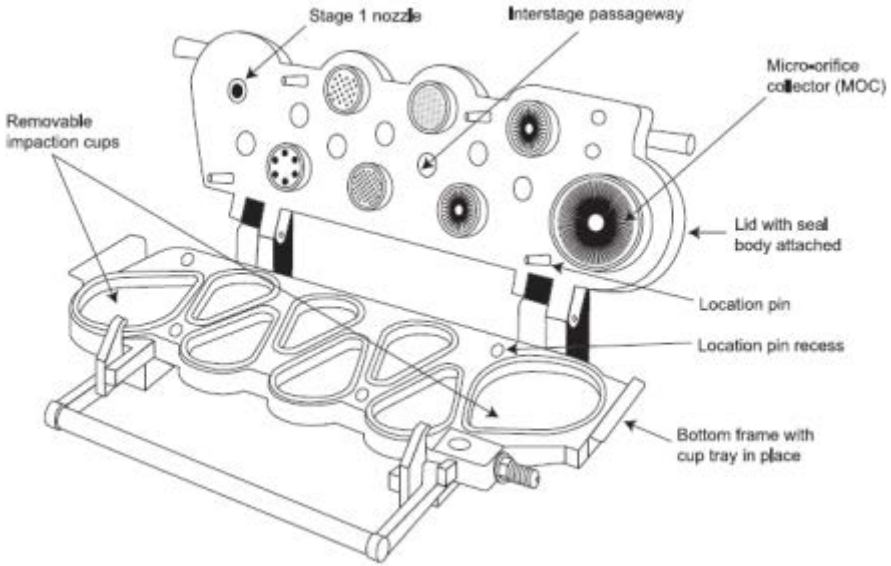
- Anderson Cascade Impactor



Impactors

- Next Generation Impactor





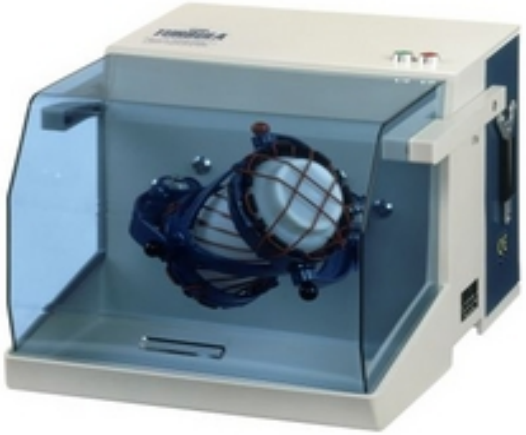
Impactors

- FPD => FPF
- Group results according to different aerodynamic diameters => detailed properties

Fast Screening Impactor
=> FPD!

