Transport across membranes:
Multiple drug resistance, mechanisms and new tools
Summer School – Bremen – 7-14/07/2007

Molecular bases for antibiotic resistance through efflux

Françoise Van Bambeke

Pharmacologie cellulaire et moléculaire
Université catholique de Louvain
Brussels, Belgium

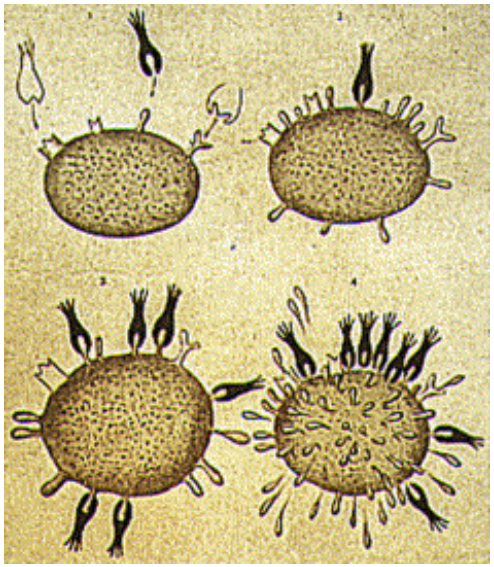
www.facm.ucl.ac.be

P. Ehrlich, a father of modern chemotherapy

Paul Ehrlich (1854-1945)

Magic bullets need to reach their target

Paul Ehrlich (1854-1945)

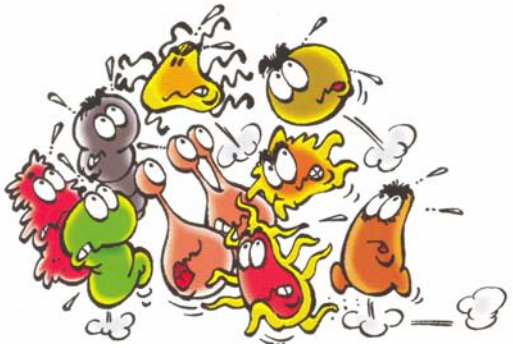


"corpora non agunt nisi fixata"

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

Chemotherapeutic agents exert toxic effects on specific target cells

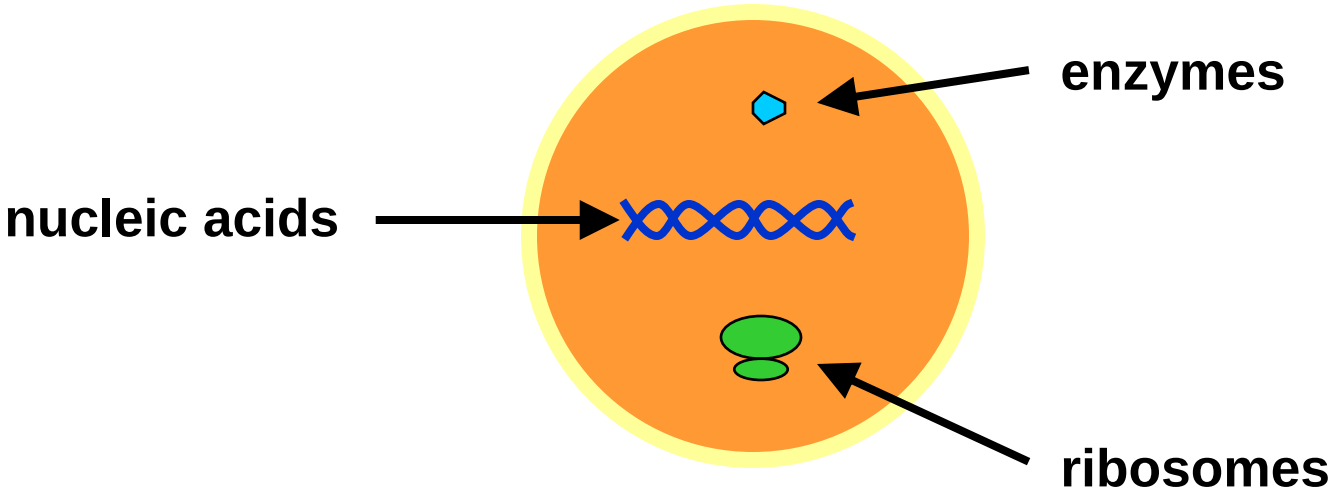
antibiotics



antifungals

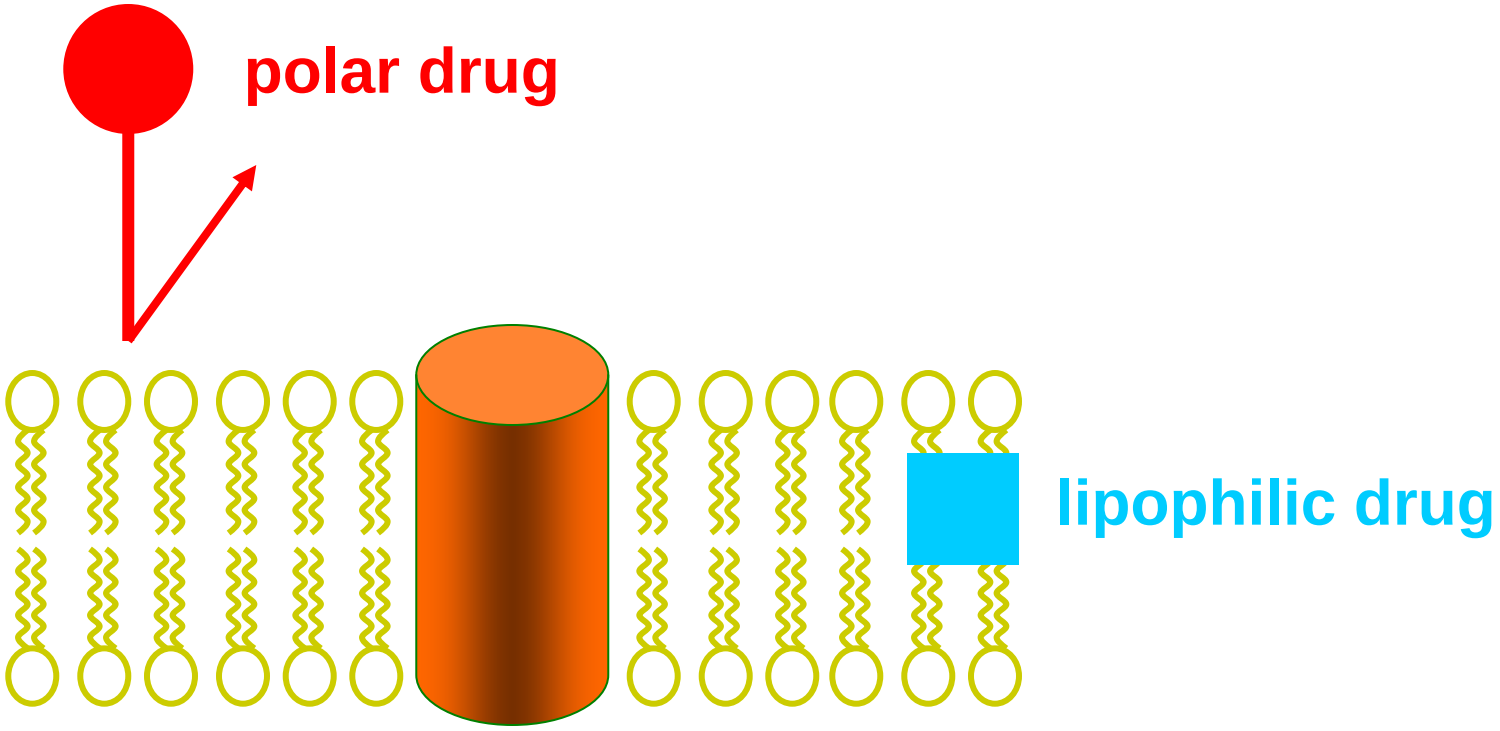
anticancer agents

Chemotherapeutic agents exert toxic effects on specific target cells



How can these drugs reach their target inside the cells ?

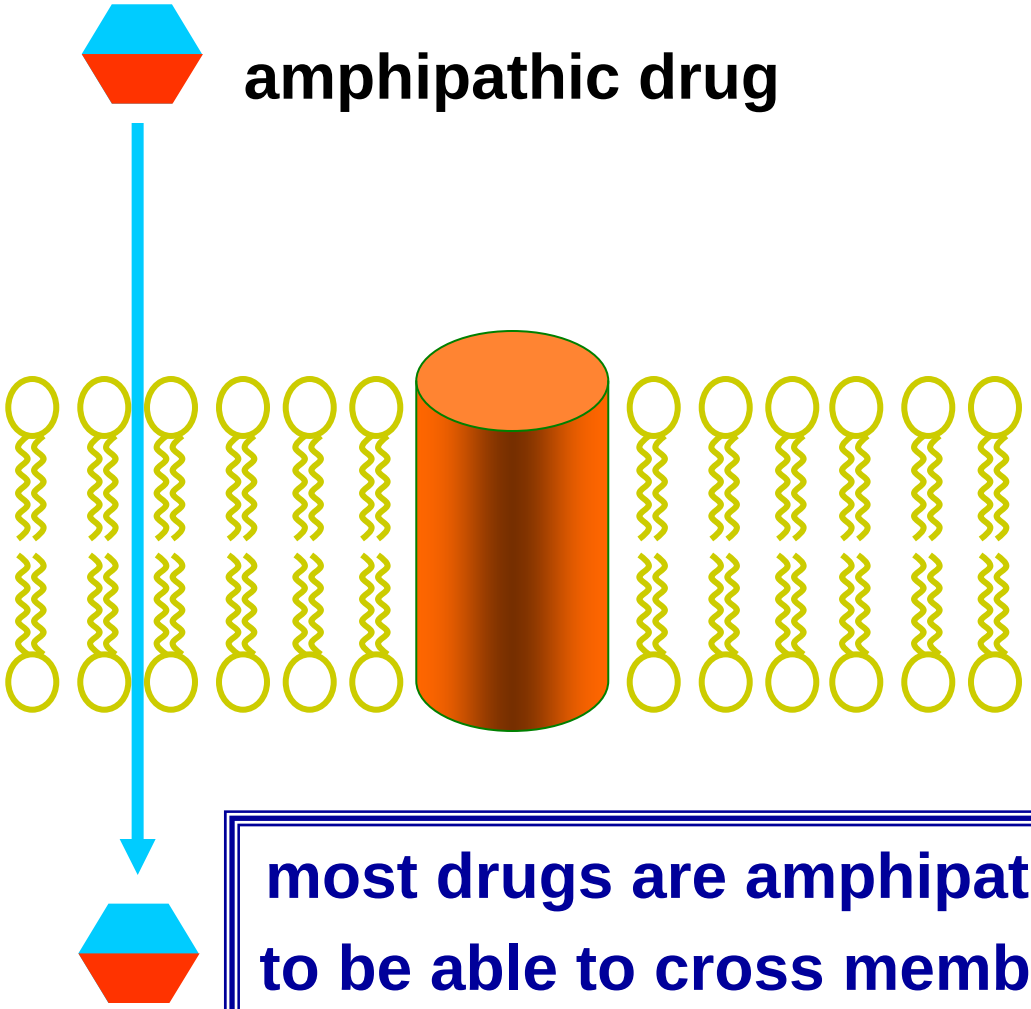
Reaching an intracellular target …



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

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

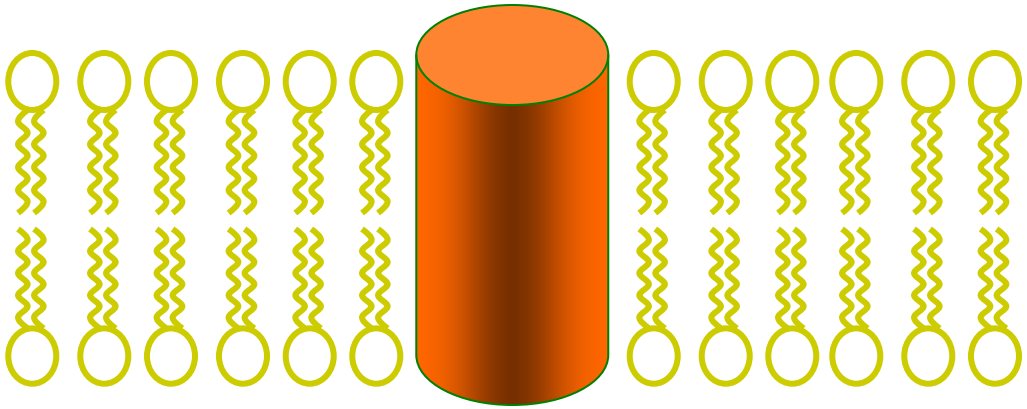
Reaching an intracellular target …

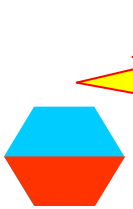


most drugs are amphipathic by design, to be able to cross membrane barriers !

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

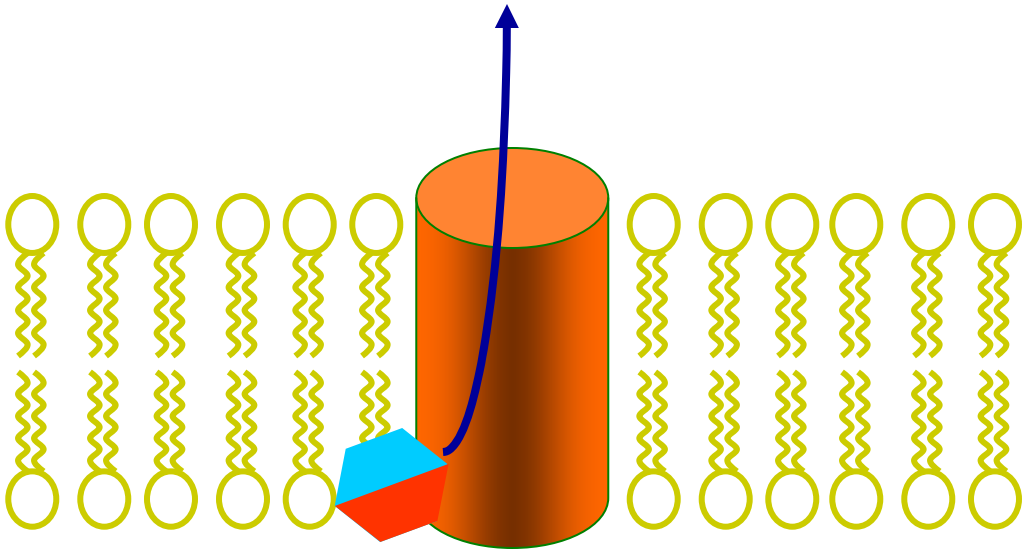
Chemotherapeutic agents exert toxic effects on specific target cells

How can cells protect themselves from these toxic substances ?

Jérome Bosch (1453-1516)

Why efflux transporters ?

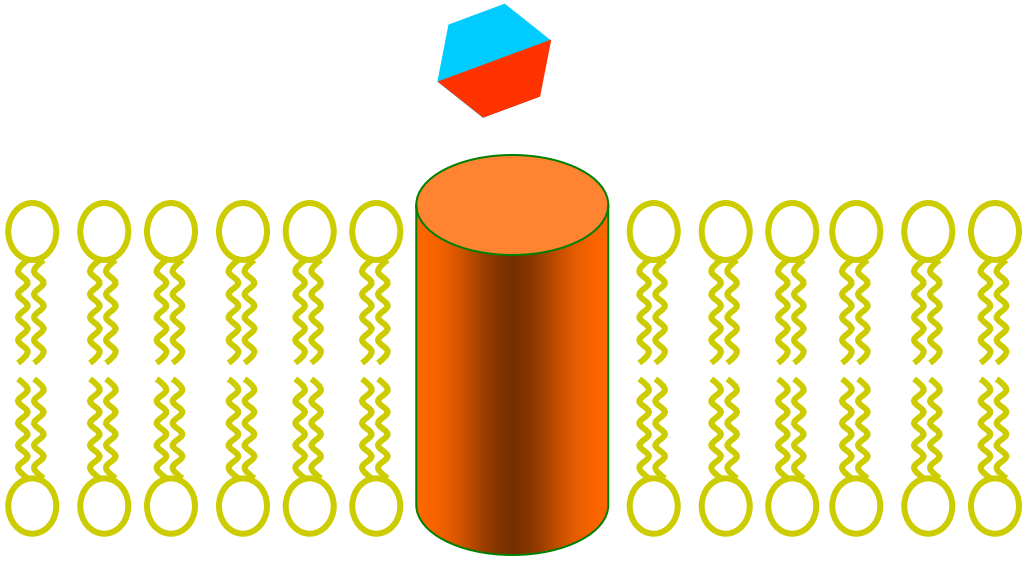
Extrusion by efflux pumps



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

Why efflux transporters ?

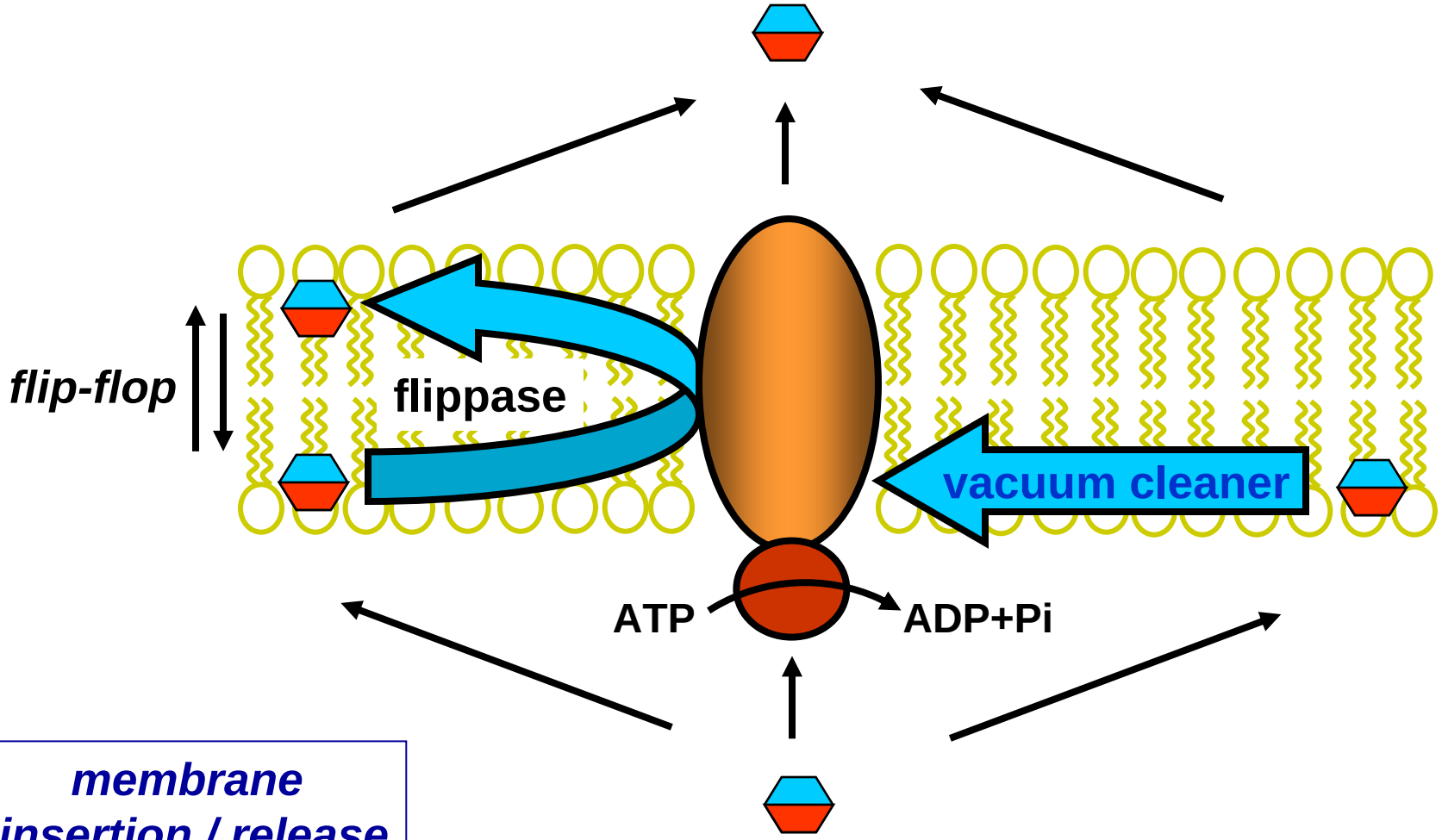
Extrusion by efflux pumps



general mean of protection
against cell invasion by diffusible molecules

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

Mechanisms of active efflux

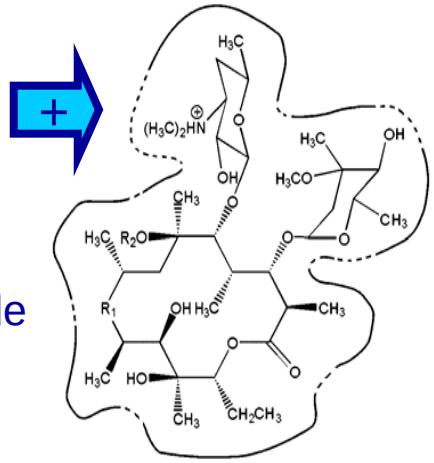


Van Bambeke *et al*. (2000) Biochem. Pharmacol. 60:457-70

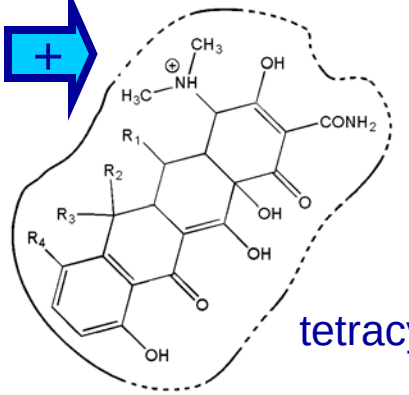
Most antibiotics are amphiphilic !

cationic amphiphiles

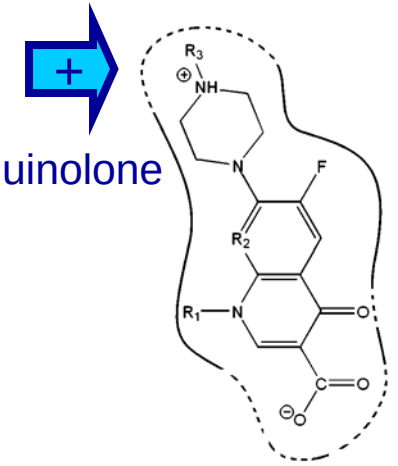
macrolide



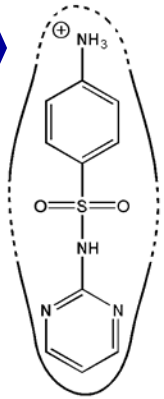
tetracycline



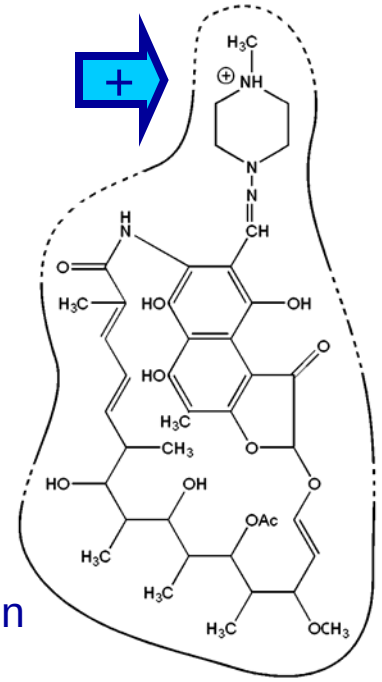
fluoroquinolone



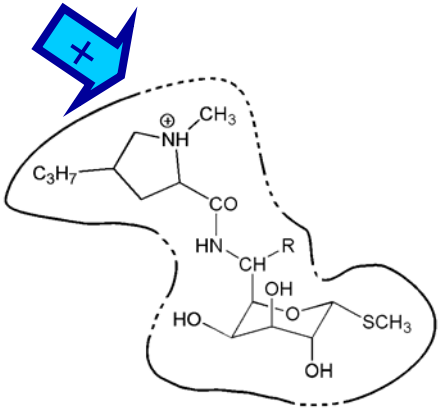
sulfamide



rifampicin



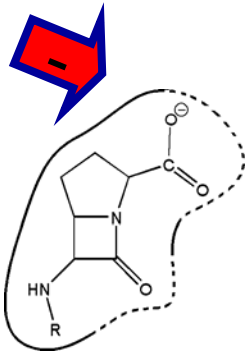
lincosamide



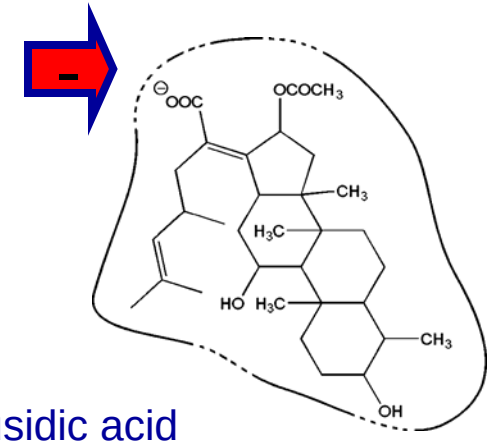
Van Bambeke *et al*. (2000) Biochem. Pharmacol. 60:457-70

Most antibiotics are amphiphilic !

