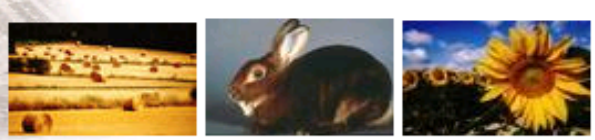
INRA Toulouse

UCL Université catholique de Louvain



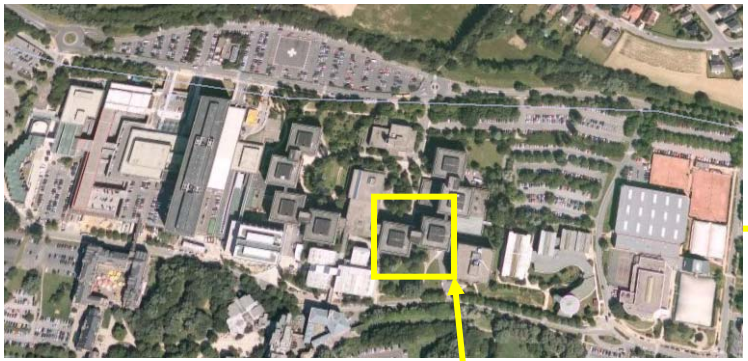
Antibiotic efflux in procaryotic and eucaryotic cells: from molecular mechanisms to pharmacological consequences

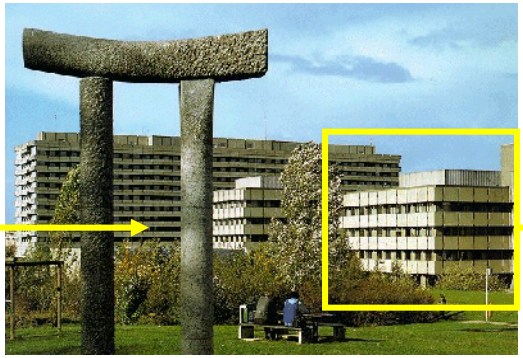
Françoise Van Bambeke, PharmD, PhD

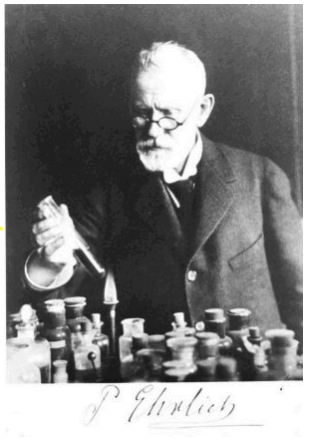
Pharmacologie cellulaire et moléculaire
Louvain Drug Research Institute
Université catholique de Louvain
Bruxelles, Belgium

www.facm.ucl.ac.be

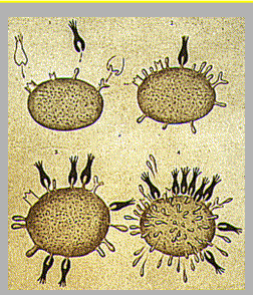
Where do I come from ?







Université catholique de Louvain
Louvain Drug Research Institute
Brussels



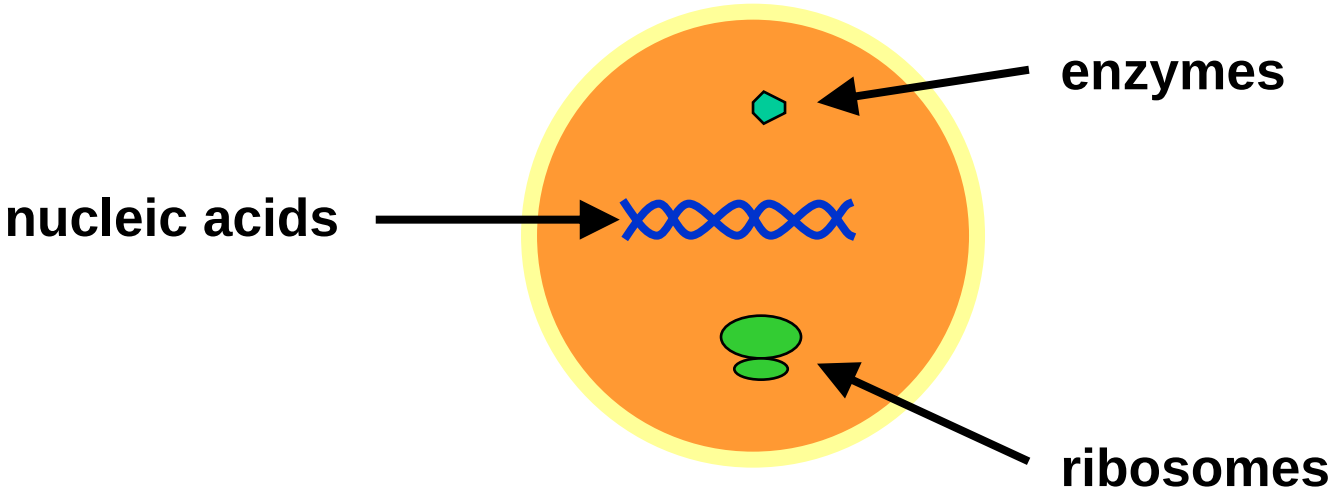
"corpora non agunt nisi fixata"

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

Why active efflux ?

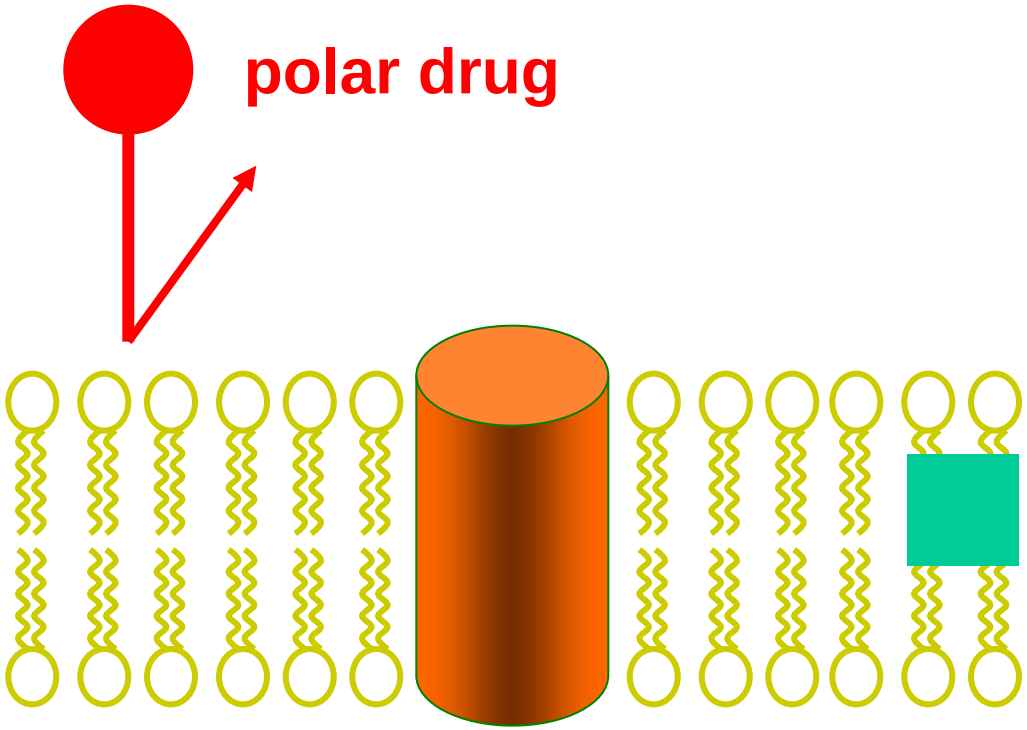
Manneken Pis, who saved Brussels from fire

Chemotherapeutic agents exert toxic effects on specific target cells



How can these drugs reach their target inside the cells ?

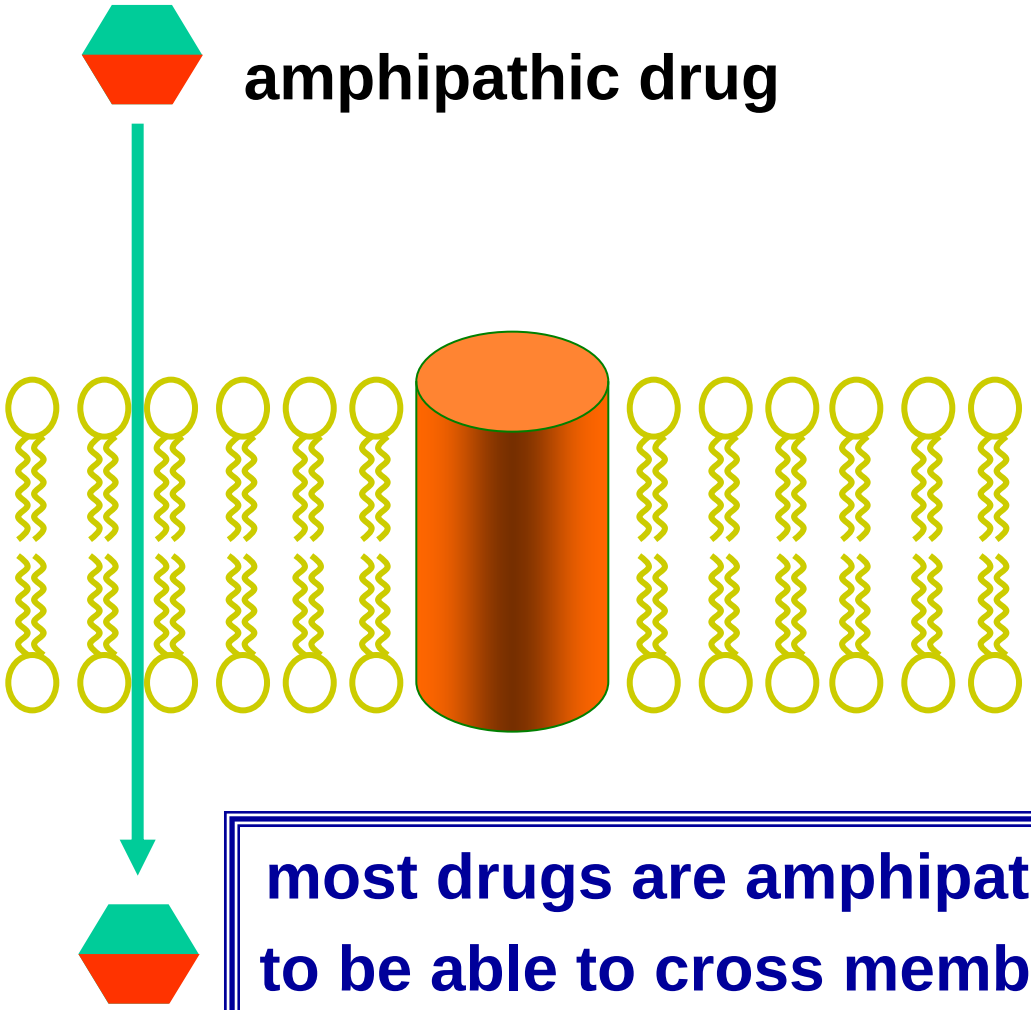
Reaching an intracellular target …



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

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

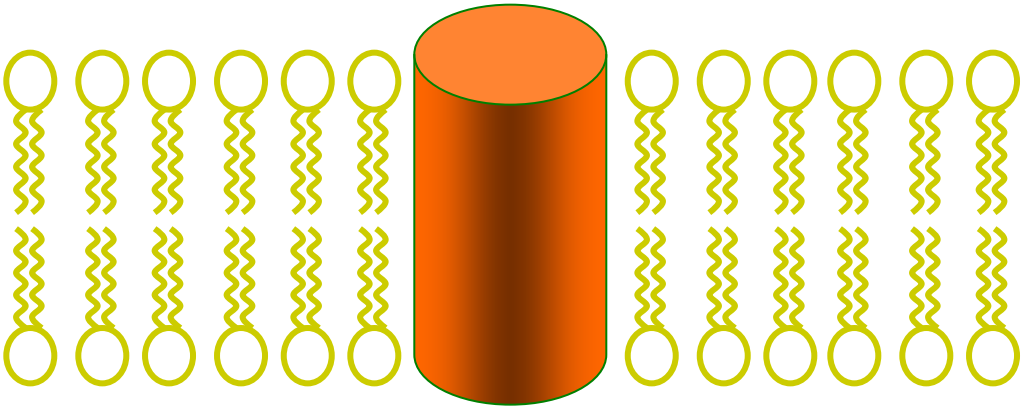
Reaching an intracellular target …

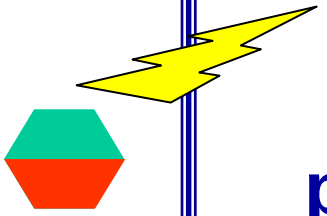


most drugs are amphipathic by design, to be able to cross membrane barriers !

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

Intracellular chemotherapeutic agents



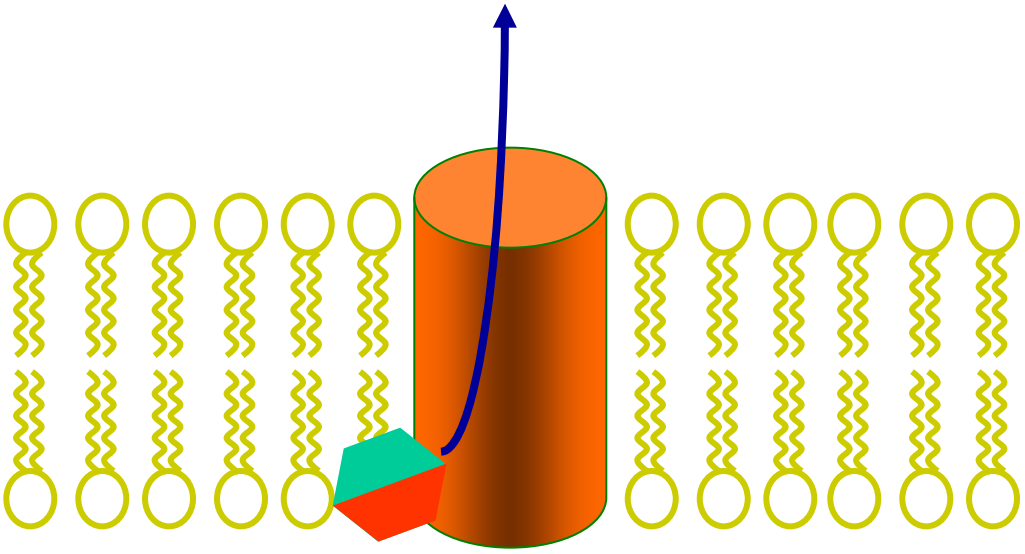


But a diffusible compound may have potentially harmful effects !

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

Why efflux transporters ?

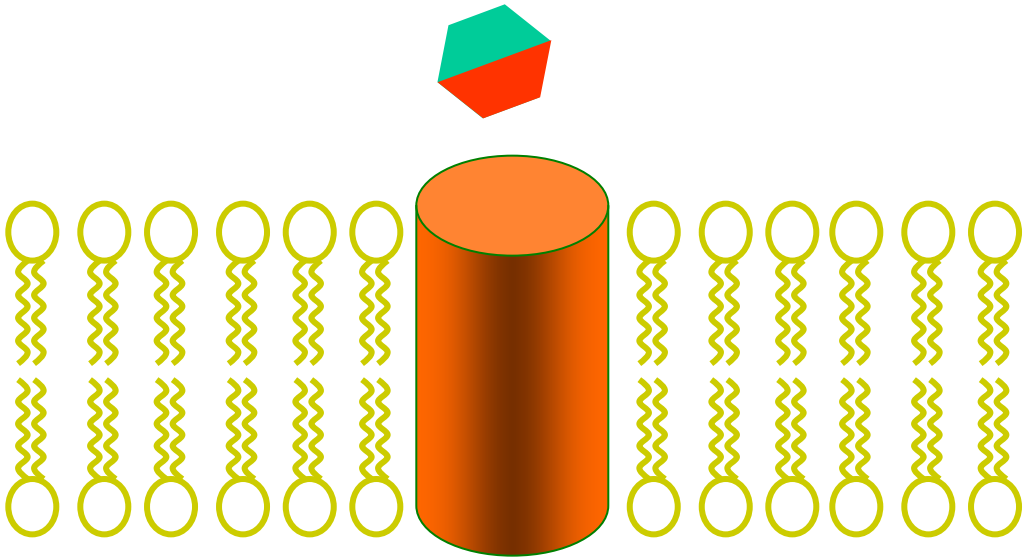
Extrusion by efflux pumps



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

Why efflux transporters ?

Extrusion by efflux pumps

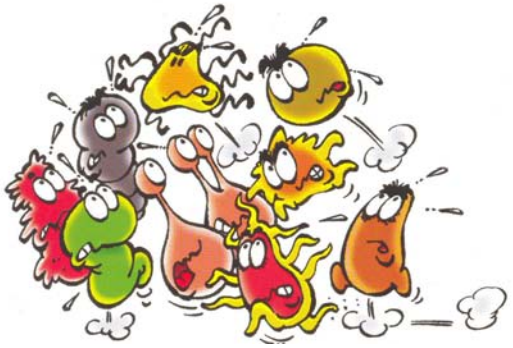


general mean of protection
against cell invasion by diffusible molecules

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

Typical ‘toxic’ diffusible substances as substrates for efflux pumps

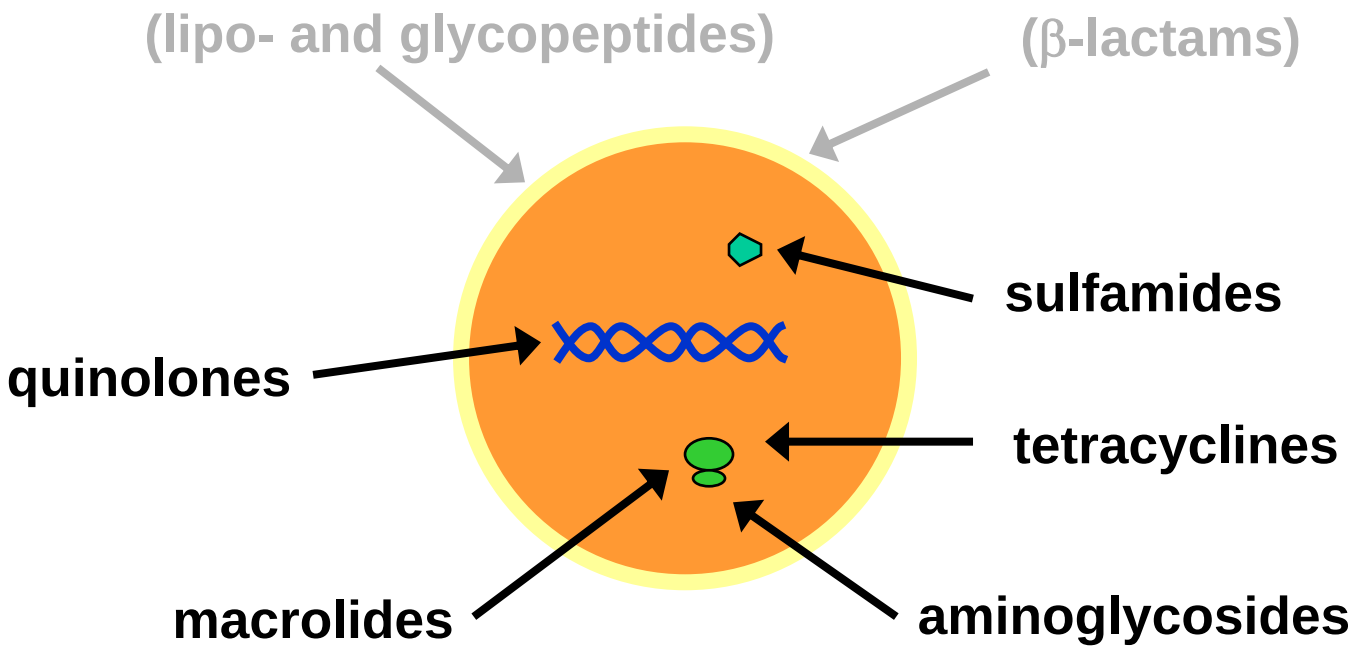
antibiotics



antifungals

anticancer agents

Most antibiotics do act on intracellular targets

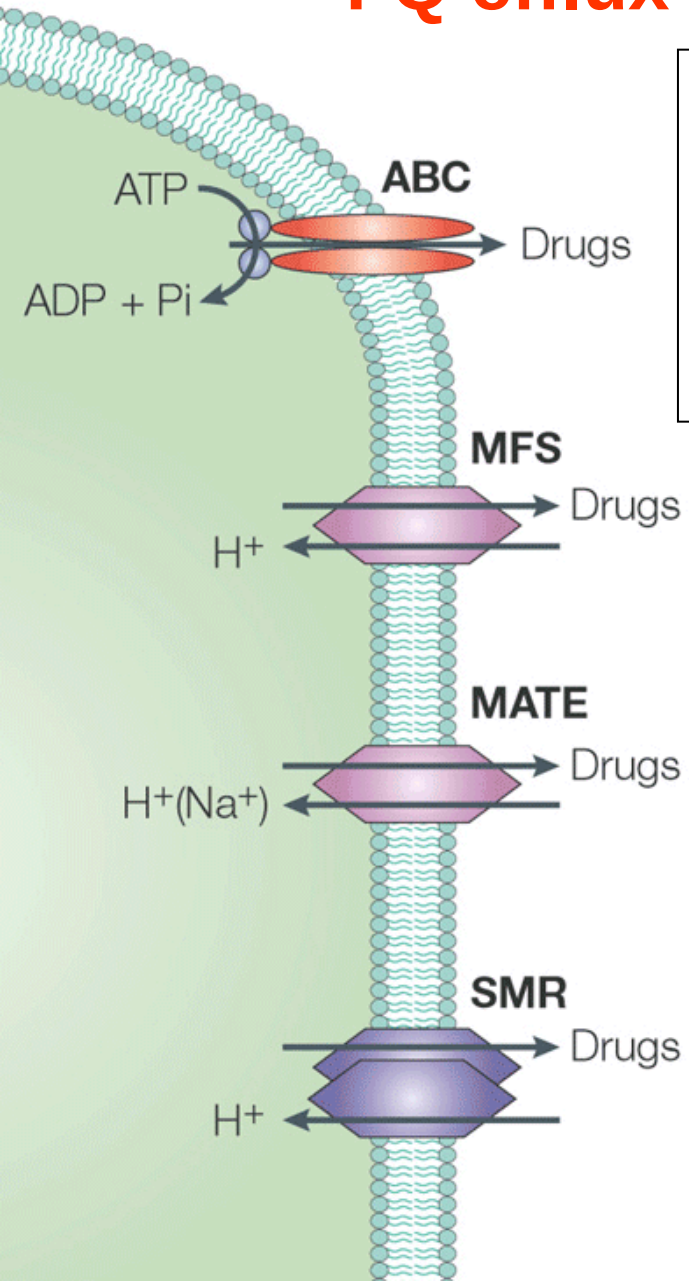


Efflux as a mechanism of resistance
by reducing antibiotic concentration inside the bacteria

Active efflux in bacteria: role in antibiotic resistance

Niagara Falls, Canada

FQ efflux pumps in *S. pneumoniae*



Primary transporters
« **A**TP-**B**inding **C**assette »

PatA/PatB

Marrer et al, AAC 2006; 50:685-93

Secondary transporters
(Proton motive force)

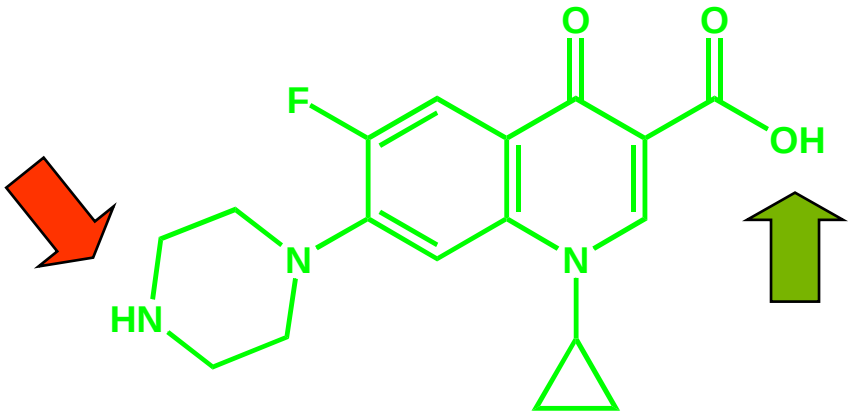
PmrA

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

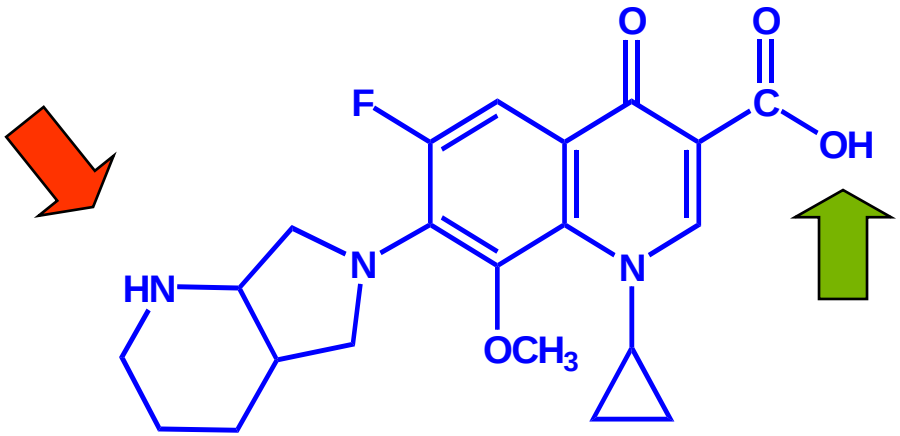
Terry et al., Nature Reviews Microbiology 2005; 3: 566-572

