

# Antibiotic resistance by efflux: from molecular aspects to clinical impact

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Notre Dame, IN – 16 September 2013



# Efflux is a Belgian specialty ....



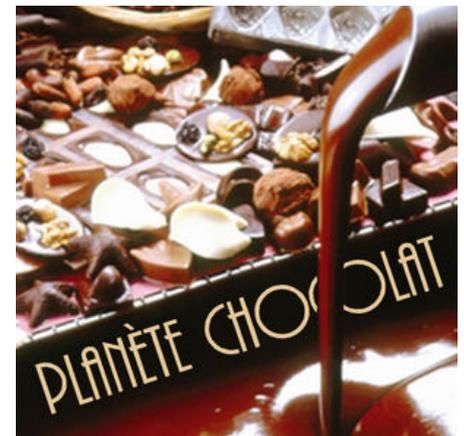
Manneken-Pis



Folon



Magritte

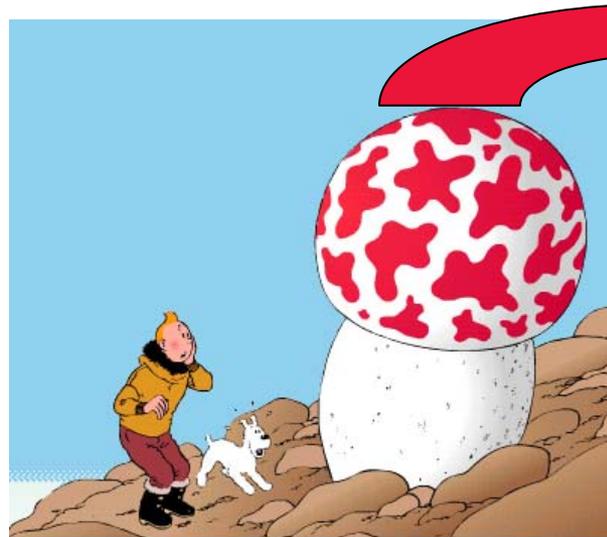
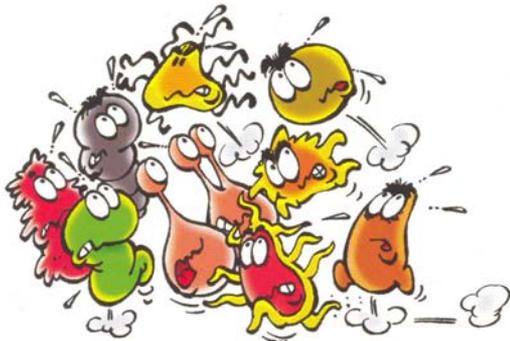
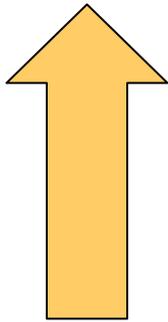


# Discovery and significance ....

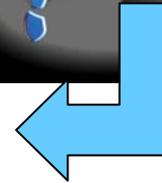
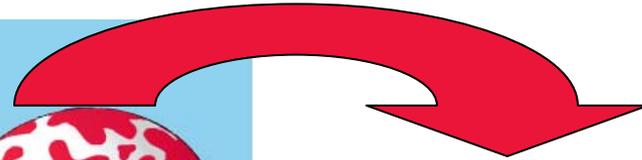


# Chemotherapeutic agents exert toxic effects on specific target cells

antibiotics

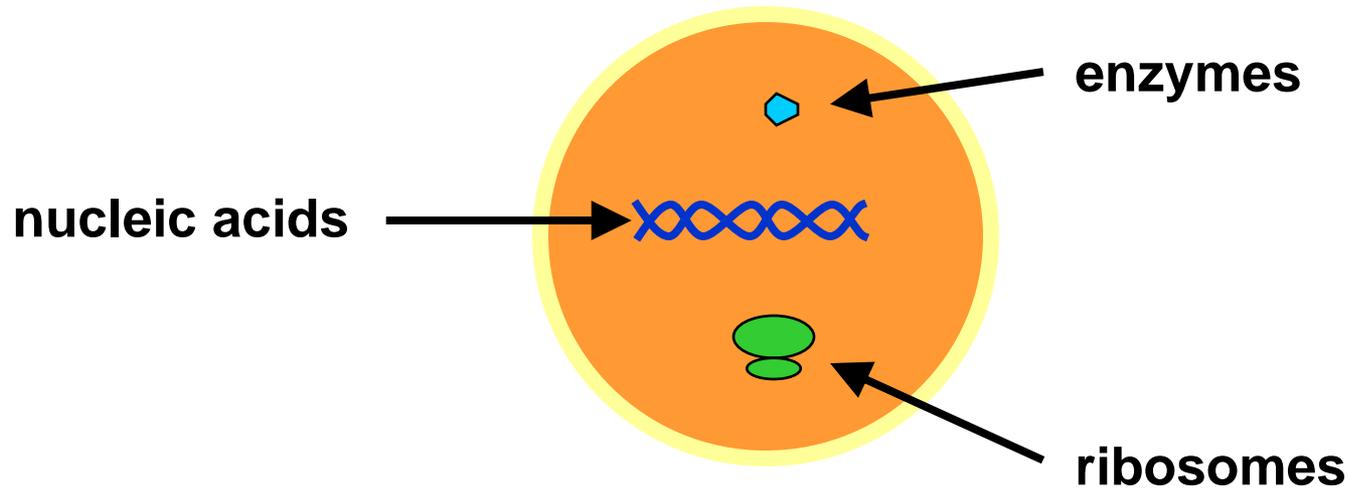


antifungals



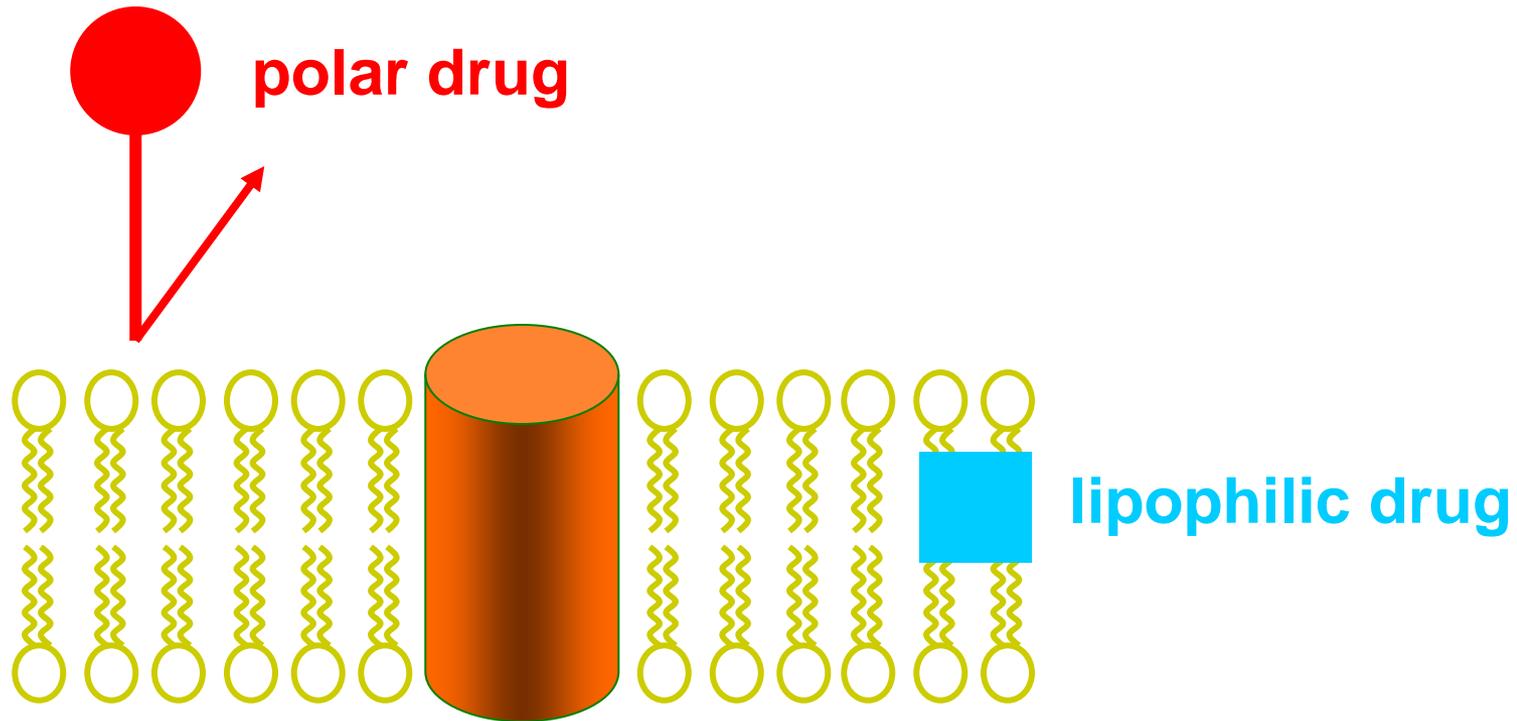
anticancer agents

# Chemotherapeutic agents exert toxic effects on specific target cells



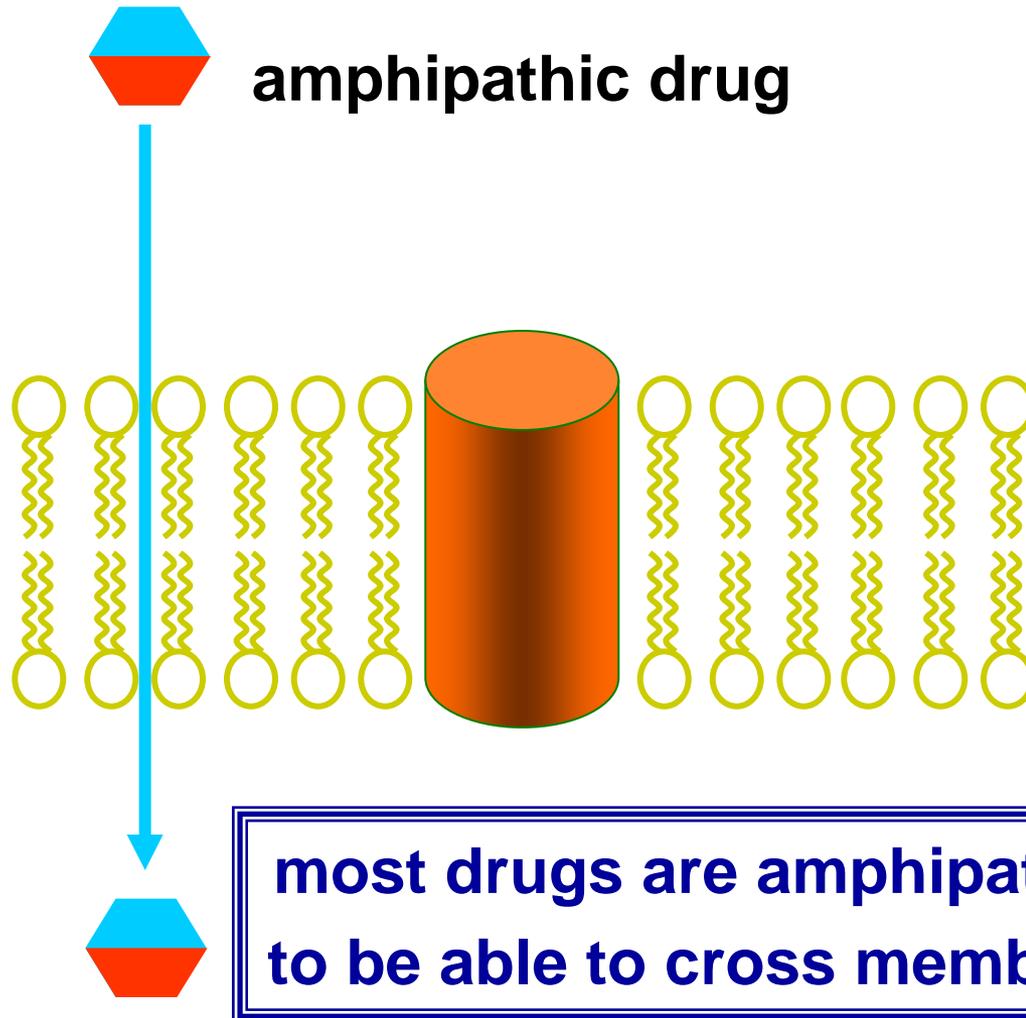
**How can these drugs reach their target inside the cells ?**

# Reaching an intracellular target ...



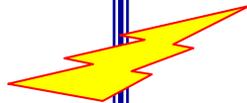
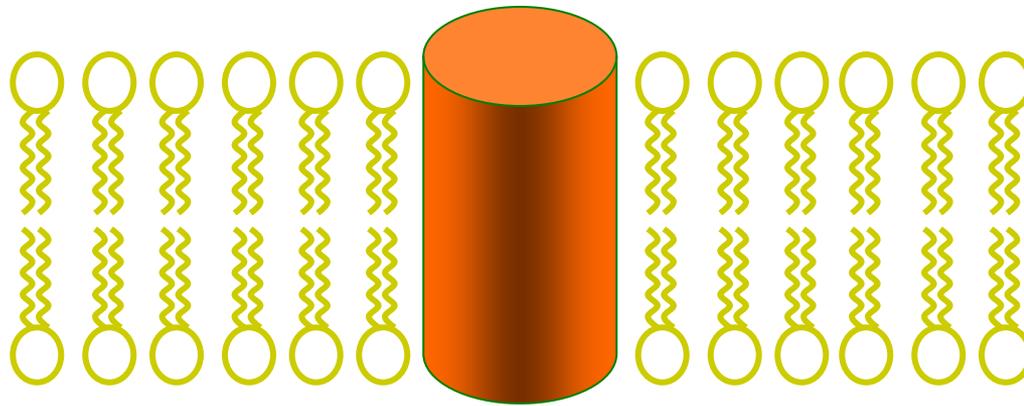
**physico-chemical properties are inadequate  
for reaching an intracellular target !**

# Reaching an intracellular target ...



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

# Intracellular chemotherapeutic agents



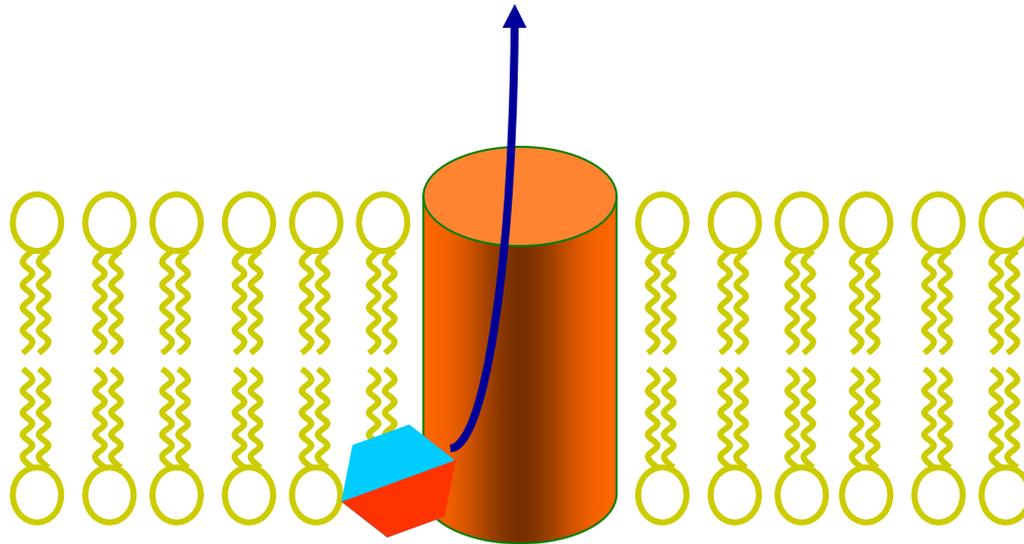
**But a diffusible compound  
may have  
potentially harmful effects !**



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

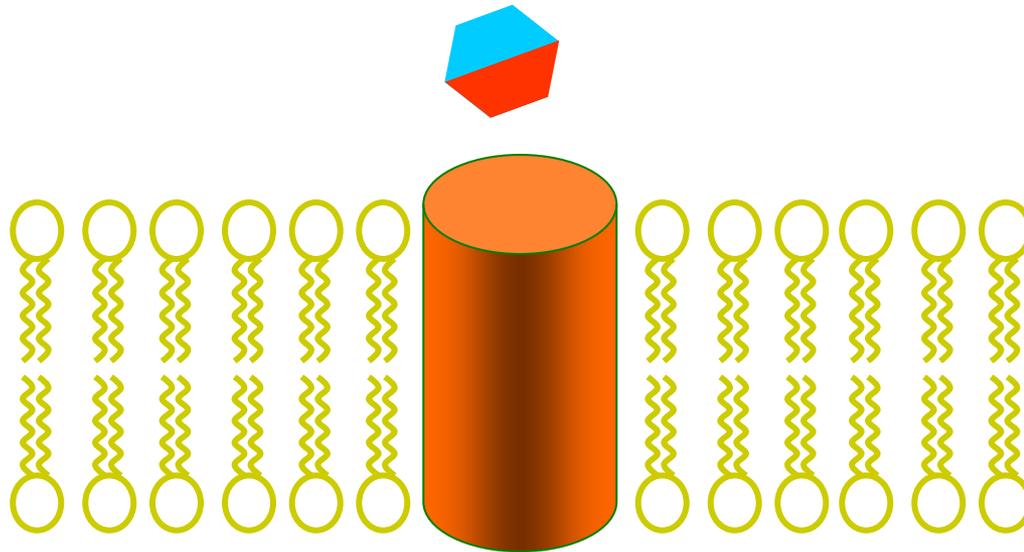
# Why efflux transporters ?

