Resistance to antibiotics: the rise of efflux...

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23 April 2015
What is in the menu?

- Brief overview of antibiotics and resistance
- Efflux: why does it exist and how has it been discovered?
- Why antibiotics?
- Main antibiotic efflux transporters
- Structure and mechanisms (examples: AcrAB-TolC)
- Antibiotic transporters important for the clinical microbiologist
- Substrate specificities
- Efflux and intrinsic susceptibility
- Efflux and clinical susceptibility and impact of treatment
- Cooperation with other mechanisms of resistance
- Inhibitors of efflux?
A very short (pictorial) (selective) survey of antibacterial chemotherapy

Cell wall
(β-lactams, glycopeptides)

Protein synthesis
tetracyclines, macrolides, aminoglycosides, …)

Replication
& transcription
(fluoroquinolones, ansamycines)

Membrane
(polymyxines, lipopeptides, …)

Enzymes
(sulfamides, diaminopyridines)
You said "antibiotic eflux"

No. of publications in PubMed with keywords: "antibiotic AND (efflux OR transporter)"
Historical landmarks …

- Successive description of efflux-mediated resistance for major classes of antibiotics

- tetracyclines
- fluoroquinolones
- macrolides
- β-lactams
- rifampin
- aminoglycosides
- linezolid
- daptomycin *

* in eucaryotic cells only (so far)
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Decreased Retention of Actinomycin D as the Basis for Cross-resistance in Anthracycline-resistant Sublines of P388 Leukemia

Makoto Inaba¹ and Randall K. Johnson²

Laboratory of Chemical Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Maryland 20014

Chart 2. Time course of uptake and efflux of actinomycin D by P388/S (○, ●), P388/ADR (△, ▲) and P388/DAU (□, ■) cells. Cells were incubated in the presence of actinomycin D, 0.04 μg/ml, for 60 min, washed, and reincubated in drug-free medium for an additional 60 min. Each point represents the mean of 3 determinations. The coefficient of variation was less than 10%.
Most chemotherapeutic agents must reach an intracellular target…

Inaba and Johnson, Cancer Res, 1977; 37:4629-34.

**Table 1**

*Subcellular distribution of [³H]actinomycin D in P388/S and P388/ADR cells after exposure to the drug (0.1 μg/ml) for 1 hr in vitro (uptake) followed 1 h incubation in drug-free medium (retention)*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Whole cells</th>
<th>Nuclear fraction</th>
<th>Mitochondrial fraction</th>
<th>Microsomal fraction</th>
<th>Cytoplasmic supernatant</th>
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<tbody>
<tr>
<td>Uptake</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>P388/S</td>
<td>1513 ± 2⁸</td>
<td>1014 ± 18 (67)⁹</td>
<td>31 ± 1 (2)</td>
<td>10 ± 1 (1)</td>
<td>409 ± 11 (27)</td>
</tr>
<tr>
<td>P388/ADR</td>
<td>672 ± 9</td>
<td>430 ± 1 (64)</td>
<td>41 ± 1 (6)</td>
<td>6 ± 0.2 (1)</td>
<td>198 ± 9 (29)</td>
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<tr>
<td>Retention</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>P388/S</td>
<td>1131 ± 3</td>
<td>766 ± 13 (68)</td>
<td>43 ± 1 (4)</td>
<td>8 ± 0.4 (1)</td>
<td>307 ± 8 (27)</td>
</tr>
<tr>
<td>P388/ADR</td>
<td>135 ± 3</td>
<td>88 ± 3 (65)</td>
<td>12 ± 3 (9)</td>
<td>2 ± 0.1 (1)</td>
<td>35 ± 1 (26)</td>
</tr>
</tbody>
</table>

⁸ Mean ± S.D.
⁹ Numbers in parentheses, percentage of total.

**Conclusion #1:** in order to survive to anticancer agents, cells "invented" efflux…
But antibiotics were first ...
Who remembers that graph?
Historical observations on tetracyclines …

Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*

(everted membrane vesicles/tetracycline transport/transposon Tn10/plasmids)

Laura McMurry, Richard E. Petrucci, Jr., and Stuart B. Levy*

Department of Molecular Biology and Microbiology and Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts 02111

Communicated by Boris Magasanik, April 21, 1980
Historical observations on tetracyclines ...

**FIG. 1.** Tetracycline uptake by *E. coli* ML308-225 (sensitive) and by R222-containing induced (resistant) cells with (○) and without (●) 1 mM DNP. Cells were grown overnight in medium A containing glucose and uptake was measured in the absence of added energy source.