Fluoroquinolone antibiotics

ciprofloxacin

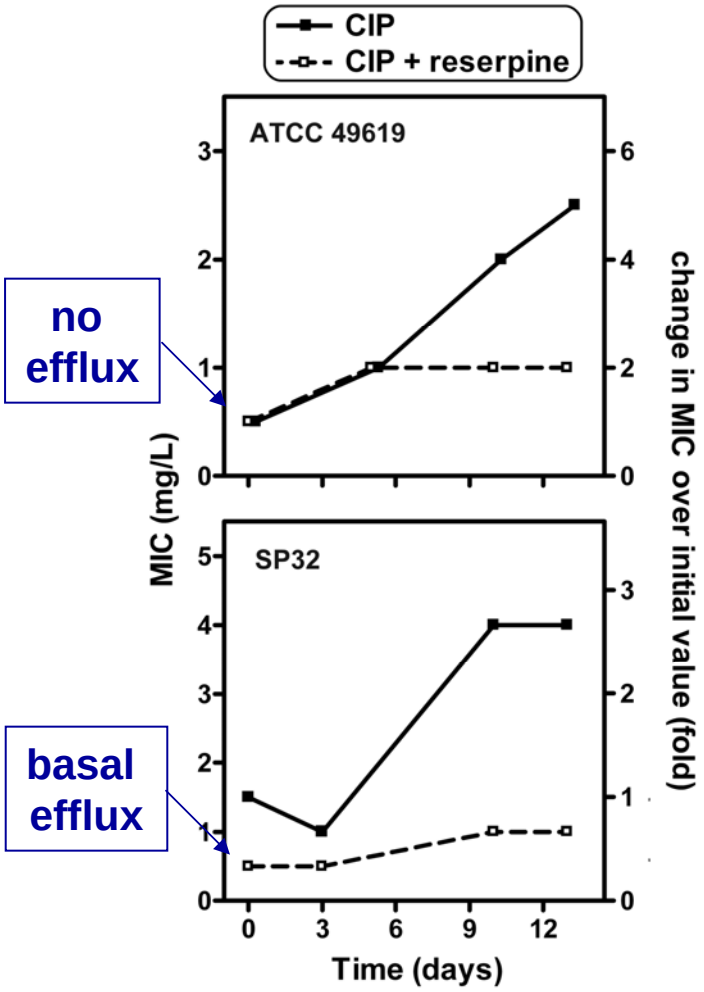


moxifloxacin



Selection of resistance by subMIC concentrations of FQ

ciprofloxacin



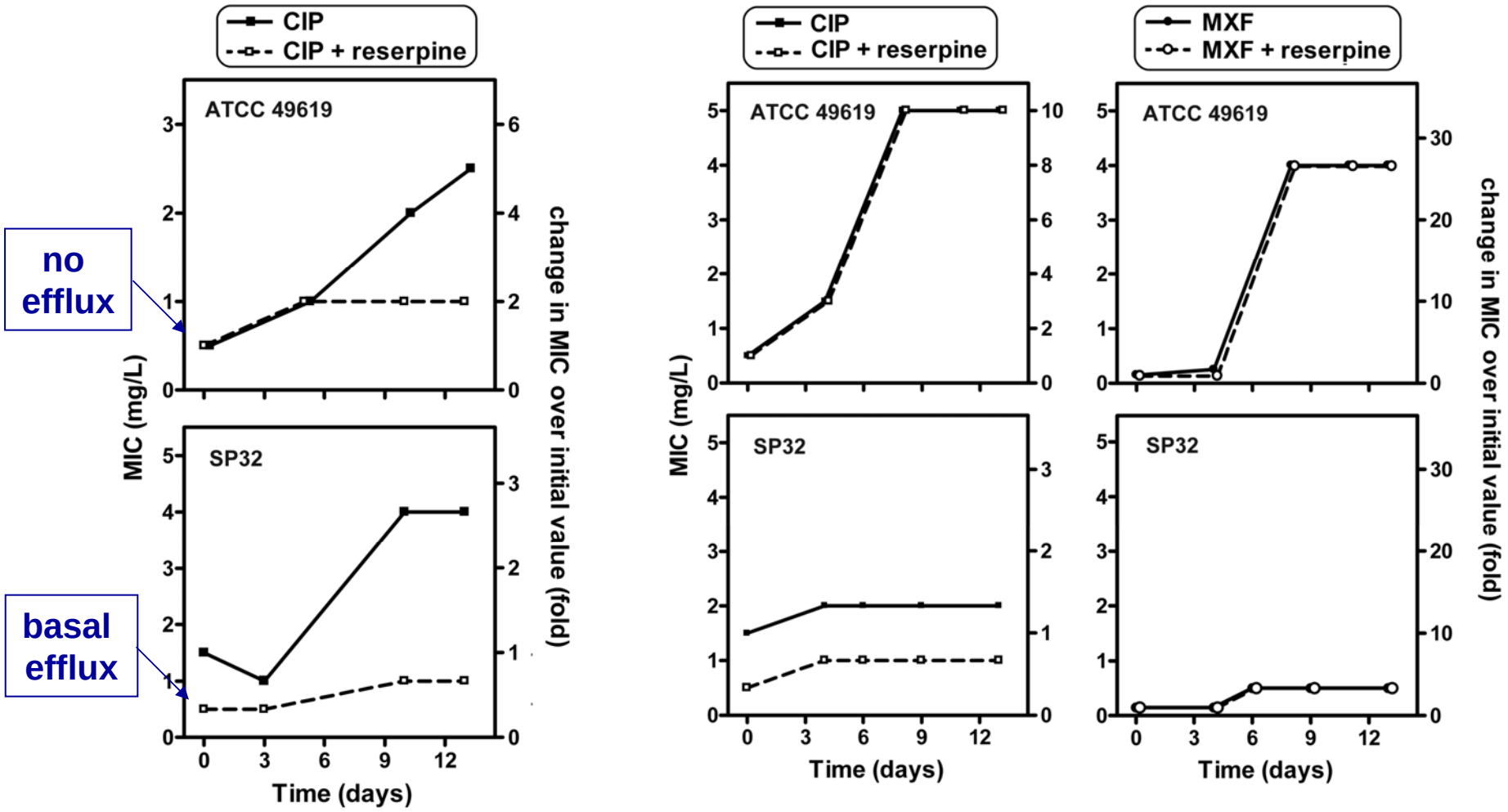
Loss of susceptibility; efflux selected by ciprofloxacin

Avrain et al, JAC (2007) 60:965-972

Selection of resistance by subMIC concentrations of FQ

ciprofloxacin

moxifloxacin

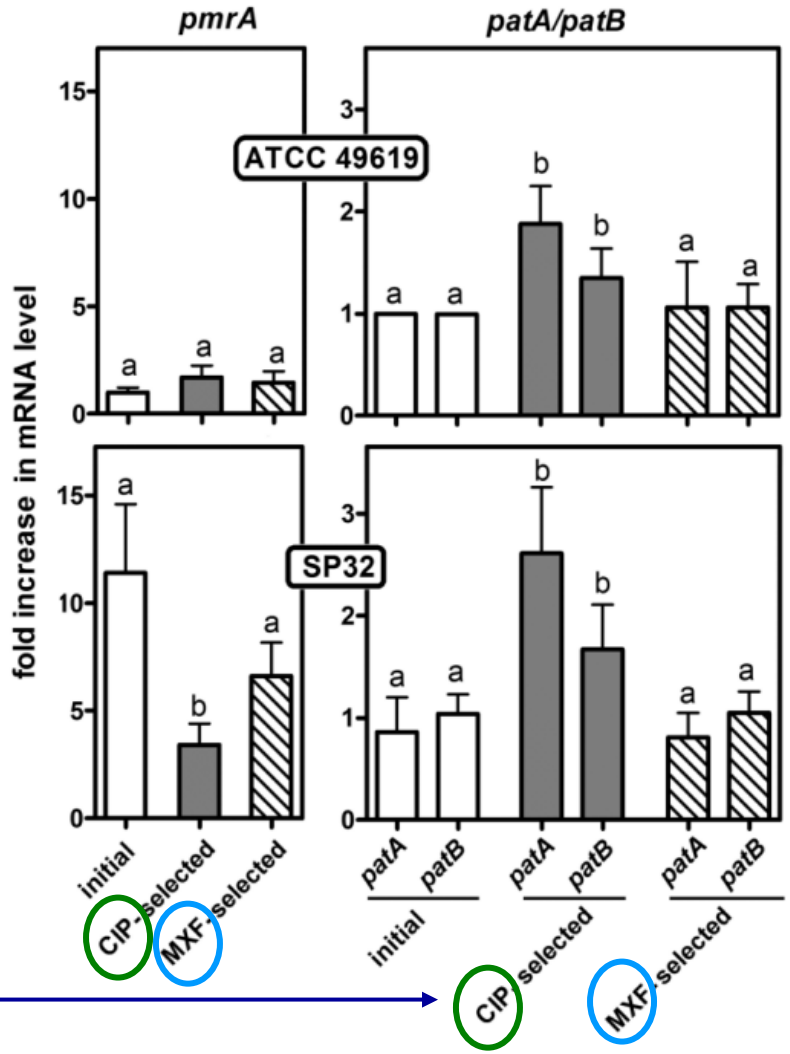


Loss of susceptibility; efflux selected by ciprofloxacin only

Avrain et al, JAC (2007) 60:965-972

Selection of resistance by subMIC concentrations of FQ

Expression of genes coding for efflux pumps in the absence of fluoroquinolones



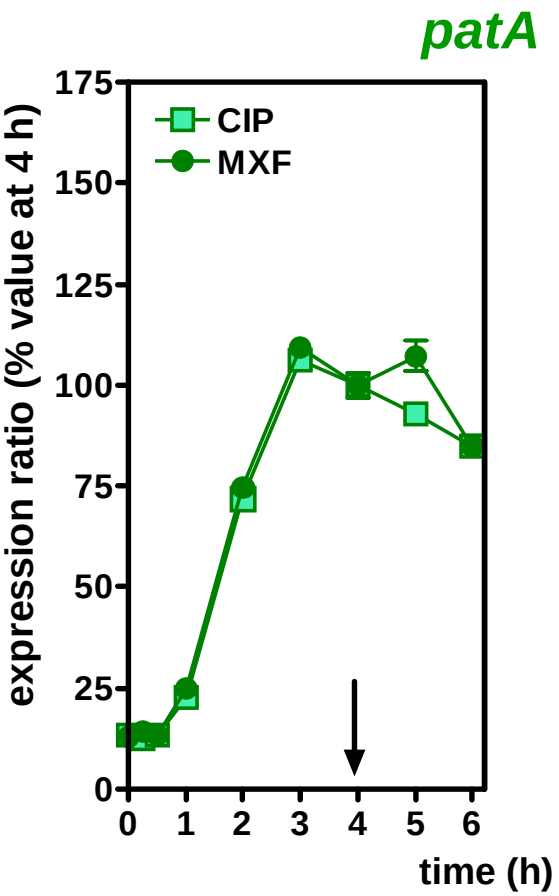
Increased efflux due to PatA/PatB

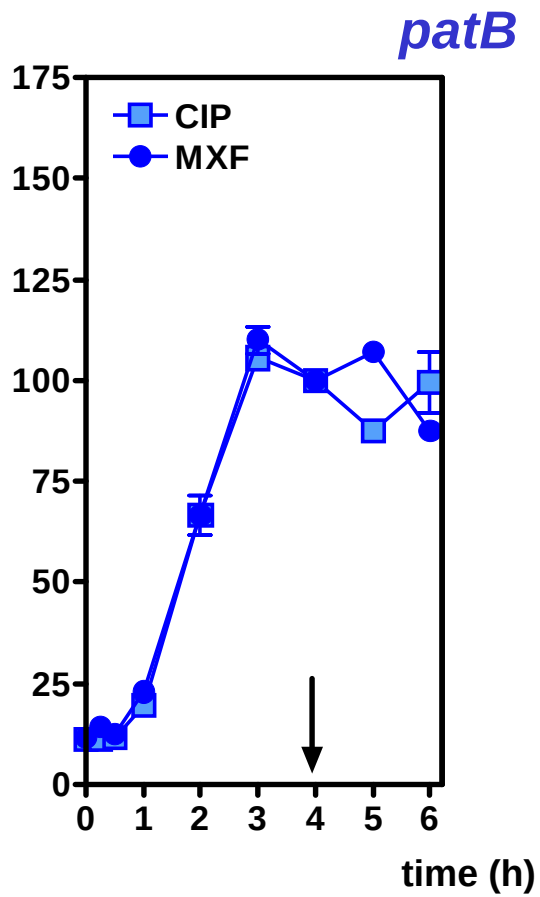
Avrain et al, JAC (2007) 60:965-972

PatA/B are inducible by fluoroquinolones

Bacteria in the presence of ½ MIC of FQ

ATCC49619



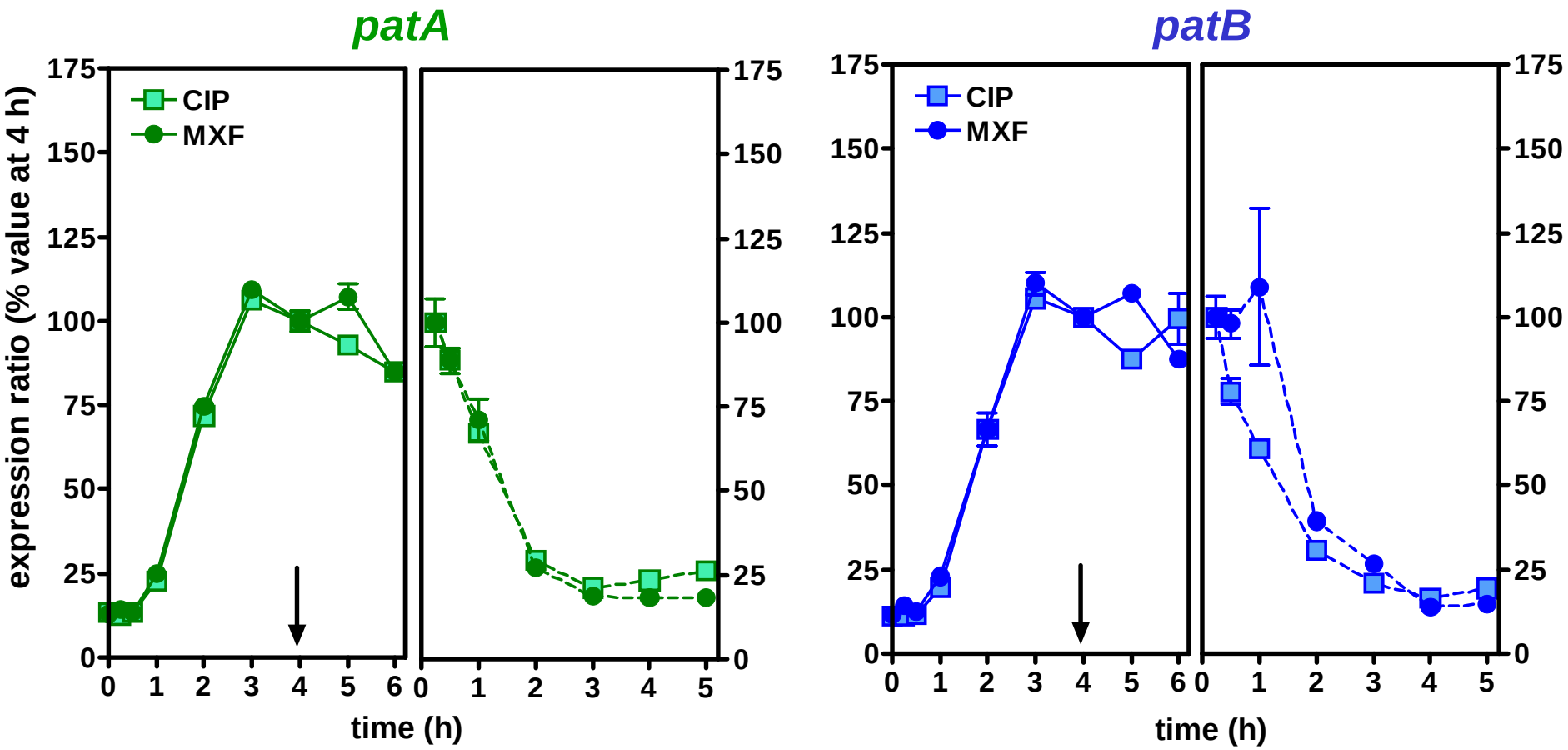


El garch et al, ECCMID (2009) O495

Kinetics of induction & reversibility

4 h with ½ MIC; up to 5 h without FQ

ATCC49619

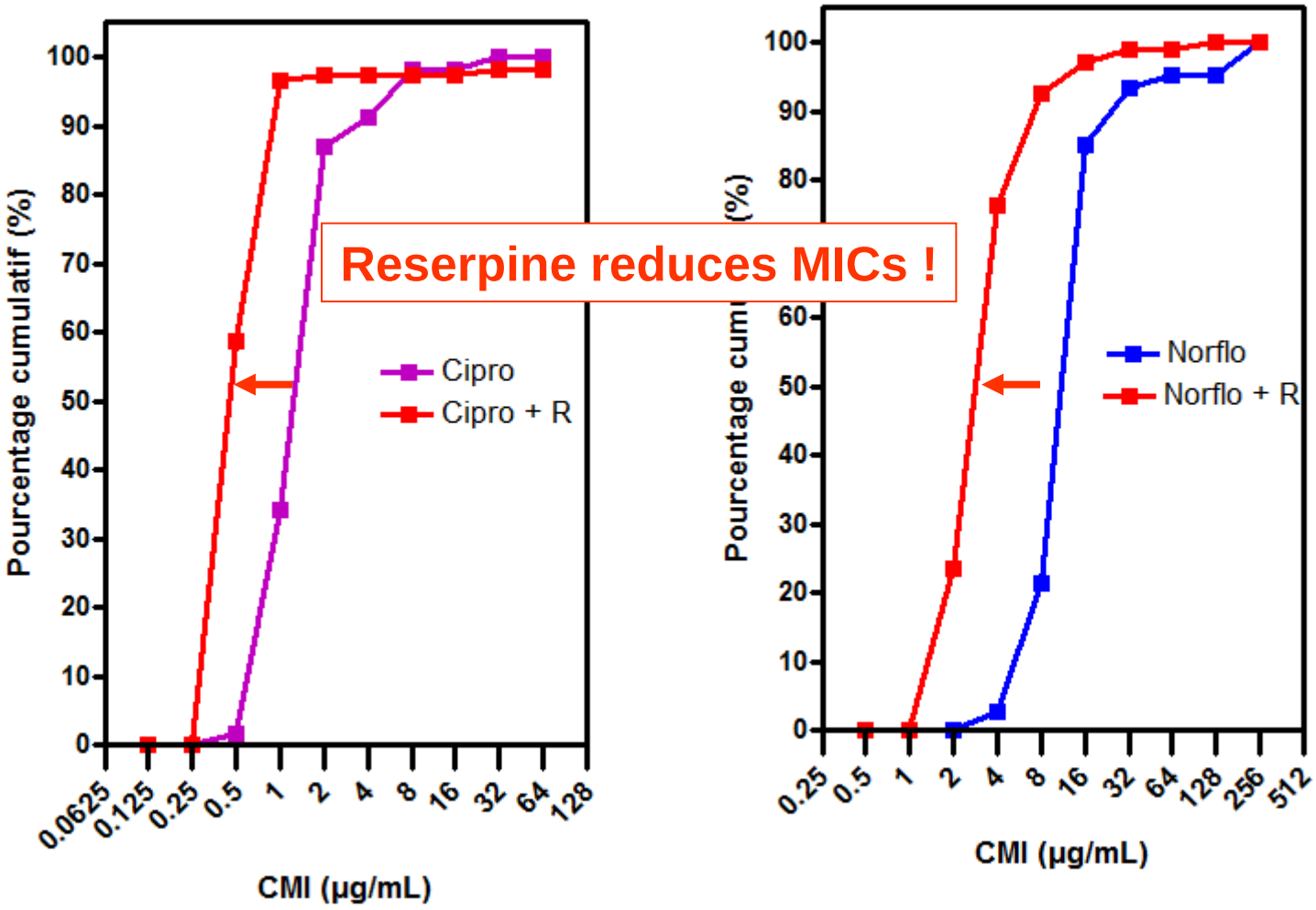


Overexpression may contribute to resistance during treatment !

El garch et al, ECCMID (2009) O495

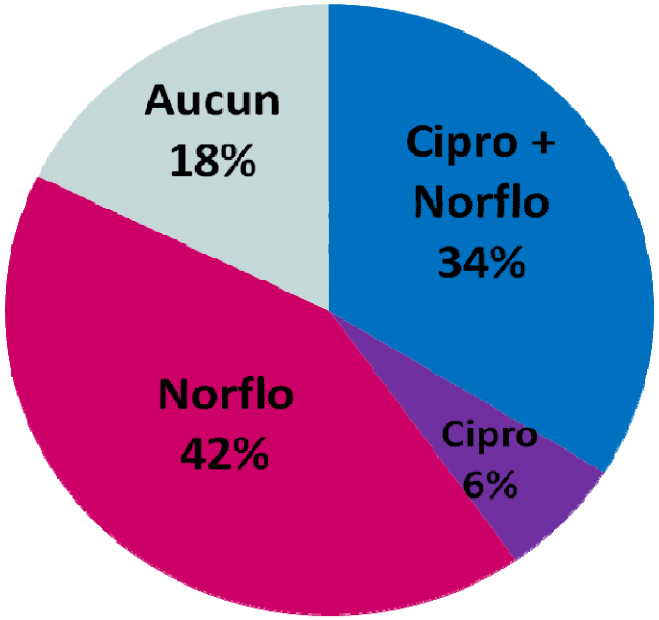
Efflux in *S. pneumoniae:* is it important in the clinics ?

MIC of fluoroquinolones in 107 strains collected from patients with AECB

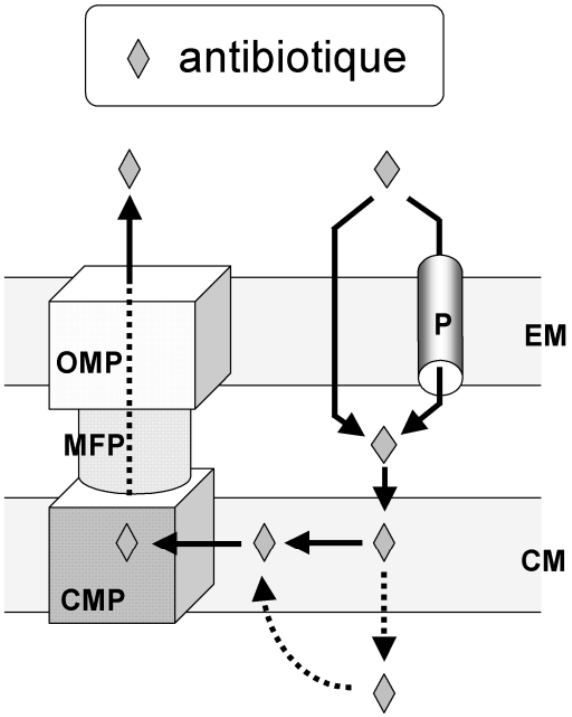


Efflux in *S. pneumoniae:* is it important in the clinics ?

