Impact of **efflux mechanisms** on susceptibility and resistance to **beta-lactam antibiotics** in *Pseudomonas aeruginosa*: beyond the usual concepts

Houssein Abdel Aziz Chalhoub

Promoter: Prof. Françoise Van Bambeke

Cellular and Molecular Pharmacology
Louvain Drug Research Institute
*Université catholique de Louvain, Brussels, Belgium*

22 December 2016
Bacteria & Humans

LA TERRE ET LA VIE - CHRONOLOGIE

-13.7 Md  -5 Md  -4 Md  -3 Md  -2 Md  -1 Md
-555 Mi  -410 Mi  -200 Mi  -65 Mi  -7 Mi

Big Bang
Terre

Eucaryotes
Procaryotes

Procaryotes:
- 3.8 Md a : les procaryotes
- 2.1 Md a : les eucaryotes
- 555 Mi a : la vie explose en diversité

Eucaryotes:
- 430 Mi a : la vie sort de l'eau

Sortie de l'eau

Faune de Burgess

Dinosaures
Hominidés
Mammifères
Bacteria: spread widely in the environment...
Bacteria: spread widely in the environment...

http://www.earthtimes.org/scitech/billion-years-old-bacterial-enzyme/1578/
“They are so small that you can’t seen them without a microscope, but they are there...”

Bacteria

http://dlb-network.com/photographyomn/pseudomonas-aeruginosa-gram-stain
Bacterial infections

Antibiotic resistance: post-antibiotic era ...

“Without urgent, coordinated action by many stakeholders, the world is headed for a post-antibiotic era, in which common infections and minor injuries which have been treatable for decades can once again kill.”

– Dr. Keiji Fukuda, WHO Assistant Director-General for Health Security
April 30, 2014
Antibiotic resistance: microbial war...
## Antibiotic resistance: revenge of the microbes

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**Sir Alexander Fleming**  
Alfred Eisenstaedt—Time & Life Pictures/Getty Images
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Antibiotic resistance: revenge of the microbes

Clinical use

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<th>Year of Introduction</th>
<th>Year of Resistance</th>
</tr>
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http://xombit.com/2013/02/nadal-magnus-demoledor-liftado
Pseudomonas aeruginosa

1-5 µm
*Pseudomonas aeruginosa*: moving by the use of flagella...

From: https://www.youtube.com/watch?v=a_5FToP_mMY
A clip from the NOVA production, "Judgment Day."
*Pseudomonas aeruginosa*: takes advantage of a sick person...

http://www.healthcare2z.org/word
Opportunistic pathogens: Lung infections in Cystic Fibrosis patients

realheroesbecomeangels.blogspot.com
**Pseudomonas aeruginosa:**
Lung infections in **Cystic Fibrosis** patients

Classes of antipseudomonal antibiotics

- **β-lactams**
- Aminoglycosides
- Quinolones
- Polymyxins

Picture from: https://en.wikipedia.org/wiki/Intravenous_therapy
Classes of antipseudomonal antibiotics

- Aztreonam
- Aminoglycosides (Tobramycin)
- Quinolones (Levofloxacin)
- Polymyxins (colistin)

Classes of antipseudomonal antibiotics

Quinolones

Picture from: http://www.dailymail.co.uk/health/article-3430291
Pseudomonas aeruginosa: Lung infections in Cystic Fibrosis patients


Annual Data Report 2014  Cystic Fibrosis Foundation Patient Registry

Multidrug-Resistant Pseudomonas aeruginosa
Classes of antipseudomonal antibiotics

**β-lactams**

**Penicillins:**
Ticarcillin / clavulanic acid
Piperacillin / Tazobactam

**Carbapenems:**
Meropenem
Imipenem

**Cephalosporins:**
Ceftazidime, cefepime

**Monobactam:** Aztreonam

Picture from: https://en.wikipedia.org/wiki/Intravenous_therapy
**P. aeruginosa** and resistance mechanisms to β-lactam antibiotics

- Downregulating / mutating porins [OprD] 
  β-lactams (imipenem, meropenem…)

http://thestir.cafemom.com/big_kid/181864/expired_medicine_dates_still_safe
*P. aeruginosa* and resistance mechanisms to β-lactam antibiotics

Active efflux [MexAB-OprM, MexXY-OprM, MexEF-OprN, MexCD-OprJ]

β-lactams (ticarcillin, meropenem...)

