Antibiotic resistance by efflux: from molecular aspects to clinical impact

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University of Notre Dame (College of Science), Notre Dame, IN – 16 September 2013
Efflux is a Belgian specialty ....

Manneken-Pis

Magritte

Folon

Planète Chocolat
Discovery and significance ....
Chemotherapeutic agents exert toxic effects on specific target cells.
Chemotherapeutic agents exert toxic effects on specific target cells

How can these drugs reach their target inside the cells?

Reaching an intracellular target …

physico-chemical properties are inadequate for reaching an intracellular target!

Reaching an intracellular target ...

most drugs are amphipathic by design, to be able to cross membrane barriers!

Intracellular chemotherapeutic agents

But a diffusible compound may have potentially harmful effects!

Why efflux transporters?

Extrusion by efflux pumps

Why efflux transporters?

Extrusion by efflux pumps

general mean of protection
against cell invasion by diffusible molecules

Most antibiotics are amphiphilic!

Most antibiotics are amphiphilic!

**anionic amphiphiles**

\( \beta \)-lactam

fusidic acid

fluoroquinolone

*Van Bambeke et al. (2000) Biochem. Pharmacol. 60:457-70*
Antibiotic efflux transporters are ubiquitous.

Mesaros et al. (2005) Louvain médical. 124:308-20
## Antibiotics as substrates of efflux pumps

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>bacteria</th>
<th>fungi</th>
<th>superior eucaryotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram (+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram (-)</td>
<td></td>
<td></td>
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<td><img src="image" alt="Blue Circle" /></td>
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<td><img src="image" alt="Blue Circle" /></td>
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<tr>
<td>chloramphenicol</td>
<td><img src="image" alt="Blue Circle" /></td>
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<td><img src="image" alt="Blue Circle" /></td>
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<tr>
<td>rifamycins</td>
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<td><img src="image" alt="Blue Circle" /></td>
<td><img src="image" alt="Blue Circle" /></td>
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<tr>
<td>sulfamides</td>
<td><img src="image" alt="Blue Circle" /></td>
<td><img src="image" alt="Blue Circle" /></td>
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<tr>
<td>trimethoprim</td>
<td><img src="image" alt="Blue Circle" /></td>
<td><img src="image" alt="Blue Circle" /></td>
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<tr>
<td>fluoroquinolones</td>
<td><img src="image" alt="Blue Circle" /></td>
<td><img src="image" alt="Blue Circle" /></td>
<td><img src="image" alt="Blue Circle" /></td>
</tr>
</tbody>
</table>

*Van Bambeke et al. (2000) Biochem. Pharmacol. 60:457-70*
Antibiotic efflux and resistance: time line …
Efflux: from molecular recognition to cellular impact
Efflux as a mechanism of resistance in Gram-positive bacteria

**spectrum**

**narrow**

Specific for one (or a few) families of drugs

- **PatA/PatB of S. pneumoniae** → FQ, chl
- **MsrA of S. epidermidis** → erythromycin

**broad**

- **ABC**
- **MFS**

- **NorA of S. aureus** → FQ, Tet, chl
- **MefE of S. pneumoniae** → ML
- **PmrA of S. pneumoniae** → FQ
- **MefA of S. pyogenes** → ML
FQ efflux pumps in *S. pneumoniae* – *S. aureus*

**Primary transporters**

« ATP-Binding Cassette »

- **PatA/PatB** (Sp)
  - Marrer et al, AAC 2006; 50:685-93

**Secondary transporters**

(Proton motive force)

- **PmrA** (Sp)
- **NorA** (Sa)
Efflux as a mechanism of resistance in Gram-negative bacteria

- **narrow spectrum**: specific for one (or a few) families of drugs
  - TetA of *E. coli* → Tet
  - MsrA of *S. epidermidis* → erythromycin

- **broad spectrum, conferring cross-resistance**
  - MexAB-OprM of *P. aeruginosa* → β-lac, FQ,Tet, ML, chl, rif, sulf
  - AcrAB-TolC of *E. coli* → β-lac, FQ,Tet, ML, chl, rif, sulf
Efflux and resistance in *P. aeruginosa*