Suspected efflux based on phenotypic analysis



Efflux and resistance in *P. aeruginosa*



CM: cytoplasmic membrane
(membrane cytoplasmique)

EM: external membrane
(membrane externe)

P: porin
(porine)

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

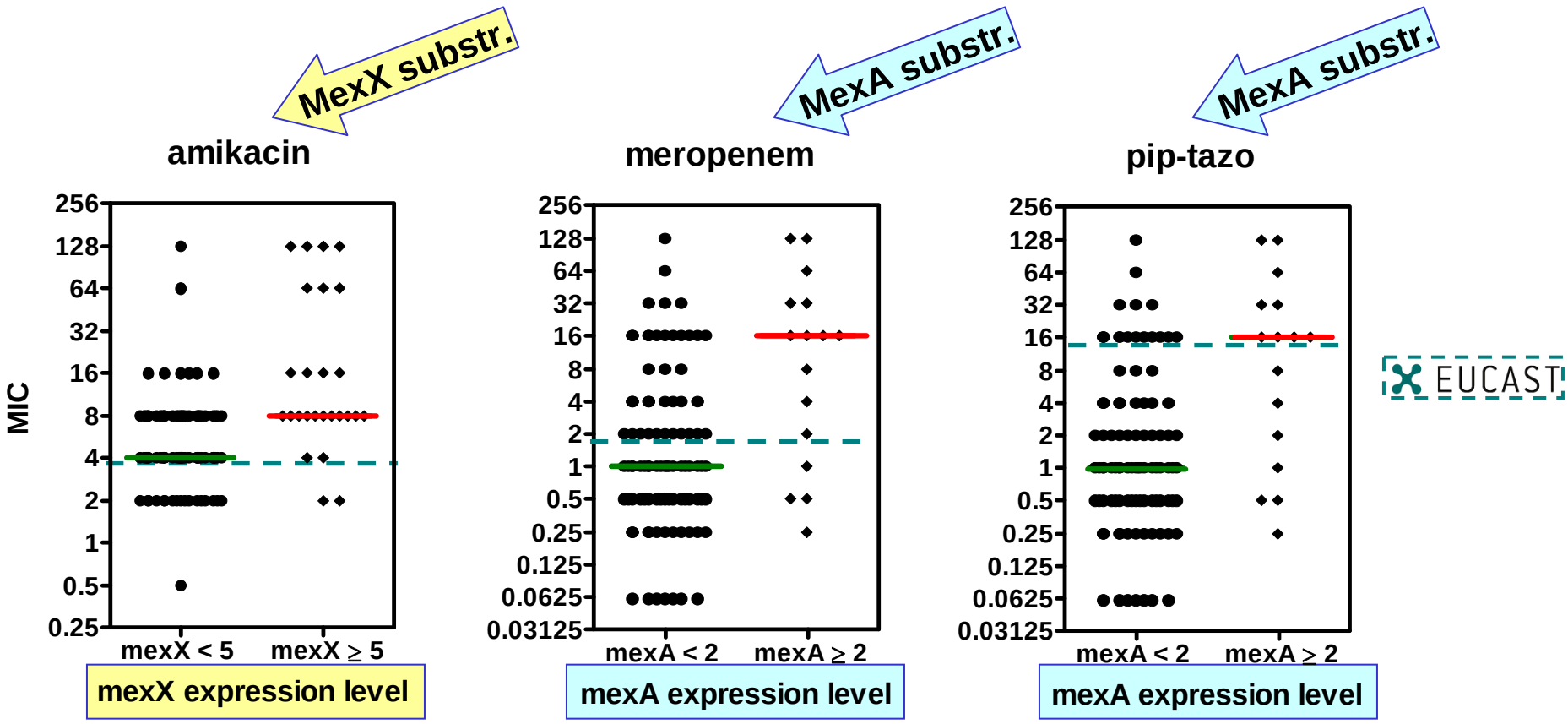
MFP: membrane fusion protein
(protéine de fusion [entre membranes])

OMP: outer membrane protein
(protéine de membrane externe)

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

Efflux and low level of resistance in *Pseudomonas aeruginosa*

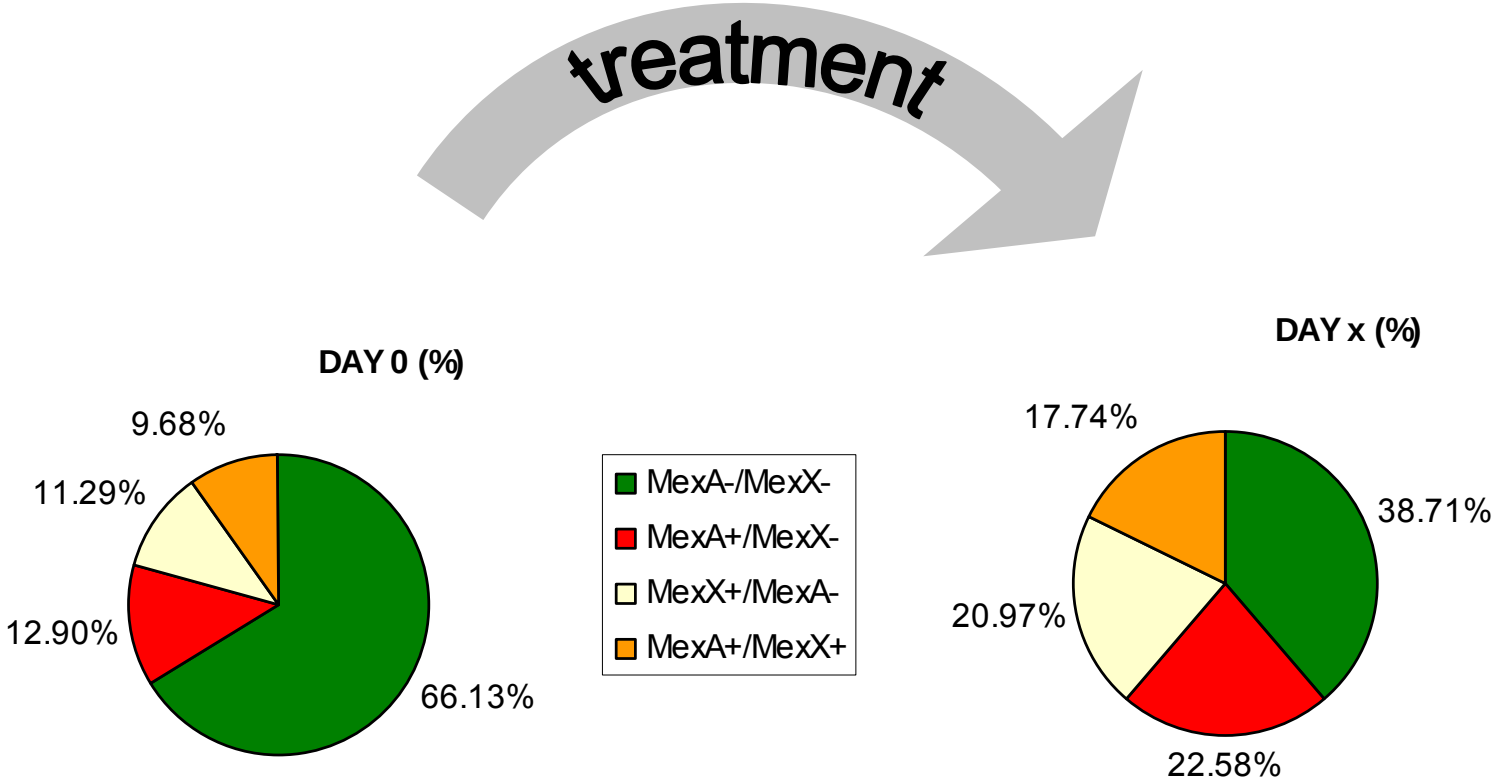
MICs vs EUCAST breakpoints for 109 *P. aeruginosa* without or with efflux mechanisms, isolated from ICU patients (VAP)



Riou et al, ECCMID 2010

Efflux selection during treatment

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



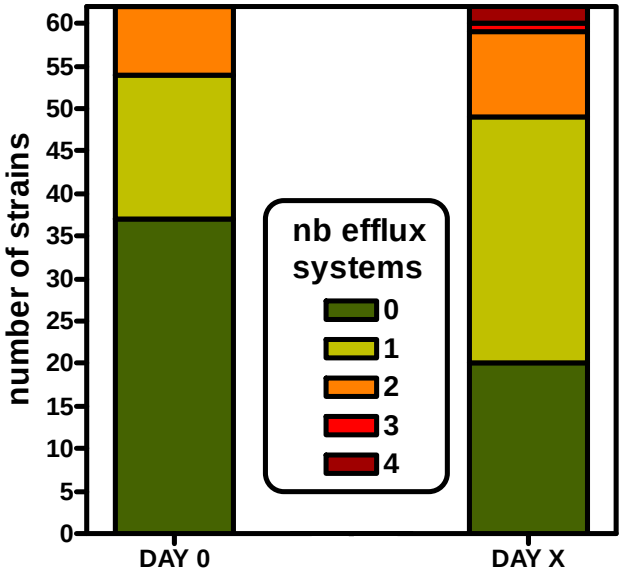
Riou et al, ECCMID 2010

Efflux selection during treatment

Antipseudomonal antibiotics received by the patients during treatment

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

global influence of treatment



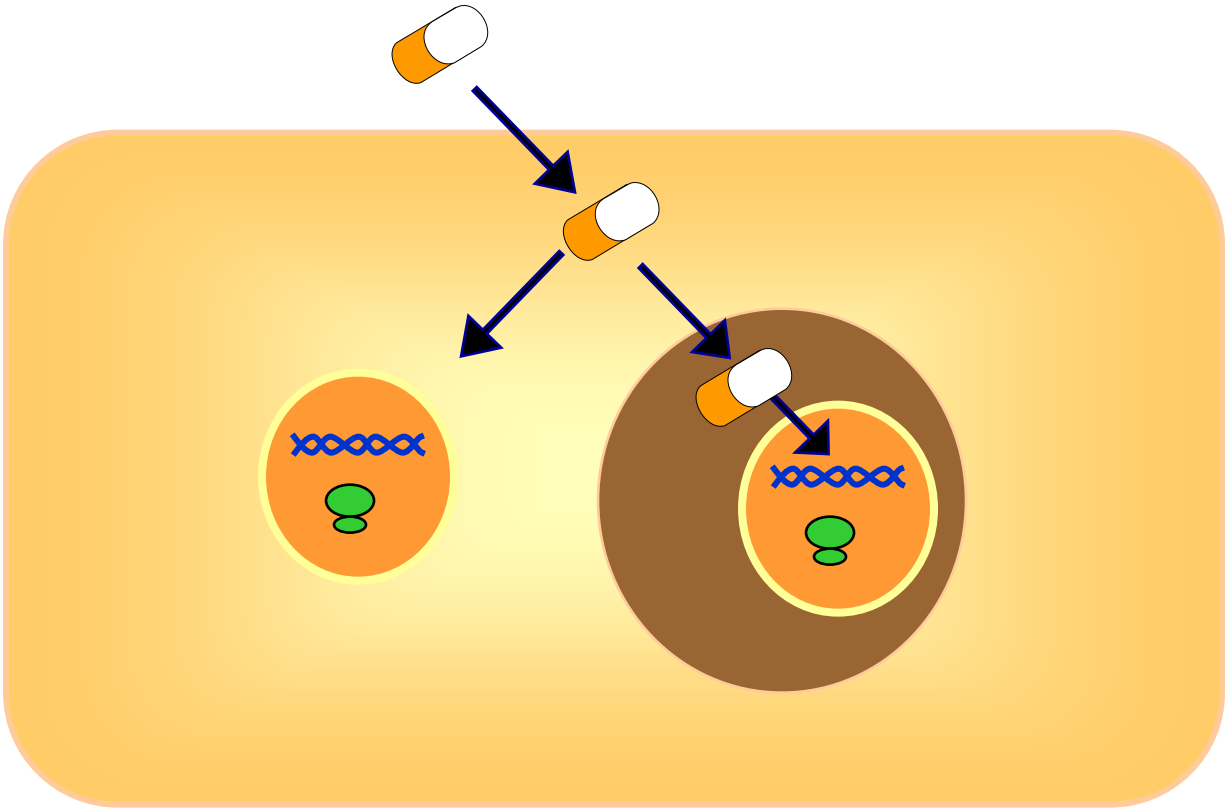
number of efflux systems detected at day 0 and day X

Riou et al, ECCMID 2010

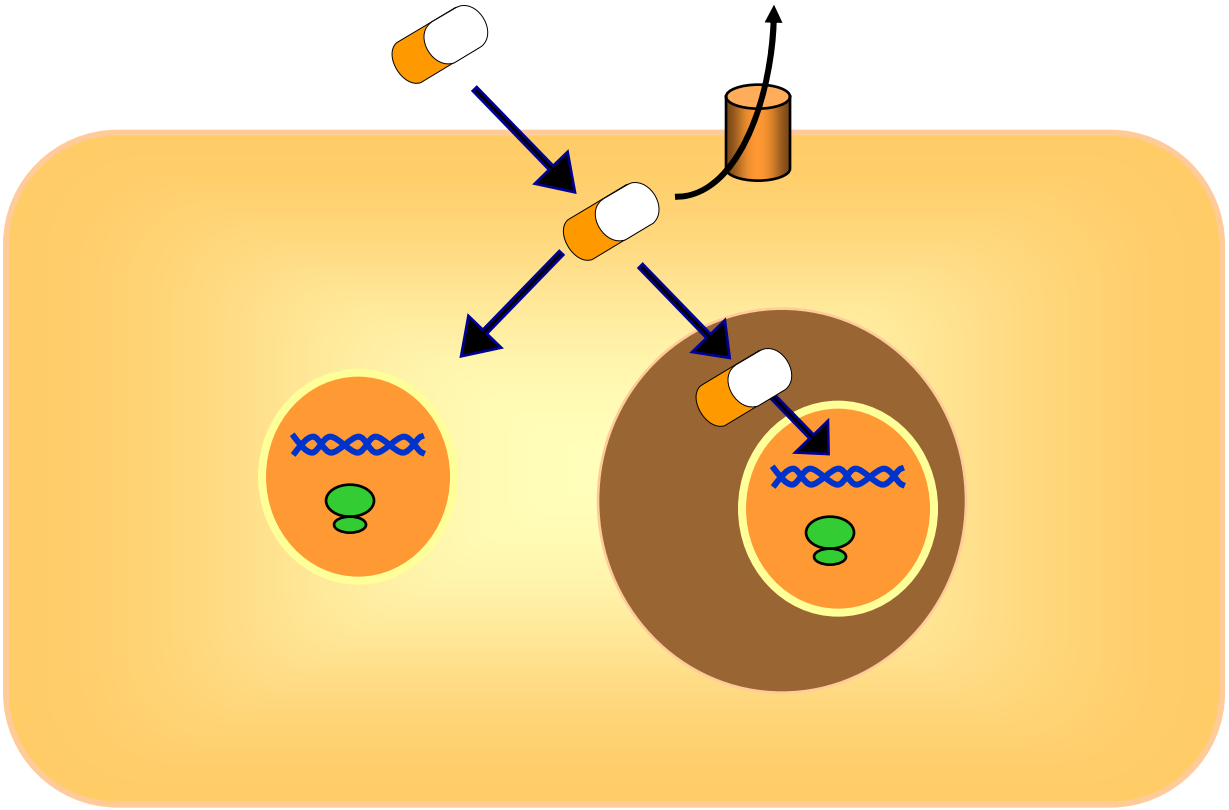
Active efflux in eucaryotic cells: role in antibiotic PK/PD

Old Faithful Geyser

Target accessibility is critical for intracellular activity



Active efflux reduces antibiotic cellular concentration



Consequences of antibiotic efflux from eucaryotic cells

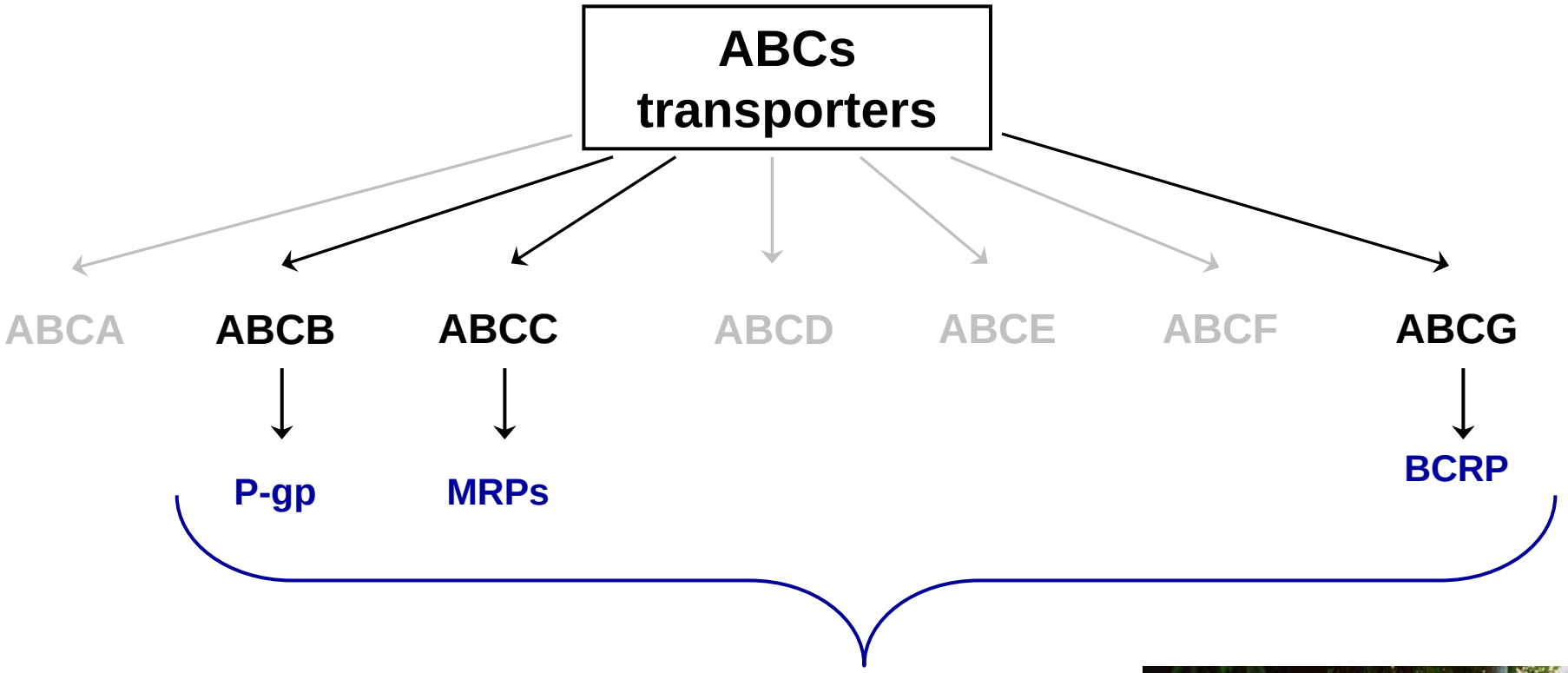
- **alteration of pharmacokinetics**
  - single cell: accumulation, localization
  - whole organism: absorption, distribution, elimination
- **alteration of pharmacodynamics**
  - cellular level: activity against intracellular bacteria
  - body level: drug concentration in the infected compartment

Van Bambeke et al, JAC (2003) 51:1067-1077

Characterization of antibiotic efflux pumps in macrophages

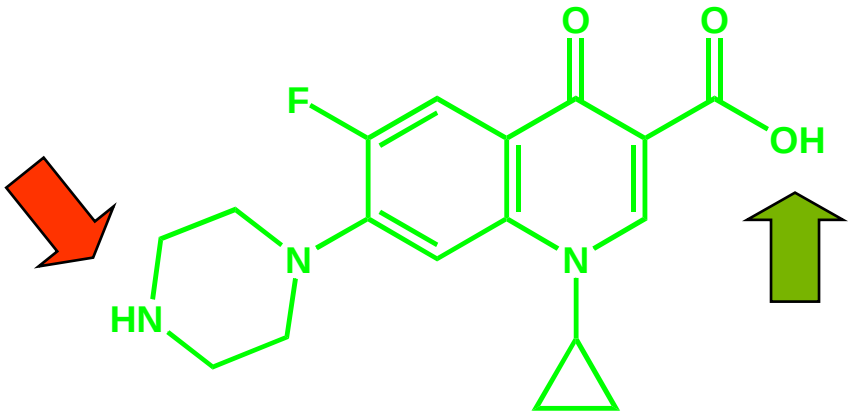
Belgian beer

ATP-Binding Cassette transporters

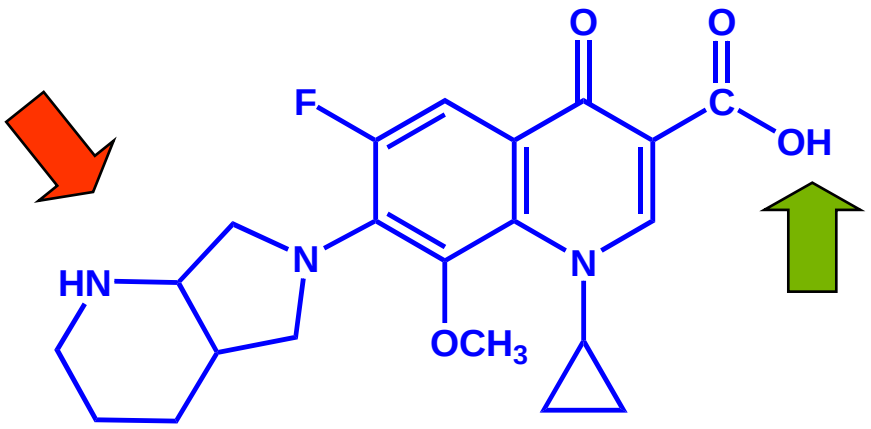


Fluoroquinolone antibiotics

ciprofloxacin

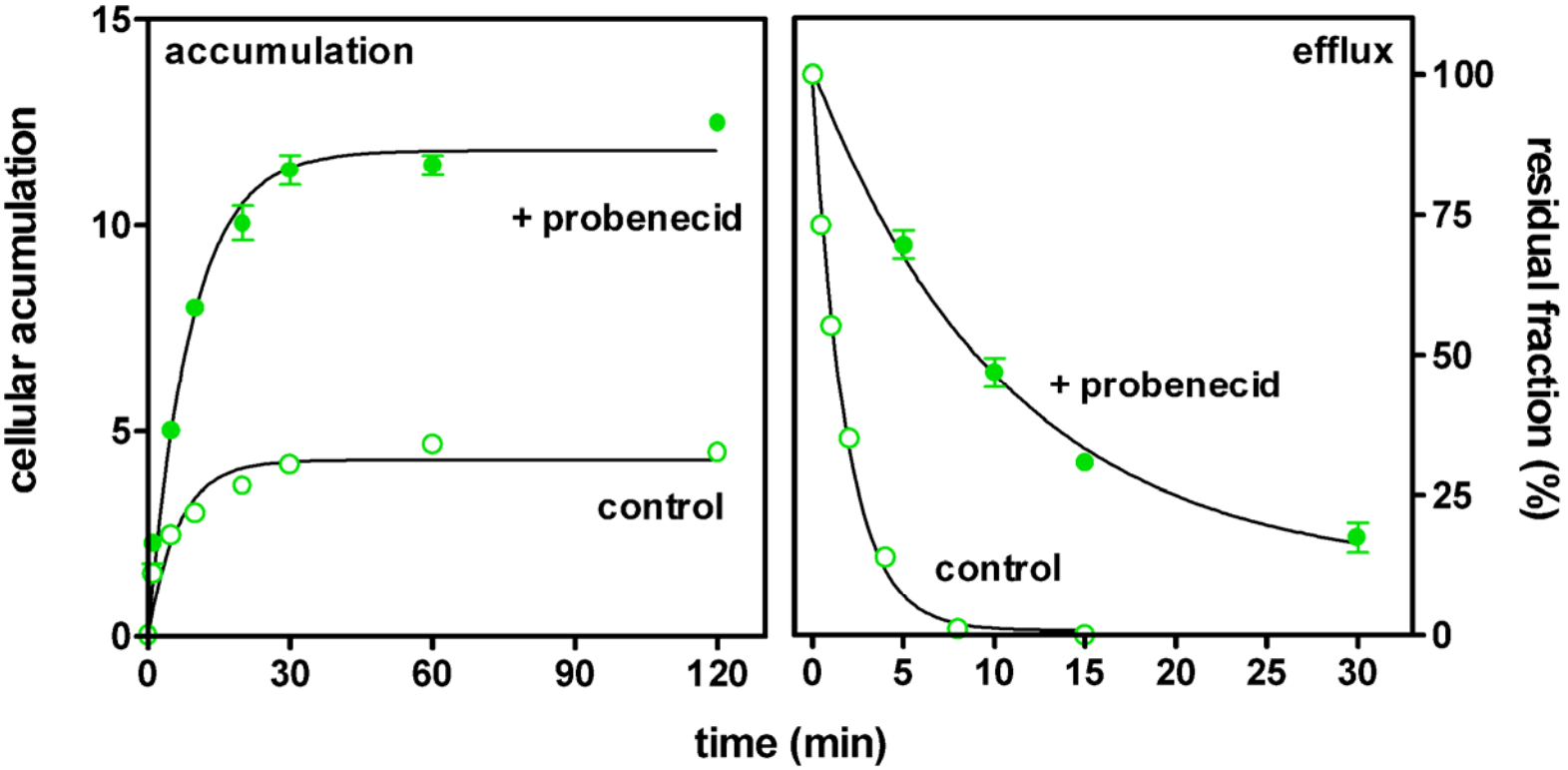


moxifloxacin



Kinetics of accumulation and efflux for ciprofloxacin

both accumulation and efflux markedly affected By probenecid (inhibitors of Mrps)

