Resistance to antibiotics: the rise of efflux…

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Cliniques universitaires SAINT-LUC UCL BRUXELLES

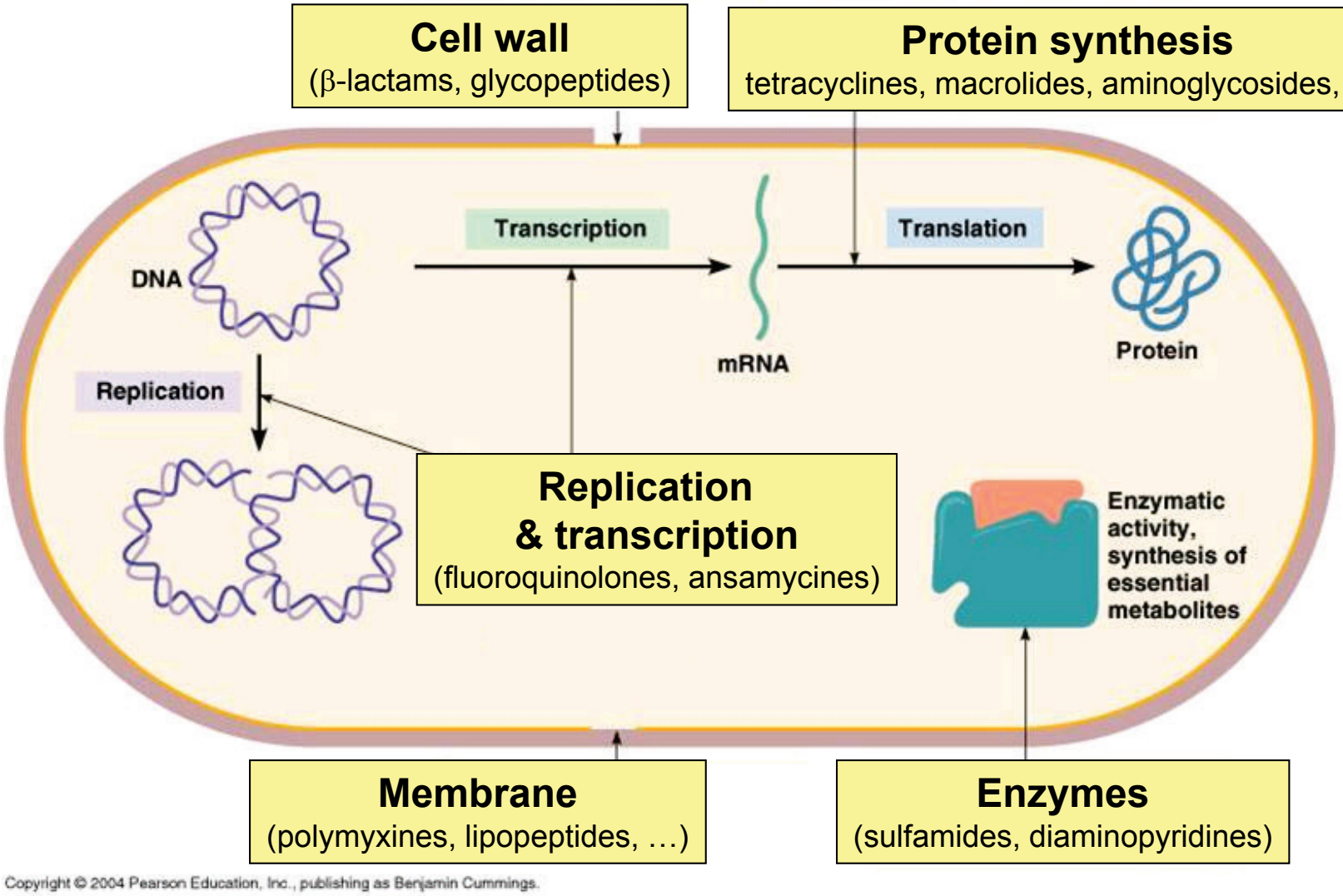
Un hôpital pour la Vi

23 April 2015

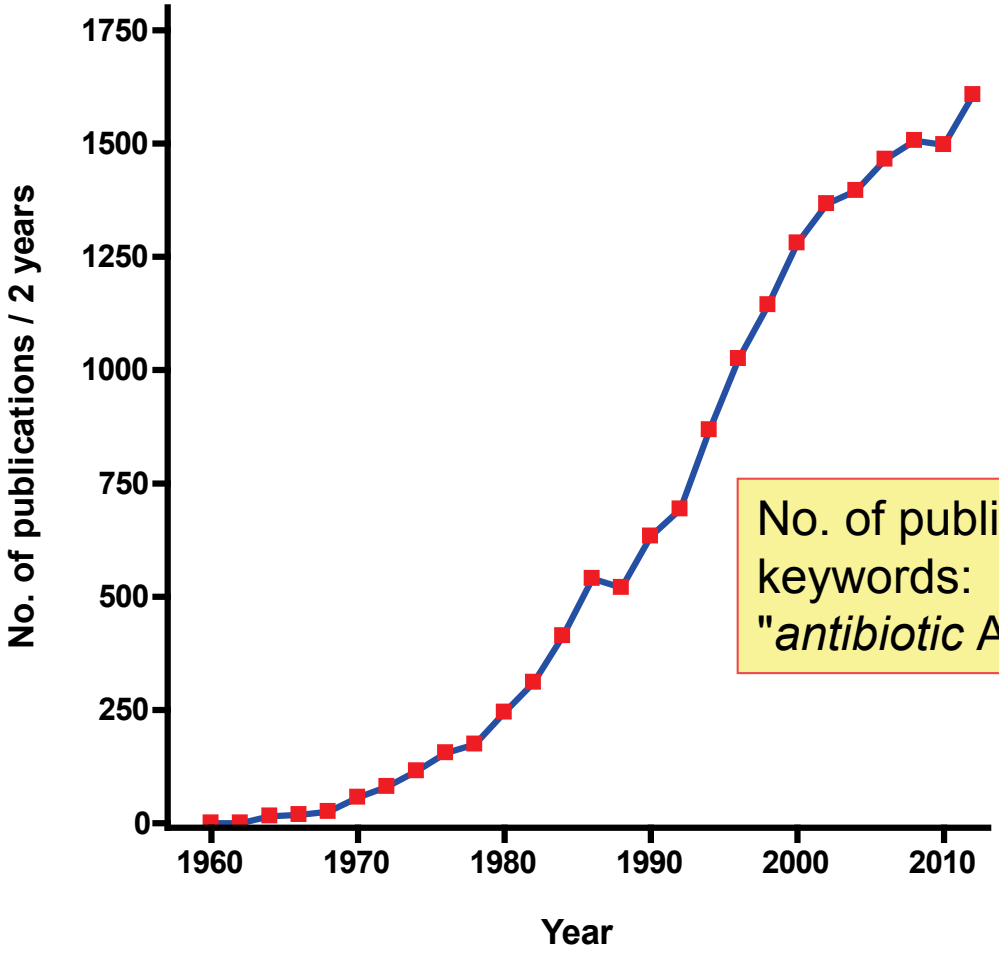
What is in the menu ?

- 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

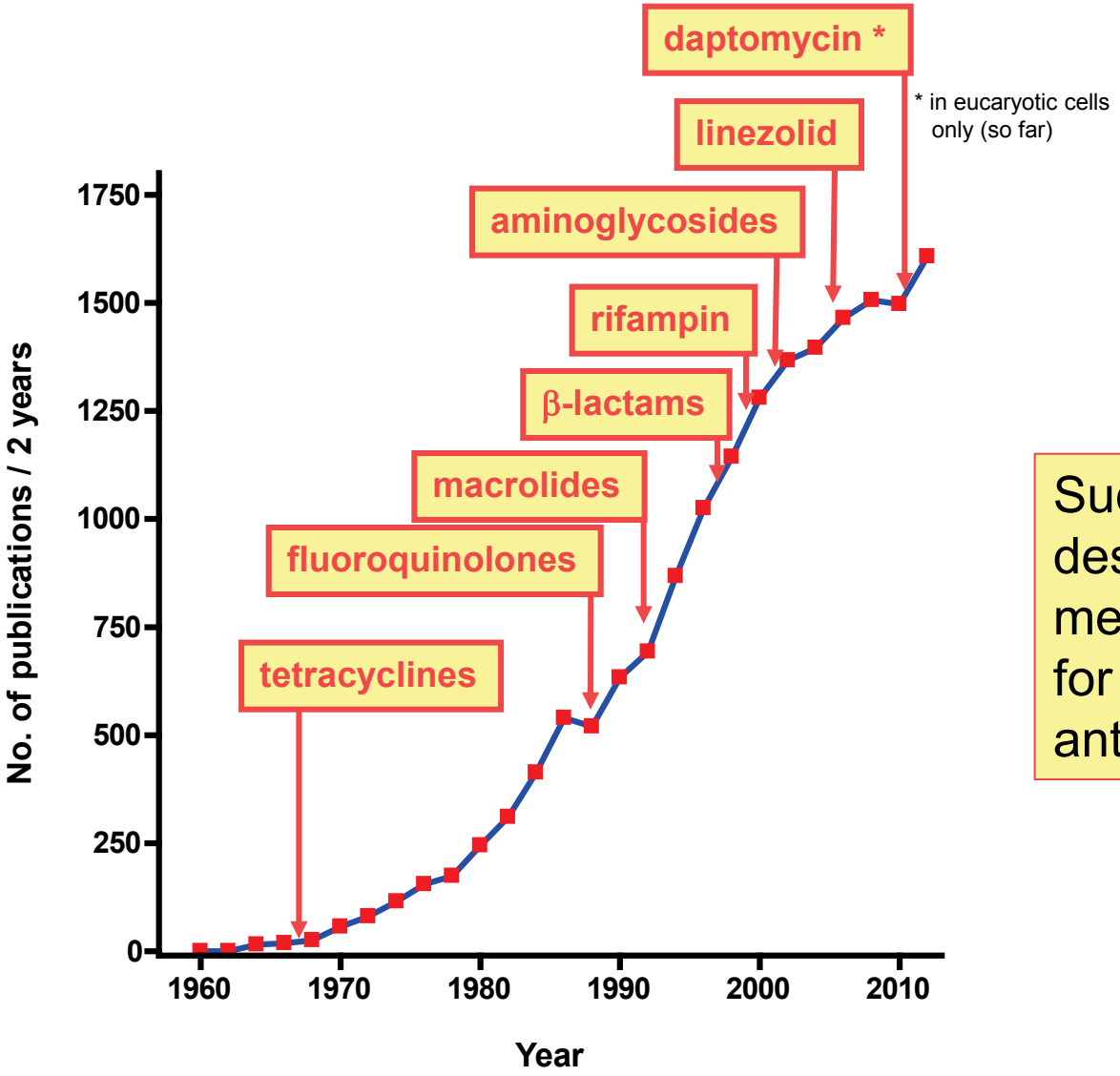


You said "antibiotic eflux"



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

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An original observation with cancer cells…

[CANCER RESEARCH 37, 4629–4634, December 1977]

Decreased Retention of Actinomycin D as the Basis for Cross-resistance in Anthracycline-resistant Sublines of P388 Leukemia

Makoto Inaba[1] and Randall K. Johnson[2]

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

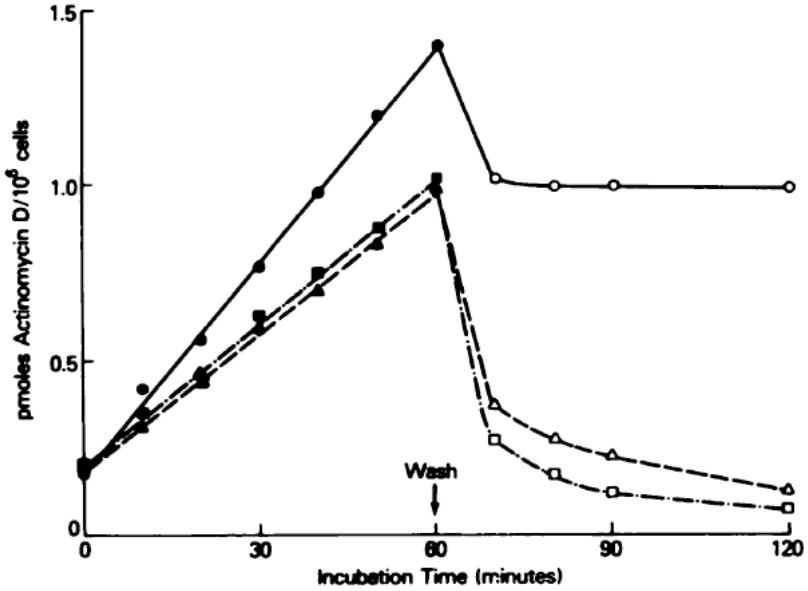

Chart 2. Time course of uptake and efflux of actinomycin D by P388/S (○, ●), P388/ADR (△, ▲) and P388/DAU (□, ■) cells. Cells were incubated in the presence of actinomycin D, 0.04 μg/ml, for 60 min, washed, and reincubated in drug-free medium for an additional 60 min. Each *point* represents the mean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

Most chemotherapeutic agents must reach an intracellular target…

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)

	Radioactivity (dpm × 10^{-3})				
Cell line	Whole cells	Nuclear fraction	Mitochondrial fraction	Microsomal fraction	Cytoplasmic supernatant
Uptake					
P388/S	1513 ± 2[a]	1014 ± 18 (67)[b]	31 ± 1 (2)	10 ± 1 (1)	409 ± 11(27)
P388/ADR	672 ± 9	430 ± 1 (64)	41 ± 1 (6)	6 ± 0.2 (1)	198 ± 9(29)
Retention					
P388/S	1131 ± 3	766 ± 13 (68)	43 ± 1 (4)	8 ± 0.4 (1)	307 ± 8 (27)
P388/ADR	135 ± 3	88 ± 3 (65)	12 ± 3 (9)	2 ± 0.1 (1)	35 ± 1 (26)

[a] Mean ± S.D.

[b] Numbers in parentheses, percentage of total.

Conclusion #1: in order to survive to anticancer agents, cells "invented" efflux…

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

But antibiotics were first ...

1: Nature. 1963 Oct 26;200:384-5.

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 …

1: Nature. 1963 Oct 26;200:384-5.

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

IZAKI K, ARIMA K.

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Who remembers that graph ?

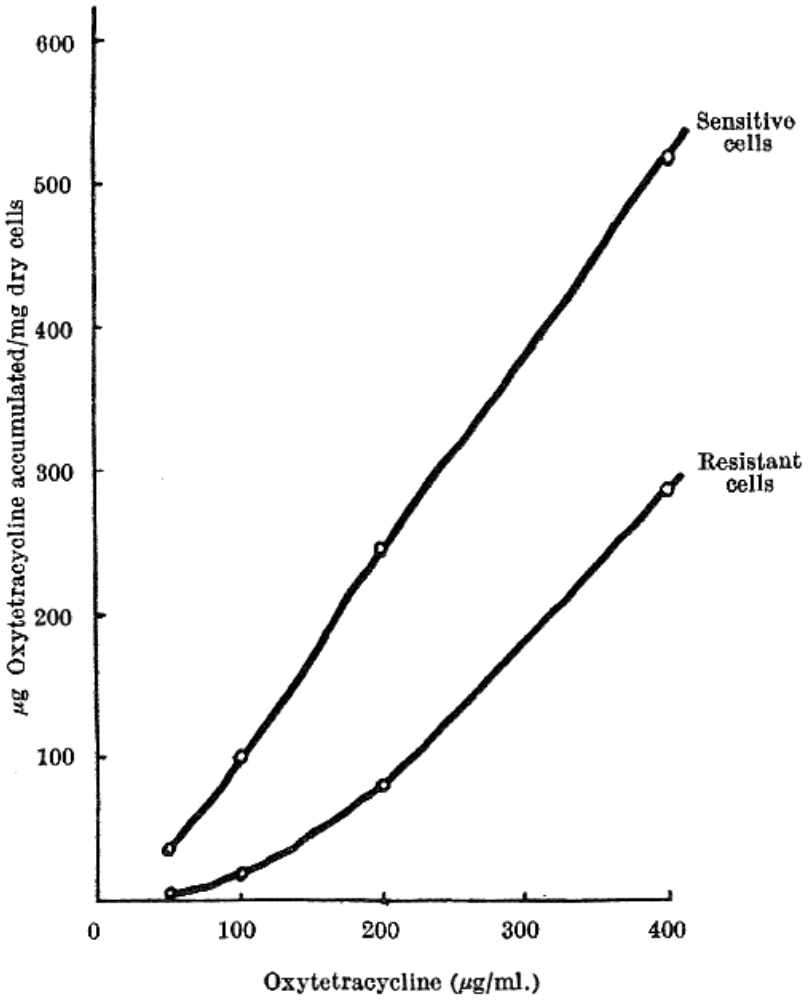


Fig. 1. Accumulation of oxytetracycline in *E. coli* K-12 at various concentrations of oxytetracycline added. The reaction mixture contains 1 ml. suspension (0·7 mg dry weight) oxytetracycline hydrochloride, 1 ml. (0·5–4·0 mg/ml.) and 1 ml. of 10 per cent (w/v) glucose, 2 per cent K_2HPO_4 and 0·1 per cent $MgSO_4.7H_2O$ respectively in a total volume of 10 ml. Incubation was carried out aerobically at 30° C for 90 min

Historical observations on tetracyclines …

15 years later...

Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 3974–3977, July 1980
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 …

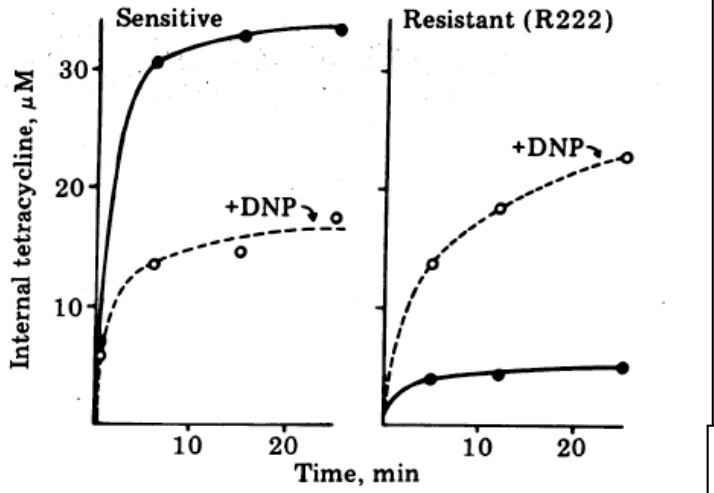


FIG. 1. Tetracycline uptake by *E. coli* ML308-225 (sensitive) and by R222-containing induced (resistant) cells with (○) and without (●) 1 mM DNP. Cells were grown overnight in medium A containing glucose and uptake was measured in the absence of added energy source.

