*Streptococcus pneumoniae* infections in a context of Chronic obstructive pulmonary disease (COPD)

Study of the factors contributing to the recurrence of the disease

Thesis public defense

Nathalie M. Vandevelde

Supervisor: Professor Françoise Van Bambeke

- Brussels, October 13, 2014 -
But ....

What is Chronic Obstructive Pulmonary Disease (COPD) ?

What are bacterial Acute Exacerbations of COPD ?

What is Streptococcus pneumoniae ?
INTRODUCTION - Chronic Obstructive Pulmonary Disease or Chronic Bronchitis

• My first COPD patient...
  - man
  - 81 years old in 2010 (birth: 1929)
  - miner during 25 years
  - former smoker (stopped in 1990, total 45 UAP)
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- Chronic cough and lung inflammation

- Bronchial obstruction : ↓ 40% of the expiratory function (GOLD 2)

- 1-2 bacterial exacerbations/ year
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**Stage Spirometric FEV₁ measures**

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<td>I</td>
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<td>Moderate COPD: 50% ≤ FEV₁ &lt; 80% predicted</td>
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<td>Severe COPD: 30% &lt; FEV₁ &lt; 50% predicted</td>
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<td>Very severe COPD: FEV₁ &lt; 30% predicted</td>
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Stratification according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD, 2014)

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Lymphangitis (see Kerley lines)
(inflammation of the lymphatic vessels associated with carcinomal lesions and bacterial infection)
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- Hypertension (157/72 mmHg)

- Heart transplant (1992), cardiomegaly

- Hypercholesteloremia

- Moderate hyperglycemia (fasting glucose 139 mg/dl)

- Overweight (BMI: 33kg/m²)

- Chronic renal failure

- Cancer

**INTRODUCTION - Chronic Obstructive Pulmonary Disease or Chronic Bronchitis**

*Lymphangitis (see Kerley lines)*
(inflammation of the lymphatic vessels associated with carcinomal lesions and bacterial infection)

*Cardiomegaly (x/y > 0.5)*
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Cancerous mass (see lung upper left lobe)
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• Who?
  - Adults >50 years
  - Men > women
  - Long-term exposition to inhaled toxic substances
  - Bronchial obstruction
  - Chronic cough
  - Emphysema
  - Lung inflammation
  - Repeated bronchial infections
  - Cardiovascular diseases (~ 60-70%)
  - Cancer (~ 30%)
  - Diabetes (~ 20-30%)

Non-reversible decrease of the respiratory function

INTRODUCTION - Chronic Obstructive Pulmonary Disease or Chronic Bronchitis

My first COPD patient...
- man
- 81 years old in 2010 (born in 1929)
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- former smoker (stopped in 1990, total 45 UAP)

- Bronchial obstruction: ↓ 40% of the expiratory function (GOLD 2)
  => SHORT & LONG-ACTING BRONCHODILATORS

- Chronic cough and lung inflammation

- 1-2 bacterial exacerbations/ year
  => ANTIBIOTICS

- Hypertension (157/72 mmHg)
  => 3 ANTI-HYPERTENSIVE DRUGS /day

- Heart transplant (1992), cardiomegaly
  => 3 ANTI-REJECTION DRUGS

- Hypercholesteloremia
  => CHOLESTEROL-LOWERING DRUGS

- Moderate hyperglycemia (fasting glucose 139 mg/dl)
  => ANTI-DIABETIC DRUGS

- Overweight (BMI: 33kg/m²)

- Chronic renal failure

- Cancer

Who?
- Adults >50 years
- Men > women

- Long-term exposition to inhaled toxic substances

- Bronchial obstruction
- Chronic cough
- Emphysema
- Lung inflammation
- Repeated bronchial infections

- Cardiovascular diseases (~ 60-70%)
- Cancer (~ 30%)
- Diabetes (~ 20-30%)

- Highly polymedicated

Non-reversible decrease of the respiratory function

Take Home Message

INTRODUCTION - Chronic Obstructive Pulmonary Disease or Chronic Bronchitis

- Non reversible decrease of the respiratory function
- Frequent acute exacerbations (AECB)
- Comorbidities

Number of deaths per year worldwide:
- 1990: 2 millions
- 2000: 3.5 millions
- 2010: 5 millions
- 2020: 5 millions
- 2030: 5 millions
- 2040: 5 millions

3rd cause of mortality around the world

---

\(a\) Lundback et al, 2003, Respir Med 97: 115-122; \(b\) Minino and Murphy, 2012, NCHS Data 1-8
INTRODUCTION - Chronic Obstructive Pulmonary Disease or Chronic Bronchitis

Non reversible decrease of the respiratory function

Frequent acute exacerbations (AECB)

Comorbidities

Number of deaths per year worldwide: 2 millions in 1990, 3.5 millions in 2010, 5 millions in 2020, 2030, 2040.