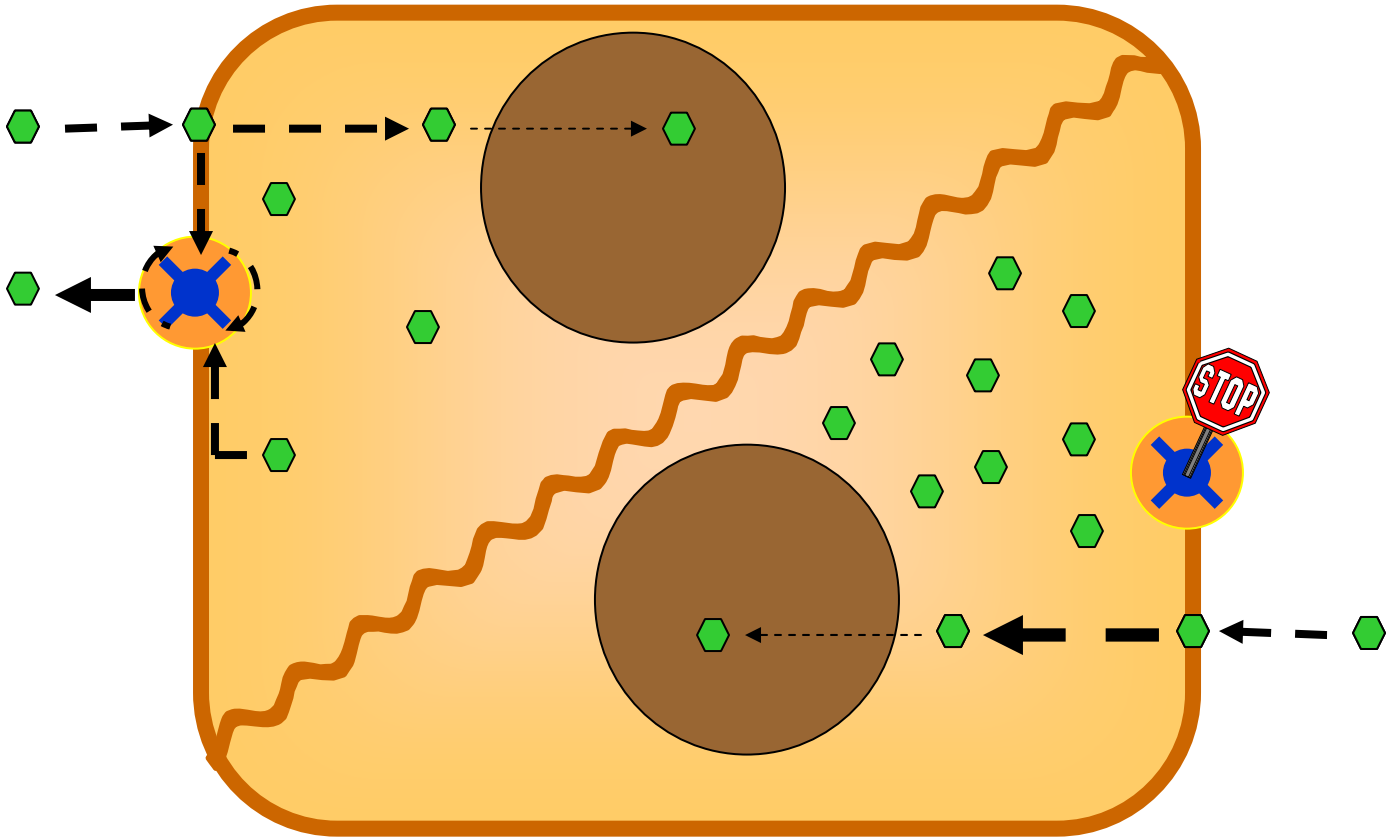


extracell. conc. 17 mg/L; probenecid 5 mM

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

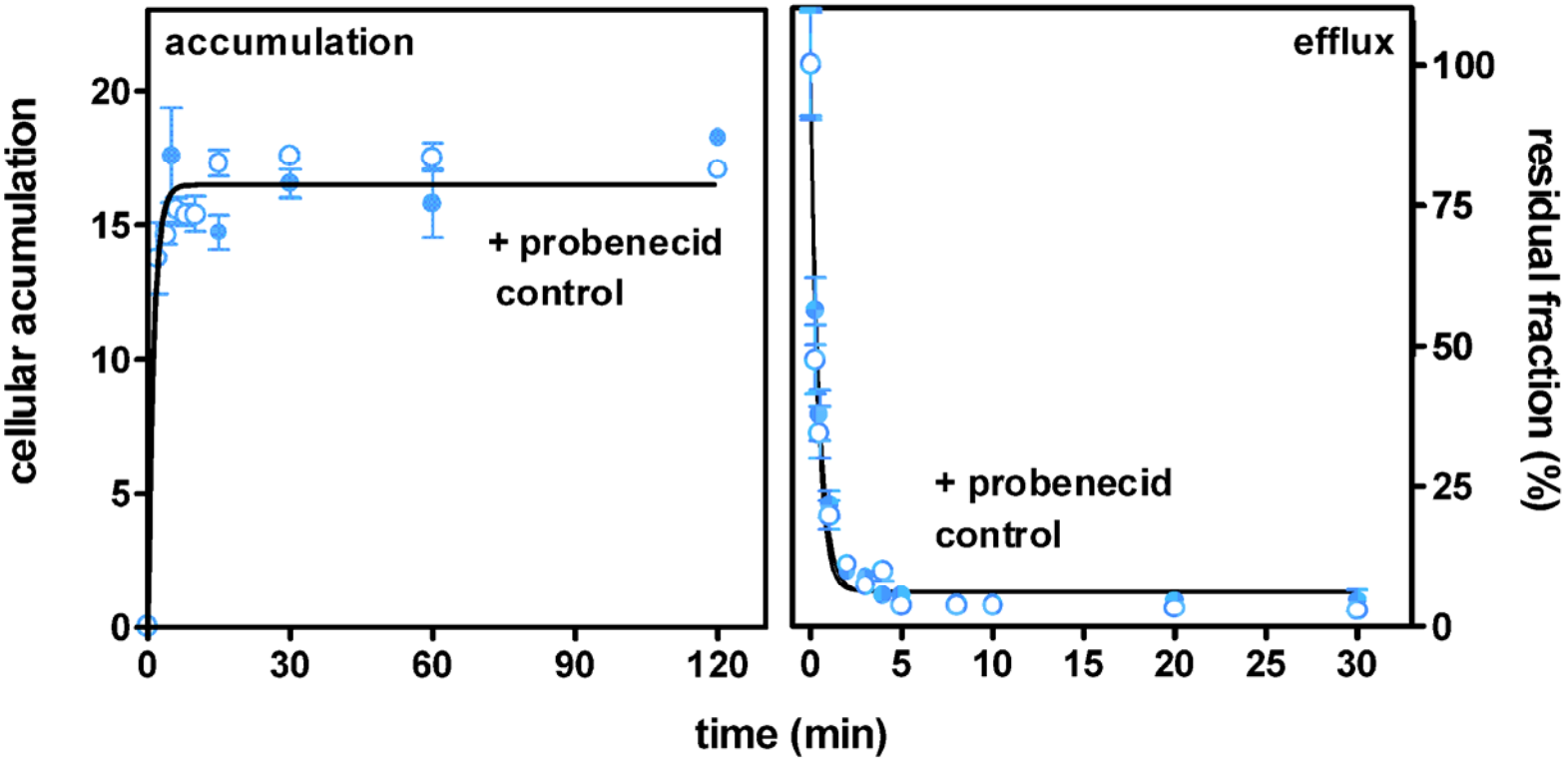
Ciprofloxacin, classical model

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



Kinetics of accumulation and efflux for moxifloxacin

neither accumulation nor efflux affected By probenecid

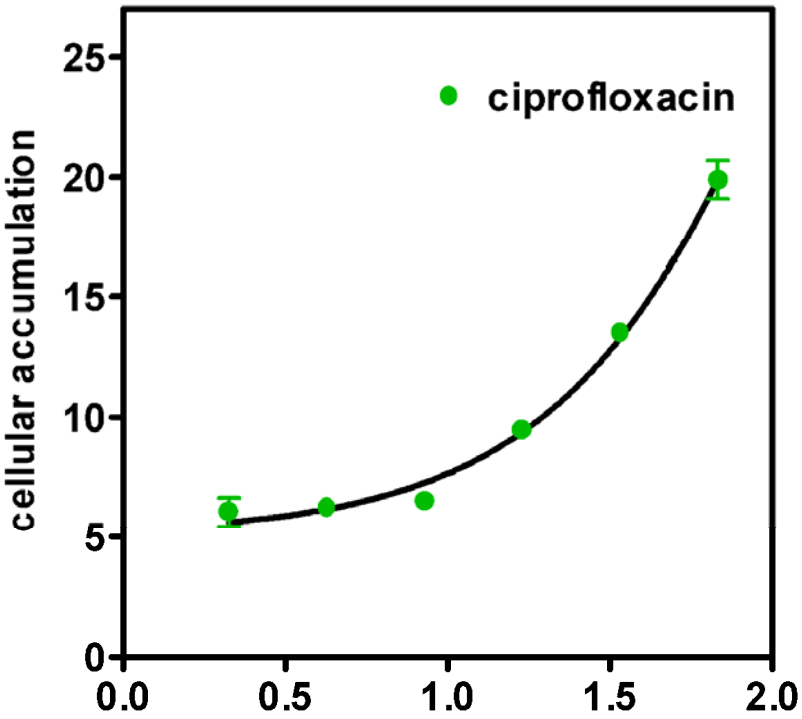


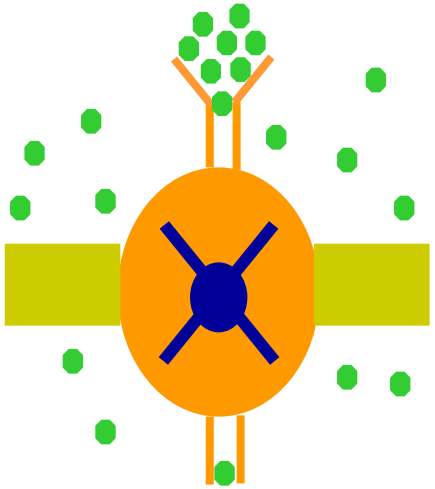
extracell. conc. 17 mg/L; probenecid 5 mM

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

Quinolones as inhibitors of ciprofloxacin efflux

- **ciprofloxacin efflux inhibited by ciprofloxacin**

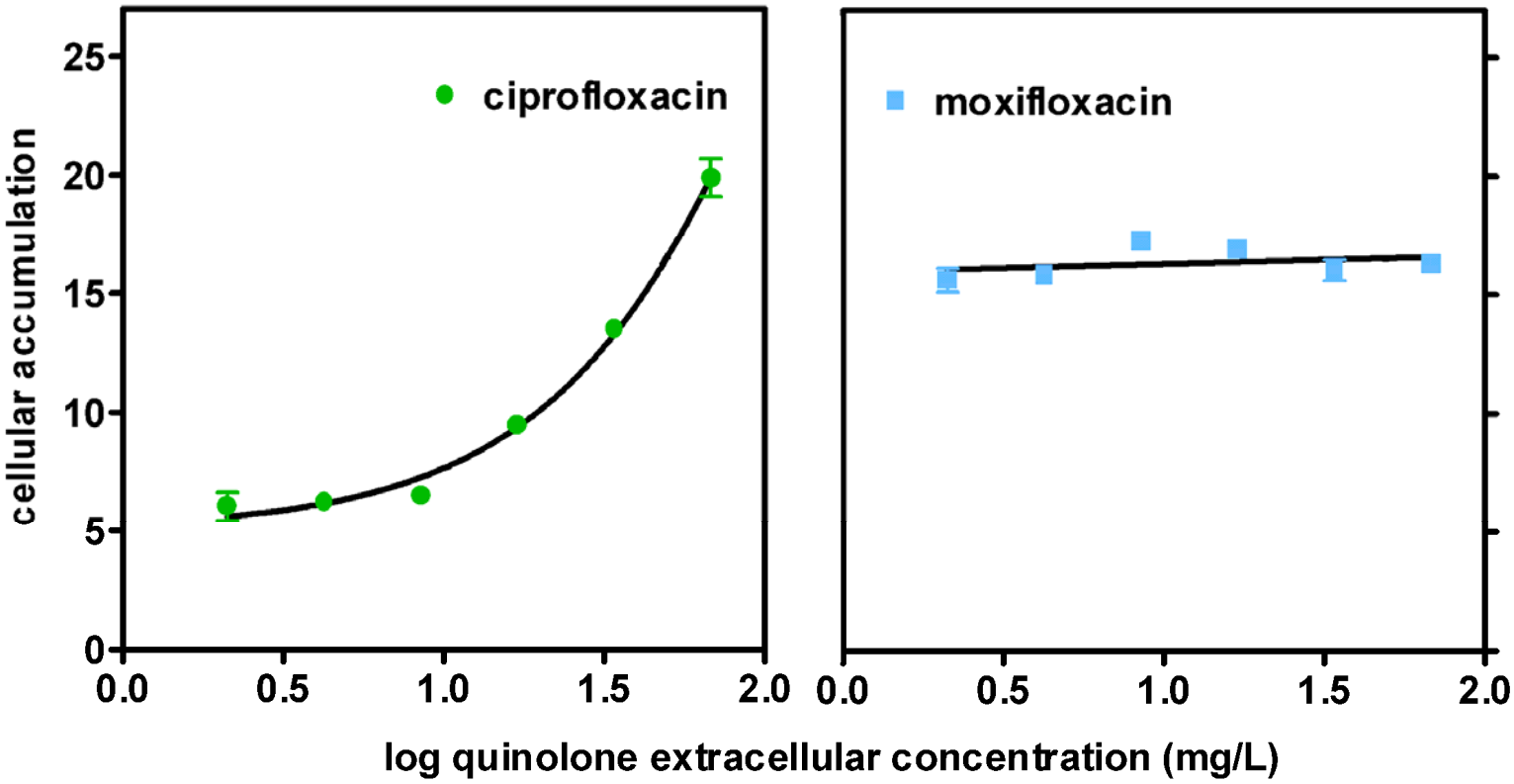




Michot et al. AAC (2005) 49:2429-37

Quinolones as inhibitors of ciprofloxacin efflux

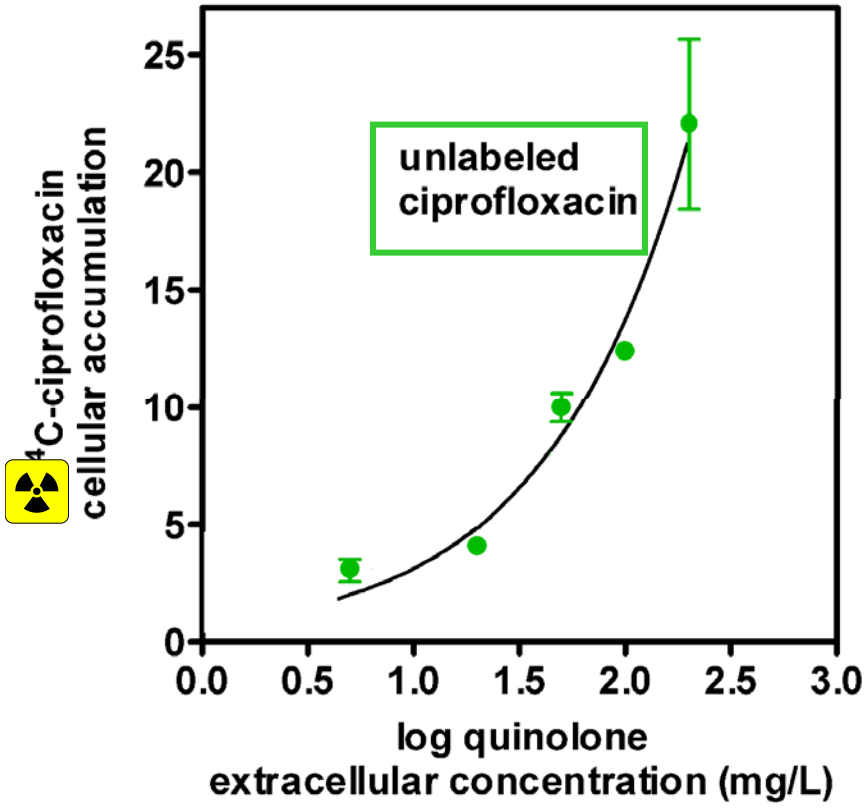
- ciprofloxacin efflux inhibited by ciprofloxacin
- moxifloxacin not affected

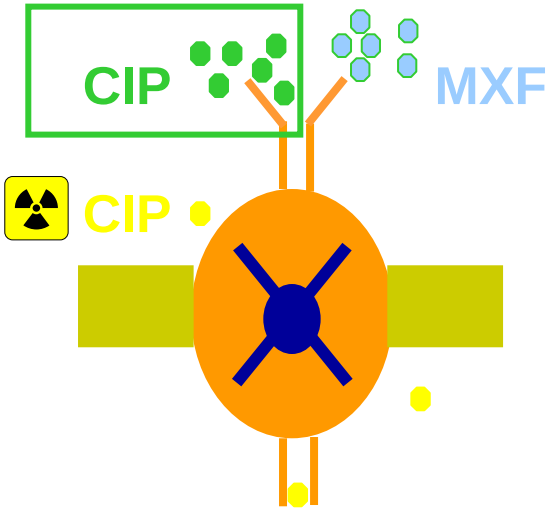


Michot et al. AAC (2005) 49:2429-37

Quinolones as inhibitors of ciprofloxacin efflux

- **ciprofloxacin efflux inhibited by ciprofloxacin**
  moxifloxacin

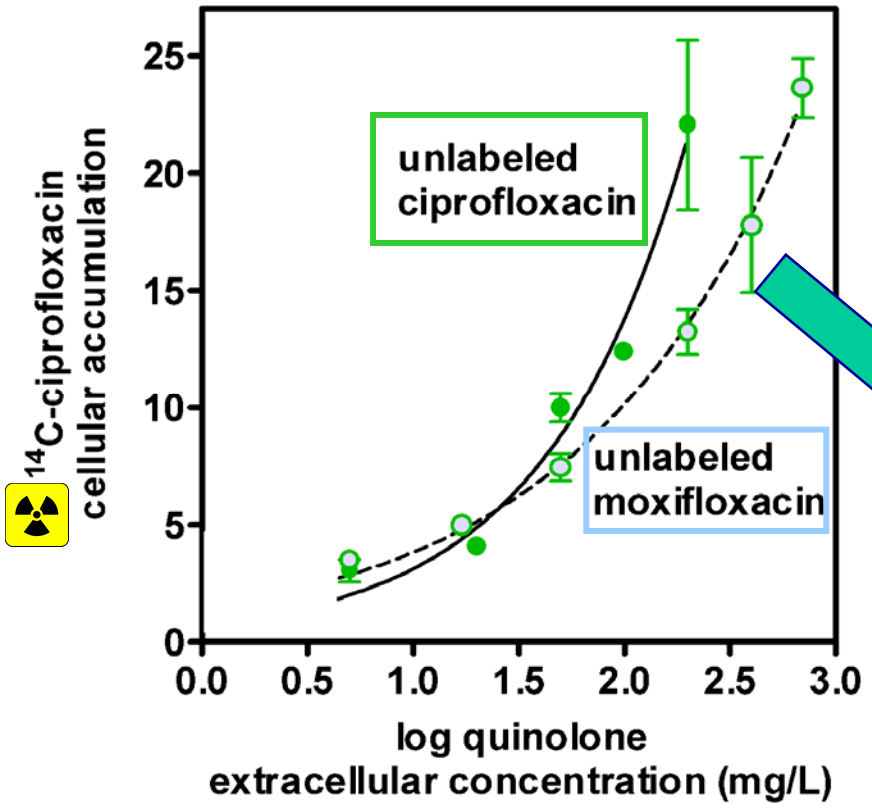


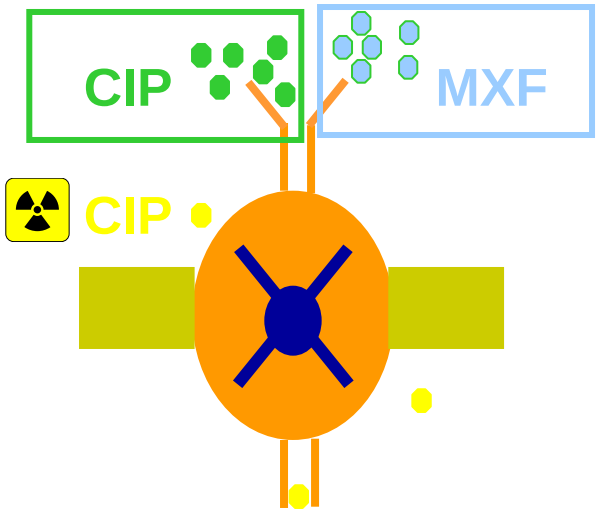


Michot et al. AAC (2005) 49:2429-37

Quinolones as inhibitors of ciprofloxacin efflux

- **ciprofloxacin efflux inhibited by ciprofloxacin**
  moxifloxacin



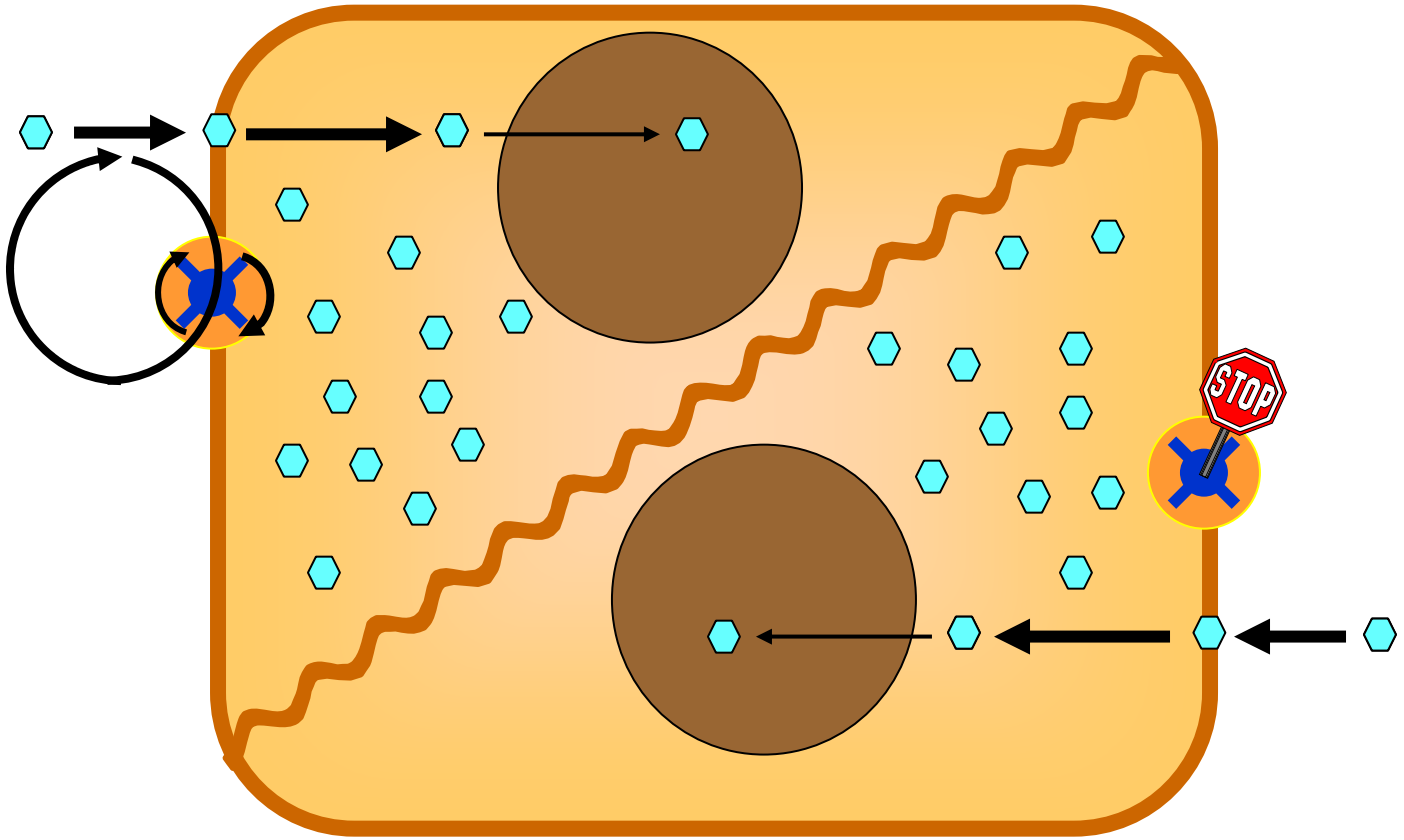


moxifloxacin also able to interact with the transporter !

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

Moxifloxacin, ‘futile-cycle’ model

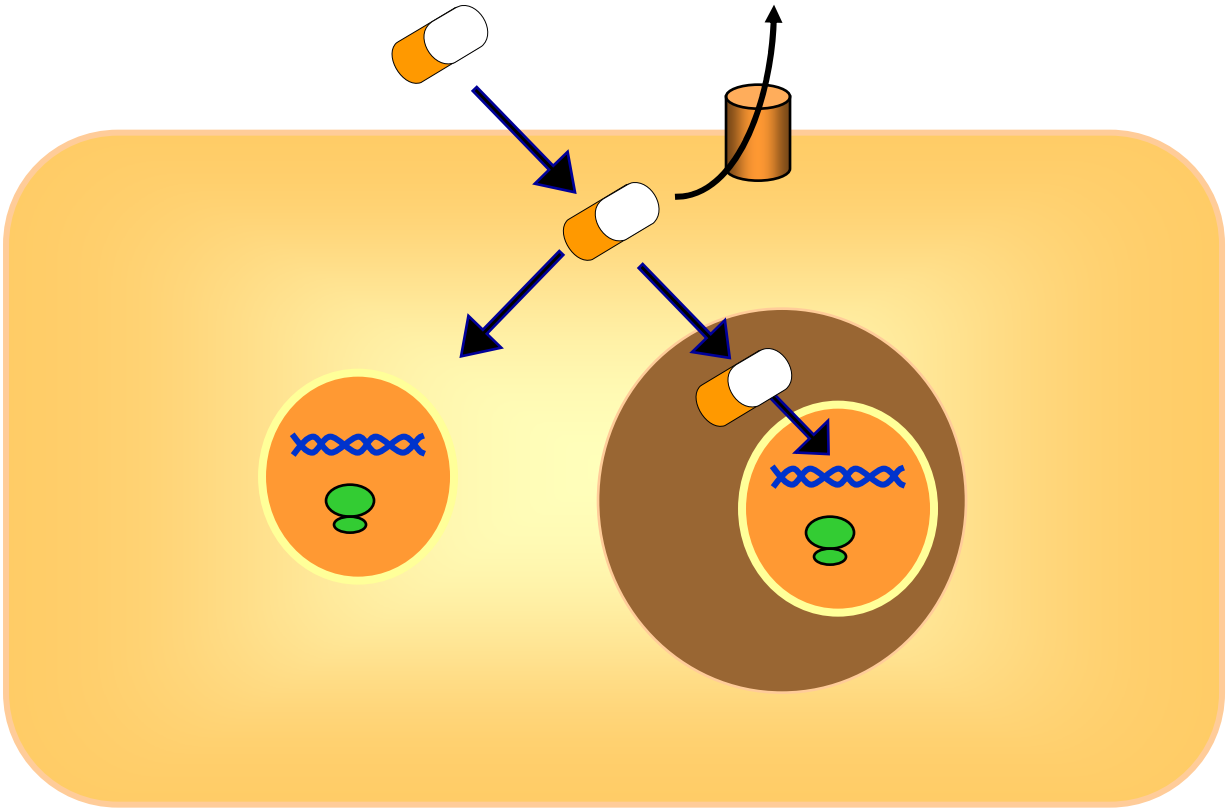
Eytan et al. (1996) JBC 271:12897-902



Influence of efflux pumps on antibiotic activity against intracellular infections