**FIG. 2.** Tetracycline (Tc) uptake by everted membrane vesicles made from sensitive ML308-225 cells and from uninduced and induced R222-containing cells. ◯, No energy; ●, D-lactate. Cells were grown in glycerol and vesicles were frozen in 5 mM Tris-HCl, pH 7.2/70 mM KCl/0.25 mM dithiothreitol/50% glycerol. The assay was done at pH 6.6.

McMurry et al., PNAS 1980; 77:3974-3977
Historical observations

FIG. 3. Effects of MDR protein substrates or inhibitors on [3H]fluconazole uptake by cells from fluconazole-susceptible (■) and fluconazole-resistant (□) cultures of C. glabrata after 80 min of incubation in the standard uptake assay; the assay was extended to 180 min for verapamil. Values are means ± standard deviations of triplicate determinations with cells from one culture.


antibiotics
1965
1980

antifungal drugs
1980
1995

1977
anticancer drugs
Historical trends …

No. of publications in PubMed with keywords: efflux AND
- bacteria
- cancer
- fungi

Year (10 years interval: 0-9)

No. of publications in 10 years
Most chemotherapeutic agents must reach an **intracellular** target...

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 …

amphipathic 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!

Why efflux transporters?

Extrusion by efflux pumps

Why efflux transporters?

Extrusion by efflux pumps

general mean of protection against cell invasion by diffusible molecules

Typical ‘toxic’ diffusible substances as substrates for efflux pumps

antibiotics

antifungals

anticancer agents
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  - Inhibitors of efflux
Most antibiotics are amphiphilic!

Neutral

chloramphenicol

Most antibiotics are amphiphilic!

- **fluoroquinolones**
- **beta-lactams**
- **fusidic acid**

Most antibiotics are amphiphilic!

cationic

- lincomamides
- macrolides
- tetracyclines
- rifampicin
- fluoroquinolones
- sulfamides

Most antibiotics are amphiphilic!
Antibiotic classes recognized by efflux pumps in different types of organisms

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>bacteria Gram (+)</th>
<th>bacteria Gram(-)</th>
<th>fungi</th>
<th>superior eucaryotes</th>
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<tbody>
<tr>
<td>β-lactams</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>fusidic acid</td>
<td></td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>macrolides</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>streptogramins</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>tetracyclines</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>aminoglycosides</td>
<td></td>
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<td>✓</td>
<td>✓</td>
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<td>chloramphenicol</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>rifamycins</td>
<td></td>
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<td>✓</td>
<td>✓</td>
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<tr>
<td>sulfamides</td>
<td></td>
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<td>✓</td>
<td>✓</td>
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<tr>
<td>trimethoprim</td>
<td></td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>fluoroquinolones</td>
<td></td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
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Structure of pumps in procaryotic cells


RND, MFS, ABC

Gram-positive

Gram-negative

AB

pore

porin

lipoprotein

periplasm

pump

H^+

Na^+

ATP

ADP
Some abbreviations

- **ABC**: ATP Binding Cassette
- **MATE**: Multi Antimicrobial Extrusion
- **MFS**: Major Facilitator Superfamily
- **RND**: Resistance Nodulation Division
- **SMR**: Small Multidrug Resistance
Antibiotic efflux transporters are ubiquitous

distribution

prokaryotes
eukaryotes

Gram (+)
Gram (-)

MATE
SMR
MFS
RND

Mesaros et al., Louvain médical (2005) 124:308-20
Efflux and resistance in pathogenic bacteria

1 bacteria → several pumps → multiresistance

1 pump → several classes of antibiotics → crossresistance

1 class of antibiotics → several pumps → efficacy of inhibitors?
A brief survey of the many transporters (2003)

Leading articles

Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy

F. Van Bambeke¹*, Y. Glupczynski², P. Plésiat³, J. C. Pechère⁴ and P. M. Tulkens¹

¹Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain, Brussels; ²Laboratoire de Microbiologie, Cliniques Universitaires de Mont-Godinne, Université Catholique de Louvain, Yvoir, Belgium; ³Laboratoire de Bactériologie, Centre Hospitalier Universitaire Jean Minjoz, Besançon, France; ⁴Département de Microbiologie, Université de Genève, Geneva, Switzerland

Keywords: antibiotic, efflux, transporters, prokaryotes, resistance
A brief survey of the many transporters (2003)

1. Gram +

Table 1. Main efflux transporters as observed in clinically important human pathogens with their corresponding antibiotic substrates²

