Antibiotic transporters: From Discovery to Clinical Implications

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Influenced largely by
• Françoise Van Bambeke, Béatrice Marquez, Youri Glupczynski (UCL) and Martine Prevost (ULB)
• my participation to the European Committee for Antibiotic Susceptibility Testing (EUCAST)

The slides are available from http://www.facm.ucl.ac.be – follow "Lectures"
Steps and Challenges of efflux in antibacterial chemotherapy

• recognizing its existence:
  is it a significant mechanism of resistance?

• which pumps …
  is antibiotic efflux different from other drug efflux?

• defining its role:
  does it need to change our vision on (and decisions about) existing antibiotics?

• setting up diagnostic techniques:
  how can we detect it today (and do we need to do this?)

• treating patients:
  – can we make non-effluxed drugs?
  – and what about efflux inhibitors?
  – is efflux important in pharmacokinetics/drug interactions?
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Historical observations on tetracyclines …


DISAPPEARANCE OF OXYTETRACYCLINE ACCUMULATION IN THE CELLS OF MULTIPLE DRUG-RESISTANT ESCHERICHIA COLI.

IZAKI K, ARIMA K.

PMID: 14087909 [PubMed - indexed for MEDLINE]

Who remembers that car?
Historical observations on tetracyclines ...

Who remembers that graph?
Resistance of *Escherichia coli* to Tetracyclines

By T. J. Franklin and A. Godfrey

Imperial Chemical Industries Ltd. (Pharmaceuticals Division),
Alderley Park, Macclesfield, Cheshire

(Received 23 March 1964)

1. A strain of *Escherichia coli* highly resistant to chlorotetacycline and partially cross-resistant to tetracycline has been isolated. 2. The nitro-reductase system of the resistant cells was inhibited to a smaller extent by chlorotetacycline than was the corresponding enzyme of sensitive cells. 3. The incorporation of leucine in vitro into the ribosomal protein of cell-free preparations from sensitive and resistant cells was equally inhibited by chlorotetacycline. 4. Resistant cells accumulated much less chlorotetacycline and tetracycline than did sensitive cells when both were cultured in the presence of these drugs. 5. The uptake of tetracycline by both sensitive and resistant *E. coli* was dependent on the presence of glucose in the medium. 6. Fractionation of cells cultured in medium containing [\(^{14}\)C]chlorotetacycline indicated that the largest proportion of radioactivity in sensitive cells was in the fraction consisting mainly of cell-wall material. There was no concentration of radioactivity in any one fraction of the resistant cells. 7. No evidence could be obtained for a specific tetracycline-excretion system in the resistant cells. 8. The significance of these results in relation to current theories of the antibiotic action of and resistance to the tetracycline drugs is discussed.
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Historical observations on tetracyclines …

Proc. Natl. Acad. Sci. USA
Biochemistry

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 6.8.

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

No. of publications in PubMed with keywords: antibiotic & efflux
Historical landmarks …

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

Successive description of efflux-mediated resistance for major antibiotics
The present situation …

- Efflux has, slowly but surely, been shown to affect most if not all major antibiotic classes …

? Are they classes that will never show efflux-mediated resistance?
May be only those which must act on bacterial surface?
? glycopeptides [vancomycin…],
? lipoglycopeptides [telavancin],
? lipopeptides [daptomycin], …
Steps and Challenges of efflux in antibacterial chemotherapy

- **recognizing its existence:**
  is it a significant mechanism of resistance?

- **which pumps ...**
  is antibiotic efflux different from other drug efflux?

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  - can we make non-effluxed drugs?
  - and what about efflux inhibitors?
  - is efflux important in pharmacokinetics/drug interactions?
Why do we have efflux?

- Polar drugs do not reach intracellular targets!
- Lipophilic drugs get stucked in the bilayer!
Reaching an intracellular target ...

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

But once inside the cell, any compound may have potentially harmful effects
Proteins with efflux capabilities may have been selected out to help cells getting rid of diffusible, potentially toxic compounds
General mechanisms of drug efflux


flip-flop
flippase
vacuum cleaner
membrane insertion / release

ATP ADP+Pi
Most antibiotics are amphiphilic!

cationic amphiphiles

macrolide

sulfamide

rifampicin

fluoroquinolone

tetracycline

lincosamide

Most antibiotics are amphiphilic!

anionic amphiphiles

β-lactam

fusidic acid

fluoroquinolone
But antibiotic efflux transporters are everywhere

Main (super)families of antibiotic transporters:
- **MATE**: Multidrug And Toxic compounds Extrusion
- **SMR**: Small Multidrug Resistance
- **MFS**: Major Facilitator Superfamily
- **ABC**: ATP Binding Cassette
- **RND**: Resistance Nodulation cell Division

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

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>bacteria</th>
<th>fungi</th>
<th>superior eucaryotes</th>
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<td>β-lactams</td>
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Antibiotic transporter spectra ...