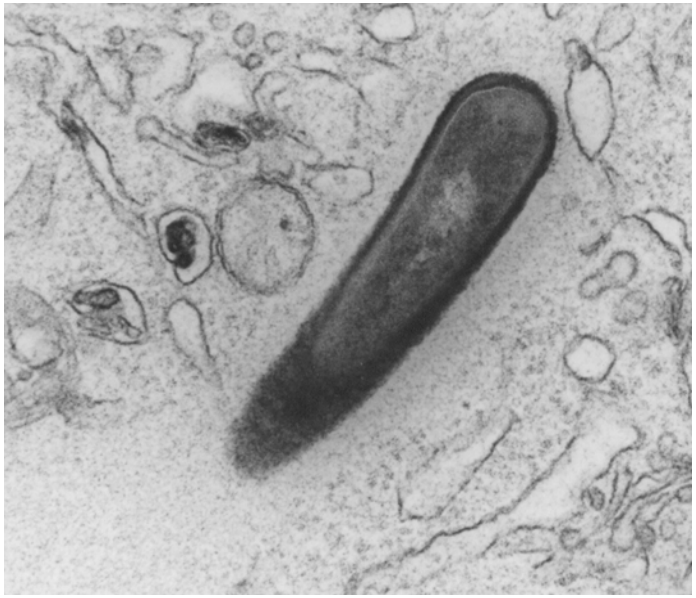
Belgian chocolate

Active efflux reduces antibiotic intracellular activity



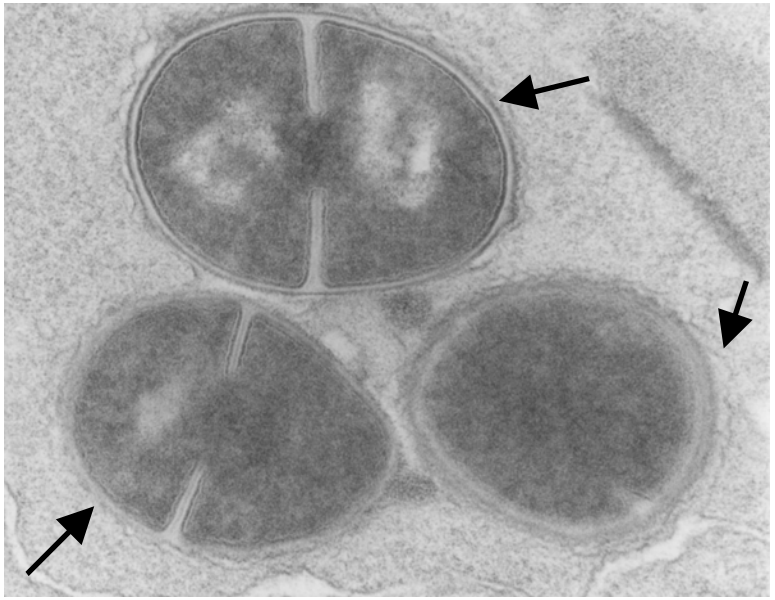
Models of intracellular infection

L. monocytogenes



cytosol

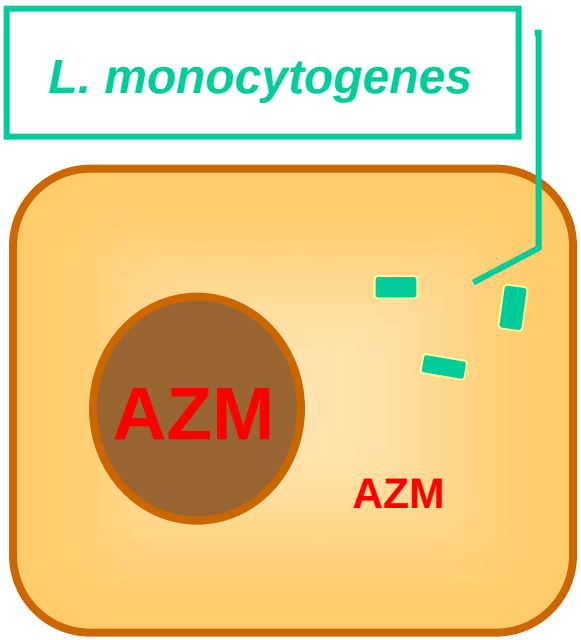
S. aureus

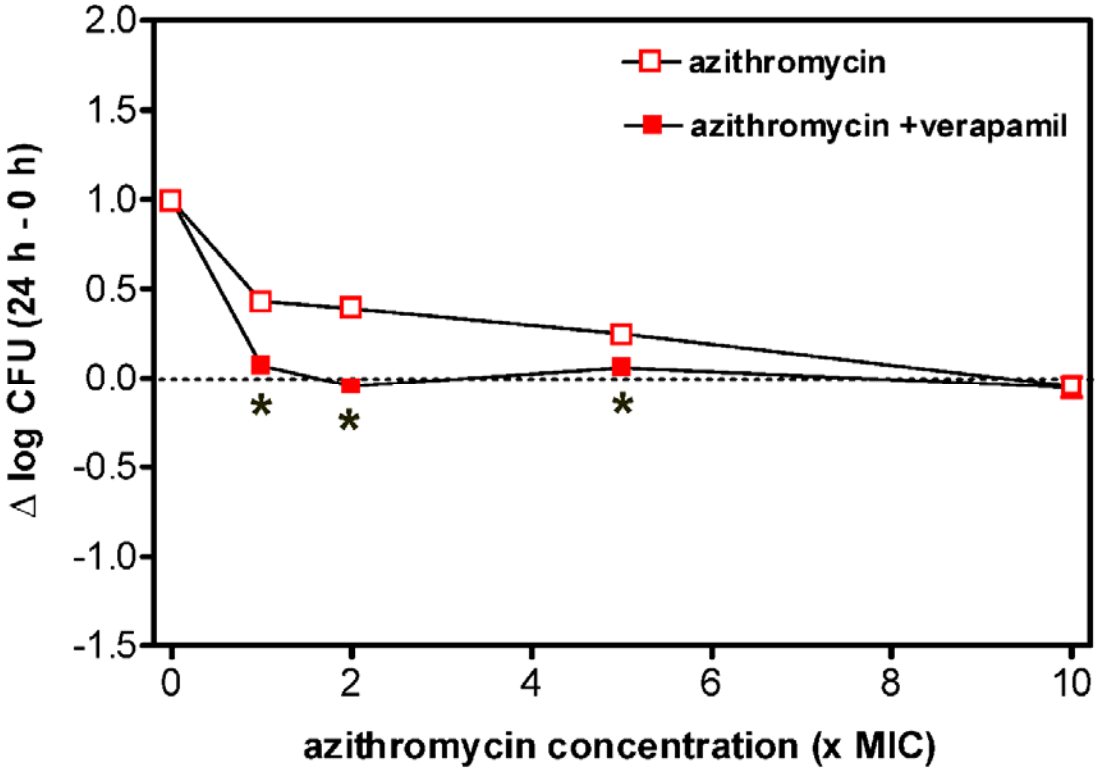


phagolysosomes

Influence of pump inhibitors on intracellular activity

azithromycin and *L. monocytogenes*



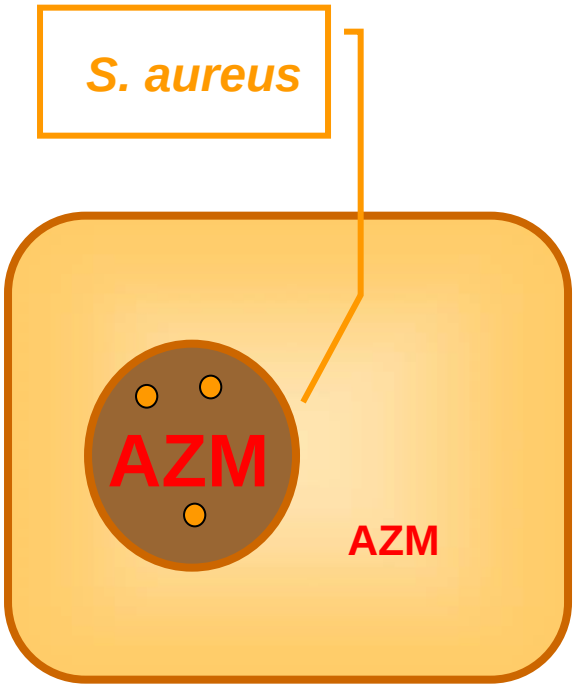


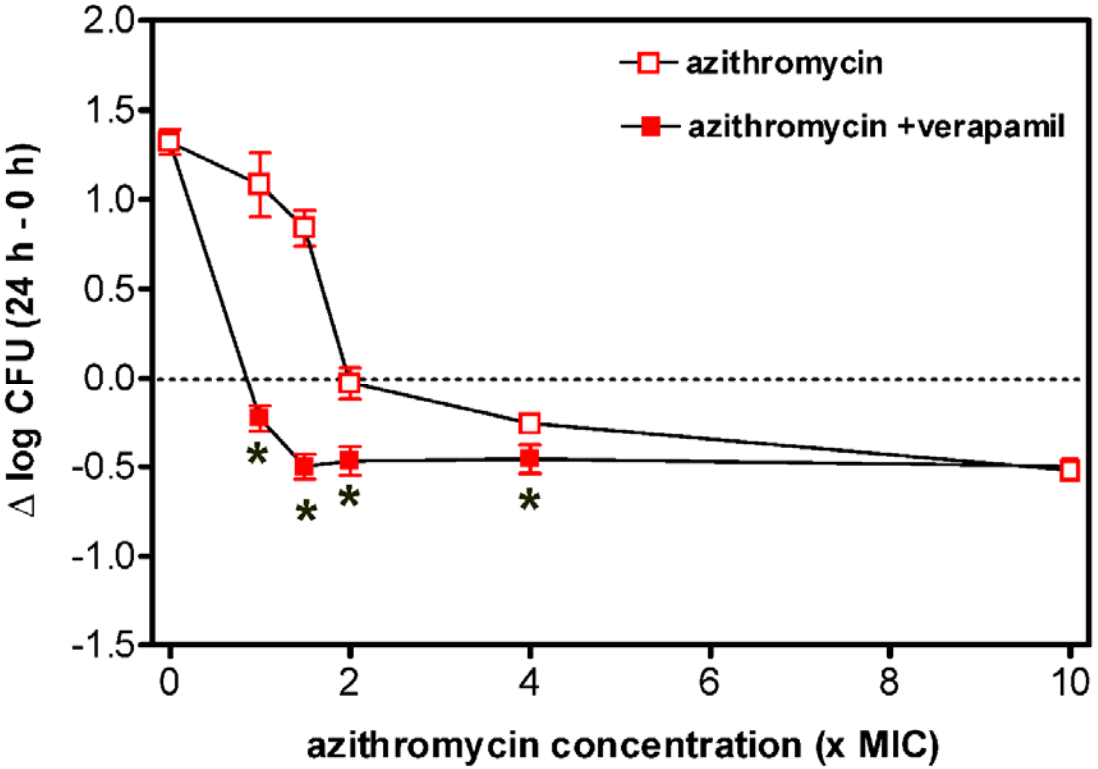
verapamil 20 µM; 24 h

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

Influence of pump inhibitors on intracellular activity

azithromycin and *S. aureus*



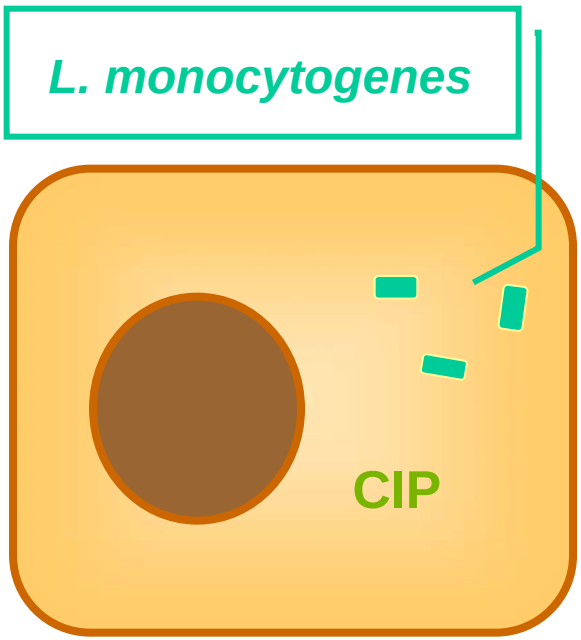


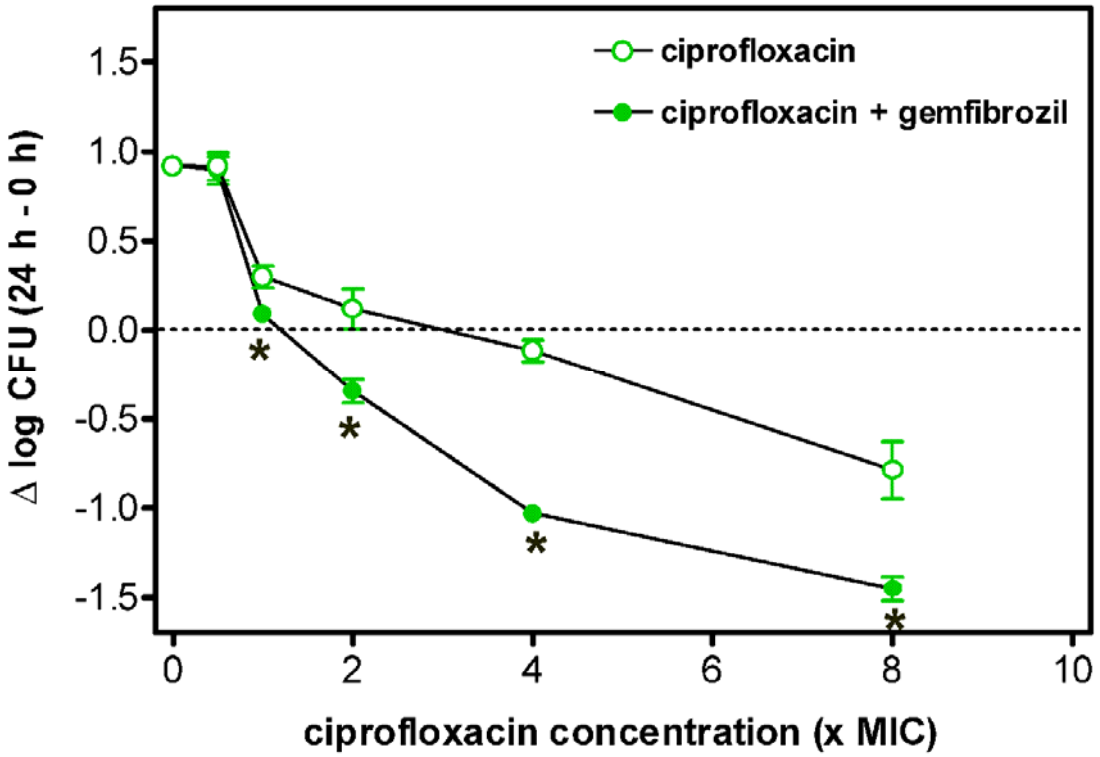
verapamil 20 µM; 24 h

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

Influence of pump inhibitors on intracellular activity

ciprofloxacin and *L. monocytogenes*



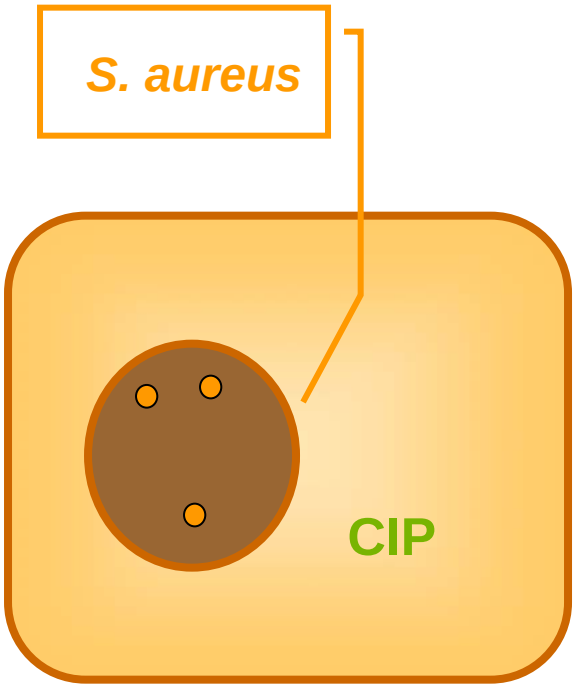


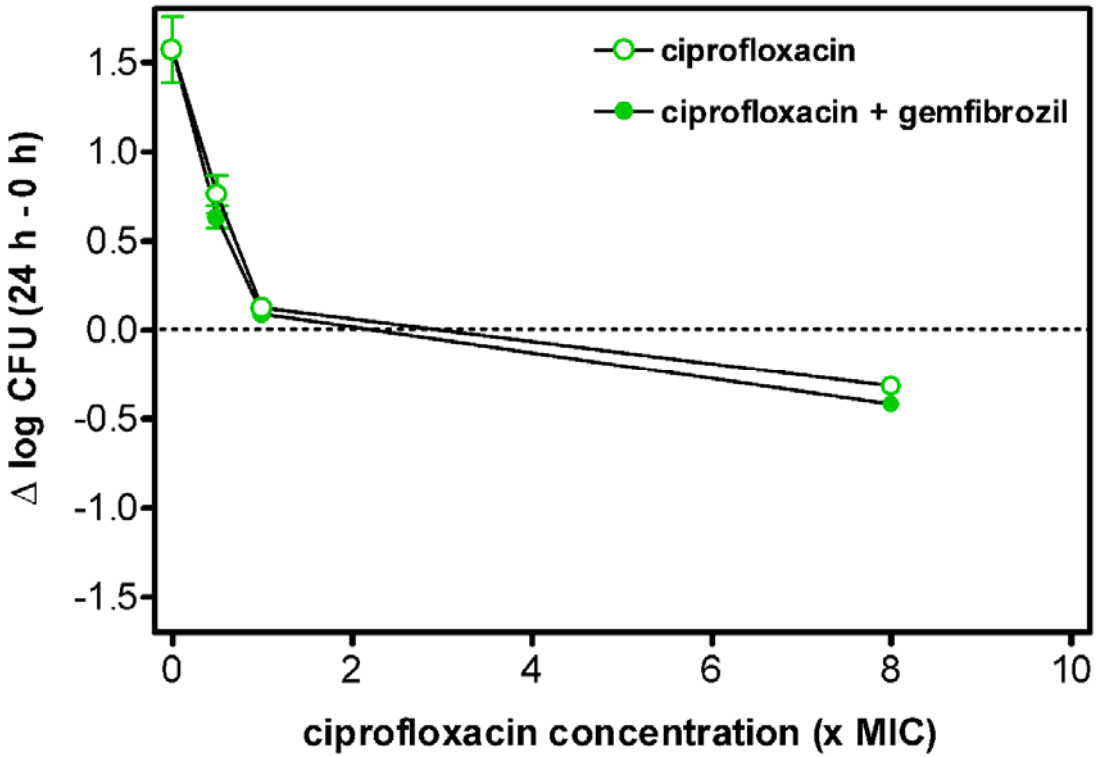
gemfibrozil 250 µM; 24 h

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

Influence of pump inhibitors on intracellular activity

ciprofloxacin and *S. aureus*



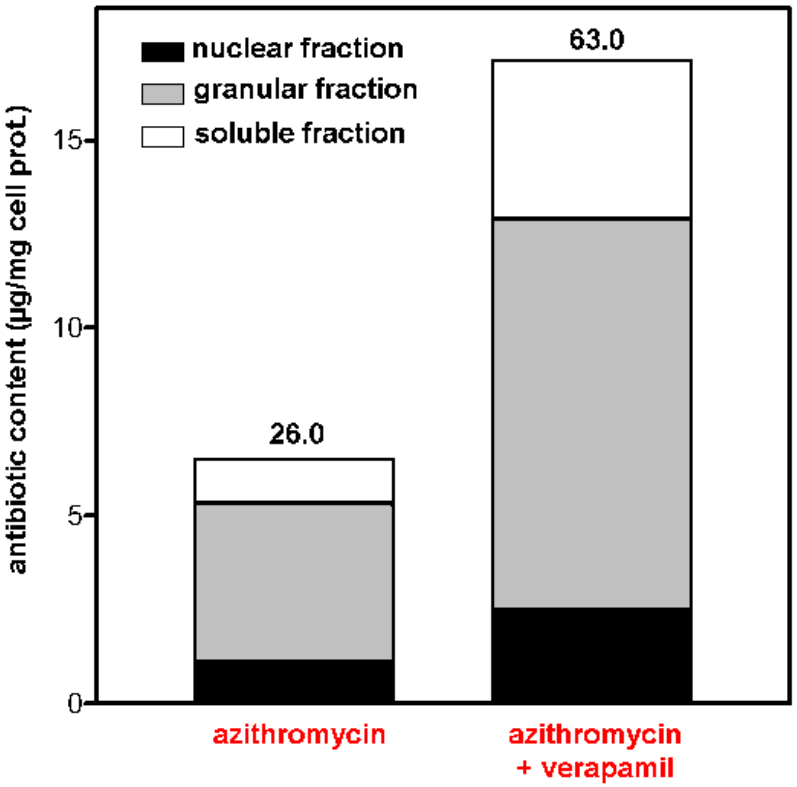


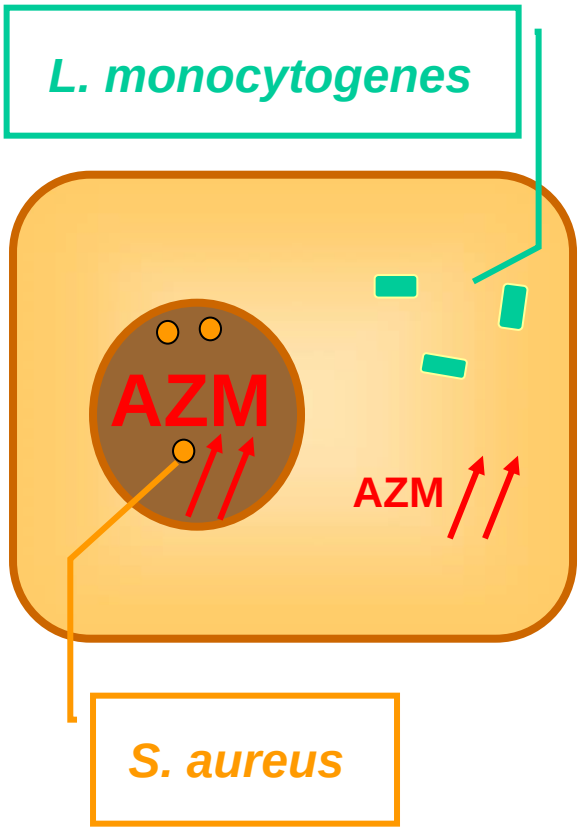
gemfibrozil 250 µM; 24 h

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

Influence of pump inhibitors on antibiotic distribution

verapamil enhances azithromycin concentration In cytosol and vacuoles

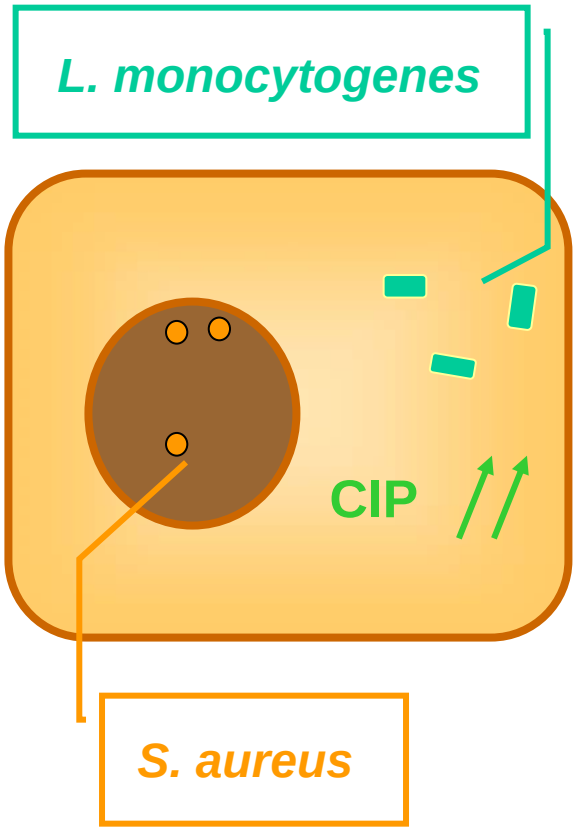


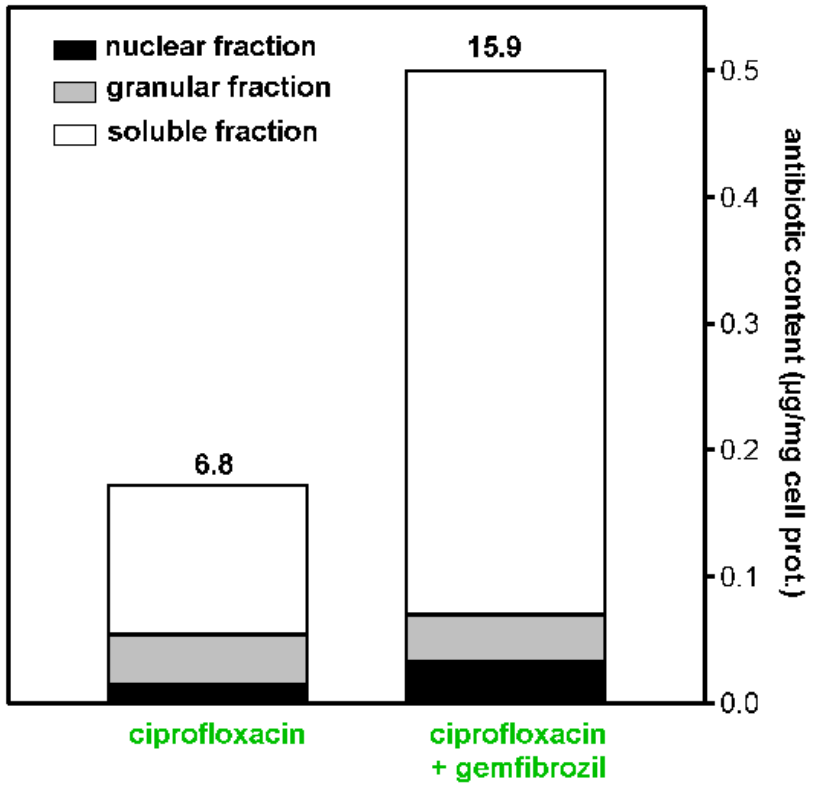


Seral et al. (2003) JAC 51:1167-73

Influence of pump inhibitors on antibiotic distribution

gemfibrozil enhances ciprofloxacin cytosolic content





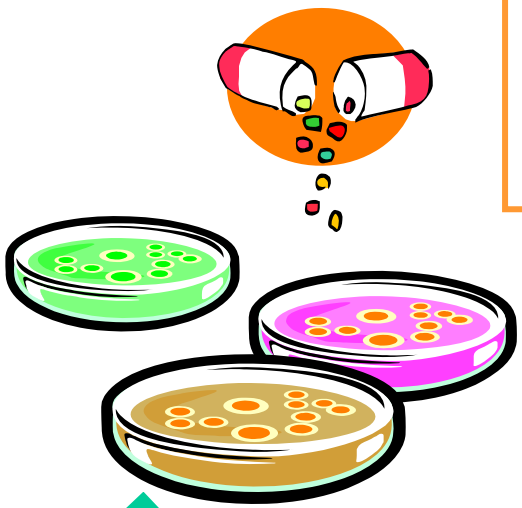
Seral et al. (2003) JAC 51:1167-73

Can we make eucaryotic cells « resistant » to antibiotics ? A way to further characterize efflux transporters

J.M. Folon, La Hulpe, Belgium

Over-expression of efflux pumps as mechanism of resistance

How to get resistant cells ?