INTRODUCTION - Acute Exacerbations of Chronic Bronchitis (AECB)

Bacterial etiology in 50 to 80% of cases

- S. pneumoniae (32%)
- nontypeable H. influenzae (34%)
- S. aureus (4%)
- E. coli (4%)
- K. pneumoniae (5%)
- M. catarrhalis (12.5%)
- typeable H. influenzae (7%)

**INTRODUCTION** - Acute Exacerbations of Chronic Bronchitis (AECB)

Bacterial etiology in 50 to 80% of cases

**Streptococcus pneumoniae** is the most predominant bacterial pathogen involved in AECOPD, regarding to all stages of COPD patients’ bronchial obstruction (GOLD 1 - 4)

- **S. pneumoniae** (32%)
- **M. catarrhalis** (12.5%)
- **typeable H. influenzae** (7%)
- **nontypeable H. influenzae** (34%)
- **K. pneumoniae** (5%)
- **S. aureus** (4%)
- **E. coli** (4%)


INTRODUCTION - *Streptococcus pneumoniae* or Pneumococcus

- **diplococci or small chains** (diameter: 0.5 - 1 µm)

- **Gram positive bacterium**

- **Several virulent factors**

---

A special life mode: growth within biofilms:

• tridimensional communities of cells embedded in a structured matrix

• adhering to inert/living surfaces

• protected from the immune system and antibiotics

• involve in up to 80% of chronic infections

Otitis media
Septicemia
Sinusitis
Meningitis
Pharyngitis
Laryngitis
Septicemia
Community acquired pneumonia (CAP)
Acute Exacerbations of Chronic Bronchitis (AECB)

INTRODUCTION - *Streptococcus pneumoniae* or Pneumococcus
INTRODUCTION - *Streptococcus pneumoniae* or Pneumococcus

- Commensal nasopharynx carriage in 5 - 10% of adults\(^a\)

- Mainly pathogenic, especially in >65 years, <2 years and immunocompromised patients\(^a\)

=> 1.6 millions of deaths every year\(^b\)

\(^a\) Perez-Trallero *et al*, 2011, AAC 55:2729-2734; \(^b\) Trappetti *et al*, 2013 Infect. Immun. 81:505-513
INTRODUCTION - *Streptococcus pneumoniae* or Pneumococcus

- Require antibiotic administration
- Especially for AECB (highly recurrent)

- But, antibiotic choices have to be appropriate...
  
  => avoid favoring/selecting resistance
  
  => safe for patients
  
  => not affected by other co-medications (drug interactions)

"Antibiotic resistance - one of the three greatest threats to human health."

World Health Organisation, 2009
THESIS OBJECTIVES

Main objectives of this thesis ...
... to understand HOW
... to find WAYS to RESTORE

Making Fundamental Research

Increasing global scientific knowledge

Investigating the Etiology of Therapeutic Failures

Offering answers to clinicians

Characterizing AECB pneumococcal strains

Determining what is/are the best therapeutic option(s) to treat each *S.p* AECB.

- Finding new markers to predict the resistance of *S.p.* strains to some antibiotics.
- Investigating the causes of antibiotic resistance.
- Setting-up new methods to better characterize the activity of antimicrobials.
RESULTS: CHAPTER 1

Acute Exacerbations of Chronic Obstructive Pulmonary Disease

Streptococcus pneumoniae

Antibiotics

Host cells

Bacteria

Patients
RESULTS: CHAPTER 1

Acute Exacerbations of Chronic Obstructive Pulmonary Disease

Streptococcus pneumoniae

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RESULTS: CHAPTER 1

3-years multicentric epidemiological and clinical study

101 AECB *Streptococcus pneumoniae* clinical isolates & patients’ medical data...
RESULTS: CHAPTER 1

- Patients' characteristics
- Susceptibility to antibiotics
- Polysaccharide Capsular antigens
- Functionnality of efflux proteins
- Ability to produce biofilm

_S. pneumoniae_ clinical isolates
RESULTS : CHAPTER 1

Susceptibility to antibiotics

Minimal inhibitory concentrations (MICs) determinations in microdilutions

Polysaccharide Capsular antigens

Functionnality of efflux proteins

Ability to produce biofilm

Patients’ characteristics

S. pneumoniae clinical isolates
Minimal inhibitory concentrations (MICs) determinations in microdilutions

Serial ANTIBIOTIC Dilutions

Minimal inhibitory concentrations (MICs):
smallest antimicrobial concentration inhibiting bacterial growth
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<th>Antibiotic classes</th>
<th>Antibiotic molecules</th>
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<td>≤0.5 / &gt;2</td>
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na: not applicable (no breakpoint defined)

* European Committee for Antibiotic Susceptibility Testing
† I: intermediate; R: resistance
‡ oral form (cefuroxime axetil)
§ not recommended for clinical use but tested here for epidemiological purposes
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*European Committee for Antibiotic Susceptibility Testing*  
*I*: intermediate; *R*: resistance  
*oral form (cefuroxime axetil)*  
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**High macrolide resistance**  
**Good susceptibility to ceftriaxone, telithromycin, moxifloxacin & levofloxacin**
## RESULTS: CHAPTER 1

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**Belgian therapeutic guidelines a**

Amoxicillin, in alternation with moxifloxacin

Good susceptibility to ceftriaxone, telithromycin, moxifloxacin & levofloxacin

High macrolide resistance

---

But...

Does it mean that these molecules are clinically active in all AECB cases?

Which molecule for patients presenting allergy to penicillins?