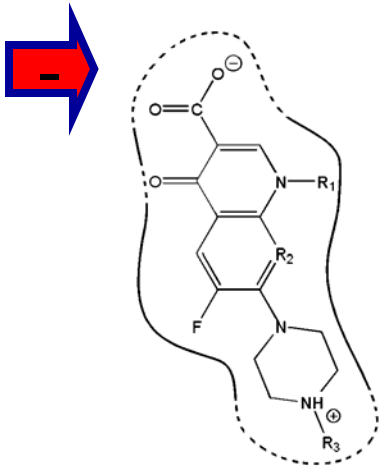
anionic amphiphiles



β-lactam



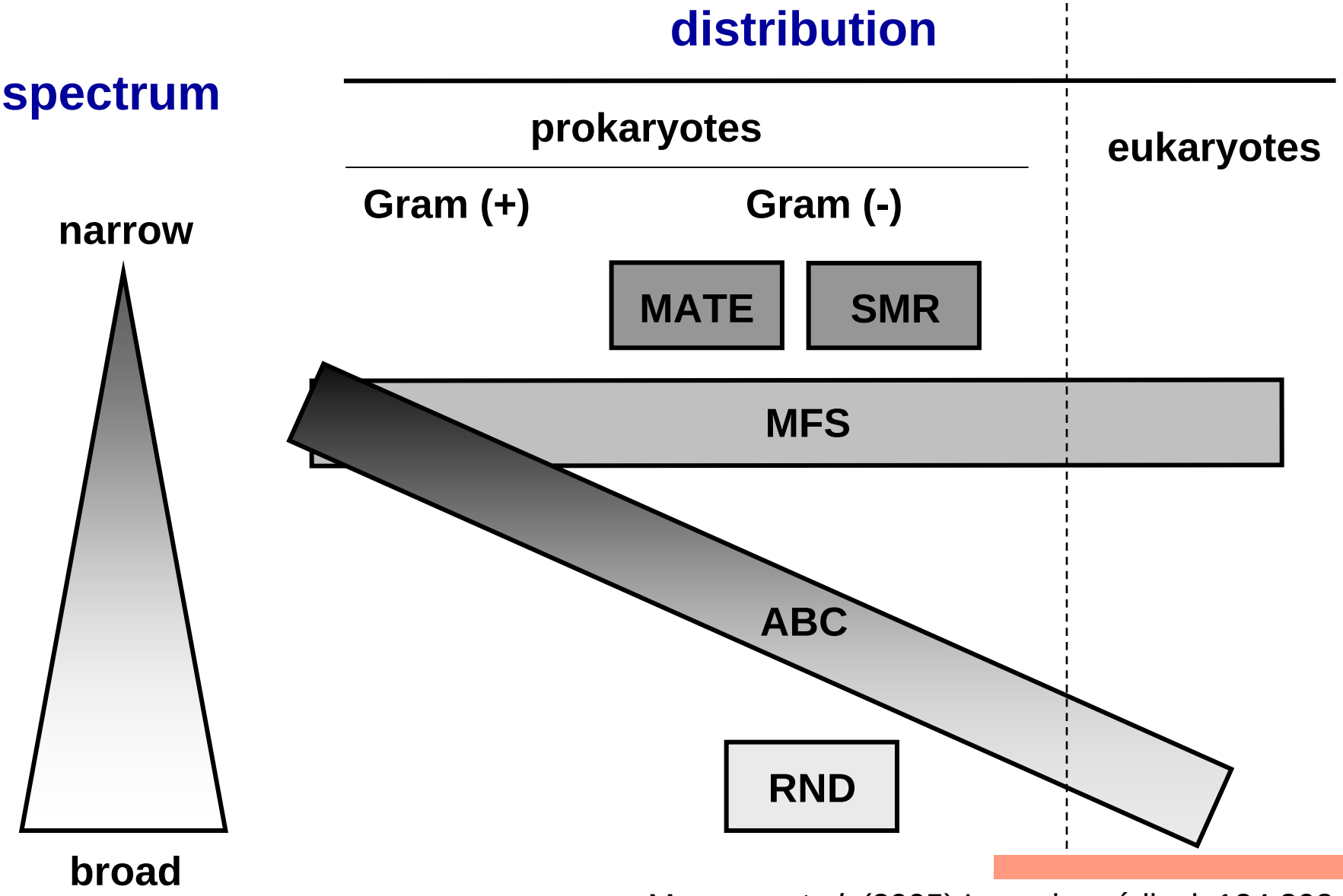
fusidic acid



fluoroquinolone

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

Antibiotic efflux transporters are ubiquitous



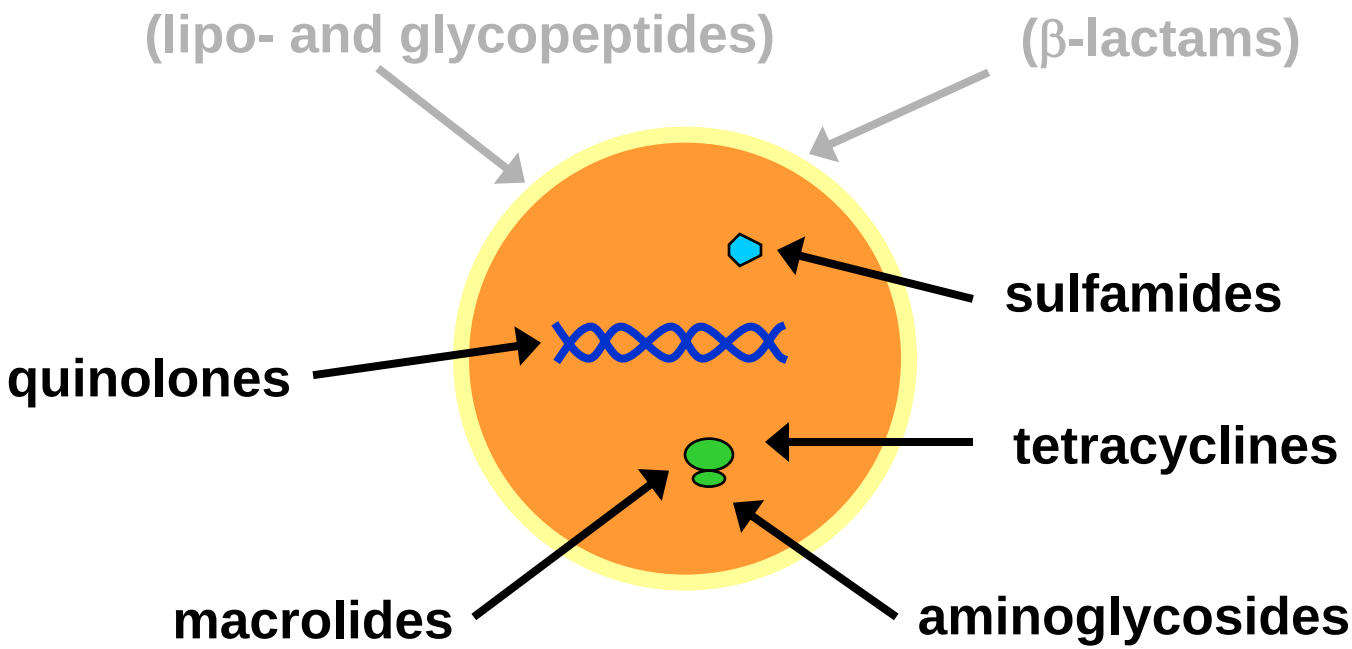
Mesaros *et al*. (2005) Louvain médical. 124:308-20

Antibiotics as substrates of efflux pumps

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

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

Most antibiotics do act on intracellular targets

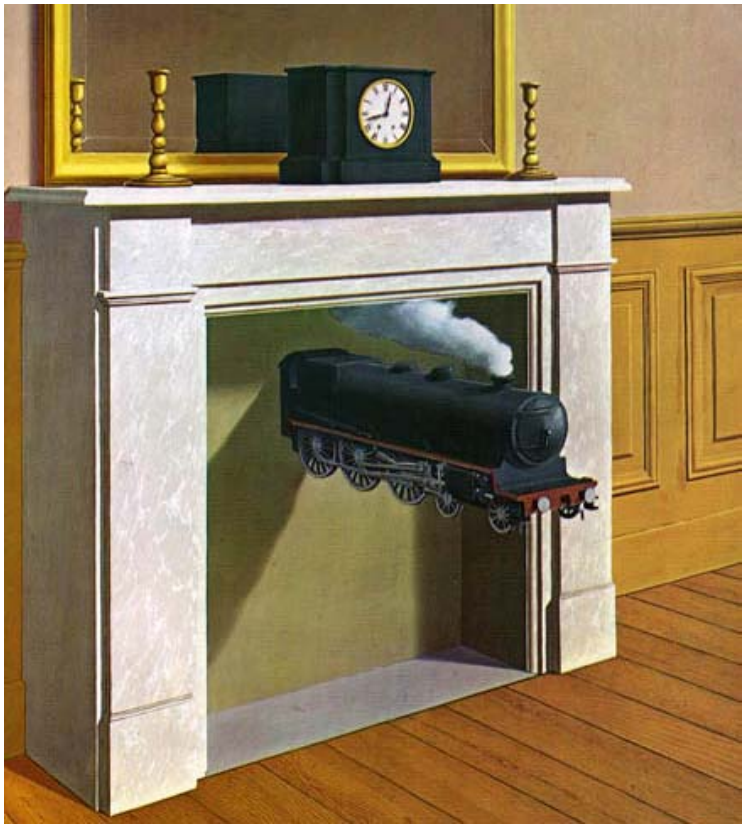


Efflux as a mechanism of resistance
by reducing antibiotic concentration inside the bacteria

Antibiotic efflux from bacteria as a mechanism of antibiotic resistance : molecular bases

Jean-Michel Folon (1934-2005)

Efflux as a mechanism of export in antibiotic producers

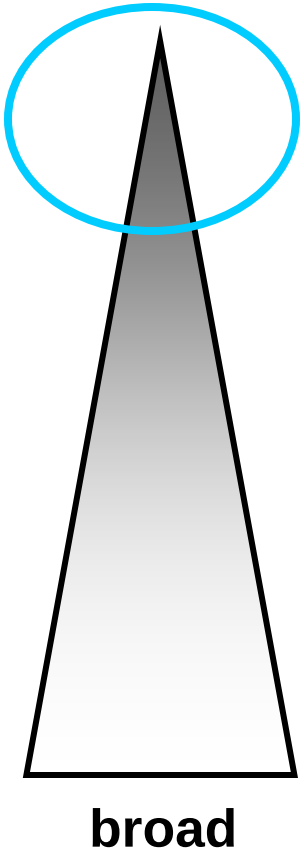


Delivery of metabolites produced by the cell

René Magritte (1898-1967)

Efflux as a mechanism of export in antibiotic producers

spectrum



specific for the produced antibiotic

ABC	MFS
OleB or C of *S. antibioticus* → oleandomycin	Ptr of *S. pristinaespiralis* → pristinamycin
SmB of *S. ambofaciens* → macrolides	LmrA of *S. lincolnensis* → lincomycin
TlrC of *S. fradiae* → tylosin	RifP of *A. mediterranei* → rifampicin

Efflux as a mechanism of export in antibiotic producers

Tylosin-biosynthetic gene cluster in *S. fradiae*

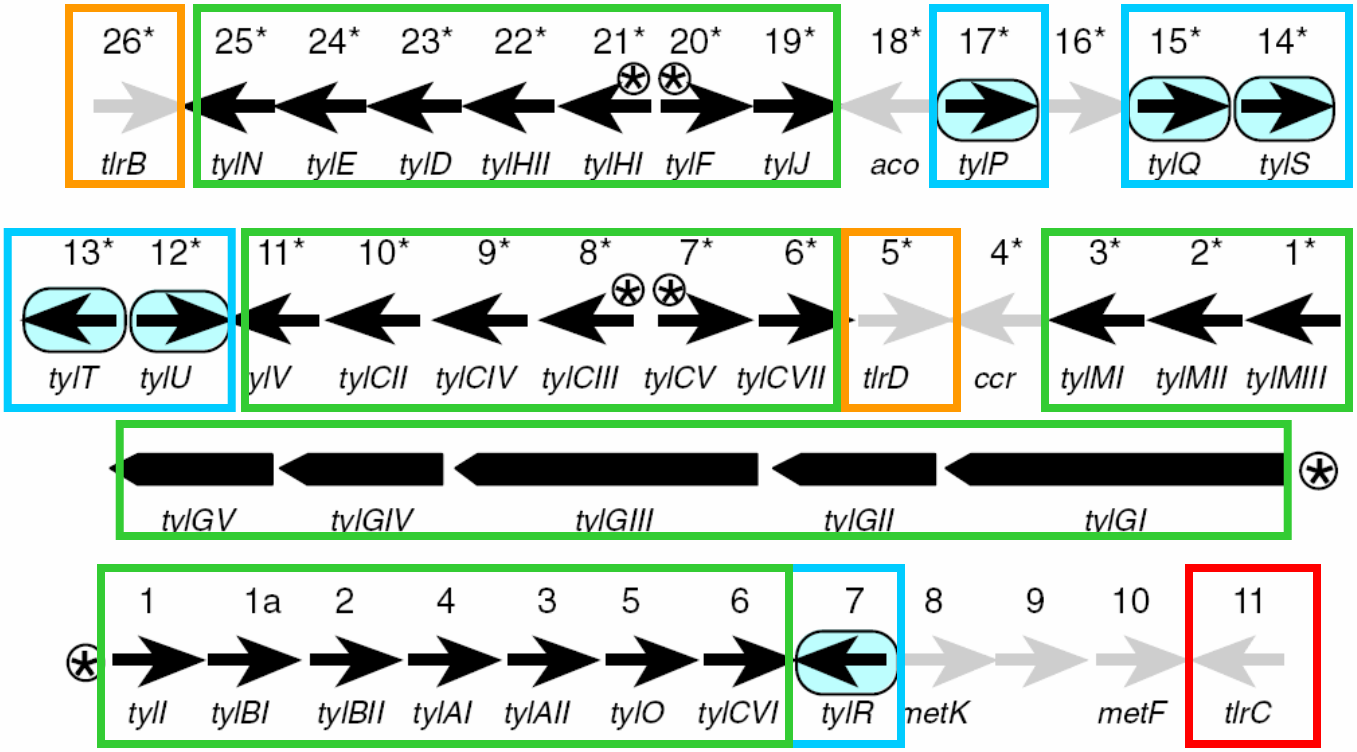


Fig. 1 The tylosin-biosynthetic gene cluster of *S. fradiae*. Not drawn to scale. The cluster occupies a contiguous portion of the genome (approximately 85 kb). Regulatory genes are outlined in *boxes*. Tylosin-biosynthetic genes are represented by *black arrows*. Resistance determinants (designated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

tylosin biosynthesis

resistance determinants

regulation

efflux

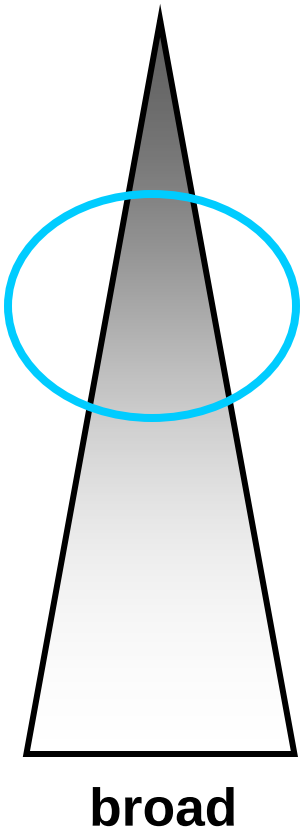
Cundliffe (2006) J. Ind. Microbiol. Biotechnol. 33:500-6

Efflux as a mechanism of antibiotic resistance in pathogenic bacteria

Pierre Paul Rubens (1530-1587)

Efflux as a mechanism of resistance in Gram-positive bacteria

spectrum



specific for one (or a few) families of drugs

ABC

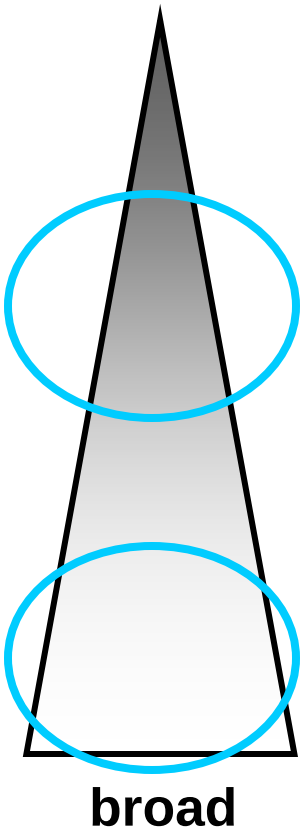
PatA/PatB of *S. pneumoniae*
→ FQ, chl
MsrA of *S. epidermidis*
→ erythromycin

MFS

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

Efflux as a mechanism of resistance in Gram-negative bacteria

spectrum



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-TolC of *E. coli*
→ β-lac, FQ,Tet, ML, chl, rif, sulf

Molecular determinants of drug efflux

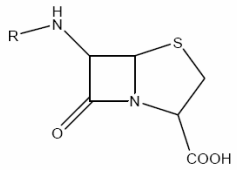
Brussels, atomium (1958; Polak)

Differences in transport between drugs within a class

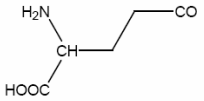
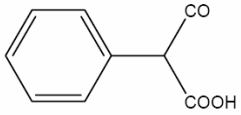
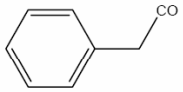
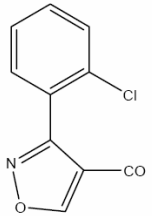
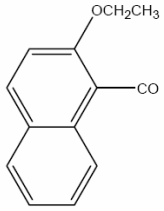
usual difference in affinity of efflux pumps towards antibiotics			
	affinity for efflux pumps		
antibiotic class	high	variable [a]	low
penicillins	nafcillin, cloxacillin, penicillin G		carbenicillin
cephalosporins	cefalotin, cefotaxime, ceftriaxone		cefazolin, cephaloridin
carbapenems	meropenem	imipenem	
macrolides	14 - and 15 - membered		16 –membered, ketolides
tetracyclines	tetracycline	minocycline glycylcyclines	
quinolones	ciprofloxacin, norfloxacin	ofloxacin, levofloxacin	cinafloxacin, gatifloxacin, gemifloxacin, moxifloxacin, garenoxacin
[a] depending on the efflux pump			

Van Bambeke *et al*. (2003) JAC 51:1055-65

Differential efflux of β-lactams by AcrB of *S. typhimurium*



Relationship between structure, lipophilicity of the side chain of selected penicillins and MIC of bacteria resistant by efflux

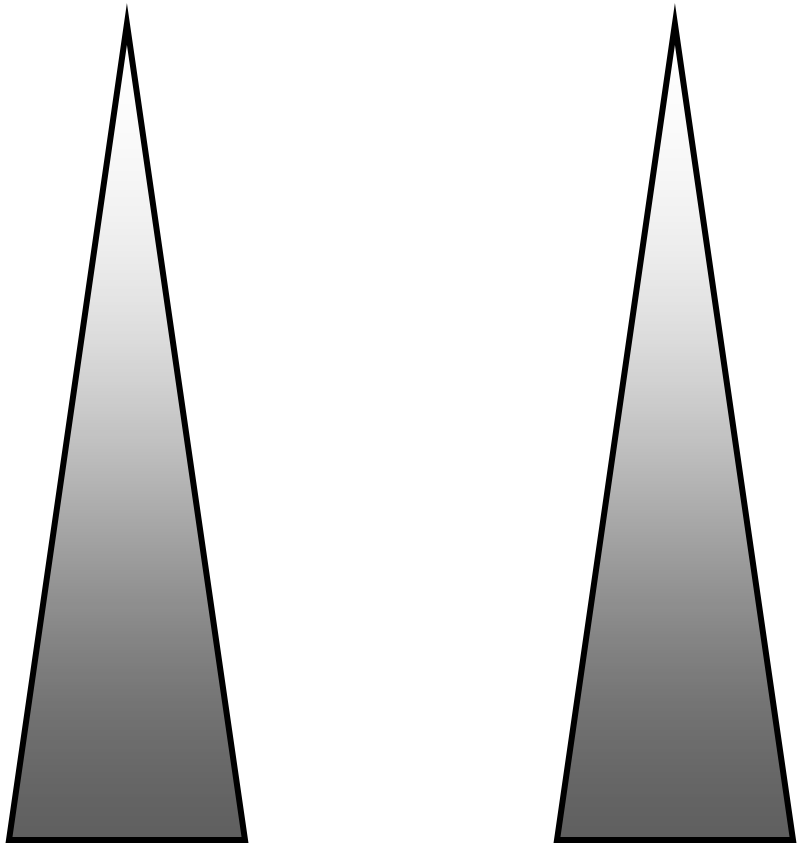
R (Side chain)	molecule	side-chain partition coefficient	MIC ratio[a]
	penicillin N	0	1
	carbenicillin	80	4
	penicillin G	270	32
	cloxacillin	890	256
	nafcillin	4200	128

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

β-lactams & broad spectrum RND

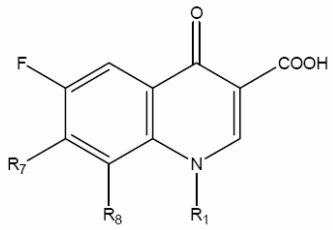
Lipophilicity of the side chain

Resistance

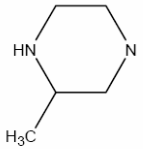
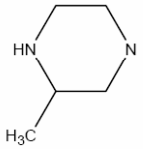
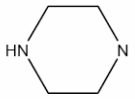
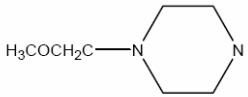
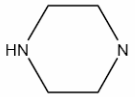


Nikaido *et al.* (1998) J. Bacteriol. 180:4686-92

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



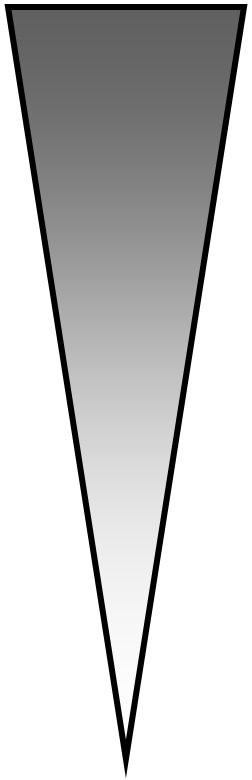
Relationship between structure, bulkiness, and hydrophobicity of selected quinolone substituents and MIC of bacteria resistant by efflux.

molecule	R_1	R_7	R_8	MIC ratio[a]
(a)	▷		OCF_3	2
(b)	▷		OCH_3	4
ciprofloxacin	▷		H	16
(c)	C_2H_5		H	16
norfloxacin	C_2H_5		H	64

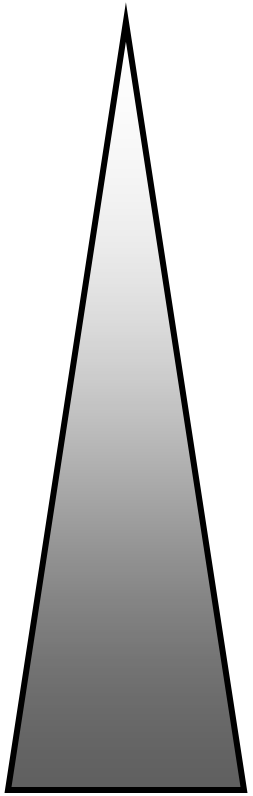
[a] ratio between the MIC of a *S. aureus* resistant mutant (NorA overproducer) and of its susceptible parental strain.

Quinolones & narrow spectrum MFS

Bulkiness in 7 and 8



Resistance

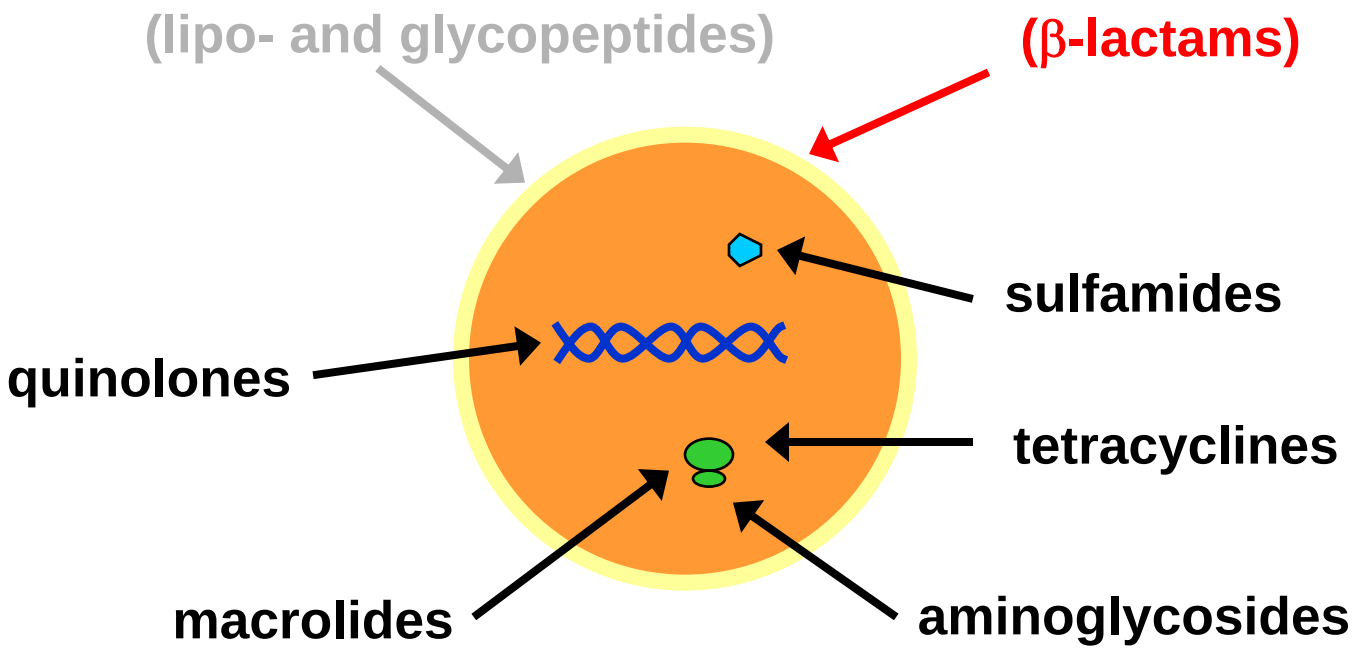


Takenouchi *et al.* (1996) AAC 40:1835-42

Unexpected antibiotic substrates

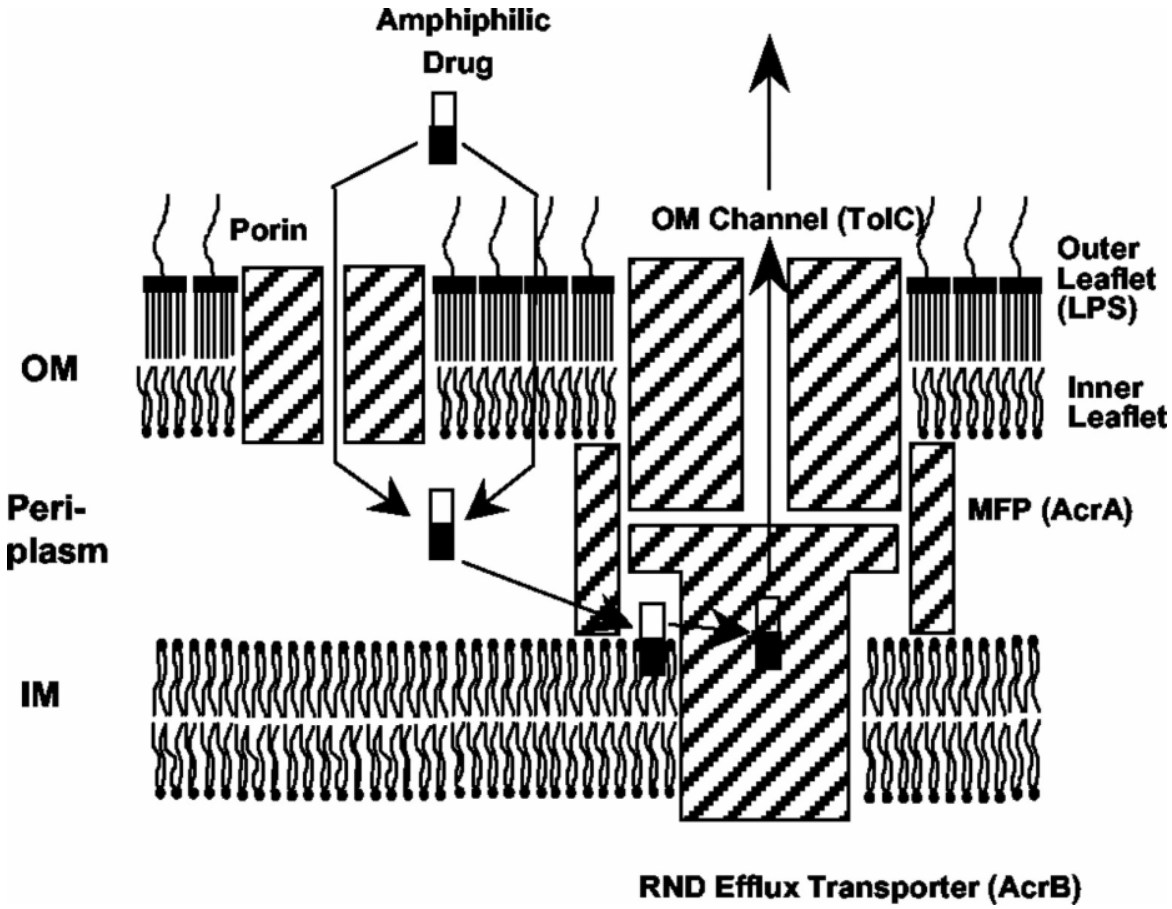
Hergé (1907-1983)