Whole bacteria

Everted membranes

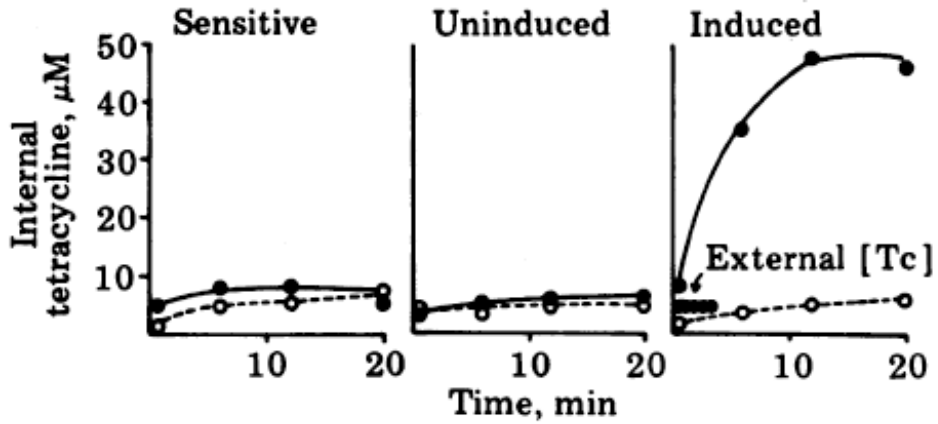


FIG. 2. Tetracycline (Tc) uptake by everted membrane vesicles made from sensitive ML308-225 cells and from uninduced and induced R222-containing cells. ○, No energy; ●, D-lactate. Cells were grown in glycerol and vesicles were frozen in 5 mM Tris·HCl, pH 7.2/70 mM KCl/0.25 mM dithiothreitol/50% glycerol.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

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

Historical observations …

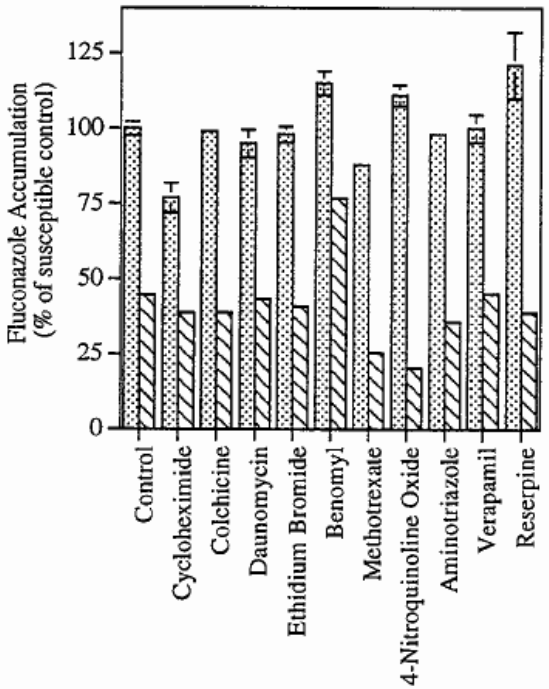


FIG. 3. Effects of MDR protein substrates or inhibitors on [^{3}H]fluconazole uptake by cells from fluconazole-susceptible (▨) and fluconazole-resistant (▧) cultures of *C. glabrata* after 80 min of incubation in the standard uptake assay; the assay was extended to 180 min for verapamil. Values are means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

Parkinson et al. Antimicrob Agents Chemother. 1995 Aug;39(8):1696-9

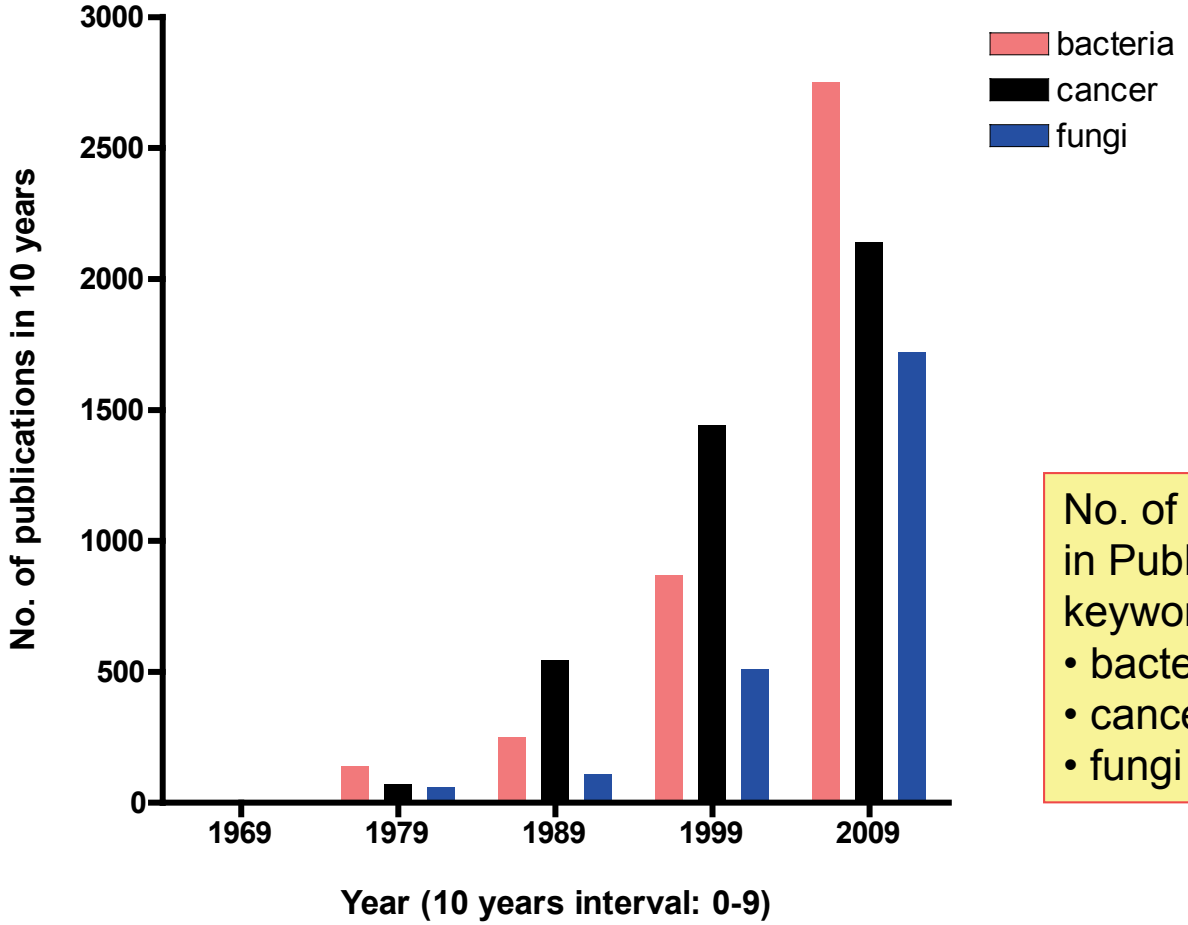
6. **De Waard, M. A., and J. G. M. Van Nistelrooy.** 1980. An energy-dependent efflux mechanism for fenarimol in a wild-type strain and fenarimol-resistant mutants of *Aspergillus nidulans*. Pestic. Biochem. Physiol. **13**:255–266.

antibiotics 1965 1980

antifungal drugs 1980 1995

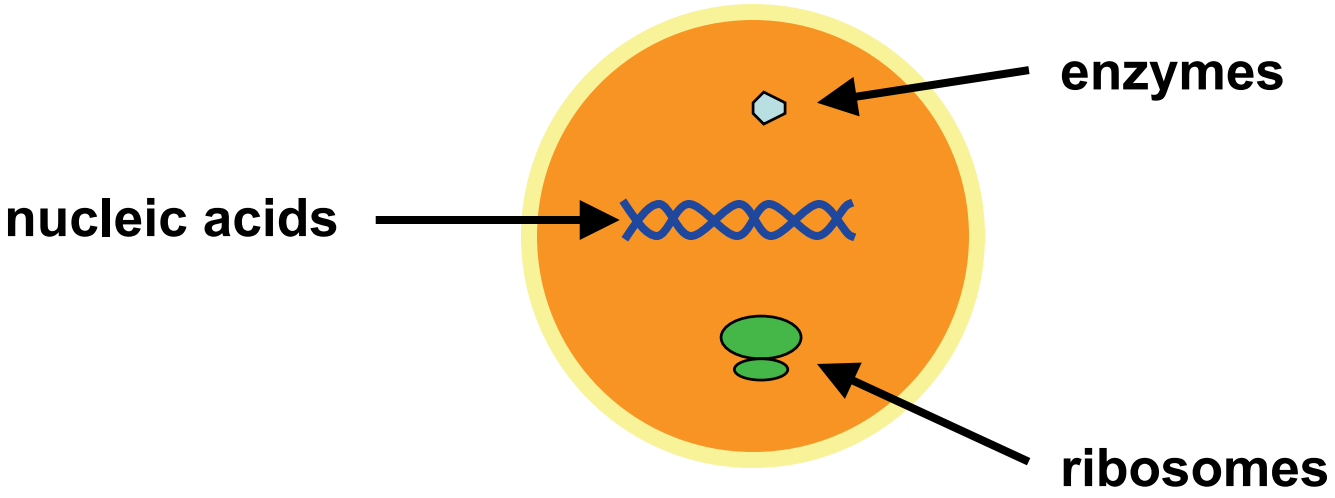
1977 anticancer drugs

Historical trends …



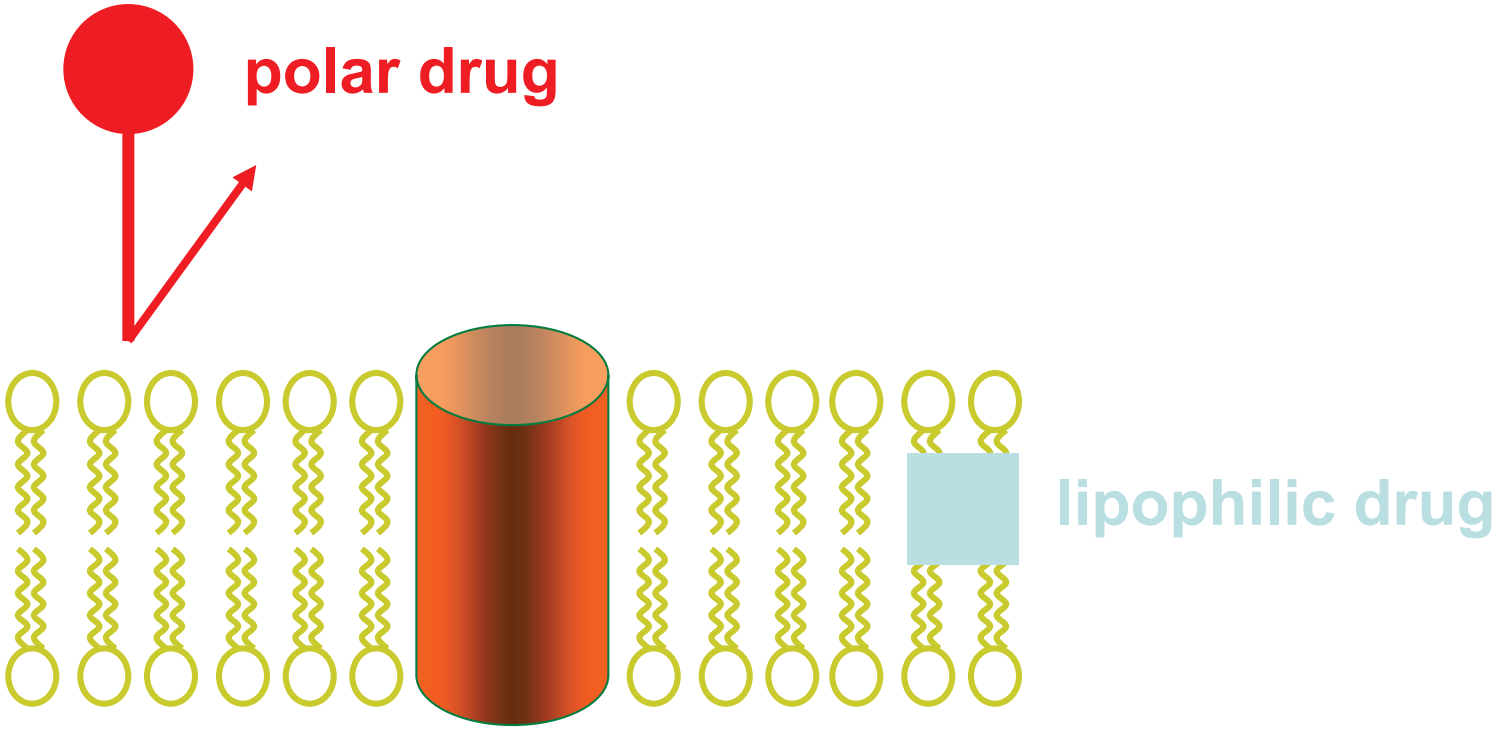
No. of publications in PubMed with keywords: efflux AND
- bacteria
- cancerl
- fungi

Most chemotherapeutic agents must reach an **intracellular** target…



How can these drugs reach their target inside the cells ?

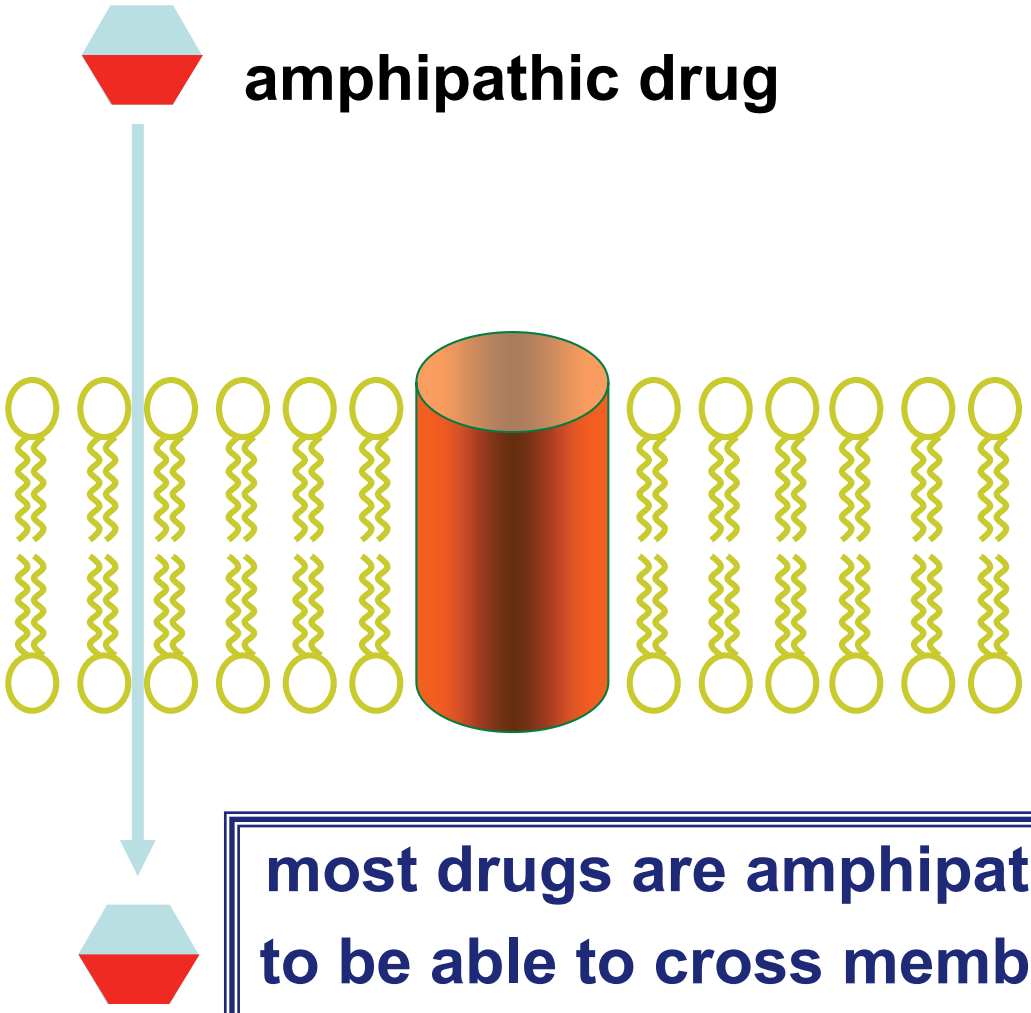
Reaching an intracellular target …



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

Van Bambeke et al., Biochem. Pharmacol (2000) 60:457-70

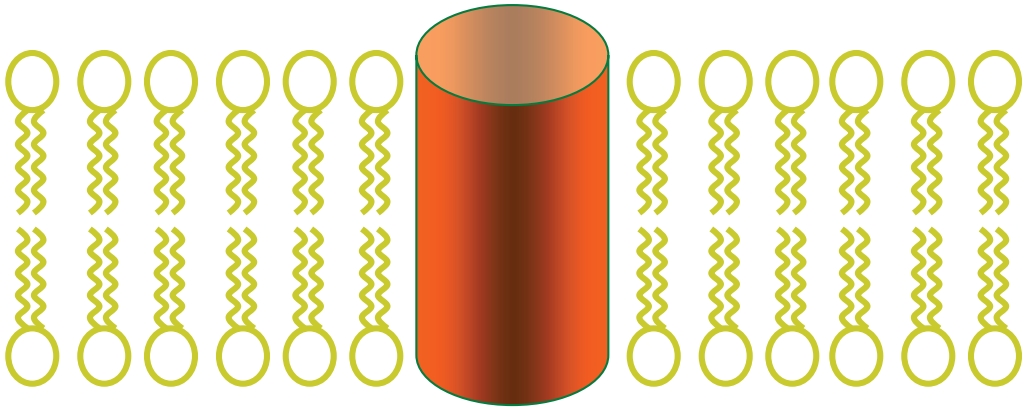
Reaching an intracellular target …



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

Van Bambeke et al., Biochem. Pharmacol (2000) 60:457-70

Intracellular chemotherapeutic agents



But a diffusible compound

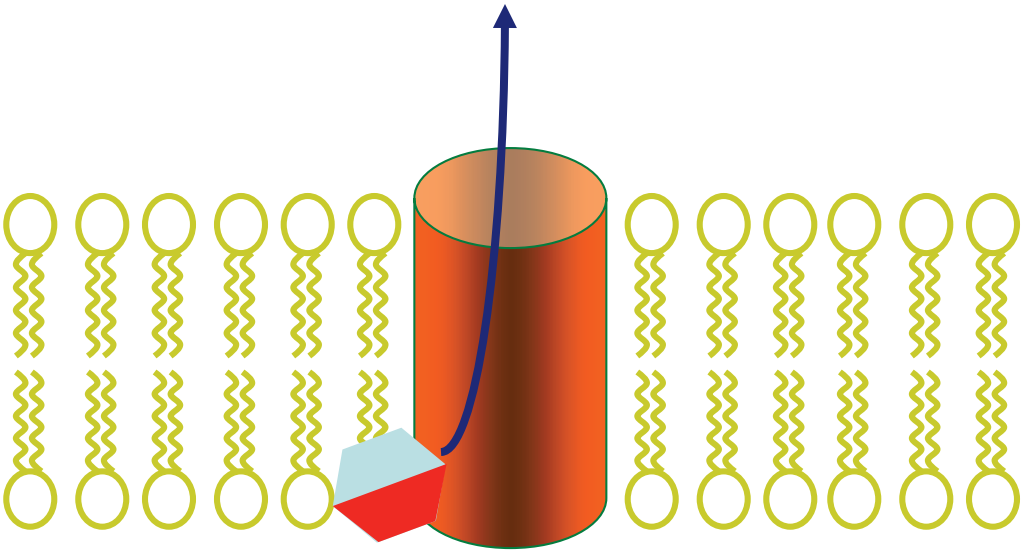
may have

potentially harmful effects !

Van Bambeke et al., Biochem. Pharmacol (2000) 60:457-70

Why efflux transporters ?