And what about the benefice-risk of fluoroquinolones in frail patients?
RESULTS: CHAPTER 1

S. pneumoniae clinical isolates

- Susceptibility to antibiotics
- Minimal inhibitory concentrations (MICs) determinations in microdilutions
- Polysaccharide Capsular antigens
- Functionnality of efflux proteins
- Ability to produce biofilm
- Patients’ characteristics
- Serotyping Antibody - Antigen reactions
Capsular polysaccharides determine the serotype

Pneumococcal phylogeny. The maximum likelihood was generated using 106,196 polymorphic sites. Fifteen monophyletic sequence clusters (SCs) are labeled, with the terminal branches of the tree colored black indicating taxa that constitute a sixteenth polyphyletic group. Within the monophyletic sequence clusters, light background shading indicates one particular serotype, with darker shading and dashed lines used to indicate groups of isolates of alternative serotypes. Croucher et al, 2013, Nat. Genet. , 45 (6): 656-63

RESULTS: CHAPTER 1

Capsular polysaccharides determine the serotype

Immuno-precipitation using antibodies specific of each serotype

The AECB clinical isolates collected were exposed to serotype-specific antibodies

Hancock L E, and Gilmore M S PNAS 2002;99:1574-1579
Isolates susceptibility to clarithromycin as a function of their serotype/serogroup

Antibiotic susceptibility to clarithromycin for all isolates as a function of their serotype (ST) / serogroup (SG) ranked from less to more susceptible. Data are presented as "Box and whiskers plots" giving the 25, 50 and 75 quartiles (boxes and horizontal line) of the MIC distributions with the lower and upper bars extending from the lowest to the highest MIC value observed. The blue and pink horizontal ribbons show the intermediate zones of clinical susceptibility according the interpretive criteria of EUCAST (from > S to > R [20]) and CLSI (from > S to < R [19]), respectively.
RESULTS: CHAPTER 1: 3-years epidemiological study

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All Serotypes/serogroups do not have the same susceptibility profile to antibiotics
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All Serotypes/serogroups do not have the same susceptibility profile to antibiotics

=> Capsular antigens may perhaps translate the strain susceptibility to AB and guide « infection-personalised » therapeutic choices
RESULTS: CHAPTER 1

Susceptibility to antibiotics

Minimal inhibitory concentrations (MICs) determinations in microdilutions

Patients' characteristics

Polysaccharide Capsular antigens

Serotyping
Antibody - Antigen reactions

S. pneumoniae clinical isolates

Functionnality of efflux proteins

Ability to produce biofilm

Static in vitro model
Thickness determined after 10 days, by biofilm staining with crystal violet
Biofilm thickness quantified over time through:
- biofilm staining with Crystal violet (CV)
- spectrophotometric measures of CV absorbance (570nm)

Fuqua and Greenberg, Nat Rev Mol Cell Biol. 2002;3(9):685-95
RESULTS: CHAPTER 1

Bacterial suspension

+ Medium
caMHB + 5% LHB

up to 20 days
37°C; 5% CO₂

+ Medium
caMHB + 5% LHB + 2% Glucose

Biofilm thickness quantified over time through
• biofilm staining with Crystal violet (CV)
• spectrophotometric measures of CV absorbance (570nm)

Biofilm maturity (days)

Biomass (Crystal violet OD 570nm)

Fuqua and Greenberg, Nat Rev Mol Cell Biol. 2002;3(9):685-95

ATCC 49619 - naïve model

R6 - induced model
RESULTS : CHAPTER 1 : 3-years epidemiological study

All pneumococcal serotypes/serogroups produce biofilm but some of them seem to be higher or lower producers => implication in cell persistence
RESULTS: CHAPTER 2

**Acute Exacerbations of Chronic Obstructive Pulmonary Disease**

- **Streptococcus pneumoniae**

- **Antibiotics** → **Bacteria**
- **Host cells**

1. **Patients**
2. **Antibiotics**
RESULTS : CHAPTER 2 : *In vitro* biofilm models
RESULTS: CHAPTER 2: *In vitro* biofilm models

In vitro biofilm models

**Induced model**

**Naive model**

![Graph showing biofilm maturity](image)

**Acv (570nm)**

**ATCC 49619 - naïve model**

**ATCC49619 - induced model**

**R6 - naïve model**

**R6 - induced model**

**Biofilm maturity (days)**

**A_{cv} (570nm)**

**0**

**2**

**4**

**6**

**8**

**10**

**12**

**0**

**50**

**100**

**150**

**200**

**250**

**300**

**350**

**Bacterial planktonic suspension from the supernatant of 6 day-old naive biofilm**

**New plate**

**+ fresh medium**
Resazurin viability assay adapted to pneumococcal biofilms

RESULTS: CHAPTER 2: *In vitro* biofilm models

Resazurin $\rightarrow$ Resorufin

C- : medium without AB
C+ : SDS 1% m/v aq. solution
RESULTS: CHAPTER 3

**Acute Exacerbations of Chronic Obstructive Pulmonary Disease**

- **Streptococcus pneumoniae**

**Antimicrobial Agents and Chemotherapy**

Antibiotic Activity against Naïve and Induced Streptococcus pneumoniae Biofilms in an *In Vitro* Pharmacodynamic Model