Two types of unexpected substrates



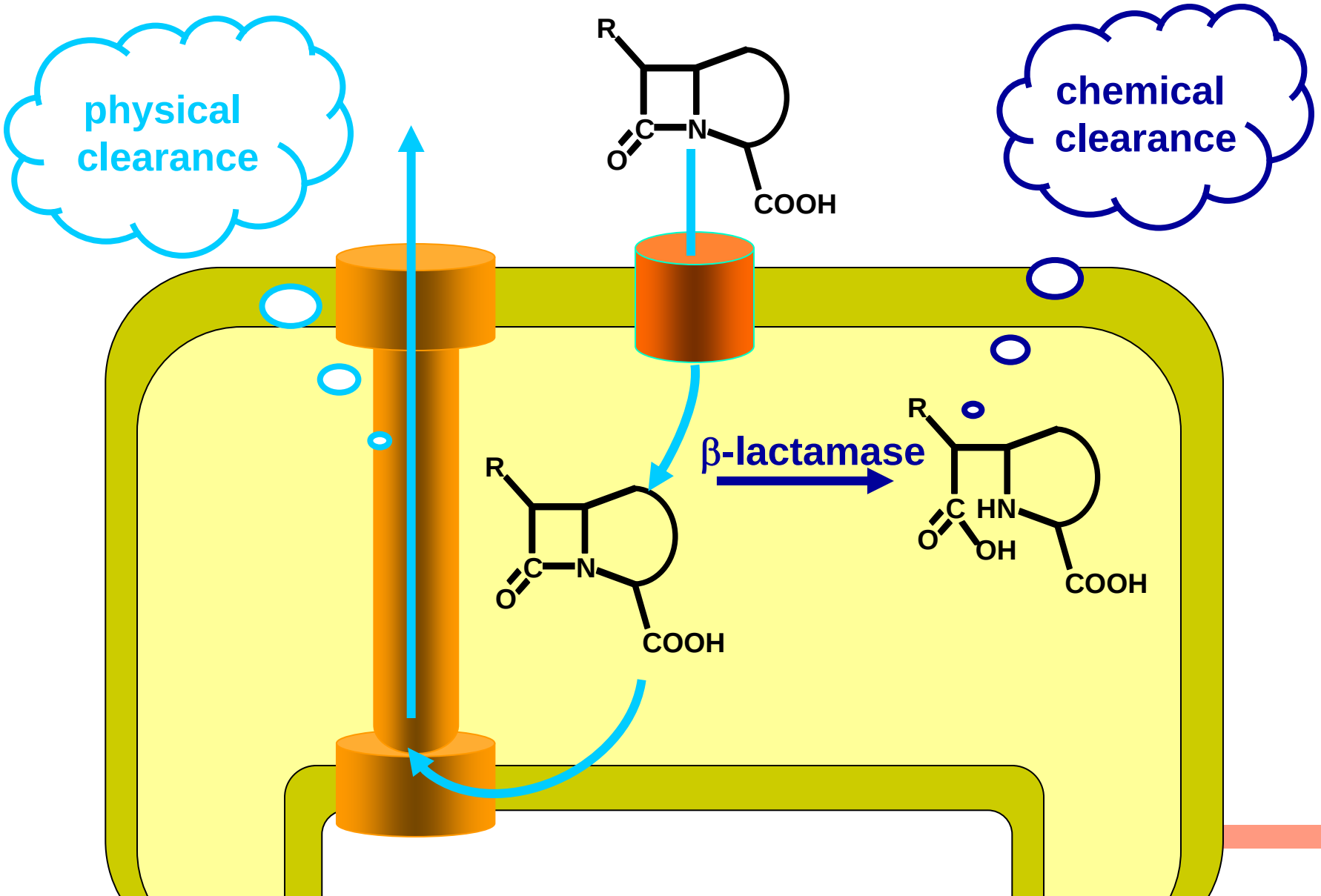
active on an extracellular target !

β-lactams as unexpected substrates for efflux pumps



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

β-lactams as unexpected substrates for efflux pumps



β-lactams as unexpected substrates for efflux pumps

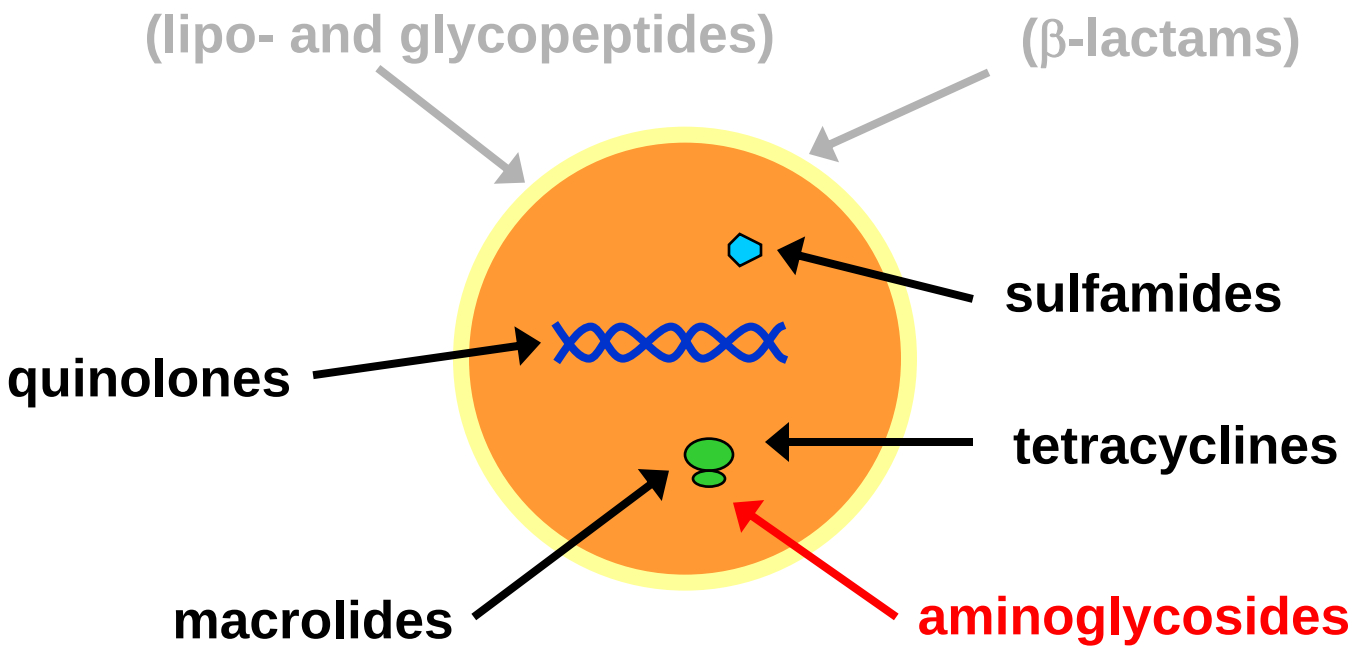
Contributions of the AmpC β-lactamase and the AcrAB Multidrug Efflux System in Intrinsic Resistance of *E. coli* to β-lactams

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

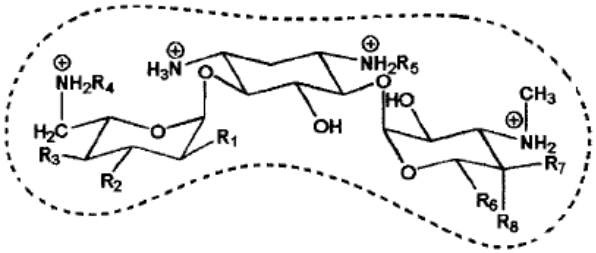
WT: intrinsic resistance !

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

Two types of unexpected substrates

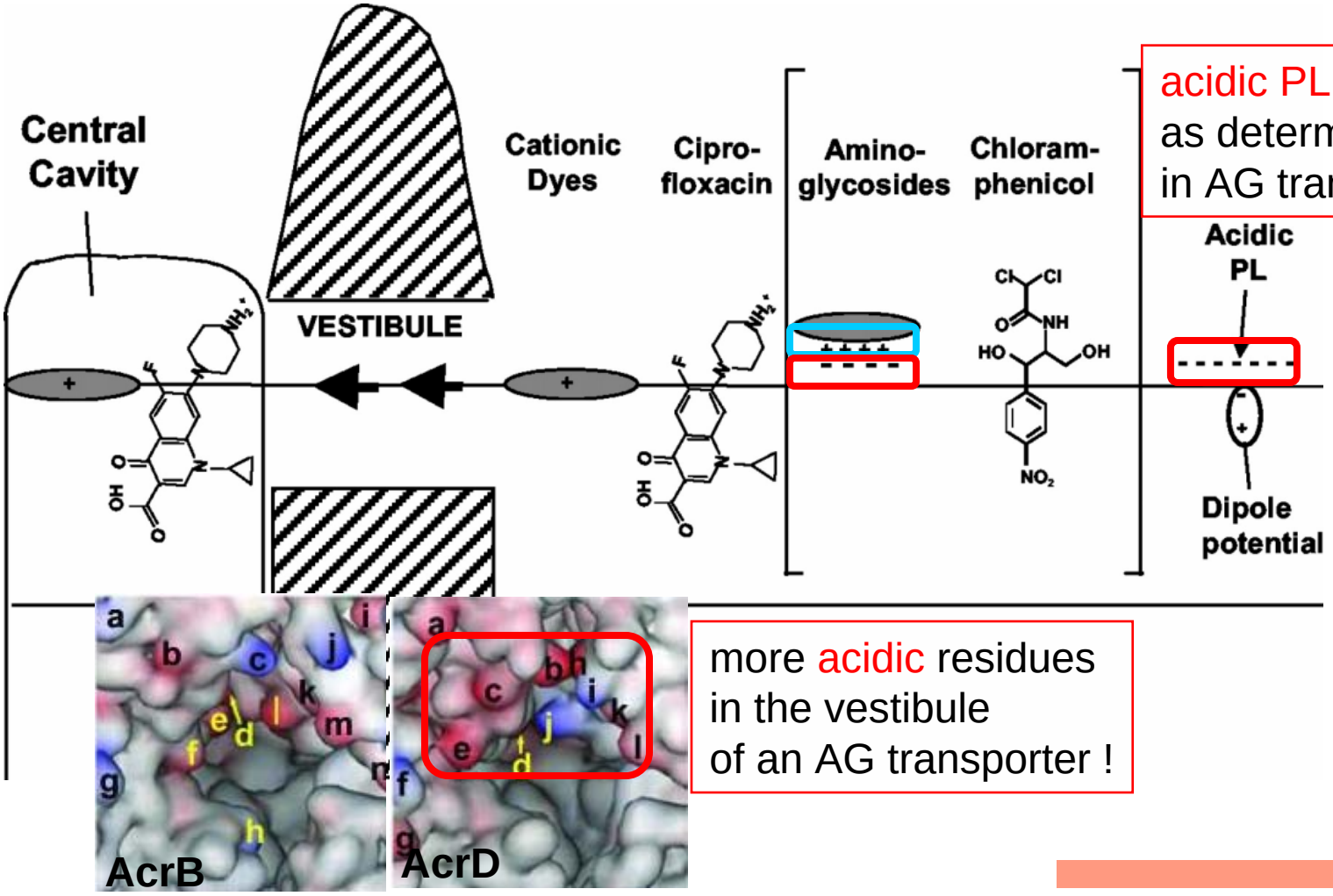


Highly hydrophilic molecules !



Aminoglycosides as unexpected substrates for efflux pumps

Composite binding site ?

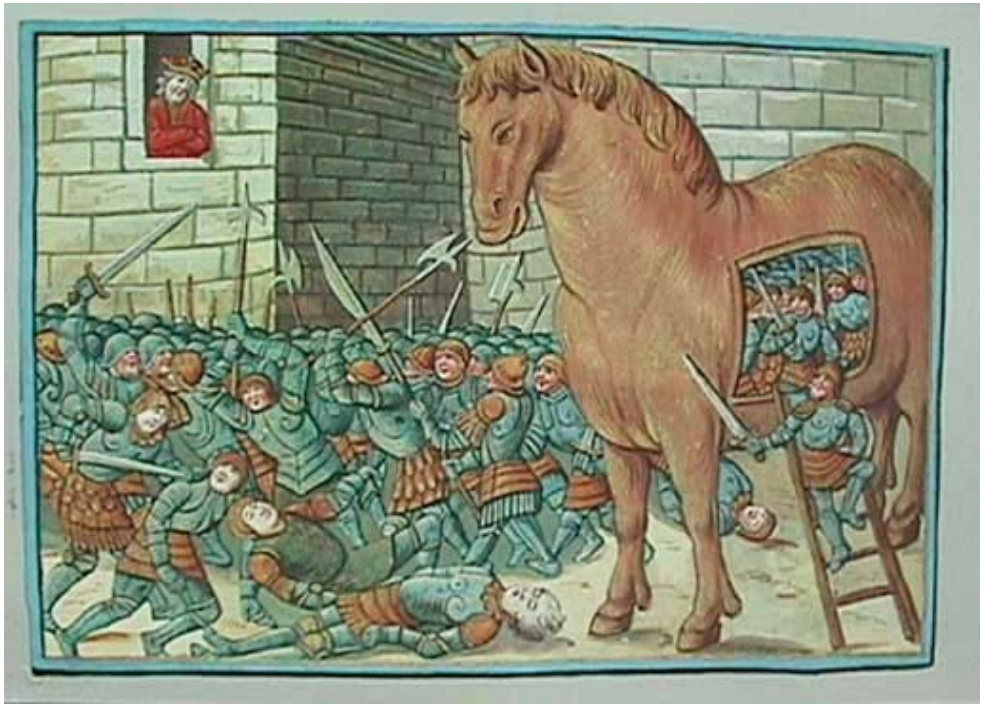


acidic PL
as determinants
in AG transport ?

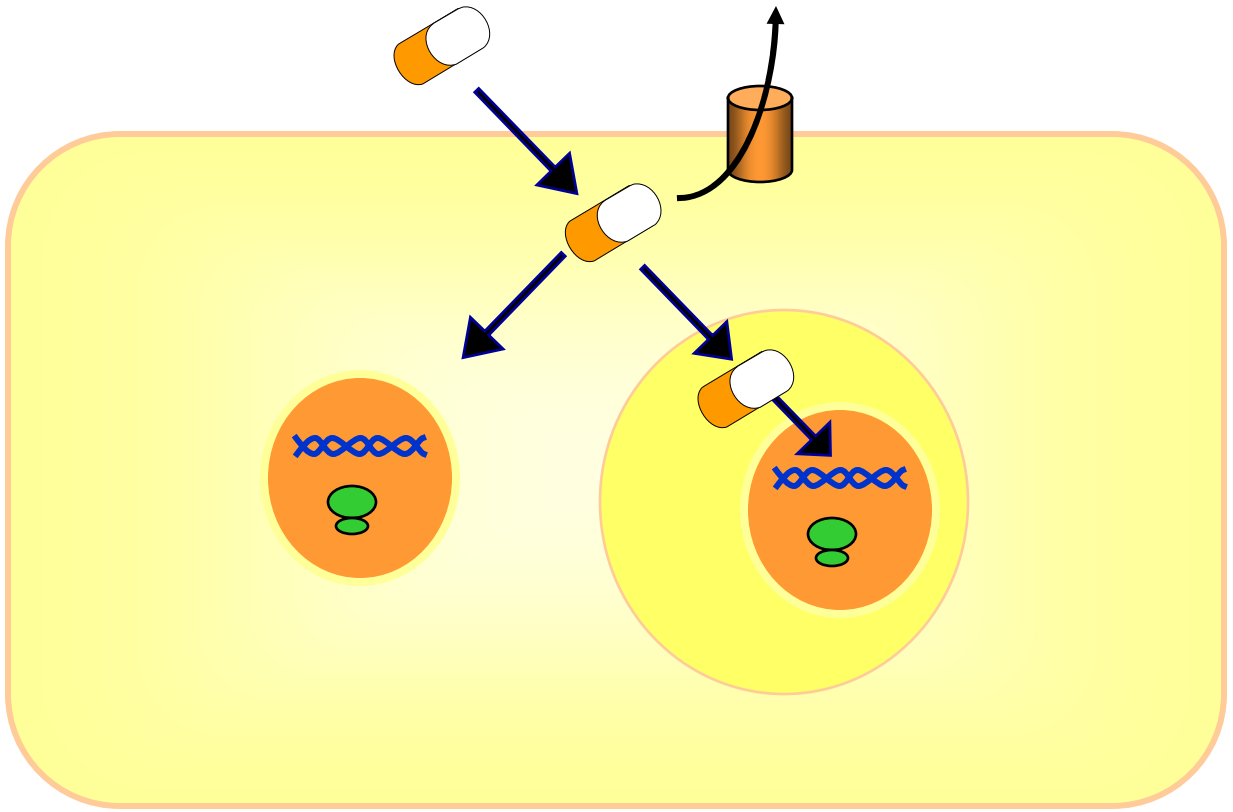
more acidic residues
in the vestibule
of an AG transporter !

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

efflux from eucaryotic cells as a mechanism of antibiotic ‘resistance’: molecular bases



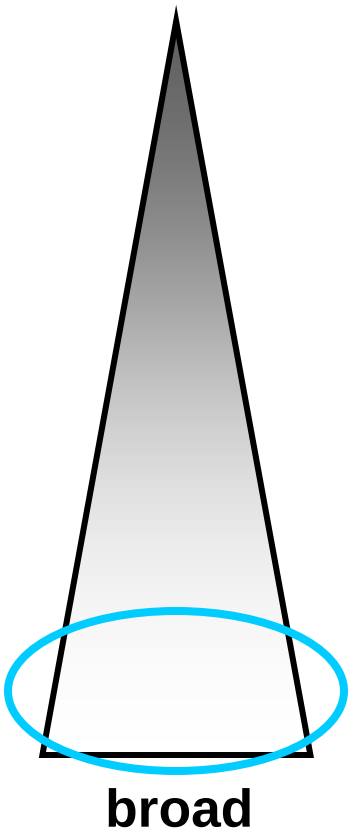
Antibiotic efflux from eucaryotic cells and intracellular ‘resistance’ ?



Reduction in intracellular drug concentration
can result in inefficacy against intracellular bacteria

Antibiotics as substrates of MDR efflux pumps

spectrum



Broad spectrum, several classes of drugs

ABC

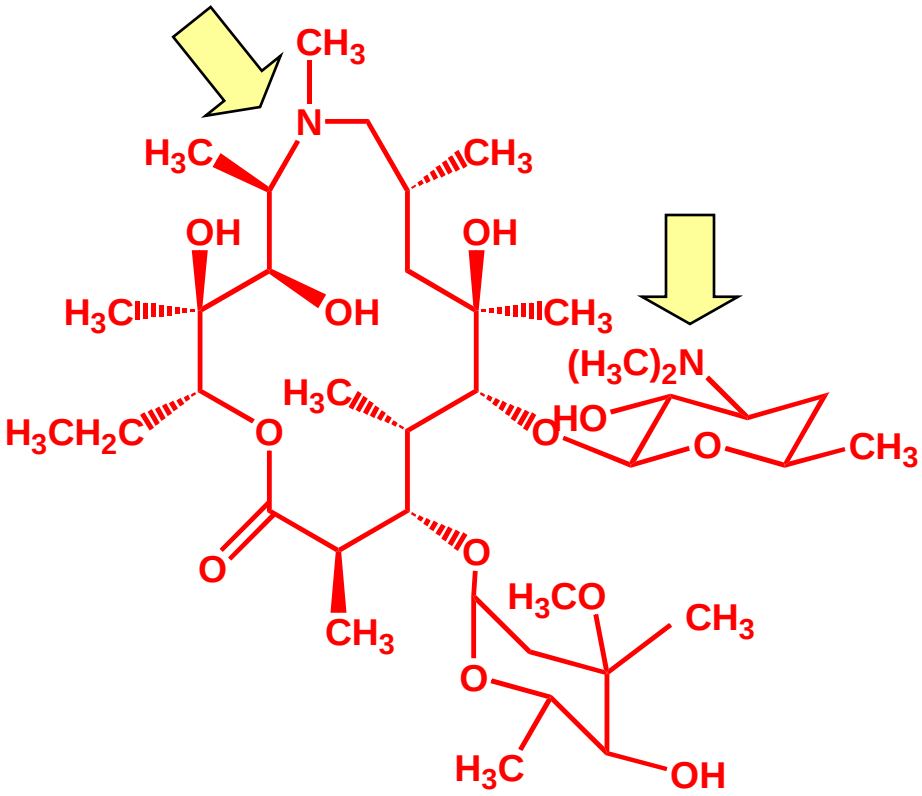
- P-glycoprotein
  - → macrolides, rifampicin
  - → anticancer agents
  - → antidepressants, antiepileptics
  - → digoxin
  - → …
- MRP
  - → fluoroquinolones, β-lactams
  - → antiviral agents
  - → anticancer agents
  - → drug conjugates
  - → …

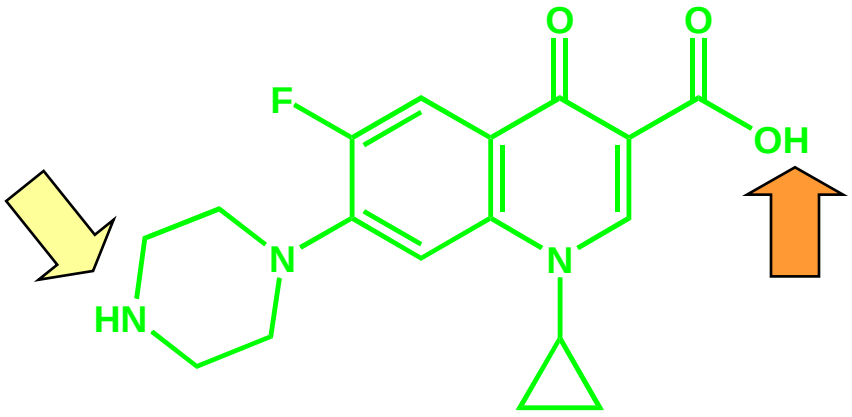
Phenotypic description of antibiotic transport by MDR pumps

Paul Bury (1922-2005)