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Transporter</th>
<th>Superfamily</th>
<th>TC number</th>
<th>β-lactams</th>
<th>Antibiotics</th>
<th>Q</th>
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<tr>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>peni</td>
<td>ceph</td>
<td>carb</td>
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<tr>
<td><em>S. aureus</em></td>
<td>NorA⁷</td>
<td>MFS</td>
<td>2.A.1.2.10</td>
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<tr>
<td></td>
<td>TetK-L⁵⁹</td>
<td>MFS</td>
<td>2.A.1.3.6</td>
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<tr>
<td></td>
<td>MdeA⁶⁰</td>
<td>MFS</td>
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<tr>
<td></td>
<td>MsrA⁶</td>
<td>ABC</td>
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<tr>
<td></td>
<td>MefE⁶¹</td>
<td>MFS</td>
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<tr>
<td></td>
<td>PmrA⁶²</td>
<td>MFS</td>
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<td></td>
<td>TetK-L</td>
<td>MFS</td>
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<tr>
<td></td>
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<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>MdrL²³</td>
<td>MFS</td>
<td></td>
<td>-23</td>
<td>+23</td>
<td>-23</td>
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<tr>
<td></td>
<td>Lde⁶⁴</td>
<td>MFS</td>
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<td></td>
<td>TetK-L</td>
<td>MFS</td>
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<tr>
<td></td>
<td>Mmr⁶⁵</td>
<td>SMR</td>
<td>2.A.7.1.2.</td>
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<tr>
<td><em>L. monocytogenes</em></td>
<td></td>
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<tr>
<td><em>Mycobacterium tuberculosis</em></td>
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<tr>
<td><em>Enterococcus spp.</em></td>
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</tbody>
</table>
A brief survey of the many transporters (2003)

2. Gram - (part #1)

Table 1. Main efflux transporters as observed in clinically important human pathogens with their corresponding antibiotic substrates

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Transporter</th>
<th>Super-family</th>
<th>TC number&lt;sup&gt;b&lt;/sup&gt;</th>
<th>β-lactams</th>
<th>Antibiotics</th>
<th>Q</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>peni</td>
<td>ceph</td>
<td>carb</td>
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<tr>
<td><em>H. influenzae</em></td>
<td>TetB, K</td>
<td>MFS</td>
<td>2.A.6.2.5</td>
<td>+72</td>
<td>+72</td>
<td>+72</td>
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<tr>
<td></td>
<td>AcrB-like</td>
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<td>2.A.6.2.5</td>
<td>+72</td>
<td>+72</td>
<td>+72</td>
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<td><em>Neisseria gonorrhoeae</em></td>
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<td>+74</td>
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<td>FloR&lt;sup&gt;75&lt;/sup&gt;</td>
<td>MFS</td>
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<td><em>Shigella dysenteriae</em></td>
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<td>SMR</td>
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<td><em>E. coli</em></td>
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<td>+79</td>
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</table>
A brief survey of the many transporters (2003)

2. Gram - (part #2)

Table 1. Main efflux transporters as observed in clinically important human pathogens with their corresponding antibiotic substrates.a

<table>
<thead>
<tr>
<th>Pathogen</th>
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<th>Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>SmeE^94</td>
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<td>+95</td>
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<td></td>
<td>CmlA^96</td>
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<td>2.A.1.2.3</td>
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<td>+95</td>
<td></td>
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<tr>
<td>TetA,C,E</td>
<td>MFS</td>
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<td>+96</td>
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<td>MexB^97</td>
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<td>2.A.6.2.6</td>
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<td>+99</td>
<td>+99</td>
<td>+95</td>
</tr>
<tr>
<td>MexD^102</td>
<td>RND</td>
<td></td>
<td>+101</td>
<td>+72</td>
<td>+100</td>
<td></td>
</tr>
<tr>
<td>MexF^103</td>
<td>RND</td>
<td></td>
<td>+104</td>
<td>+104</td>
<td>+100</td>
<td></td>
</tr>
<tr>
<td>MexK^105</td>
<td>RND</td>
<td></td>
<td>+105</td>
<td>+105</td>
<td>+105</td>
<td></td>
</tr>
<tr>
<td>MexY^106</td>
<td>RND</td>
<td></td>
<td>+101</td>
<td>+101</td>
<td>+101</td>
<td></td>
</tr>
</tbody>
</table>

ABC, ATP binding cassette superfamily; MATE, multi-antimicrobial extrusion; MFS, major facilitator superfamily; RND, resistance nodulation division; SMR, small multidrug resistance; peni, penicillins; ceph, cephalosporins; carb, carbapenems; m-bac, monobactams, inhib β-ase, inhibitors of β-lactamases; FA, fusidic acid; AG, aminoglycosides; Tet, tetracyclines; OX, oxazolidinones; ML, macrolides; SG, synergistsin, LM, lincosamines; CHL, chloramphenicol; RIF, rifampicin; Q, quinolones; NAL, nalidixic acid; FQ, fluoroquinolones; SM, sulfamides; TMP, trimethoprim.