Nathalie M. Vandevelde, Paul M. Tulkens and Françoise Van Bambeke
RESULTS: CHAPTER 3: Pharmacodynamic studies of antibiotic activity

Ex: ATCC 49619 biofilms - MXF
- - : 2 days-old
— : 11 days-old

Moxifloxacin
Naive model

Survival

Log10 MIC broth
% Residual viability (of the ctrl)

Biofilm thickness

Log10 MIC broth
% Residual matrix (of the ctrl)

Ex: ATCC 49619 biofilms - MXF
- - : 2 days-old
— : 11 days-old

Data were used to fit a sigmoid function (Hill equation, slope factor set to 1) by non-linear regression

<table>
<thead>
<tr>
<th>Biofilm model</th>
<th>Effect on bacterial survival</th>
<th>Effect on biofilm thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}$ (%) &amp; 95% CI</td>
<td>$E_{\text{max}}$ (%) &amp; 95% CI</td>
</tr>
<tr>
<td>2 days naïve</td>
<td>74.07 (65.42 to 82.72) /A</td>
<td>56.23/A</td>
</tr>
<tr>
<td>11 days naïve</td>
<td>42.18 (36.11 to 48.25) /B</td>
<td>3.78/B</td>
</tr>
</tbody>
</table>

Stat. analysis: unpaired, two-tailed t-test for comparisons between maturity stages, values with different letters are significantly different from each other (P<0.05)

Matrix effect: ↓ Efficacy & Potency
Comparison of antibiotic maximal efficacies (E_{max}) expressed as percentages reduction in viability (left panels) or biomass (right panels) as compared to the control (no antibiotic) for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619. Values were calculated using the Hill equation of the concentration-response curves and are presented as means ± SEM. Statistical analyses: one-way ANOVA with Tukey post test for multiple comparisons; values with different letters are significantly different from each other (p<0.05). Small letters: comparison between antibiotic for each type of biofilm; caps letters: comparison between different types of biofilms for each antibiotic.

• Matrix effect : biofilm maturity and induction

• More active drugs : moxifloxacin (MXF), Levofloxacin (LVX) and solithromycin (SOL)
RESULTS

• spontaneous matrix disassembly (at old maturity stages) restores antibiotic activity, even for poorly active AB

• promoting this phenomenon may be an appealing strategy for improving antibiotic activity towards pneumococcal biofilms

Yvan Diaz Iglesias, Françoise Van Bambeke, Nathalie M. Vandevelde (ESGB 2014)
RESULTS: CHAPTER 4

Acute Exacerbations of Chronic Obstructive Pulmonary Disease

Streptococcus pneumoniae

Antibiotics

Host cells

Bacteria

Patients
Bronchodilators improve antibiotic activity against pneumococcal biofilms.

Nathalie M. Vandevelde, Paul M. Tulkens, Giulio G. Muccioli, Françoise Van Bambeke
**RESULTS : CHAPTER 4**

1. **Biofilm growth in different media**

   *Ref. medium “m”:* caMHB + 5%LHB + 2% Glc
   
   *m + SALBUTAMOL 7.25mg/L* *(β2-agonist)*

   (*) Concentration in the Epithelial lining fluid (ELF)

---

Kinetics of biofilm formation (biomass, as evaluated by crystal violet absorbance OD_{570nm}) by the reference capsulated strain ATCC49619 in the naive (dotted line; open symbols) and induced (full line; closed symbols) models when cultivated in control conditions (black), or in medium supplemented with 7.25 mg/L salbutamol (blue). All values are means ± standard error of the mean (SEM) of 3 to 26 experiments (each made 12 times; when not visible, the SEM bars are smaller than the size of the symbols). Data were used to fit a sigmoidal dose-response function.
RESULTS: CHAPTER 4

1. Biofilm growth in different media

Ref. medium “m”: caMHB + 5%LHB + 2% Glc

m + SALBUTAMOL 7.25mg/L* (β2-Agonist)

(*) Concentration in the Epithelial lining fluid (ELF)

2. Biofilm treatment in absence of BD

ANTIBIOTICS

Ex: MOXIFLOXACIN
Fluoroquinolone >> DNA replication

BRONCHODILATORS (BD)

ANTIBIOTICS

S. pneumoniae BIOFILMS

2 reference strains
1 AECB clinical isolate

Moxifloxacin
Levofloxacin
Solithromycin

Interactions

Interactions
Comparison of antibiotic maximal efficacies on viability or biomass for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619 grown under control conditions or in supplemented media.

Comparison of antibiotic maximal efficacies (E_max) expressed as percentages reduction in viability (left panels) or biomass (right panels) as compared to the control (no antibiotic) for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619 treated with moxifloxacin. Values were calculated using the Hill equation of the concentration-response curves and are presented as means ± SD. Statistical analyses: one-way ANOVA with Tukey post test for multiple comparisons; values with different letters are significantly different from each other (p<0.05). Small letters: comparison between biofilm grown conditions for each antibiotic; caps letters: comparison between different types of biofilms for each biofilm culture medium.

