Molecular bases for antibiotic resistance through efflux

Françoise Van Bambeke

Pharmacologie cellulaire et moléculaire
Université catholique de Louvain
Brussels, Belgium

www.facm.ucl.ac.be
P. Ehrlich, a father of modern chemotherapy

Paul Ehrlich (1854-1945)
Magic bullets need to reach their target

"The goal is … to find chemical substances that have special affinities for pathogenic organisms and that, like magic bullets, go straight to their targets"

"corpora non agunt nisi fixata"

Paul Ehrlich (1854-1945)
Chemotherapeutic agents exert toxic effects on specific target cells.

- Antibiotics
- Antifungals
- Anticancer agents
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 ...

amphiphatic drug

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!

Chemotherapeutic agents exert toxic effects on specific target cells.

How can cells protect themselves from these toxic substances?
Why efflux transporters?

Extrusion by efflux pumps

Why efflux transporters?

Extrusion by efflux pumps

general mean of protection against cell invasion by diffusible molecules

Mechanisms of active efflux

Most antibiotics are amphiphilic!

cationic amphiphiles

- macrolide
- sulfamide
- tetracycline
- fluoroquinolone
- rifampicin
- lincosamide

Most antibiotics are amphiphilic!

Anionic amphiphiles

β-lactam

Fusidic acid

Fluoroquinolone

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>
<td></td>
</tr>
<tr>
<td>β-lactams</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>fusidic acid</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>macrolides</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>streptogramins</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>tetracyclines</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>aminoglycosides</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>chloramphenicol</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>rifamycins</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>sulfamides</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>trimethoprim</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>fluoroquinolones</td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
<td><img src="https://example.com/circle.png" alt="Circle" /></td>
</tr>
</tbody>
</table>

Most antibiotics do act on intracellular targets

(lipo- and glycopeptides)  (β-lactams)

quinolones  sulfamides

macrolides  tetracyclines

aminoglycosides

Efflux as a mechanism of resistance by reducing antibiotic concentration inside the bacteria
Antibiotic efflux from bacteria as a mechanism of antibiotic resistance: molecular bases
Efflux as a mechanism of export in antibiotic producers

Delivery of metabolites produced by the cell

René Magritte (1898-1967)
Efflux as a mechanism of export in antibiotic producers

Spectrum

Narrow

Specific for the produced antibiotic

ABC

OleB or C of S. antibioticus
→ oleandomycin
SmB of S. ambofaciens
→ macrolides
Tlrc of S. fradiae
→ tylosin

MFS

Ptr of S. pristinaespiralis
→ pristinamycin
LmrA of S. lincolnensis
→ lincomycin
RifP of A. mediterranei
→ rifampicin

Broad
Efflux as a mechanism of export in antibiotic producers

Tylosin-biosynthetic gene cluster in *S. fradiae*

Fig. 1 The tylosin-biosynthetic gene cluster of *S. fradiae*. Not drawn to scale. The cluster occupies a contiguous portion of the genome (approximately 85 kb). Regulatory genes are outlined in boxes. Tylosin-biosynthetic genes are represented by black arrows. Resistance determinants (designated 'trl'), ancillary genes and others that are unassigned are represented as grey arrows. The full complement of biosynthetic genes could, in principle, be expressed from three pairs of divergent promoters (stars) via operon control.

Efflux as a mechanism of antibiotic resistance in pathogenic bacteria

Pierre Paul Rubens (1530-1587)
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

specific for one (or a few) families of drugs

ABC

MFS

NorA of *S. aureus* → FQ,Tet, chl
MefE of *S. pneumoniae* → ML
PmrA of *S. pneumoniae* → FQ
MefA of *S. pyogenes* → ML
Efflux as a mechanism of resistance in Gram-negative bacteria

- **narrow spectrum**:
  - MFS
  - TetA of *E. coli* → Tet
  - MsrA of *S. epidermidis* → erythromycin

- **broad spectrum, conferring cross-resistance**:
  - RND
  - 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

Specific for one (or a few) families of drugs
Molecular determinants of drug efflux

Brussels, atomium (1958; Polak)
## Differences in transport between drugs within a class

### Usual difference in affinity of efflux pumps towards antibiotics

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>Affinity for efflux pumps</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
</tr>
<tr>
<td>Penicillins</td>
<td>nafcillin, cloxacillin, penicillin G</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>cefalotin, cefotaxime, ceftaxone</td>
</tr>
<tr>
<td>Carbapenems</td>
<td>meropenem</td>
</tr>
<tr>
<td>Macrolides</td>
<td>14 - and 15 - membered</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>tetracycline</td>
</tr>
<tr>
<td>Quinolones</td>
<td>ciprofloxacin, norfloxacin</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ depending on the efflux pump