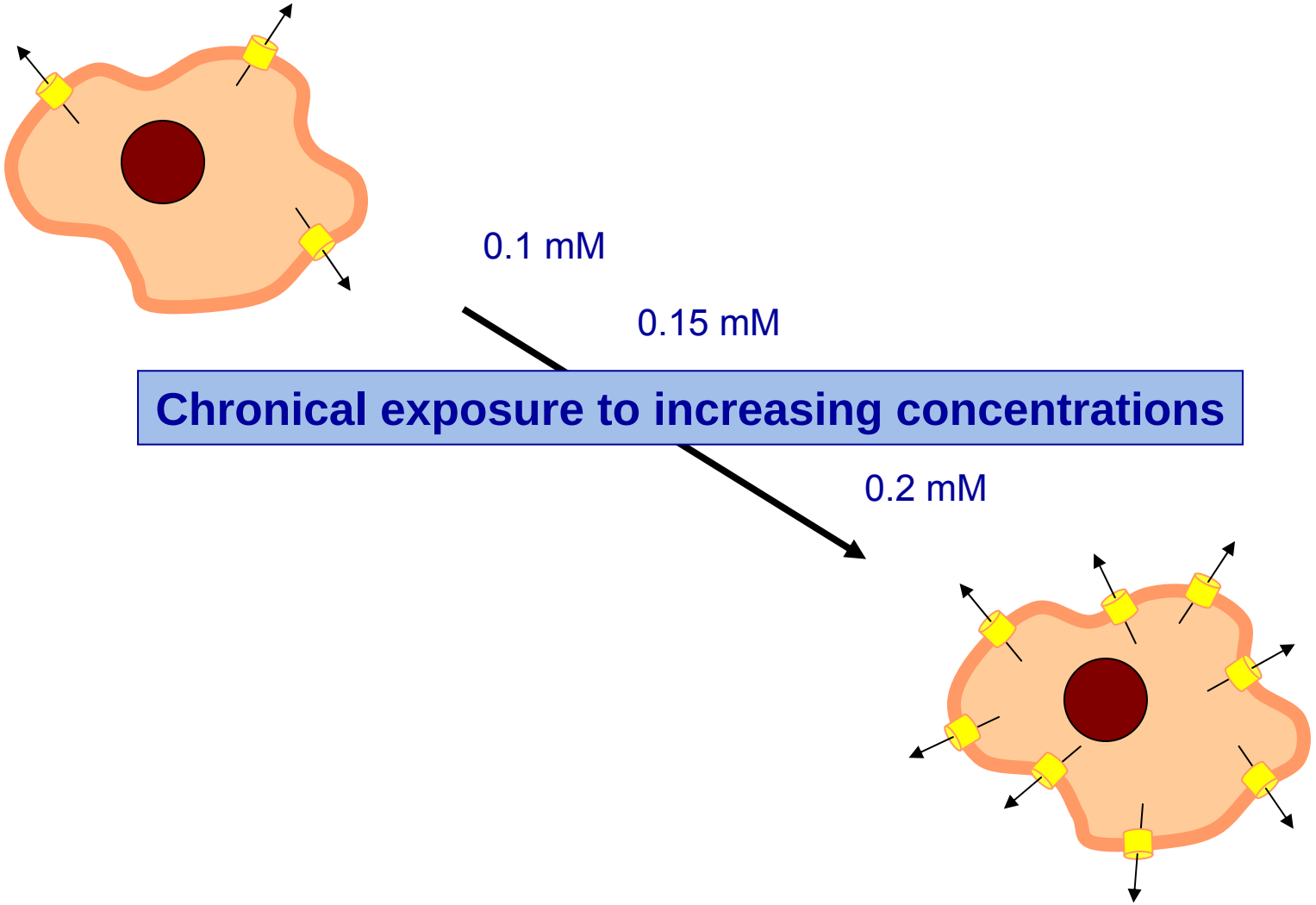
- step-wise increase in AB concentration
- several passages at each step

time and drug consumming!

multifactorial multidrug resistance

Gottesman et al, Methods Enzymol. (1998) 292: 248-58

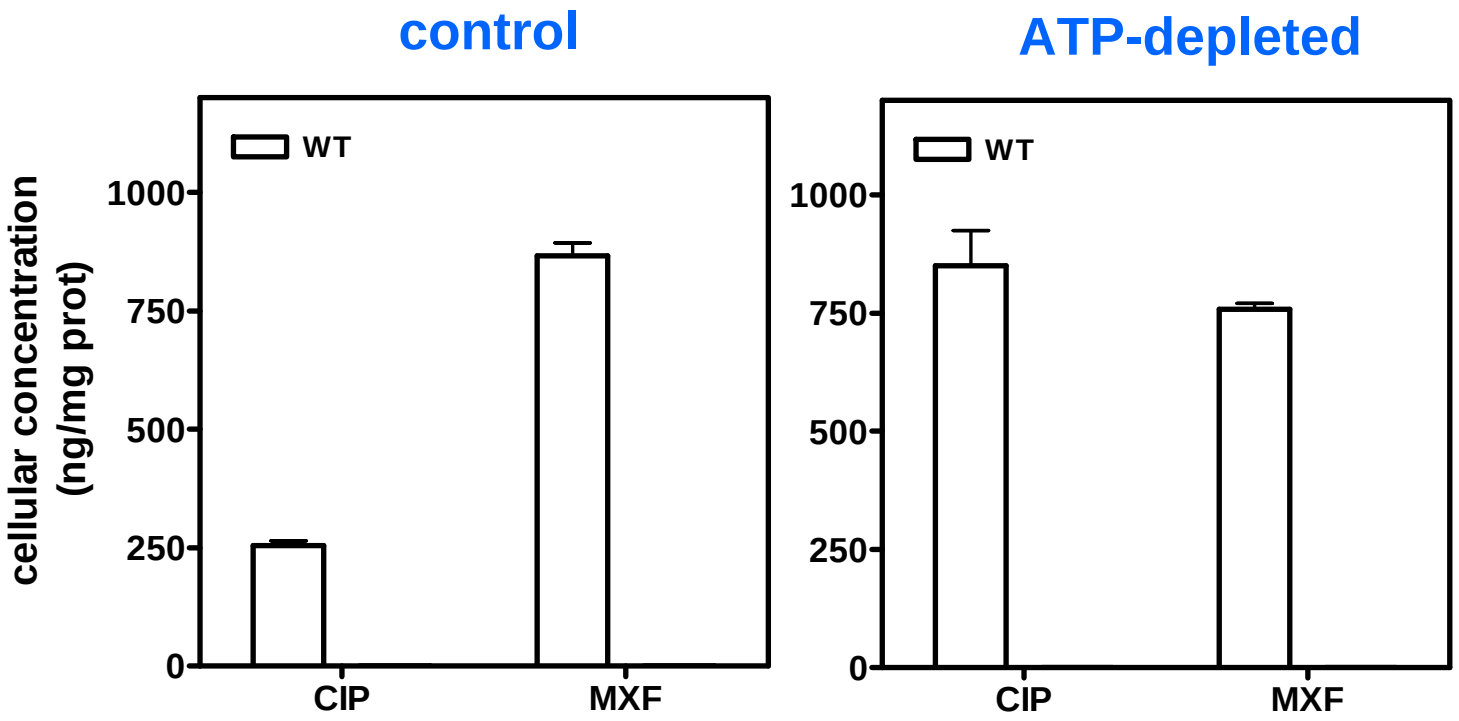
Identification of the ciprofloxacin transporter: « resistant » cells as a tool



Michot et al., Antimicrob. Ag. Chemother. (2006) 50:1689-1695

Ciprofloxacin « resistant » cells: phenotypic analysis

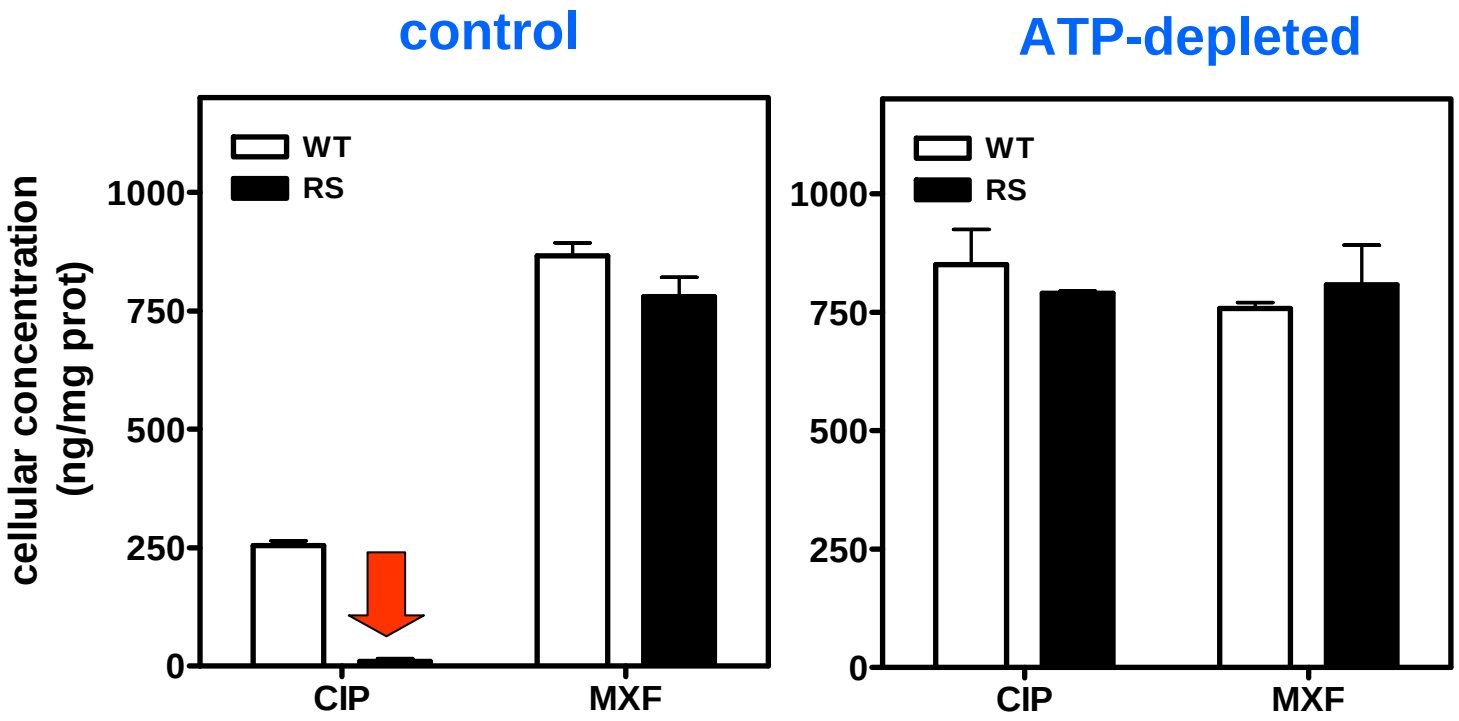
ATP-dependent reduction in cell accumulation of CIP; MXF non affected



Michot et al., Antimicrob. Ag. Chemother. (2006) 50:1689-1695

Ciprofloxacin « resistant » cells: phenotypic analysis

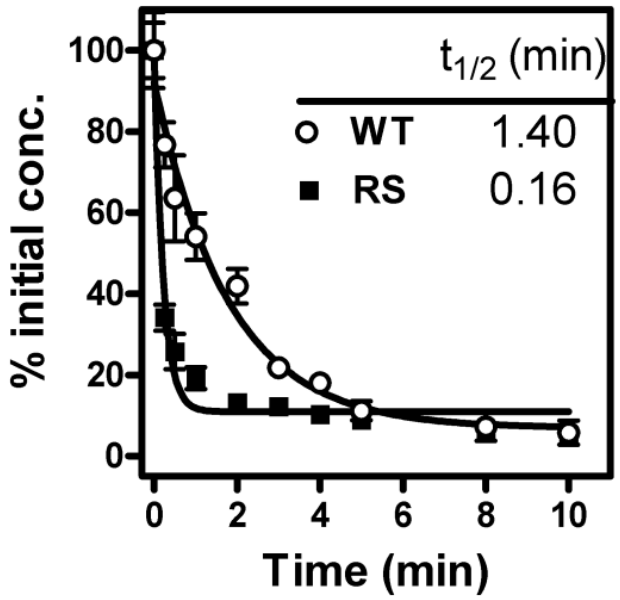
ATP-dependent reduction in cell accumulation of CIP; MXF non affected



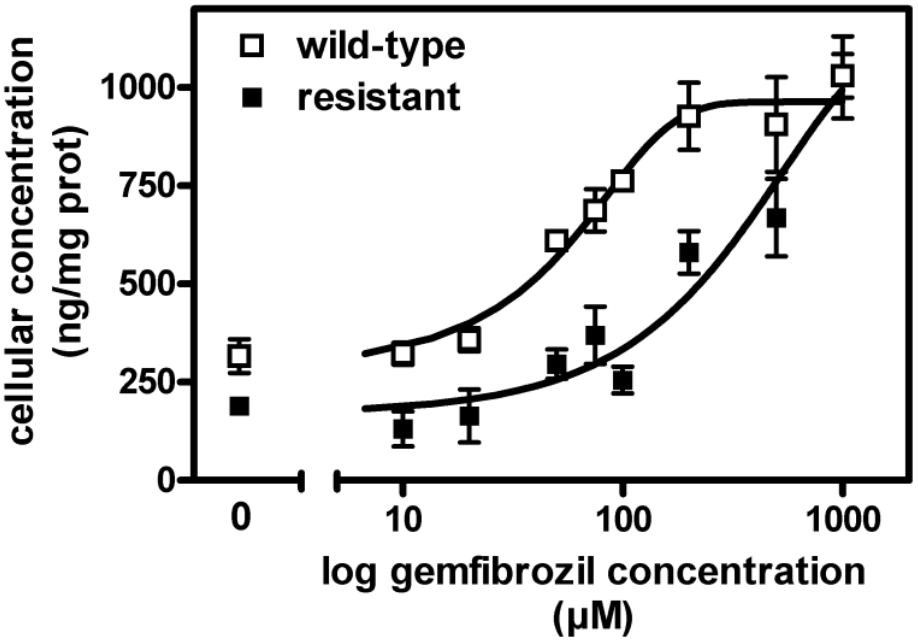
Michot et al., Antimicrob. Ag. Chemother. (2006) 50:1689-1695

Ciprofloxacin « resistant » cells: phenotypic analysis

↗ efflux rate



↗ IC_{50} gemfibrozil

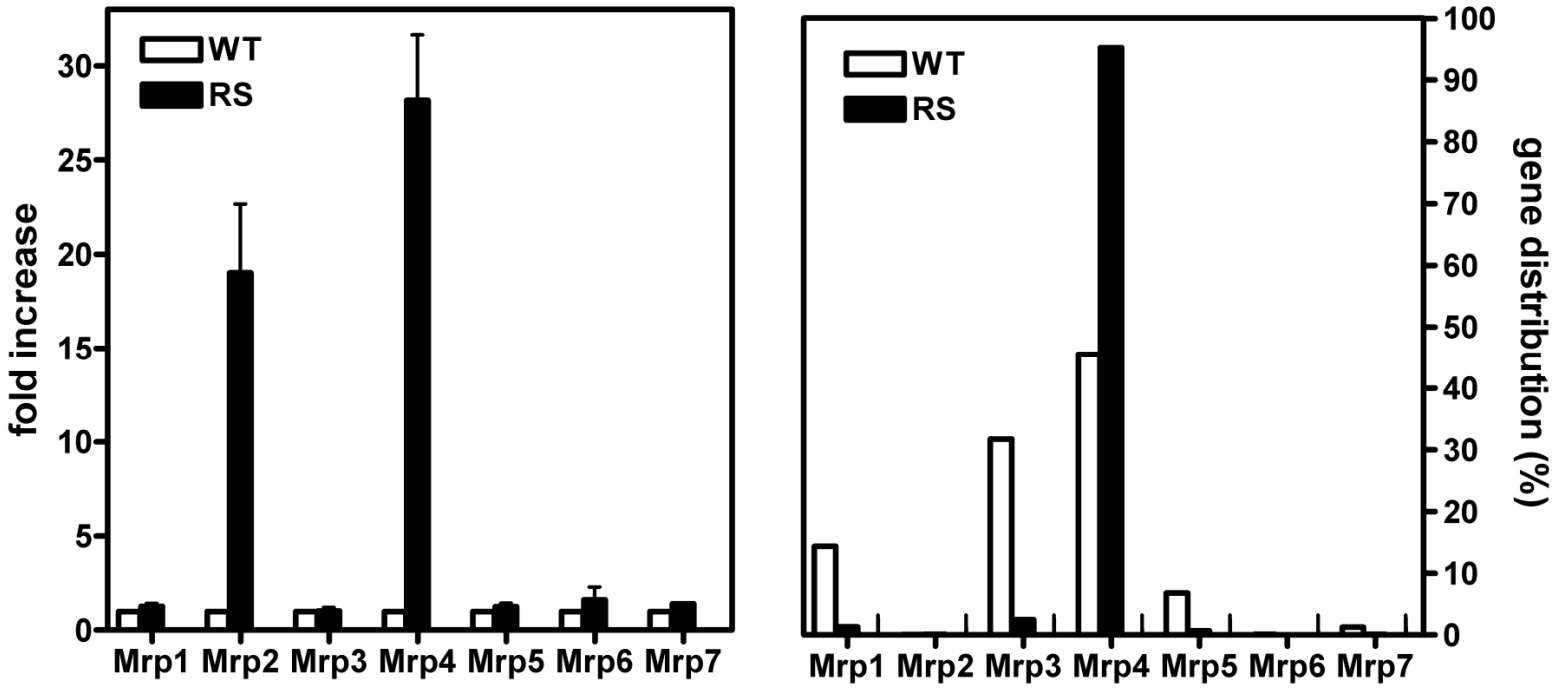


Marquez et al., Antimicrob. Ag. Chemother. (2009) 53:2410-6

Ciprofloxacin « resistant » cells: genotypic analysis

ARNm levels (Real-Time PCR)

↗ expression Mrp2 and Mrp4, but Mrp4 predominates



Marquez et al., Antimicrob. Ag. Chemother. (2009) 53:2410-6

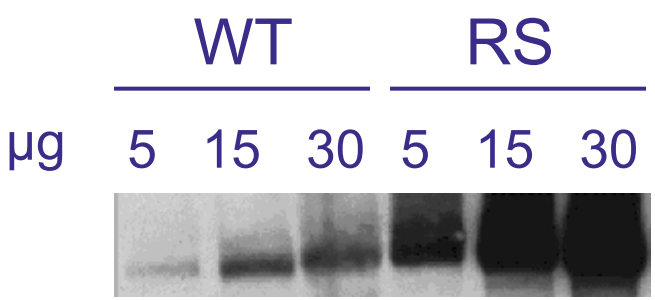
Ciprofloxacin « resistant » cells: proteomic analysis

detection of the proteins by

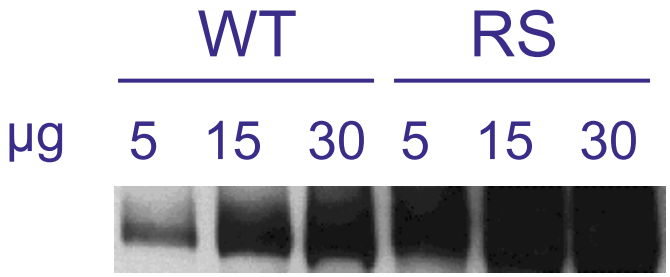
Western-Blot of membrane fraction

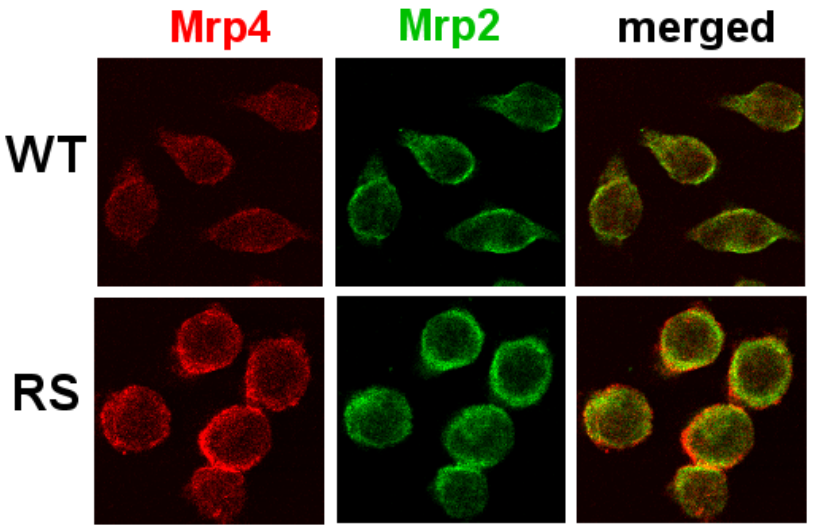
Confocal microscopy

Mrp2



Mrp4

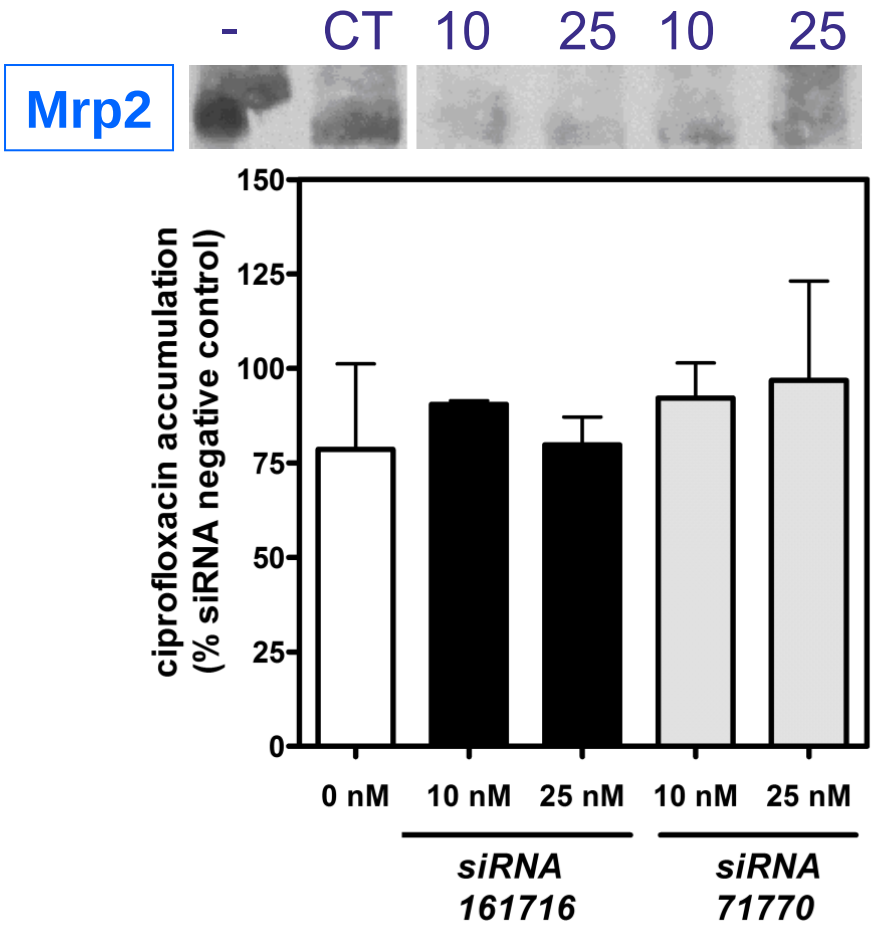


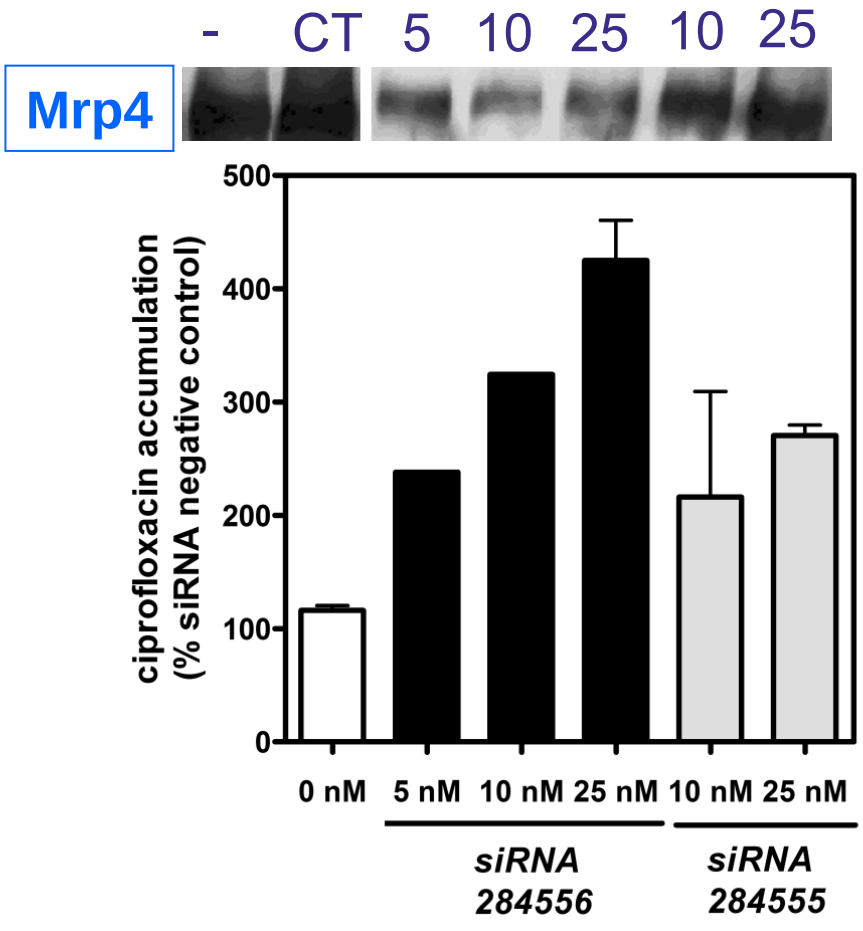


Marquez et al., Antimicrob. Ag. Chemother. (2009) 53:2410-6

Ciprofloxacin « resistant » cells : which transporter ?