Different mixers used

1) Planetary mixer



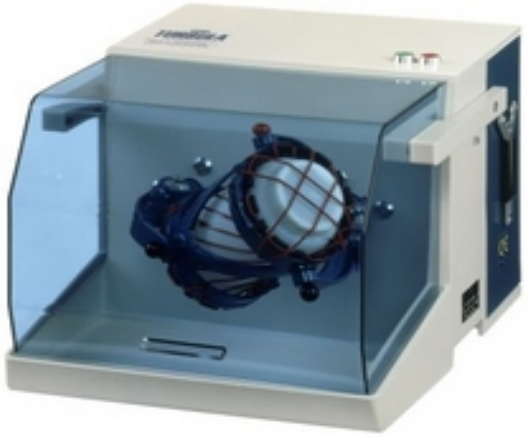
2) Turbula

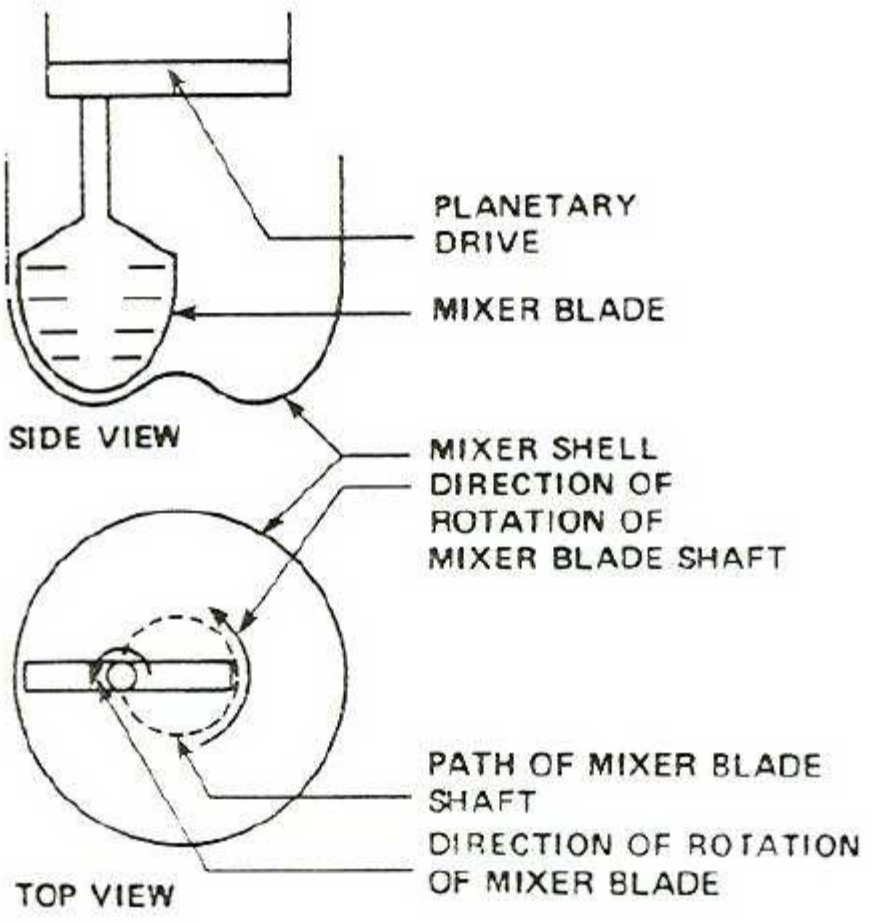
3) High-shear mixer

Different mixers for formulations

- Planetary mixer
  - uniformity is achieved by the movement of the planetary mixer blades
  - intense circulation => destruction of the agglomerates of cohesive powder.

- Turbula
  - three-dimensional motion
  - rotation, translation and inversion as per the geometric theory according to Schatz.





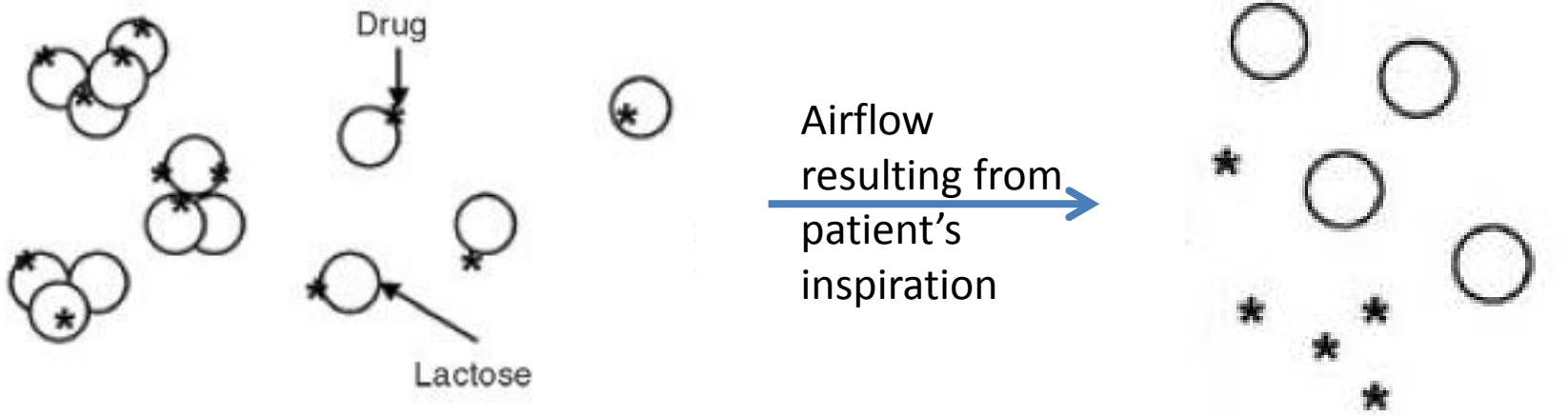
Représentation schématique d'un mélangeur planétaire [175].