**β-lactamases: antibiotic degradation**

- Extended-Spectrum-β-Lactamases: ceftazidime
- Cephalosporinase AmpC: ceftazidime
- Carbapenemase: meropenem, ceftazidime

**P. aeruginosa** and resistance mechanisms to β-lactam antibiotics

- **β-lactamases** (cephalosporinases AmpC-type, carbapenemases, ESBLs...)
- Downregulating / mutating porins [OprD]
- Active efflux [MexAB-OprM, MexXY-OprM, MexEF-OprN, MexCD-OprJ]
Impact of **efflux mechanisms** on susceptibility and resistance to **beta-lactam antibiotics** in *Pseudomonas aeruginosa*: beyond the usual concepts

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Cellular and Molecular Pharmacology

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22 December 2016
Main objectives of the thesis

1. Activity of beta-lactam antibiotics against *Pseudomonas aeruginosa* from patients with cystic fibrosis

2. Role of efflux versus other resistance mechanisms against:
   a. Meropenem (carbapenems)
   b. Ceftazidime / avibactam (new β-lactamases inhibitor)
   c. Temocillin (old beta-lactam antibiotic)
International collection of *P. aeruginosa* from patients with cystic fibrosis chronically exposed to different antipseudomonal agents.

→ **Bacterial isolates:** 333 clinically relevant isolates were obtained from:

- Dr Michael Tunney (Queen’s University of Belfast, UK): n=99;
- Drs Anne Vergison / Olivier Denis (Hôpital Erasme, Brussels, Belgium): n=88;
- Prof. Patrick Plésiat (CHRU Besançon, Besançon, France): n=80;
- Prof. Barbara Kahl (University of Münster, Münster, Germany): n=68.

Antibacterial activity of beta-lactams against *P. aeruginosa* from cystic fibrosis patients

<table>
<thead>
<tr>
<th>Breakpoint (mg/L)</th>
<th>Ticarcillin</th>
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<td>8</td>
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- A microorganism is defined as susceptible or resistant by a level of antimicrobial activity associated with a high likelihood of therapeutic success or therapeutic failure, respectively.

Antibacterial activity of beta-lactams against *P. aeruginosa* from cystic fibrosis patients

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- **Summary.** Imipenem and meropenem were the most active antibiotics...

Meropenem
(β-lactam)

- Stable to AmpC and ESBL but not carbapenemases.
High-level resistance to meropenem

*Pseudomonas*
(cystic fibrosis patients)

Meropenem MIC = 64-128 mg/L

Related publications: Chalhoub et al, Intern. J. Antimicrob. Ag. in press
High-level resistance to meropenem

Comparison with *Pseudomonas* from hospital acquired pneumonia

- *Pseudomonas* (cystic fibrosis patients)
  - Meropenem MIC = 64-128 mg/L

- *Pseudomonas* (Hospital acquired pneumonia)
  - Meropenem MIC = 128 mg/L

Related publications: Chalhoub *et al*, Intern. J. Antimicrob. Ag. in press
High-level resistance to meropenem

Comparison with *Pseudomonas* from hospital acquired pneumonia

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*Pseudomonas* (cystic fibrosis patients)

Meropenem MIC = 64-128 mg/L

*Pseudomonas* (Hospital acquired pneumonia)

Meropenem MIC = 128 mg/L

Related publications: Chalhoub et al, Intern. J. Antimicrob. Ag. in press
Meropenem + efflux inhibitor in *Pseudomonas*

Adapted from Jason Sello, Brown university, 2011
High-level resistance to meropenem