Biofilm development in the presence of SALBUTAMOL improves the antibiotic bactericidal activity towards pneumococcal sessile cells, without any effect on biomass.
• The Neuraminidase A: the main enzymes involved in pneumococcal biofilm formation

• Cleaving terminal sialic acid sugar residues from polysaccharides chains (at the surface of host eukaryotic cells and bacteria)

=> creating receptors for prokaryote-prokaryote or prokaryote-eukaryote adhesion

RESULTS: CHAPTER 4

http://www.twiv.tv/virus-entry-into-cell

http://www.twiv.tv/virus-entry-into-cell

• Changes in the enzymatic activity are translated by modifications in free sialic acid concentration in the extracellular compartment.

RESULTS: CHAPTER 4

Influence of salbutamol (SAL; 7.25 mg/L) or of salbutamol (7.25 mg/L) combined with zanamivir (250 mg/L) (SAL+ZAN) on the concentration of free sialic acid in the biofilm supernatant as determined for each strain individually and, for each of them, for 2-days (left histogram) and 11-days (right histogram) naive and induced biofilms. Statistical analysis (one-way ANOVA with Tukey’s post-test): in each group, bars with different letters indicate significant differences between media (p < 0.05).

Salbutamol induces a loss of matrix cohesion translated by an increases of free sialic acid levels in supernatant.
Influence of salbutamol (SAL; 7.25 mg/L) or of salbutamol (7.25 mg/L) combined with zanamivir (250 mg/L) (SAL+ZAN) on the concentration of free sialic acid in the biofilm supernatant as determined for each strain individually and, for each of them, for 2-days (left histogram) and 11-days (right histogram) naive and induced biofilms. Statistical analysis (one-way ANOVA with Tukey’s post-test): in each group, bars with different letters indicate significant differences between media (p < 0.05).

Salbutamol induces a loss of matrix cohesion translated by an increases of free sialic acid levels in supernatant

Reverted by zanamivir, an inhibitor of the neuraminidase A implicated in changes of matrix tridimensional structure
Amounts of sialic acid (mg/L) released from S. pneumoniae collected from the supernatant of 2-days old naive biofilms made by reference strains ATCC49619 by purified Arthrobacter ureafaciens α-(2→3,6,8,9)-neuraminidase alone or in the presence of salbutamol, zanamivir, or their combination. CTRL (full bars): control conditions (no addition); SAL, ZAN, SAL+ZAN: addition of salbutamol, zanamivir, or their combination at the concentrations indicated in the abscissa. Statistical analysis (one-way ANOVA with Tukey’s post-test): in each group, bars with different letters indicate significant differences between conditions (p < 0.05). Salbutamol and/or zanamivir concentrations used for biofilm culture during studies of biofilm development and antibiotic activity are represented with stippled bars, other concentrations used in this experiment are represented with open bars.

**Purified Arthrobacter ureafaciens α-(2→3,6,8,9)-neuraminidase**

- Salbutamol increases the neuraminidase activity
- Zanamivir inhibits the neuraminidase activity
- Salbutamol - Zanamivir antagonism
RESULTS: CHAPTER 4

**BRONCHODILATORS (BD)**

**S. pneumoniae BIOFILMS**

2 reference strains
1 AECB clinical isolate

**ANTIBIOTICS**

- Moxifloxacin
- Levofloxacin
- Solithromycin

---

1. **Biofilm growth in different media**

**Ref. medium “m”:** caMHB + 5%LHB + 2% Glc

- m + SALBUTAMOL 7.25mg/L* (β2-Agonist)
- +/- ZANAMIVIR 250 mg/L **(NanA inhibitor)**

(*) Concentration in the Epithelial lining fluid (ELF)

(**) Neuraminidase inhibitory concentration

---

2. **Biofilm treatment in absence of BD**

**ANTIBIOTICS**

Ex: MOXIFLOXACIN

Fluoroquinolone >> DNA replication
Comparison of antibiotic maximal efficacies on viability or biomass for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619 grown under control conditions or in supplemented media.

The SALBUTAMOL-mediated improve of antibiotic bactericidal activity is probably related to loss of matrix cohesion, dependent on the NanA activity.
RESULTS: CHAPTER 4

Biofilm growth in different media

Ref. medium “m”: cAMHB + 5% LHB + 2% Glc

m + IPRATROPIUM 1.45mg/L* (mus. antag.)

(*) Concentration in the Epithelial lining fluid (ELF)

Biofilm disassembly with matrix loss from day 8 in the naive model and from day 7 in the induced model.

Kinetics of biofilm formation (biomass, as evaluated by crystal violet absorbance OD_{570nm}) by the reference capsulated strain ATCC49619 in the naive (dotted line; open symbols) and induced (full line; closed symbols) models when cultivated in control conditions (black), or in medium supplemented with 1.45 mg/L ipratropium (orange). All values are means ± standard error of the mean (SEM) of 3 to 26 experiments (each made 12 times; when not visible, the SEM bars are smaller than the size of the symbols). Data were used to fit a sigmoidal dose-response function whenever possible (dotted straight lines are used when changes in OD_{570nm} occurred abruptly).
Free sialic acid may be considered as a marker of matrix cohesion.