Extrusion by efflux pumps



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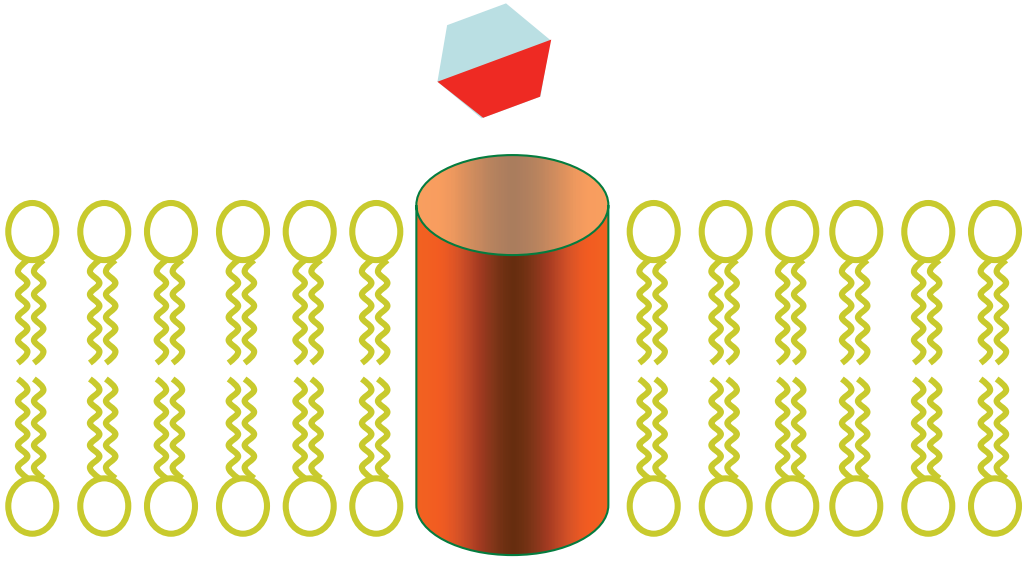
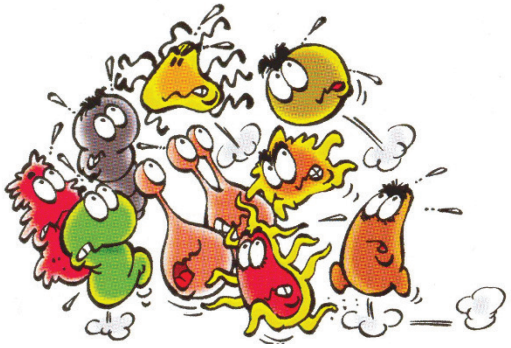


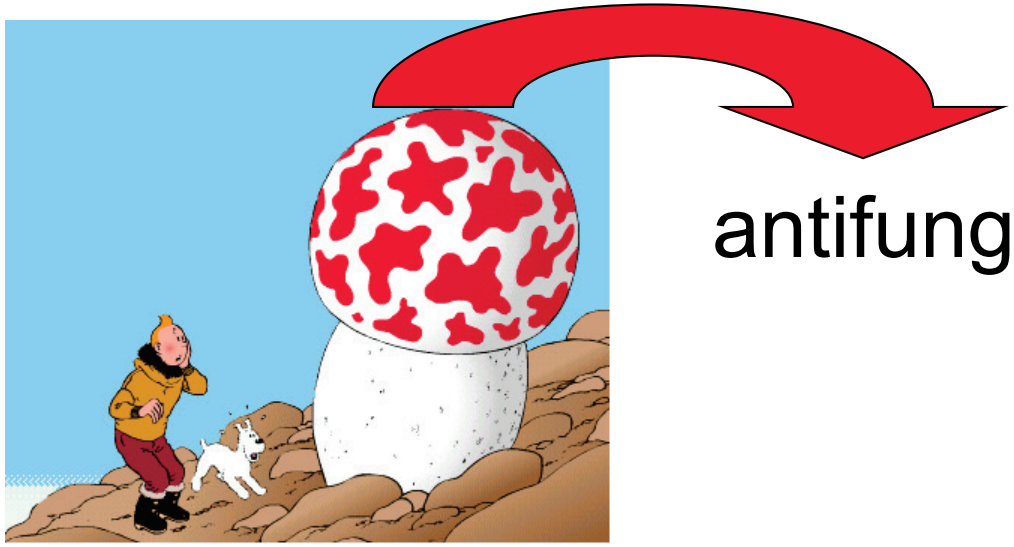
general mean of protection
against cell invasion by diffusible molecules

Van Bambeke et al., Biochem. Pharmacol (2000) 60:457-70

Typical ‘toxic’ diffusible substances as substrates for efflux pumps

antibiotics





antifungals

anticancer agents

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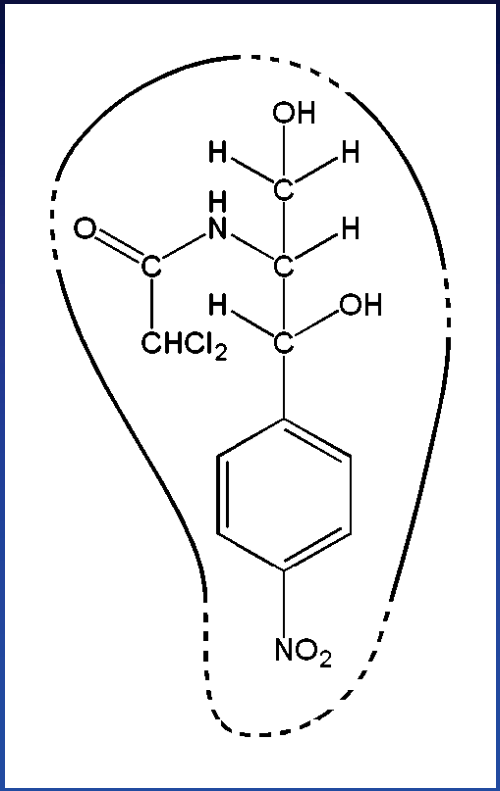
Most antibiotics are amphiphilic !

Neutral



apolar

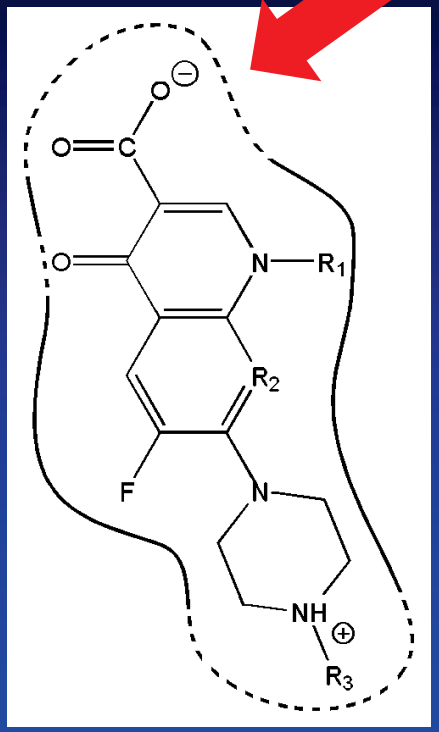
polar

chloramphenicol

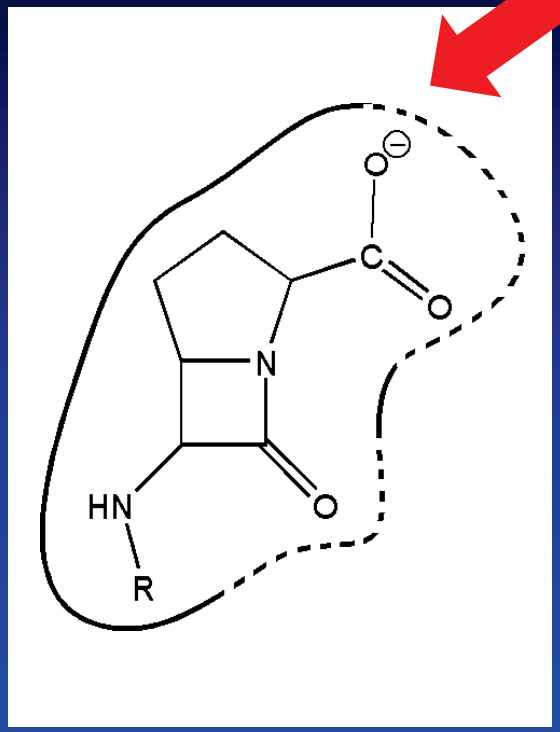
Van Bambeke *et al*. Biochem. Pharmacol. (2000) 60: 457-470

Most antibiotics are amphiphilic !

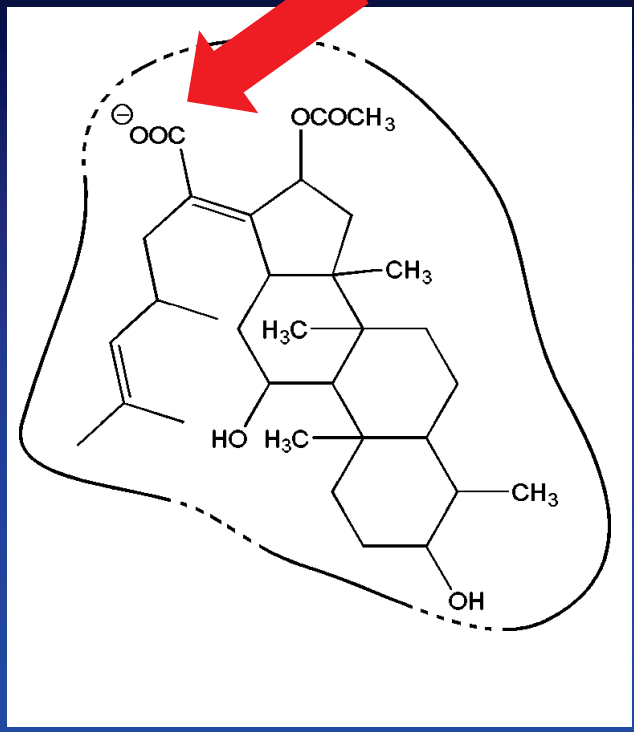
anionic



fluoroquinolones



beta-lactams

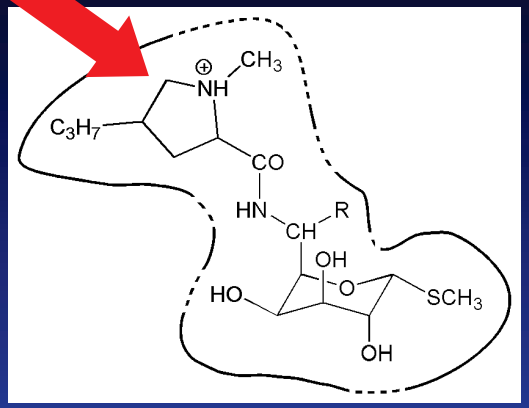


fusidic acid

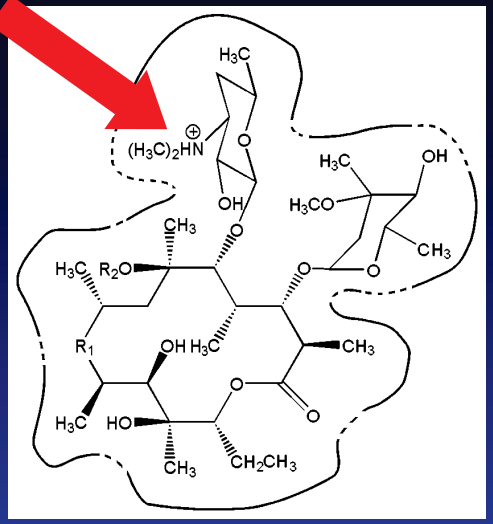
Van Bambeke *et al*. Biochem. Pharmacol. (2000) 60: 457-470

Most antibiotics are amphiphilic !

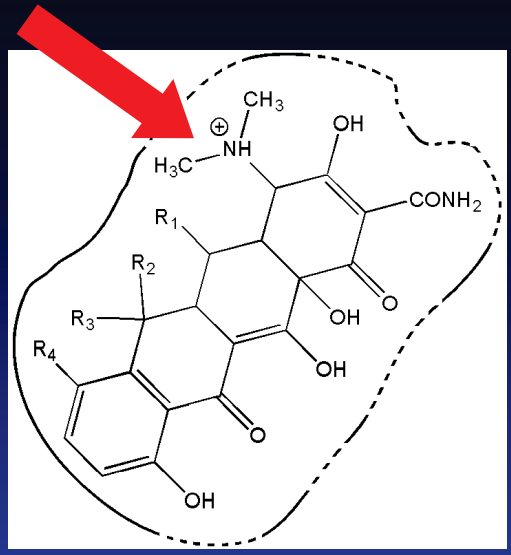
cationic



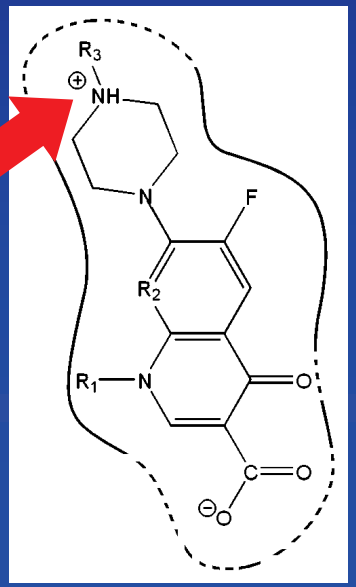
lincosamides



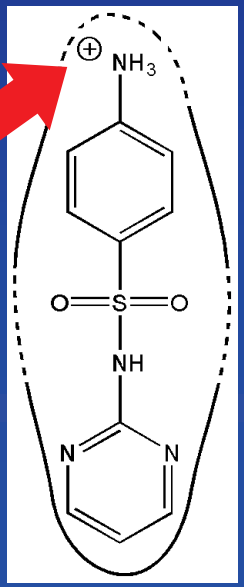
macrolides



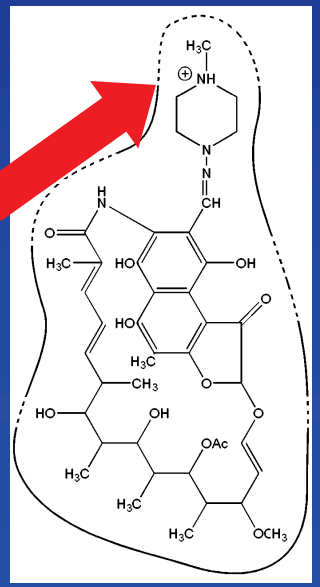
tetracyclines



fluoroquinolones



sulfamides



rifampicin

Antibiotic classes recognized by efflux pumps in different types of organisms

Antibiotic class	bacteria Gram (+)	bacteria Gram(-)	fungi	superior eucaryotes
β-lactams	●	●	●	●
fusidic acid		●		
macrolides	●	●	●	●
streptogramins	●			●
tetracyclines	●	●	●	●
aminoglycosides		●	●	
chloramphenicol	●	●	●	
rifamycins				●
sulfamides			●	
trimethoprim		●		
fluoroquinolones	●	●		●

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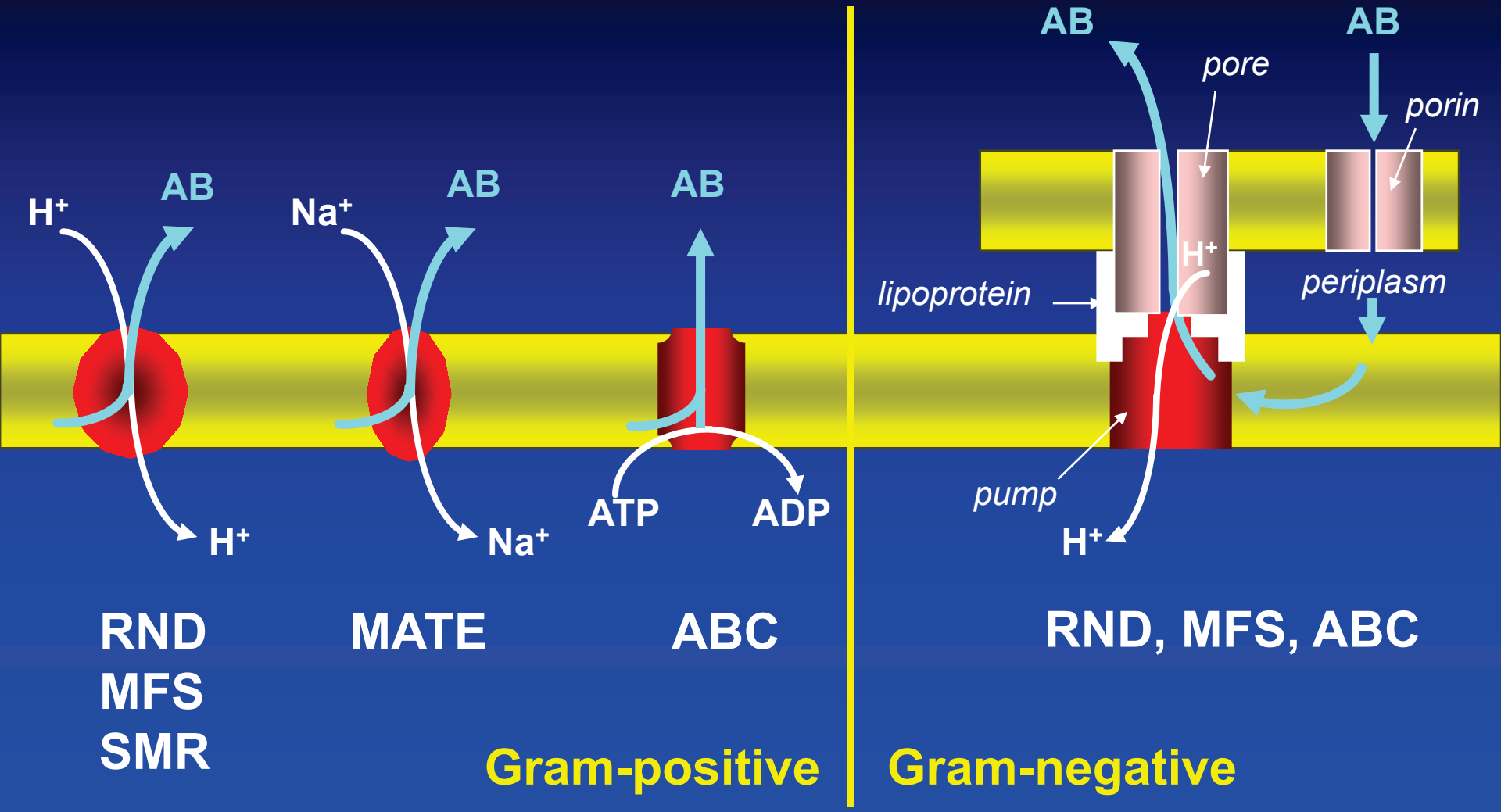
Structure of pumps in procaryotic cells