Comparison with *Pseudomonas* from hospital acquired pneumonia

*Pseudomonas* (cystic fibrosis patients)

Meropenem MIC = 64-128 mg/L

*Pseudomonas* (Hospital acquired pneumonia)

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High-level resistance to meropenem

Comparison with *Pseudomonas* from hospital acquired pneumonia

*Pseudomonas* (cystic fibrosis patients)
Meropenem MIC = 64-128 mg/L

*Pseudomonas* (Hospital acquired pneumonia)
Meropenem MIC = 128 mg/L

Similarities and differences in the resistance profiles of *Pseudomonas* strains from cystic fibrosis (CF) and hospital-acquired pneumonia (HAP) patients are highlighted in the graph. The figure shows the Minimum Inhibitory Concentration (MIC) of meropenem for different strains.

**Key:***
- MexAB-OprM
- MexXY-OprM
- MexCD-OprJ

**Carbapenemases and Resistance Levels:**
- **- PAβN:** Strains without PAβN
- **+ PAβN:** Strains with PAβN

**MEM MIC (mg/L):**
- **2**
- **4**
- **8**
- **16**
- **32**
- **64**
- **128**
- **256**

**Comparison:**
- Strains from CF patients with carbapenemases (-) show lower resistance levels compared to those with carbapenemases (+).
- Strains from HAP patients with carbapenemases (+) show higher resistance levels.

**Related publications:**
High-level resistance to meropenem

Comparison with *Pseudomonas* from hospital acquired pneumonia

- *Pseudomonas* (cystic fibrosis patients)
- *Pseudomonas* (Hospital acquired pneumonia)

Sequencing *OprD* genes confirmed these results (premature stop mutations)

Related publications: Chalhoub et al, Intern. J. Antimicrob. Ag. in press
Impact of **efflux mechanisms** on susceptibility and resistance to **beta-lactam antibiotics** in *Pseudomonas aeruginosa*:

**beyond the usual concepts**

1- [Efflux + / porin -] are enough to confer high-level resistance to meropenem.

Related publications: Chalhoub *et al*, Intern. J. Antimicrob. Ag. in press
High-level resistance to ceftazidime

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AmpC cephalosporinases
inhibitor: avibactam

Ceftazidime/avibactam activity in the whole collection of isolates (n = 333)

- Susceptible isolates to ceftazidime increased from 36% to 76% after addition of avibactam [4mg/L].

Promising results for cystic fibrosis patients

Ceftazidime/avibactam activity in the whole collection of isolates (n = 333)

24 % resistance to this new combination !?

The mechanism of resistance to ceftazidime/avibactam combination

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(Prepared for submission as a correspondence to the Journal of Antimicrobial Chemotherapy)

Chalhoub et al., Poor membrane permeability impedes avibactam activity in Pseudomonas aeruginosa
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<td>PAO1 OprD-mutant</td>
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Chalhoub *et al.*, Poor membrane permeability impedes avibactam activity in *Pseudomonas aeruginosa*
The mechanism of resistance to ceftazidime/avibactam combination

MexAB-OprM, MexEF-OprN efflux systems overexpression

OprD loss

Cephalosporinases

Avibactam

Impact of **efflux mechanisms** on susceptibility and resistance to **beta-lactam antibiotics** in *Pseudomonas aeruginosa*:

**beyond the usual concepts**

1- [Efflux + / porin -] are enough to confer high-level resistance to meropenem

2- [Efflux + / porin -] are pre-existing as resistance mechanisms to avibactam, which is not used yet in CF patients !!
Impact of **efflux mechanisms** on susceptibility and resistance to **beta-lactam antibiotics** in *Pseudomonas aeruginosa*:

**beyond the usual concepts**

Is there any antibiotic resistant to beta-lactamases, and not affected by efflux systems and OprD porin downregulation ??
Impact of **efflux mechanisms** on susceptibility and resistance to **beta-lactam antibiotics** in *Pseudomonas aeruginosa*:

**beyond the usual concepts**
Temocillin (β-lactam)

- Derived from the class of carboxypenicillins (ticarcillin, carbenicillin)
- Withdrawn due to lack of activity against wild-type strains of *Pseudomonas aeruginosa*, Gram-positive organisms and anaerobes.
- Not used against *Pseudomonas*.
Temocillin: stable against β-lactamases

Born in the 1989’s (UK)
Temocillin versus ticarcillin in the active site of β-lactamases

Adapted from:
- Cubist pharmaceuticals
Temocillin & Efflux in *P. aeruginosa*

In 2012
FACM Group, LDRI, Belgium

<table>
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<th>Isogenic strains of <em>PA</em></th>
<th>MIC (mg/L)</th>
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<tbody>
<tr>
<td></td>
<td>temocillin</td>
<td>ticarcillin</td>
</tr>
<tr>
<td>Wild type PAO1</td>
<td>512</td>
<td>64</td>
</tr>
<tr>
<td>PAO1 <em>delta mexAB</em></td>
<td>2</td>
<td>1</td>
</tr>
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**Delta mexAB** Susceptible

Temocillin
Temocillin & Efflux in *P. aeruginosa*

Δ *mexAB*

Susceptible

Natural mutations of these efflux pumps can be observed in isolates from patients with cystic fibrosis !!!


Temocillin considered as inactive against *Pseudomonas* showed surprising activity !!!

<table>
<thead>
<tr>
<th></th>
<th>TIC</th>
<th>Temocillin</th>
<th>TZP</th>
<th>CAZ</th>
<th>Meropenem</th>
<th>Ceftazidime + avibactam</th>
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<td>76</td>
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<td>51</td>
<td>24</td>
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# proposed breakpoint for temocillin in Belgium: 16 mg/L

Temocillin + efflux inhibitor in *Pseudomonas*

- Activity of temocillin was improved in the presence of the efflux inhibitor
- % susceptibility increased from ~30 to 55%

Speed of efflux by MexAB-OprM pumps

- Activity of temocillin was improved in the presence of the efflux pump inhibitor.
- Lowest efflux speed for isolates with temocillin MICs < 128mg/L.

Sequencing *mexA/B* genes in *P. aeruginosa* from cystic fibrosis patients

OprM-gated porin

MexA-Linker

MexB-exporter protein

http://learn.genetics.utah.edu/content/cells/organelles/
https://genedoe.wordpress.com/tag/dna/
Sequencing *mexA/B* genes in *P. aeruginosa* from cystic fibrosis patients

<table>
<thead>
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<th>Major mutations</th>
<th>Minor mutations</th>
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<tr>
<td>-DNA Fragment Deletions</td>
<td>-Synonymous mutations</td>
</tr>
<tr>
<td>-Stop mutations</td>
<td>-Missense mutations</td>
</tr>
</tbody>
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Mutations in \textit{mexA/B} genes in \textit{P. aeruginosa} as a function of temocillin activity

(e) MIC vs. mutations

(f) NPN efflux vs. mutations

Modelisation *mexB* mutations in the same clone isolated from 3 German patients

MexB mutant (truncated + aberrant protein)  
Temocillin MIC = 8 mg/L

MexB mutant (AA substitutions)  
Temocillin MIC = 256 mg/L

MexB mutant (AA substitutions)  
Temocillin MIC = 512 mg/L

Truncated MexB and low efflux speed *but high temocillin MIC*!

- **Truncated MexB** (M720_Q1046del): temocillin MIC = **128 mg/L**

<table>
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<tr>
<th>Strains</th>
<th>MIC (mg/L)</th>
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<tr>
<td><em>delta mexB</em></td>
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<tr>
<td>Wild type</td>
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</table>

Influx of temocillin inside *Pseudomonas*??