The ipratropium-mediated biofilm disassembly is accompanied by a major release of free sialic acid levels in supernatant.
RESULTS: CHAPTER 4

**S. pneumoniae BIOFILMS**

**Interactions**

2 reference strains
1 AECB clinical isolate

**BRONCHODILATORS (BD)**

**Interactions**

**ANTIBIOTICS**

- Moxifloxacin
- Levofloxacin
- Solithromycin

1. Biofilm growth in different media
   - **Ref. medium “m”**: caMHB + 5%LHB + 2% Glc
   - **m + IPRATROPIUM 1.45mg/L** *(musc. antag.)*

2. Biofilm treatment in absence of BD
   - Ex: MOXIFLOXACIN
   - Fluoroquinolone ≫ DNA replication

(*) Concentration in the Epithelial lining fluid (ELF)
Comparison of antibiotic maximal efficacies on viability or biomass for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619 grown under control conditions or in supplemented media.
Inhibition of pneumococcal choline-binding proteins and cell growth by esters of bicyclic amines
Beatriz Maestro1, Ana González2, Pedro García3 and Jesus M. Sanz1
1 Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Elche, Spain
2 Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain

Keywords
antibacterial resistance; similar dihydroxyl (CD); inhibition of bacterial growth; repeat protein; Streptococcus pneumoniae

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Received 18 October 2009, revised 6 November 2009, accepted 8 November 2009

Streptococcus pneumoniae is one of the major pathogens worldwide. The use of currently available antibiotics to treat pneumococcal disease is hampered by increasing resistance levels; also, capsular polysaccharide-based vaccines is of limited efficacy. Therefore, it is desirable to find targets for the development of new antimicrobial drugs specifically designed to fight pneumococcal infections. Choline-binding proteins are a family of polypeptides, found in all S. pneumoniae strains, that take part in important physiological processes of this bacterium. Among them are several enzymes hydrolyzing whose enzymatic activity is usually inhibited by an excess of choline. Using a simple chromatographic procedure, we have identified several choline analogs able to strongly interact with the choline-binding module (C-LyTA) of the major autolysin of S. pneumoniae. Two of these compounds (atropine and ipratropium) display a higher binding affinity to C-LyTA than choline, and also increase the stability of the protein. CD and fluorescence spectroscopy analyses revealed that the conformational changes of C-LyTA upon binding of these alkaloids are different to those induced by choline, suggesting a different mode of binding. In vitro inhibition assays of three pneumococcal, choline-dependent cell wall lytic enzymes also demonstrated a greater inhibitory efficiency of these molecules. Moreover, atropine and ipratropium strongly inhibited in vivo pneumococcal growth, altering cell morphology and reducing cell viability, a very different response than that observed upon addition of an excess of choline. These results may open up the possibility of the development of bicyclic amines as new antimicrobials for use against pneumococcal pathologies.

Details of the ipratropium recognition by CbpF. The views show the interactions between CbpF and ipratropium. The residues forming the binding site are drawn as capped sticks. Carbon atoms of the ligand are in green. Hydrogen bonds are shown as dashed lines. (A) Ipratropium recognition at the cavity between dp6 and p1 repeats. (B) Ipratropium recognition at the cavity between p5 and the C-terminal. (C) Ipratropium recognition at the cavity between dp1 and dp2 repeats.

Crystal structures of CbpF complexed with atropine and ipratropium reveal clues for the design of novel antimicrobials against Streptococcus pneumoniae (Silva-Martin et al, 2014)
Acute Exacerbations of Chronic Obstructive Pulmonary Disease

**Streptococcus pneumoniae**

**DISCUSSION**

Antibiotics → Bacteria → Patients

**1** → **2** → **3** → **4** → **5**
Are some non-antibiotic drugs deleterious for anti-bacterial therapy?

**DISCUSSION**

- **Antihypertensive calcium channel blocker (verapamil)**
  - ↓ *in vitro* *E. coli* susceptibility to ampicillin *(Gunics et al, 2000)*

- **Mucolytic agent (N-acetylcystein)**
  - ↓ *in vitro* *S. aureus, P. aeruginosa, K. pneumoniae, E. coli* susceptibilities to many AB *(Goswani et al, 2010; Garcia et al, 2012)*

- **Non-steroidal anti-inflammatory drugs (salicylates)**
  - ↓ *in vitro* *S. aureus, K. pneumoniae, E. coli* susceptibilities to fluoroquinolones, through the induction of efflux *(Riordan et al, 2007; Tavio et al, 2004)*

- **Benzodiazepines (diazepam)**
  - ↓ *in vitro* *S. aureus, K. pneumoniae, E. coli* susceptibilities to fluoroquinolones, through the induction of efflux *(Tavio et al, 2004; Tavio et al, 2012; Vandevelde NM., Van Obbergh M. et al, ICAAC 2013)*

- **Antipsychotics (haloperidol)**
  - ↓ *in vitro* *E. coli* susceptibilities to fluoroquinolones, through the induction of efflux *(Tavio et al, 2012)*
• My first COPD patient...