Van Bambeke et al. (2003) JAC 51:1055-65
Differential efflux of β-lactams by AcrB of *S. typhimurium*

<table>
<thead>
<tr>
<th>R (Side chain)</th>
<th>molecule</th>
<th>side-chain partition coefficient</th>
<th>MIC ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂N</td>
<td>penicillin N</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>HOOC</td>
<td>carbenicillin</td>
<td>80</td>
<td>4</td>
</tr>
<tr>
<td>CO</td>
<td>penicillin G</td>
<td>270</td>
<td>32</td>
</tr>
<tr>
<td>CO</td>
<td>cloxacillin</td>
<td>890</td>
<td>256</td>
</tr>
<tr>
<td>CO</td>
<td>nafcillin</td>
<td>4200</td>
<td>128</td>
</tr>
</tbody>
</table>

* ratio between the MIC of a *S. typhimurium* resistant mutant (AcrAB-ToIC overproducer) and of its susceptible parental strain

**β-lactams & broad spectrum RND**

Lipophilicity of the side chain

Resistance

### Differential efflux of quinolones by NorA of *S. aureus*

**Quinolones & narrow spectrum MFS**

- **Bulkiness in 7 and 8**
- **Resistance**

### Relationship between structure, bulkiness, and hydrophobicity of selected quinolone substituents and MIC of bacteria resistant by efflux.

<table>
<thead>
<tr>
<th>molecule</th>
<th>( R_1 )</th>
<th>( R_7 )</th>
<th>( R_4 )</th>
<th>MIC ratio(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>△</td>
<td><img src="image1" alt="Chemical structure" /></td>
<td>OCF(_3)</td>
<td>2</td>
</tr>
<tr>
<td>(b)</td>
<td>△</td>
<td><img src="image2" alt="Chemical structure" /></td>
<td>OCH(_3)</td>
<td>4</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>△</td>
<td><img src="image3" alt="Chemical structure" /></td>
<td>H</td>
<td>16</td>
</tr>
<tr>
<td>(c)</td>
<td>( \text{C}_2\text{H}_5 )</td>
<td><img src="image4" alt="Chemical structure" /></td>
<td>H</td>
<td>16</td>
</tr>
<tr>
<td>norfloxacin</td>
<td>( \text{C}_2\text{H}_6 )</td>
<td><img src="image5" alt="Chemical structure" /></td>
<td>H</td>
<td>64</td>
</tr>
</tbody>
</table>

\(^a\) ratio between the MIC of a *S. aureus* resistant mutant (NorA overproducer) and of its susceptible parental strain.

**Takenouchi et al. (1996) AAC 40:1835-42**
Unexpected antibiotic substrates
Two types of unexpected substrates

(lipo- and glycopeptides) (β-lactams)

quinolones

macrolides

tetracyclines

sulfamides

aminoglycosides

active on an extracellular target!
β-lactams as unexpected substrates for efflux pumps

Yu et al. (2003) J. Bacteriol. 185:5657-64
β-lactams as unexpected substrates for efflux pumps

- Physical clearance
- Chemical clearance

β-lactamase
Contributions of the AmpC β-lactamase and the AcrAB Multidrug Efflux System in Intrinsic Resistance of *E. coli* to β-lactams

<table>
<thead>
<tr>
<th>Efflux</th>
<th>β-lactamase</th>
<th>CMI carbenicillin</th>
<th>CMI ofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>0.05</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>12.5</td>
<td>0.2</td>
</tr>
<tr>
<td>+++</td>
<td>-</td>
<td>50</td>
<td>1.56</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>100</td>
<td>0.05</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>200</td>
<td>0.39</td>
</tr>
<tr>
<td>+++</td>
<td>+</td>
<td>400</td>
<td>1.56</td>
</tr>
</tbody>
</table>

WT: intrinsic resistance!

Mazzariol *et al.* (2000) AAC 44:1387-1390
Two types of unexpected substrates

(lipo- and glycopeptides) (β-lactams)

- quinolones
- sulfamides
- tetracyclines
- macrolides
- aminoglycosides

Highly hydrophilic molecules!
Aminoglycosides as unexpected substrates for efflux pumps

Yu et al. (2003) J. Bacteriol. 185:5657-64

Composite binding site?

acidic PL as determinants in AG transport?

more acidic residues in the vestibule of an AG transporter!
efflux from eucaryotic cells as a mechanism of antibiotic ‘resistance’: molecular bases
Antibiotic efflux from eucaryotic cells and intracellular ‘resistance’?

Reduction in intracellular drug concentration can result in inefficacy against intracellular bacteria.
Antibiotics as substrates of MDR efflux pumps

**spectrum**

- **narrow**
  - P-glycoprotein
    - macrolides, rifampicin
    - anticancer agents
    - antidepressants, antiepileptics
    - digoxin
    - ...
  - MRP
    - fluoroquinolones, β-lactams
    - antiviral agents
    - anticancer agents
    - drug conjugates
    - ...