## Extrusion by efflux pumps



# Why efflux transporters ?

## Extrusion by efflux pumps

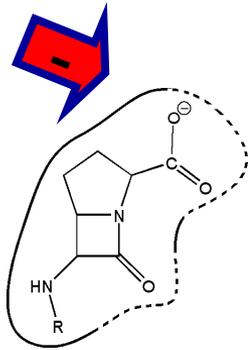


**general mean of protection  
against cell invasion by diffusible molecules**

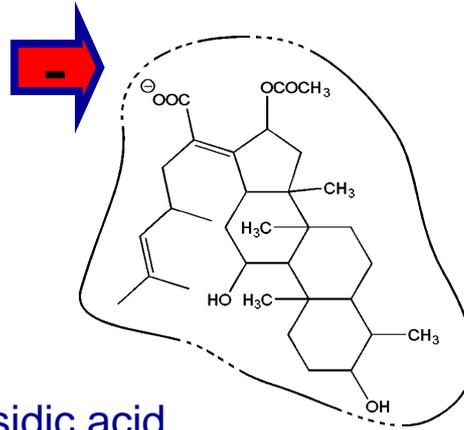


# Most antibiotics are amphiphilic !

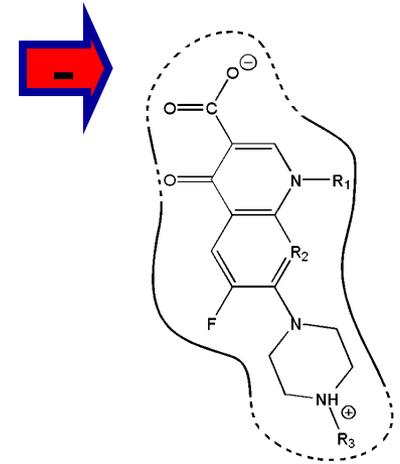
## anionic amphiphiles



$\beta$ -lactam

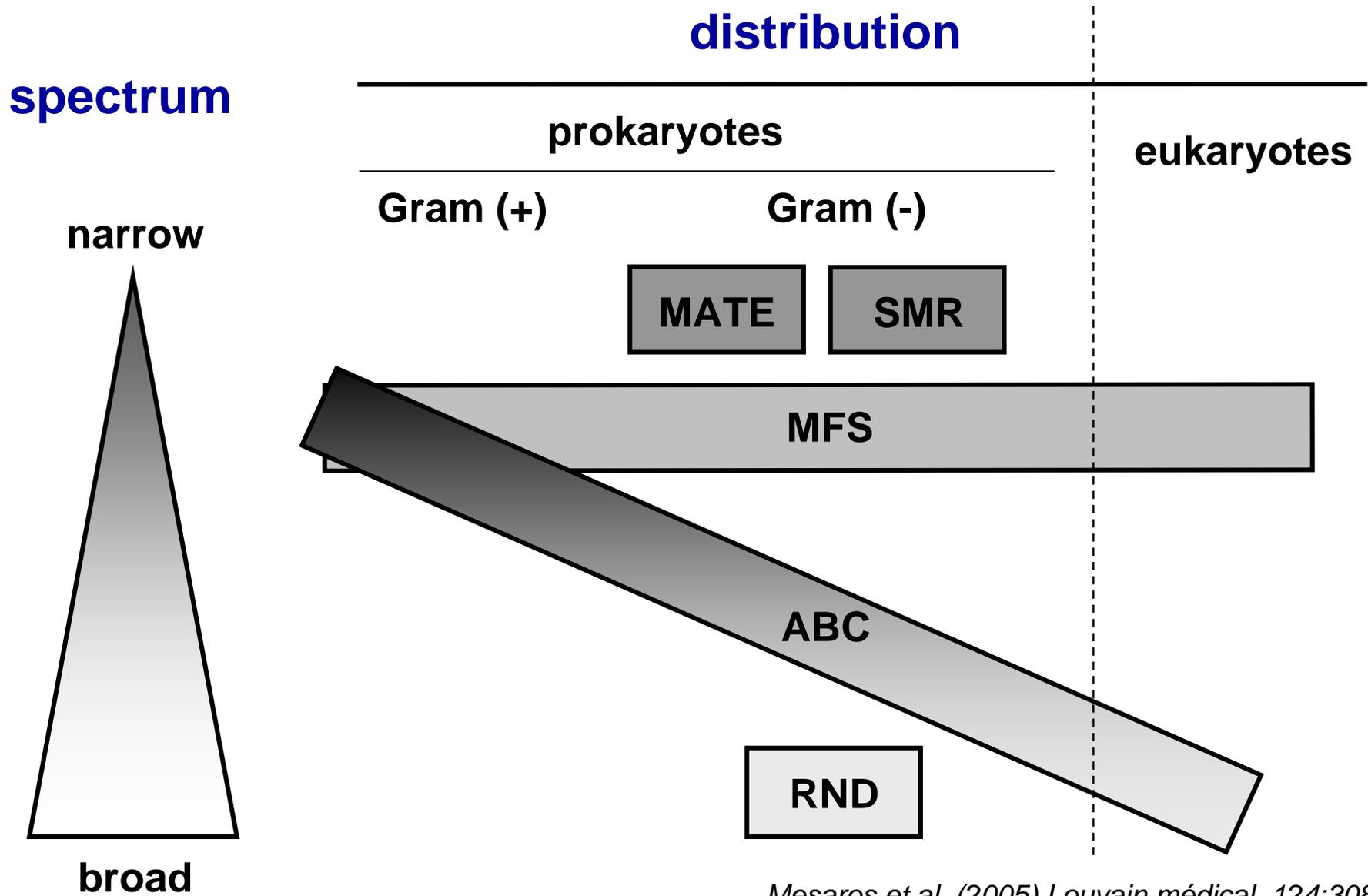


fusidic acid



fluoroquinolone

# Antibiotic efflux transporters are ubiquitous



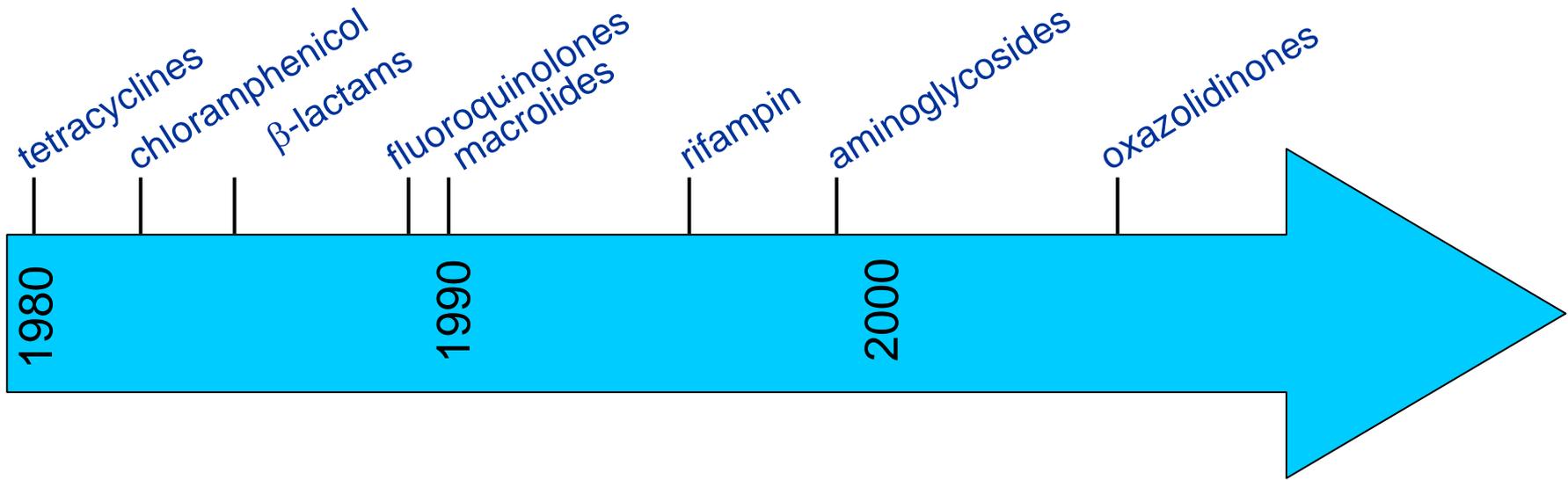
Mesaros et al. (2005) *Louvain médical*. 124:308-20

# Antibiotics as substrates of efflux pumps

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

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

# Antibiotic efflux and resistance: time line ...



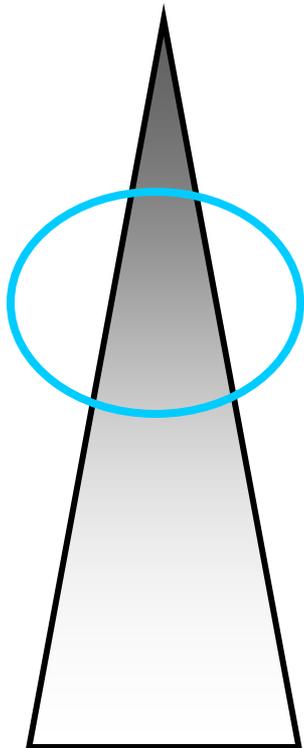
# Efflux: from molecular recognition to cellular impact



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

## spectrum

narrow



specific for one (or a few) families of drugs

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*

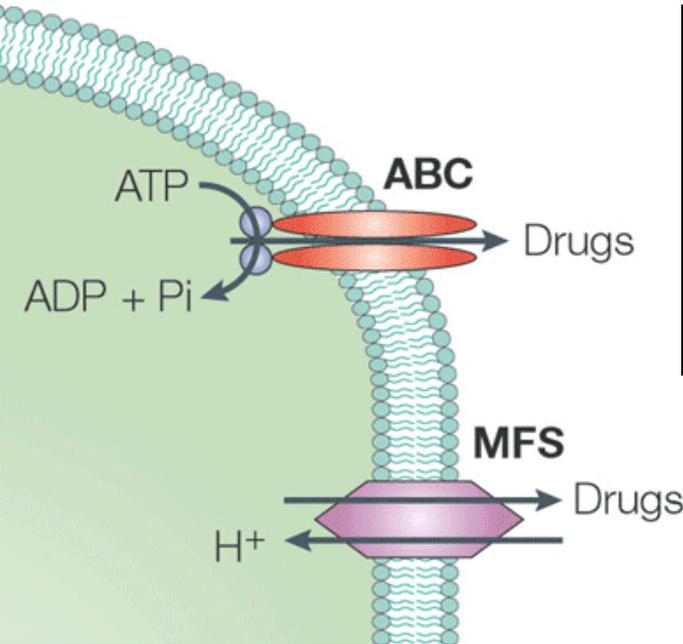
→ FQ

MefA of *S. pyogenes*

→ ML

broad

# 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)

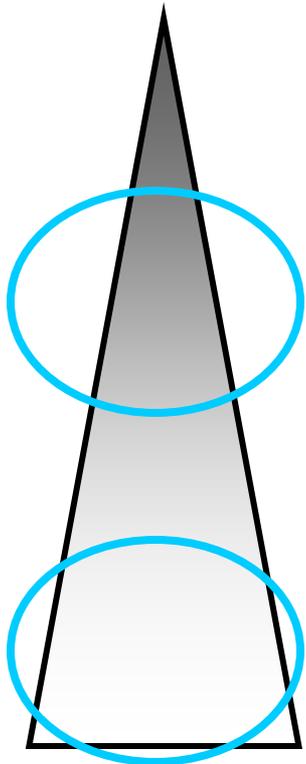
*Gill et al, AAC 1999; 43:187-9*



# 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*

→  $\beta$ -lac, FQ, Tet, ML, chl, rif, sulf

AcrAB-TolC of *E. coli*

→  $\beta$ -lac, FQ, Tet, ML, chl, rif, sulf

broad

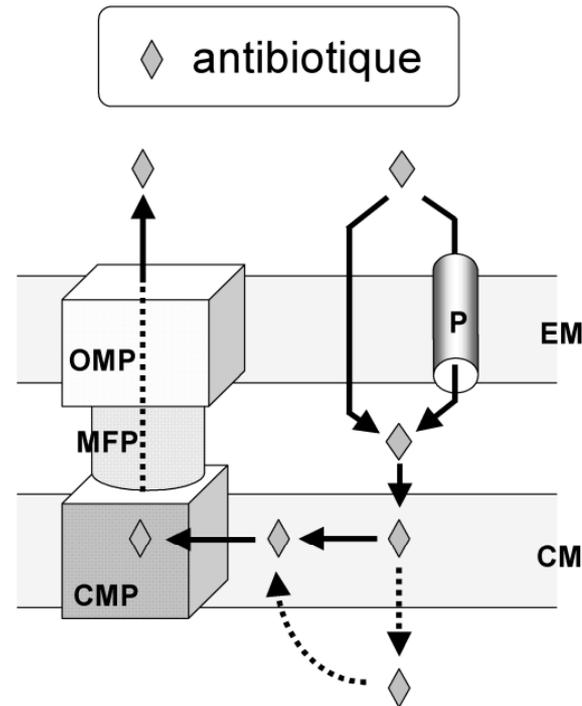
# Efflux and resistance in *P. aeruginosa*

Constitutive  
basal expression  
overexpressed  
upon induction

MexB	MexY
MexA	MexX
OprM	OprM

No basal  
expression;  
expression  
upon induction

MexD	MexF
MexC	MexE
OprJ	OprN



CM: cytoplasmic membrane  
(*membrane cytoplasmique*)

EM: external membrane  
(*membrane externe*)

P: porin  
(*porine*)