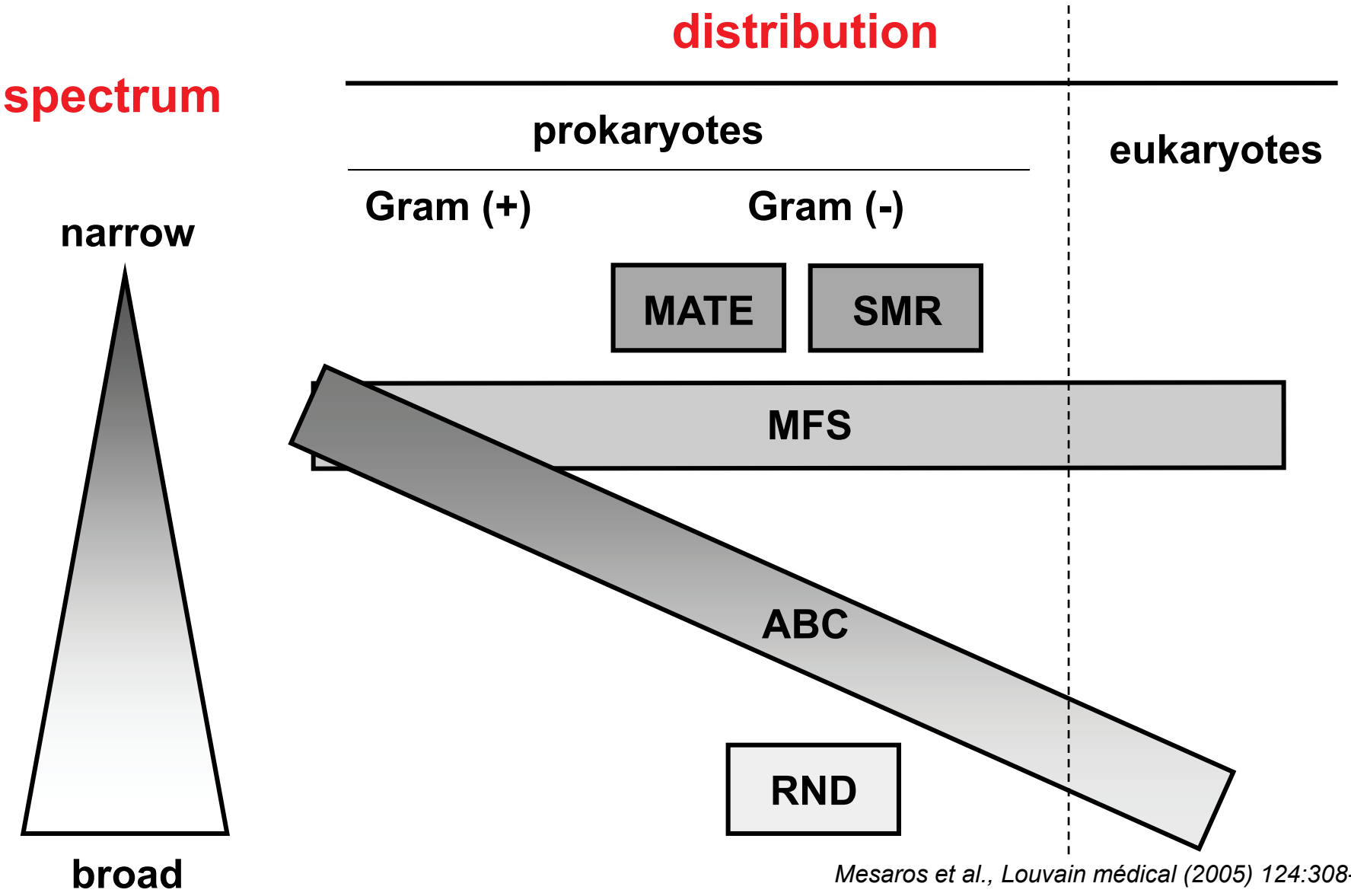
Van Bambeke et *al*. JAC (2003) 51: 1055-1065



Some abbreviations

- **ABC**: **A**TP **B**inding **C**assette
- **MATE**: **M**ulti **A**n**T**imicrobial **E**xtrusion
- **MFS**: **M**ajor **F**acilitator **S**uperfamily
- **RND**: **R**esistance **N**odulation **D**ivision
- **SMR**: **S**mall **M**ultidrug **R**esistance

Antibiotic efflux transporters are ubiquitous



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)

Journal of Antimicrobial Chemotherapy (2003) **51**, 1055–1065
DOI: 10.1093/jac/dkg224
Advance Access publication 14 April 2003

JAC

Leading articles

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

F. Van Bambeke[1]*, Y. Glupczynski[2], P. Plésiat[3], J. C. Pechère[4] and P. M. Tulkens[1]

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

				Antibiotics																
				β-lactams													Q			
Pathogen	Transporter	Super-family	TC number[b]	peni	ceph	carb	m-bac	inhib β-ase	FA	AG	Tet	OX	ML	SG	LM	CHL	RIF	NAL	FQ	SM	TMP
S. aureus	NorA[7]	MFS	2.A.1.2.10													+[58]			+[58]		
	TetK-L[59]	MFS	2.A.1.3.6								+[31]										
	MdeA[60]	MFS								+[60]											
	MsrA[6]	ABC	3.A.1.121.1										+[6]								
S. pneumoniae	MefE[61]	MFS											+[61]								
	PmrA[62]	MFS																	+[62]		
	TetK-L	MFS									+[31]										
Streptococcus pyogenes	MefA[63]	MFS	2.A.1.21.2										+[63]		+[63]						
L. monocytogenes	MdrL[23]	MFS		–[23]	+[23]					–[23]	–[23]		+[23]		+[23]						
	Lde[64]	MFS																	+[64]		
	TetK-L	MFS									+[31]										
Mycobacterium tuberculosis	Mmr[65]	SMR	2.A.7.1.2.										+[65]								
	TetK-L	MFS									+[31]										
	DrrB[66]	ABC	3.A.1.105.1																+[66]		
Enterococcus spp.	Mef?[67]	MFS											+[67]								
	TetK-L	MFS									+[31]										
	EmeA[68]	MFS											+[68]		+[68]				+[68]		
	Lsa[69]	ABC												+[69]	+[69]						

A brief survey of the many transporters (2003)

2. Gram - (part #1)

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Pathogen	Transporter	Super-family	TC number[b]	peni	ceph	carb	m-bac	inhib β-ase	FA	AG	Tet	OX	ML	SG	LM	CHL	RIF	NAL	FQ	SM	TMP
H. influenzae	TetB, K	MFS									$+^{31}$										
	AcrB-like	RND											$+^{70}$				$+^{70}$				
Neisseria gonorrhoeae	MtrD[71]	RND	2.A.6.2.5	$+^{72}$					$+^{72}$		$+^{72}$		$+^{72}$			$+^{72}$	$+^{72}$				
Salmonella spp.	AcrB[73]	RND		$+^{74}$	$+^{74}$				$+^{74}$		$+^{74}$		$+^{74}$			$+^{74}$	$+^{74}$	$+^{74}$	$+^{74}$		
	TetA-D	MFS									$+^{31}$										
	FloR[75]	MFS														$+^{75}$					
Shigella dysenteriae	TetA-D	MFS									$+^{31}$										
E. coli	EmrE[76]	SMR	2.A.7.1.3								$+^{77}$		$+^{77}$							$+^{77}$	
	YdhE[78]	MATE	2.A.66.1.3													$+^{79}$			$+^{79}$		$+^{79}$
	TetA-E[80]	MFS	2.A.1.2.4								$+^{31}$										
	Bcr[81]	MFS	2.A.1.2.7								$+^{79}$									$+^{82}$	
	MdfA[83]	MFS	2.A.1.2.19							$+^{79,83}$	$+^{83}$		$+^{83}$			$+^{79,83}$	$+^{83}$		$+^{79,83}$		$+^{79}$
	YceL[84]	MFS	2.A.1.2.21																$+^{79}$		
	YidY[84]	MFS	2.A.1.2.22													$+^{79}$					
	EmrB[85]	MFS	2.A.1.3.2								$-^{85}$					$-^{85}$		$+^{85}$	$-^{85}$		
	YebQ[84]	MFS	2.A.1.3.17																		$+^{79}$
	SetA[86]	MFS	2.A.1.20.1							$+^{87}$											
	Fsr[88]	MFS	2.A.1.35.1																		$+^{79}$
	AcrB[89]	RND	2.A.6.2.2	$+^{20,90}$					$+^{72}$		$+^{72,90}$	$+^{91}$	$+^{72}$			$+^{72,90}$	$+^{72,90}$	$+^{90}$	$+^{72}$		$+^{79}$
	AcrD[84]	RND	2.A.6.2.7							$+^{79,92}$											
	AcrF[89]	RND		$+^{90}$					$+^{90}$		$+^{90}$		$+^{90}$				$+^{90}$				
	YegN	RND	2.A.6.2.12															$+^{79}$	$+^{79}$		
	YhiV	RND	2.A.6.2.13										$+^{79}$								
	MacB[93]	ABC	3.A.1.122.1										$+^{93}$								

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]

				Antibiotics																	
				β-lactams													Q				
Pathogen	Transporter	Super-family	TC number[b]	peni	ceph	carb	m-bac	inhib β-ase	FA	AG	Tet	OX	ML	SG	LM	CHL	RIF	NAL	FQ	SM	TMP
Stenotrophomonas maltophilia	SmeE[94]	RND								+[95]	+[95]		+[95]			+[95]			+[95]		
P. aeruginosa	CmlA[96]	MFS	2.A.1.2.3													+[96]					
	TetA,C,E	MFS									+[31]										
	MexB[97]	RND	2.A.6.2.6	+[98,99]	+[99]	+[98]	+[99]	+[100]	+[72]		+[72]		+[72]		+[101]	+[72]	+[72]		+[72]	+[72]	+[72]
	MexD[102]	RND		+[101]	+[72]	+[101]					+[72]		+[101]		+[101]	+[72]			+[72]		+[72]
	MexF[103]	RND				–[104]	–[104]	+[100]								+[72,104]			+[72,104]		+[72,104]
	MexK[105]	RND									+[105]		+[105]								
	MexY[106]	RND		+[101]	+[101]	+[101]				+[101]	+[101]		+[101]		+[101]	+[101]			+[101]		

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, synergistins, LM, lincosamides; CHL, chloramphenicol; RIF, rifampicin; Q, quinolones; NAL, nalidixic acid; FQ, fluoroquinolones; SM, sulfamides; TMP, trimethoprim.

[a] +, occurrence; –, absence (in both cases, through functional studies).

[b] According to the classification of Saier.[2]

A brief survey of the many transporters (2009)

Published in final edited form as:
Drugs. 2009 August 20; 69(12): 1555–1623. doi:10.2165/11317030-000000000-00000.

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*

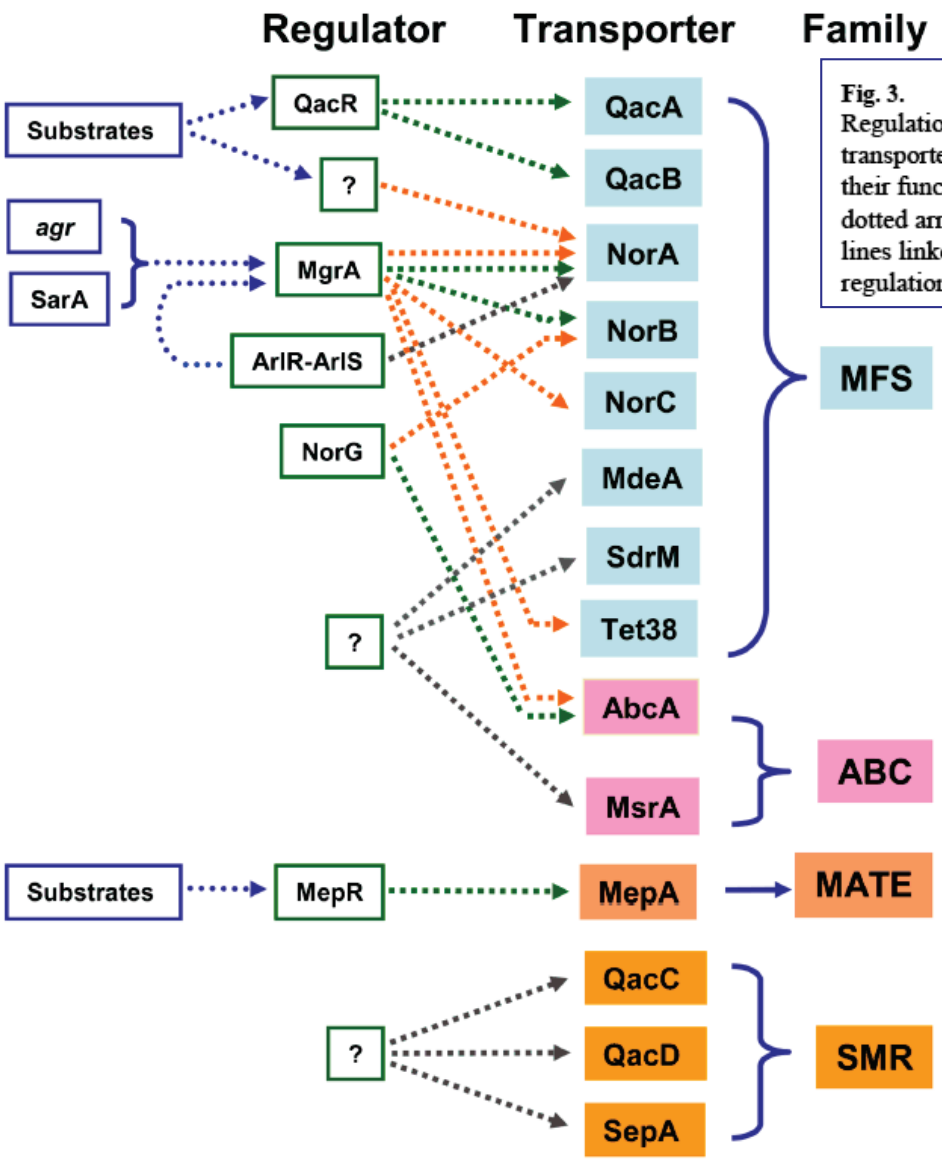
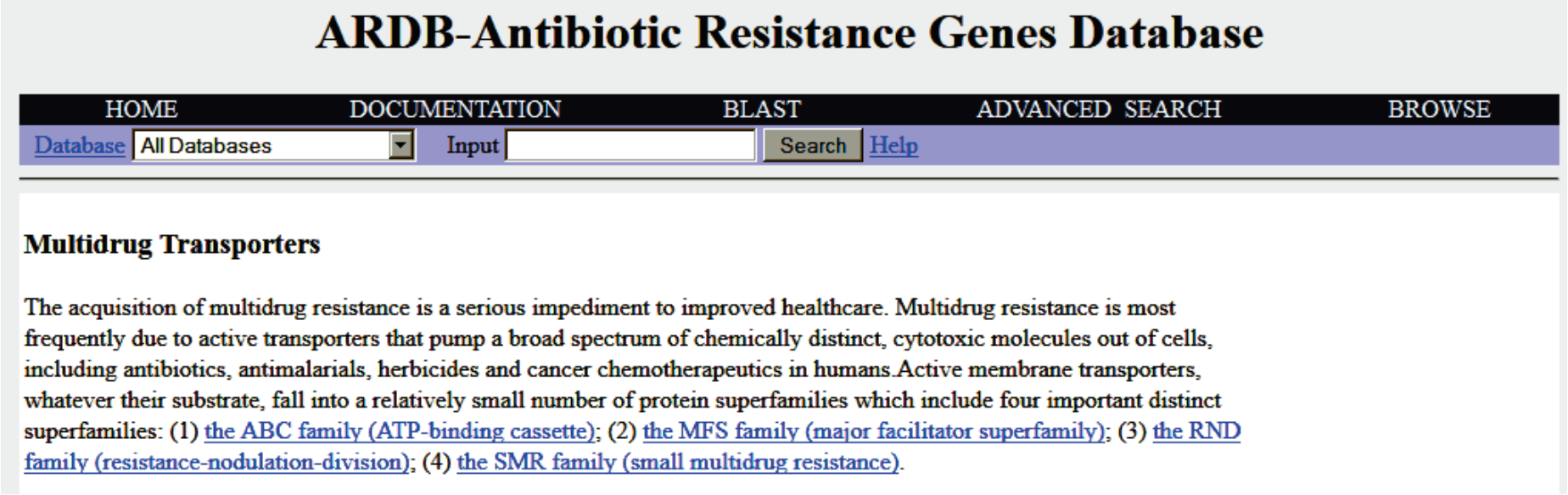


Fig. 3.
Regulation of multidrug or drug-specific efflux transporters of *S. aureus*. The efflux transporters are shown in colour blocks. All regulators are presented in the green boxes, and their functions as repressor or activator are indicated, respectively, by the green or orange dotted arrows. Unknown 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

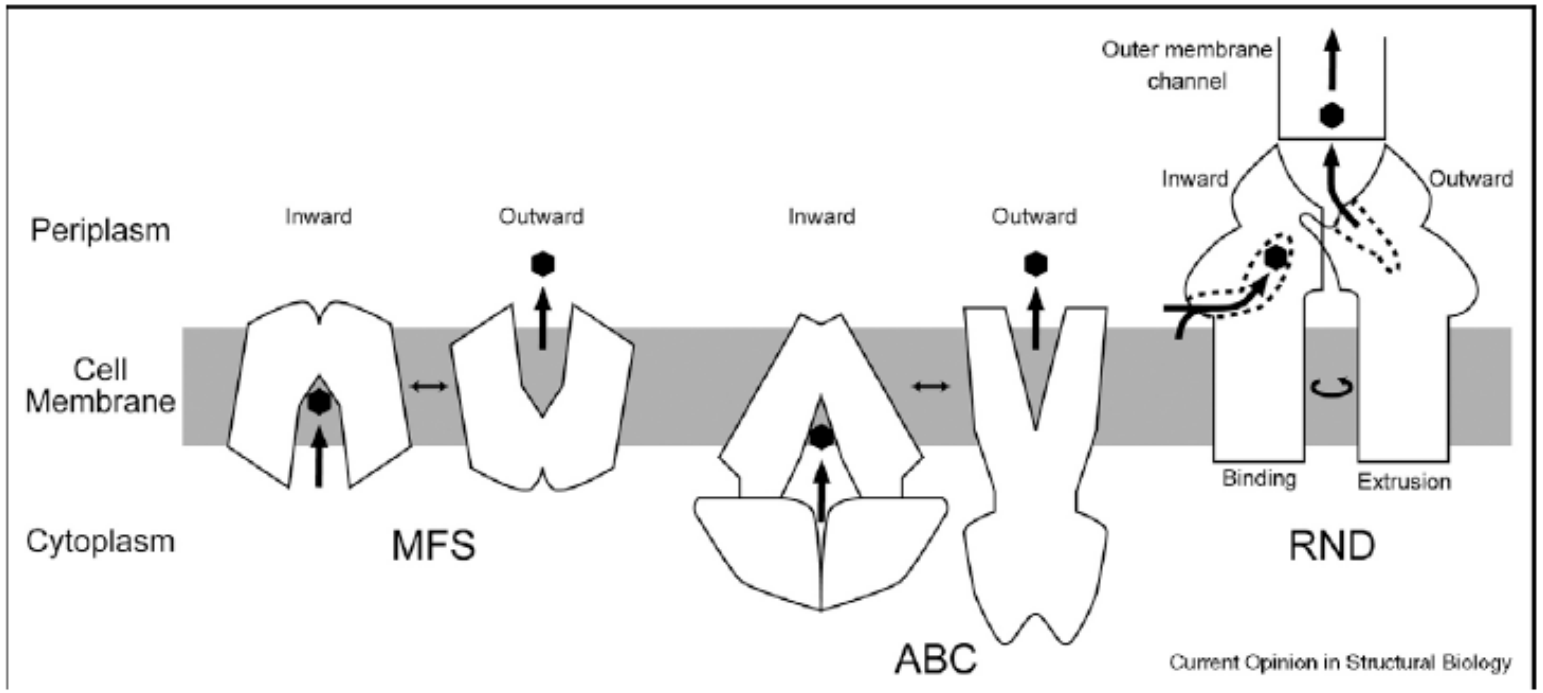


http://ardb.cbcb.umd.edu/browse/multidrug.shtml

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



Alternating access mechanism of transporter families. (*from left to right*) Schematic illustrations of MFS, ABC and RND transporter families. In the case of the RND, only two monomers ('Binding' and 'Extrusion') in the trimer are depicted. In each transporter, the inward-facing and outward-facing conformations are illustrated left and right, respectively.