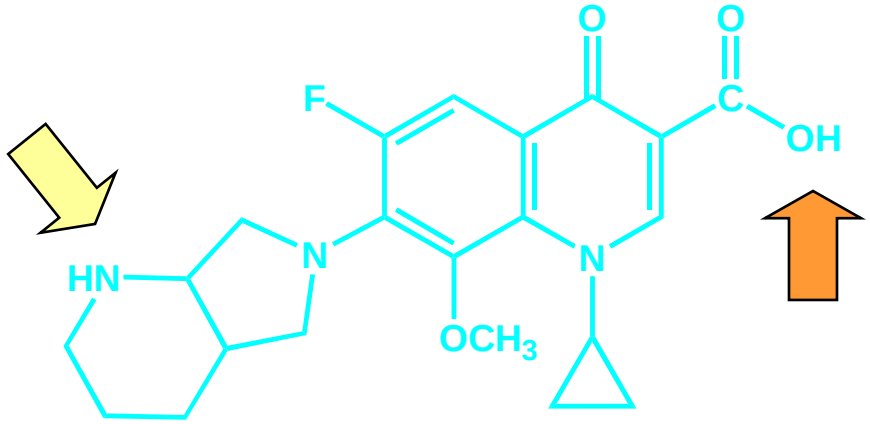
Antibiotics as substrates of MDR efflux pumps

azithromycin



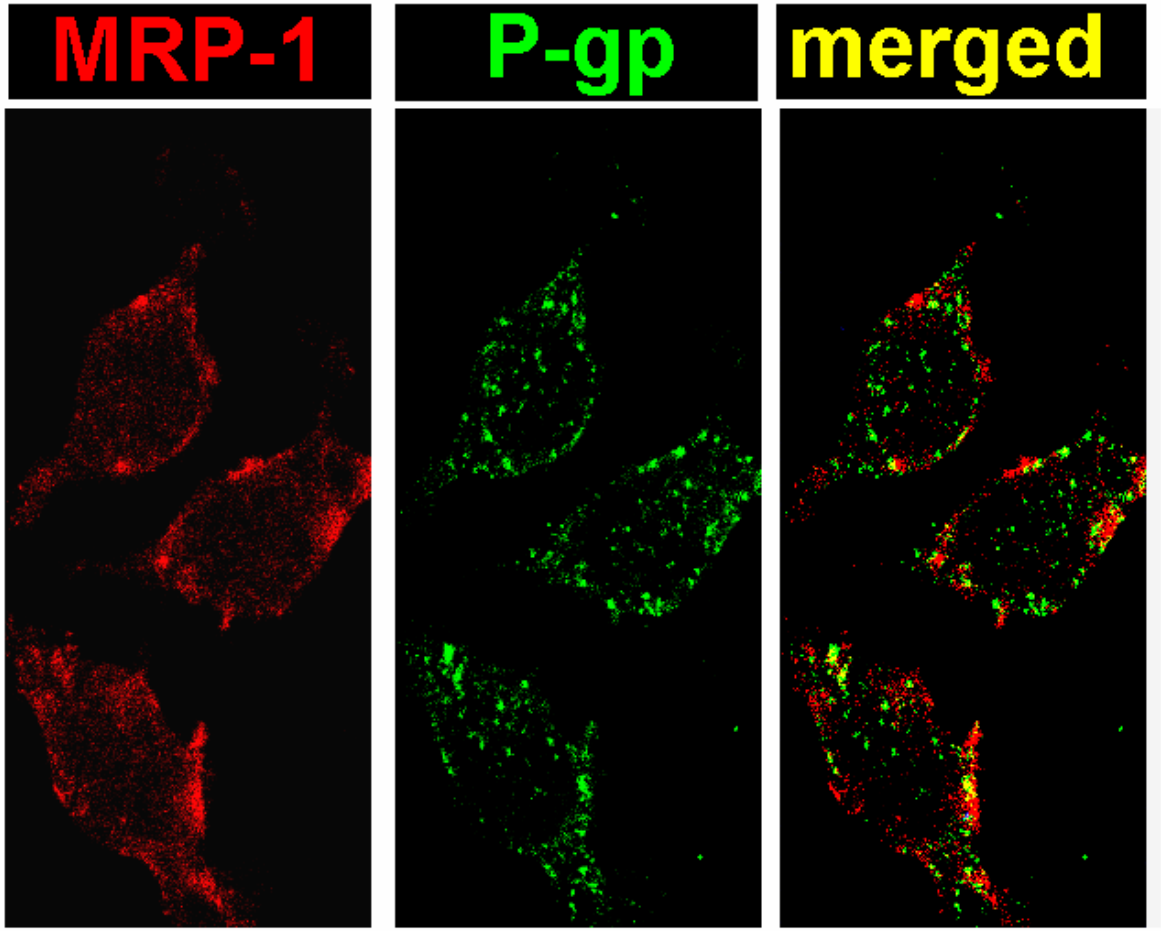


ciprofloxacin



moxifloxacin

J774 macrophages do express MDR pumps



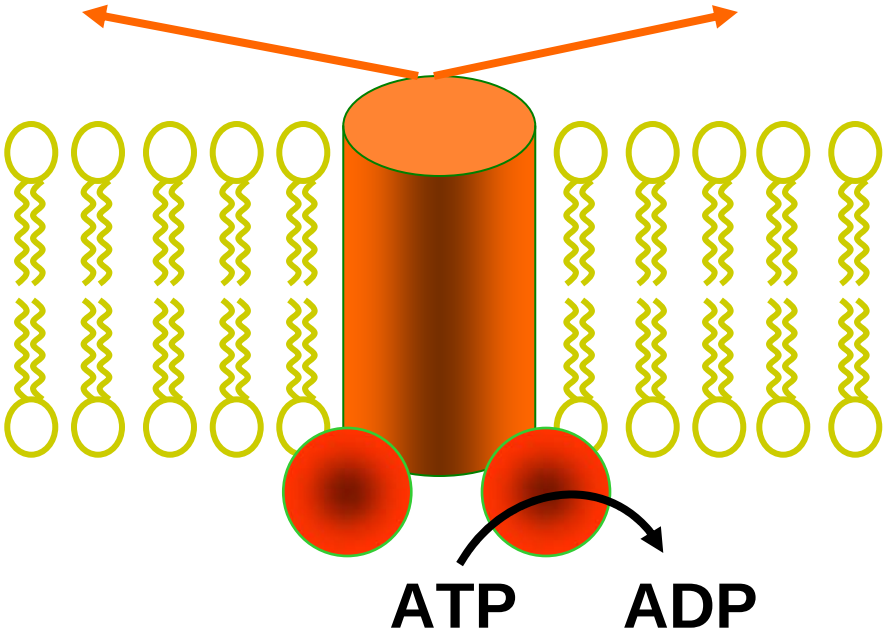
ABC multidrug transporters

cationic amphiphiles

MDR-1 (P-glycoprotein)

anionic amphiphiles

MRP1-10



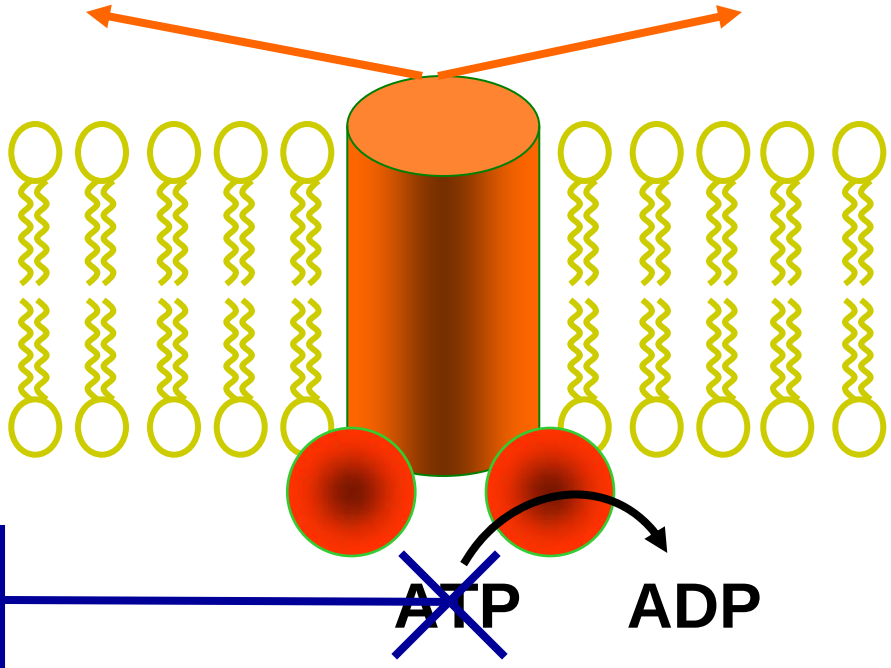
How to inhibit ABC transporters ?

cationic amphiphiles

MDR-1 (P-glycoprotein)

anionic amphiphiles

MRP1-10

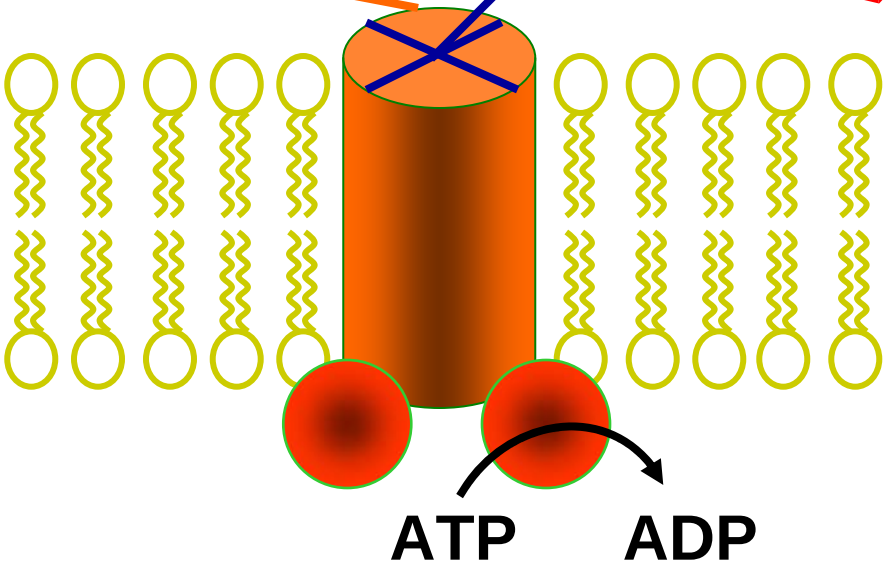


deoxyglucose
NaN_3

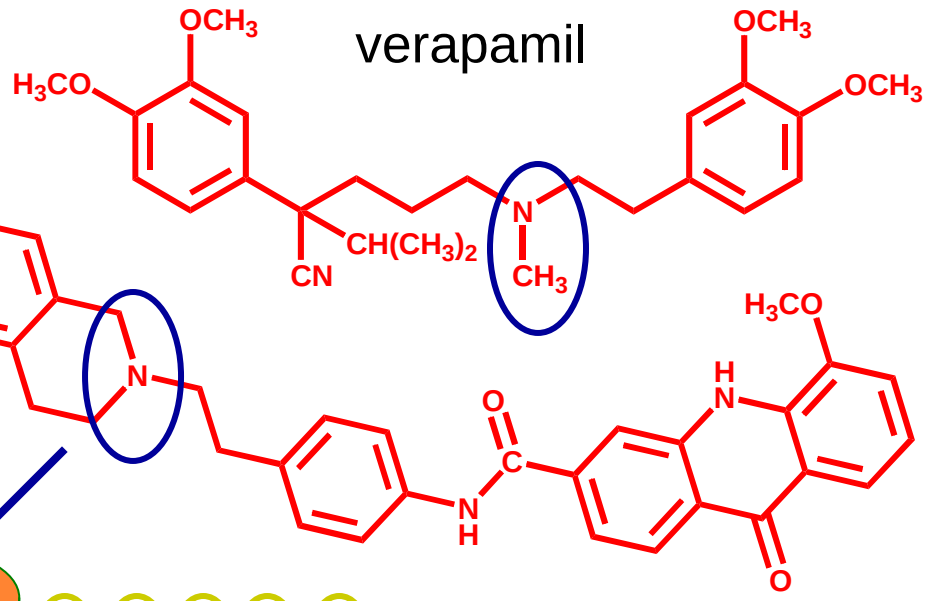
How to inhibit ABC transporters ?

cationic amphiphiles

MDR-1 (P-glycoprotein)



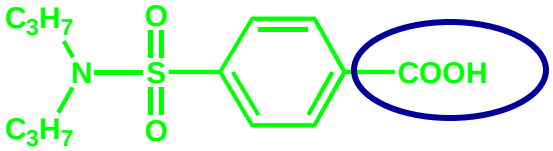
verapamil



GF120918

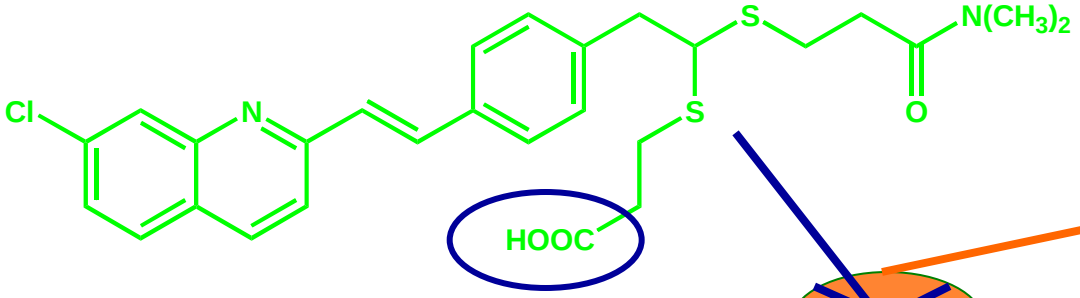
How to inhibit ABC transporters ?

probenecid

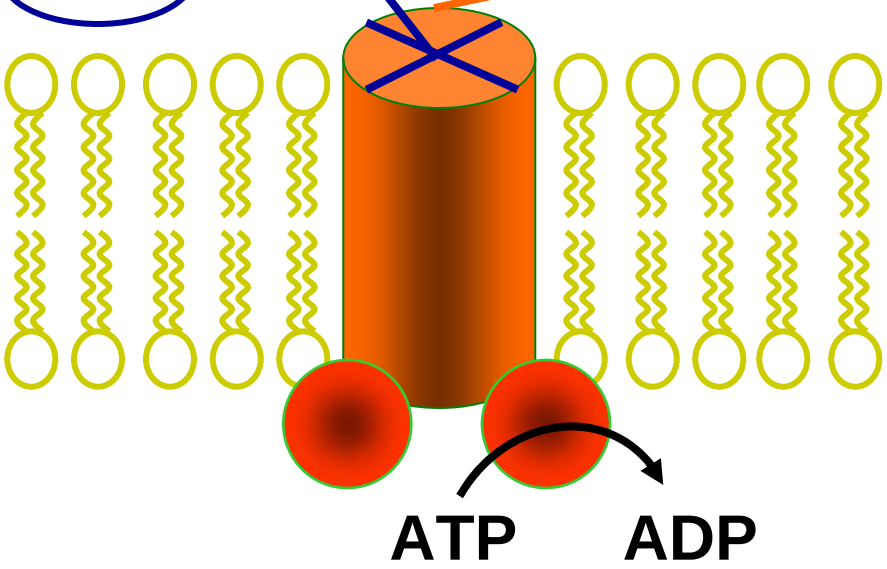


anionic amphiphiles

MRP1-10

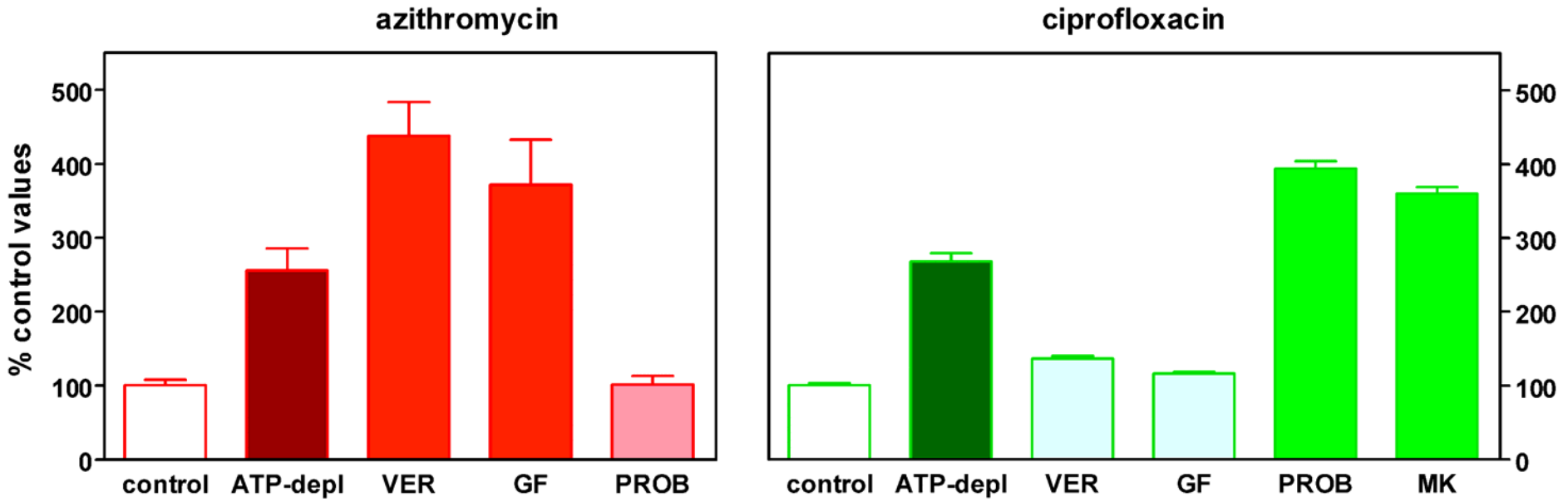


MK571



Differential recognition of antibiotics by MDR pumps

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



azithromycin & P-glycoprotein

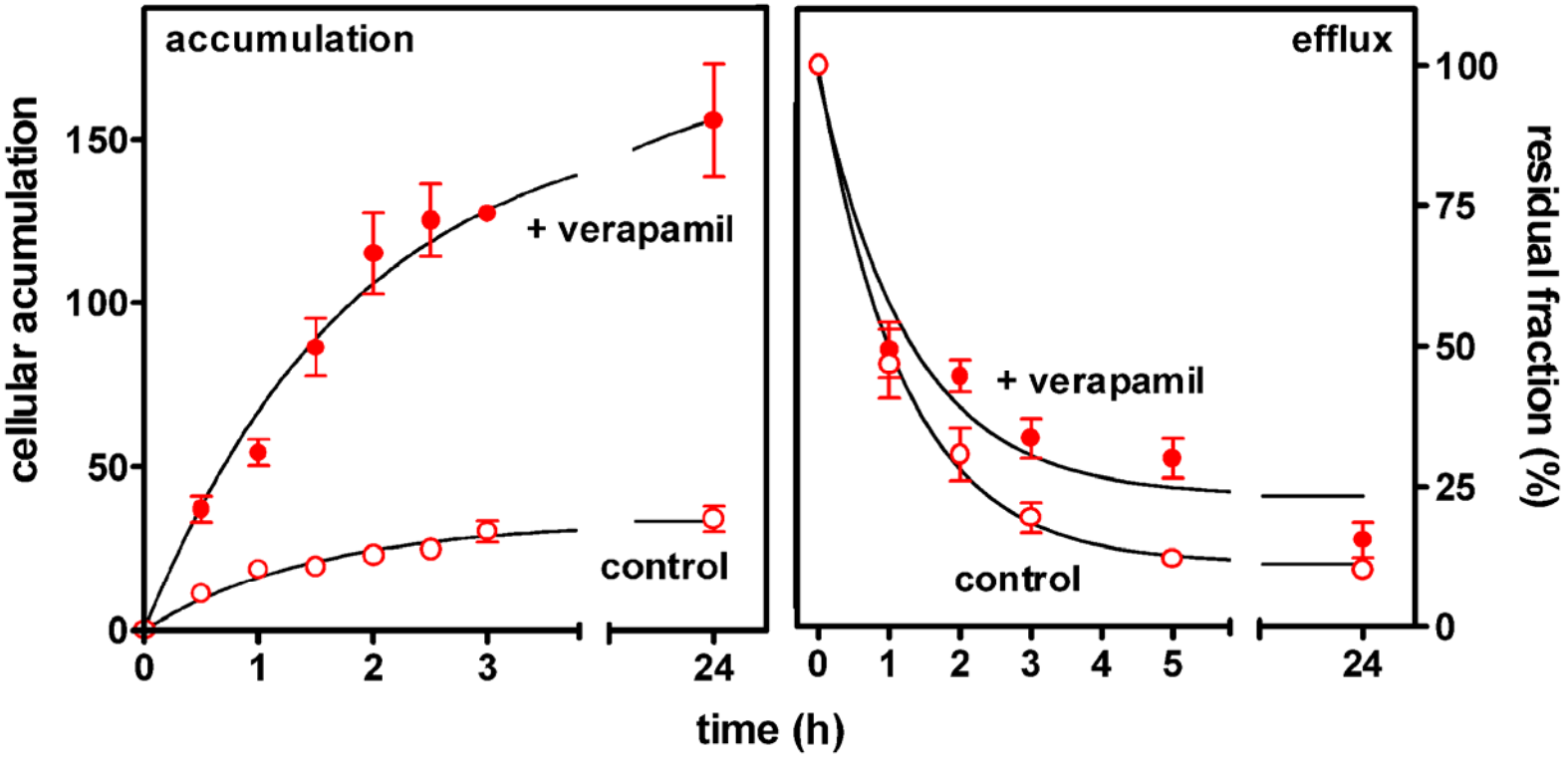
ciprofloxacin & MRP

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

Michot *et al*. AAC (2004) 48:2673-82

Kinetics of accumulation and efflux for azithromycin

accumulation markedly increased; efflux marginally affected

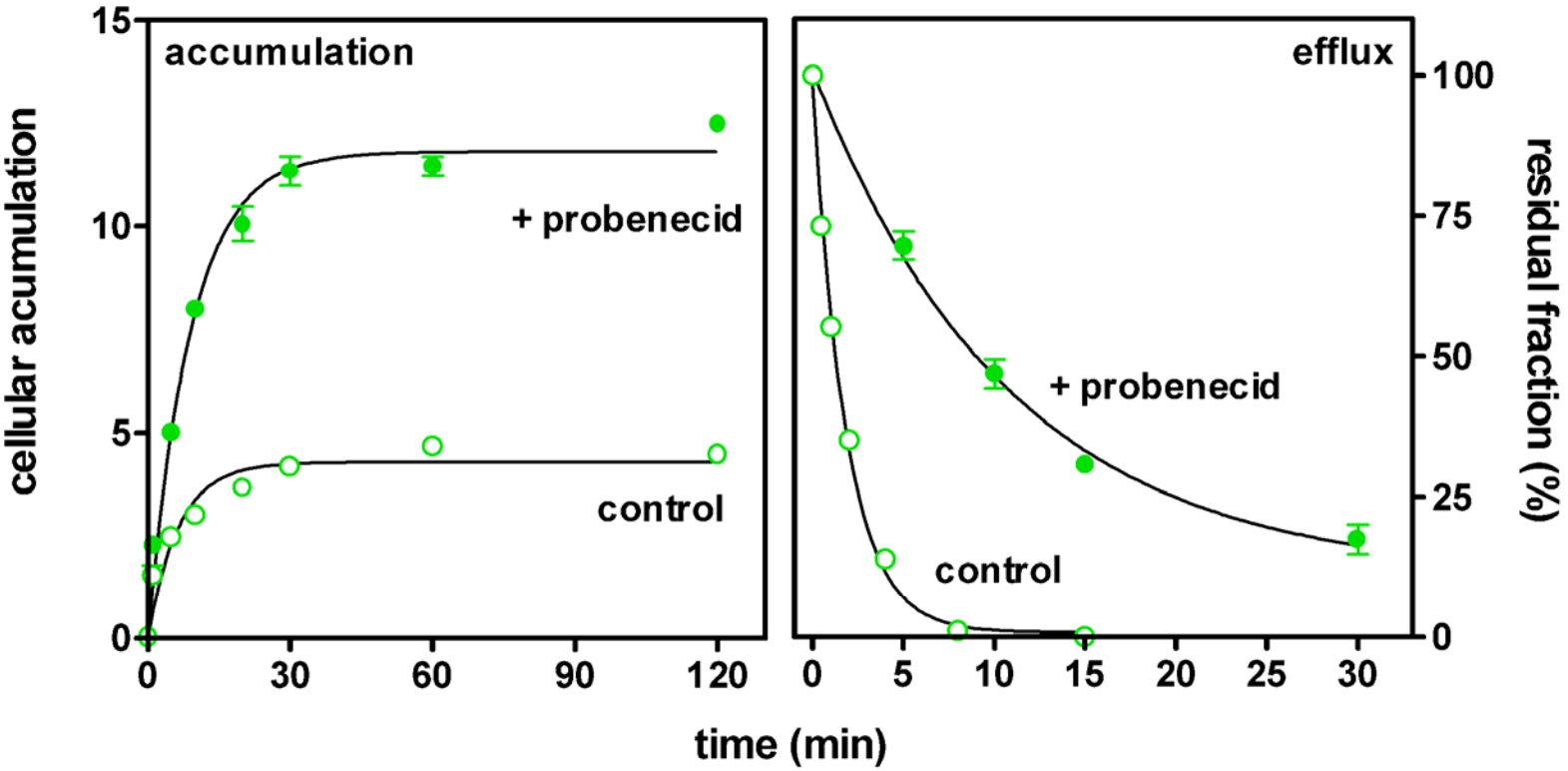


extracell. conc. 5 mg/L; verapamil 20 µM

Seral *et al.* (2003) AAC 47:1047-51

Kinetics of accumulation and efflux for ciprofloxacin

both accumulation and efflux markedly affected

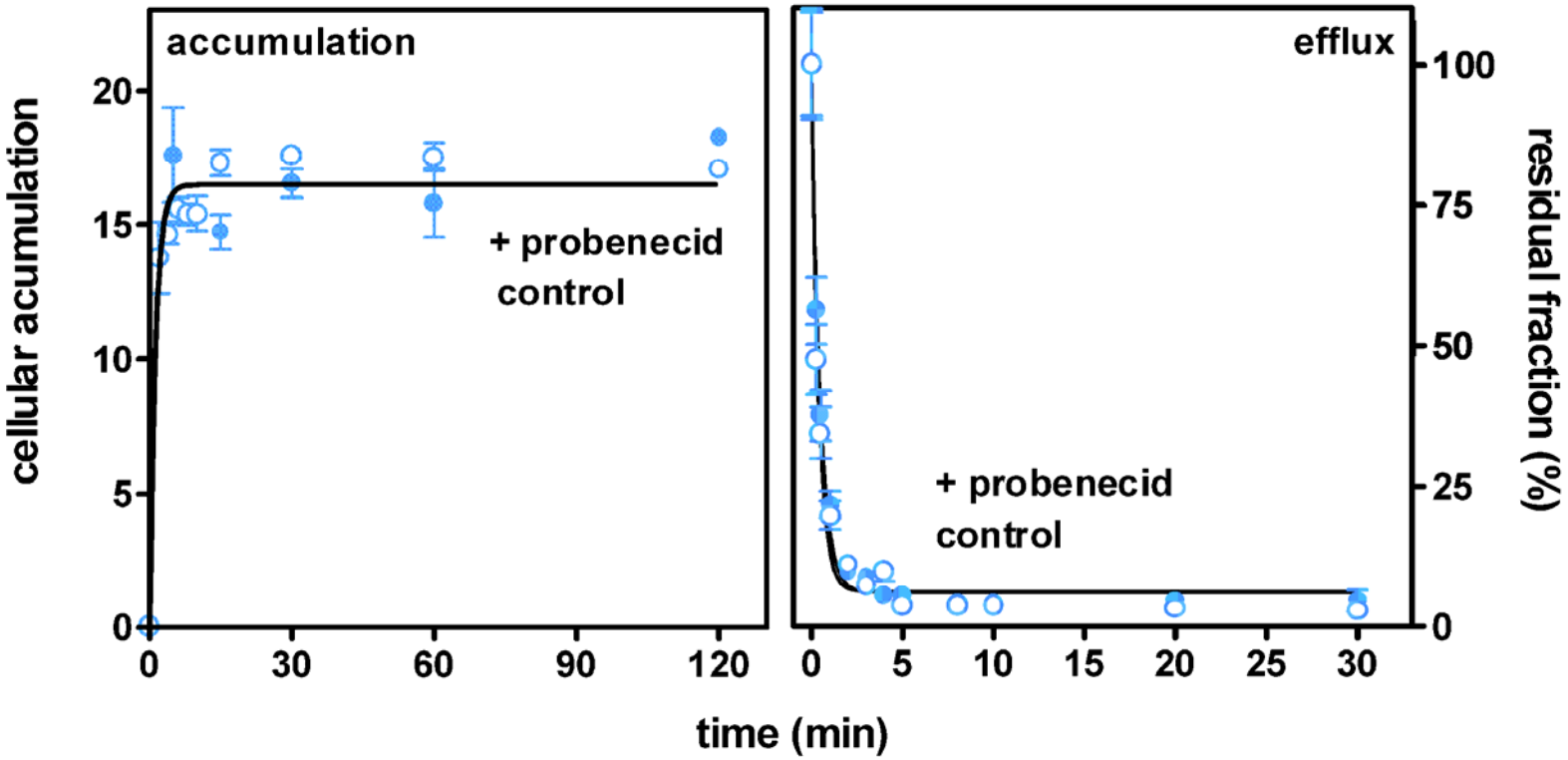


extracell. conc. 17 mg/L; probenecid 5 mM

Michot *et al*. (2004) AAC 48:2673-82

Kinetics of accumulation and efflux for moxifloxacin

neither accumulation nor efflux affected

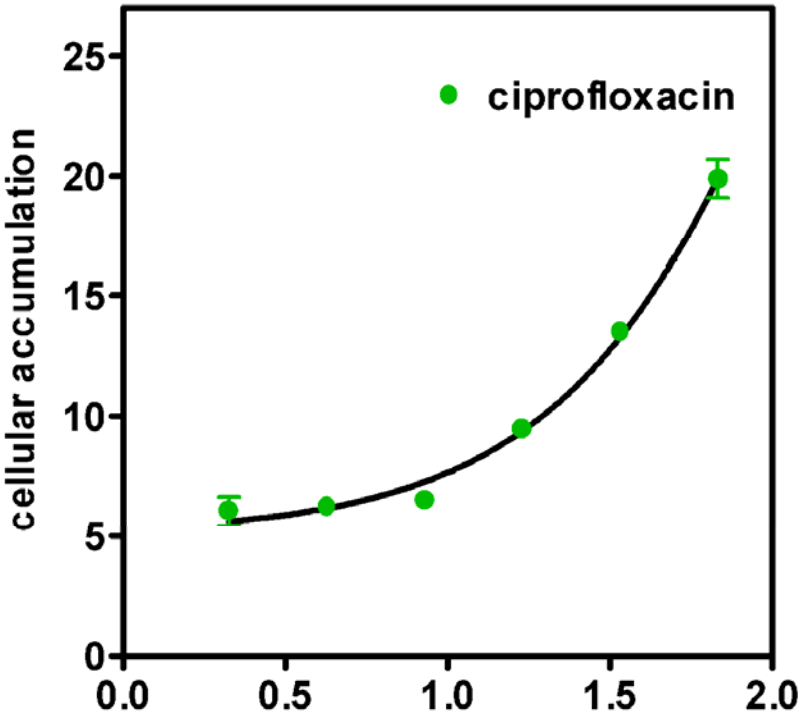


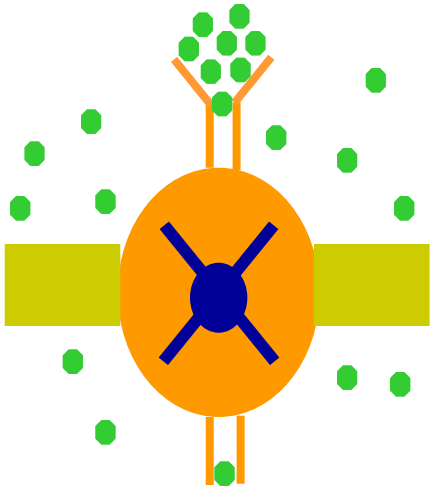
extracell. conc. 17 mg/L; probenecid 5 mM