^a, occurrence; -, absence (in both cases, through functional studies).

^aAccording to the classification of Saier.
A brief survey of the many transporters (2009)

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**Efflux-Mediated Drug Resistance in Bacteria: an Update**

Xian-Zhi Li\(^1\) and Hiroshi Nikaido\(^2\)

\(^{1}\) Human Safety Division, Veterinary Drugs Directorate, Health Products and Food Branch, Health Canada, Ottawa, Ontario K1A OK9, Canada

\(^{2}\) Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3202, USA

809 references
A brief survey of the many transporters: *S. aureus*

Fig. 3. Regulation of multidrug or drug-specific efflux transporters of *S. aureus*. The efflux transporters are shown in colour blocks. All regulators are presented in the green boxes, and their functions as repressor or activator are indicated, respectively, by the green or orange dotted arrows. Unknown regulators are marked with a question mark (?) with the dotted grey lines linked to the relevant transporters. See text and relevant references for details of the regulation.

14 distinct transporters for *S. aureus* (only) in 2009 vs. 4 in 2003
What do you wish to know?

- Specific information about antibiotic transporters in procaryotes

ARDB-Antibiotic Resistance Genes Database

Multidrug Transporters

The acquisition of multidrug resistance is a serious impediment to improved healthcare. Multidrug resistance is most frequently due to active transporters that pump a broad spectrum of chemically distinct, cytotoxic molecules out of cells, including antibiotics, antimalarials, herbicides and cancer chemotherapeutics in humans. Active membrane transporters, whatever their substrate, fall into a relatively small number of protein superfamilies which include four important distinct superfamilies: (1) the ABC family (ATP-binding cassette); (2) the MFS family (major facilitator superfamily); (3) the RND family (resistance-nodulation-division); (4) the SMR family (small multidrug resistance).

http://ardb.cbcb.umd.edu/browse/multidrug.shtml
What is in the menu?

- Brief overview of antibiotics and resistance
- Efflux: why does it exist and how has it been discovered
- Why antibiotics?
- Main antibiotic efflux transporters
- **Structure and mechanisms** (an example with AcrAB-TolC)
- Antibiotic transporters important for the clinical microbiologist
- Substrate specificities
- Efflux and intrinsic susceptibility
- Efflux and clinical susceptibility and impact of treatment
- Cooperation with other mechanisms of resistance
- Inhibitors of efflux?
Mechanisms of transport

General structure of an RND (AcrAB-TolC)

Fig. 1. An early schematic view of the tripartite pump complex. Note that amphiphilic substrates (empty and filled-in rectangles represent hydrophobic and hydrophilic parts of the molecule) are hypothesized to be captured either from the periplasm (or the periplasm–plasma membrane interface) or from the cytosol (or the cytosol–membrane interface). For the latter process, two possible pathways are envisaged: either the substrate is flipped over to the outer surface of the membrane first and then follows the regular periplasmic capture pathway, or it follows a different capture pathway from the cytosol. From [5].

Nikaido & Takatsuka, Biochimica et Biophysica Acta 1794 (2009) 769–781
AcrB in more details

(B) AcrB trimer. Each protomer is shown in cyan, mauve, and blue. The large central cavity (thick black lines) is connected to the periplasm through vestibules (thick dotted lines) between protomers. Substrate molecules (ciprofloxacin) bound to the ceiling of the central cavity are shown in green stick models. Proximal portion of the structure was cut away to reveal the presence of vestibule. Drawn by using PyMol with Protein Data Bank coordinate 1OYE.