Murakami S., Current Opinion in Structural Biology 2008, 18:459–465

General structure of an RND (AcrAB-TolC)

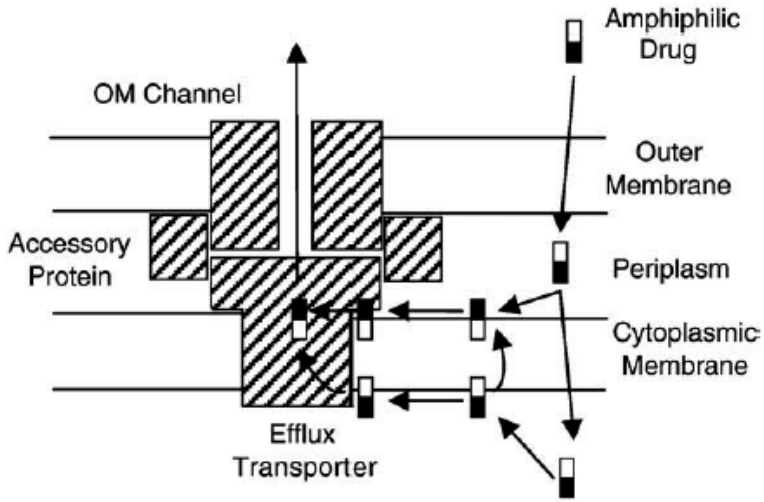
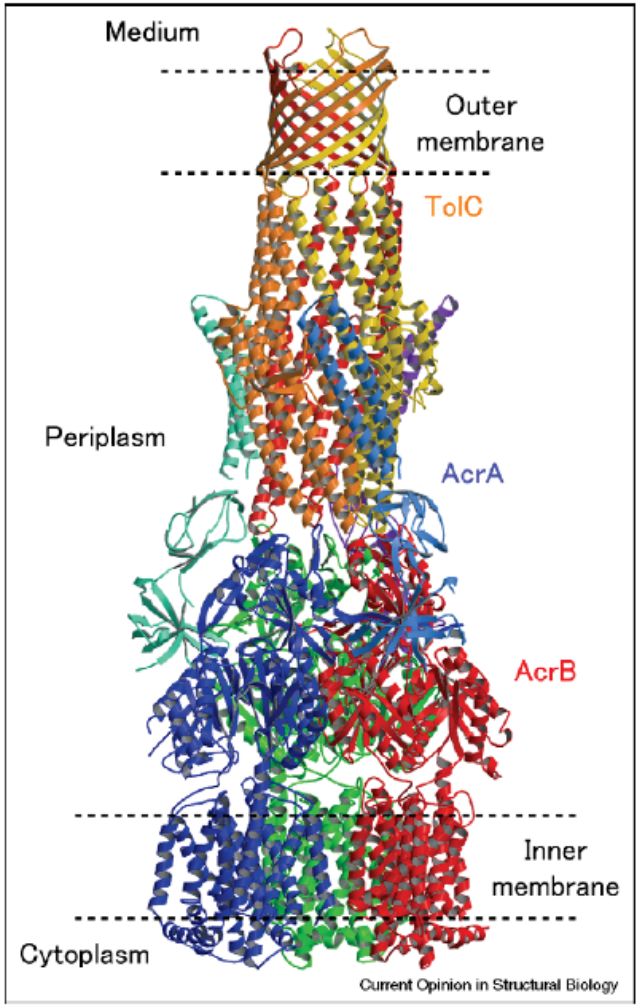


Fig. 1. An early schematic view of the tripartite pump complex. Note that amphiphilic substrates (empty and filled-in rectangles represent hydrophobic and hydrophilic parts of the molecule) are hypothesized to be captured either from the periplasm (or the periplasm–plasma membrane interface) or from 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].

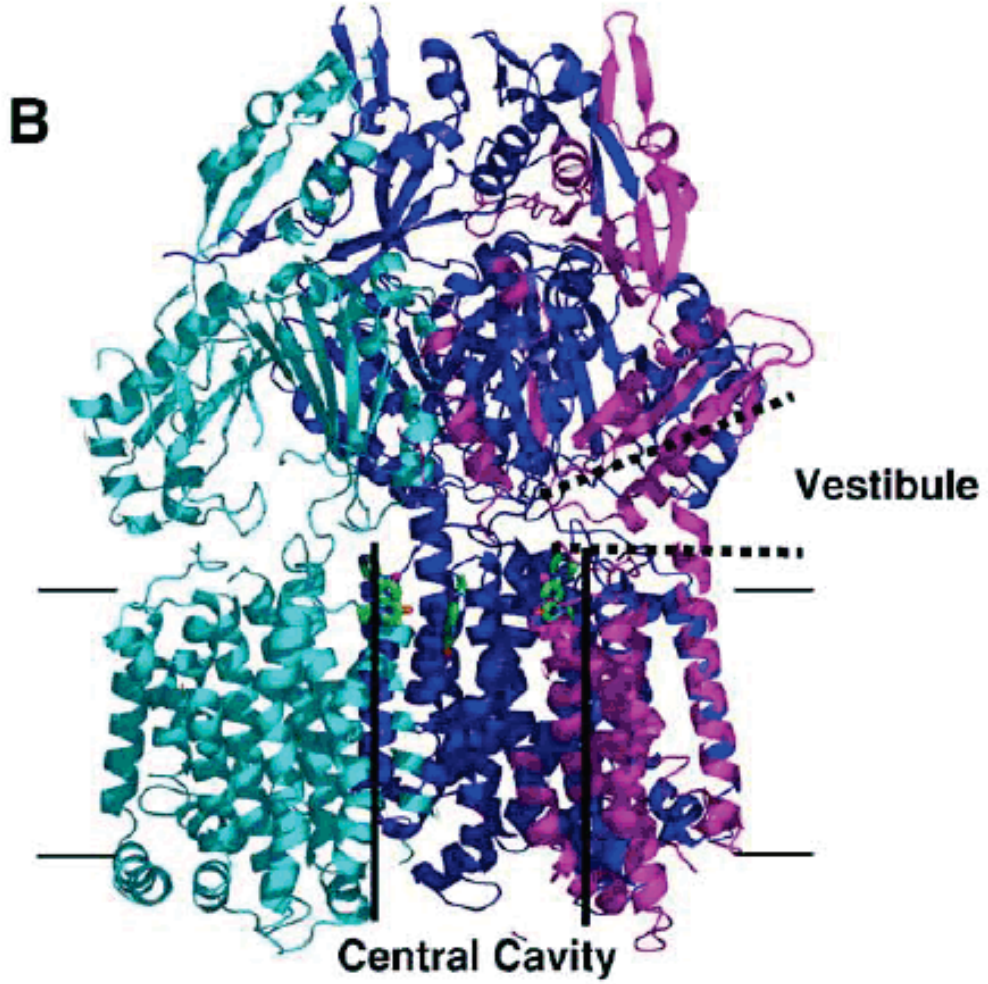
Nikaido & Takatsuka, Biochimica et Biophysica Acta 1794 (2009) 769–781



Proposed model of the AcrA–AcrB–TolC complex. Structures of AcrA [14] and TolC [12] are manually docked to AcrB with inspection according to engineered cysteine cross-linking study between AcrB–TolC [9] and AcrA–TolC [50].

Murakami S., Current Opinion in Structural Biology 2008, 18:459–465

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

Pos K. Biochimica et Biophysica Acta 1794 (2009) 782–793
Seeger et al. Curr. Drug Targets 9 (2008) 729–749.

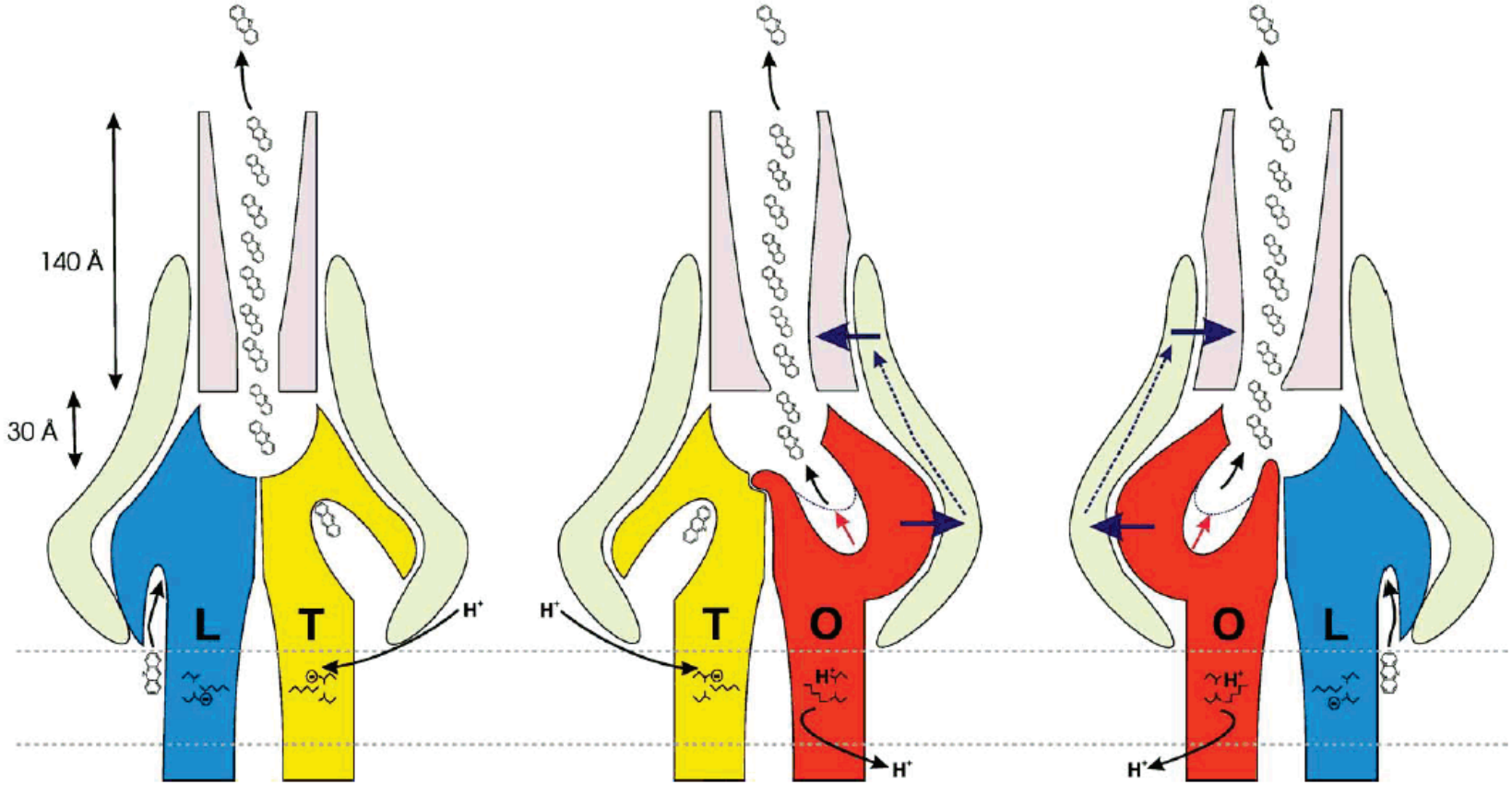


Fig. 10. Schematic representation of the AcrB alternating site functional rotation transport mechanism. The conformational states loose (L), tight (T), and open (O) are colored blue, yellow and red, respectively. Only two of the three monomers of the AcrB trimer are shown in side-view. AcrA and TolC 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 TolC (O conformation). Peristaltic transport of drugs through the AcrB tunnels (indicated by the red arrow) and through TolC in combination to the line up of drug molecules inside the AcrB funnel and the TolC 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 TolC (indicated by black arrows), which results in the movement of the proximal part of TolC and the facilitation of drug extrusion to the outside of the cell. From Seeger et al. [11] with permission.

AcrB-TolC is a **multidrug** transporter

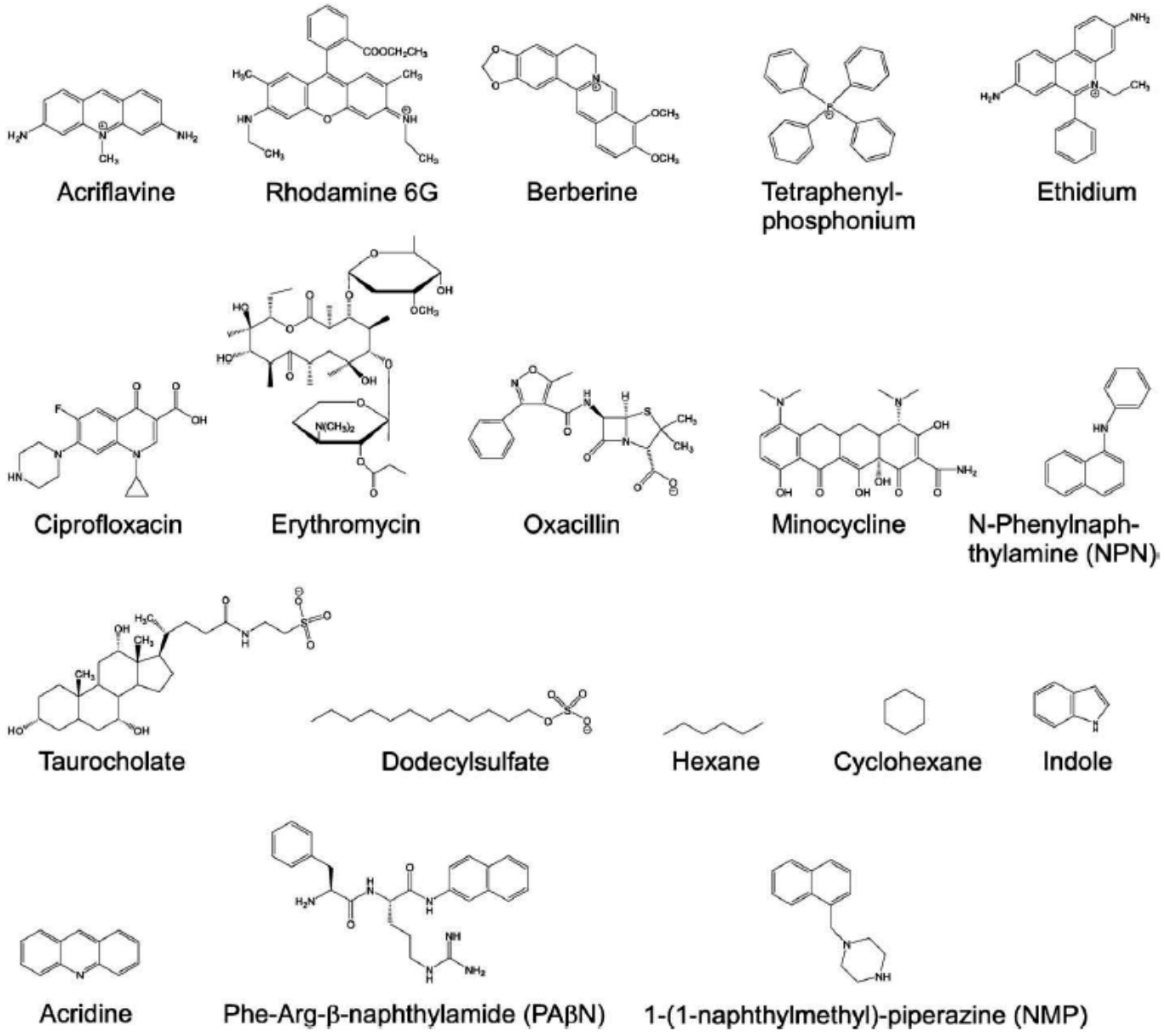


Fig. 1. Substrates and inhibitors of the AcrAB–TolC efflux system. The system confers resistance to a wide variety of noxious substances like dyes, different classes of antibiotics, detergents, bile salts and small organic molecules. Phe-Arg--naphthylamide and 1-(1-naphthylmethyl)-piperazine (NMP) inhibit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

Pos K. Biochimica et Biophysica Acta 1794 (2009) 782–793 / Seeger et al. Curr. Drug Targets 9 (2008) 729–749.

How can AcrB be a multi-drug ?

LETTER

doi:10.1038/nature10641

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

Ryosuke Nakashima[1]*, Keisuke Sakurai[1]*, Seiji Yamasaki[2], Kunihiko Nishino[3] & Akihito Yamaguchi[1,2]

Nature. 2011 Nov 27;480(7378):565-9.

- Our structures indicate that there are two discrete multisite binding pockets along the intramolecular channel.
- High-molecular-mass drugs (**rifampicin**[1], **erythromycin**[2]) 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 **minocycline**[3] and **doxorubicin**[4], 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.

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[1] 822; [2] 733; [3] 457; [4] 543

Interplay of RND and porins

Structure, Function and Regulation of Outer Membrane Proteins
The Open Microbiology Journal, 2013, Volume 7 23

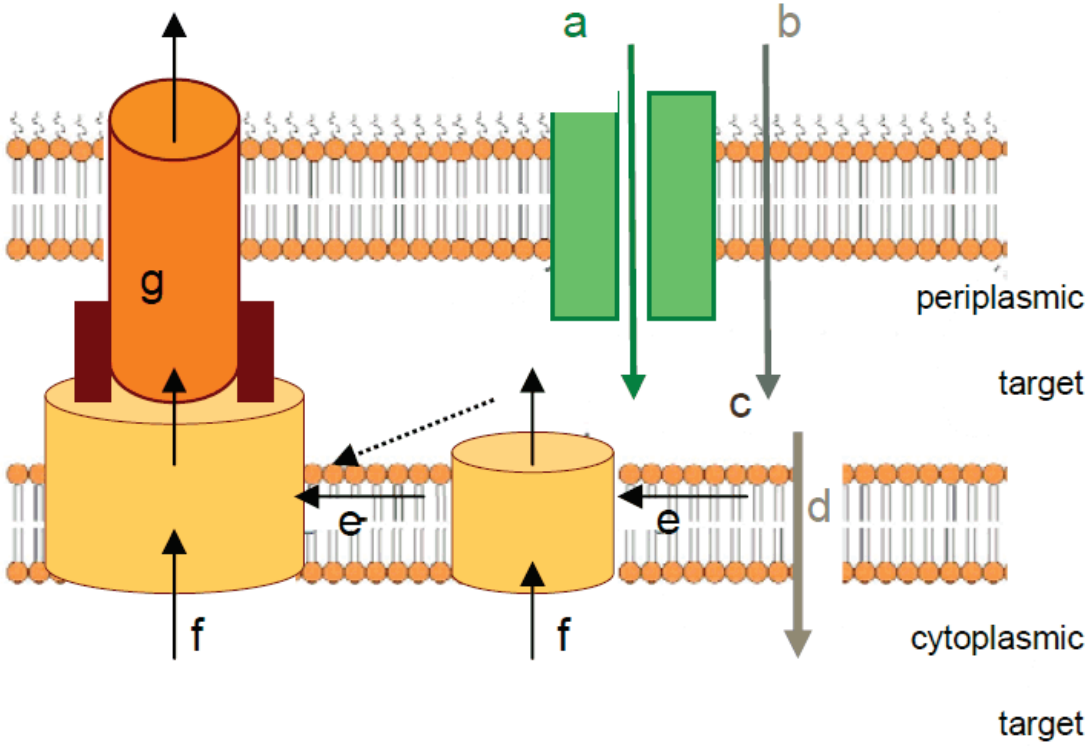

Fig. (1). Antibiotic transport through the membranes of Gram-negative bacteria (reproduced from [168]).