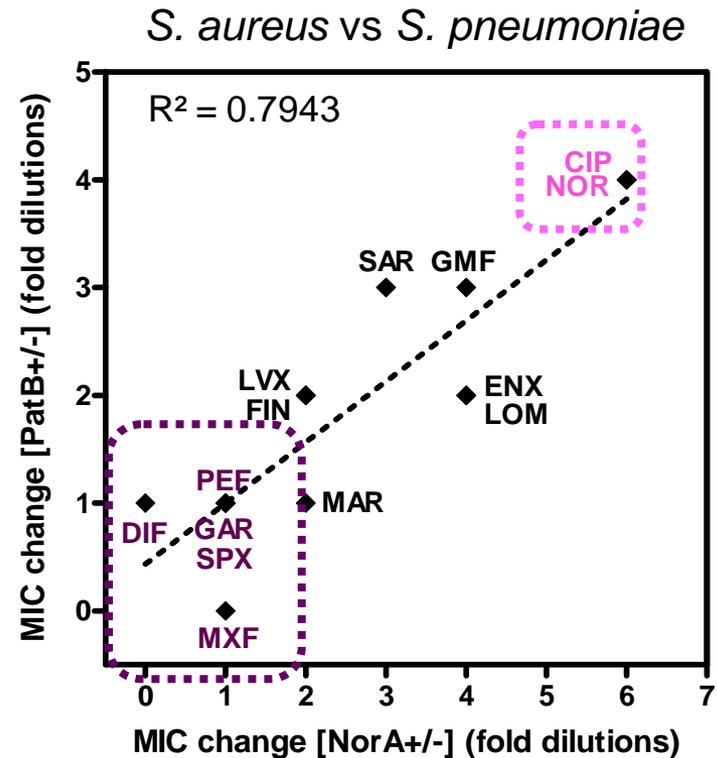
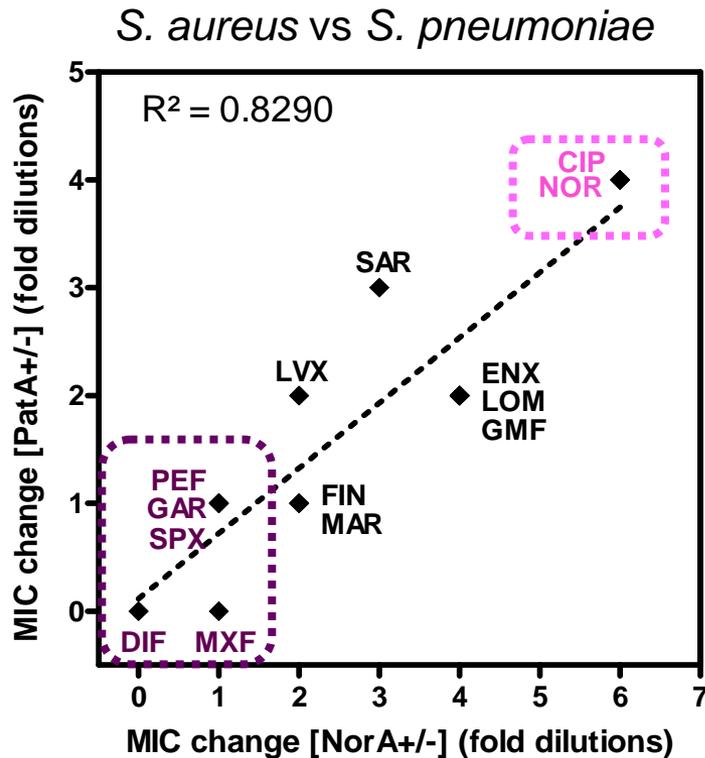
CMP: cytoplasmic membrane protein  
(*protéine de la membrane cytoplasmique*)

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

OMP: outer membrane protein  
(*protéine de membrane externe*)

# Substrate specificity of efflux pumps

14 fluoroquinolones; Gram + versus Gram +

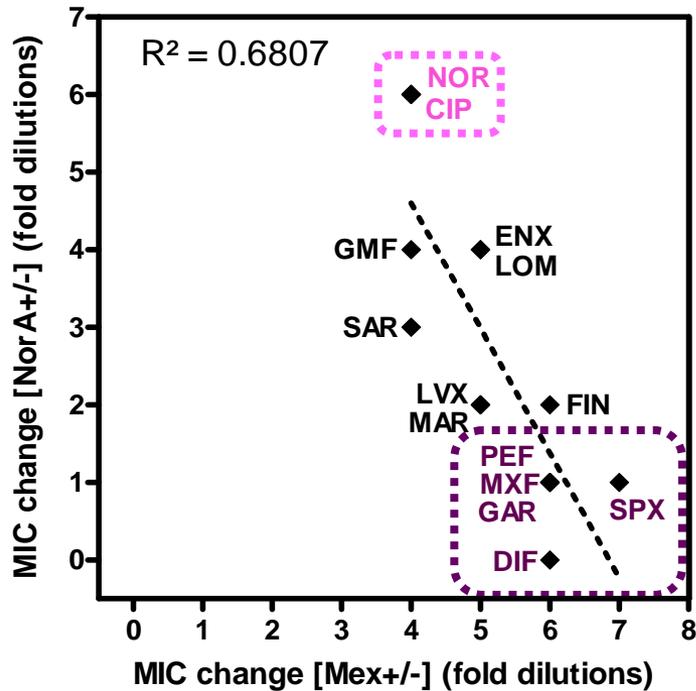


Similar recognition for non phylogenetically-related transporters

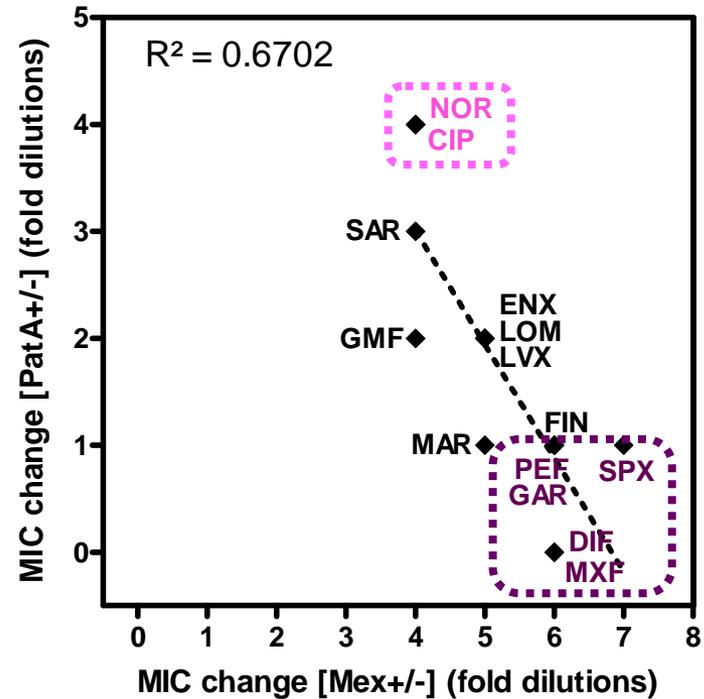
# Substrate specificity of efflux pumps

14 fluoroquinolones; Gram + versus Gram -

*P. aeruginosa* vs *S. aureus*



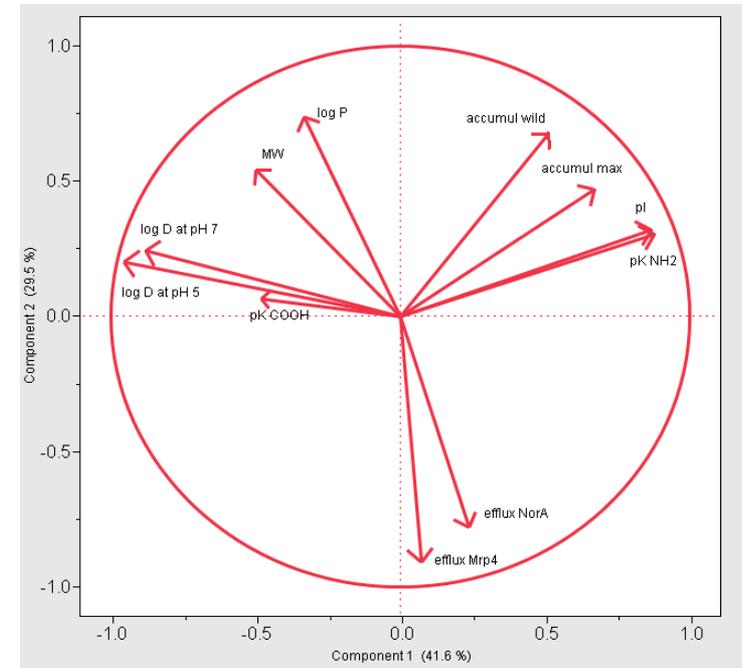
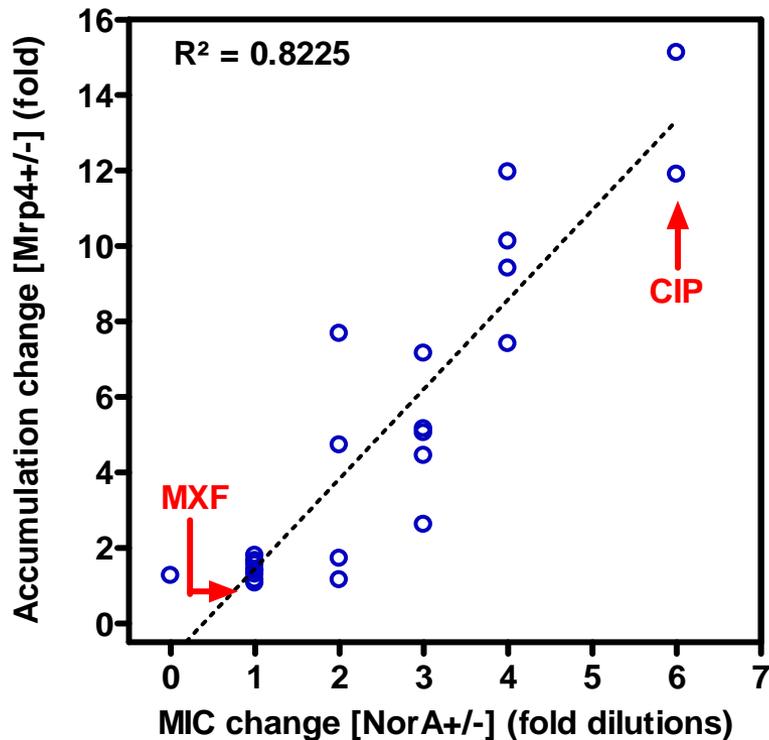
*P. aeruginosa* vs *S. pneumoniae*



All fluoroquinolones are substrates for broad spectrum transporters from Gram -

# Substrate specificity of efflux pumps

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



Principal component analysis of the correlations between biophysical properties of fluoroquinolones and susceptibility to efflux

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

# Cooperation between prokaryotic and eucaryotic transporters to reduce FQ activity against intracellular bacteria



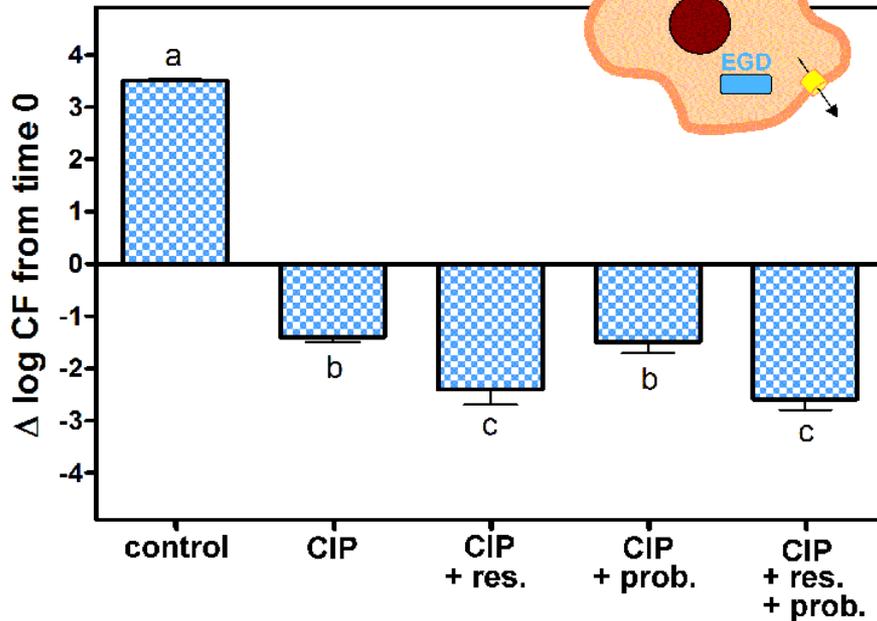
MIC of *Listeria* strains and effect of reserpine

		MIC (mg/L)			
		EGD		CLIP	
quinolone		Res. (-)	Res. (+)	Res. (-)	Res. (+)
	<b>CIP</b>		<b>1.2</b>	<b>1.0</b>	<b>5.0</b>
<b>MXF</b>		<b>0.6</b>	<b>0.6</b>	<b>0.5</b>	<b>0.25</b>