Nikaido & Takatsuka, Biochimica et Biophysica Acta 1794 (2009) 769–781
Proposed AcrB drug / H⁺ exchange

Fig. 10. Schematic representation of the AcrB alternating site functional rotation transport mechanism. The conformational states loose (L), tight (T), and open (O) are colored blue, yellow and red, respectively. Only two of the three monomers of the AcrB trimer are shown in side-view. AcrA and ToIC are indicated in light green and grey, respectively. The proposed proton translocation site (D407, D408, and K940) is indicated in the membrane part of each monomer. In the first state of the cycle (from left to right), a monomer binds a substrate (acridine) in its transmembrane domain (L conformation), subsequently transports the substrate from the transmembrane domain to the hydrophobic binding pocket (conversion to T conformation) and finally releases the substrate in the funnel toward ToIC (O conformation). Peristaltic transport of drugs through the AcrB tunnels (indicated by the red arrow) and through ToIC in combination to the line up of drug molecules inside the AcrB funnel and the ToIC channel would account for a strict unidirectional movement towards the outside of the cell. The conversion from the T monomer to the O monomer conformation is suggested to be the major energy-requiring (proton motive force-dependent) step in this functional rotation cycle and requires the binding of a proton to the proton translocation site (D407, D408, and K940) from the periplasm. The release of a proton from the proton translocation site to the cytoplasm might occur during conversion from the O monomer to the L monomer (as depicted) or from the latter to the T monomer. AcrA is expected to participate in the transduction of the conformational changes from AcrB to ToIC (indicated by black arrows), which results in the movement of the proximal part of ToIC and the facilitation of drug extrusion to the outside of the cell. From Seeger et al. [11] with permission.
AcrB-ToIC is a multidrug transporter

Fig. 1. Substrates and inhibitors of the AcrAB-ToIC efflux system. The system confers resistance to a wide variety of noxious substances like dyes, different classes of antibiotics, detergents, bile salts and small organic molecules. Phe-Arg-β-naphthylamide and 1-(1-naphthylmethyl)-piperazine (NMP) inhibit RND/MFP/OMF efflux systems. From Seeger et al. [11] with permission.

How can AcrB be a multi-drug?

LETTER

Structures of the multidrug exporter AcrB reveal a proximal multisite drug-binding pocket

Ryosuke Nakashima1*, Kelsuke Sakurai1*, Seiji Yamasaki2, Kunihiko Nishino3 & Akihito Yamaguchi1,2


• Our structures indicate that there are two discrete multisite binding pockets along the intramolecular channel.

• High-molecular-mass drugs (rifampicin1, erythromycin2) first bind to the proximal pocket in the access state and are then forced into the distal pocket in the binding state by a peristaltic mechanism involving subdomain movements that include a shift of the Phe-617 loop.

• By contrast, low-molecular-mass drugs, such as minocycline3 and doxorubicin4, travel through the proximal pocket without specific binding and immediately bind to the distal pocket.

• The presence of two discrete, high-volume multisite binding pockets contributes to the remarkably broad substrate recognition of AcrB.

1 822; 2 733; 3 457; 4 543
Interplay of RND and porins

*Fig. (1). Antibiotic transport through the membranes of Gram-negative bacteria (reproduced from [168]).*

Rosner JL, Martin RG.
What is in the menu?

- Brief overview of antibiotics and resistance
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- Substrate specificities
- Efflux and intrinsic susceptibility
- Efflux and clinical susceptibility and impact of treatment
- Cooperation with other mechanisms of resistance
- Inhibitors of efflux?
Efflux as a significant mechanism of resistance in Gram-positive bacteria

- **Spectrum**
  - **Narrow**: specific for one (or a few) families of drugs
  - **Broad**: specific for one (or a few) families of drugs

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

- **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)
  *Gill et al., AAC 1999; 43:187-9*

- **NorA** (Sa)
  *Gill et al., AAC 1999; 43:187-9*

*Terry et al., Nature Reviews Microbiology 2005; 3: 566-572*
Efflux as a significant mechanism of resistance in Gram-negative bacteria

**Spectrum**

- **Narrow**
  - MFS
  - TetA of *E. coli* → Tet

- **Broad**
  - 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
Efflux and resistance in *P. aeruginosa*

<table>
<thead>
<tr>
<th>Efflux Proteins</th>
<th>Expression Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>MexB, MexY</td>
<td>Constitutive basal expression; overexpressed upon induction</td>
</tr>
<tr>
<td>MexA, MexX</td>
<td>No basal expression; expression upon induction</td>
</tr>
<tr>
<td>OprM, OprM</td>
<td>No basal expression; expression upon induction</td>
</tr>
</tbody>
</table>

**Diagram:**
- OMP: outer membrane protein
- CMP: cytoplasmic membrane protein
- EM: external membrane
- MFP: membrane fusion protein
- P: porin