Rosner JL, Martin RG.
J Bacteriol 2009; 19: 5283-92

What is in the menu ?

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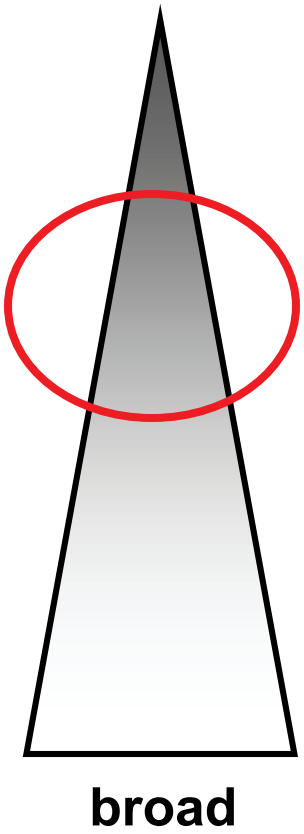
- Efflux and clinical susceptibility and impact of treatment
- Cooperation with other mechanisms of resistance
- Inhibitors of efflux ?

Efflux as a significant mechanism of resistance in Gram-positive bacteria

spectrum



specific for one (or a few) families of drugs

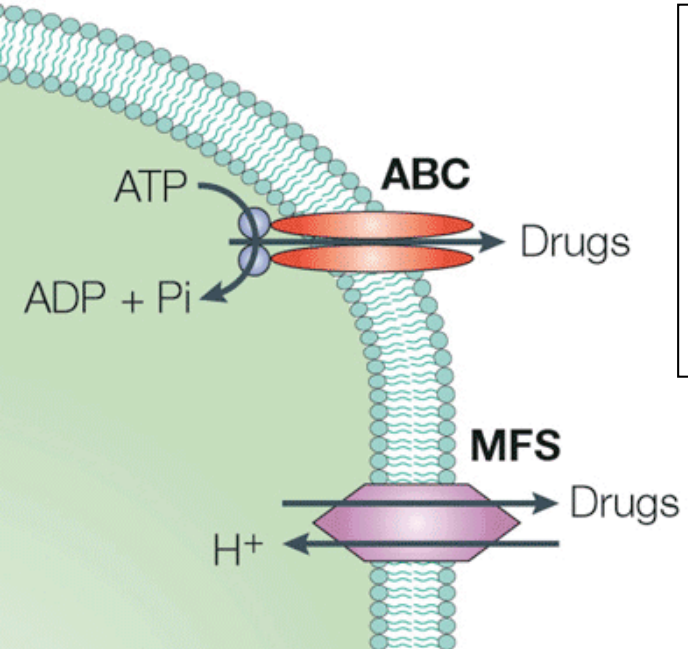
ABC

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
« **A**TP-**B**inding **C**assette »

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

Secondary transporters
(Proton motive force)

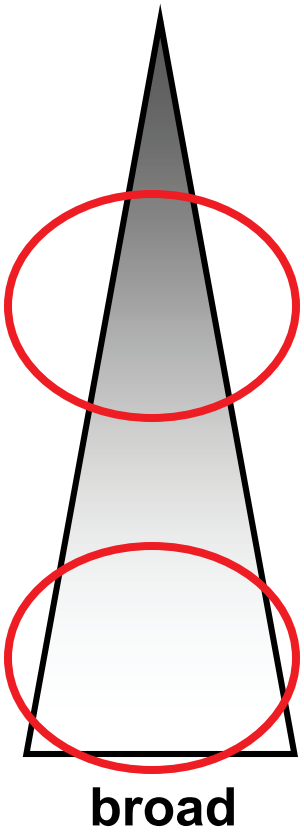
PmrA (Sp)
Gill et al, AAC 1999; 43:187-9

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

Terry et al., Nature Reviews Microbiology 2005; 3: 566-572

Efflux as a a significant mechanism of resistance in Gram-negative bacteria

spectrum



specific for one (or a few) families of drugs

MFS

TetA of *E. coli*
→Tet

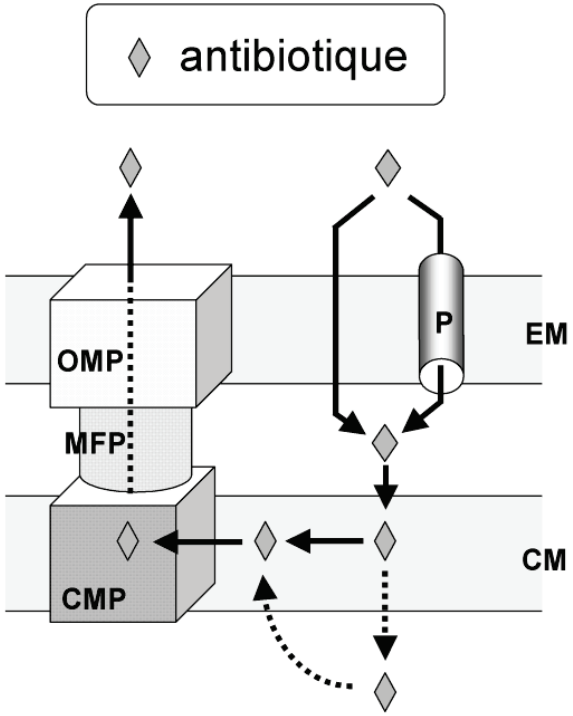
broad spectrum, conferring cross-resistance

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

Efflux and resistance in *P. aeruginosa*

Constitutive basal expression overexpressed upon induction		No basal expression; expression upon induction	
MexB	MexY	MexD	MexF
MexA	MexX	MexC	MexE
OprM	OprM	OprJ	OprN



CM: cytoplasmic membrane
(membrane cytoplasmique)

CMP: cytoplasmic membrane protein
(protéine de la membrane cytoplasmique)

EM: external membrane
(membrane externe)

MFP: membrane fusion protein
(protéine de fusion [entre membranes])

P: porin
(porine)

OMP: outer membrane protein
(protéine de membrane externe)

Mesaros et al. (2005) Louvain médical. 124:308-20

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- Inhibitors of efflux

Early data with β-lactams

TABLE 3. Apparent contribution of multidrug efflux to MIC

β-Lactam	$MIC_{wt}/MIC_{\Delta acrAB}$ in *E. coli* K-12[a]	MIC_{wt}/MIC_{acr} in *S. typhimurium*[b]	Side chain lipophilicity[c]
Cloxacillin	128	256	890
Oxacillin	512	ND	ND
Mezlocillin	32	ND	ND
Piperacillin	16	ND	ND
Cefuroxime	16	ND	55[d]
Carbenicillin	4	4	80
Penicillin G	2	32	270
Cefoxitin	4	4	130
Cephaloridine	2	2	130
Ceftriaxone	1	2	6
Cefsulodin	1	1	80[e]
Cefmetazole	1	1	2
Cefazolin	1	1	0.5
Cefepime	1	ND	6
Cefpirome	1	1	6
Imipenem	1	1	0.3

[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).

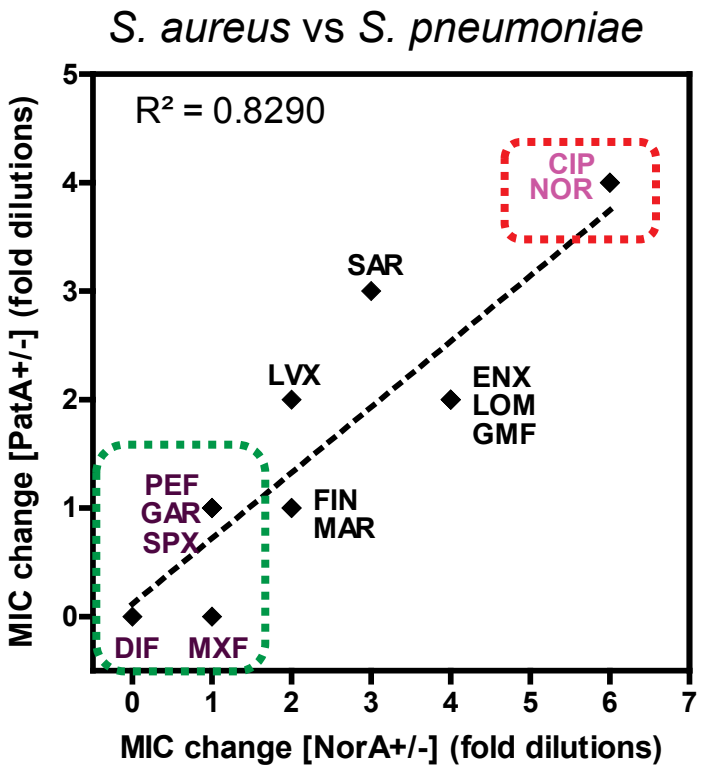
logD pH 7.4 = 2.25 (REAXYS)

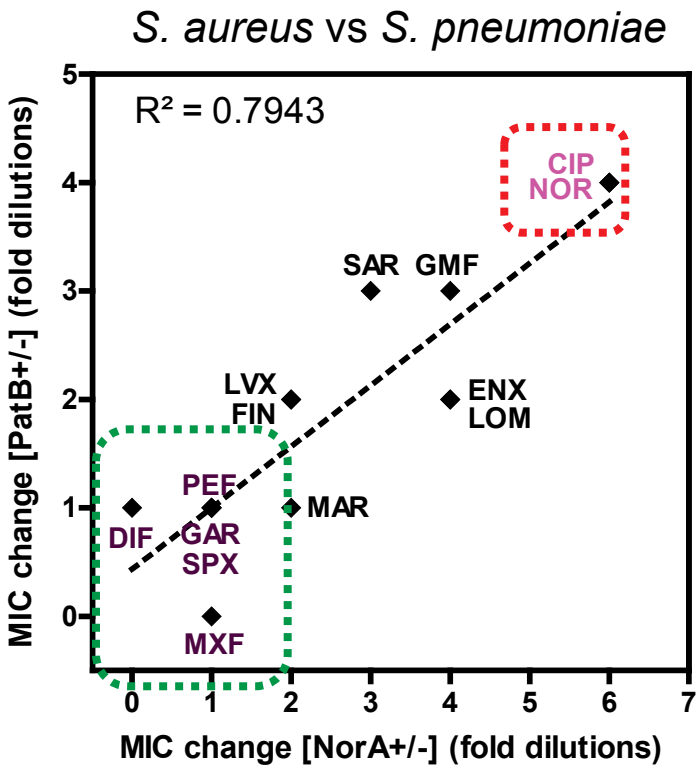
logD pH 7.4 = 0.25 (REAXYS)

Mazzariol et al. Antimicrob Agents Chemother. 2000 May;44(5):1387-90.

Substrate specificity of efflux pumps

14 fluoroquinolones; Gram + *versus* Gram +





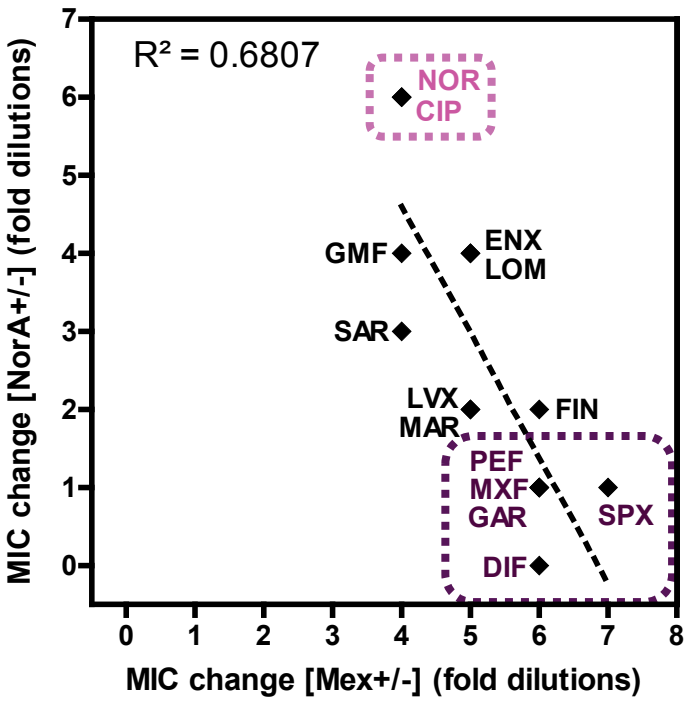
Similar recognition for non phylogenitically-related transporters

Vallet et al. ECCMID 2011

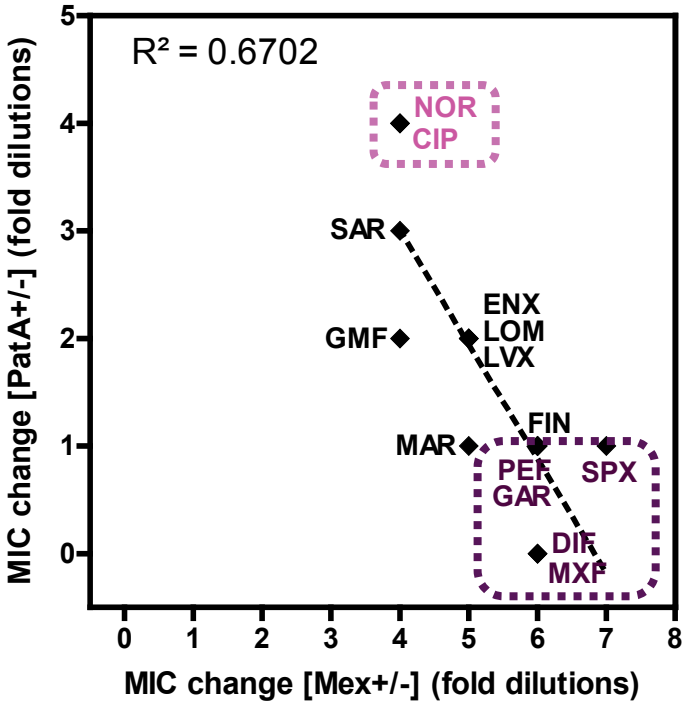
Substrate specificity of efflux pumps

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

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Pseudomonas and penem efflux

Mex pumps			MICs					
AB	CD	XY	MERO	IMI	BIA	PANI	FARO	RITI
-	-	-	0.032	0.25	0.25	0.25	1	2
+ *	-	-	**0.25**	1	0.5	4	512	128
++	-	-	**1**	0.25	0.25	1	4096	256
-	++	-	**0.25**	0.125	0.063	0.25	16	4
-	-	++	0.063	0.25	0.25	2	4	8

* clinical isolate, basal level of expression

Okamoto *et al*. J. Infect. Chemother (2002) 8: 371-373

Okamoto *et al.* AAC (2002) 46:2696-2699

Pseudomonas and penem efflux

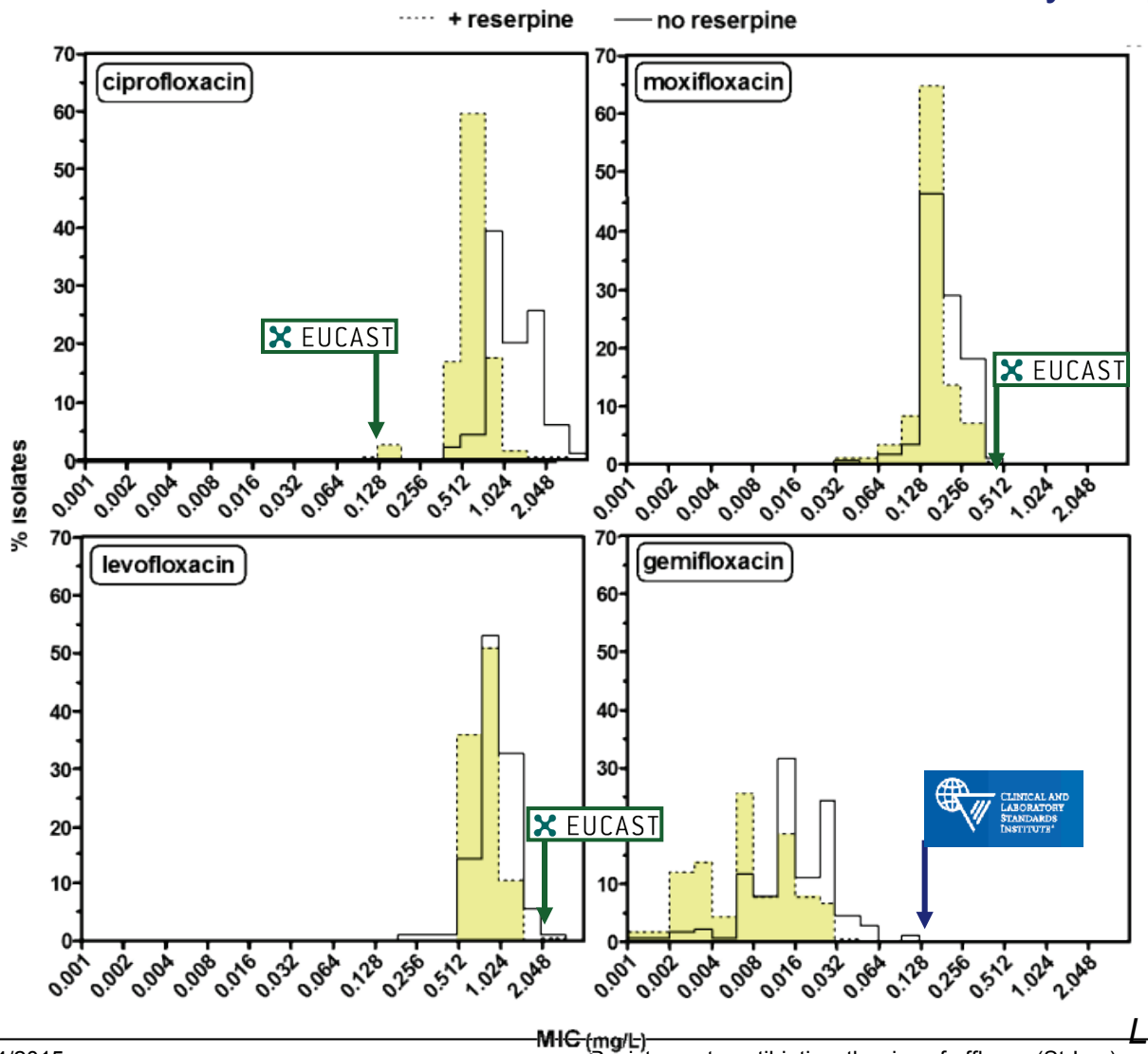
Mex pumps			MICs					
AB	CD	XY	MERO	IMI	BIA	PANI	FARO	RITI
-	-	-	0.032	0.25	0.25	0.25	1	2
+ *	-	-	0.25	1	0.5	4	512	128
++	-	-	1	0.25	0.25	1	4096	256
-	++	-	0.25	0.125	0.063	0.25	16	4
-	-	++	0.063	0.25	0.25	2	4	8

* clinical isolate, basal level of expression

Okamoto *et al*. J. Infect. Chemother (2002) 8: 371-373
Okamoto *et al*. AAC (2002) 46:2696-2699