Different mixers for formulations

- High-shear mixer
  - High shear force (impeller and chopper)

Formulation development : main challenges

- Lactose as a carrier (Respitose®)



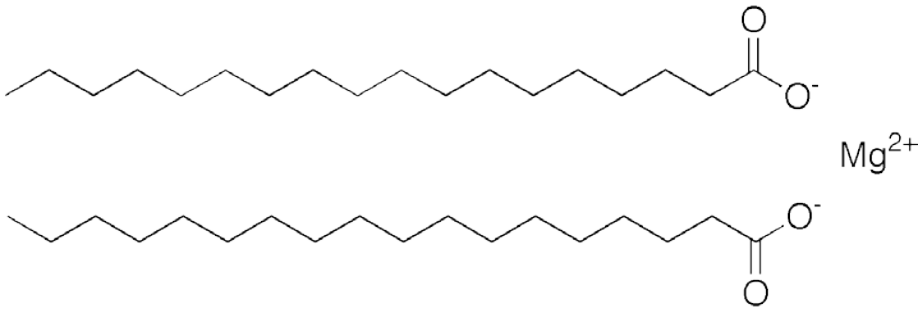
• Drug particles need to be sufficiently attracted to the carrier during mixing to support blend homogeneity, device filling and formulation stability.

• Yet the active ingredient must be readily detached from the carrier upon inhalation to form a fine particle cloud.

→ Balance of inter-particulate forces

Formulation principles

- Addition of Magnesium Stearate (MgSt) as a lubricant



Advantage :
Help flowability and handling during manufacture

Used in pharmaceutical tablets (0.2% to 5% in composition)
Approved for usage in DPI (BREO ELLIPTA, GSK) (1% of MgSt)

Addition of MgSt

The Influence of Force Control Agents on the Cohesive-Adhesive Balance in Dry Powder Inhaler Formulations†

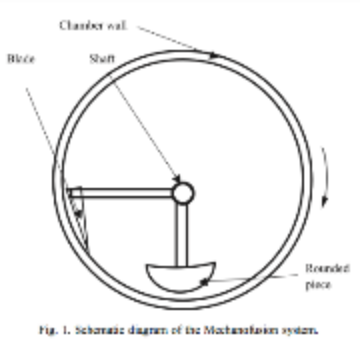


Fig. 1. Schematic diagram of the Mechanofusion system.

P. Begat and R. Price[1]
Pharmaceutical Technology Research Group
Department of Pharmacy & Pharmacology
University of Bath••
H. Harris, D.A.V. Morton
and J.N. Staniforth
Vectura Ltd.••••

KONA 23:109–121 (2005)

- Preconditioning of MgSt with drugs (using Mechanofusion) before addition of lactose

  => homogenous blend, better delivery

Formulation development : parameters monitored

1. **Homogeneity** => homogenous blend => **Uniformity of delivered dose**
2. **Flowability** => Measure of tapped density => calculate Hausner ratio, $H = \rho_T/\rho_B$

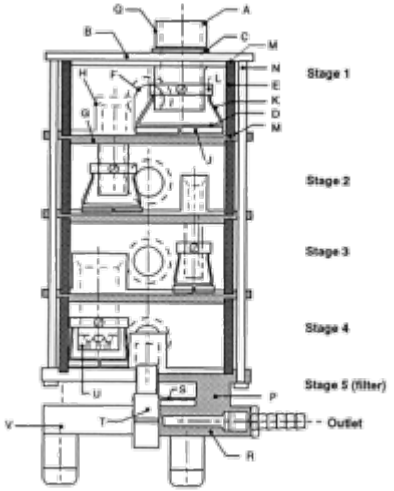
Hausner ratio	Flow character
1.00-1.11	Excellent
1.12-1.18	Good
1.19-1.25	Fair
1.26-1.34	Passable
>1.35	Poor to very poor

(European Pharmacopeia, Chapter 2.9.36)

Formulation development : parameters monitored

1. **Homogeneity** => homogenous blend => **Uniformity of delivered dose**
2. **Flowability** => Measure of tapped density => calculate Hausner ratio, $H = \rho_T/\rho_B$
3. **Pulmonary deposition** => Aerodynamic assessment of fine particles **=> Impactors!**

Multi-Stage Liquid Impinger (MLI)



Formulation development : parameters monitored

1. **Homogeneity** => homogenous blend => **Uniformity of delivered dose**
2. **Flowability** => Measure of tapped density => calculate Hausner ratio, $H = \rho_T/\rho_B$
3. **Pulmonary deposition** => Aerodynamic assessment of fine particles

- Fine particle dose (FPD) = mass of the particle with $d_a < 5$ µm (in mg) => pulmonary deposition!

- **Fine particle fraction (FPF)** = ratio of FPD to the total nominal dose (in %)

1) Homogeneity

- Homogenous blend => **Uniformity of delivered dose**
- 10 samples of the formulation powders were taken for which the exact weights were noted (around 42mg, equivalent to the filling weight in a capsule).
- These samples are analyzed by the analytical method above for their TOB and CLR content.