Specific extinction of gene expression by siRNA

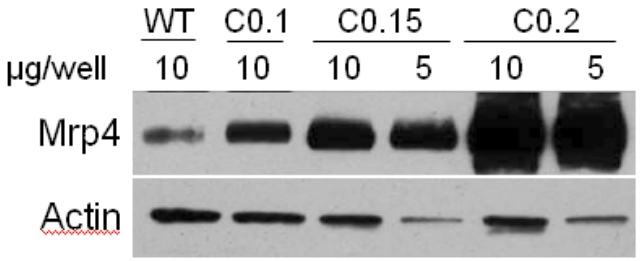


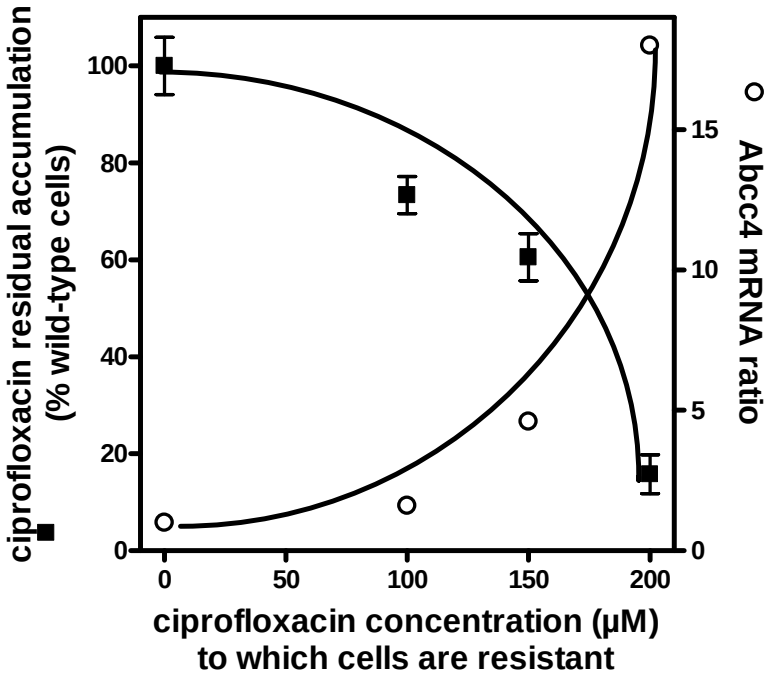


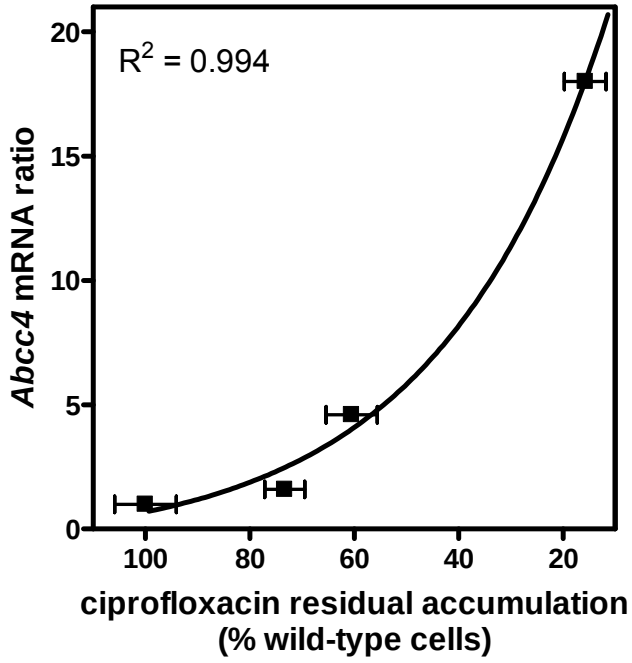
Marquez et al., Antimicrob. Ag. Chemother. (2009) 53:2410-6

Acquisition of resistance is a stepwise process

Accumulation and Mrp4 expression during selection of resistance

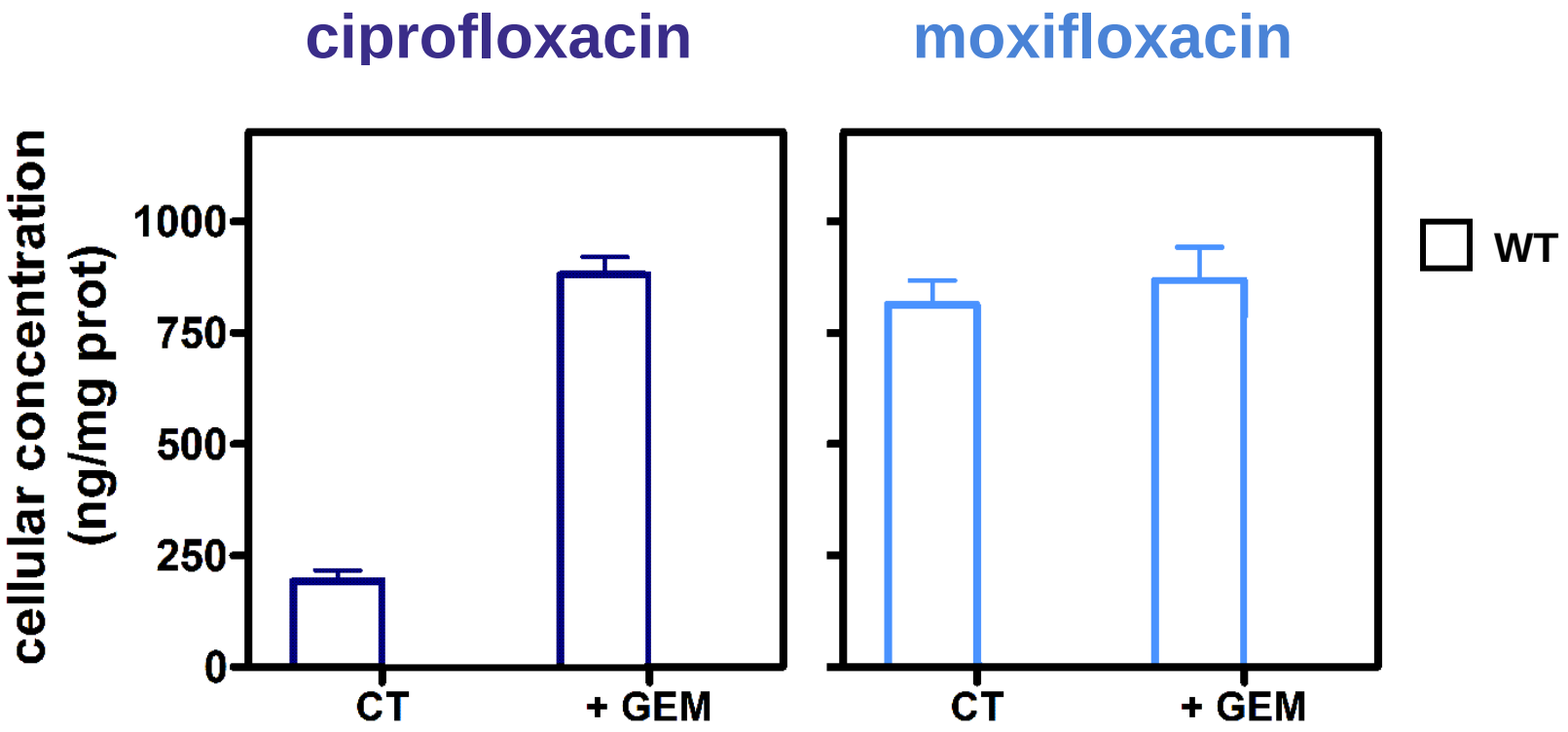






Can we select for moxifloxacin « resistant » cells ?

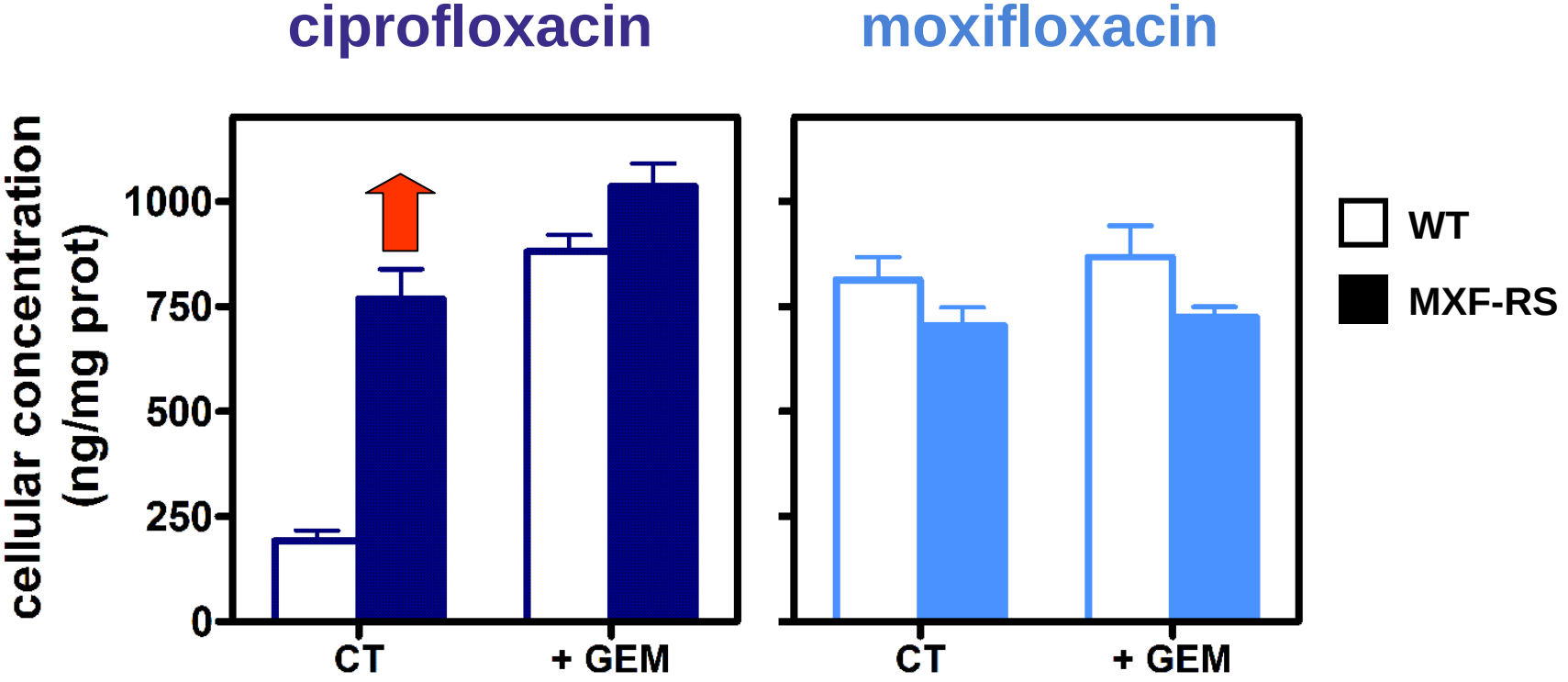
FQ accumulation and gemfibrozil effect



Vallet et al., FEBS-ABC meeting. (2010)

Can we select for moxifloxacin « resistant » cells ?

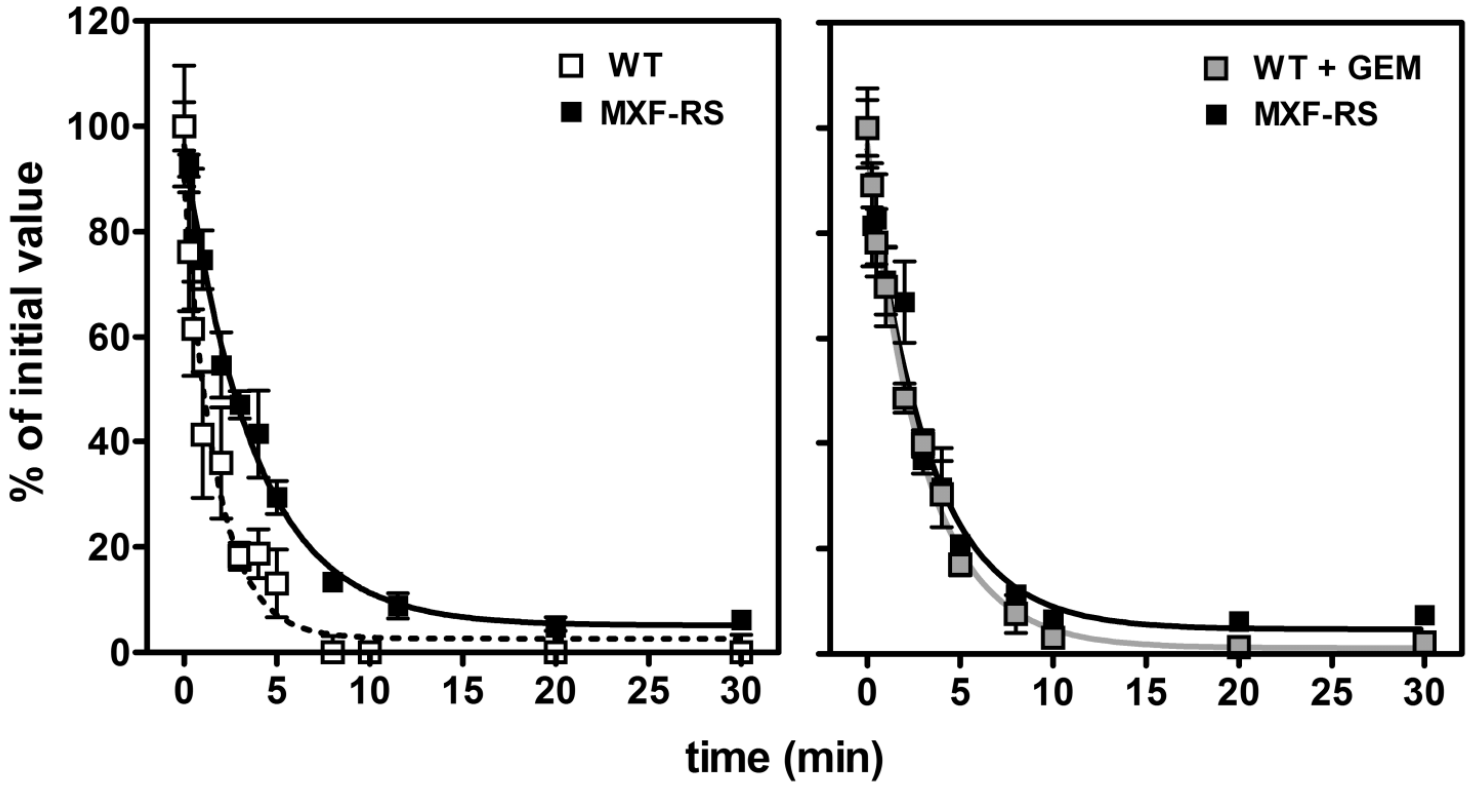
FQ accumulation and gemfibrozil effect



Vallet et al., FEBS-ABC meeting. (2010)

Can we select for moxifloxacin « resistant » cells ?

Ciprofloxacin efflux



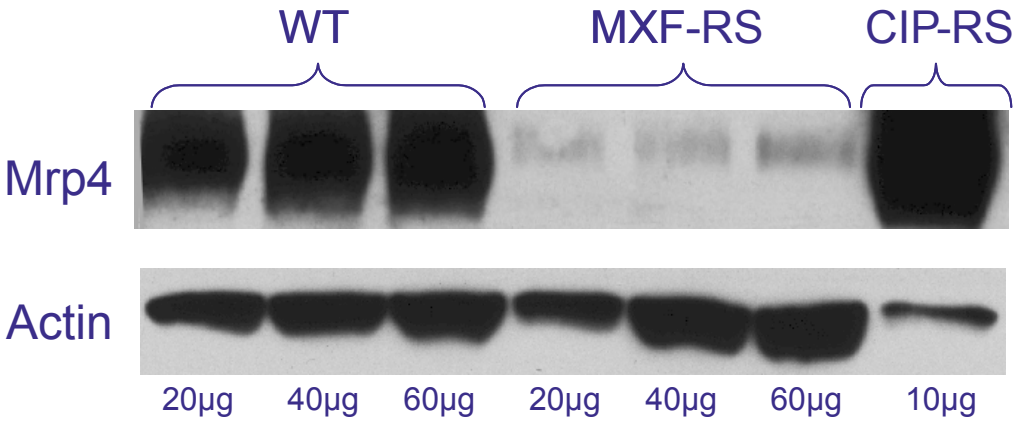
$T_{1/2}$ WT >> $T_{1/2}$ WT+GEM = $T_{1/2}$ MXF-RS

Vallet et al., FEBS-ABC meeting. (2010)

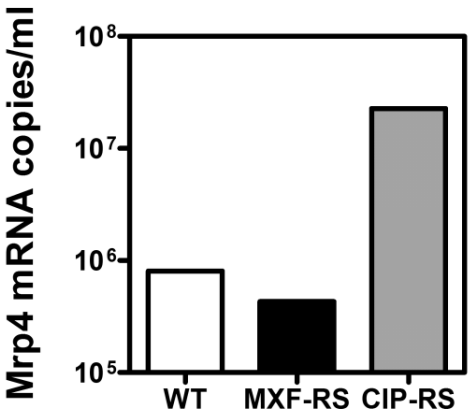
Moxifloxacin-exposed cells are « anti » resistant!

Mrp4 expression

Western-Blot

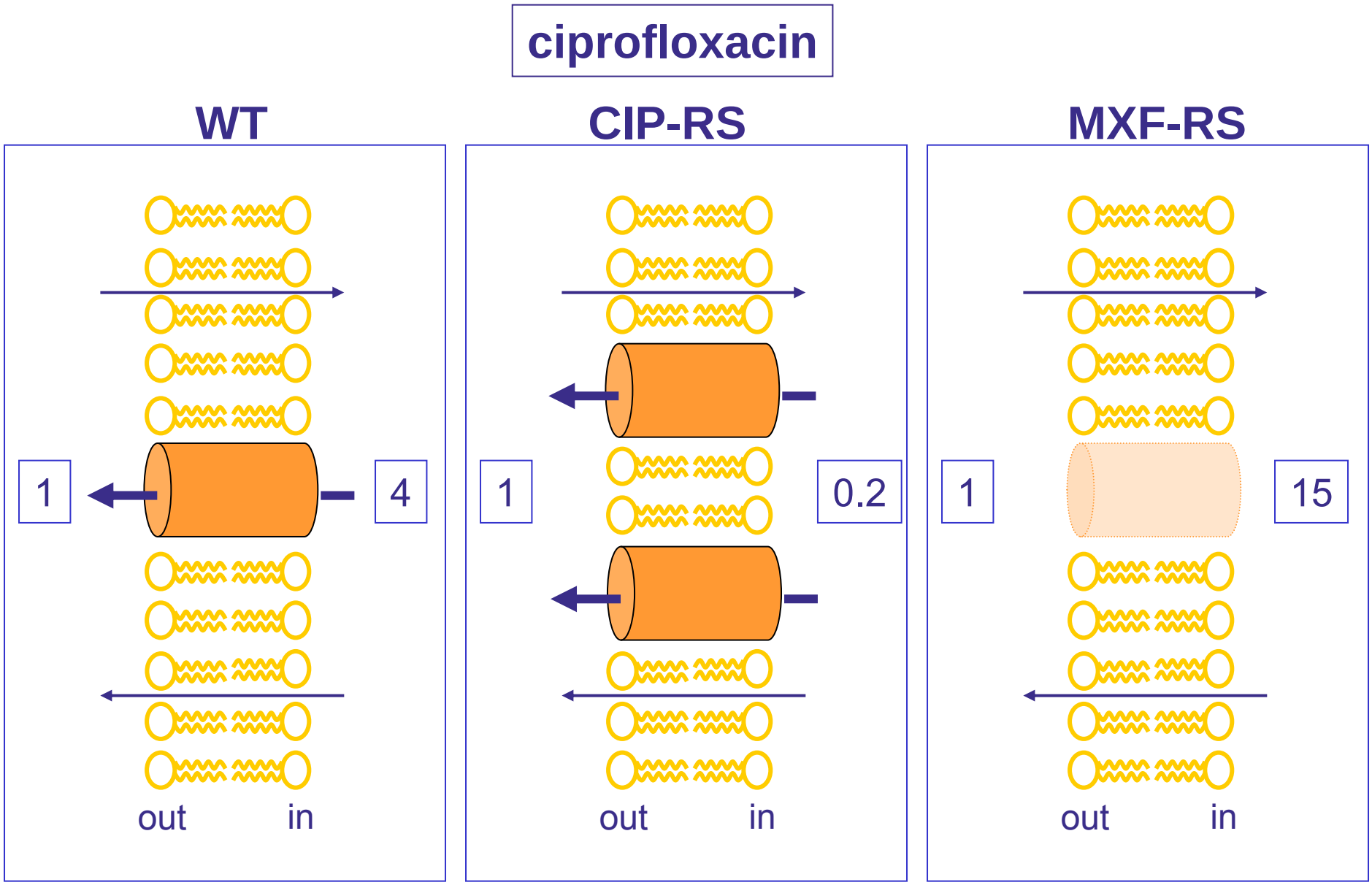


ARNm (Real Time PCR)



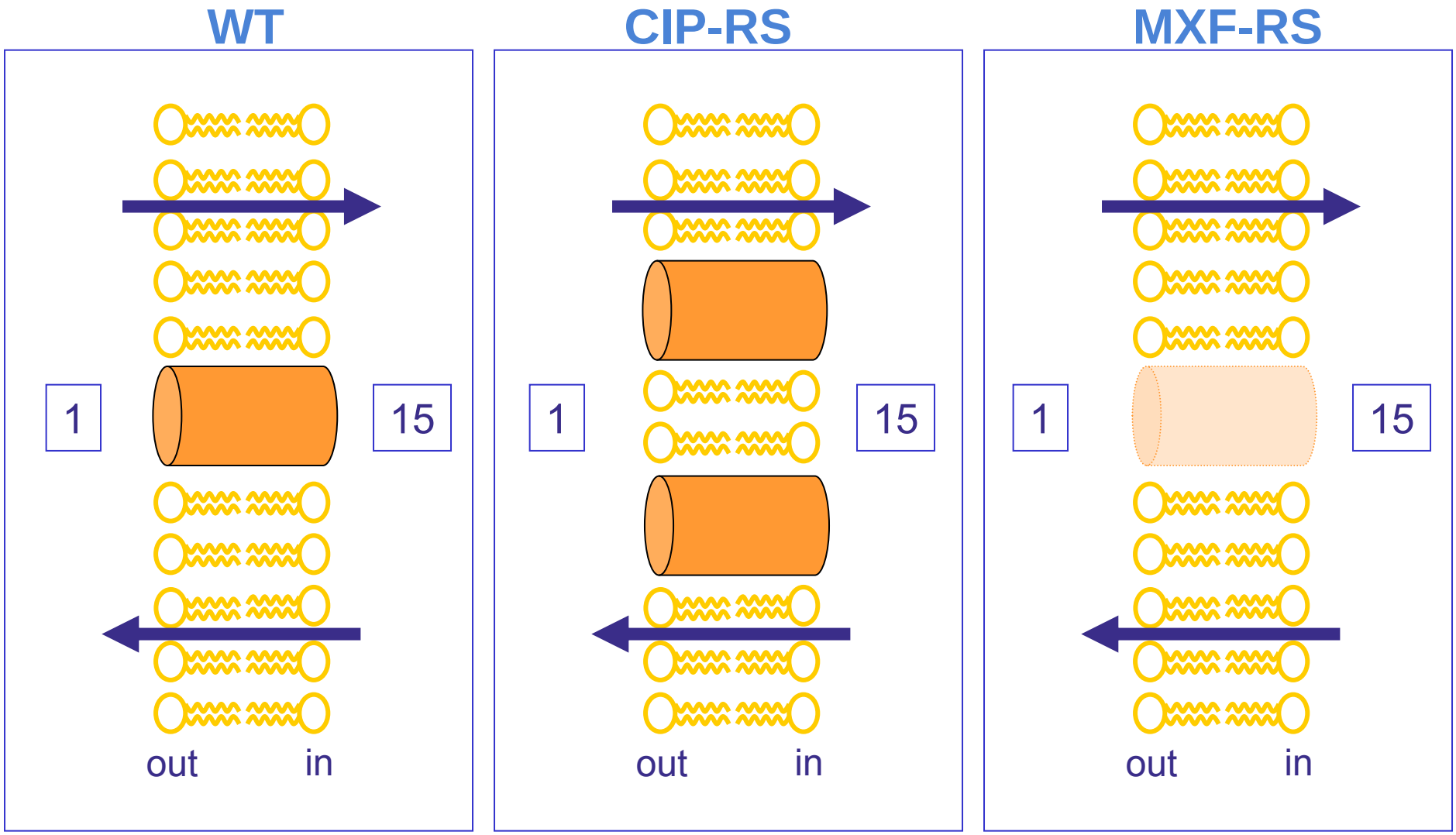
Vallet et al., FEBS-ABC meeting. (2010)

Fluoroquinolone transport: model



Fluoroquinolone transport: model

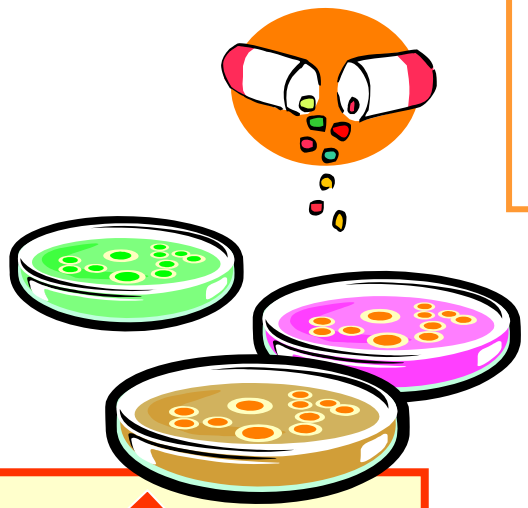
moxifloxacin



Efflux pumps … and what next ?

How to get resistant cells ?