**Constitutive basal expression**
- MexB
- MexA
- OprM

**Overexpressed upon induction**
- MexY
- MexX
- MexM
- MexF
- MexE
- OprJ
- OprN

**No basal expression; expression upon induction**
- MexD
- MexC

**Abbreviations**
- CM: cytoplasmic membrane
  - (membrane cytoplasmique)
- EM: external membrane
  - (membrane externe)
- P: porin
  - (porine)
- CMP: cytoplasmic membrane protein
  - (protéine de la membrane cytoplasmique)
- MFP: membrane fusion protein
  - (protéine de fusion [entre membranes])
- OMP: outer membrane protein
  - (protéine de membrane externe)

*Mesaros et al. (2005) Louvain médical. 124:308-20*
Substrate specificity of efflux pumps

14 fluoroquinolones; Gram + versus Gram +

S. aureus vs S. pneumoniae

Similar recognition for non phylogenetically-related transporters

Vallet et al. ECCMID 2011
Substrate specificity of efflux pumps

14 fluoroquinolones; Gram + versus Gram -

All fluoroquinolones are substrates for broad spectrum transporters from Gram -

Vallet et al. (2011) ECCMID
Substrate specificity of efflux pumps

24 fluoroquinolones; Gram + (NorA) versus eucaryotic transporter (Mrp4)

- Correlation between FQ transport by eukaryotic and procaryotic transporters
- No simple correlation between recognition by transporters and physicochemical properties

Dupont et al. (2012) ECCMID
Cooperation between prokaryotic and eucaryotic transporters to reduce FQ acativity against intracellular bacteria

**MIC of *Listeria* strains and effect of reserpine**

<table>
<thead>
<tr>
<th>Quinolone</th>
<th>EGD</th>
<th>CLIP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Res. (-)</td>
<td>Res. (+)</td>
</tr>
<tr>
<td>CIP</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>MXF</td>
<td>0.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Cooperation between prokaryotic and eucaryotic transporters to reduce FQ acativity against intracellular bacteria

Wild-type cells and bacteria

Bacterial efflux is expressed intracellularly

Lismond et al., AAC (2008) 52:3040-46
Cooperation between prokaryotic and eucaryotic transporters to reduce FQ acativity against intracellular bacteria

Bacteria overproducing efflux pumps for ciprofloxacin

Bacteria AND cells overproducing efflux pumps for ciprofloxacin

Bacterial and eukaryotic efflux cooperate to reduce ciprofloxacin intracellularly activity

Lismond et al., AAC (2008) 52:3040-46
Cooperation between prokaryotic and eucaryotic transporters to reduce FQ acativity against intracellular bacteria

Bacteria overproducing efflux pumps for ciprofloxacin

Bacterial and eukaryotic efflux do not affect the activity of moxifloxacin

Lismond et al., AAC (2008) 52:3040-46
Efflux: from molecular recognition to cellular impact

- selection of molecules that are poor substrates for efflux may prove useful

- Molecular determinants for recognition by efflux pumps need to be identified to design poor substrates
Role of antibiotic efflux in epidemiology and resistance ....
Does efflux mean « resistance » vs. epidemiological breakpoints?

MICs vs breakpoints for 183 *S. pneumoniae* isolated from CAP

- Efflux (+) strains considered as susceptible
- FQ with high intrinsic activity can be substrates for efflux!

*Lismond et al., JAC (2011) 66:948-951*
Does efflux mean « resistance » vs. epidemiological breakpoints?