Michot *et al*. AAC (2005) 49:2429-37

Quinolones as inhibitors of ciprofloxacin efflux

- **ciprofloxacin efflux inhibited by ciprofloxacin**

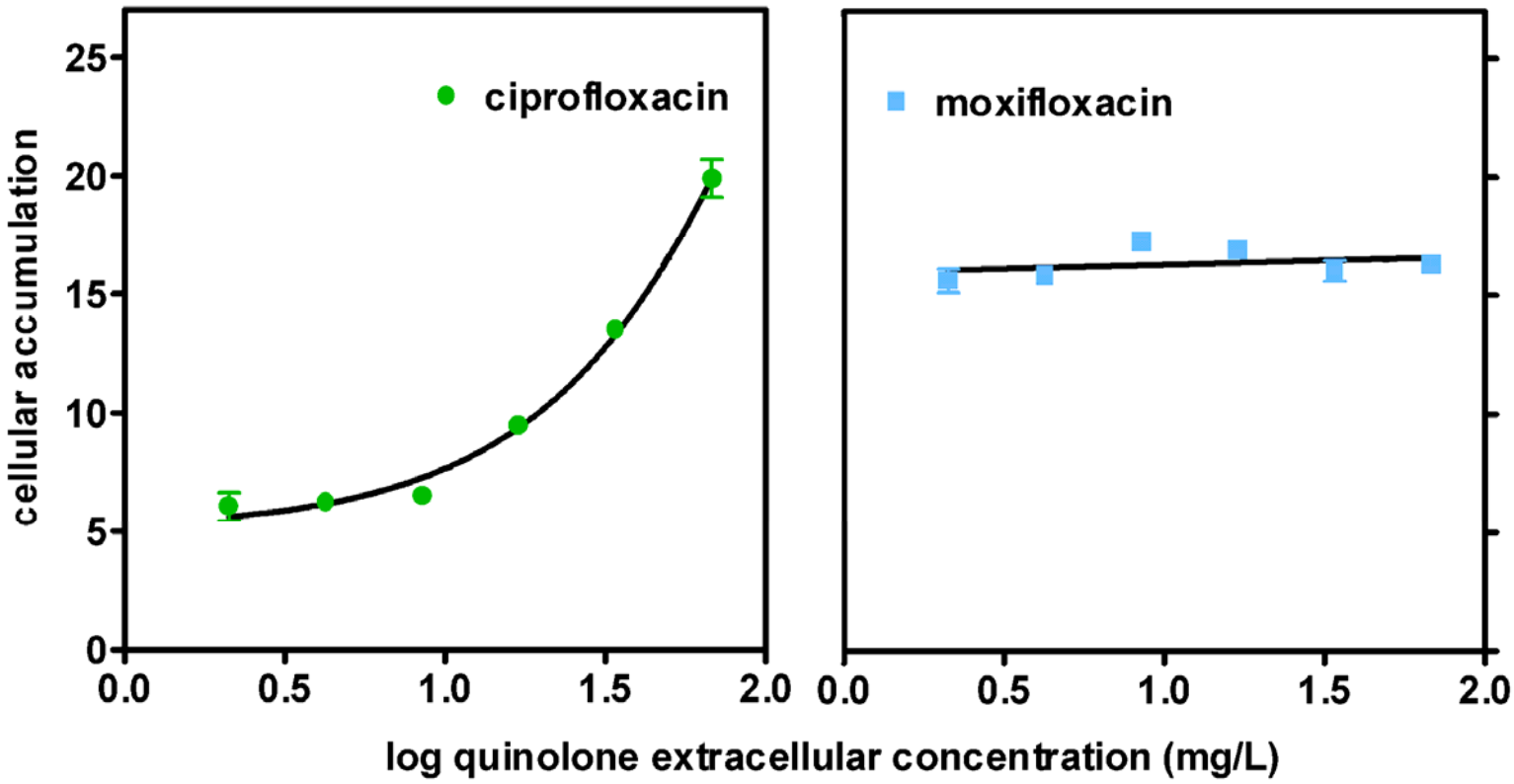




Michot *et al*. AAC (2005) 49:2429-37

Quinolones as inhibitors of ciprofloxacin efflux

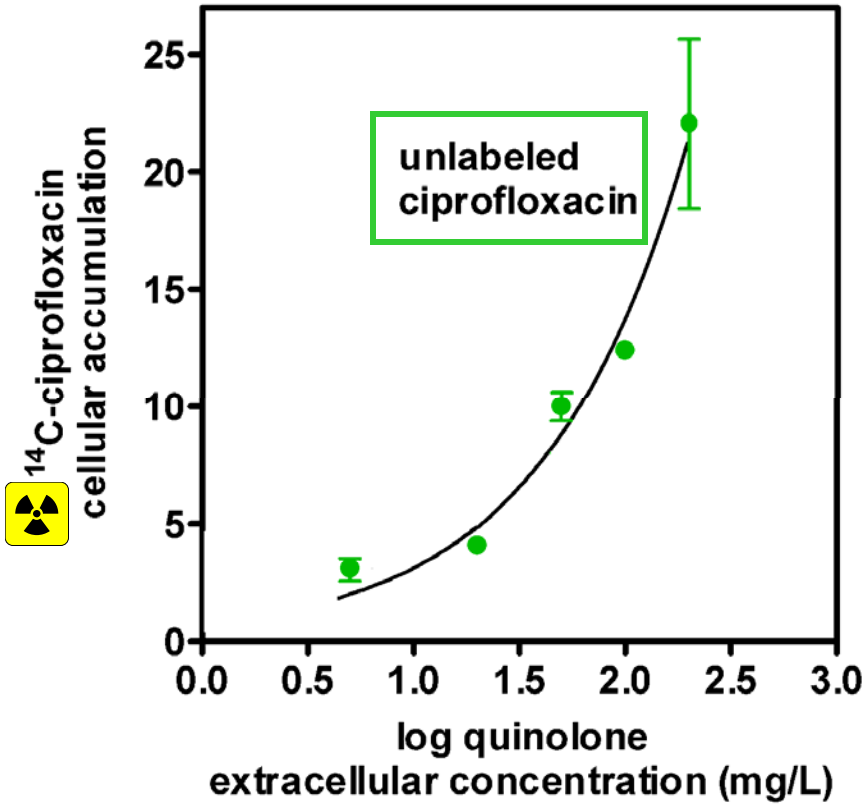
- ciprofloxacin efflux inhibited by ciprofloxacin
- moxifloxacin not affected

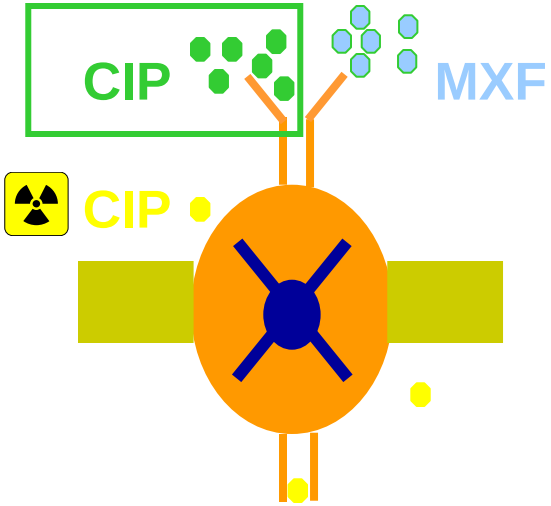


Michot *et al*. AAC (2005) 49:2429-37

Quinolones as inhibitors of ciprofloxacin efflux

- **ciprofloxacin efflux inhibited by ciprofloxacin**
  moxifloxacin

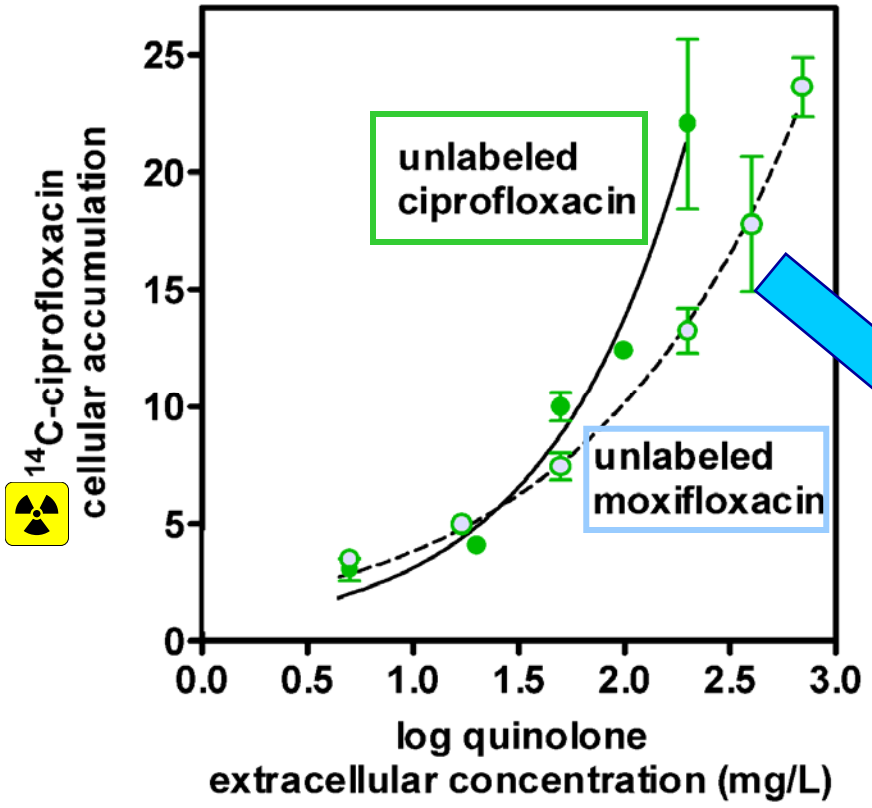


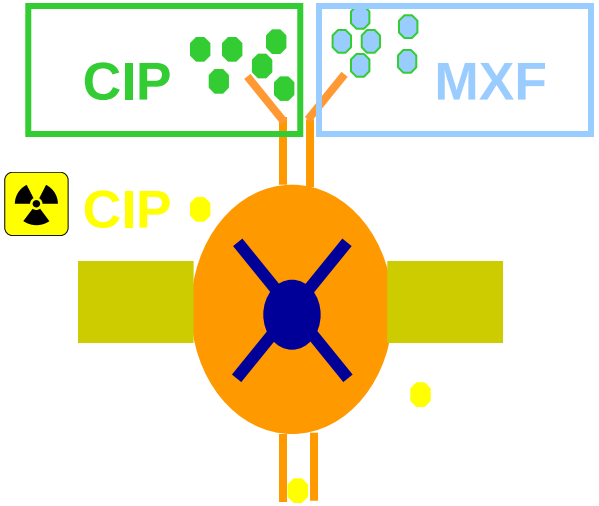


Michot *et al*. AAC (2005) 49:2429-37

Quinolones as inhibitors of ciprofloxacin efflux

- **ciprofloxacin efflux inhibited by ciprofloxacin**
  moxifloxacin



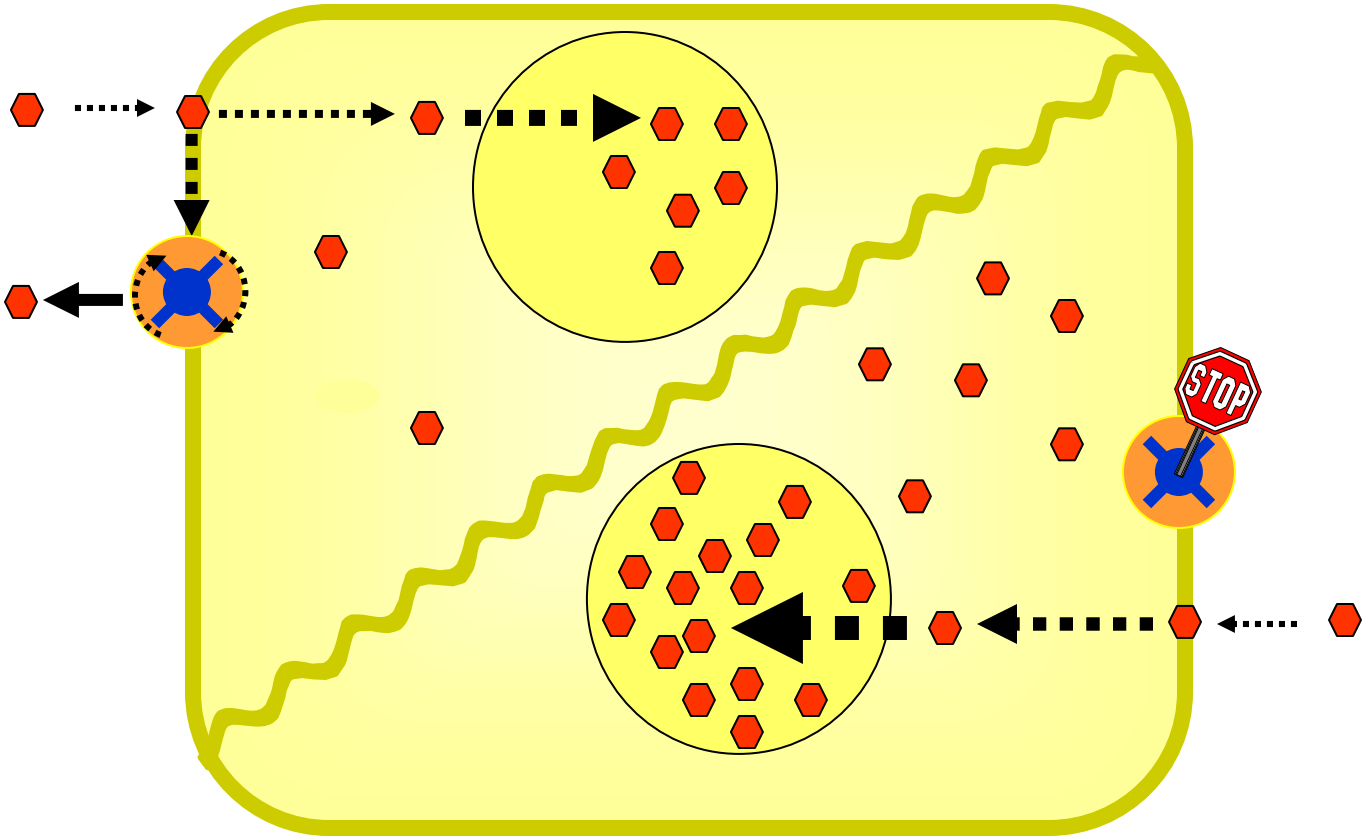


moxifloxacin also able to interact with the transporter !

Michot *et al*. AAC (2005) 49:2429-37

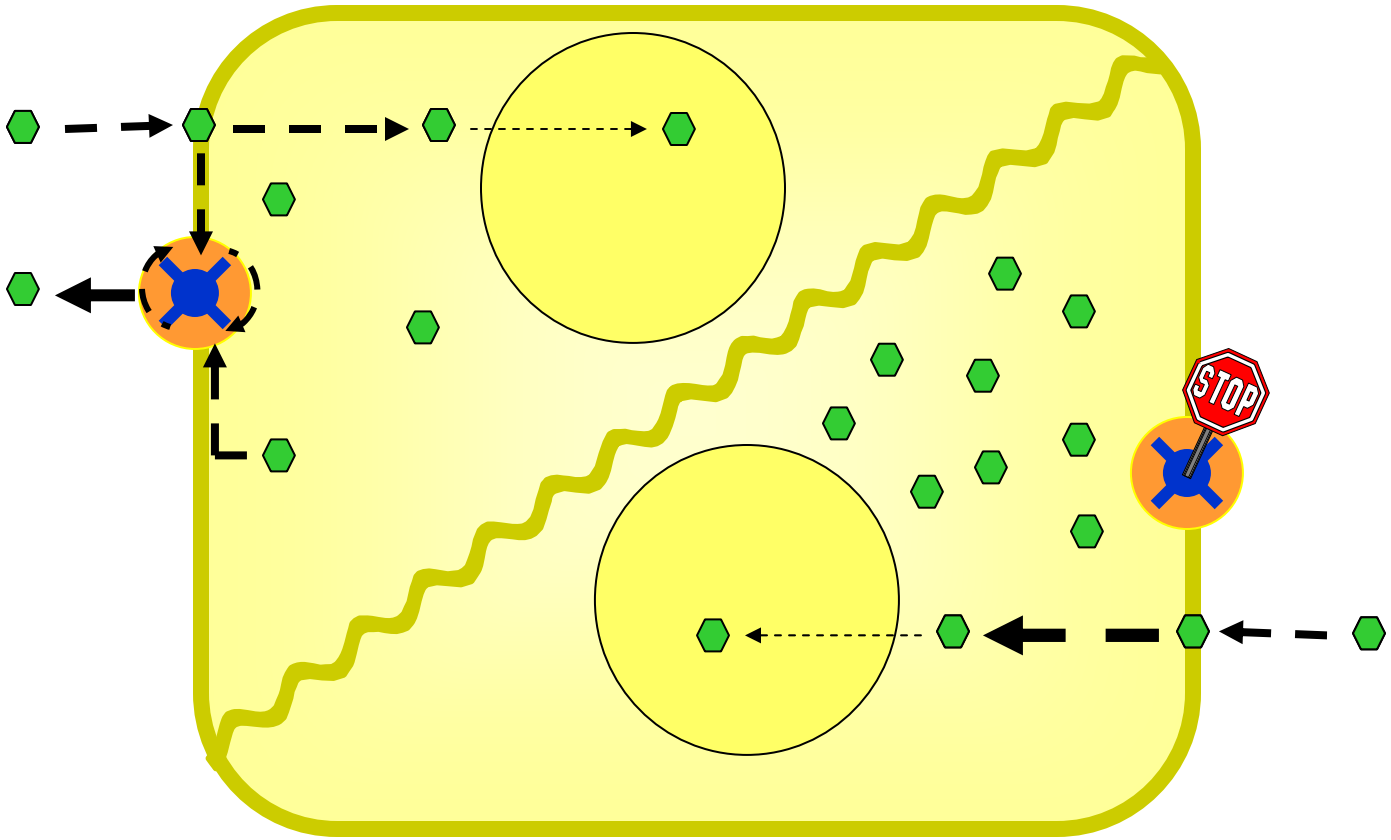
Azithromycin, ‘kick-back’ model

Gaj *et al*. (1998) Biochem. Pharmacol. 55:1199-211



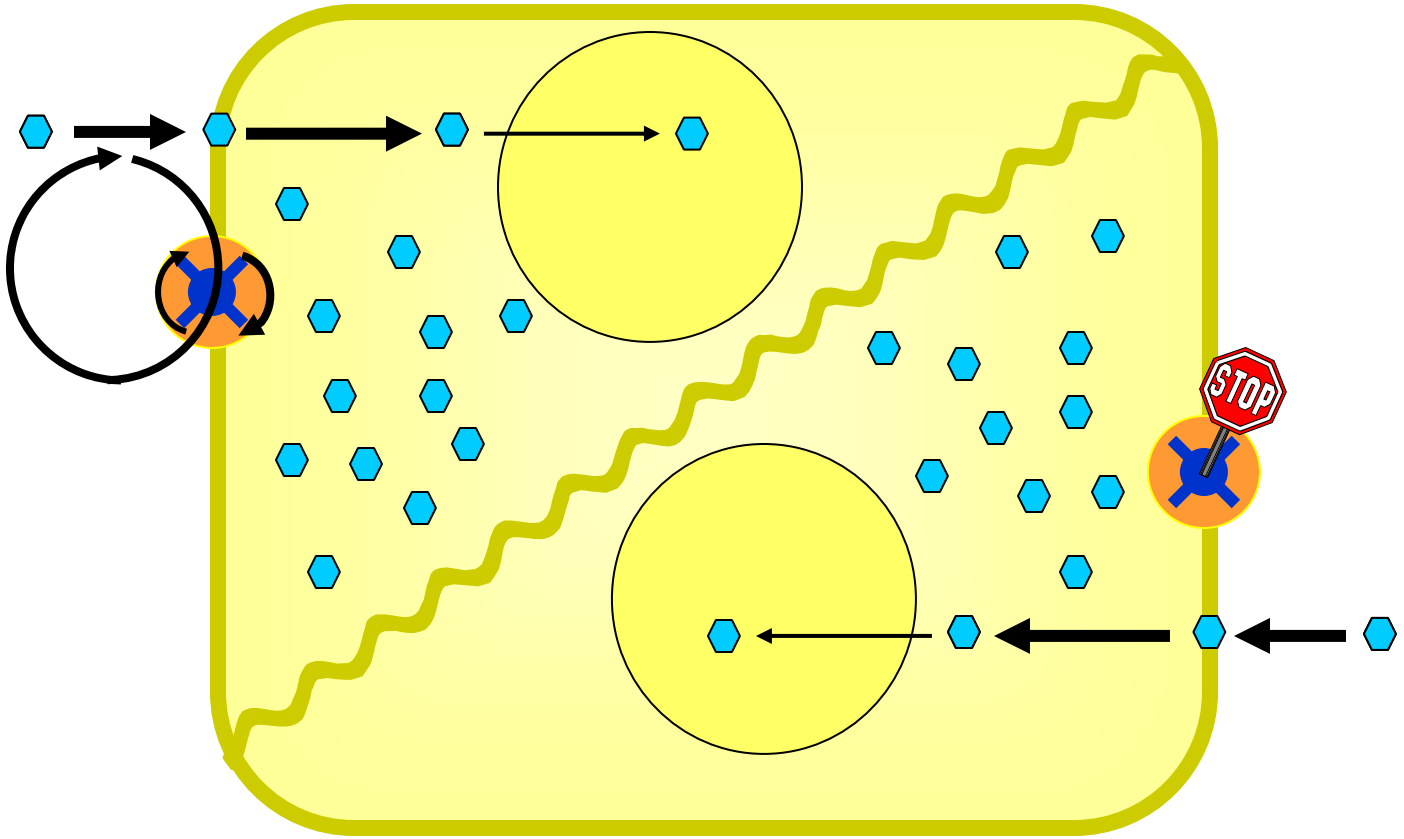
Ciprofloxacin, classical model

Kolaczkowski & Goffeau (1997) Pharmacol. Ther. 76:219-42



Moxifloxacin, ‘futile-cycle’ model

Eytan *et al*. (1996) JBC 271:12897-902



Pharmacological consequences of antibiotic transport

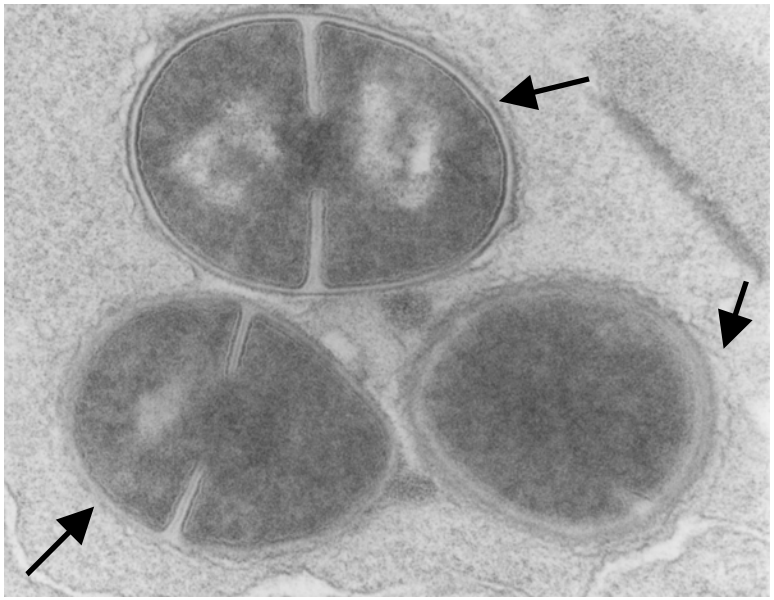
Paul Delvaux (1897-1994)