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

Strain	Description	Efflux characteristics					MIC (mg/L)	
		Gene expression level						
		mexA [a]	*mexX* [a]	*oprM* [a]	*mexC* [b]	*mexE* [b]	temocillin (+ PAβN [c])	ticarcillin (+ PAβN [c])
Reference strain								
PAO1		1	1	1	-	-	256-512 (64)	32 (16)
Engineered strains								
CB 536	PAO1 Δ*mexCD-oprJ*	1.09	1.65	ND	-	+	128 (16)	8 (1)
CB603	PAO1 Δ*mexEF-oprN*	1.21	1.02	0.51	-	-	128 (32)	16 (16)
CB602	PAO1 *mexXY::FRT*	1.10	*0.06*	0.55	-	+	64 (16)	16 (16)
PAO1 mexAB	PAO1 *mexAB::FRT*	*0* [m]	1.08	ND	-	+	4 (2)	2 (2)

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

Buyck et al, J. Antimicrob. Chemother. (2012) 67(3):771-5

Intrinsic resistance of *Pseudomonas* to temocillin

But temocillin is used successfully in Cystic Fibrosis patients …

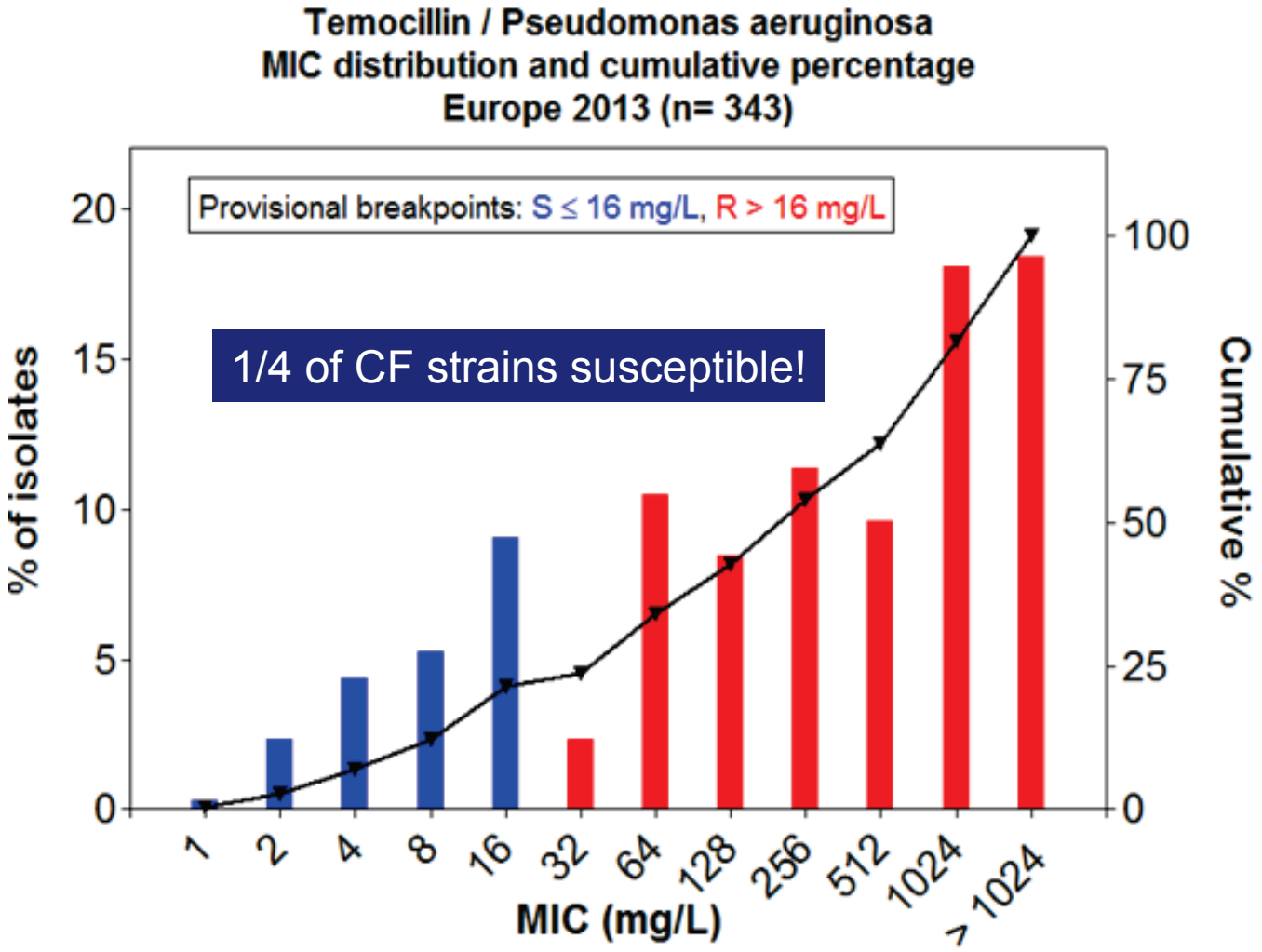
			Efflux characteristics, alterations				MIC (mg/L)	
			mexA	MexA	*mexB*	MexB	temocillin	ticarcillin
Clinical isolates from cystic fibrosis patients								
3020S	[d]		—	—	—	—	128	16
3020R	[d]	isogenic to 3020S with deletion in *mexA*	Δ 112 nt (370–482)	aberrant	—	—	2	1
3525			—	—	—	—	512	32
3807		isogenic to 3525 with mutation in *mexA*	G214A	G72S	—	—	32	4
2715	[d]	mutation in *mexA*	A590G	Y197C	—	—	32	2
616		mutation in *mexA*	C752T	S251F	—	—	1	0.5
2729	[d]	deletion in *mexA*	Δ 8 nt (576–583)	aberrant	—	—	2	1
2933	[d]	deletion in *mexA*	Δ 1 nt (870)	aberrant	—	—	2	0.5
2998	[d]	deletion in *mexA*	C205T	truncated	—	—	2	0.25
2721	[d]	deletion in *mexA*	Δ 1 nt (860)	aberrant	—	—	1	0.25
2716	[d]	mutation in *mexB*	—	—	A776T	Q259L	1	0.5
2804	[d]	deletion in *mexB*	—	—	Δ 1 nt (2147)	aberrant	4	1
2858	[d]	deletion in *mexB*	—	—	Δ 1 nt (494)	aberrant	1	0.5
3066		deletion in *mexB*	—	—	G2364A	truncated	1	0.5

Natural mutations in MexAB-OprM restore temocillin activity

Buyck et al, J. Antimicrob. Chemother. (2012) 67(3):771-5

Intrinsic resistance of *Pseudomonas* to temocillin

Is this clinically relevant ?



Chalhoub, unpublished

Conditions modulating efflux and susceptibility

Azithromycin is widely and successfully used in Cystic Fibrosis patients

J Antimicrob Chemother 2011; **66**: 968–978
doi:10.1093/jac/dkr040 Advance Access publication 2 March 2011

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]

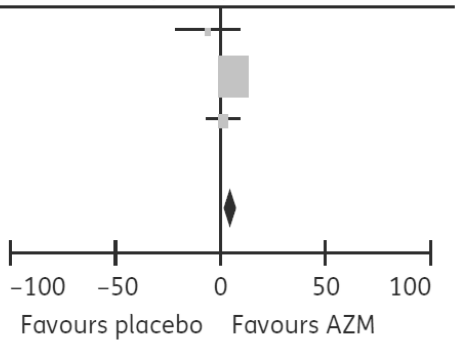
Conclusions: Long-term use of azithromycin can improve lung function, especially for *P. aeruginosa*-colonized CF patients. There was no evidence of increased adverse events with azithromycin. More data are needed to verify the best azithromycin regimen and to evaluate other macrolides in CF patients.

FEV_1% change in *P. aeruginosa*-infected patients

	AZM			Placebo				Mean difference
Study or subgroup	**mean**	**SD**	**total**	**mean**	**SD**	**total**	**weight**	**IV, fixed, 95% CI**
Clement 2006[10]	−8.7	11.5	9	−2.7	21	10	4.4%	−6.00 [−21.03, 9.03]
Saiman 2003[12]	4.4	13.6	87	−1.8	10.7	98	77.9%	6.20 [2.64, 9.76]
Steinkamp 2008[8]	−3.7	13.3	21	−5	10.1	17	17.8%	1.30 [−6.14, 8.74]
Total (95% CI)			**117**			**125**	**100.0%**	**4.80 [1.66, 7.94]**

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

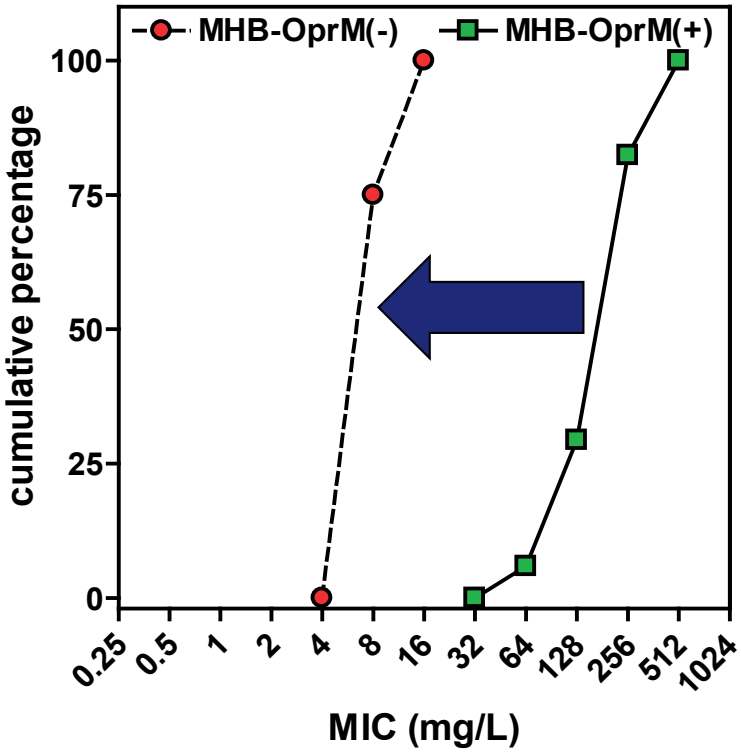
Test for overall effect: $Z = 3.00$ ($P = 0.003$)



BUT *Pseudomonas* is supposedly intrinsically resistant ….

Intrinsic resistance of *Pseudomonas* to macrolides

Is *Pseudomonas* « intrinsically » resistant to macrolides ?



Major role
of constitutively-expressed
transporters!

Buyck et al. Clin Infect Dis. 2012; 55:534-42

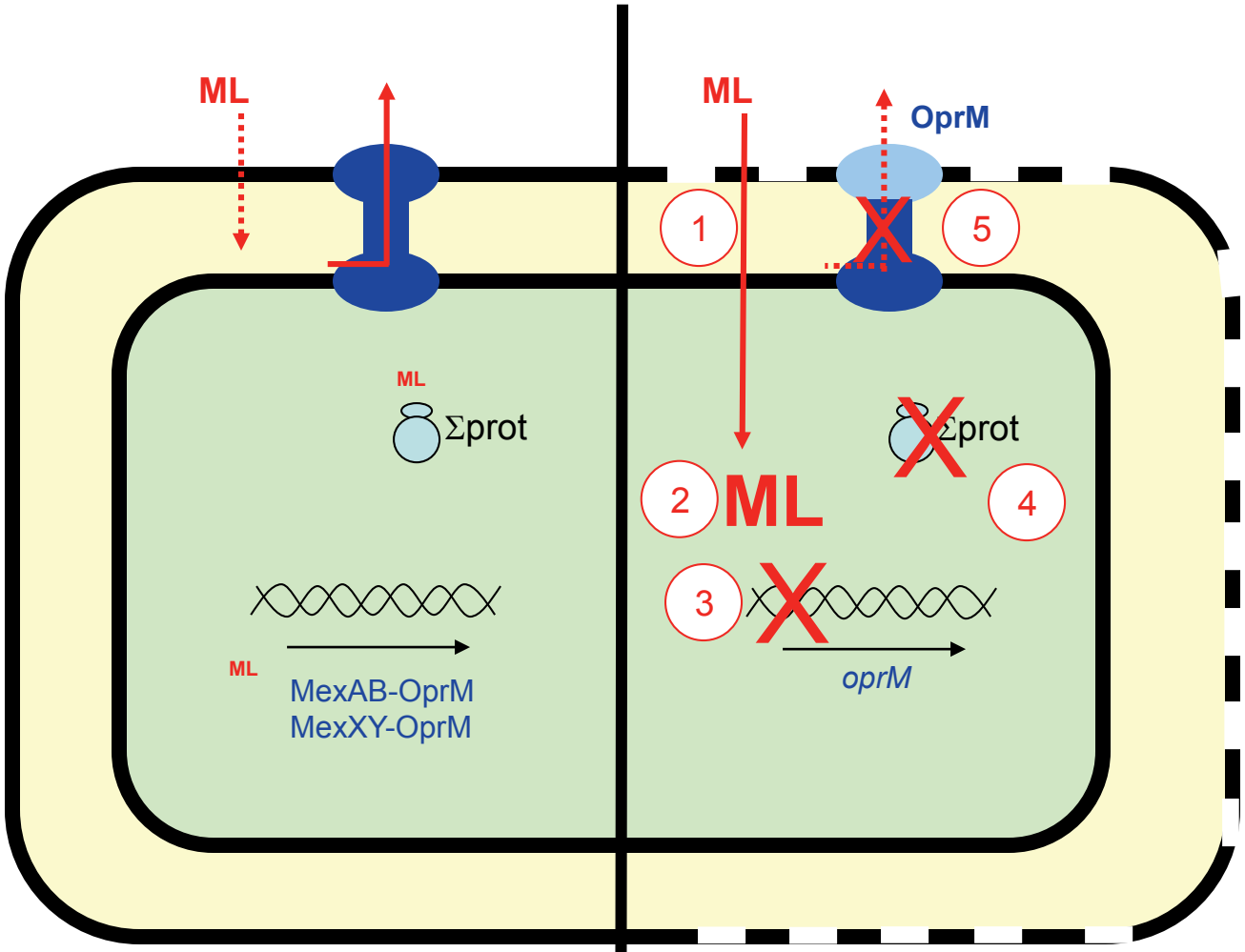
An intriguing observation …

Antibiotic	MIC (mg/L)		
	CA-MHB		RPMI-1640
	pH 7.4	pH 5.5	
Aminoglycosides			
Gentamicin	2	8	4
Amikacin	4	64	4
Tobramycin	1	8	1
β-lactams			
Piperacillin/Tazobactam	16	16	16
Cefepime	4	8	4
Ceftazidime	2	4	2
Aztreonam	8	16	8
Meropenem	1	1	2
Fluoroquinolones			
Ciprofloxacin	0.125	0.25	0.125
Polymyxins			
Colistin	1	2	2
Azithromycin	128	>512	16

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

Buyck et al. Clin Infect Dis. 2012; 55:534-42

Why do macrolides express their activity against *P. aeruginosa* in « eukaryotic » media ?



Buyck et al.
Clin Infect Dis. 2012; 55:534-42

What is in the menu ?

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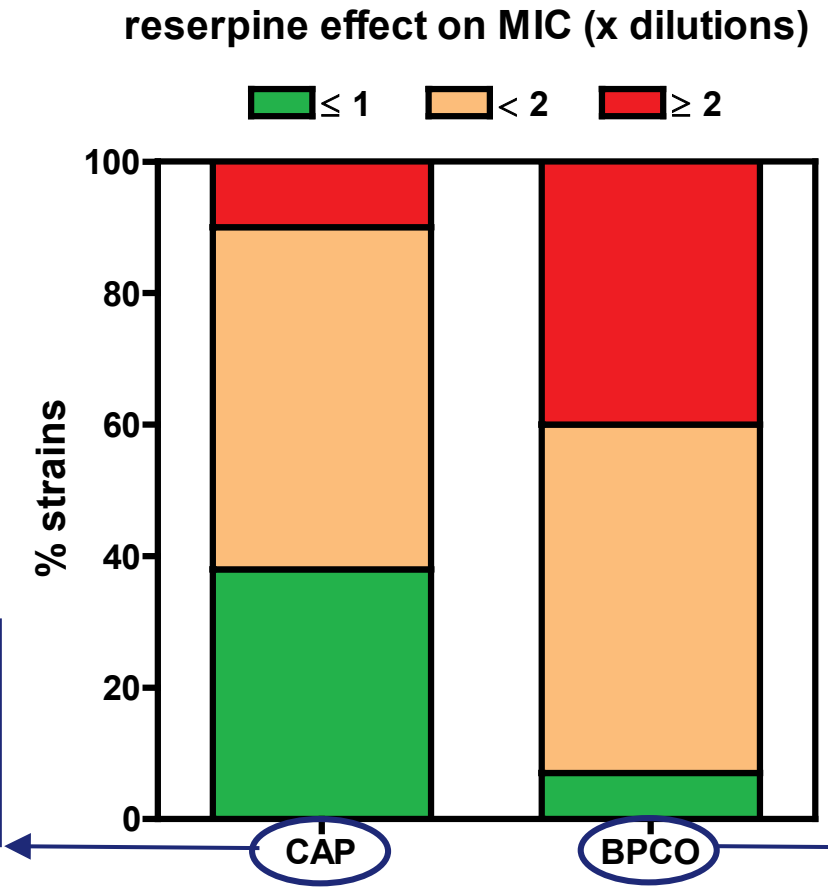
- Antibiotic transporters important for the clinical microbiologist
- Substrate specificities
- Efflux and intrinsic susceptibility
- **Efflux and clinical susceptibility and impact of treatment**
- Cooperation with other mechanisms of resistance
- Cooperation between procaryotic and eucaryotic transporters

Efflux of fluroquinolones in *S. pneumoniae:* is transporter more expressed in patients chronically treated