MICs vs EUCAST breakpoints for 109 *P. aeruginosa* without or with efflux mechanisms, isolated from ICU patients (VAP)

Riou et al, ECCMID 2010
Role of antibiotic efflux in epidemiology and resistance …

- Efflux confers globally low level of resistance, but it can bring MICs above susceptibility breakpoints

- Molecules with high intrinsic activity (low MIC) can be excellent substrates for efflux … with a risk of seeing their MICs increasing rapidly
Role of antibiotic efflux in intrinsic resistance ....
Intrinsic resistance of *Pseudomonas* to temocillin

Temocillin [6-\(\alpha\)-methoxy-ticarcillin], a carbapenem-sparing drug ….

\[
\text{stable to } \beta\text{-lactamases, including ESBL} \\
\rightarrow \text{Treatment of infections by Gram(-)}
\]

BUT *Pseudomonas* is intrinsically resistant to temocillin ….
Intrinsic resistance of *Pseudomonas* to temocillin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Efflux characteristics</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gene expression level</td>
<td>temocillin (+ PAßN °)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>mexA</em> <em>a</em></td>
<td><em>mexX</em> <em>a</em></td>
</tr>
<tr>
<td><strong>Reference strain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Engineered strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB 536</td>
<td>PAO1 <em>Delta</em>mexCD-oprJ</td>
<td>1.09</td>
<td>1.65</td>
</tr>
<tr>
<td>CB603</td>
<td>PAO1 <em>Delta</em>mexEF-oprN</td>
<td>1.21</td>
<td>1.02</td>
</tr>
<tr>
<td>CB602</td>
<td>PAO1 <em>mexXY::FRT</em></td>
<td>1.10</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>PAO1 mexAB</strong></td>
<td><strong>PAO1 mexAB::FRT</strong></td>
<td><em>0 m</em></td>
<td>1.08</td>
</tr>
</tbody>
</table>

MexAB-OprM mutants are highly susceptible! → Efflux responsible for intrinsic resistance

Intrinsic resistance of *Pseudomonas* to temocillin

But temocillin is used successfully in Cystic Fibrosis patients …

Natural mutations in MexAB-OprM restore temocillin activity

---

**Efflux characteristics, alterations**

<table>
<thead>
<tr>
<th>Clinical isolates from cystic fibrosis patients</th>
<th>Efflux characteristics, alterations</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3020S</td>
<td>MexA MexB</td>
<td>temocillin</td>
</tr>
<tr>
<td>3020R</td>
<td>MexA MexB</td>
<td>ticarcillin</td>
</tr>
<tr>
<td>3525</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>3807</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>2715</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>616</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>2729</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>2933</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>2998</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>2721</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>2716</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>2804</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>2858</td>
<td>MexA MexB</td>
<td></td>
</tr>
<tr>
<td>3066</td>
<td>MexA MexB</td>
<td></td>
</tr>
</tbody>
</table>

**Natural mutations in MexAB-OprM restore temocillin activity**

Intrinsic resistance of *Pseudomonas* to temocillin

Is this clinically relevant?

1/4 of CF strains susceptible!

*Chalhoub, unpublished*
Intrinsic resistance of *Pseudomonas* to macrolides

Azithromycin is widely and successfully used in Cystic Fibrosis patients

Effectiveness and safety of macrolides in cystic fibrosis patients: a meta-analysis and systematic review

Yun Cai\(^1\), Dong Chai\(^1\), Rui Wang\(^1\), Nan Bai\(^1\), Bei-Bei Liang\(^1\) and Youning Liu\(^2\)

**Conclusions:** Long-term use of azithromycin can improve lung function, especially for *P. aeruginosa*-colonized CF patients. There was no evidence of increased adverse events with azithromycin. More data are needed to verify the best azithromycin regimen and to evaluate other macrolides in CF patients.