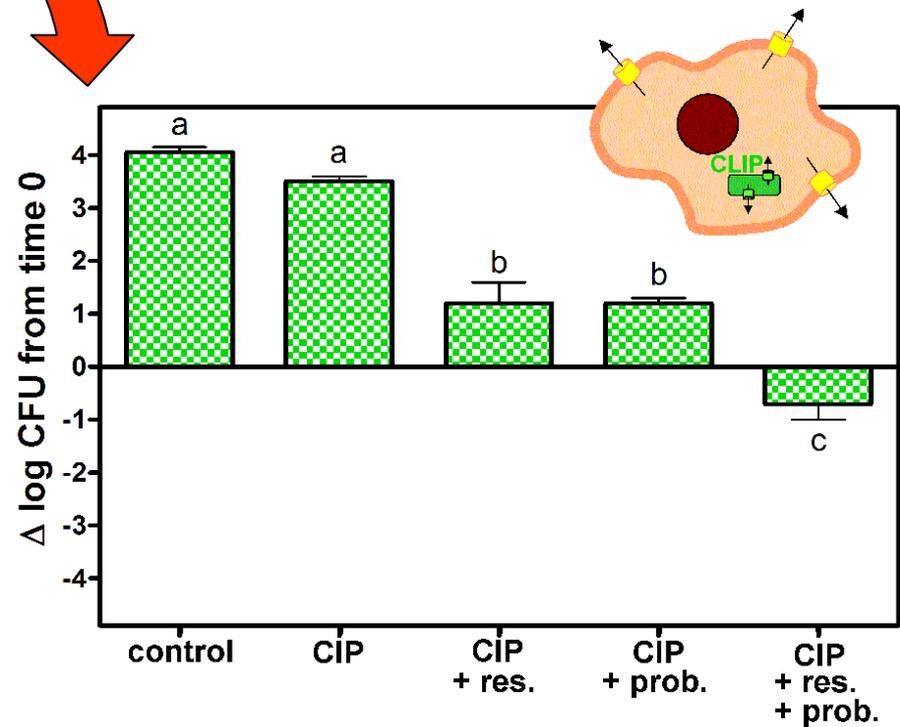
Lismond et al., AAC (2008) 52:3040-46

# Cooperation between prokaryotic and eucaryotic transporters to reduce FQ activity against intracellular bacteria

Wild-type cells and bacteria



bacteria overproducing efflux pumps for ciprofloxacin

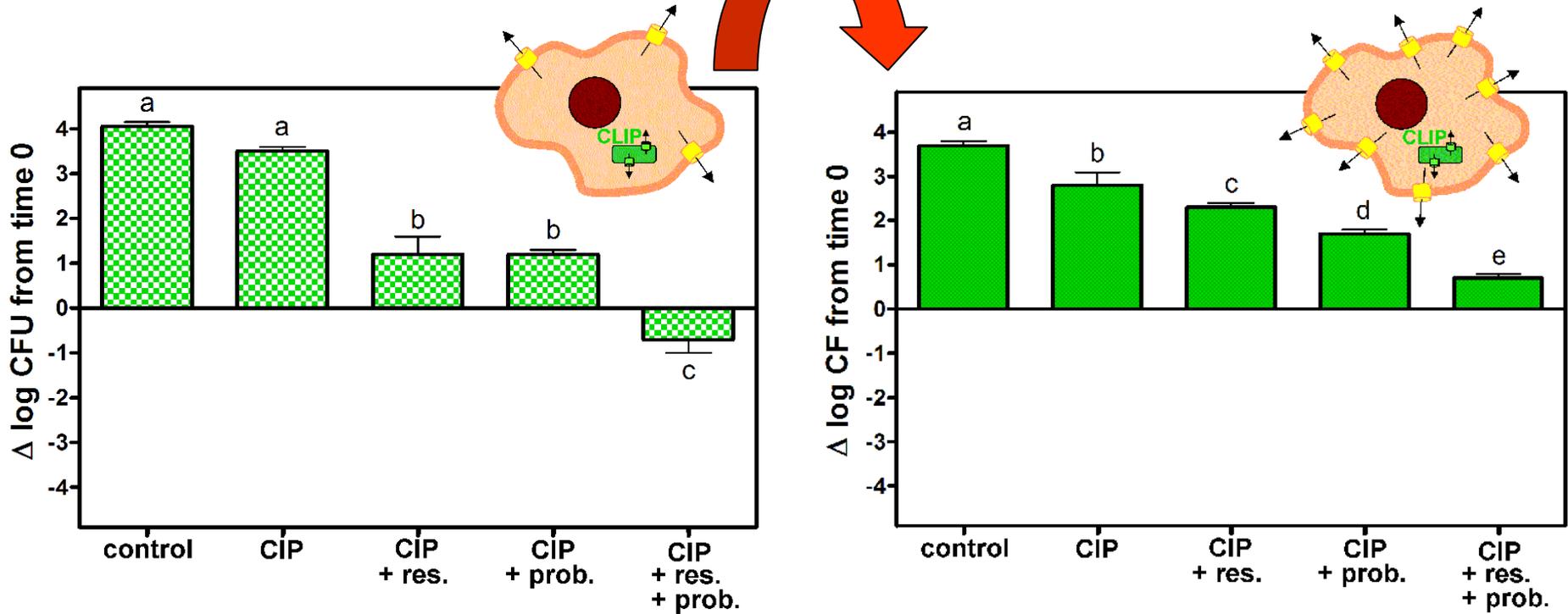


Bacterial efflux is expressed intracellularly

# Cooperation between prokaryotic and eucaryotic transporters to reduce FQ activity against intracellular bacteria

bacteria overproducing efflux pumps for ciprofloxacin

Bacteria AND cells overproducing efflux pumps for ciprofloxacin

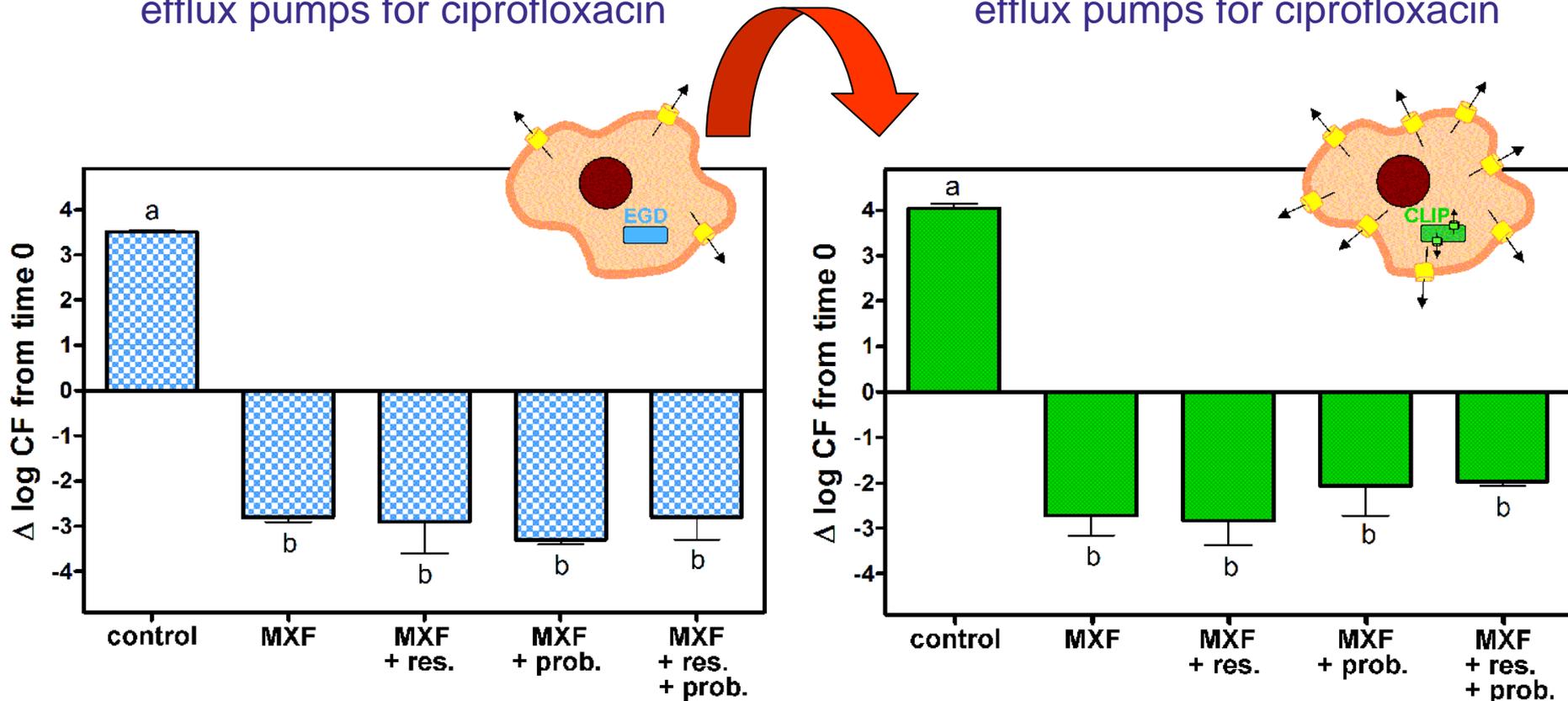


Bacterial and eukaryotic efflux cooperate to reduce ciprofloxacin intracellularly activity

# Cooperation between prokaryotic and eucaryotic transporters to reduce FQ activity against intracellular bacteria

bacteria overproducing efflux pumps for ciprofloxacin

Bacteria AND cells overproducing efflux pumps for ciprofloxacin



**Bacterial and eukaryotic efflux do not affect the activity of moxifloxacin**

# Efflux: from molecular recognition to cellular impact

- selection of molecules that are poor substrates for efflux may prove useful
- Molecular determinants for recognition by efflux pumps need to be identified to design poor substrates

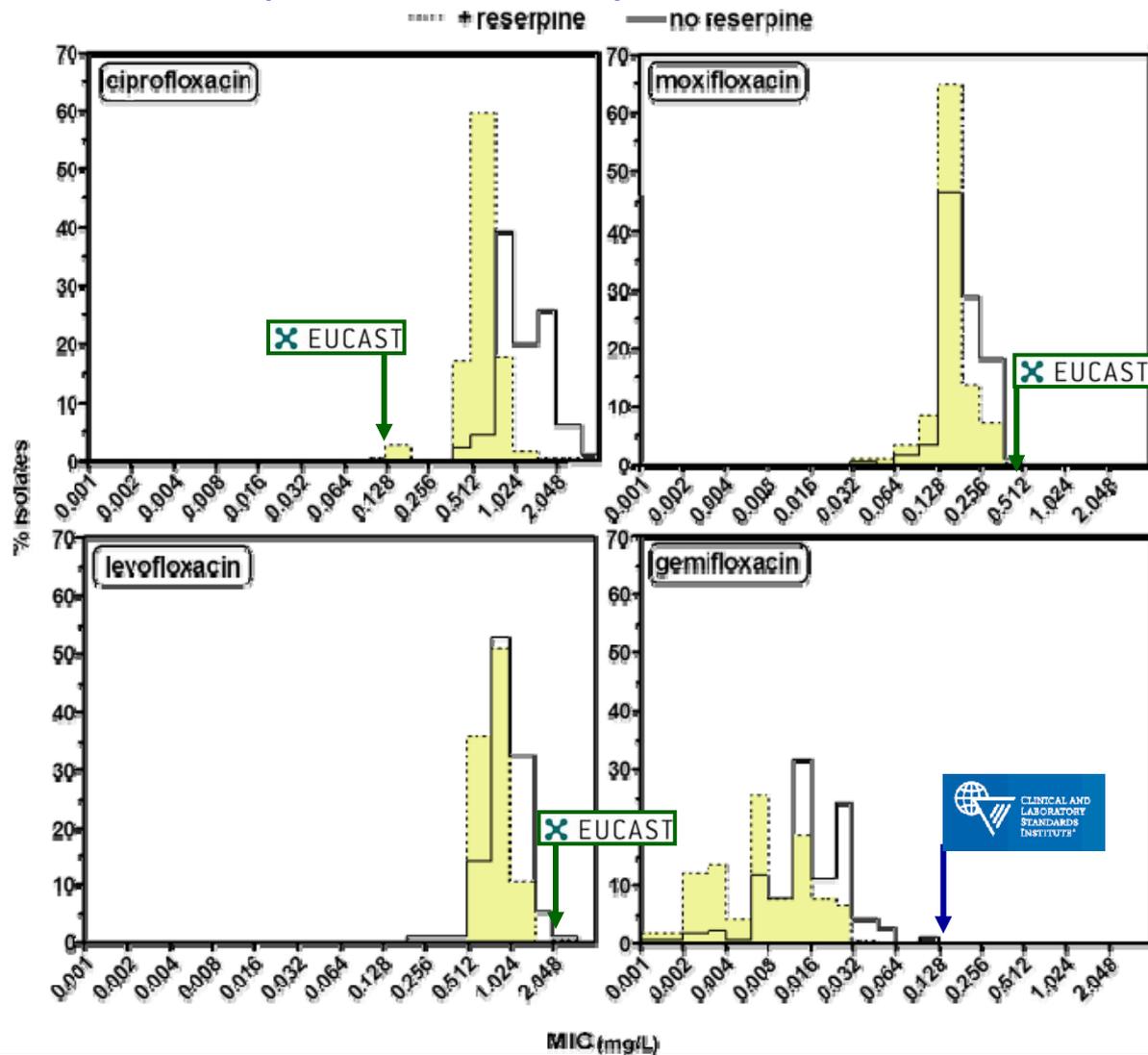


# Role of antibiotic efflux in epidemiology and resistance ....



# Does efflux mean « resistance » vs. epidemiological breakpoints ?

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



- Efflux (+) strains considered as susceptible

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

Lismond et al., JAC (2011) 66:948-951

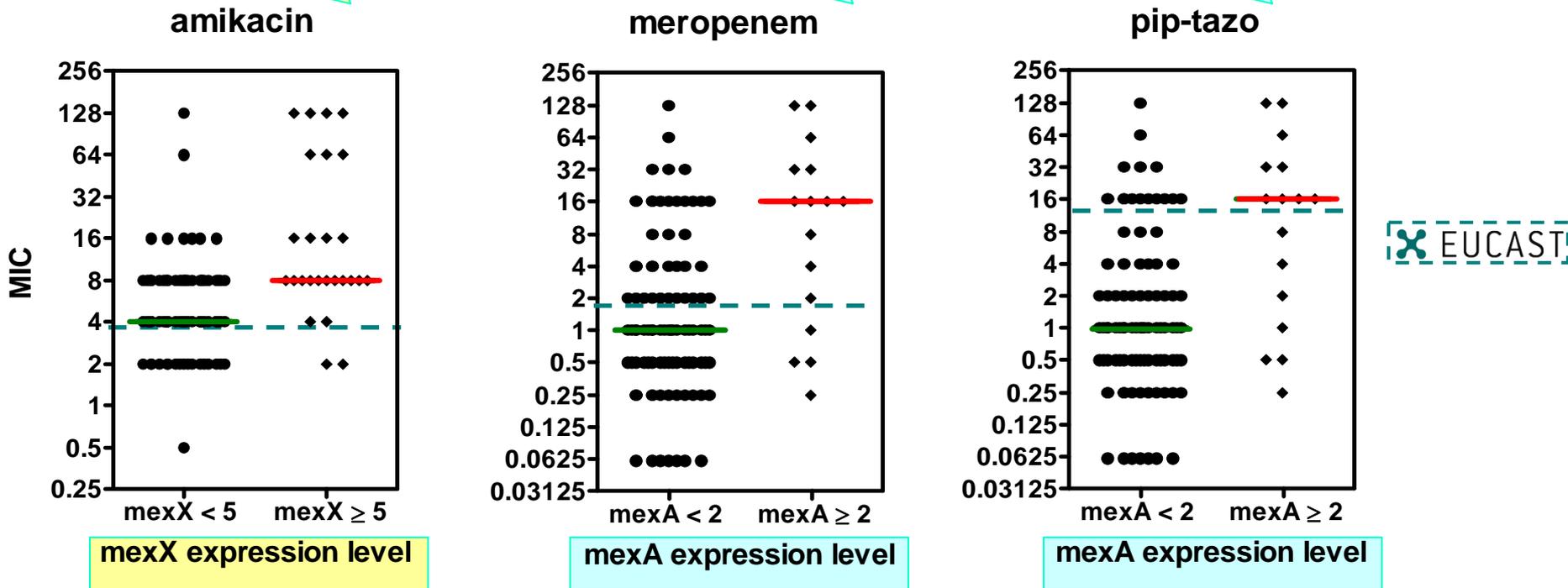
# Does efflux mean « resistance » vs. epidemiological breakpoints ?

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

MexX substr.

MexA substr.

MexA substr.



# Role of antibiotic efflux in epidemiology and resistance ....

- Efflux confers globally low level of resistance, but it can bring MICs above susceptibility Bkpts
- Molecules with high intrinsic activity (low MIC) can be excellent substrates for efflux ... with a risk of seeing their MICs increasing rapidly

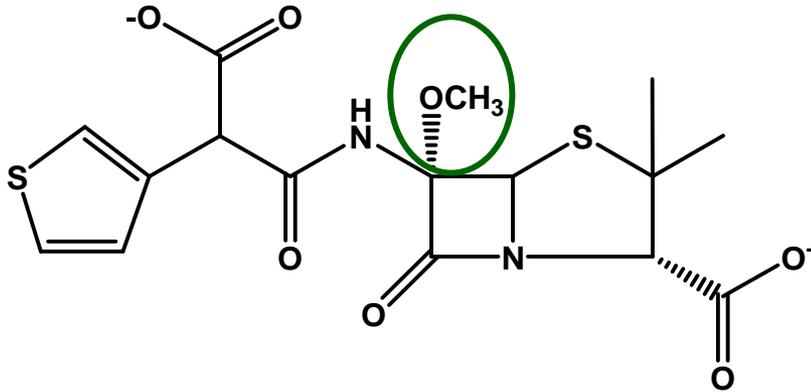


# Role of antibiotic efflux in intrinsic resistance ....



# Intrinsic resistance of *Pseudomonas* to temocillin

Temocillin [6- $\alpha$ - methoxy-ticarcillin], a carbapenem-sparing drug ....



stable to  $\beta$ -lactamases, including ESBL  
→ Treatment of infections by Gram(-)

**BUT *Pseudomonas* is intrinsically resistant to temocillin ....**

# Intrinsic resistance of *Pseudomonas* to temocillin

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

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 ...