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



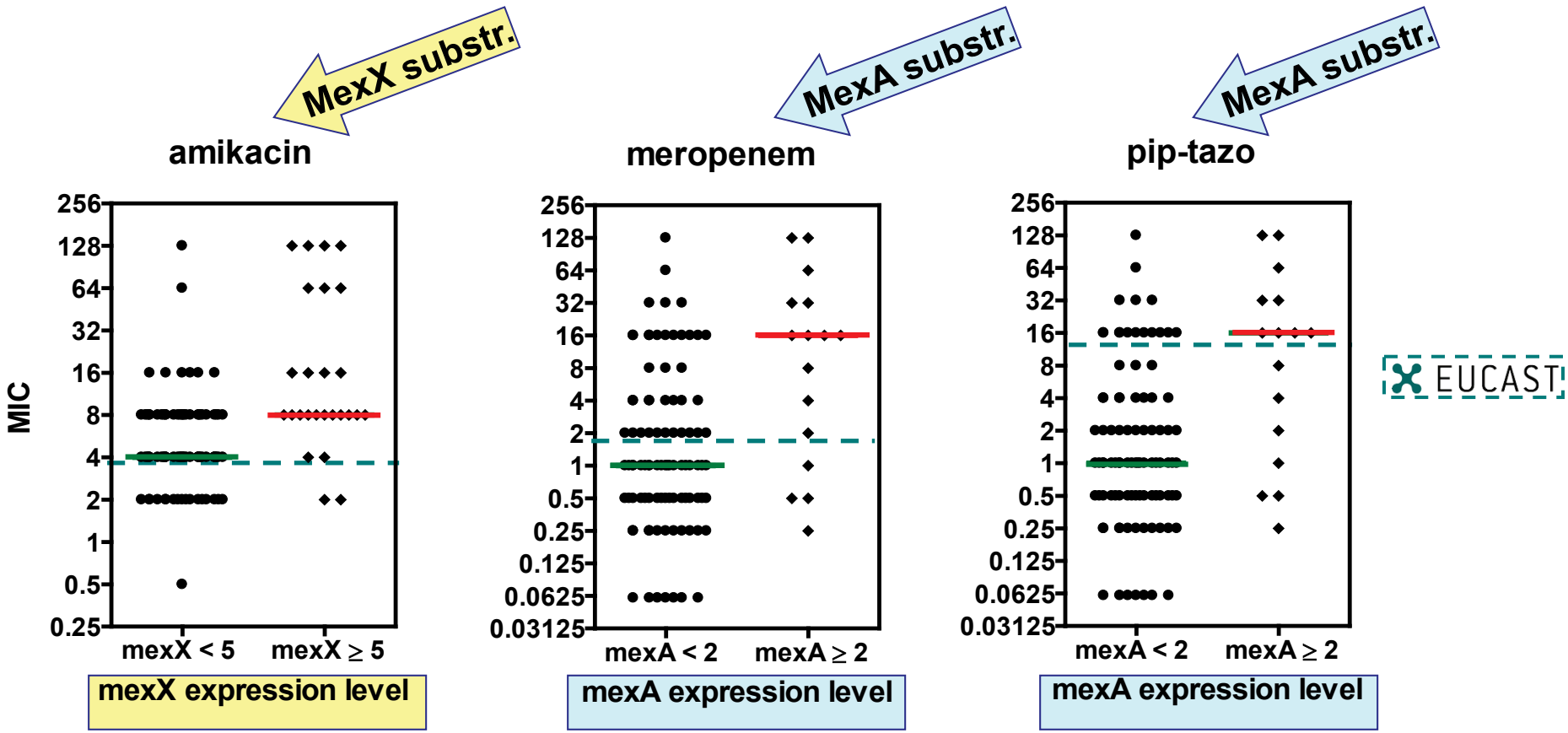
acute pathology
⬇
« one shot »
antibiotic exposure

chronic pathology
⬇
repetitive
antibiotic exposures

Lismond & Degives, unpublished

Impact of efflux on clinical susceptibility of *P. aeruginosa*

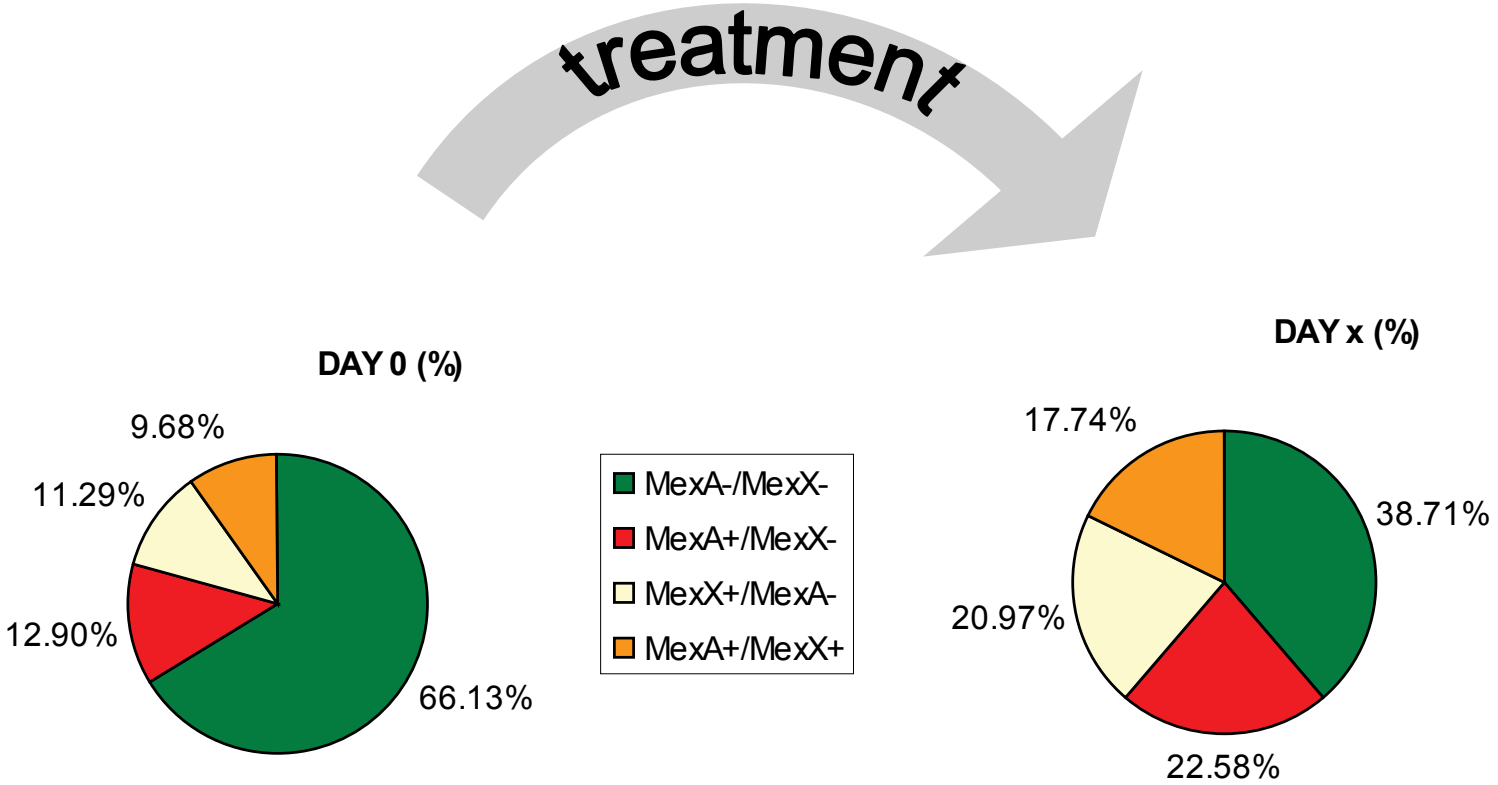
MICs vs EUCAST breakpoints for 109 *P. aeruginosa* without or with efflux mechanisms, isolated from ICU patients (VAP)



Riou et al, ECCMID 2010

Increase of *P.* aeruginosa during treatment: is efflux involved ?

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



Riou et al, ECCMID 2010
Riou et al. submitted for publication

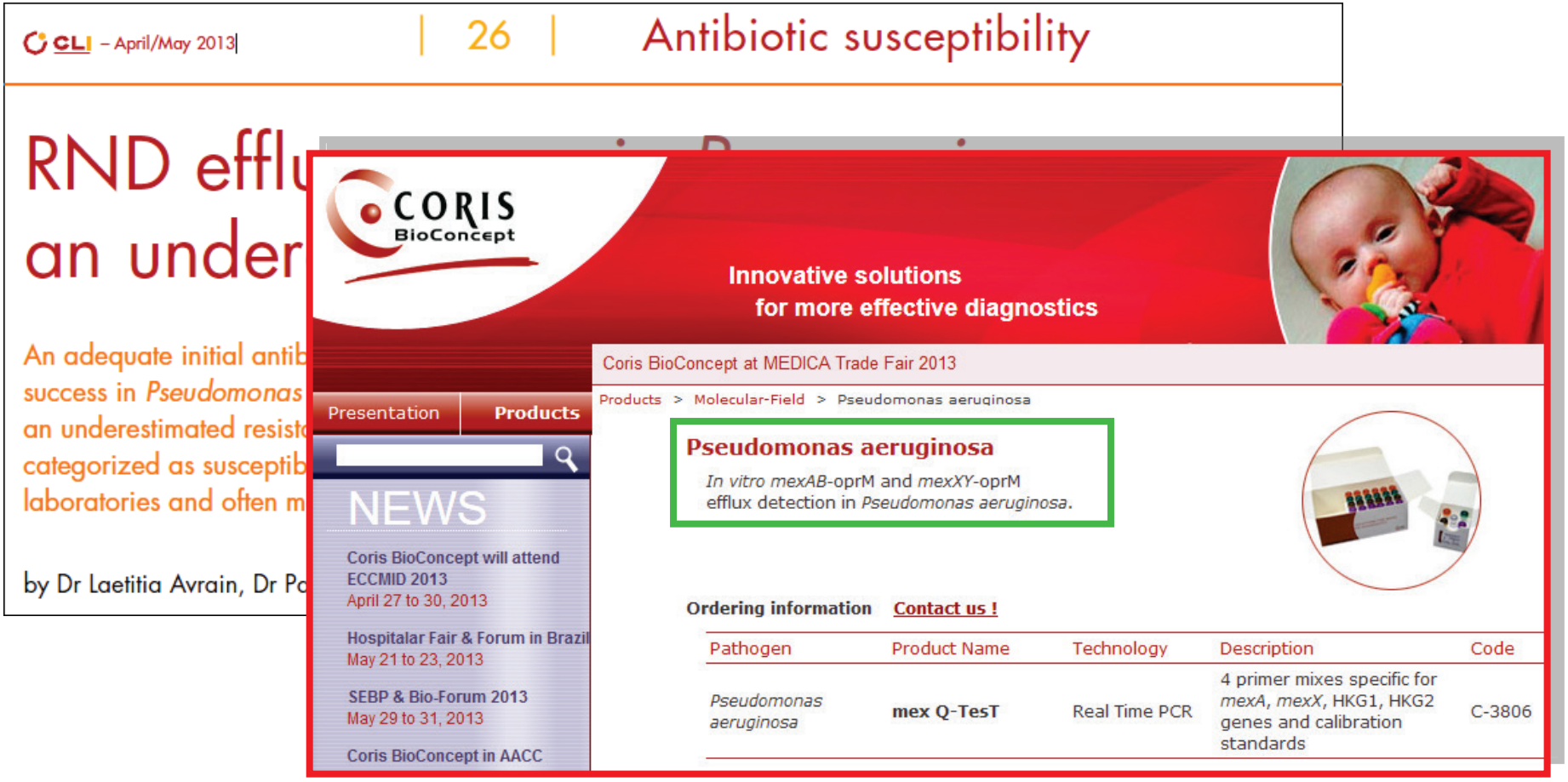
Early diagnosis could be implemented in the clinics

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



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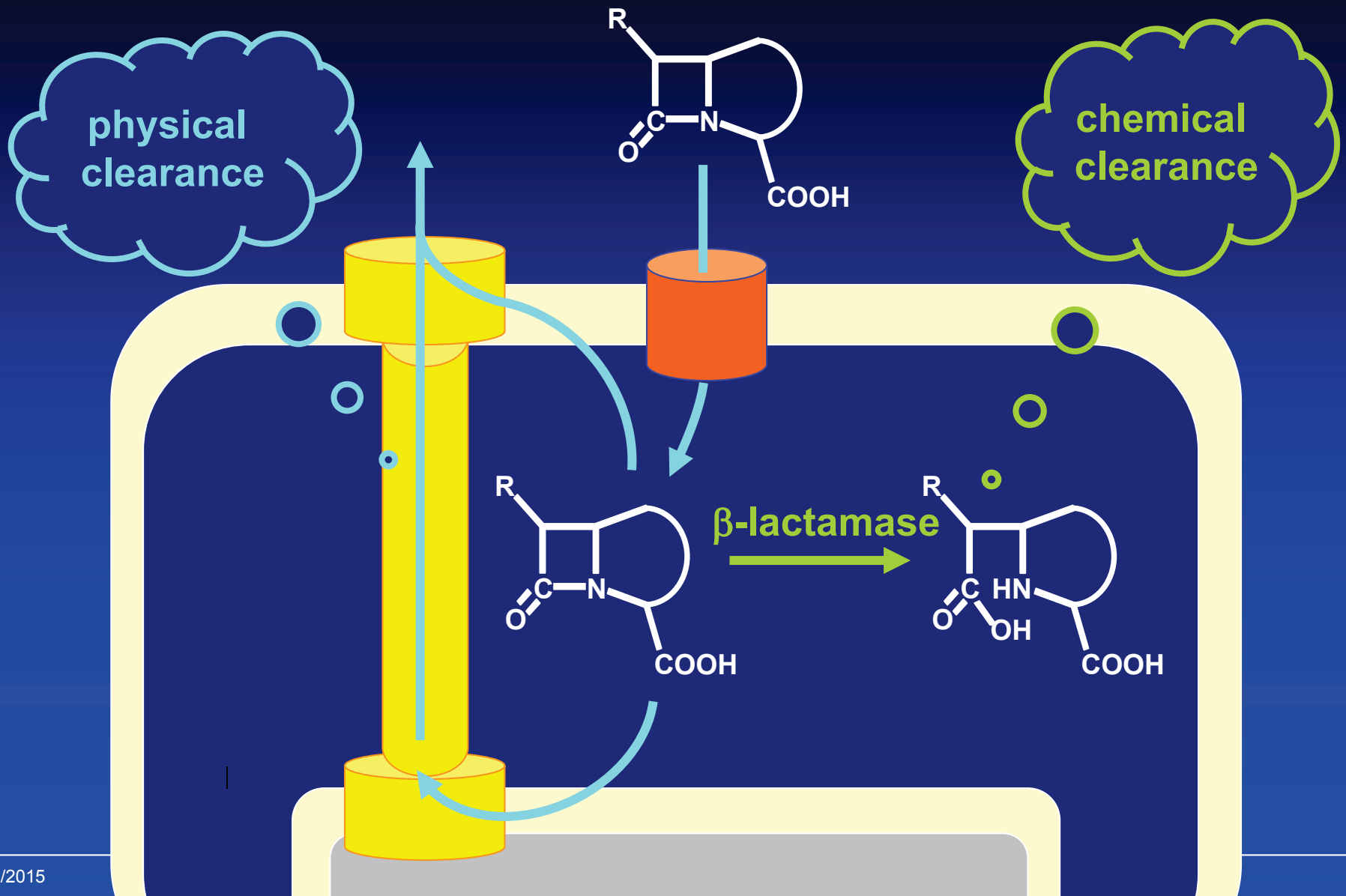
- **Cooperation between procaryotic and eucaryotic transporters**

Efflux cooperates with other mechanisms of bacterial resistance



Efflux cooperates with other mechanisms of resistance

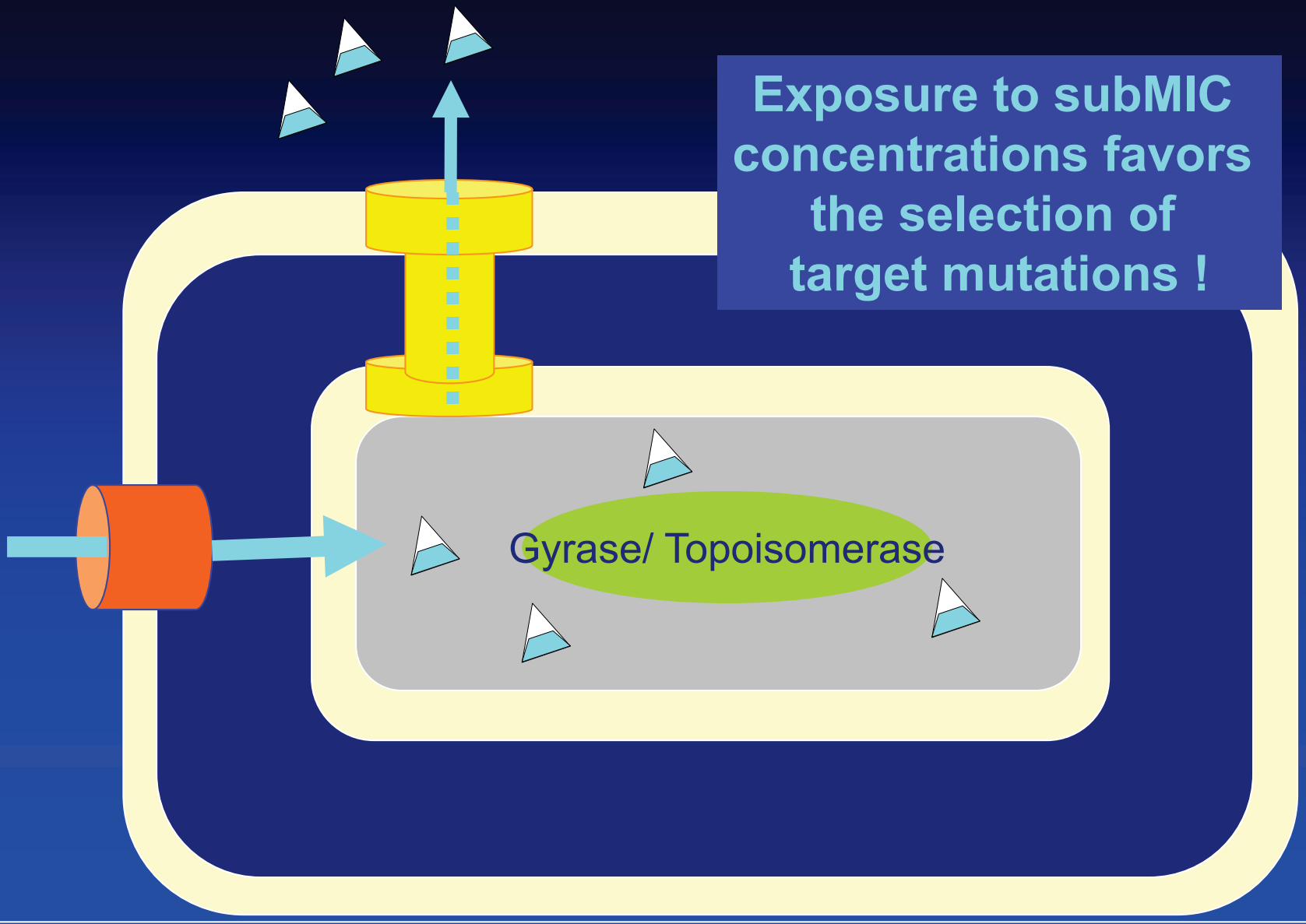
Contributions of the AmpC β-lactamase and the AcrAB Multidrug Efflux System in Intrinsic Resistance of *E. coli* to β -lactams

Efflux	β-lactamase	CMI carbenicillin	CMI ofloxacin
-	-	0.2	0.05
+	**-**	**12.5**	**0.2**
+++	-	**50**	1.56
-	+	100	0.05
+	+	200	0.39
+++	**+**	**400**	1.56

WT: intrinsic resistance !

Mazzariol *et al*, AAC (2000) 44:1387-1390

Efflux and selection of resistance to FQ



Efflux and selection of resistance

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

Pump status	LVX MIC	Frequency of LVX-resistant mutants
WT	0.25	2×10^7 - 4×10^7
Δ mexAB-oprM	0.015	2×10^7 - 4×10^7
Δ mexCD-oprJ	0.25	2×10^7 - 4×10^7
Δ mexEF-oprN	0.25	2×10^7 - 4×10^7
Δ mexAB-oprM;Δ mexEF-oprN	0.015	2×10^7 - 10^7
Δ mexCD-oprJ; Δ mexEF-oprN	0.25	2×10^6
Δ mexAB-oprM; Δ mexCD-oprJ	0.015	1×10^9
Δ mexAB-oprM; Δ mexCD-oprJ; Δ mexEF-oprN	0.015	**$<1 \times 10^{11}$**

Lomovskaya *et al*,
AAC (1999) 43:1340-1346

Selection of mutants in FQ target undetectable if ALL pumps are disrupted

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- 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 that 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…