**Legend:**
- ♦ antibiotique

**Notes:**
- Mesaros et al. (2005) Louvain médical. 124:308-20
What is in the menu?

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- Efflux and intrinsic susceptibility
- Efflux and clinical susceptibility and impact of treatment
- Cooperation with other mechanisms of resistance
- Inhibitors of efflux
Early data with $\beta$-lactams

<table>
<thead>
<tr>
<th>$\beta$-Lactam</th>
<th>$\text{MIC}<em>{\text{wt}} / \text{MIC}</em>{\Delta\text{acrAB}}$ in $E. \text{coli}$ K-12$^a$</th>
<th>$\text{MIC}<em>{\text{wt}} / \text{MIC}</em>{\text{acr}}$ in $S. \text{typhimurium}$</th>
<th>Side chain lipophilicity$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloxacillin</td>
<td>128</td>
<td>256</td>
<td>890</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>512</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mezlocillin</td>
<td>32</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>16</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>16</td>
<td>ND</td>
<td>55$^d$</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>4</td>
<td>4</td>
<td>80</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>2</td>
<td>32</td>
<td>270</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>4</td>
<td>4</td>
<td>130</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>2</td>
<td>2</td>
<td>130</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Cefsulodin</td>
<td>1</td>
<td>1</td>
<td>80$^e$</td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Cefepime</td>
<td>1</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>Cepirome</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td><strong>Imipenem</strong></td>
<td>1</td>
<td>1</td>
<td><strong>0.3</strong></td>
</tr>
</tbody>
</table>

$^a$ Based on Table 1 data. wt, wild type.

$^b$ From reference 18.

$^c$ Expressed as the calculated octanol-water partition coefficient. From reference 18.

$^d$ Calculated as described in reference 18.

$^e$ Although the phenyl group shows a moderate lipophilicity, insertion of this side chain may be prevented by the presence of a negatively charged group next to it (18).

Substrate specificity of efflux pumps

14 fluoroquinolones; Gram + versus Gram +

S. aureus vs S. pneumoniae

Vallet et al. ECCMID 2011

Similar recognition for non phylogenetically-related transporters
Substrate specificity of efflux pumps

14 fluoroquinolones; Gram + versus Gram -

P. aeruginosa vs S. aureus

P. aeruginosa vs S. pneumoniae

All fluoroquinolones are substrates for broad spectrum transporters from Gram -

Vallet et al. (2011) ECCMID
What is in the menu?

- Brief overview of antibiotics and resistance
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- Substrate specificities
- **Efflux and intrinsic susceptibility**
- Efflux and clinical susceptibility and impact of treatment
- Cooperation with other mechanisms of resistance
- Inhibitors of efflux?
## Pseudomonas and penem efflux

<table>
<thead>
<tr>
<th>Mex pumps</th>
<th>MICs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB CD XY</td>
<td>MERO IMI BIA PANI FARO RITI</td>
</tr>
<tr>
<td>- - -</td>
<td>0.032 0.25 0.25 0.25 1 2</td>
</tr>
<tr>
<td>+ * - -</td>
<td>0.25 1 0.5 4 512 128</td>
</tr>
<tr>
<td>++ - -</td>
<td>1 0.25 0.25 1 4096 256</td>
</tr>
<tr>
<td>- ++ -</td>
<td>0.25 0.125 0.063 0.25 16 4</td>
</tr>
<tr>
<td>- - ++</td>
<td>0.063 0.25 0.25 2 4 8</td>
</tr>
</tbody>
</table>

* clinical isolate, basal level of expression

Pseudomonas and penem efflux

<table>
<thead>
<tr>
<th>Mex pumps</th>
<th>MICs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MERO</td>
</tr>
<tr>
<td>AB CD XY</td>
<td></td>
</tr>
<tr>
<td>- - -</td>
<td>0.032</td>
</tr>
<tr>
<td>+ * - -</td>
<td>0.25</td>
</tr>
<tr>
<td>++ - -</td>
<td>1</td>
</tr>
<tr>
<td>- ++ -</td>
<td>0.25</td>
</tr>
<tr>
<td>- - ++</td>
<td>0.063</td>
</tr>
</tbody>
</table>