OprD family, 19 members of carboxylate channels

Porin (Front view)

Porin (Extracellular side)

http://sbcb.bioch.ox.ac.uk/memprotmd/beta/protein/pdbid/3sys
Temocillin & the 19 Gates (OprD porin family) of *P. aeruginosa*

Outer membrane carboxylate channels OccD/K in *P. aeruginosa*

Figure 1. Crystal structures of Occ channels show a wide variety in pore sizes. Transparent surface representations from the extracellular side for OccD1–D3 (blue) and OccK1–K6 (salmon). The channels are shown in identical orientations. This and other structure figures were made with PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC).

doi:10.1371/journal.pbio.1001242.g001
**oprD**-single mutant of *P. aeruginosa*

- **OprD porin** is not utilized by temocillin!

<table>
<thead>
<tr>
<th>Strains</th>
<th>Temocillin</th>
<th>MEM</th>
<th>IPM</th>
</tr>
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<tr>
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<tr>
<td>PA::oprD</td>
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<td>3</td>
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Blocking OccK1 in occK2-single mutant of *P. aeruginosa*

- OccK1/OccK2 porins are involved in the uptake of temocillin

<table>
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<tr>
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Overexpression of OccK1 or OccK2 in porin-deficient *E. coli*

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<th>IPM</th>
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<tr>
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<tr>
<td>+OccK2</td>
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Overexpression of OccK1 or OccK2 in porin-deficient *E. coli*

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<td>20</td>
</tr>
<tr>
<td>+OccK2</td>
<td>1</td>
<td>1.5</td>
<td>22</td>
</tr>
</tbody>
</table>
Exopolysaccharides (alginate) from *P. aeruginosa*


Exopolysaccharides (alginate-like) ??

The diffusion of beta-lactam antibiotics through mixed gels of cystic fibrosis-derived mucin and Pseudomonas aeruginosa alginate.
Bolister N¹, Basker M, Hodges NA, Marriott C.

Figure 2. Rate of diffusion of ticarcillin through gels containing alginate alone (■) and alginate/mucin mixtures (○), mean ± s.d. shown.
Exopolysaccharide abundance in cultures of clinical isolates as a function of temocillin MICs

Summary (temocillin in cystic fibrosis)

✔ Temocillin resistance is mainly due to active MexAB efflux system

with participation of exopolysaccharides production and/or

OccK1/OccK2-porin loss!

Impact of **efflux mechanisms** on susceptibility and resistance to **beta-lactam antibiotics** in *Pseudomonas aeruginosa*:

- **Beyond the usual concepts**

1- [Efflux + / porin -] are enough to confer high-level resistance to meropenem

2- [Efflux + / porin -] are pre-existing as resistance mechanisms to avibactam, which is not used yet in CF patients !!

3- Mutations in MexAB-OprM efflux pumps appear as a mechanism of restored susceptibility to temocillin in *Pseudomonas* !!
Conclusion

- Efflux is a key mechanism of resistance for *Pseudomonas* from CF patients.

- Porins are important players if associated with efflux.

- Bacterial outer membrane is sufficiently strong barrier against β-lactams.
Conclusion

- Several paralogous efflux and porin genes to accommodate and extrude different substrates.

- *Pseudomonas* invades different territories with enough fitness and adaptability.
Conclusion

- Temocillin should be included in CF antimicrobial susceptibility testing.

- Temocillin could be also used as carbapenem sparing agent against *Pseudomonas* with OprD porin mutations!

General perspectives (research)

- Check the prevalence of mutations in *mexA/mexB* genes in *Pseudomonas* from a recent CF population (2016/2017).

- Determine whether *mexA/mexB* mutations in CF are beneficial for the activity of temocillin only or could also affect other substrates.
Perspectives (management of CF infections)

- Develop fast techniques to screen epidemic and risky clones.
- Prevent intra- and inter-hospital dissemination of epidemic *Pseudomonas* clones.
- Establishment of early appropriate and targeted antimicrobial therapies.
Acknowledgments

Cystic Fibrosis centres
Acknowledgments

Professor Mathias Winterhalter

Dr Hector Rodriguez-Villalobos
Acknowledgments

Welcome
Acknowledgments

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