Models of intracellular infection

L. monocytogenes

cytosol

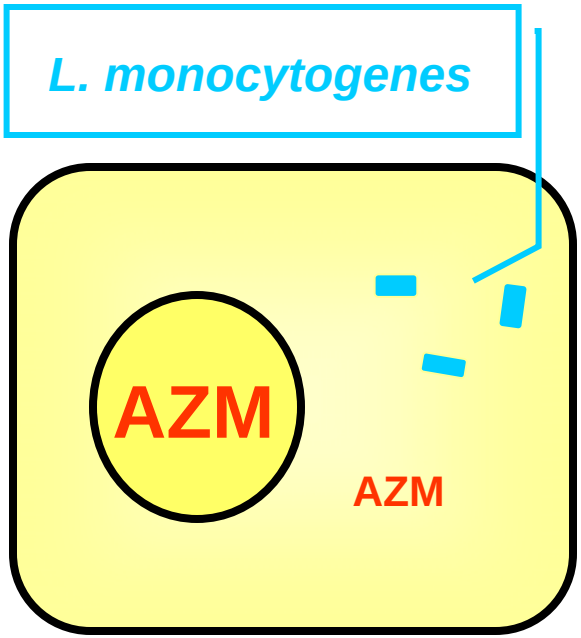
S. aureus

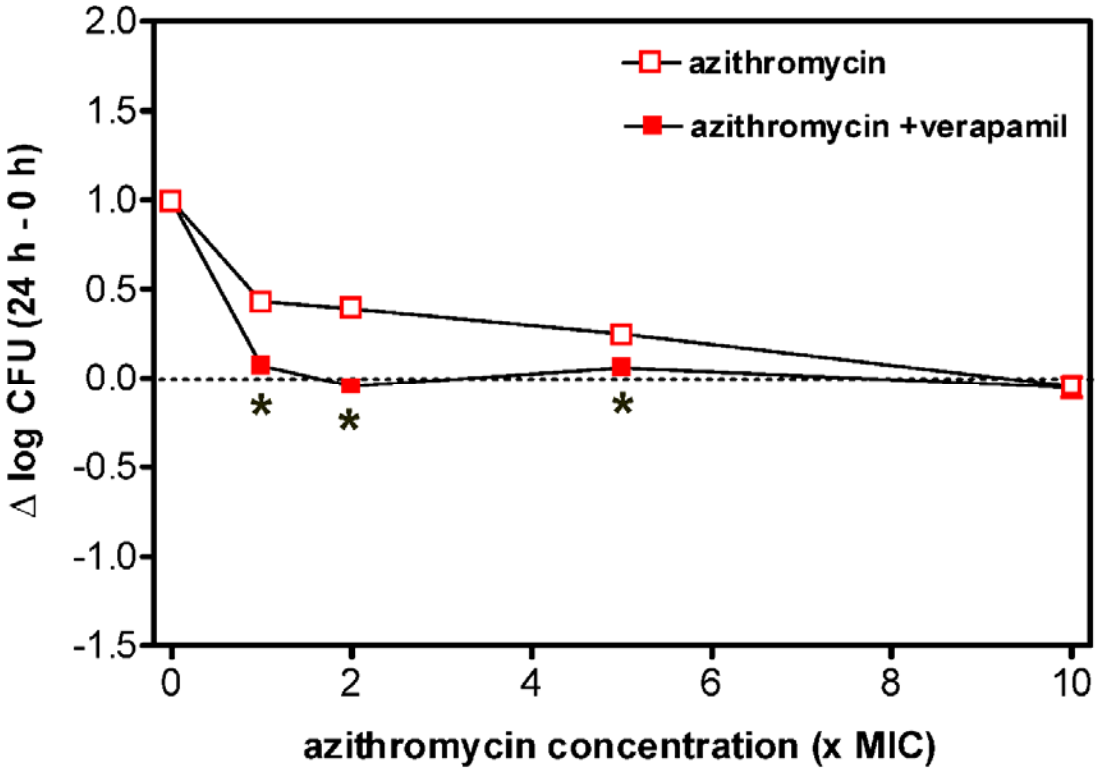


phagolysosomes

Influence of pump inhibitors on intracellular activity

azithromycin and *L. monocytogenes*



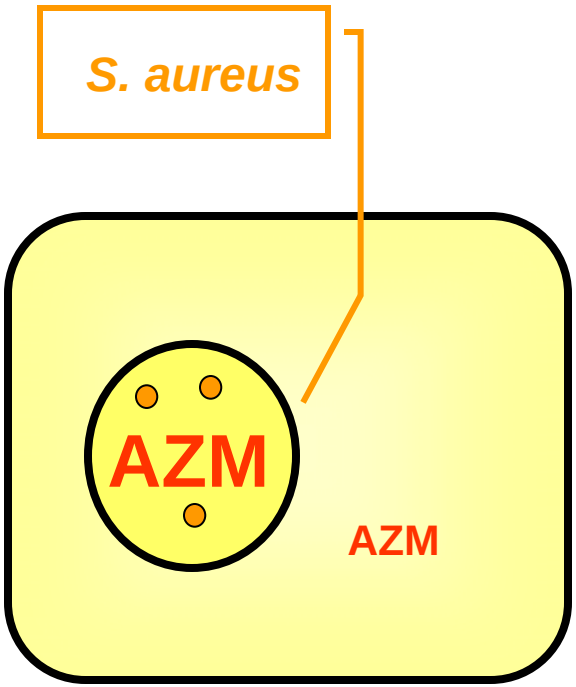


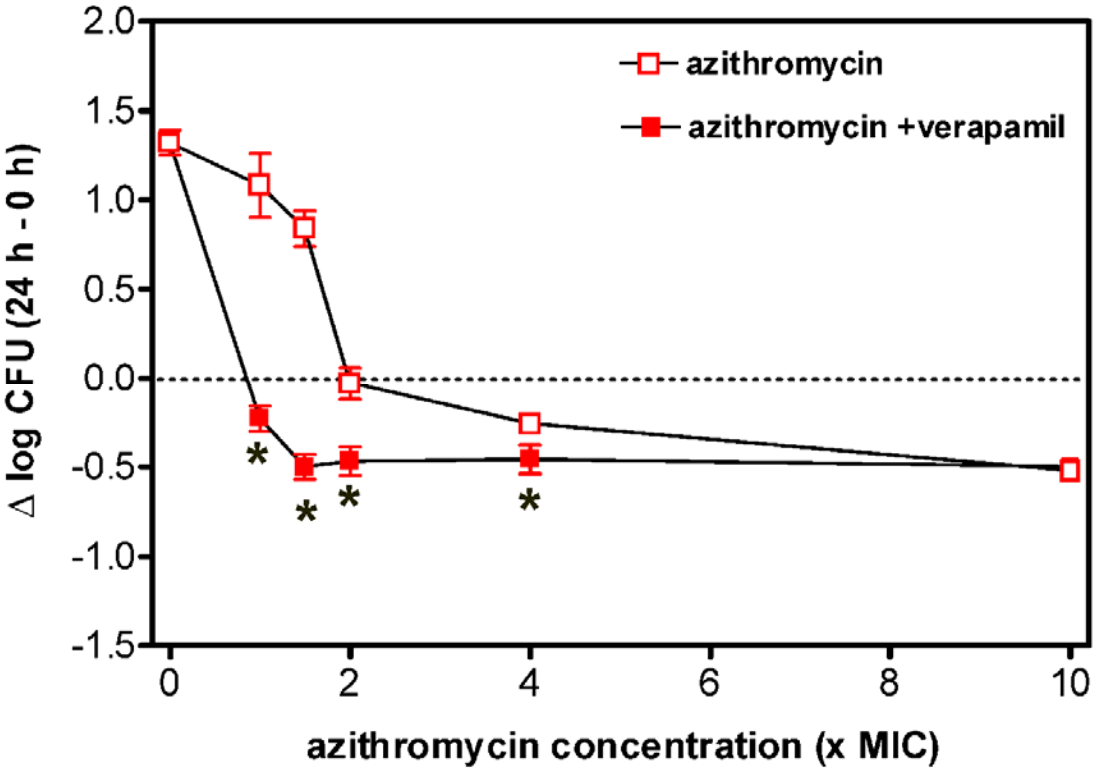
verapamil 20 µM; 24 h

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

Influence of pump inhibitors on intracellular activity

azithromycin and *S. aureus*



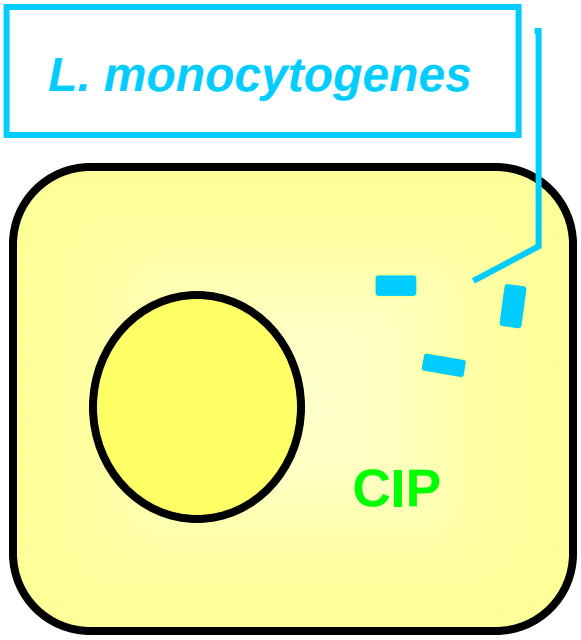


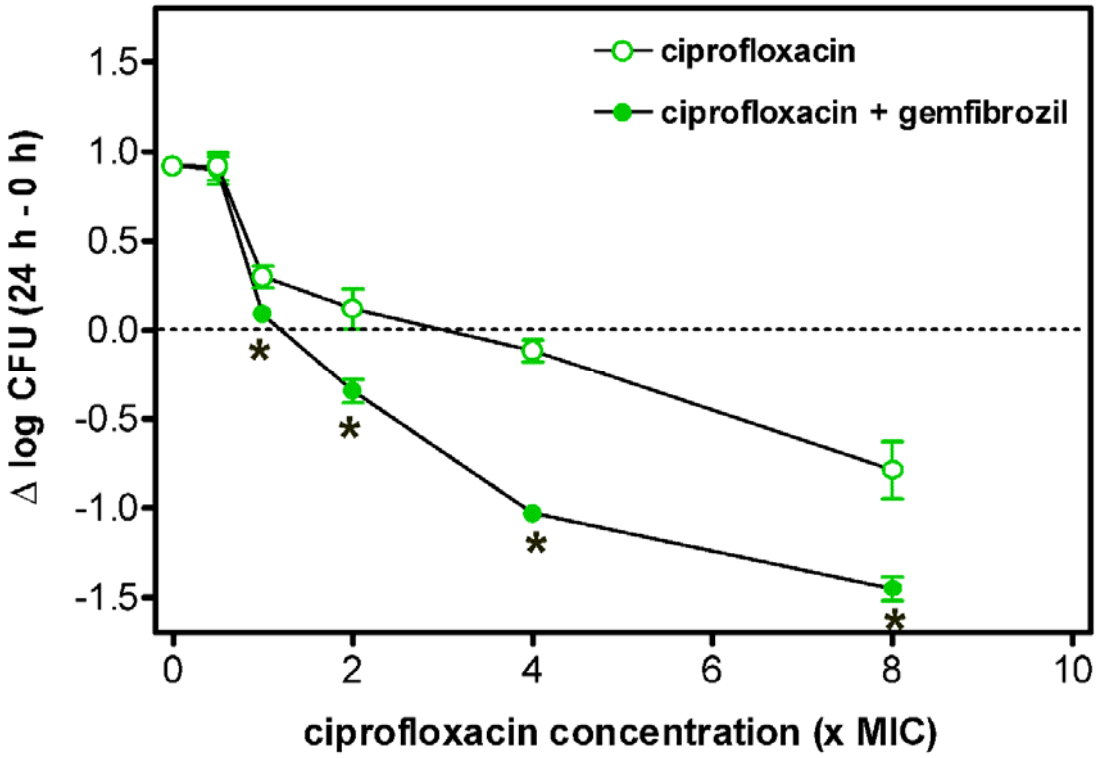
verapamil 20 µM; 24 h

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

Influence of pump inhibitors on intracellular activity

ciprofloxacin and *L. monocytogenes*



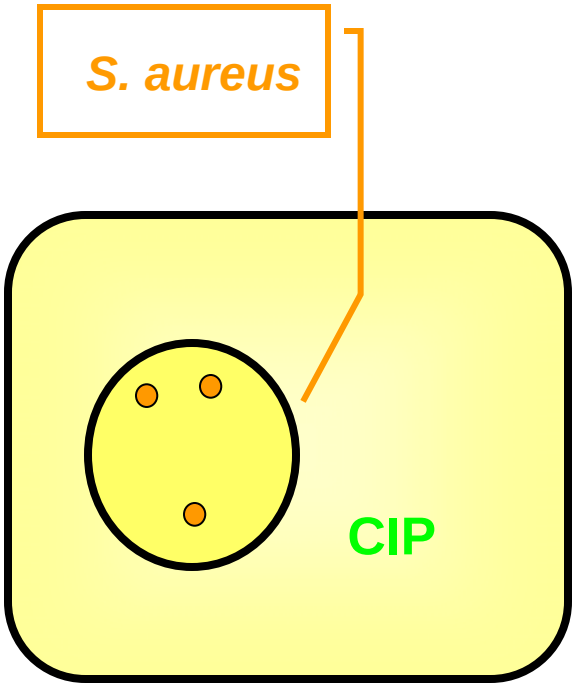


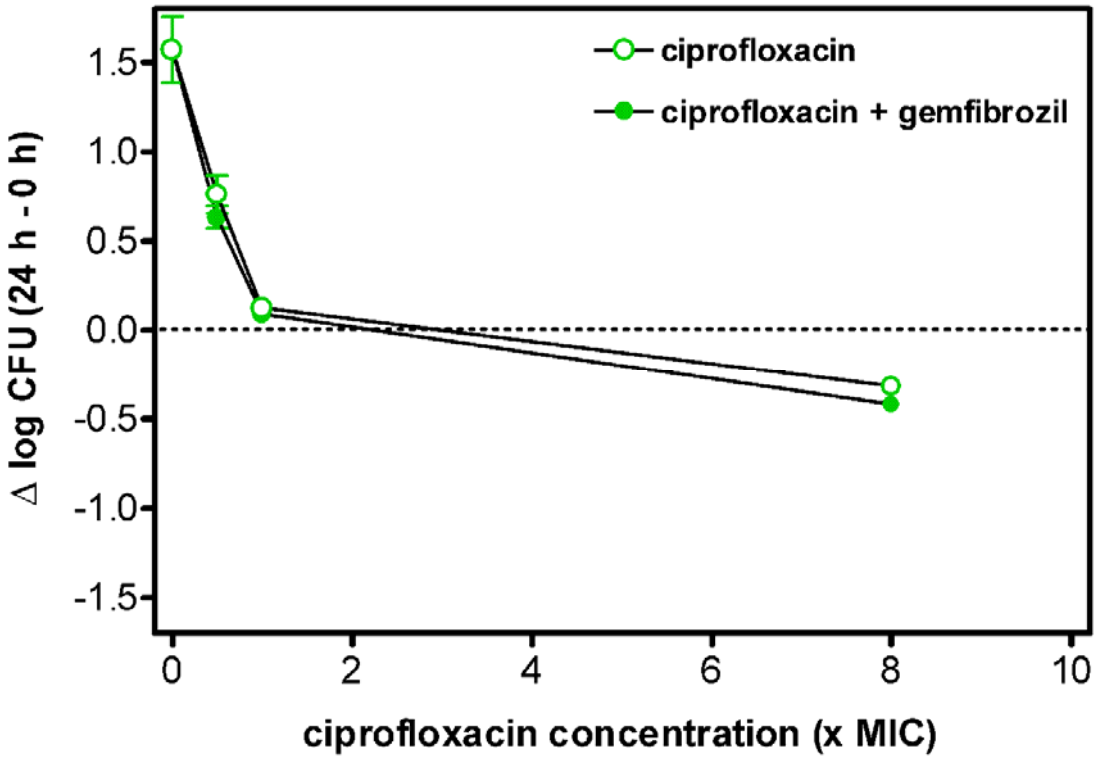
gemfibrozil 250 µM; 24 h

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

Influence of pump inhibitors on intracellular activity

ciprofloxacin and *S. aureus*



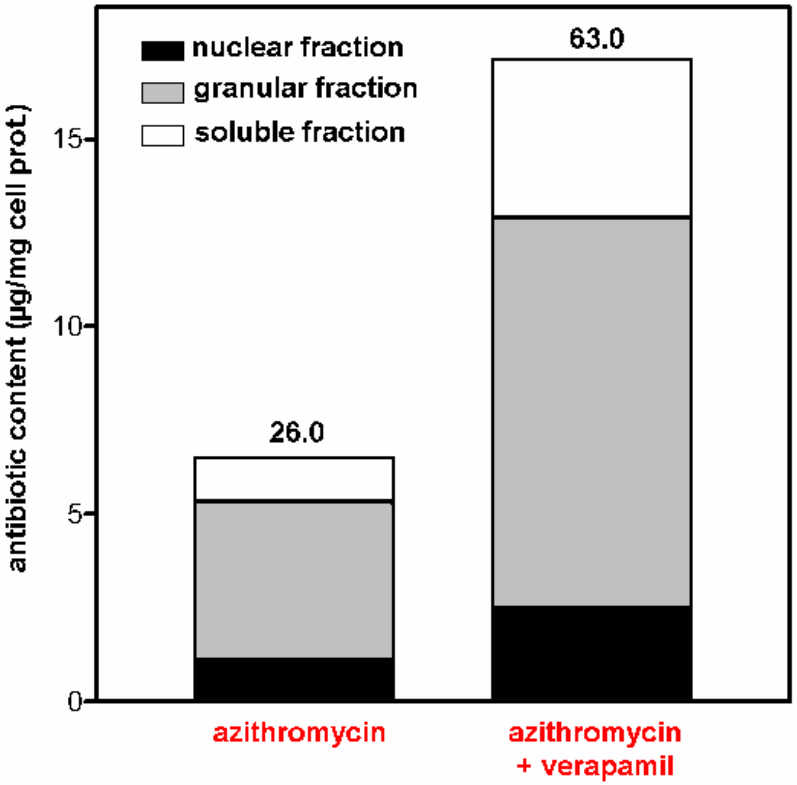


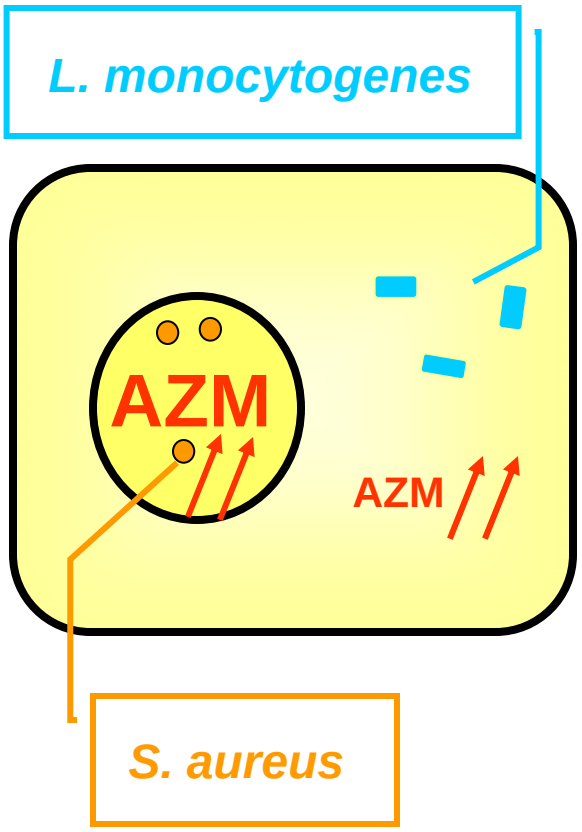
gemfibrozil 250 µM; 24 h

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

Influence of pump inhibitors on antibiotic distribution

verapamil enhances azithromycin concentration In cytosol and vacuoles

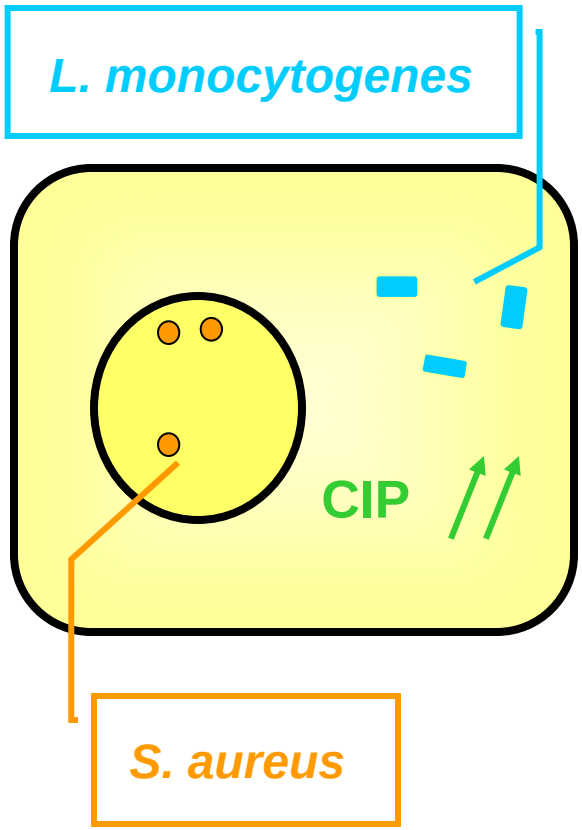


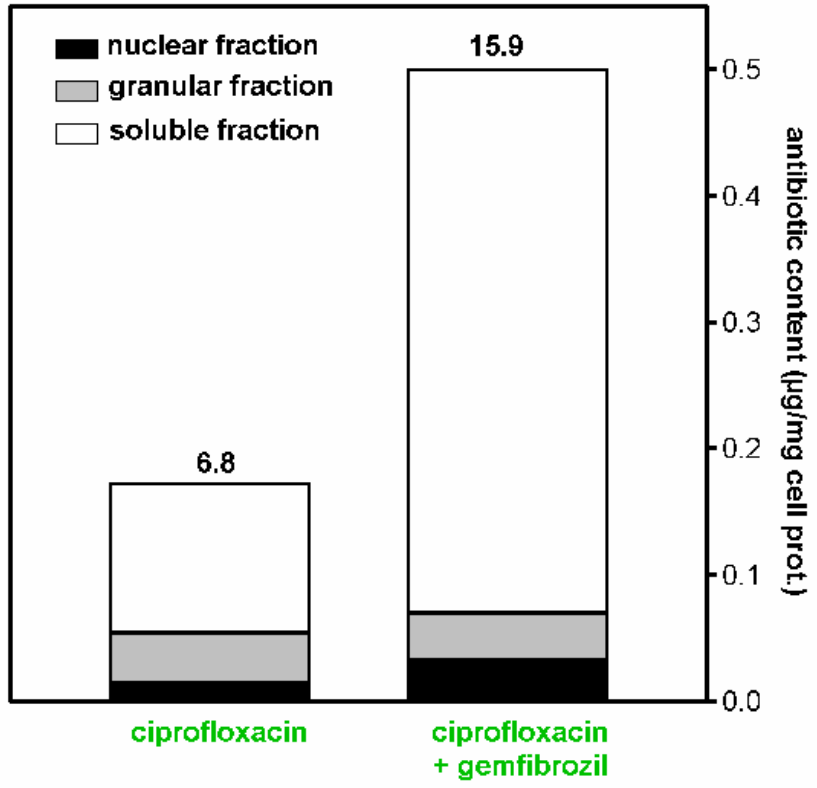


Seral *et al.* (2003) JAC 51:1167-73

Influence of pump inhibitors on antibiotic distribution

gemfibrozil enhances ciprofloxacin cytosolic content





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

Unexpected antibiotic substrates

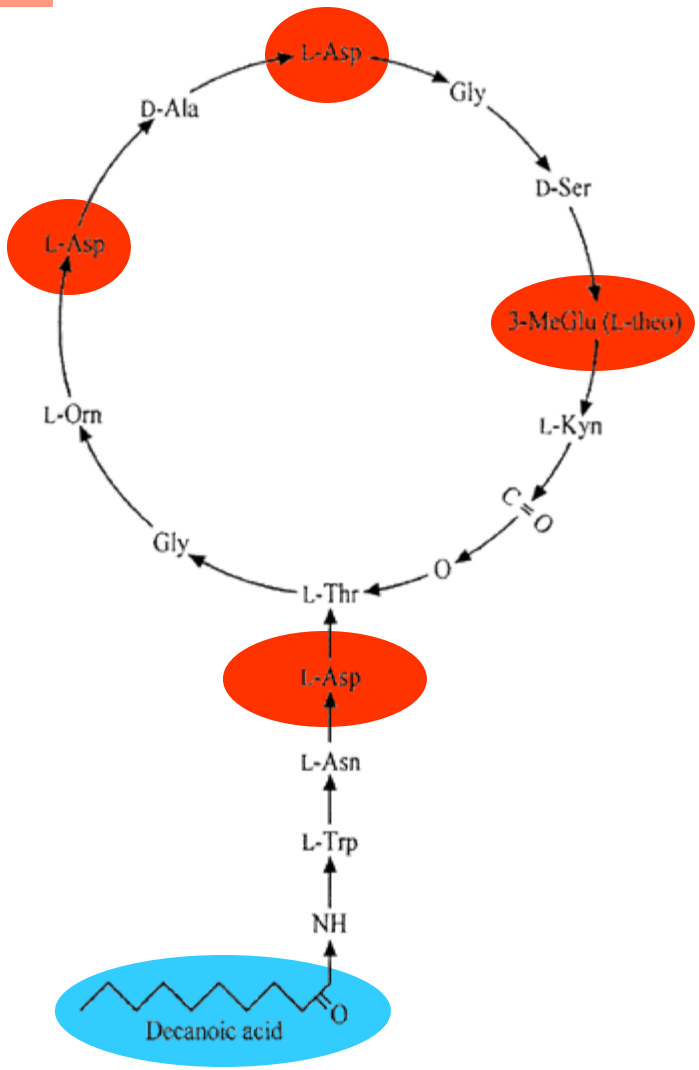
Peyo (1928-1992)

Unexpected substrate

daptomycin

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

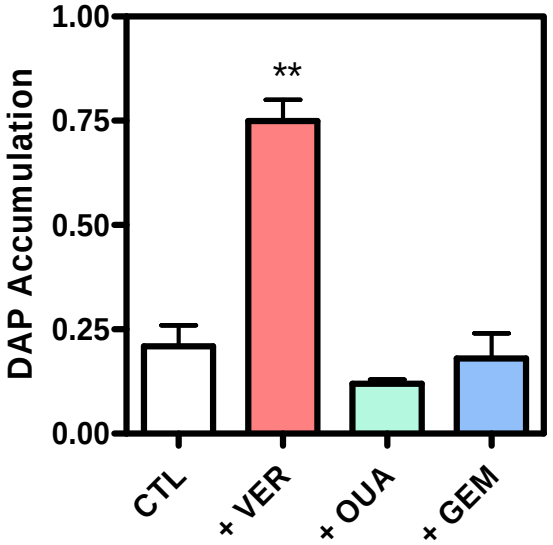
Highly polar molecule !