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>AZM mean</th>
<th>AZM SD</th>
<th>Placebo mean</th>
<th>Placebo SD</th>
<th>weight</th>
<th>IV, fixed, 95% CI</th>
<th>Mean difference IV, fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clement 2006(^10)</td>
<td>-8.7</td>
<td>11.5</td>
<td>-2.7</td>
<td>21</td>
<td>10</td>
<td>-6.00 [-21.03, 9.03]</td>
<td></td>
</tr>
<tr>
<td>Saiman 2003(^12)</td>
<td>4.4</td>
<td>13.6</td>
<td>-1.8</td>
<td>10.7</td>
<td>98</td>
<td>6.20 [2.64, 9.76]</td>
<td></td>
</tr>
<tr>
<td>Steinkamp 2008(^8)</td>
<td>-3.7</td>
<td>13.3</td>
<td>-5</td>
<td>10.1</td>
<td>17</td>
<td>1.30 [-6.14, 8.74]</td>
<td></td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>117</td>
<td>125</td>
<td>100.0%</td>
<td>4.80 [1.66, 7.94]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: \(\chi^2 = 3.43, df = 2 (P = 0.18); I^2 = 42\%\)

Test for overall effect: \(Z = 3.00 (P = 0.003)\)

BUT *Pseudomonas* is intrinsically resistant ....
Intrinsic resistance of *Pseudomonas* to macrolides

Azithromycin is widely and successfully used in Cystic Fibrosis patients

Intrinsic resistance of *Pseudomonas* to macrolides

Is *Pseudomonas* « intrinsically » resistant to macrolides?

Major role of constitutively-expressed transporters!

---

An intriguing observation ...

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>CA-MHB pH 7.4</th>
<th>CA-MHB pH 5.5</th>
<th>RPMI-1640 pH 5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aminoglycosides</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Amikacin</td>
<td>4</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>1</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td><strong>β-lactams</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piperacillin/Tazobactam</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Cefepime</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>8</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Meropenem</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Fluoroquinolones</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.125</td>
<td>0.25</td>
<td>0.125</td>
</tr>
<tr>
<td><strong>Polymyxins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colistin</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

| Azithromycin        | 128           | >512          | 16              |

Macrolides regain activity against *P. aeruginosa* in « eukaryotic » media

Why do macrolides express their activity in « eukaryotic » media?

Buyck et al.  
*Clin Infect Dis. 2012; 55:534-42*
Why do macrolides express their activity in « eukaryotic » media?

![Diagram showing outer membrane permeability and macrolide activity](image)

- **Why do macrolides express their activity in « eukaryotic » media?**
- **Diagram:**
  - **NPN and nitrocefin permeability**
  - **Outer membrane permeability (%)**
  - **ML (Macrolides)**
  - **OprM (Outer membrane porin)**
  - **MexAB-OprM**
  - **MexXY-OprM**
  - **High MIC (Minimum Inhibitory Concentration)** in MHB (Minimum Bacterial Concentration) RPMI (Reduced Protein Medium)
  - **Low MIC** in RPMI (Reduced Protein Medium)

**Legend:**
- A: MHB
- B: BAL
- C: RPMI
- a: MHB
- b: RPMI

---

16-09-2013
University of Notre-Dame, Notre Dame, IN
Why do macrolides express their activity in « eukaryotic » media?

ML

ML

OprM

MexAB-OprM

MexXY-OprM

ML

Σprot

ML

Σprot

ML

ML

ML

ML

MHB RPMI

1

2

0

4

8

C14-CLR accumulation (ng/mg of prot.)

MHB RPMI

Time (h)

0 4 8

Low MIC

High MIC

*
Why do macrolides express their activity in « eukaryotic » media?

1. oprM
2. MexAB-OprM
3. MexXY-OprM
4. oprM

RPMI-1640
CA MHB

no AZM
AZM 1 mg/L

oprM relative expression

MHB high MIC
RPMI low MIC
Why do macrolides express their activity in « eukaryotic » media?

- **ML**
  - oprM
  - X

- **Σprot**
  - MexAB-OprM
  - MexXY-OprM
  - oprM

- **ML**
  - high MIC
  - MHB

- **ML**
  - low MIC
  - RPMI

**Graph:**
- **3H-Leucin incorporation (% control)** vs. **Azithromycin concentration (mg/L)**
- **CA-MHB**
- **RPMI**

Legend:
- **MIC**
- **CA-MHB**
- **RPMI**
Why do macrolides express their activity in « eukaryotic » media?
Intrinsic resistance of *Pseudomonas* to macrolides

Is this medium effect clinically relevant?