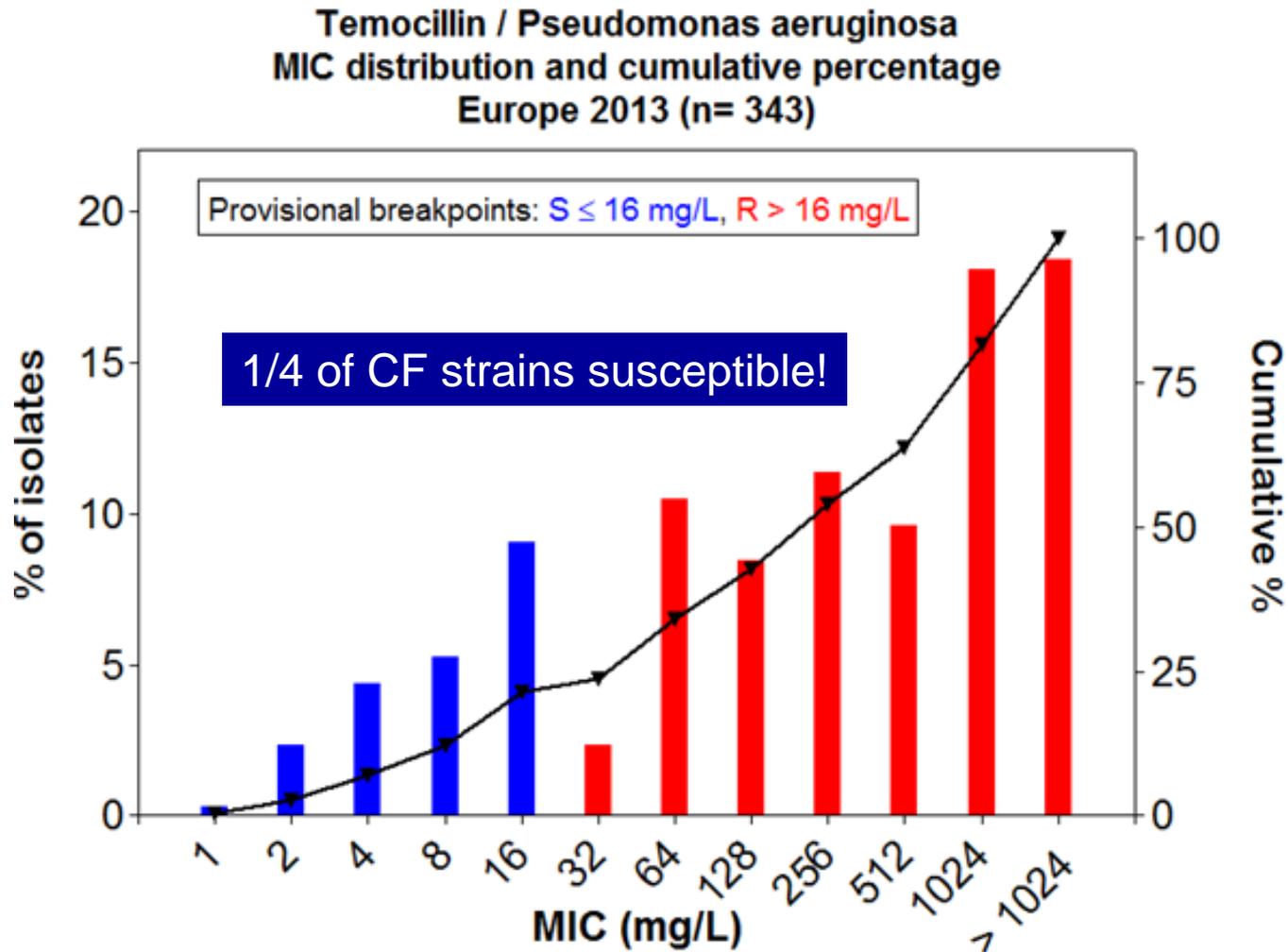
		Efflux characteristics, alterations				MIC (mg/L)	
		<i>mexA</i>	MexA	<i>mexB</i>	MexB	temocillin	ticarillin
Clinical isolates from cystic fibrosis patients							
3020S	<sup>d</sup>	—	—	—	—	128	16
3020R	<sup>d</sup>	Δ 112 nt (370–482)	aberrant	—	—	2	1
3525		—	—	—	—	512	32
3807		G214A	G72S	—	—	32	4
2715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

# Intrinsic resistance of *Pseudomonas* to macrolides

Azithromycin is widely and successfully used in Cystic Fibrosis patients

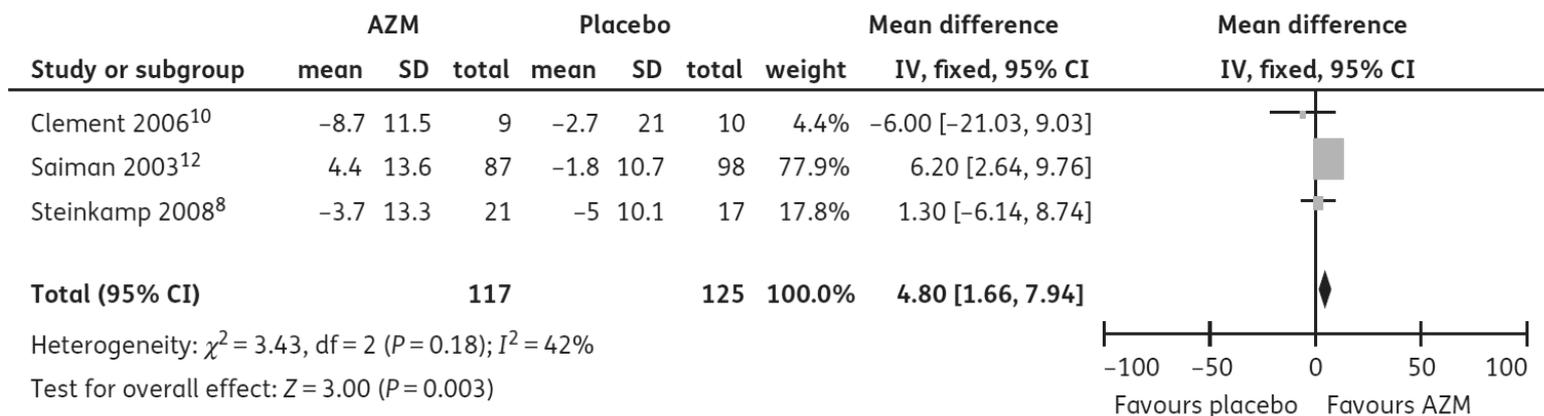
*J Antimicrob Chemother* 2011; **66**: 968–978  
doi:10.1093/jac/dkr040 Advance Access publication 2 March 2011

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

Yun Cai<sup>1</sup>, Dong Chai<sup>1</sup>, Rui Wang<sup>1\*</sup>, Nan Bai<sup>1</sup>, Bei-Bei Liang<sup>1</sup> and Youning Liu<sup>2</sup>

**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.

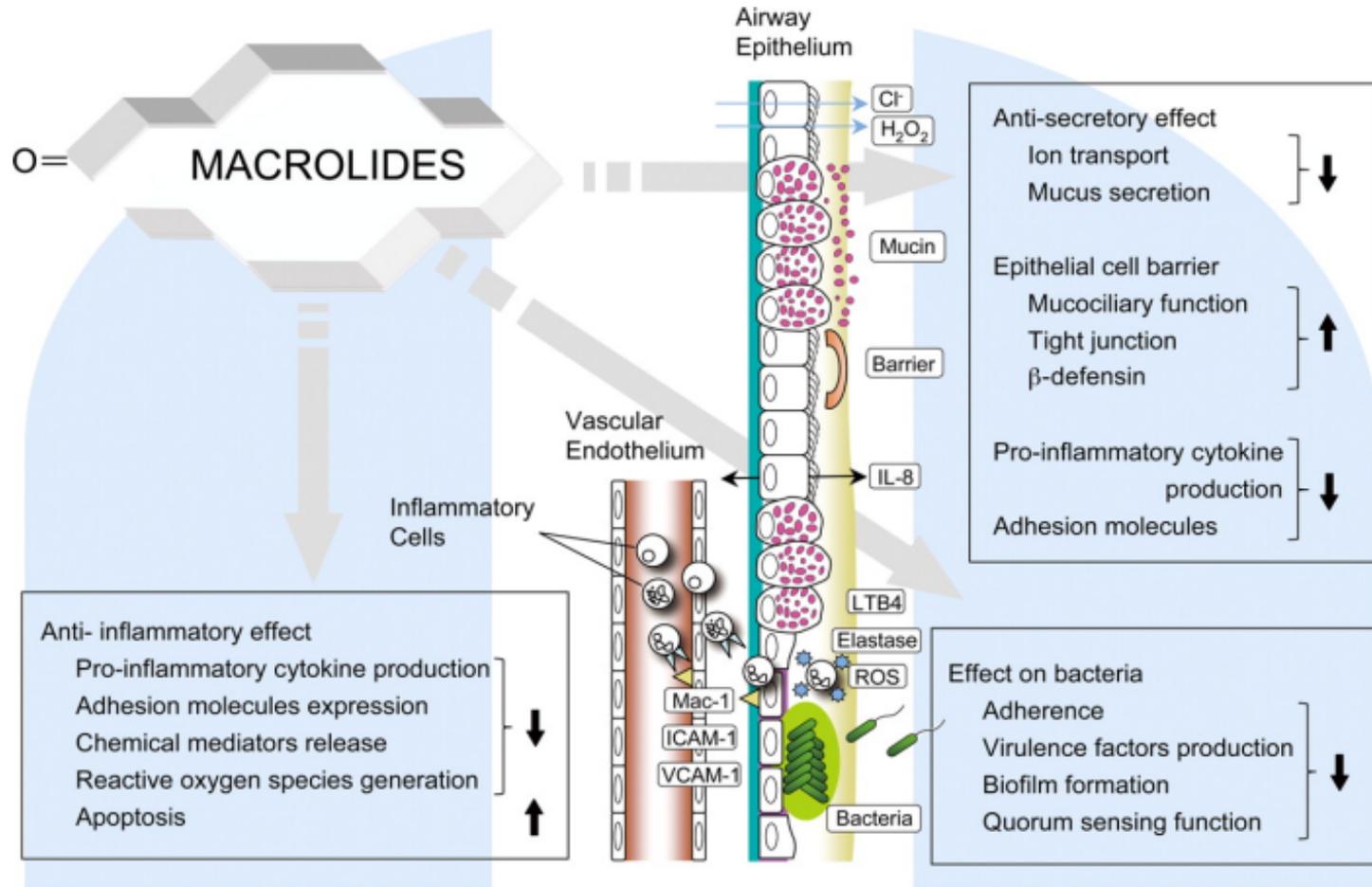
FEV<sub>1</sub>% change in *P. aeruginosa*-infected patients



**BUT *Pseudomonas* is intrinsically resistant ...**

# Intrinsic resistance of *Pseudomonas* to macrolides

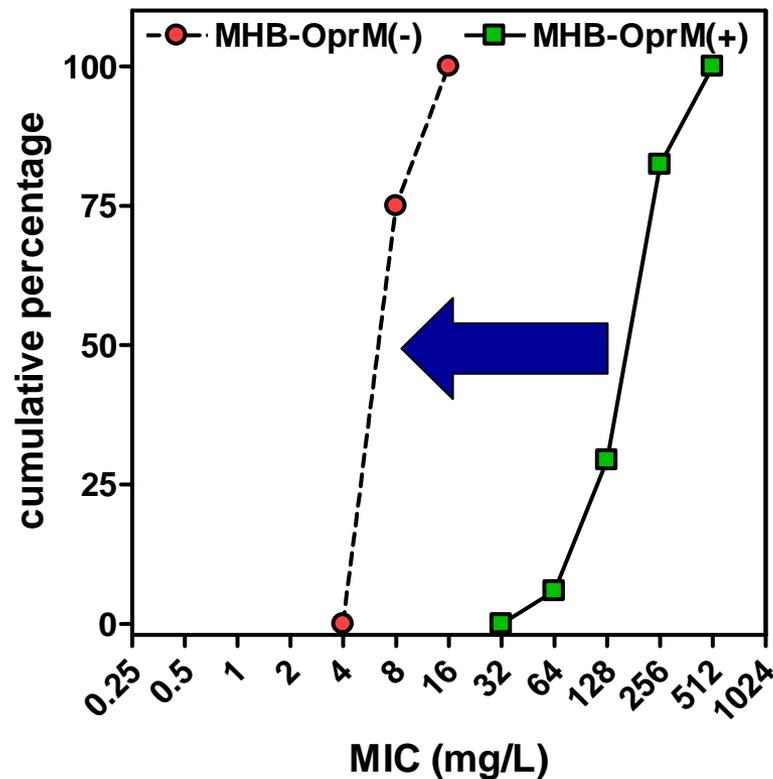
Azithromycin is widely and successfully used in Cystic Fibrosis patients



Kanoh & Rubin; Clin. Microbiol. Rev. (2010) 23:590-615

# Intrinsic resistance of *Pseudomonas* to macrolides

Is *Pseudomonas* « intrinsically » resistant to macrolides ?



Major role  
of constitutively-expressed  
transporters!

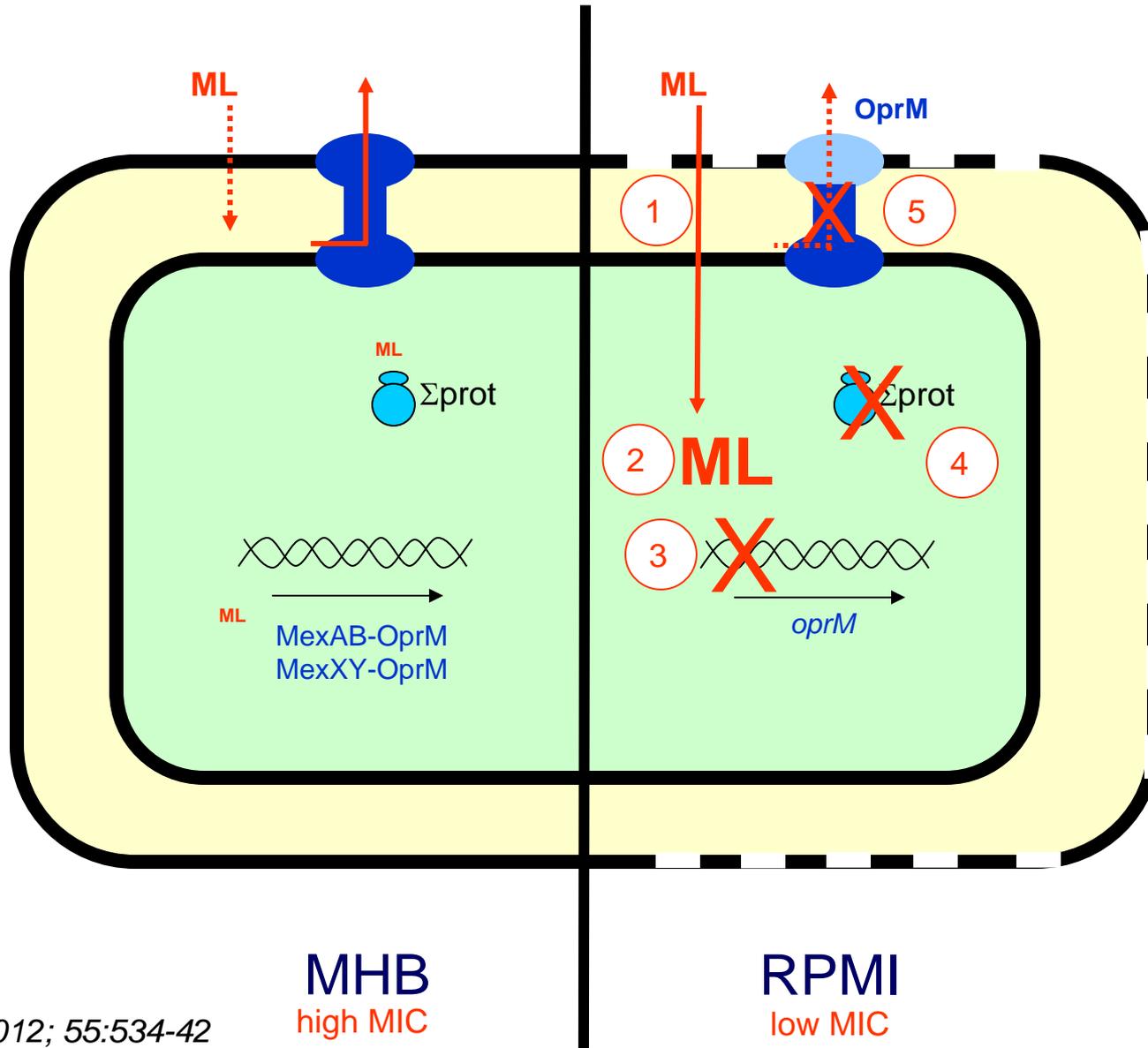
# An intriguing observation ...