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<tr>
<th>Spectrum</th>
<th>prokaryotes</th>
<th>eukaryotes</th>
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<tbody>
<tr>
<td>Gram (+)</td>
<td>MATE</td>
<td>SMR</td>
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<tr>
<td>Gram (-)</td>
<td>MFS</td>
<td>ABC</td>
</tr>
<tr>
<td>broad</td>
<td></td>
<td>RND</td>
</tr>
<tr>
<td>narrow</td>
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</table>

Mesaros et al. (2005) Louvain médical. 124:308-20
Efflux as a mechanism of export in antibiotic producers

**spectrum**

**narrow**

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

**broad**

- LmrA of *S. lincolnensis* → lincomycin
- RifP of *A. mediterranei* → rifampicin
- SmB of *S. ambofaciens* → macrolides
- Tlrc of *S. fradiae* → tylosin

*ABC* and *MFS* specific for the produced antibiotic
Efflux as a mechanism of resistance in Gram-positive bacteria

**Spectrum**

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

**Broad**
- MFS
  - NorA of *S. aureus* → FQ, Tet, chl
  - MefE of *S. pneumoniae* → ML
  - PmrA of *S. pneumoniae* → FQ
  - MefA of *S. pyogenes* → ML

Specific for one (or a few) families of drugs
Efflux as a mechanism of resistance in Gram-negative bacteria

Spectrum

Narrow

Specific for one (or a few) families of drugs

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-ToIC of *E. coli* → β-lac, FQ, Tet, ML, chl, rif, sulf
Topological organization of efflux transporters in procaryotes

H⁺

ATP

ADP

RND

MFSSMR

MATE

ABC

H⁺ Na⁺

pump

periplasm

lipoprotein

Gram-positive

Gram-negative

Common antibiotic-efflux pumps in eucaryotes (1/2)

- **Multiple Drug Resistance (PgP)**

**TOPOLOGY**

**MECHANISM**

**ANTIBIOTICS**

- tetracyclines
- fluoroquinolones
- macrolides
- lincosamides
- rifampicin
- daptomycin
- chloramphenicol
- aminoglycosides
Common antibiotic-efflux pumps in eucaryotes (2/2)

- Multidrug Resistance Proteins (MRP)

**TOPOLOGY**

**MECHANISM**

**ANTIBIOTICS**

- fluoroquinolones
- tetracyclines
- macrolides
Molecular determinants of antibiotic efflux

- lipophilicity
- modulation by (small) structural changes
- anionic / cationic character
- capacity to bind to phospholipids
- and … multi-ligand binding sites …
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&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂N CH COOH</td>
<td>penicillin N</td>
<td>0</td>
<td>1</td>
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<tr>
<td>CO</td>
<td>carbenicillin</td>
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<td>4</td>
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<td></td>
<td>penicillin G</td>
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<td>32</td>
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<tr>
<td></td>
<td>cloxacillin</td>
<td>890</td>
<td>256</td>
</tr>
<tr>
<td>Cl</td>
<td>nafoilin</td>
<td>4200</td>
<td>128</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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 fluoroquinolones by NorA of *S. aureus*

<table>
<thead>
<tr>
<th>molecule</th>
<th>R₁</th>
<th>R₇</th>
<th>R₈</th>
<th>MIC ratio²</th>
</tr>
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<tbody>
<tr>
<td>(a)</td>
<td>⬤</td>
<td>⬤</td>
<td>OCF₃</td>
<td>2</td>
</tr>
<tr>
<td>(b)</td>
<td>⬤</td>
<td>⬤</td>
<td>OCH₃</td>
<td>4</td>
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<tr>
<td>Ciprofloxacin</td>
<td>⬤</td>
<td>⬤</td>
<td>H</td>
<td>16</td>
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<tr>
<td>(c)</td>
<td>C₂H₅</td>
<td>⬤</td>
<td>H</td>
<td>16</td>
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<tr>
<td>Norfloxacin</td>
<td>C₂H₅</td>
<td>⬤</td>
<td>H</td>
<td>64</td>
</tr>
</tbody>
</table>

² 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
Moxifloxacin vs. ciprofloxacin for *S. pneumoniae* in Brussels: influence of reserpine ... (MFS [PmrA ?])