- man
- 81 years old in 2010 (born in 1929)
- miner during 25 years
- former smoker (stopped in 1990, total 45 UAP)

- Bronchial obstruction: ↓ 40% of the expiratory function (GOLD 2)
  => SHORT & LONG-ACTING BRONCHODILATORS

- Chronic cough and lung inflammation

- 1-2 bacterial exacerbations/year
  => ANTIBIOTICS

- Hypertension (157/72 mmHg)
  => 3 ANTI-HYPERTENSIVE DRUGS/day

- Heart transplant (1992), cardiomegaly
  => 3 ANTI-REJECTION DRUGS

- Hypercholesteloremia
  => CHOLESTEROL-LOWERING DRUGS

- Moderate hyperglycemia (fasting glucose 139 mg/dl)
  => ANTI-DIABETIC DRUGS

- Overweight (BMI : 33kg/m²)

- Chronic renal failure

- Cancer
Thank you !!
• Ma promotrice de thèse, le Professeur Françoise Van Bambeke

• The members of the jury and thesis committee

• Le Professeur Paul Tulkens

• Le Professeur Marie-Paule Mingeot

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• Les membres du groupe de recherche CLIP

• Les autres doctorants et post-doctorants du LDRI

• Les chercheurs avec qui j’ai travaillé:
  Dr. Julia Bauer
  Professeur Giulio G. Muccioli

• Ma famille

Merci à tous du fond du cœur !! 😊
RESULTS: CHAPTER 5: Set up of an NADH oxidation-based viability assay

A/ Schematic representation of the differences existing between NADH oxidase (NOX) release in the case of bactericidal versus bacteriostatic antibiotic activities.

B/ NADH and NAD⁺ chemical structures.

C/ Schematic representation of NAD⁺ and NADH UV absorbance spectra with maximal absorbances values measured at respectively 260 and 339nm.

R = ribose-P-P-ribose-adenine
RESULTS: CHAPTER 5: Set up of an NADH oxidation-based viability assay

Dose-response curves of moxifloxacin (MXF, full symbols) and clarithromycin (CLR, open symbols) activities on bacterial survival in planktonic cultures and 2-days old biofilms made with strain ATCC 49619, after 24 hours of incubation with increasing concentrations of antibiotics. The ordinate shows the change in viability as a percentage of the control value (no antibiotic present) and measured by the decrease in resorufin fluorescence, in NADH absorbance and in CFU. All values are means ± SD of 2 to 6 independent experiments. When not visible, the error bars are smaller than the size of the symbols.
RESULTS : CHAPTER 5 : Set up of an NADH oxidation-based viability assay

GAPDH : glyceraldehyde–phosphate dehydrogenase
PK : pyruvate kinase
LDH : lactate dehydrogenase
PDH : pyruvate dehydrogenase
NOX : NADH oxidase
PTA : phosphate acetyltransferase
ACK : acetylphosphokinase
RESULTS: CHAPTER 5: Set up of an NADH oxidation-based viability assay

Yamamoto et al, 2006 - *S. agalactiae*
Yesilkaya et al, 2009 - *S. pneumoniae*
Ramos-Montanez et al, 2010 - *S. pneumoniae*

**GAPDH:** glyceraldehyde–phosphate dehydrogenase
**PK:** pyruvate kinase
**LDH:** lactate dehydrogenase
**PDH:** pyruvate dehydrogenase
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RESULTS : CHAPTER 5 : Set up of an NADH oxidation-based viability assay

- Anaerobic conditions
- acidic pH
- Glucose (blood,...)

- Aerobic conditions
- neutral pH
- Galactose (lung and nasopharynx mucosal sites)

Yamamoto et al, 2006 - S. agalactiae
Yesilkaya et al, 2009 - S. pneumoniae
Ramos-Montanez et al, 2010 - S. pneumoniae

GAPDH : glyceraldehyde -phosphate dehydrogenase
PK : pyruvate kinase
LDH : lactate dehydrogenase
PDH : pyruvate dehydrogenase
NOX: NADH oxidase
PTA : phosphate acetyltransferase
ACK : acetylphosphokinase
ADHE : acetaldehyde dehydrogenase
ADH : alcohol dehydrogenase
RESULTS : CHAPTER 5 : Set up of an NADH oxidation-based viability assay

- Anaerobic conditions
- acidic pH
- Glucose (blood,...)

- Aerobic conditions
- (O<sub>2</sub>)
- neutral pH
- Galactose (lung and nasopharynx mucosal sites)

Adapted from Yesilkaya et al, 2009 - S. pneumoniae
Ramos-Montanez et al, 2010 - S. pneumoniae
## Structures – Betalactams

<table>
<thead>
<tr>
<th>Amoxicillin</th>
<th>Cefuroxime</th>
<th>Ceftriaxone</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Amoxicillin Structure" /></td>
<td><img src="image2.png" alt="Cefuroxime Structure" /></td>
<td><img src="image3.png" alt="Ceftriaxone Structure" /></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>structures</th>
<th>d-aladala</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image4.png" alt="D-Ala-D-Ala" /></td>
<td></td>
</tr>
</tbody>
</table>

- **Amoxicillin**
- **Cefuroxime**
- **Ceftriaxone**
Structures – Macrolides & kétolides

Clarithromycine

Azithromycine

Telithromycine

Solithromycine

NH₂

↓ ocular and muscular side effects

Increased activity

Increased stability

Absence de cladinose:
- stabilité en milieu acide
- pas d’induction MLS₆

Chaine latérale:
- liaison au domaine II
- liaison aux ribosomes méthylés
- pas de reconnaissance par les pompes à efflux de S. pneumoniae
- pharmacocinétique

Carbamate:
- activité accrue

Increased activity

Increased stability
Structures - Quinolones

- Moxifloxacin
- Pradofloxacin
- Gemifloxacin
- Levofloxacin
- Marbofloxacin
- Delafloxacin
Diazepam: anxiolytic & spasmolytic benzodiazepine, binding to the GABA<sub>A</sub> receptors

Efflux modulation by diazepam leading to resistance in *S. pneumoniae*?