2) Flowability

- Measure of tapped density => calculate Hausner ratio, **H = ρB/ρT**

Hausner ratio	Flow character
1.00-1.11	Excellent
1.12-1.18	Good
1.19-1.25	Fair
1.26-1.34	Passable
>1.35	Poor to very poor

(European Pharmacopeia, Chapter 2.9.36)

3) Pulmonary deposition : Aerodynamic assessment of fine particles

- Fine particle dose (FPD) = mass of the particle with $d_a < 5$ µm (in mg) => pulmonary deposition!

  d_a = aerodynamic diameter = diameter of the spherical particle with a density of 1000 kg/m3 and the same settling velocity as the irregular particle

- Fine particle fraction (FPF) = ratio of FPD to the total nominal dose (in %)

  total dose of drug prescribed

How do we measure FPD? => Impactors!

TOBI® Podhaler® : Reference treatment

TOBI® Podhaler™ Is Designed for Patients on the Go

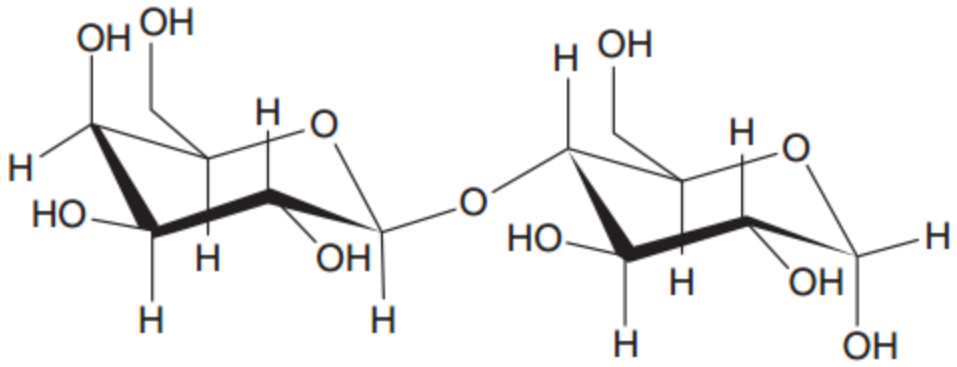
Each TOBI Podhaler 28-day pack provides everything needed for one treatment cycle

28 mg of TOB per capsule

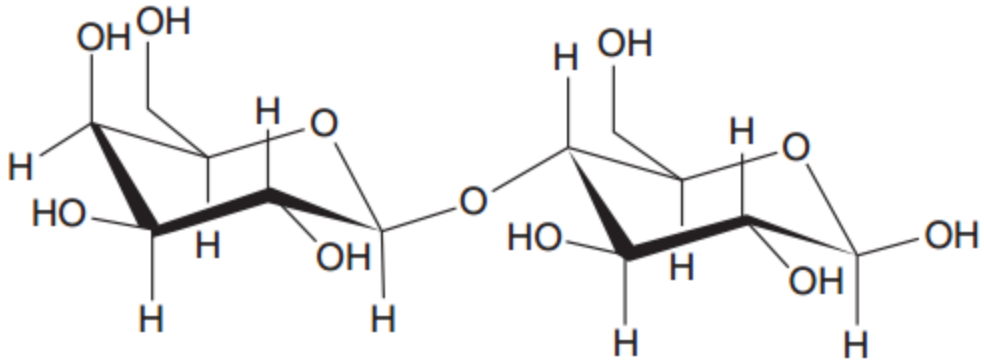
*Four capsules for inhalation in the morning and 4 capsules for inhalation in the evening.

http://www.tobipodhaler.com

Approved by FDA in March 2013



α-Lactose



β-Lactose

Figure 7.2 *The anomers of lactose molecule*

RESUME

Treatment	Species	Estimated delivered dose (mg/kg/day)	Daily administration	Necropsy at	Toxicity	
Tobra/Clari	rats	17.8/1.7	28 days	Day 29	Dose dependent lesions in the respiratory tract and kidney	Females more affected. Partial recovery after 28 days.
Tobra/Clari	rats	49.6/5.2	28 days	Day 29		
Tobra/Clari	rats	154.8/13.5	28 days	Day 29		
Tobra	rats	164	28 days	Day 29		
Clari	rats	15	28 days	Day 29	Well tolerated	
Tobra/Clari	dogs	59.9/4.6	28 days	Day 29	Dose dependent lesions in the respiratory tract and kidney	Partial recovery after 28 days.
Tobra/Clari	dogs	116.5/8.80	28 days	Day 29		
Tobra/Clari	dogs	228.8/17.9	28 days	Day 29		Premature death females.
Tobra	dogs	224.9	28 days	Day 29		
Clari	dogs	21	28 days	Day 29	Well tolerated	