A. Lismond et al., ECCMID 2008
Aminoglycosides as unexpected substrates for efflux pumps (RND)

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

Composite binding site?
Another unexpected substrate

Daptomycin

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

Highly polar molecule!

Polyanionic cyclic peptide

Lipophilic chain
Daptomycin is substrate of P-gp

Daptomycin accumulation in macrophages is inversely proportional to P-gp activity and expression level.

Lemaire et al. AAC 2007; 51(8):2748-57
Putative mechanism of daptomycin transport by P-gp

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

Lemaire et al. AAC 2007; 51(8):2748-57
Multi-ligand binding sites: the binding domain of AcrB (RND) in *E. coli*

- The methyl moiety of the 7-dimethylamino group and the C-ring of minocycline interact with Phe 178 and Phe 615, respectively. Two oxygen atoms of the 1-oxo and 2-amido groups interact with Asn 274.
- Doxorubicin interacts with Phe 615, similar to minocycline, but interacts with Gln 176 and Phe 617 instead of Asn 274 and Phe 178, respectively.

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Impact of efflux on bacterial susceptibility

• Bacterial efflux
  – cooperation with other mechanisms of resistance, leading to high level resistance
  – suboptimal exposure of the antibiotic target, leading to selection of less susceptible mutants
  – low level resistance if alone, but nearing the limit of the PK/PD breakpoint (and new EUCAST breakpoints)

• Efflux from eucaryotic cells
  – decrease of intracellular activity
β-lactams: cooperation between efflux and β-lactamase(s) in Gram-negative bacteria

chemical clearance

physical clearance

β-lactamase
Efflux and selection of resistance to Floroquinolones

Exposure to subMIC concentrations favors the selection of target mutations!

Gyrase/ Topoisomerase

Illustration for Gram (-) but also existing in Gram (+)
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?
# Efflux and resistance in pathogenic bacteria

<table>
<thead>
<tr>
<th>bacteria</th>
<th>efflux pump</th>
<th>MFS</th>
<th>RND</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>NorA</td>
<td>MFS</td>
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<tr>
<td></td>
<td>TetK-L</td>
<td>MFS</td>
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<td>S. pneumoniae</td>
<td>mefE</td>
<td>MFS</td>
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<td></td>
<td>pmrA</td>
<td>MFS</td>
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<td></td>
<td>TetK-L</td>
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<td>S. pyogenes</td>
<td>mefA</td>
<td>MFS</td>
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<td>L. monocytogenes</td>
<td>mdrL</td>
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<td>M. tuberculosis</td>
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<td>H. influenzae</td>
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<td></td>
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<td>N. gonorrhoeae</td>
<td>MtrCDE</td>
<td>RND</td>
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<tr>
<td>Salmonella</td>
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- MFS: Multidrug efflux system
- RND: Resistance-nodulating system
- SMR: Specific multidrug resistance system
Fluoroquinolones and *P. aeruginosa* at the Gaslhuysberg Academic Hospital (Belgium)

Limit of usefulness of levofloxacin (PK/PD)

Efflux and first mutants

J. van Eldere, 2003
Antibiotic efflux from eucaryotic cells and "intracellular resistance"

Reduction in intracellular drug concentration can result in inefficacy against intracellular bacteria
Models of intracellular infection

**L. monocytogenes**

**S. aureus**

cytosol

phagolysosomes
Influence of an inhibitor * of an eucaryotic antibiotic transporter (MRP) on the intracellular activity of an antibiotic ciprofloxacin and *L. monocytogenes*

*Seral et al. (2003) JAC 51:1167-73*

*gemfibrozil 250 µM; 24 h*
Influence of an inhibitor * of an eucaryotic antibiotic transporter (P-gp) on the intracellular activity of an antibiotic azithromycin and *S. aureus*

* verapamil 20 µM; 24 h

Seral et al. (2003) JAC 51:1167-73
Cooperation between bacterial and eucaryotic efflux pumps…
Cooperation between bacterial and eucaryotic efflux

ciprofloxacin

<table>
<thead>
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<th>wild-type bacteria</th>
<th>resistant bacteria</th>
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Lismond et al. (2006) ICAAC-A1108 and submitted
Steps and Challenges of efflux in antibacterial chemotherapy

• **recognizing its existence:**
  is it a significant mechanism of resistance?