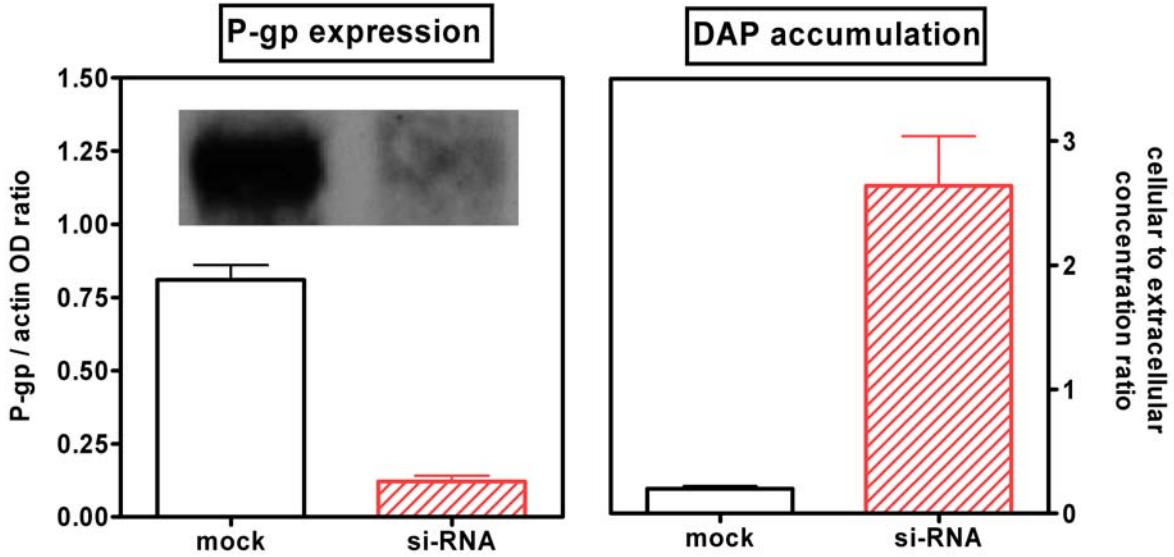
polyanionic
cyclic peptide

lipophilic chain

Daptomycin is substrate of P-gp



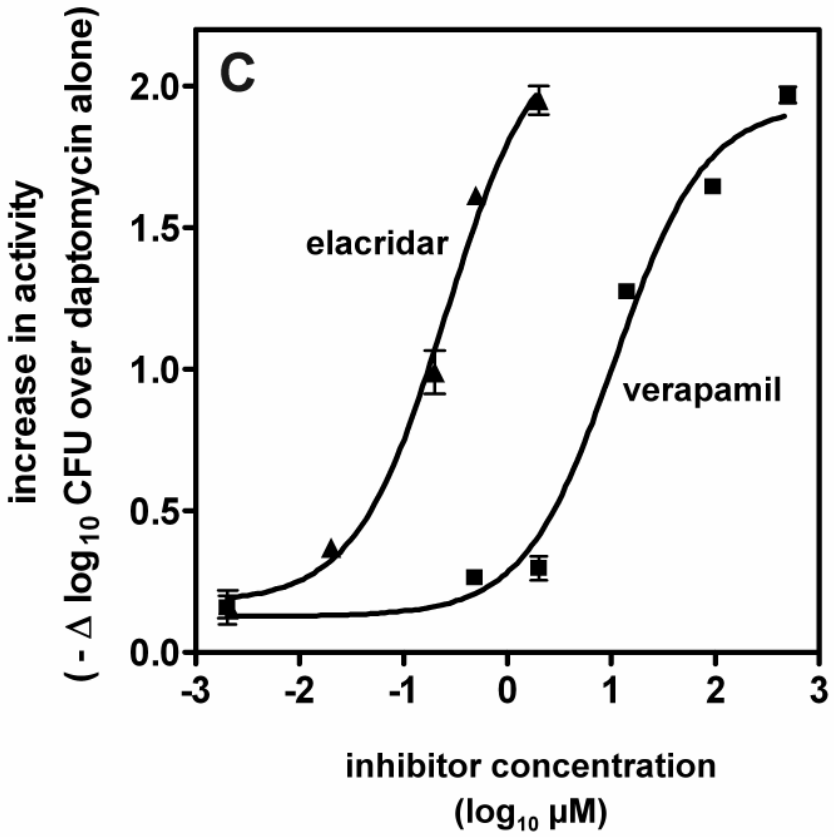
Daptomycin accumulation proportional to P-gp activity and expression level



Lemaire *et al.* (2007) AAC - Epub

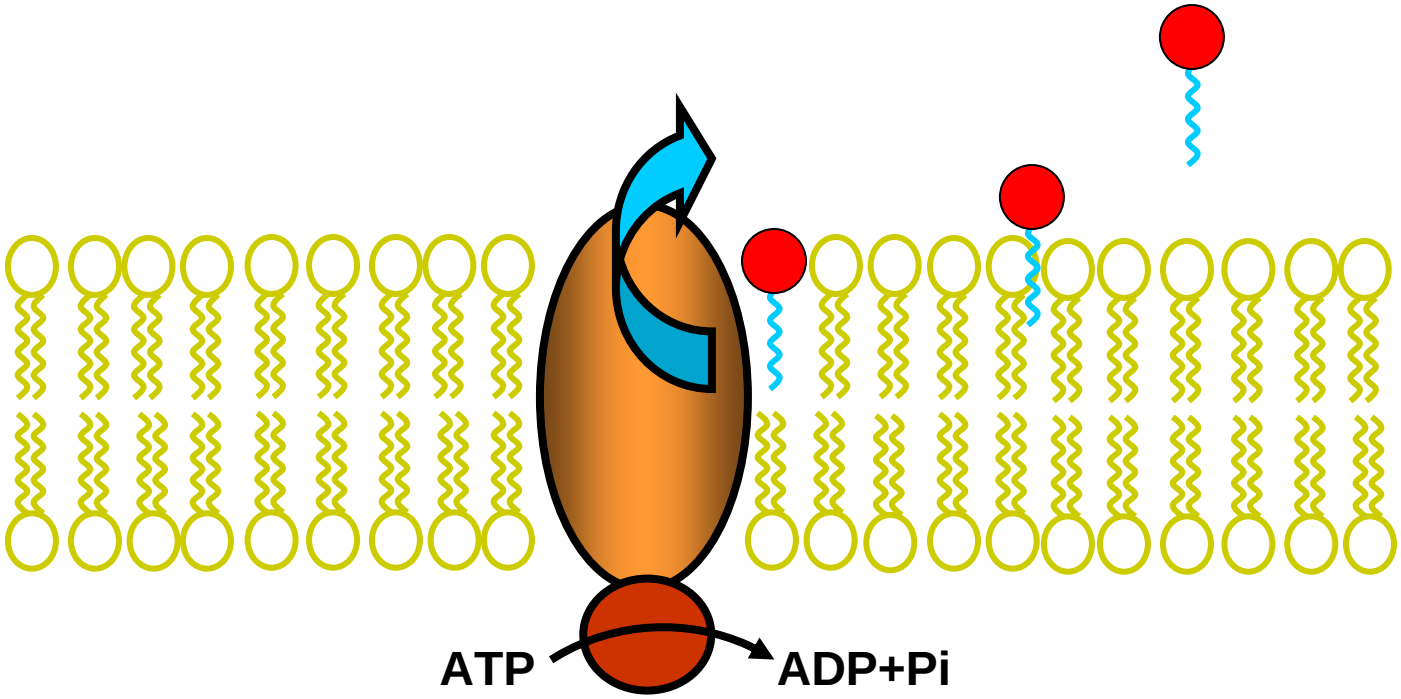
Daptomycin is substrate of P-gp

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



Lemaire *et al.* (2007) AAC - Epub

Putative mechanism of daptomycin transport by P-gp



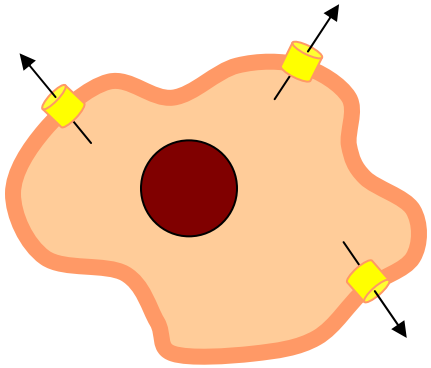
anchoring in the membrane towards the hydrophobic chain
and extrusion from the membrane

Lemaire *et al.* (2007) AAC - Epub

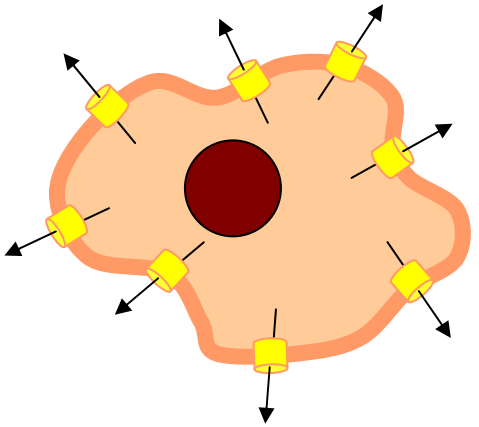
Can we make eukaryotic cells resistant to antibiotics ?

Victor Horta (1861-1947)

Can we make eukaryotic cells resistant to antibiotics ?

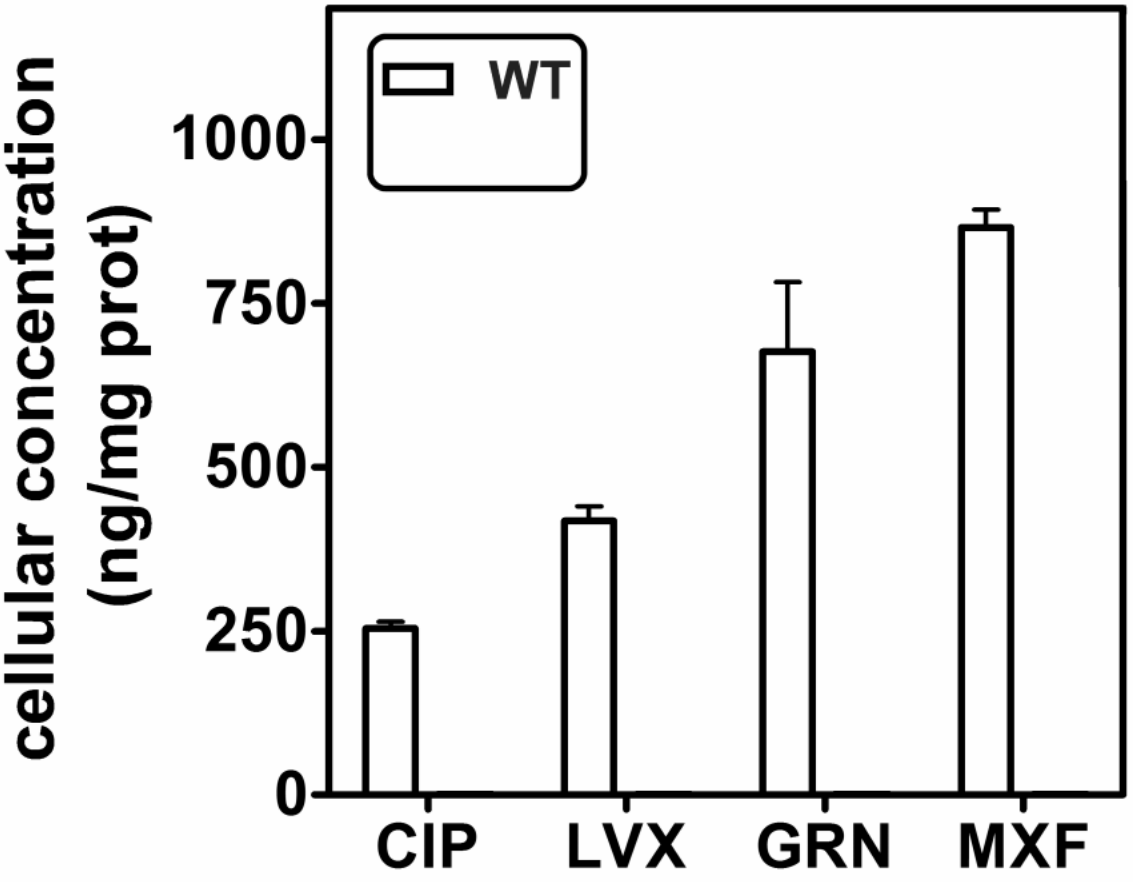


Chronical exposure of J774 macrophages
to increasing concentrations of ciprofloxacin



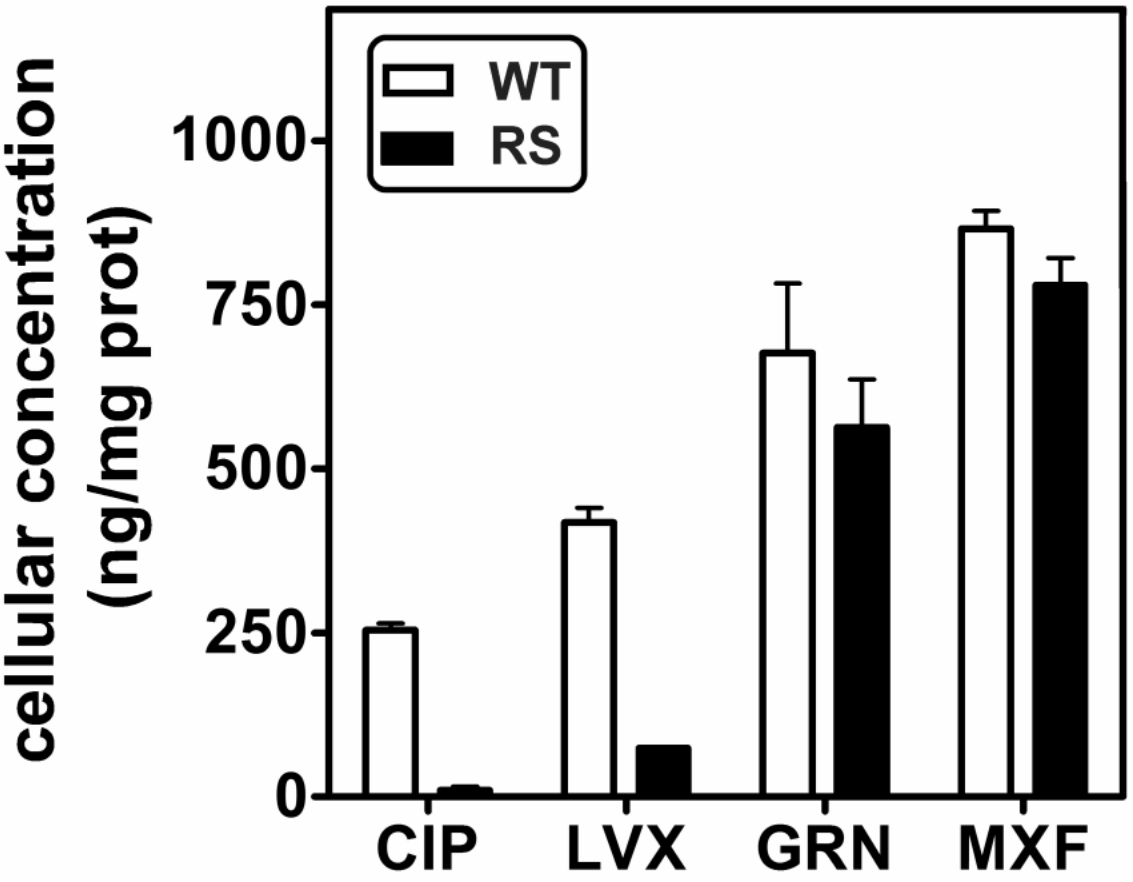
Michot *et al.* (2006) AAC 50:1689-95

Reduced drug accumulation in resistant macrophages



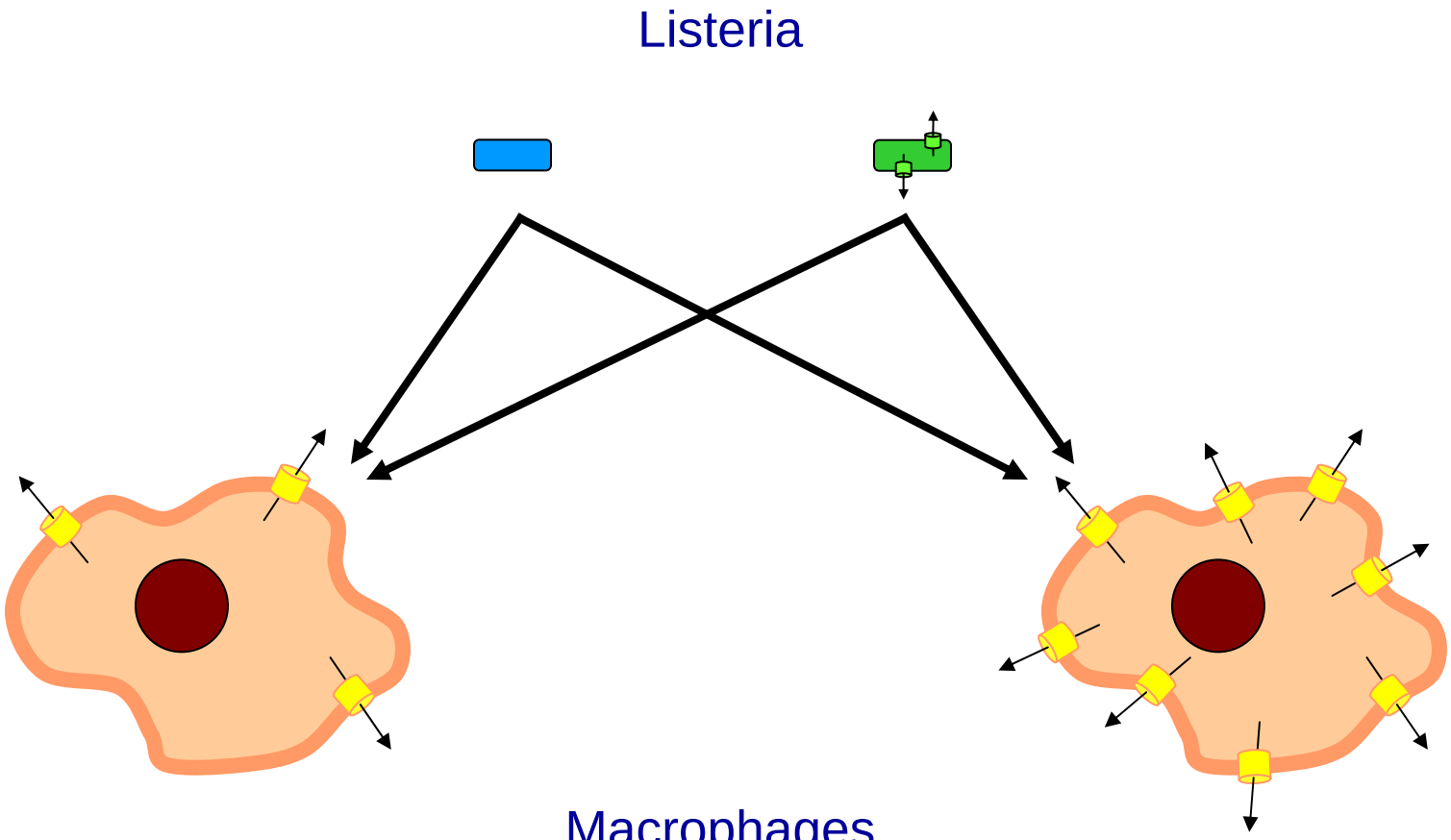
Michot *et al.* (2006) AAC 50:1689-95

Reduced drug accumulation in resistant macrophages



Michot *et al.* (2006) AAC 50:1689-95

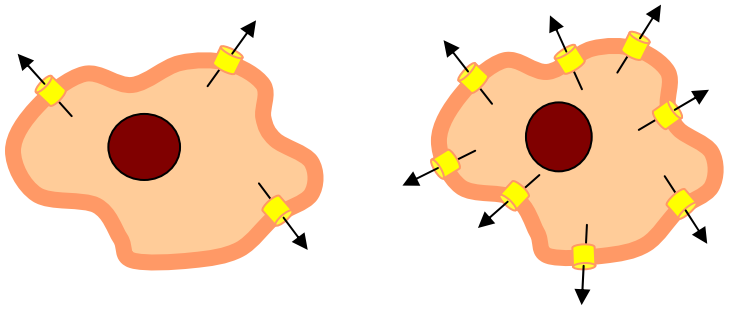
Coworking between bacteria and macrophage pumps to reduce ciprofloxacin activity



Coworking between bacteria and macrophage pumps to reduce ciprofloxacin activity

Listeria

Macrophages



FQ	MIC (mg/L)			
	EGD		CLIP	
	Res. (-)	Res. (+)	Res. (-)	Res. (+)
CIP	**1.2**	**1.0**	**5.0**	**1.0**
MXF	**0.6**	**0.6**	**0.5**	**0.25**

CIP R; MXF S

FQ	Cellular concentration (ng/mg prot)			
	WT		RS	
	Prob. (-)	Prob. (+)	Prob. (-)	Prob. (+)
CIP	**72**	**263**	**23**	**159**
MXF	**262**	**208**	**241**	**257**

CIP R; MXF S

Lismond *et al.* (2006) ICAAC-A1108

Coworking between bacteria and macrophage pumps to reduce ciprofloxacin activity

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

FQ	MIC (mg/L)			
	EGD		CLIP	
	Res. (-)	Res. (+)	Res. (-)	Res. (+)
CIP	**1.2**	**1.0**	**5.0**	**1.0**
MXF	**0.6**	**0.6**	**0.5**	**0.25**

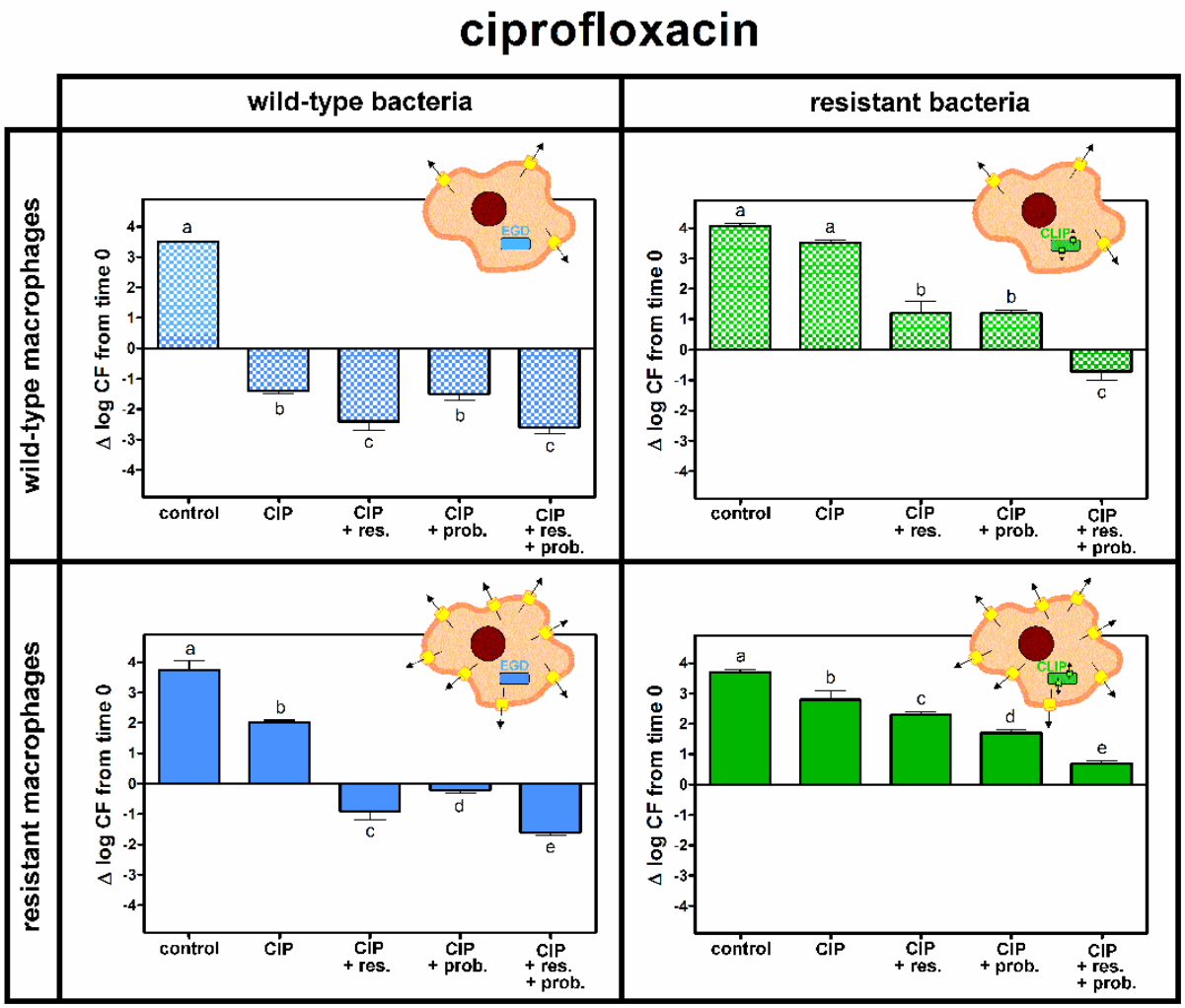
CIP R; MXF S

FQ	Cellular concentration (ng/mg prot)			
	WT		RS	
	Prob. (-)	Prob. (+)	Prob. (-)	Prob. (+)
CIP	**72**	**263**	**23**	**159**
MXF	**262**	**208**	**241**	**257**