- **broad**

**Broad spectrum, several classes of drugs**
Phenotypic description of antibiotic transport by MDR pumps
Antibiotics as substrates of MDR efflux pumps

azithromycin

ciprofloxacin

moxifloxacin
J774 macrophages do express MDR pumps
ABC multidrug transporters

- Cationic amphiphiles
- Anionic amphiphiles
- MDR-1 (P-glycoprotein)
- MRP1-10

ATP → ADP
How to inhibit ABC transporters?

cationic amphiphiles

MDR-1 (P-glycoprotein)

anionic amphiphiles

MRP1-10

deoxyglucose

\( \text{NaN}_3 \)

ATP → ADP
How to inhibit ABC transporters?

cationic amphiphiles

MDR-1 (P-glycoprotein)

ATP → ADP
How to inhibit ABC transporters?

- probenecid
- anionic amphiphiles
- MK571

MRP1-10

ATP → ADP
Differential recognition of antibiotics by MDR pumps

Influence of ATP-depletion and pump inhibitors on accumulation at equilibrium

extracell. conc. 5 mg/L; AZM 3 h; CIP 2 h

Kinetics of accumulation and efflux for azithromycin

accumulation markedly increased; efflux marginally affected

extracell. conc. 5 mg/L; verapamil 20 µM

Seral et al. (2003) AAC 47:1047-51
Kinetics of accumulation and efflux for ciprofloxacin

both accumulation and efflux markedly affected

extracell. conc. 17 mg/L; probenecid 5 mM

Michot et al. (2004) AAC 48:2673-82
Kinetics of accumulation and efflux for moxifloxacin

neither accumulation nor efflux affected

eextracell. conc. 17 mg/L; probenecid 5 mM

Michot et al. AAC (2005) 49:2429-37
Quinolones as inhibitors of ciprofloxacin efflux

- ciprofloxacin efflux inhibited by ciprofloxacin

Michot et al. AAC (2005) 49:2429-37
Quinolones as inhibitors of ciprofloxacin efflux

- ciprofloxacin efflux inhibited by ciprofloxacin
- moxifloxacin not affected

Michot et al. AAC (2005) 49:2429-37
Quinolones as inhibitors of ciprofloxacin efflux

- ciprofloxacin efflux inhibited by ciprofloxacin and moxifloxacin

Michot et al. AAC (2005) 49:2429-37
Quinolones as inhibitors of ciprofloxacin efflux

- ciprofloxacin efflux inhibited by ciprofloxacin, moxifloxacin

Michot et al. AAC (2005) 49:2429-37
Azithromycin, ‘kick-back’ model

Ciprofloxacin, classical model

Moxifloxacin, ‘futile-cycle’ model

Eytan et al. (1996) JBC 271:12897-902
Pharmacological consequences of antibiotic transport
Models of intracellular infection

*L. monocytogenes*  
S. aureus

*cytosol*  
*phagolysosomes*
Influence of pump inhibitors on intracellular activity

azithromycin and *L. monocytogenes*

verapamil 20 µM; 24 h

Seral et al. (2003) JAC 51:1167-73
Influence of pump inhibitors on intracellular activity

azithromycin and S. aureus

verapamil 20 µM; 24 h

Seral et al. (2003) JAC 51:1167-73
Influence of pump inhibitors on intracellular activity
ciprofloxacin and *L. monocytogenes*

*gemfibrozil 250 µM; 24 h*

Seral *et al.* (2003) JAC 51:1167-73
Influence of pump inhibitors on intracellular activity

ciprofloxacin and S. aureus

gemfibrozil 250 µM; 24 h

S. aureus

"Seral et al. (2003) JAC 51:1167-73"
Influence of pump inhibitors on antibiotic distribution

verapamil enhances azithromycin concentration
In cytosol and vacuoles

Seral et al. (2003) JAC 51:1167-73
Influence of pump inhibitors on antibiotic distribution

gemfibrozil enhances ciprofloxacin cytosolic content

Seral et al. (2003) JAC 51:1167-73
Unexpected antibiotic substrates

Peyo (1928-1992)
Unexpected substrate

Log P = -4
Log D = -9.5 at pH 7

Highly polar molecule!

Lipophilic chain

Daptomycin

Polyanionic cyclic peptide
Daptomycin is substrate of P-gp

Daptomycin accumulation proportional to P-gp activity and expression level

Lemaire et al. (2007) AAC - Epub
Daptomycin is substrate of P-gp

Daptomycin intracellular activity is increased in the presence of P-gp inhibitors

Lemaire et al. (2007) AAC - Epub
Putative mechanism of daptomycin transport by P-gp

anchoring in the membrane towards the hydrophobic chain and extrusion from the membrane

Lemaire et al. (2007) AAC - Epub
Can we make eukaryotic cells resistant to antibiotics?
Can we make eukaryotic cells resistant to antibiotics?