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

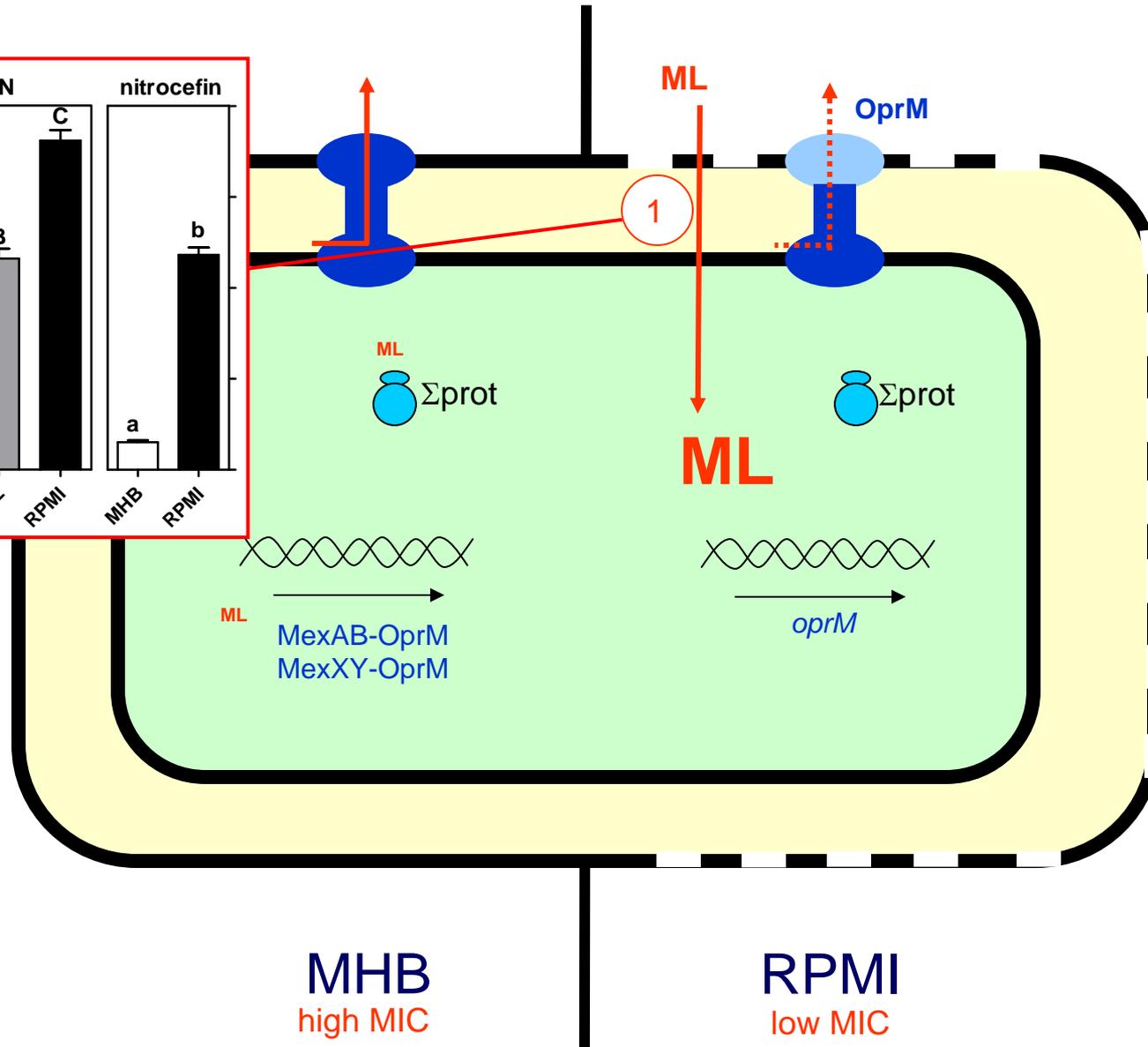
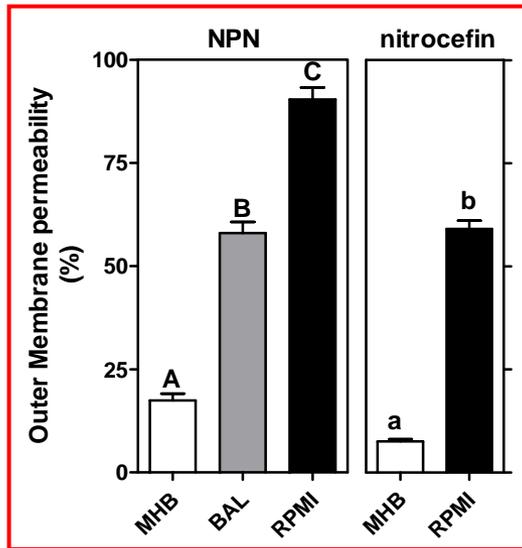
Macrolides regain activity against *P. aeruginosa* in « eukaryotic » media

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

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



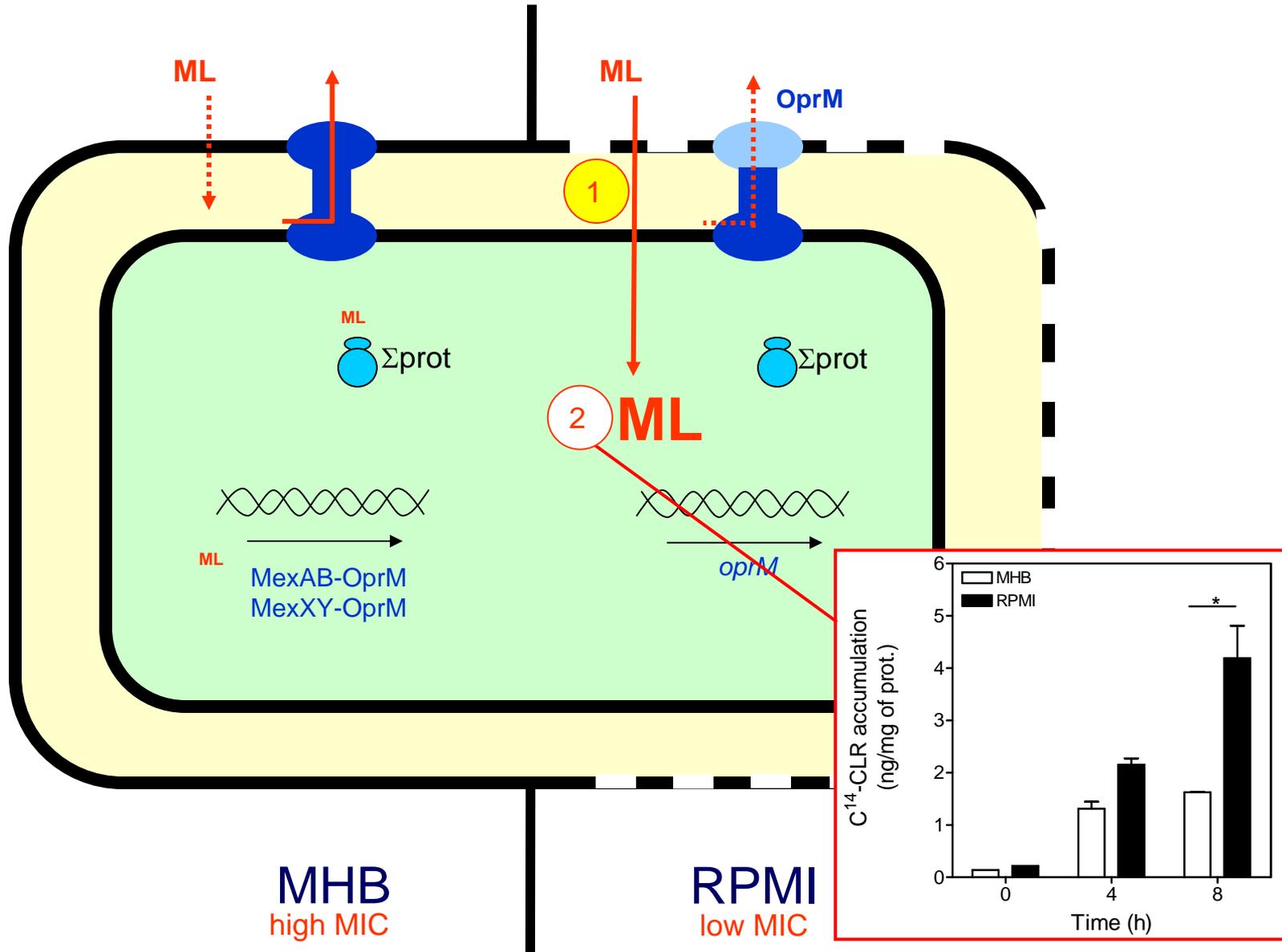
# Why do macrolides express their activity in « eukaryotic » media ?



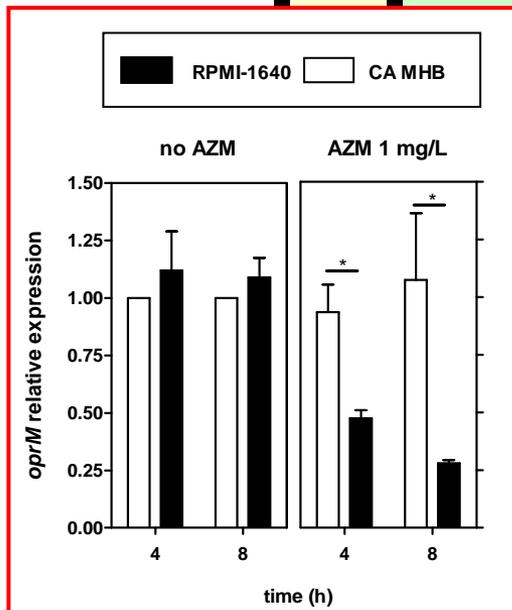
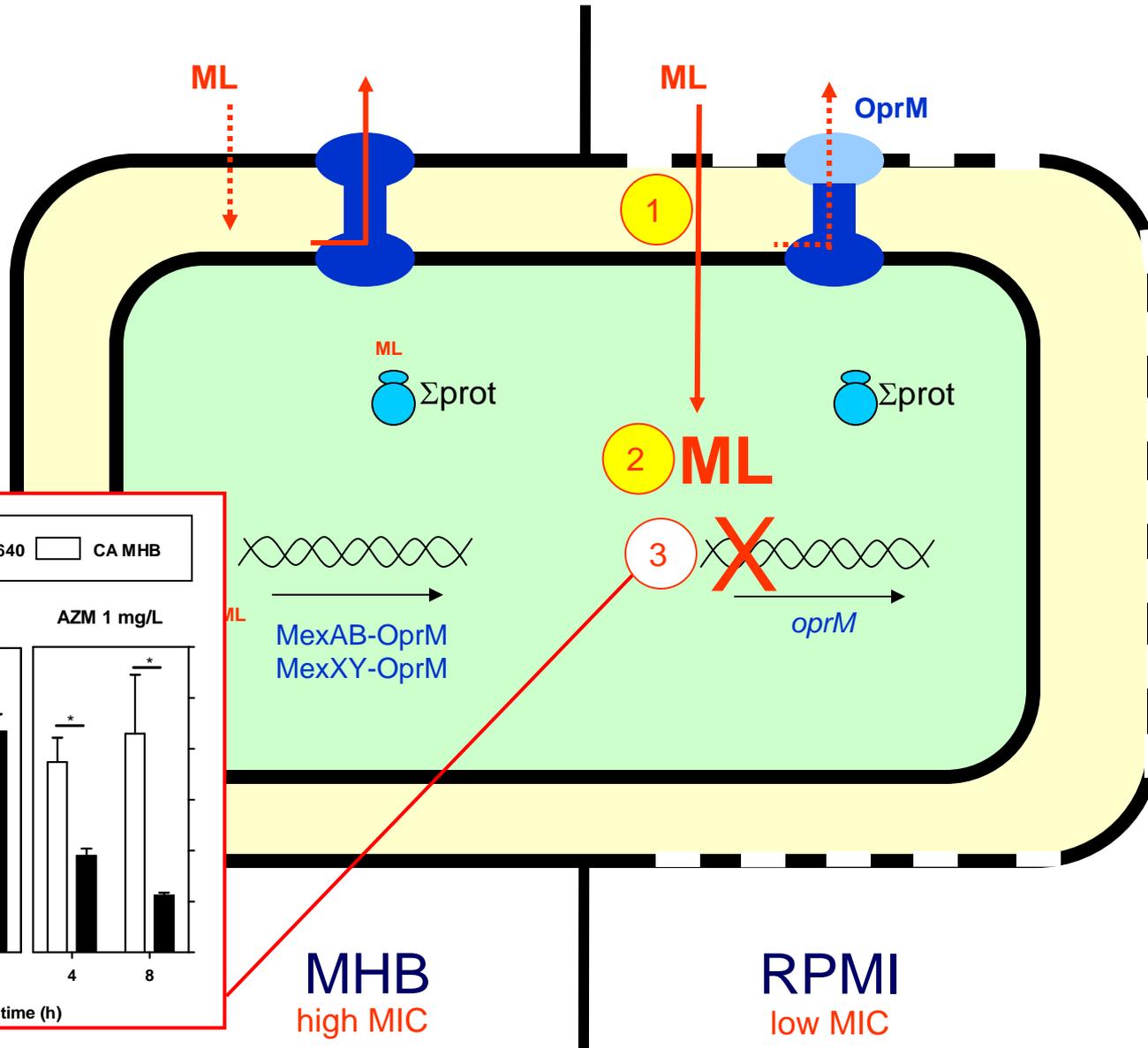
MHB  
high MIC