* clinical isolate, basal level of expression

**S. pneumoniae** and fluoroquinolones

MIC distribution for 184 isolates from community-acquired pneumonia

- **Efflux (+) strains considered as susceptible**

- **FQ with high intrinsic activity can be substrates for efflux!**

Lismond et al., JAC (2011) 66:948-951
**P. aeruginosa and temocillin**

**Pseudomonas aeruginosa and 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 (+ PAβN)</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>Reference strain</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PAO1</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Engineered strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th><em>mexA</em> <em>a</em></th>
<th><em>mexX</em> <em>a</em></th>
<th><em>oprM</em> <em>a</em></th>
<th><em>mexC</em> <em>b</em></th>
<th><em>mexE</em> <em>b</em></th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB 536</td>
<td>PAO1 Δ<em>mexCD-oprJ</em></td>
<td>1.09</td>
<td>1.65</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>128 (16)</td>
</tr>
<tr>
<td>CB603</td>
<td>PAO1 Δ<em>mexEF-oprN</em></td>
<td>1.21</td>
<td>1.02</td>
<td>0.51</td>
<td>-</td>
<td>-</td>
<td>128 (32)</td>
</tr>
<tr>
<td>CB602</td>
<td>PAO1 <em>mexXY::FRT</em></td>
<td>1.10</td>
<td>0.06</td>
<td>0.55</td>
<td>-</td>
<td>+</td>
<td>64 (16)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th><em>mexA</em> <em>a</em></th>
<th><em>mexX</em> <em>a</em></th>
<th><em>oprM</em> <em>a</em></th>
<th><em>mexC</em> <em>b</em></th>
<th><em>mexE</em> <em>b</em></th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1 <em>mexAB</em></td>
<td>PAO1 <em>mexAB::FRT</em></td>
<td>0 m</td>
<td>1.08</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>4 (2)</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 …

<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></td>
<td></td>
</tr>
<tr>
<td>3020R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3525</td>
<td>isogenic to 3020S with deletion in mexA</td>
<td>128</td>
</tr>
<tr>
<td>3807</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>2715</td>
<td></td>
<td>512</td>
</tr>
<tr>
<td>616</td>
<td>isogenic to 3525 with mutation in mexA</td>
<td>32</td>
</tr>
<tr>
<td>2729</td>
<td>mutation in mexA</td>
<td>32</td>
</tr>
<tr>
<td>2933</td>
<td>deletion in mexA</td>
<td>1</td>
</tr>
<tr>
<td>2998</td>
<td>deletion in mexA</td>
<td>2</td>
</tr>
<tr>
<td>2721</td>
<td>deletion in mexA</td>
<td>2</td>
</tr>
<tr>
<td>2716</td>
<td>mutation in mexB</td>
<td>1</td>
</tr>
<tr>
<td>2804</td>
<td>deletion in mexB</td>
<td>4</td>
</tr>
<tr>
<td>2858</td>
<td>deletion in mexB</td>
<td>1</td>
</tr>
<tr>
<td>3066</td>
<td>deletion in mexB</td>
<td>1</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!
Conditions modulating efflux and susceptibility

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 Cai¹, Dong Chai¹, Rui Wang¹*, Nan Bai¹, Bei-Bei Liang¹ and Youning Liu²

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 (SD)</th>
<th>Placebo mean (SD)</th>
<th>Mean difference</th>
<th>Mean difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clement 2006¹⁰</td>
<td>-8.7 (11.5)</td>
<td>-2.7 (21)</td>
<td>4.4 %</td>
<td>-6.00 [-21.03, 9.03]</td>
</tr>
<tr>
<td>Saiman 2003¹²</td>
<td>4.4 (13.6)</td>
<td>-1.8 (10.7)</td>
<td>77.9 %</td>
<td>6.20 [2.64, 9.76]</td>
</tr>
<tr>
<td>Steinkamp 2008⁸</td>
<td>-3.7 (13.3)</td>
<td>-5 (10.1)</td>
<td>17.8 %</td>
<td>1.30 [-6.14, 8.74]</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>
</tr>
</tbody>
</table>

FEV₁% change in P. aeruginosa-infected patients

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 supposedly intrinsically resistant ....
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>RPMI-1640 pH 5.5</th>
<th>RPMI-1640 pH 5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</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>β-lactams</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>Fluoroquinolones</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>Polymyxins</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 against *P. aeruginosa* in « eukaryotic » media?

![Diagram of macrolide activity and efflux systems](image)

- **ML**: Macrolides
- **Σprot**: Total protein
- **Σprot**: Total protein
- **MexAB-OprM**, **MexXY-OprM**: Efflux systems
- **OprM**: Efflux pump
- **oprM**: Efflux pump gene
- **MHB**: Medium containing high MIC
- **RPMI**: Medium containing low MIC

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*Buyck et al.*
*Clin Infect Dis. 2012; 55:534-42*
What is in the menu?