CIP R; MXF S

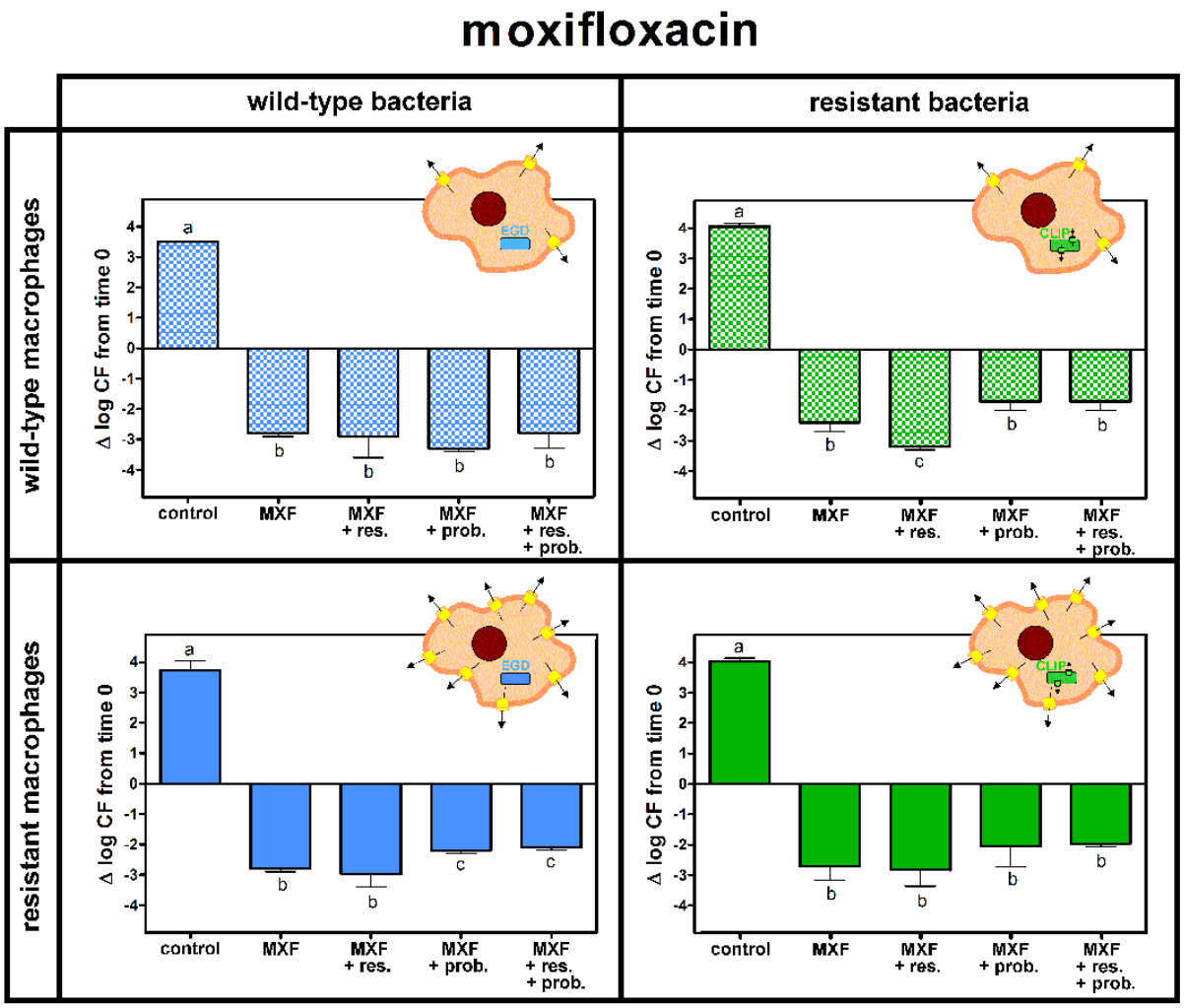
Lismond *et al.* (2006) ICAAC-A1108

Coworking between bacteria and macrophage pumps to reduce ciprofloxacin activity



Lismond *et al.* (2006) ICAAC-A1108

No effect of bacteria and macrophage pumps on moxifloxacin activity



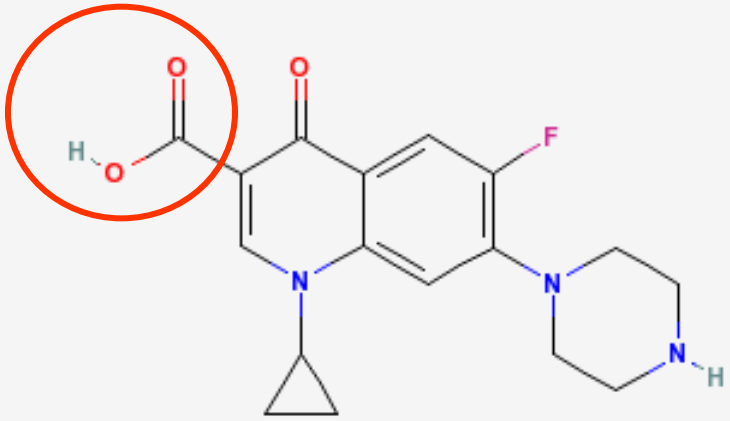
Lismond *et al.* (2006) ICAAC-A1108

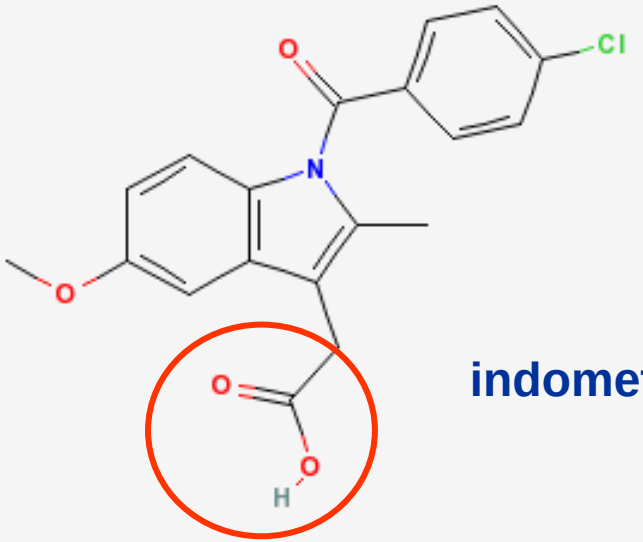
Competition for transporters as a mechanism of drug interaction

Pieter Breughel (1525-1569)

Ciprofloxacin versus NSAIDs

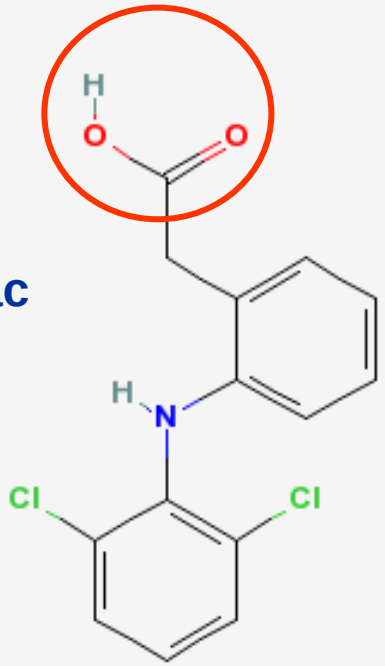
ciprofloxacin



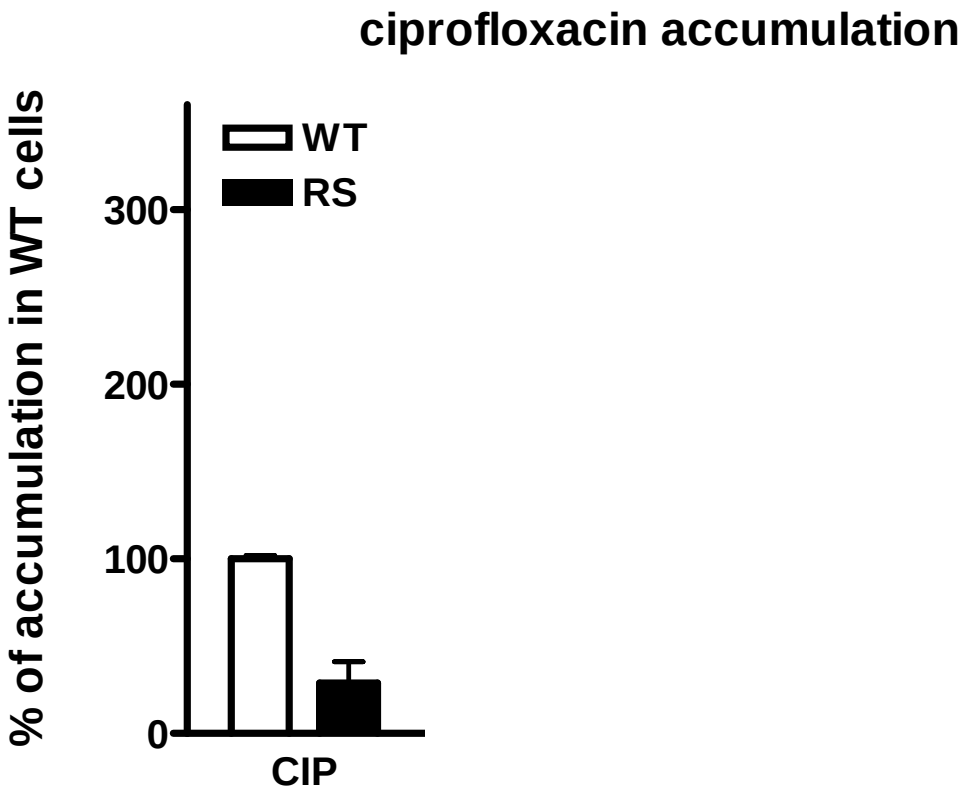


indomethacin

diclofenac

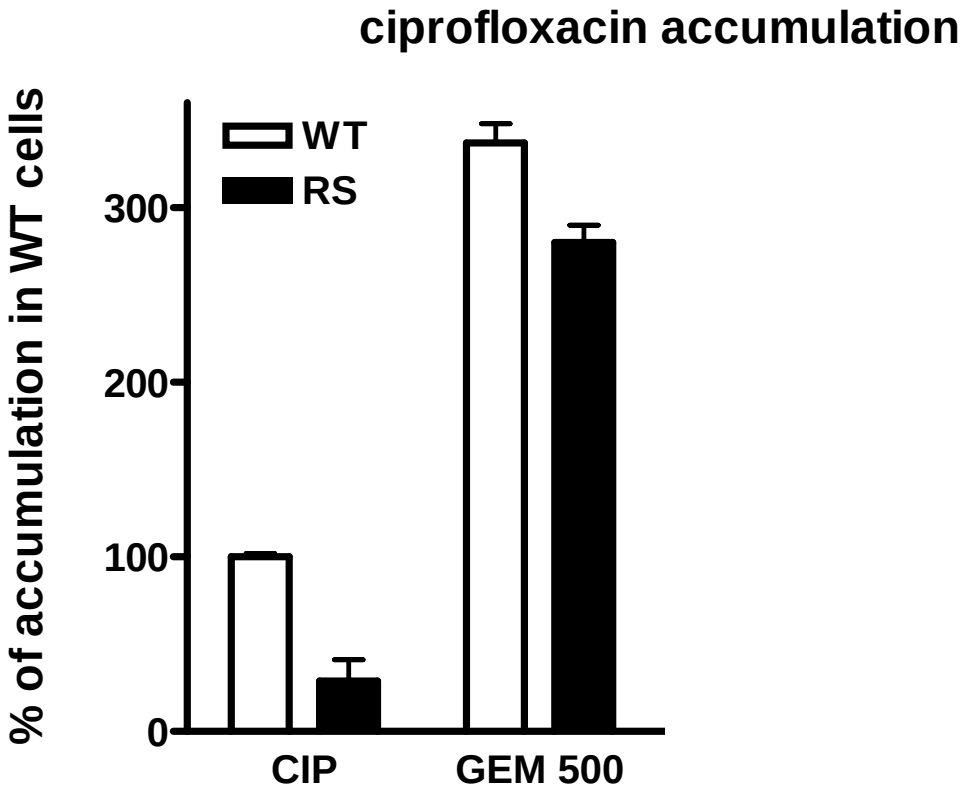


Ciprofloxacin versus NSAIDs



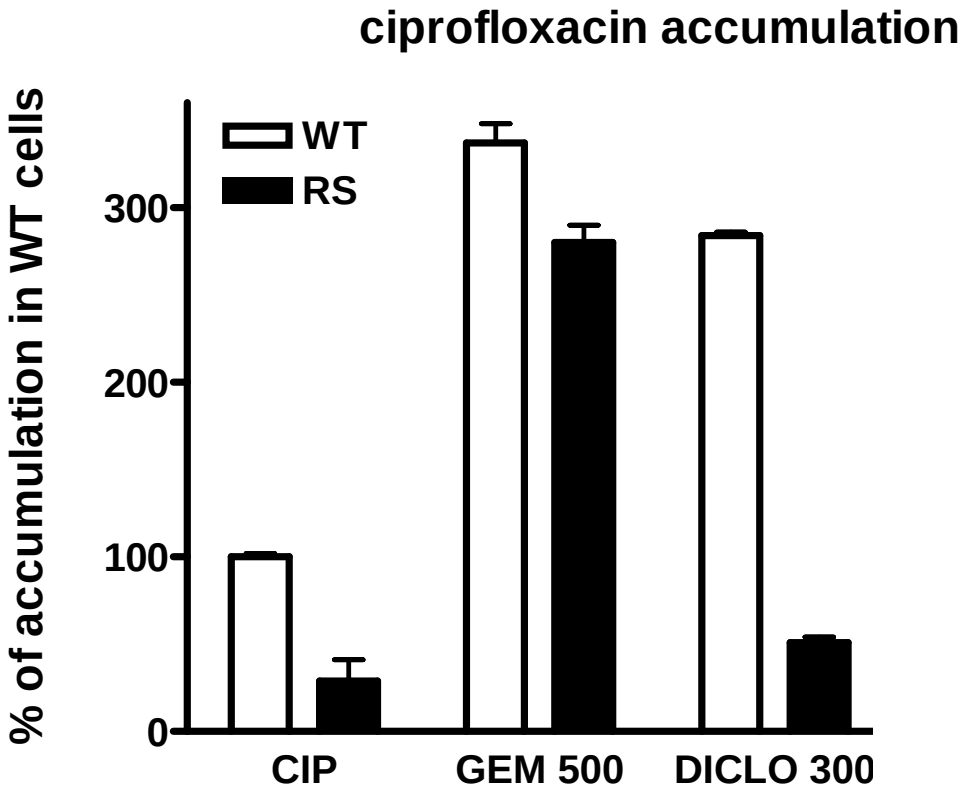
Huygue *et al.* Unpublished data

Ciprofloxacin versus NSAIDs



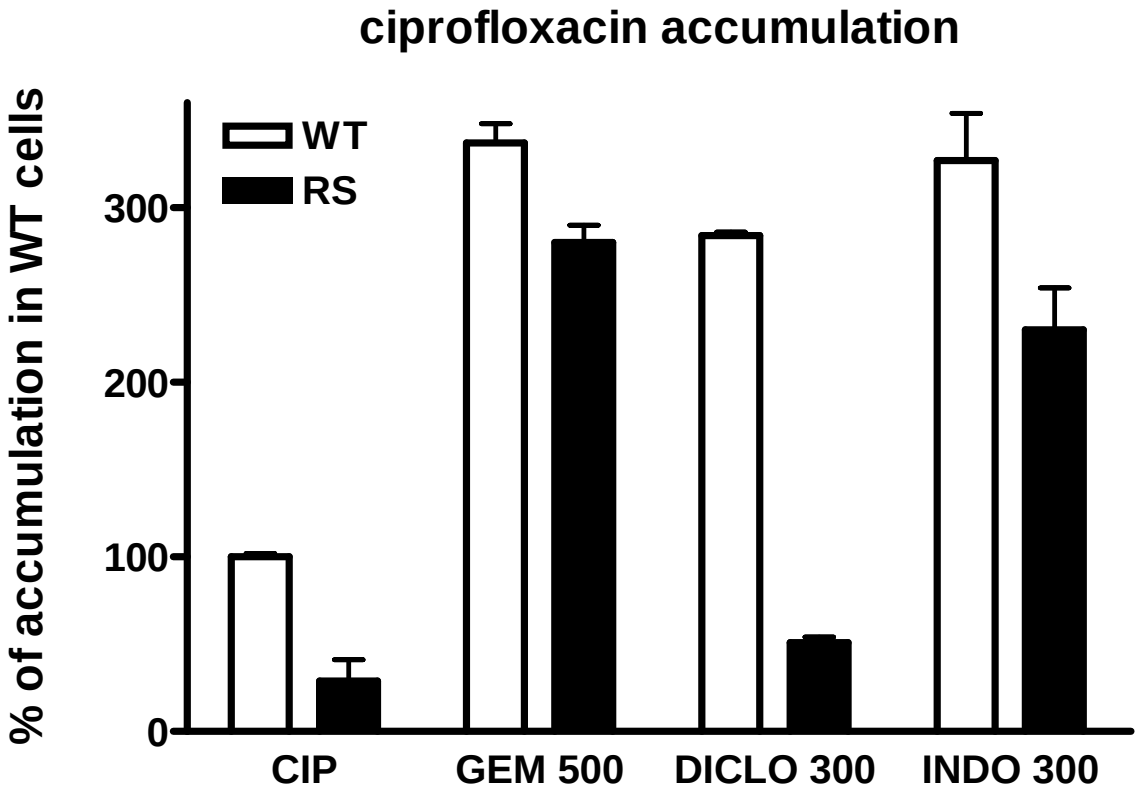
Huygue *et al.* Unpublished data

Ciprofloxacin versus NSAIDs



Huygue *et al.* Unpublished data

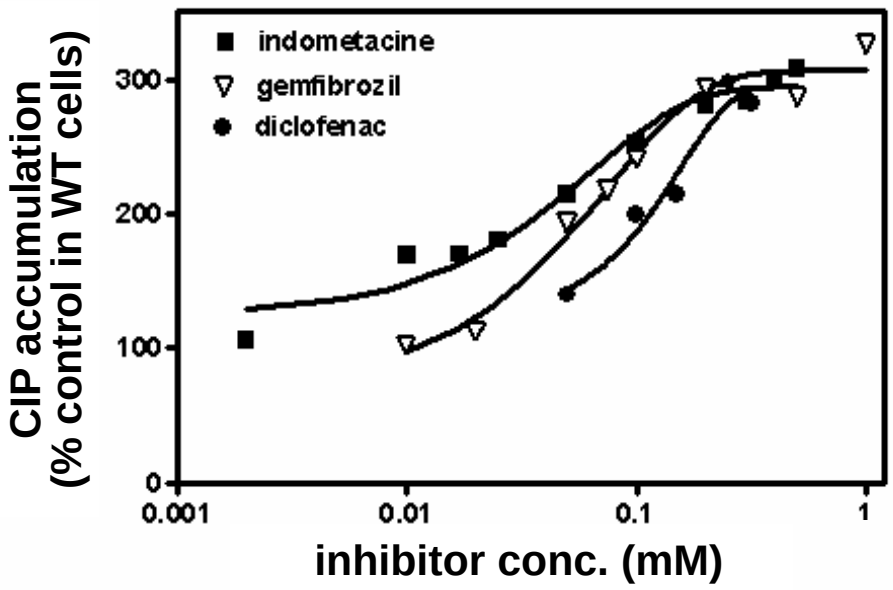
Ciprofloxacin versus NSAIDs



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

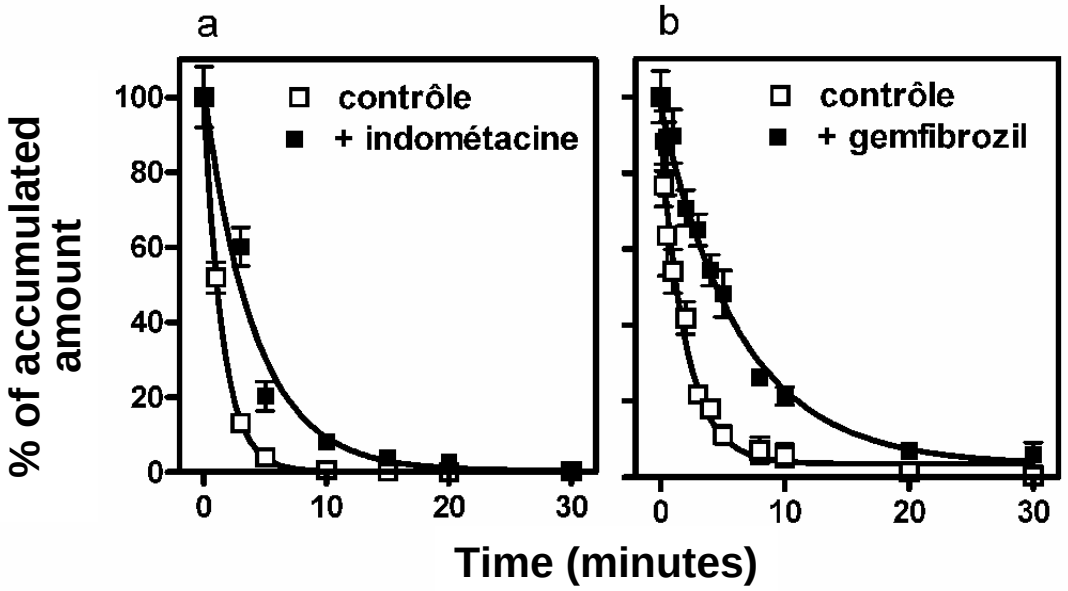
Huygue *et al.* Unpublished data

NSAIDs as inhibitors of ciprofloxacin efflux



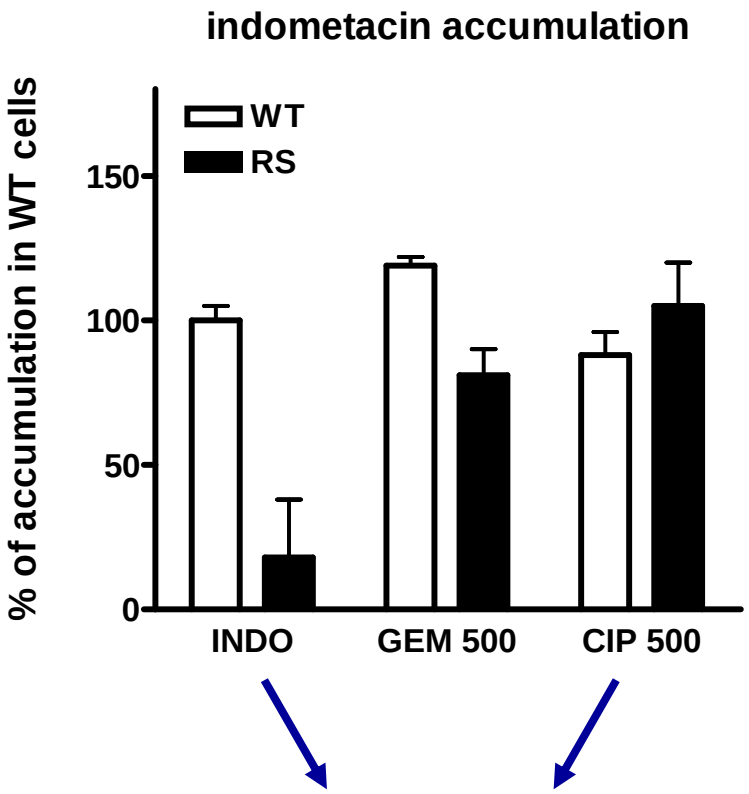
Dose-dependent effect on accumulation

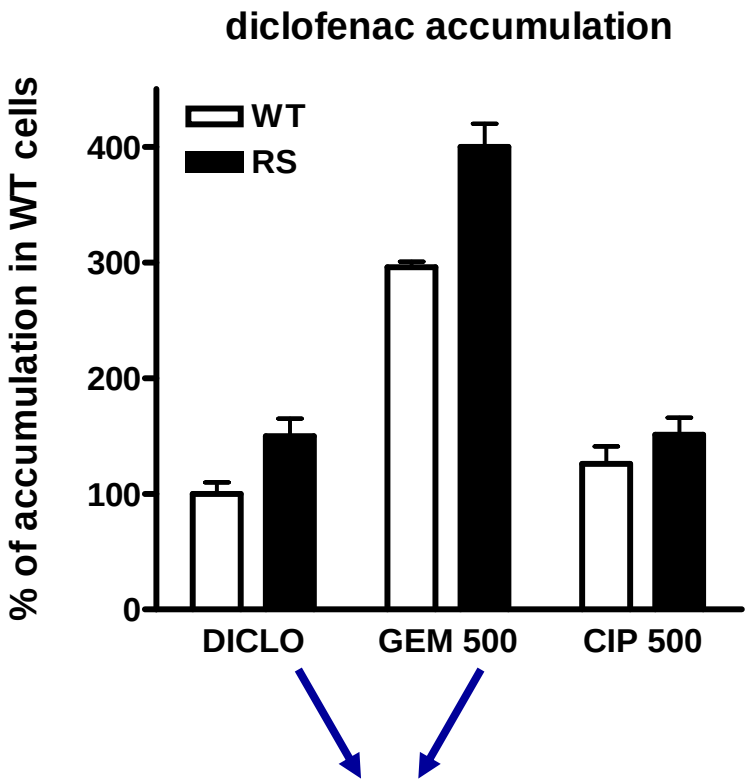
related to slowed-down efflux



Huygue *et al.* Unpublished data

NSAIDs versus Ciprofloxacin





indomethacin shares a same transporter with ciprofloxacin

diclofenac is substrate of another gemfibrozil-inhibitable transporter

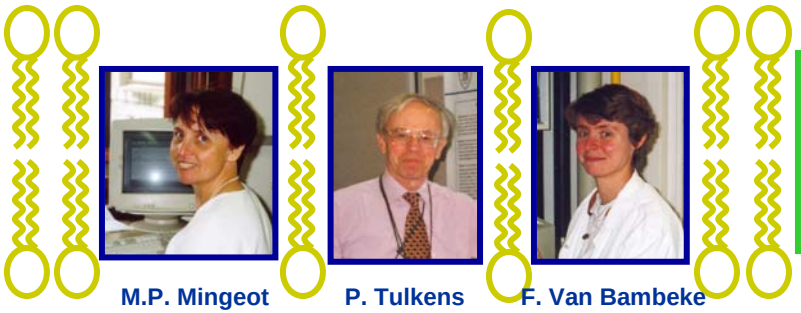
Huygue *et al.* Unpublished data

Conclusion: avenues for the future

Do we need to include
'transport' studies
with bacteria and eucaryotic cells
in the early development
of new antibiotics ?

René Magritte (1898-1967)

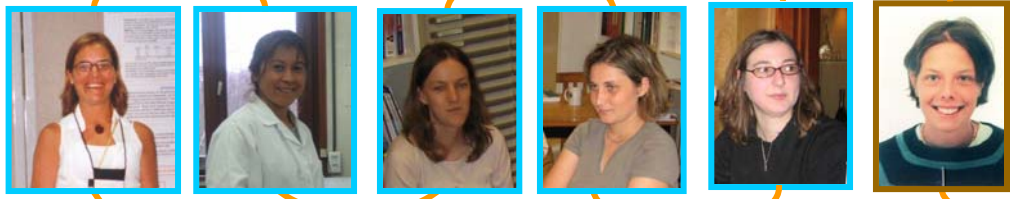
The past and present efflux team in Brussels



M.P. Mingeot P. Tulkens F. Van Bambeke

J.M. Michot H. Chanteux A. Lismond S. Lemaire H. Bensikaddour F. Huyghe

HOOC



C. Seral N. Caceres B. Marquez N. Mesaros L. Avrain M. Heremans

H_2N

F. Renoird M.C. Cambier C. Misson M. Vergauwen N. Couwenbergh

seniors
students
PhD students
post-docs
technicians