Diazepam → modifications of bacterial behaviour<sup>a,b,c,d</sup>

---


---

Fluoroquinolone efflux pumps

Diazepam

Antibiotic

Efflux pump

Bacterial membrane

Therapeutic target

---

% COPD patients

<table>
<thead>
<tr>
<th>Intake</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>yes</td>
<td>36%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>64%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

Diagram:

- Diazepam: anxiolytic & spasmolytic benzodiazepine, binding to the GABA<sub>A</sub> receptors
- Efflux modulation by diazepam leading to resistance in *S. pneumoniae*?
Planctonic cultures: which impact of diazepam on bacterial efflux?

Bacterial growth conditions:

Medium: caMHB + 5%LHB ± DZP 1µg/ml (~ Human peak plasma concentration [0,2-1,5µg/ml]) ; 37°C ; 5% CO₂
Suspensions diluted everyday with fresh medium to OD₆₂₀nm = 0,1 (0,5 McF)

Evaluation of the bacterial susceptibility to fluoroquinolones

- MICs (*) measures in microdilutions (96-well plates)
- In absence vs presence of reserpine (10mg/L), an efflux pump inhibitor
- Fluoroquinolones tested: ex: - Norfloxacin (FQ substrate of efflux pumps PatA/Bᵃ,c,d)
  - Moxifloxacin (not substrate of efflux pumps a,c,d)

(*) MIC: Minimal inhibitory concentration:
Smallest antibiotic concentration able to inhibit bacterial growth

\[\text{MIC} = \text{Minimal inhibitory concentration}\]

\[\text{Smallest antibiotic concentration able to inhibit bacterial growth}\]

---

\(^a\) El Garch et al,2010; \(^b\) Drug Information Handbook 18\(^{th}\) Edition; \(^c\) Lismond et al, 2011; \(^d\) Piddock et al, 2002 b
Planctonic cultures: which impact of diazepam on bacterial efflux?

Results:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Days of culture</th>
<th>Growth medium</th>
<th>Measures of the MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NOR</td>
</tr>
<tr>
<td>ATCC 49619</td>
<td>0</td>
<td>CTRL m</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m + DZP 1µg/ml</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>CTRL m</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m + DZP 1µg/ml</td>
<td>32</td>
</tr>
</tbody>
</table>

- Susceptibility to NOR < MXF
- Significant impact on bacterial susceptibility to norfloxacin (substrate of efflux pumps): induction of an efflux phenotype by Diazepam after 8 days of culture *
- No significant impact of Diazepam on moxifloxacin activity (not substrate of efflux pumps)

(*) Also observed for other FQ substrate of efflux pumps
1. Biofilm formation in different media:

- Reference medium: «m»: caMHB + 5% LHB + 2% glucose

- medium supplemented with diazepam (Human Peak Plasma Concentration $^a$): Medium «DZP»: m + **Diazepam 1µg/ml** (Valium® Sol. for injection i.v. / i.r)

2. Biofilms treatment with antibiotics (in absence of diazepam)

---

$^a$ Drug Information Handbook 18th Edition
Results: Antibiotic pharmacodynamic studies in polytherapies

Ex: ATCC 49619 biofilms
- - : 2 days-old, m
- - : 11 days-old, m
- - : 2 days-old, m+DZP 1µg/ml
- - : 11 days-old, m+DZP 1µg/ml

Survival

Biofilm mass

Moxifloxacin
Not substrate of efflux pumps

Norfloxacin
Substrate of efflux pumps

Moxifloxacin
Norfloxacin
Results: Antibiotic pharmacodynamic studies in polytherapies

Ex: ATCC 49619 biofilms
- - : 2 days-old, m
- : 11 days-old, m
- - : 2 days-old, m+DZP 1µg/ml
- : 11 days-old, m+DZP 1µg/ml

Survival

Biofilm mass

Moxifloxacin
Not substrate of efflux pumps

Norfloxacin
Substrate of efflux pumps

Log10 MIC broth
% Residual viability (of the ctrl - )

Log10 MIC broth
% Residual matrix (of the ctrl - )

Log10 MIC broth
% Residual viability (of the ctrl - )

Log10 MIC broth
% Residual matrix (of the ctrl - )

Results: Antibiotic pharmacodynamic studies in polytherapies
CIP efflux in >30% of isolates

Comparison of biofilm production after 10 days of culture for strains resistant to both clarithromycin and azithromycin and to ciprofloxacin using EUCAST interpretive criteria and grouped according to the absence (open symbols) or the presence (closed symbols) of efflux as detected for clarithromycin and azithromycin by dissociation of susceptibilities with clindamycin, and for ciprofloxacin by a 2-fold decrease in MICs upon addition of reserpine (10 mg/L). No correlation between efflux and biofilm thickness was seen unpaired t-test (with of without Welch correction).

No correlation between efflux and biofilm production