![Graph showing cumulative percentage of MIC values for pneumonia and cystic fibrosis strains in MHB and RPMI media.](image)

CF strains = 345
pneumonia strains = 48

Mustafa, unpublished
Role of antibiotic efflux in intrinsic resistance ....

• Inactivating efflux may reveal antibiotic activity and could be a useful tool when developing new drugs

• Bacterial responsiveness to antibiotics may be highly different in the host than in the test tube
Role of efflux pumps in the clinics …
Efflux in \textit{S. pneumoniae}: is it important in the clinics?

Suspected efflux based on phenotypic analysis (CIP MIC +/- reserpine)

<table>
<thead>
<tr>
<th>Origin of Isolates</th>
<th>% Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsc{ap}</td>
<td>183 strains</td>
</tr>
<tr>
<td>B\textsc{pco}</td>
<td>107 strains</td>
</tr>
</tbody>
</table>

\textit{Lismond \& Degives, unpublished}
Efflux in *S. pneumoniae*: is it important in the clinics?

Identification of FQ transporters in clinical isolates

Inactivation of *patA* or *patB* as efficient as reserpine to reduce MIC
- responsible for FQ efflux in clinical isolates
- work as heterodimers

*Lismond et al, ECCMID 2010*
Efflux in *S. pneumoniae*: is it important in the clinics?

SubMICs concentrations of fluoroquinolones may induce efflux systems…

El Garch et al., JAC (2010) 65:2076-82

Optimal dosing is needed!
Efflux in *P. aeruginosa*: is it important in the clinics?

Prevalence of MexA and MexX overexpressers in 62 phylogenetically-related pairs of *P. aeruginosa* isolated from ICU patients (VAP)

Riou et al, ECCMID 2010
Efflux selection in *P. aeruginosa* during treatment

Antipseudomonal antibiotics received by the patients during treatment

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>no. patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperacillin-tazobactam (TZP)</td>
<td>26</td>
</tr>
<tr>
<td>Amikacin (AMK)</td>
<td>22</td>
</tr>
<tr>
<td>Meropenem (MEM)</td>
<td>20</td>
</tr>
<tr>
<td>Cefepime (CEF)</td>
<td>19</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>6</td>
</tr>
</tbody>
</table>

Antibiotic treatment selects for efflux-mediated resistance!

**Number of efflux systems detected at day 0 and day X**

**Global influence of treatment**

*Riou et al, ECCMID 2010*
MIC during treatment

Increases in MICs of antibiotics used in empirical antipseudomonal therapy between D0 and DX of treatment

... and this is associated with increase in MICs!

Early diagnosis should be implemented in the clinics

RND efflux pumps in *P. aeruginosa*: an underestimated resistance mechanism

An adequate initial antibiotic therapy is a key determinant of therapeutic success in *Pseudomonas aeruginosa*-infected patients. Antibiotic efflux is an underestimated resistance mechanism because it may occur in strains categorized as susceptible. It is rarely or not at all diagnosed in routine laboratories and often masked by high-level resistance mechanisms.

by Dr Laetitia Avrain, Dr Pascal Mertens and Professor Françoise Van Bambeke
Role of efflux pumps in the clinics …

- Efflux is present in clinical isolates and may be induced upon exposure to suboptimal antibiotic concentrations

- Detecting efflux in clinical isolates may help optimizing antibiotic selection and dosing
Antibiotic resistance by efflux: from molecular aspects to clinical impact

Take home message

**My molecular point of view:** Appropriate in vitro models may help you selecting antibiotics that are not affected by efflux

**My clinical point of view:** Early diagnosis of resistance mechanisms and optimal dosing are keys to success
Efflux and bacteria in our team ...

- L. Avrain
- M. Riou
- J. Buyck
- H. Chalhoub
- H. Mustafa
- F. el Garch
- C. Vallet
- A. Lismond
- V. Mohymont
- F. Degives
- N. Vandevelde

www.facm.ucl.ac.be