RPMI  
low MIC

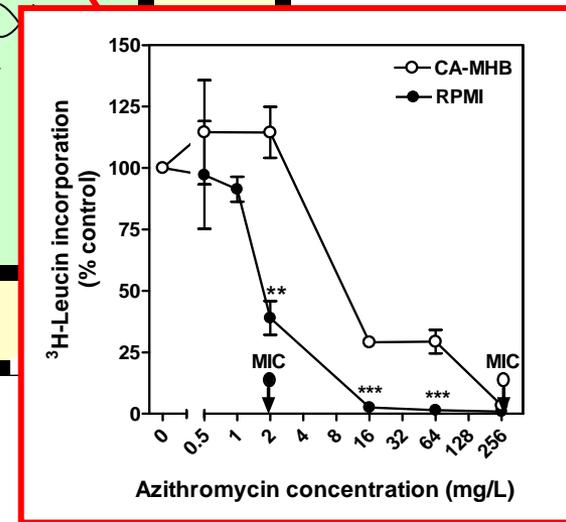
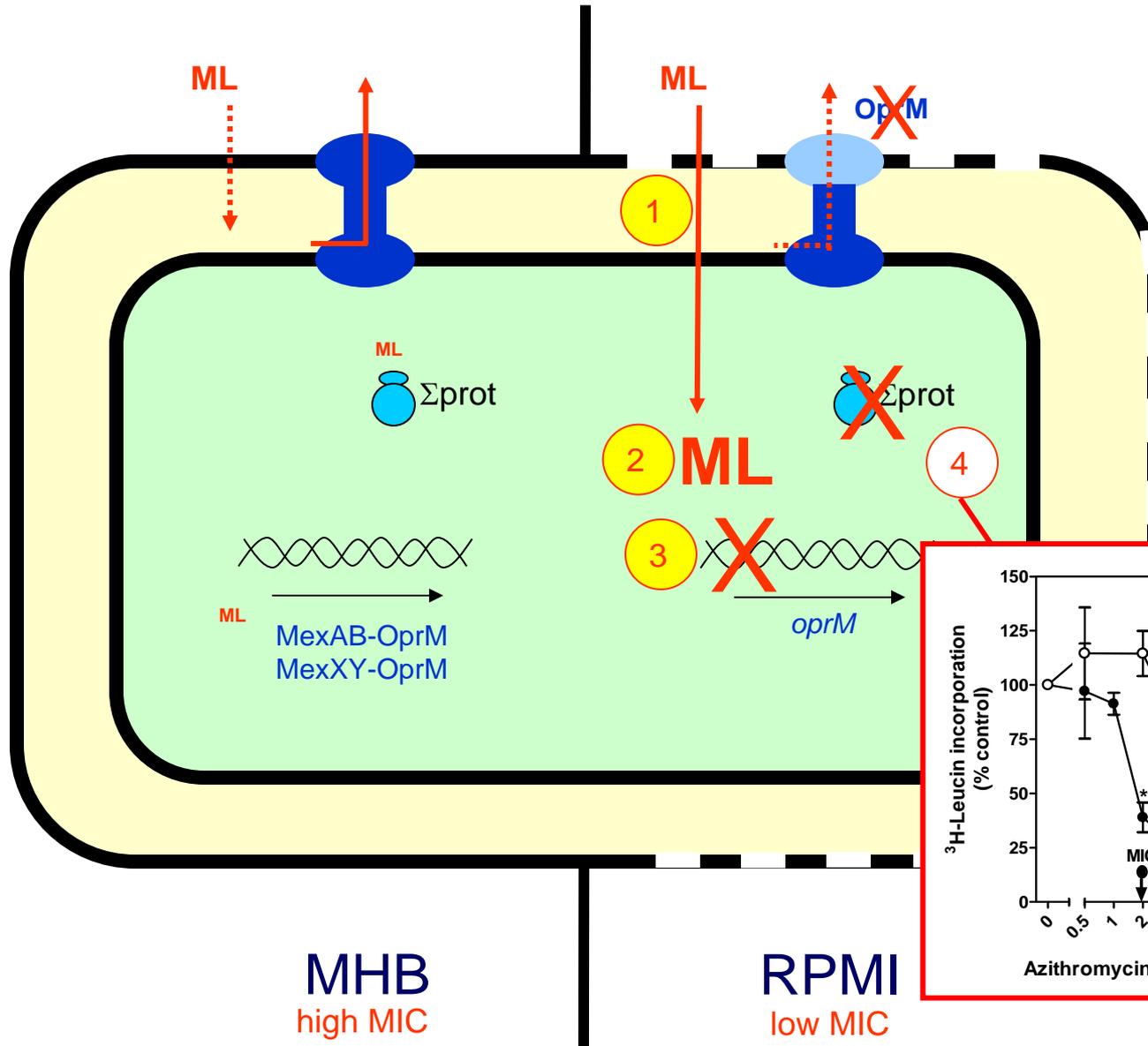
# Why do macrolides express their activity in « eukaryotic » media ?



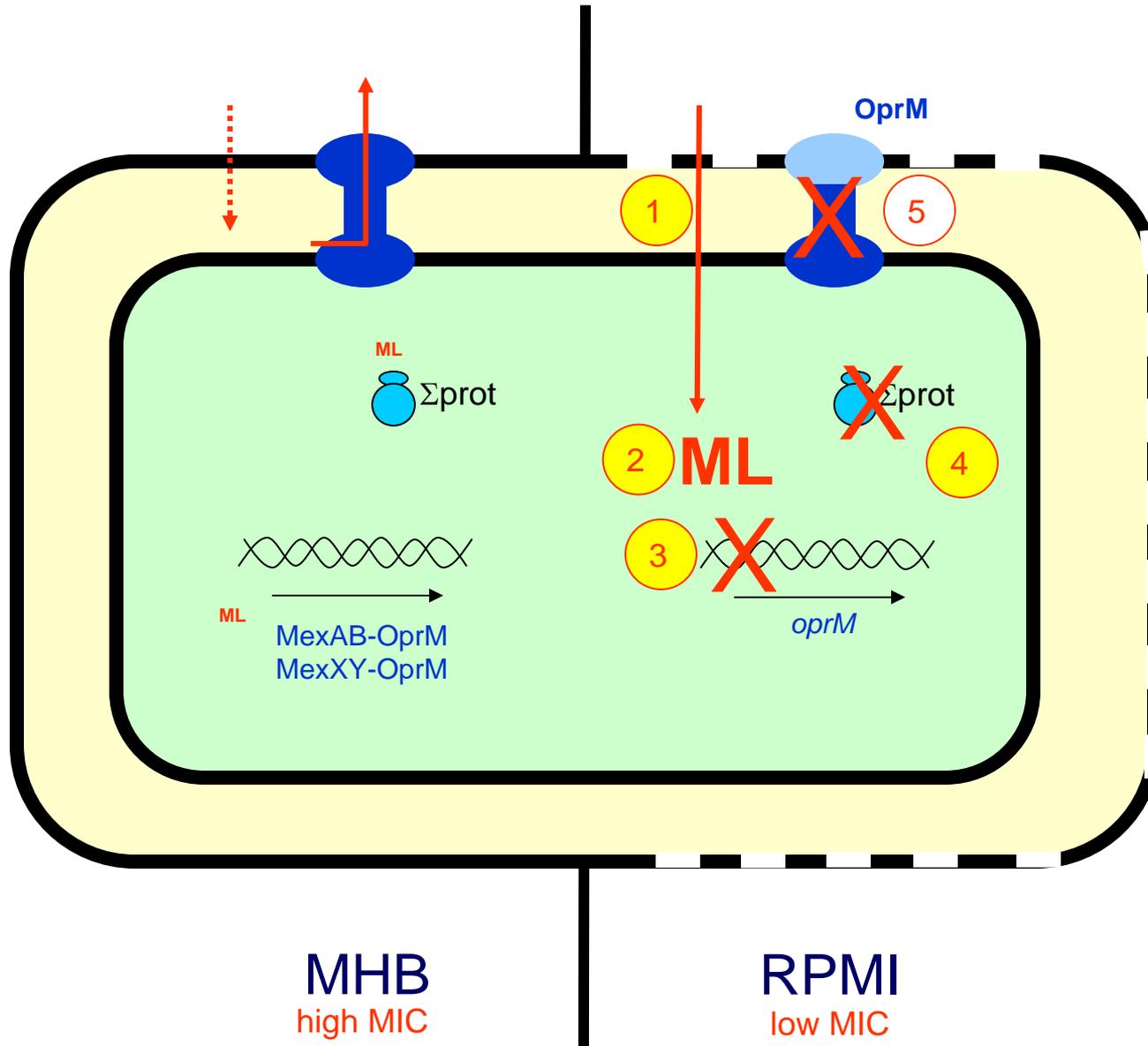
# Why do macrolides express their activity in « eukaryotic » media ?



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

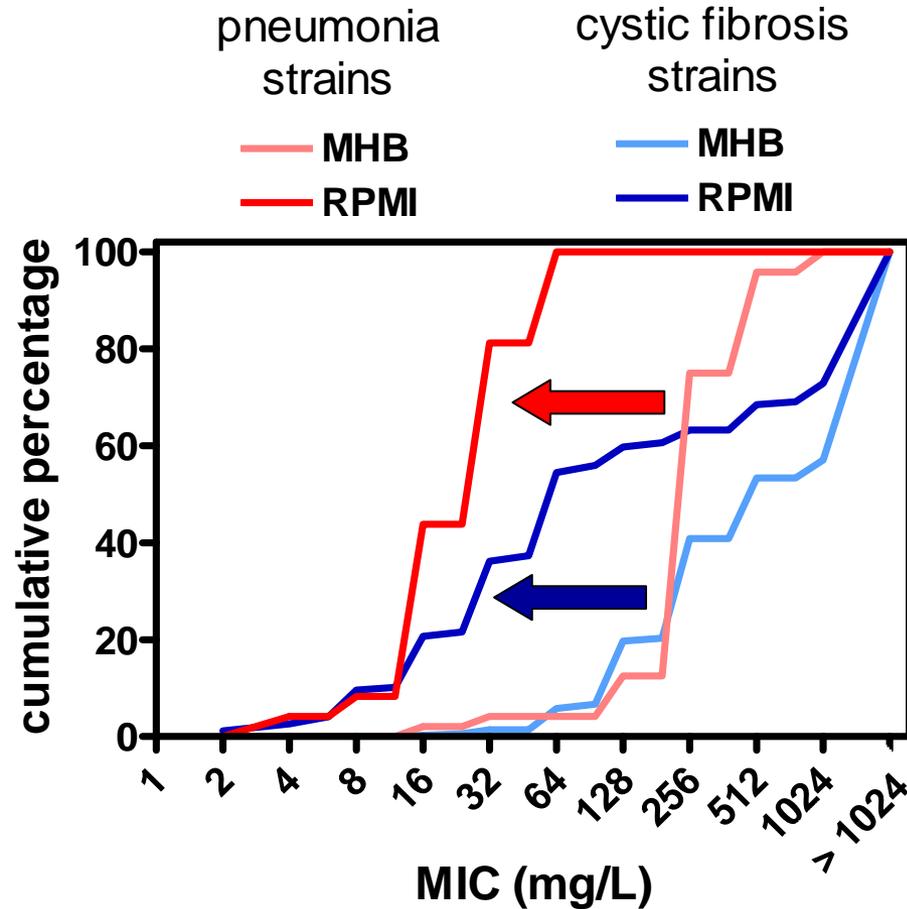


# Why do macrolides express their activity in « eukaryotic » media ?



# Intrinsic resistance of *Pseudomonas* to macrolides

Is this medium effect clinically relevant ?



CF strains = 345  
pneumonia strains = 48

Mustafa, unpublished

# Role of antibiotic efflux in intrinsic resistance ....



- Inactivating efflux may reveal antibiotic activity and could be a useful tool when developing new drugs
- Bacterial responsiveness to antibiotics may be highly different in the host than in the test tube

# Role of efflux pumps in the clinics ...

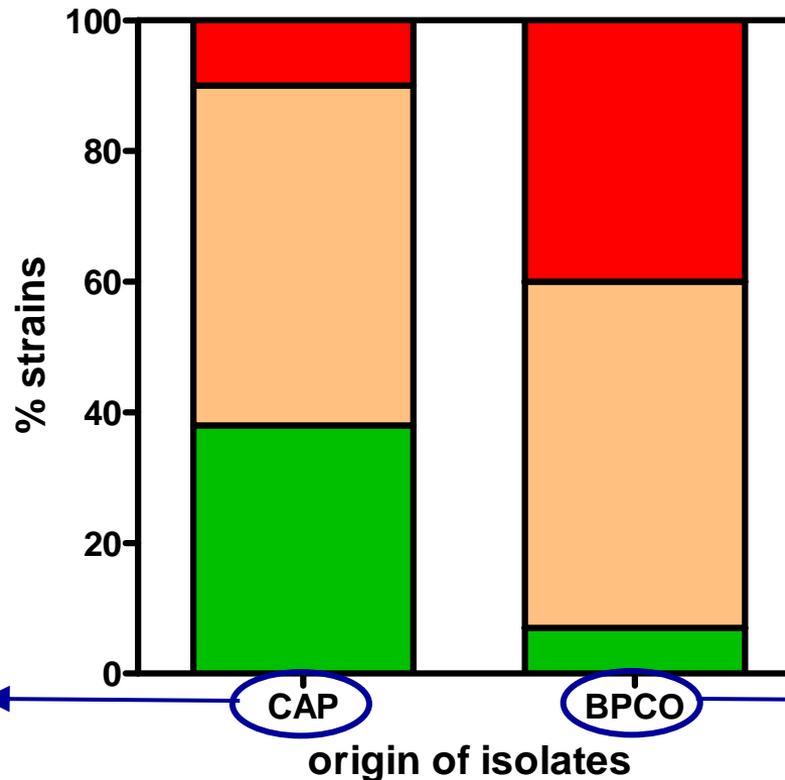


# Efflux in *S. pneumoniae*: is it important in the clinics ?

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

reserpine effect on MIC (x dilutions)