• **which pumps ...**
  is antibiotic efflux different from other drug efflux?

• **defining its role:**
  does it need to change our vision on (and decisions about) existing antibiotics?

• **setting up diagnostic techniques:**
  how can we detect it today (and do we need to do this?)

• **treating patients:**
  – can we make non-effluxed drugs?
  – and what about efflux inhibitors?
  – is efflux important in pharmacokinetics/drug interactions?
Diagnostic approaches …

doi:10.1093/jac/dkl504
Advance Access publication 8 February 2007

A combined phenotypic and genotypic method for the detection of Mex efflux pumps in Pseudomonas aeruginosa

Narcisa Mesaros¹, Youri Glupczynski², Laëtitia Avrain¹, Nancy E. Caceres¹, Paul M. Tulkens¹* and Françoise Van Bambeke¹

¹Unité de Pharmacologie cellulaire et moléculaire, Brussels, Université catholique de Louvain, UCL 7370 avenue E. Mounier 73, B-1200 Bruxelles, Belgium; ²Laboratoire de Microbiologie, Cliniques universitaires UCL de Mont-Godinne, avenue G. Therasse 1, B-5530 Yvoir, Belgium
Correlation between the level of expression (PCR) of constitutive Mex pumps and the effect of PAβN on the MIC of reporter antibiotics (carbenicillin for mexA and gentamicin for mexX).

Data are grouped in two quadrants of potentially different diagnostic significance:

- lower left, no or minimally meaningful efflux-mediated decrease of susceptibility
- upper right, efflux is likely to be the cause of the decreased susceptibility.

Steps and Challenges of efflux in antibacterial chemotherapy

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  – is efflux important in pharmacokinetics/drug interactions?
Tigecycline…

- truly made to resist efflux-mediated resistance in Gram(-) bacteria (and also *S. aureus*)
- broad spectrum including MRSA (MIC < 2 mg/L) and VISA
- tet(M) [ribosomal protection] or tet(K) [efflux] have no discernible effect on MICs (AAC 2006 Feb;50(2):505-10).
- approved by the FDA in 2005 and by the EMEA in 2006 for use in patients with complicated skin infections, skin-structure infections and intra-abdominal infections
The final selection of tigecycline was the result of a systematic research to combine the hydrophobic moiety AND the additional aminogroup in a substituent attached to position 9

### Table 2. In Vitro Antibacterial Activity of Compounds 13–25.

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<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>E. coli UBMS 88-1 Tet B</th>
<th>E. coli PRP1 Tet A</th>
<th>E. coli J3272 Tet C</th>
<th>E. coli J3272 Tet D</th>
<th>E. coli UBMS 90-4 Tet M</th>
<th>E. coli UBMS 90-5 sensitive</th>
<th>S. aureus UBMS 88-7 Tet K</th>
<th>S. aureus UBMS 90-1 Tet M</th>
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Can we make **clinically-useful** inhibitors of bacterial efflux?

Inhibitors of Bacterial Efflux Pumps as Adjuvants in Antibiotic Treatments and Diagnostic Tools for Detection of Resistance by Efflux

François Van Bambeke$^{1,*}$, Jean-Marie Pagès$^2$ and Ving J. Lee$^3$

$^1$Unité de Pharmacologie cellulaire et moléculaire, Université catholique de Louvain, Brussels, Belgium  
$^2$EA2197 Enveloppe Bactérienne, Perméabilité et Antibiotiques, Faculté de Médecine, Université de la Méditerranée, Marseille, France; $^3$CB Research and Development, Inc. (Adesits, Inc), New Castle, DE, USA

Practical applications and feasibility of efflux pump inhibitors in the clinic—A vision for applied use

Olga Lomovskaya, Keith A. Bostian *

Mpx Pharmaceuticals Inc., 3030 Bunker Hill Street, San Diego, CA 92109, United States
Clinically useful inhibitors of antibiotic efflux pumps: 
the bottom line …

It will be difficult because…

• Many procaryotic transporters have eucaryotic homologues;
• antibiotics are (for that reason or another …) substrate to many of them… (sometimes in an unanticipated fashion)
• and so will probably be many inhibitors…

Van Bambeke et al. (2003) 
Is efflux important in pharmacokinetics?

Yes, ...
Let us believe in pumps…
(each has its own set of challenges)

the fire pump…
(save your life)

the gas pump…
(keeps you moving)

the pump for everything …
(white product)

the little amateur one…
but with great expectations …
The past and present "pump team" at UCL

http://www.facm.ucl.ac.be