Chronical exposure of J774 macrophages to increasing concentrations of ciprofloxacin

Michot et al. (2006) AAC 50:1689-95
Reduced drug accumulation in resistant macrophages

Michot et al. (2006) AAC 50:1689-95
Reduced drug accumulation in resistant macrophages

Michot et al. (2006) AAC 50:1689-95
Coworking between bacteria and macrophage pumps to reduce ciprofloxacin activity.
Coworking between bacteria and macrophage pumps to reduce ciprofloxacin activity

<table>
<thead>
<tr>
<th>FQ</th>
<th>MIC (mg/L)</th>
<th>EGD</th>
<th>CLIP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Res. (-)</td>
<td>Res. (+)</td>
<td>Res. (-)</td>
</tr>
<tr>
<td>CIP</td>
<td>1.2</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>MXF</td>
<td>0.6</td>
<td>0.6</td>
<td>0.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FQ</th>
<th>Cellular concentration (ng/mg prot)</th>
<th>WT</th>
<th>RS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prob. (-)</td>
<td>Prob. (+)</td>
<td>Prob. (-)</td>
</tr>
<tr>
<td>CIP</td>
<td>72</td>
<td>263</td>
<td>23</td>
</tr>
<tr>
<td>MXF</td>
<td>262</td>
<td>208</td>
<td>241</td>
</tr>
</tbody>
</table>

Lismond et al. (2006) ICAAC-A1108
Coworking between bacteria and macrophage pumps to reduce ciprofloxacin activity

<table>
<thead>
<tr>
<th>FQ</th>
<th>MIC (mg/L)</th>
<th>EGD</th>
<th>CLIP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Res. (−)</td>
<td>Res. (+)</td>
</tr>
<tr>
<td>CIP</td>
<td>1.2</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>MXF</td>
<td>0.6</td>
<td>0.6</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Same substrate specificity of the MFS procaryotic pump and of the ABC eucaryotic pump!

<table>
<thead>
<tr>
<th>FQ</th>
<th>Cellular concentration (ng/mg prot)</th>
<th>WT</th>
<th>RS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prob. (−)</td>
<td>Prob. (+)</td>
<td>Prob. (−)</td>
</tr>
<tr>
<td>CIP</td>
<td>72</td>
<td>263</td>
<td>23</td>
</tr>
<tr>
<td>MXF</td>
<td>262</td>
<td>208</td>
<td>241</td>
</tr>
</tbody>
</table>

Lismond et al. (2006) ICAAC-A1108
Coworking between bacteria and macrophage pumps to reduce ciprofloxacin activity

Lismond et al. (2006) ICAAC-A1108
No effect of bacteria and macrophage pumps on moxifloxacin activity

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>wild-type bacteria</th>
<th>resistant bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ log CF from time 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild-type macrophages</td>
<td>control</td>
<td>MXF</td>
<td>MXF + res.</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>MXF</td>
<td>MXF + res.</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>resistant macrophages</td>
<td>control</td>
<td>MXF</td>
<td>MXF + res.</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>MXF</td>
<td>MXF + res.</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>b</td>
<td>c</td>
</tr>
</tbody>
</table>

Lismond et al. (2006) ICAAC-A1108
Competition for transporters as a mechanism of drug interaction

Pieter Breughel (1525-1569)
Ciprofloxacin versus NSAIDs

ciprofloxacin

indomethacin

diclofenac
Ciprofloxacin versus NSAIDs

Huygge et al. Unpublished data
Ciprofloxacin versus NSAIDs

Huygue et al. Unpublished data
Ciprofloxacin versus NSAIDs

Huygue et al. Unpublished data
Ciprofloxacin versus NSAIDs

Indomethacin and, to a lesser extent, diclofenac, are inhibitors of ciprofloxacin transport.

Huygue et al. Unpublished data
NSAIDs as inhibitors of ciprofloxacin efflux

Dose-dependent effect on accumulation

related to slowed-down efflux

Huygue et al. Unpublished data
NSAIDs versus Ciprofloxacin

Indomethacin shares a same transporter with ciprofloxacin

Diclofenac is substrate of another gemfibrozil-inhibitable transporter

Huygue et al. Unpublished data
Conclusion: avenues for the future

Do we need to include ‘transport’ studies with bacteria and eucaryotic cells in the early development of new antibiotics?
The past and present efflux team in Brussels


C. Seral  N. Caceres  B. Marquez  N. Mesares  L. Avrain  M. Heremans  F. Renoird  M.C. Cambier  C. Misson  M. Vergauwen  N. Couwenbergh

seniors  students  PhD students  post-docs  technicians