- Brief overview of antibiotics and resistance
- Efflux: why does it exist and how has it been discovered
- Why antibiotics?
- Main antibiotic efflux transporters
- Structure and mechanisms (an example with AcrAB-TolC)
- Antibiotic transporters important for the clinical microbiologist
- Substrate specificities
- Efflux and intrinsic susceptibility
- **Efflux and clinical susceptibility and impact of treatment**
- Cooperation with other mechanisms of resistance
- Cooperation between procaryotic and eucaryotic transporters
Efflux of fluoroquinolones in *S. pneumoniae*: is transporter more expressed in patients chronically treated

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

- **Reserpine effect on MIC (x dilutions)**
  - Green: ≤ 1
  - Orange: < 2
  - Red: ≥ 2

- **% strains**
  - 0
  - 20
  - 40
  - 60
  - 80
  - 100

- **Origin of isolates**
  - **CAP**: 183 strains
  - **BPCO**: 107 strains

- **Acute pathology**: « one shot » antibiotic exposure
- **Chronic pathology**: repetitive antibiotic exposures

*Lismond & Degives, unpublished*
Impact of efflux on clinical susceptibility of *P. aeruginosa*

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

Riou et al, ECCMID 2010
Increase of *P. aeruginosa* during treatment: is efflux involved?

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

Riou et al, ECCMID 2010
Riou et al. submitted for publication
Early diagnosis could 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
Early diagnosis could be implemented in the clinics.

RND efflux, an underestimated resistance mechanism...
What is in the menu?

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- Efflux and clinical susceptibility and impact of treatment
- **Cooperation with other mechanisms of resistance**
- Cooperation between procaryotic and eucaryotic transporters
Efflux cooperates with other mechanisms of bacterial resistance
Efflux cooperates with other mechanisms of resistance

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>

Mazzariol *et al*, AAC (2000) 44:1387-1390
Efflux and selection of resistance to FQ

Exposure to subMIC concentrations favors the selection of target mutations!
### Efflux and selection of resistance

#### Frequency of Levofloxacin-resistant mutants in *Pseudomonas aeruginosa* with deletions of the efflux pump operons

<table>
<thead>
<tr>
<th>Pump status</th>
<th>LVX MIC</th>
<th>Frequency of LVX-resistant mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.25</td>
<td>$2 \times 10^7 - 4 \times 10^7$</td>
</tr>
<tr>
<td>$\Delta$ mexAB-oprM</td>
<td>0.015</td>
<td>$2 \times 10^7 - 4 \times 10^7$</td>
</tr>
<tr>
<td>$\Delta$ mexCD-oprJ</td>
<td>0.25</td>
<td>$2 \times 10^7 - 4 \times 10^7$</td>
</tr>
<tr>
<td>$\Delta$ mexEF-oprN</td>
<td>0.25</td>
<td>$2 \times 10^7 - 4 \times 10^7$</td>
</tr>
<tr>
<td>$\Delta$ mexAB-oprM; $\Delta$ mexEF-oprN</td>
<td>0.015</td>
<td>$2 \times 10^7 - 10^7$</td>
</tr>
<tr>
<td>$\Delta$ mexCD-oprJ; $\Delta$ mexEF-oprN</td>
<td>0.25</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>$\Delta$ mexAB-oprM; $\Delta$ mexCD-oprJ</td>
<td>0.015</td>
<td>$1 \times 10^9$</td>
</tr>
<tr>
<td>$\Delta$ mexAB-oprM; $\Delta$ mexCD-oprJ; $\Delta$ mexEF-oprN</td>
<td>0.015</td>
<td>$&lt;1 \times 10^{11}$</td>
</tr>
</tbody>
</table>


Selection of mutants in FQ target undetectable if ALL pumps are disrupted
What is in the menu?

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- Efflux and intrinsic susceptibility
- Efflux and clinical susceptibility and impact of treatment
- Cooperation with other mechanisms of resistance
- Inhibitors of efflux?
And now, can we make inhibitors of efflux?

- There are a LARGE number of inhibitors.
- Many are endowed with other pharmacological activities that appear already at lower concentrations than what is needed to impair efflux (e.g., reserpine).
- Others are very effective but also very toxic (e.g., Phenylalanine-arginine-β-naphthylamide [PAβN; MC-MC-207110]).
- The search for microbiologically-active and safe-to-host inhibitors is ongoing but with little “drug” success so far…