■  $\leq 1$    ■  $< 2$    ■  $\geq 2$



acute pathology  
↓  
« one shot »  
antibiotic exposure

chronic pathology  
↓  
repetitive  
antibiotic exposures

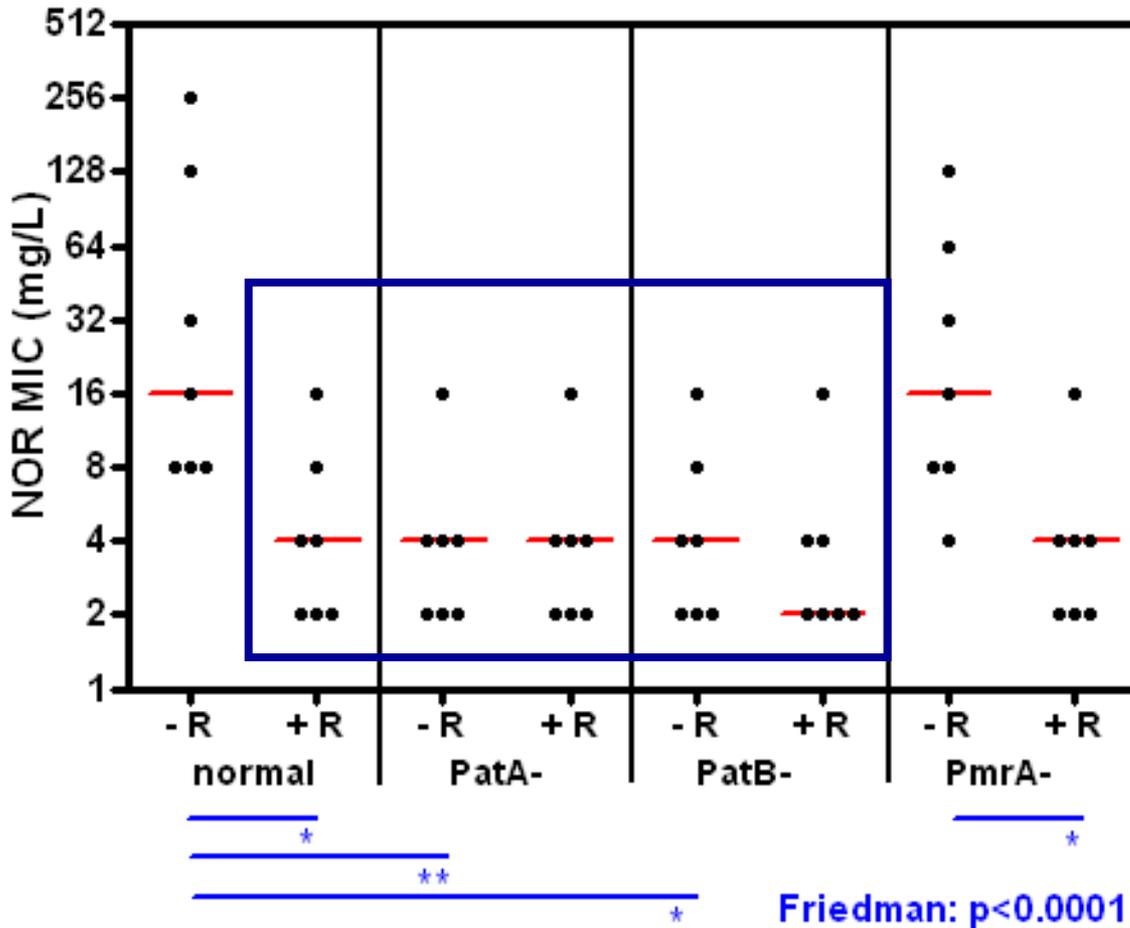
183 strains

107 strains

Lismond & Degives, unpublished

# Efflux in *S. pneumoniae*: is it important in the clinics ?

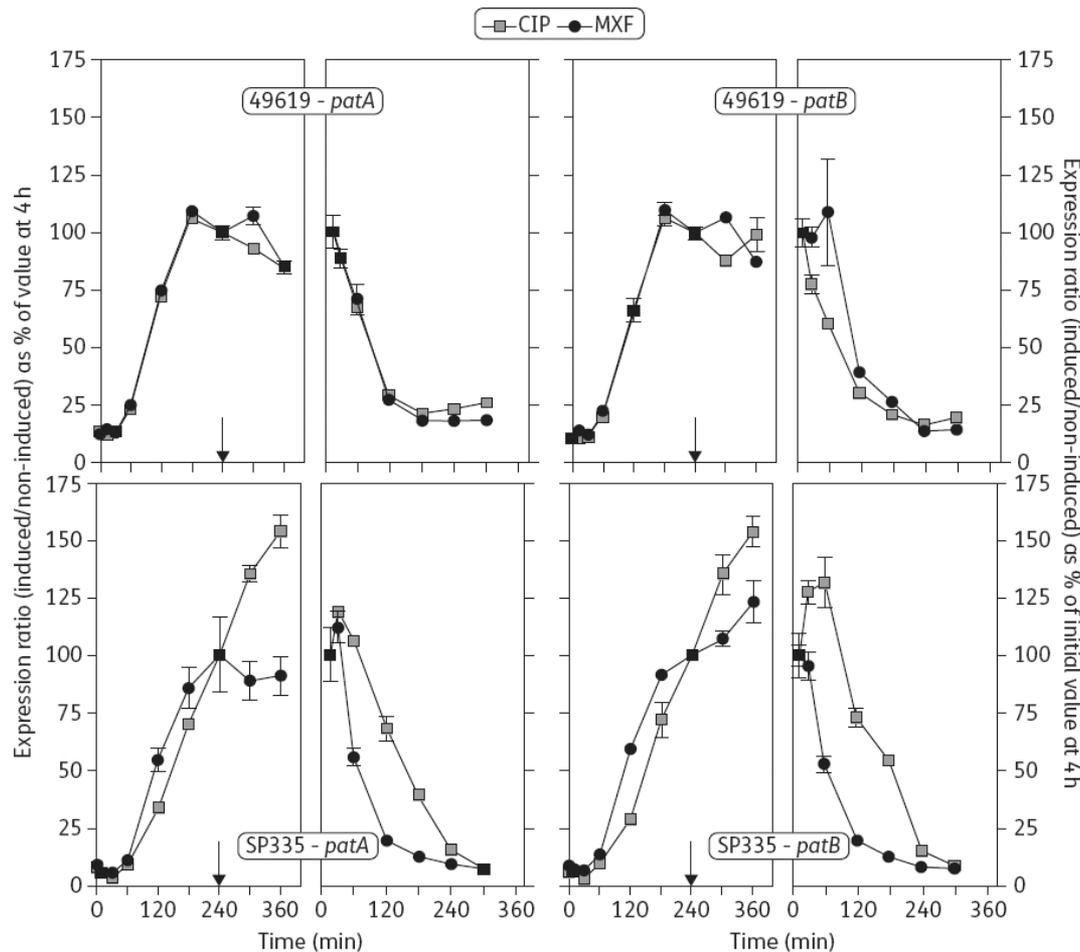
Identification of FQ transporters in clinical isolates



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

# Efflux in *S. pneumoniae*: is it important in the clinics ?

SubMICs concentrations of fluoroquinolones may induce efflux systems...

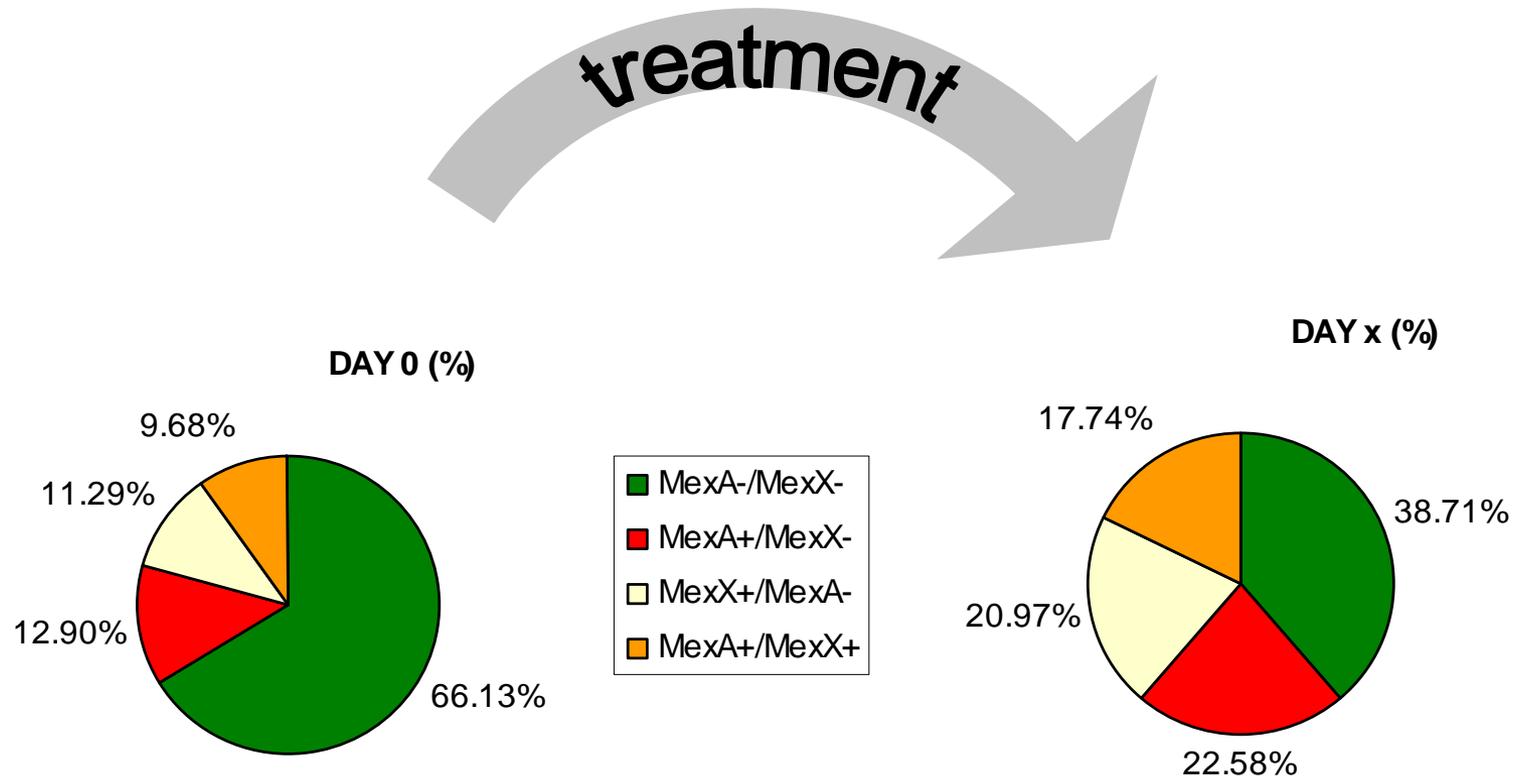


Optimal dosing  
is needed!

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

# Efflux in *P. aeruginosa*: is it important in the clinics ?

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



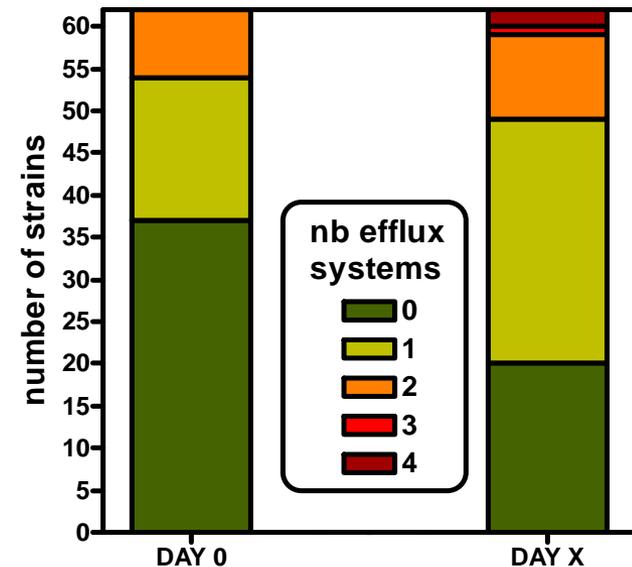
# Efflux selection in *P. aeruginosa* during treatment

Antipseudomonal antibiotics  
received by the patients during  
treatment

Antibiotic	no. patients	69% combinations
Piperacillin-tazobactam (TZP)	26	
Amikacin (AMK)	22	
Meropenem (MEM)	20	
Cefepime (CEF)	19	
Ciprofloxacin (CIP)	6	

Antibiotic treatment selects for  
efflux-mediated resistance !

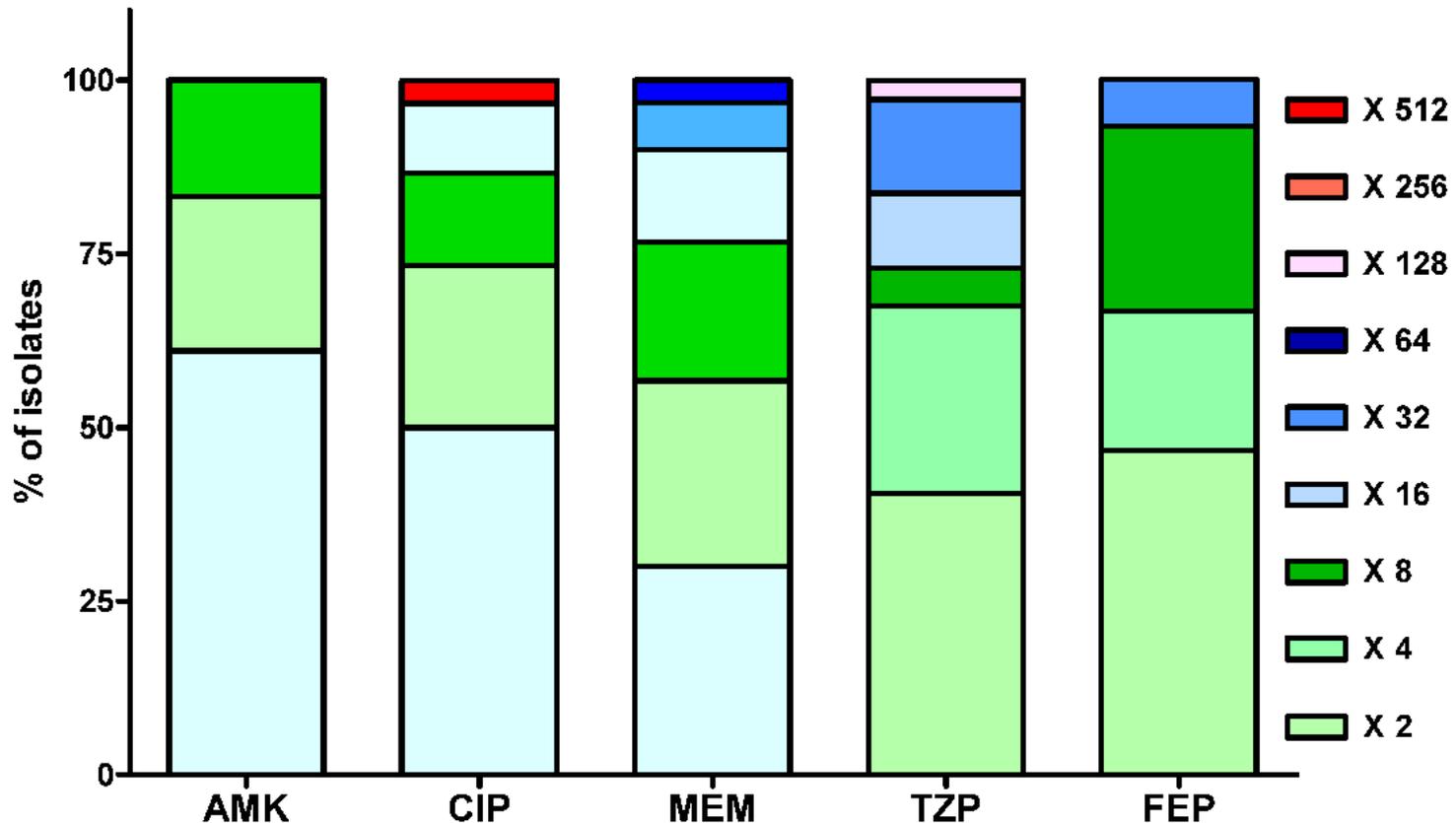
global influence  
of treatment



number of efflux systems  
detected at day 0 and day X

# MIC during treatment

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



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

# Early diagnosis should be implemented in the clinics

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Antibiotic susceptibility

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

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

by Dr Laetitia Avrain, Dr Pascal Mertens and Professor Françoise Van Bambeke

# Role of efflux pumps in the clinics ...



- Efflux is present in clinical isolates and may be induced upon exposure to suboptimal antibiotic concentrations
- Detecting efflux in clinical isolates may help optimizing antibiotic selection and dosing

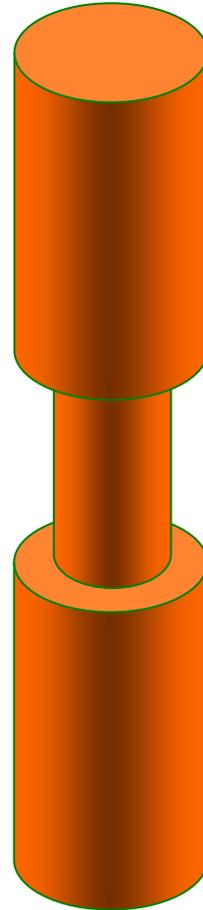
# Antibiotic resistance by efflux: from molecular aspects to clinical impact

## Take home message

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



**My clinical point of view:**  
Early diagnosis of resistance mechanisms and optimal dosing are keys to success



# Efflux and bacteria in our team ...