- step-wise increase in AB concentration
- several passages at each step

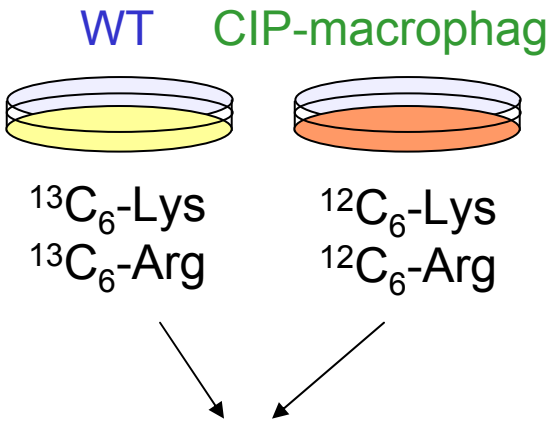


time and drug consumming!

multifactorial multidrug resistance

Gottesman et al, Methods Enzymol. (1998) 292: 248-58

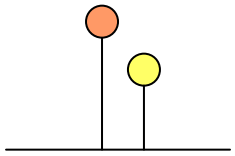
Stable Isotope Labeling Aminoacid in Culture



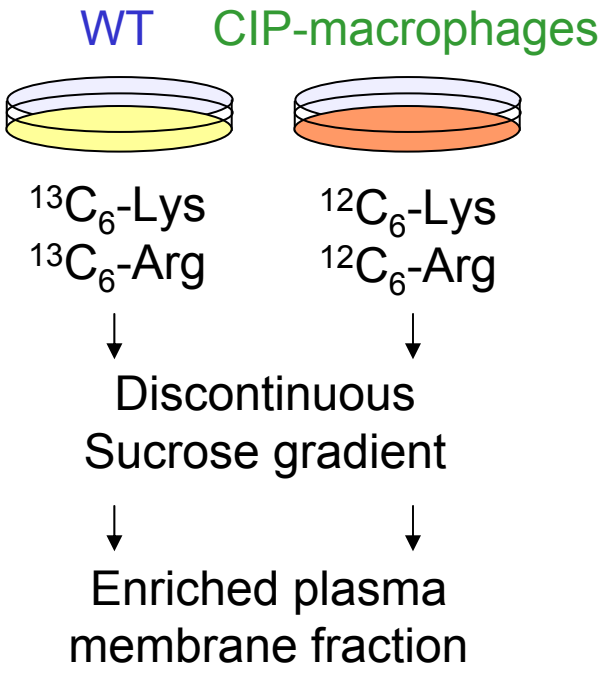
sample mixing 1:1

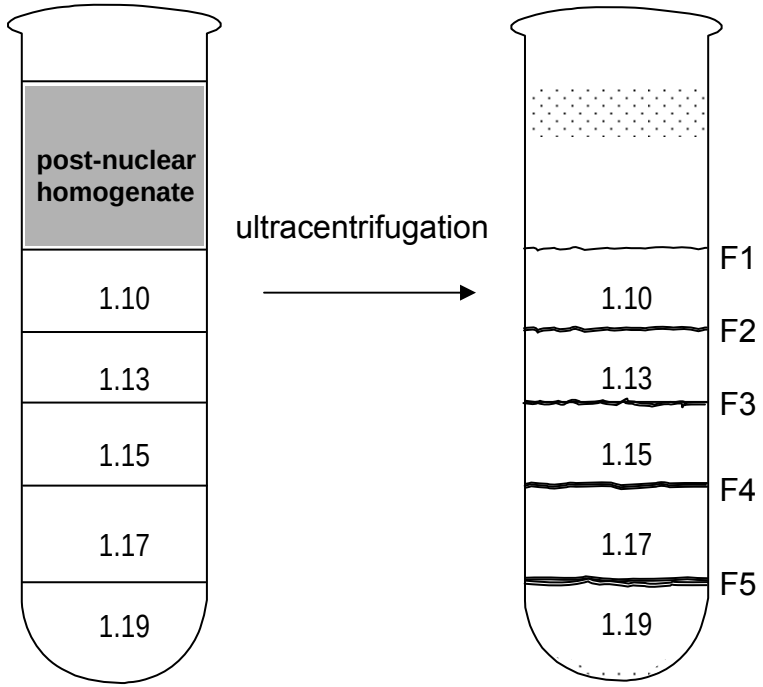
protein digestion

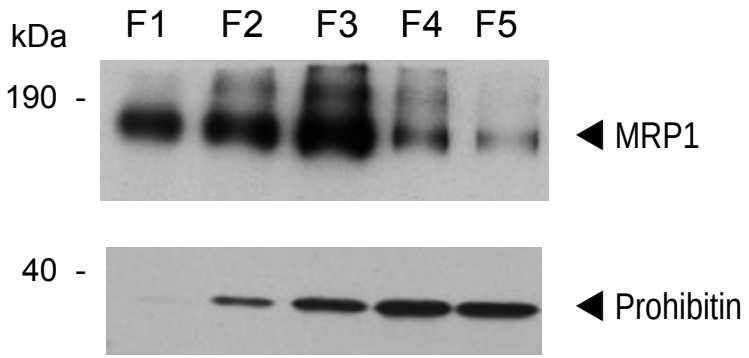
identification in mass spectrometry
and determination of the relative abundance



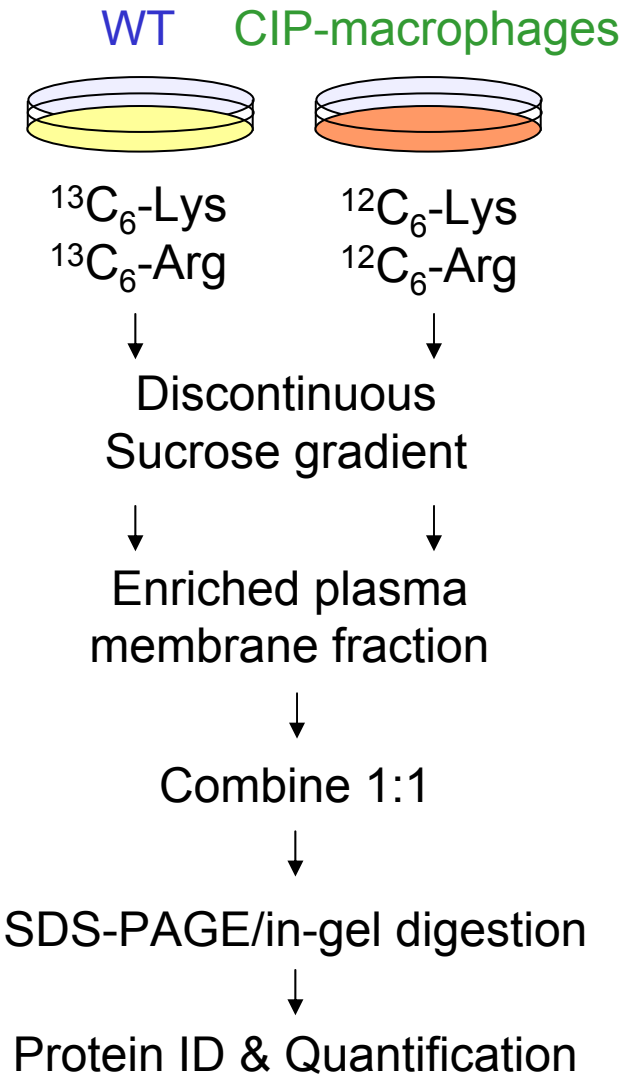
Stable Isotope Labeling Aminoacid in Culture

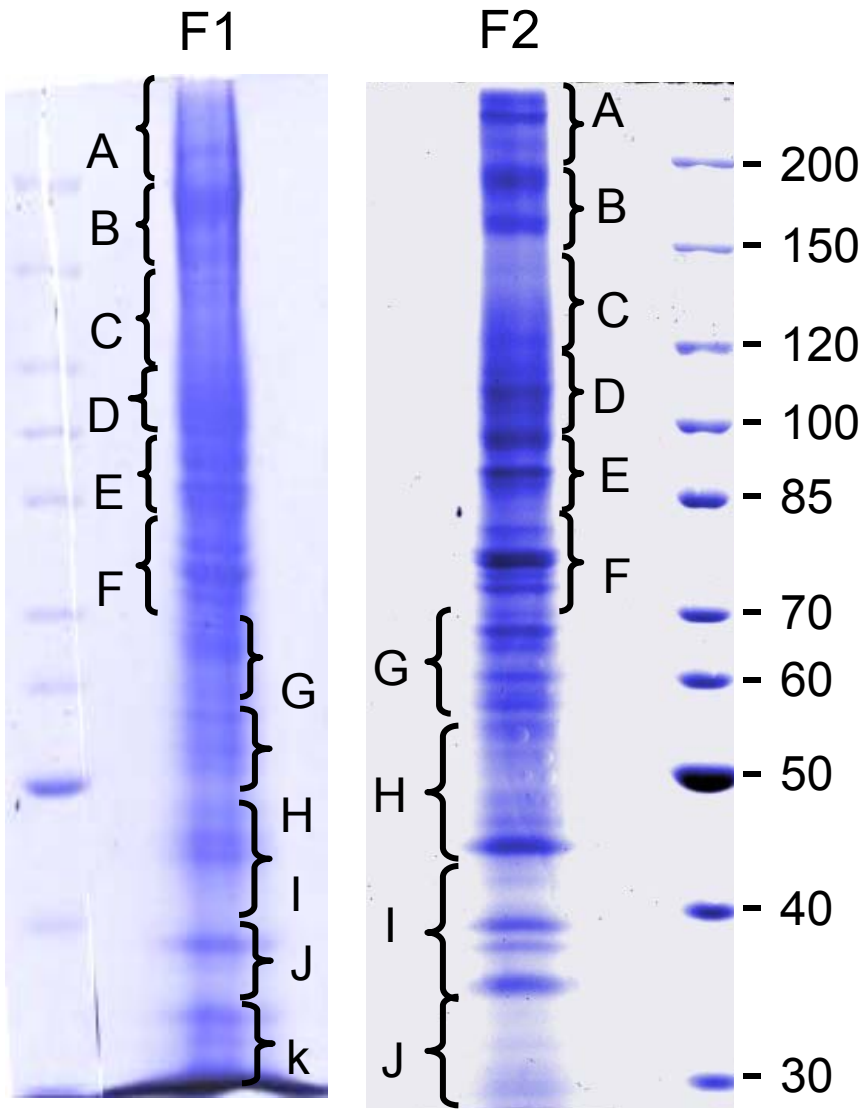






Stable Isotope Labeling Aminoacid in Culture





SILAC: global data

⇨ Identification of 900 proteins with 3 uniques peptides

Among proteins detected in both fractions

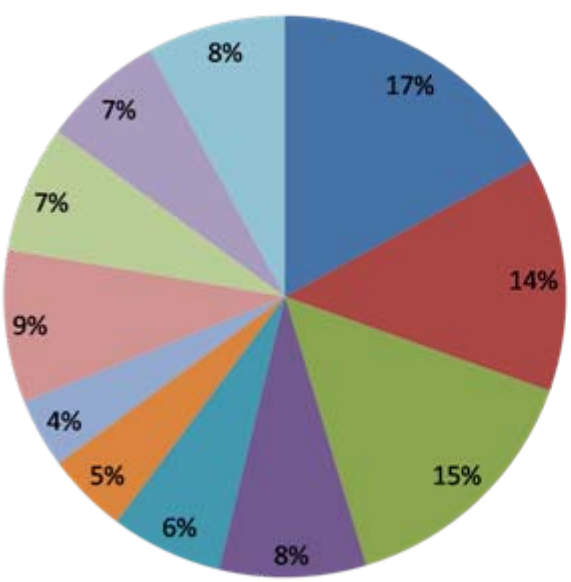
- ↳ 15 ↗ expression in CIP-macrophages as compared to WT
- ↳ 13 ↘ expression in CIP-macrophages as compared to WT

Among proteins detected in one of the two fractions

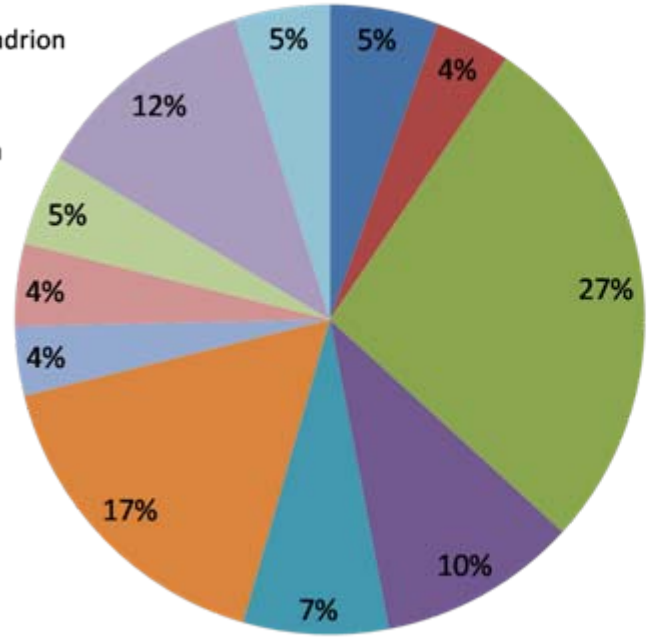
- ↳ 37/36 ↗ expression in CIP-macrophages as compared to WT
- ↳ 29/34 ↘ expression in CIP-macrophages as compared to WT

Marquez et al, FEBS 2009

Proteins with modified expression in CIP-resistant cells

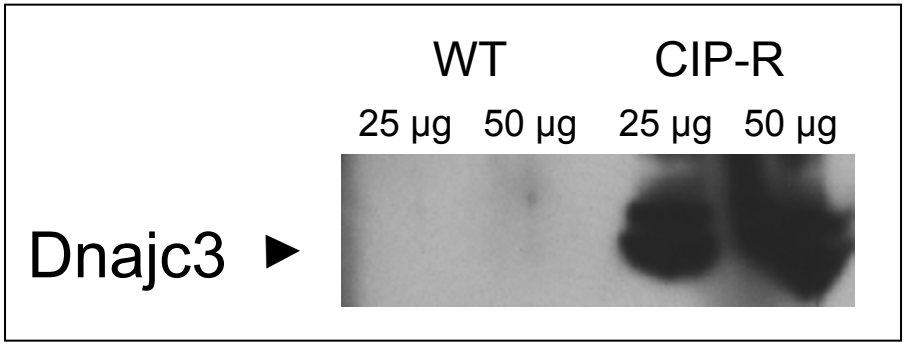


- Plasma membrane
- Unknown membrane protein
- ER/Golgi
- Endosome/Lysosome
- Cytoskeleton
- Ribosome
- Extracellular
- Nucleus
- Mitochondrion
- Cytosol
- Unknown



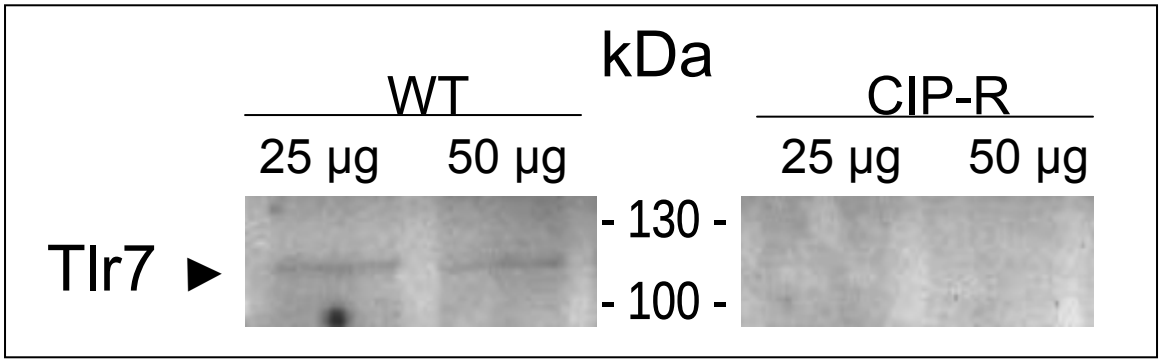
- Cell adhesion and localization
- Immune response
- Transport
- Signal transduction
- Cytoskeleton organization
- Protein metabolism
- Carbohydrate metabolism
- Lipid metabolism
- Nucleid acid metabolism
- Other
- Unknown

Proteins with modified expression in CIP-resistant cells



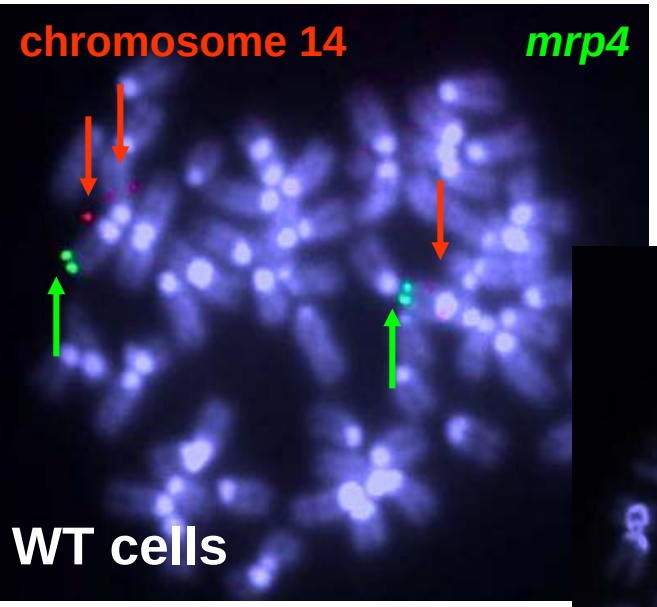
On the same chromosome as *mrp4* !

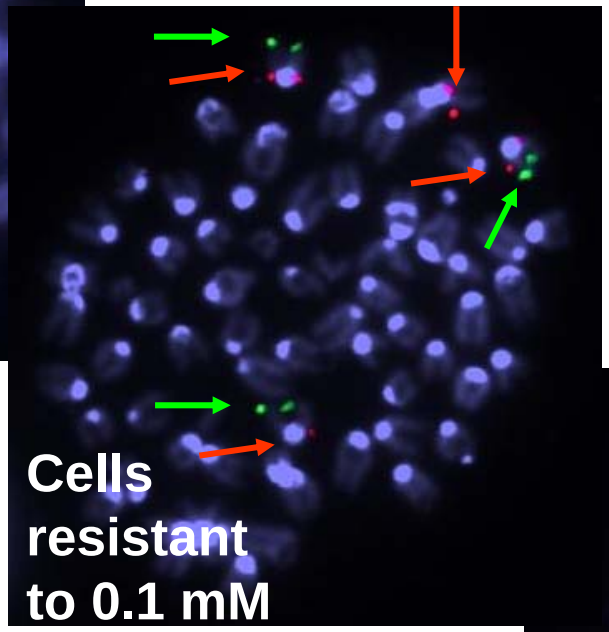
Transports nucleosides/tides (substrates for Mrp4 !)

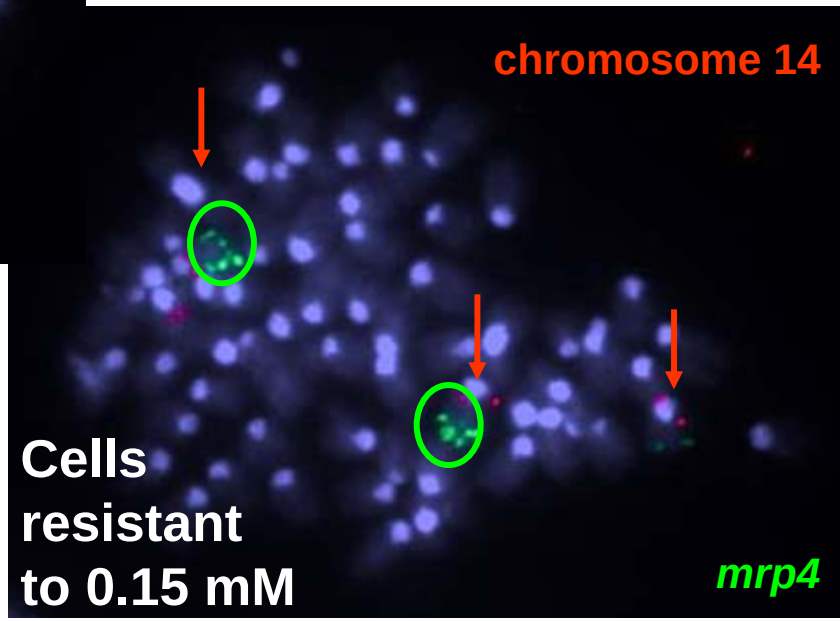


Gene amplification in CIP-resistant cells

FISH analysis



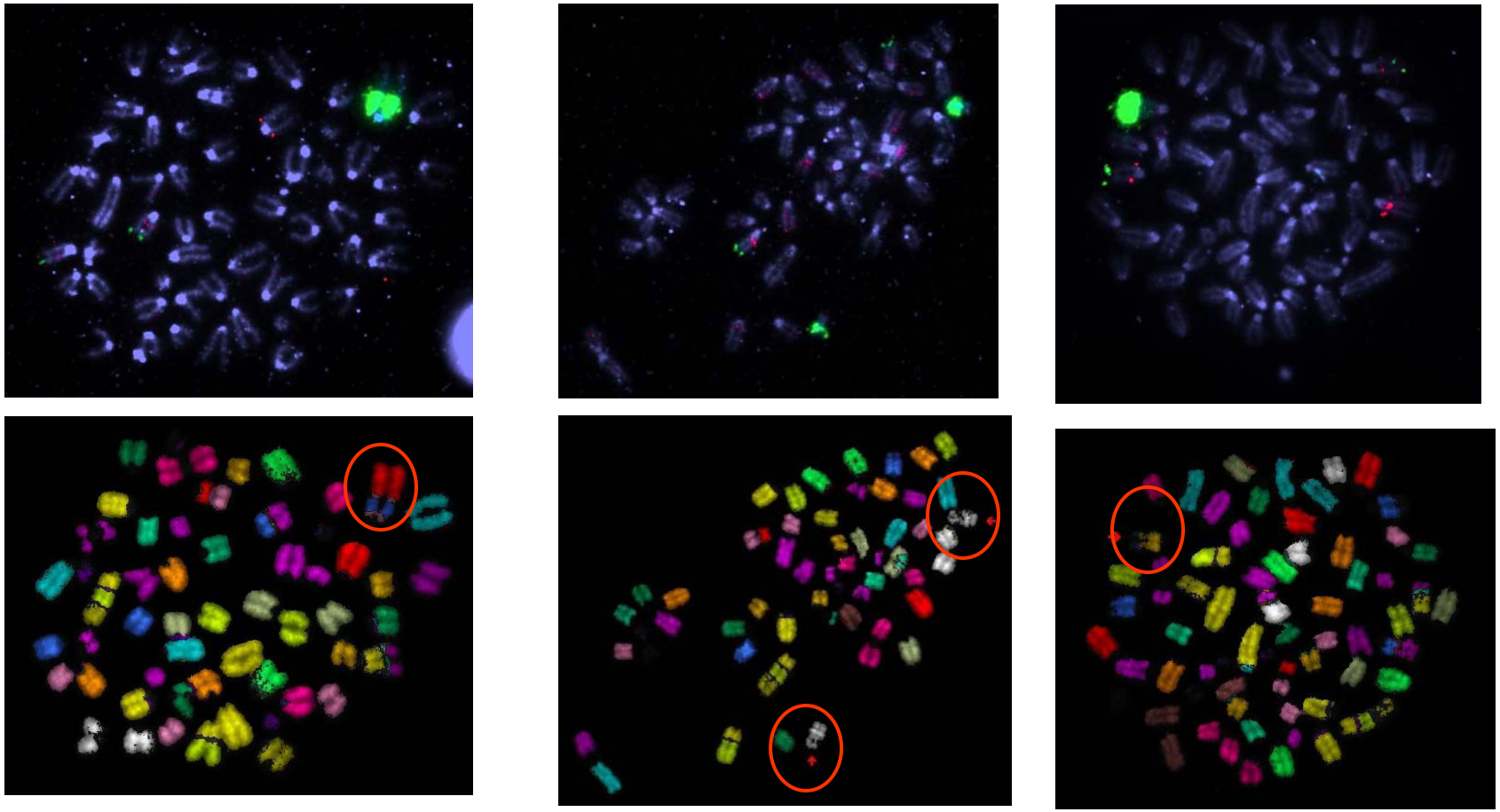




Gene amplification in CIP-resistant cells

mFISH analysis of CIP-R cells

Heterogeneity of cell population !

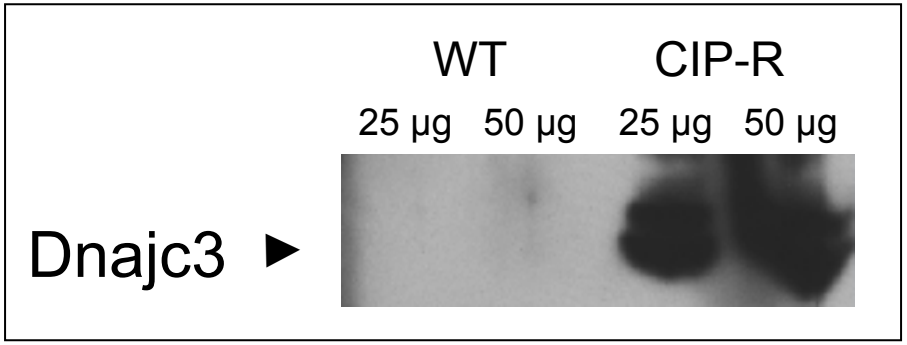


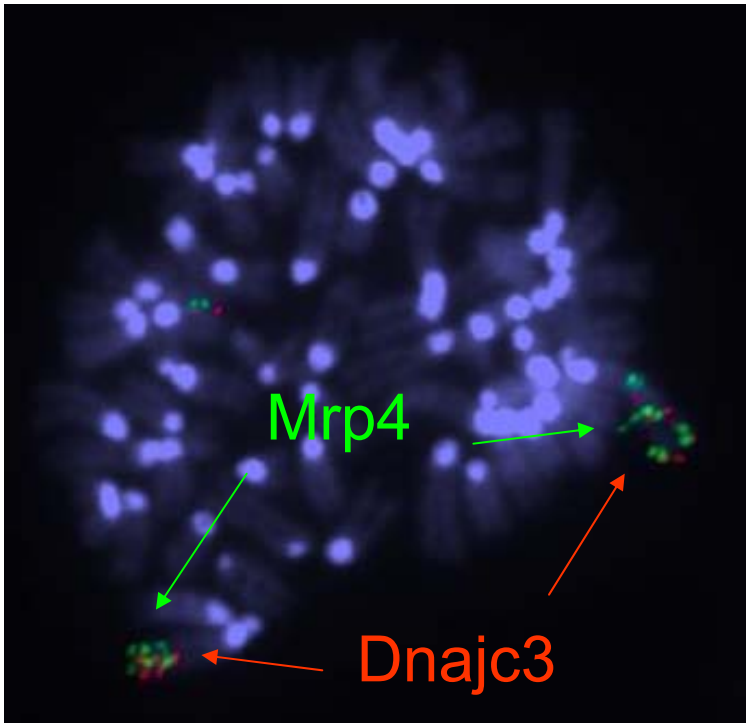
chromosome 5 | chromosome 13 | chromosome 16

Gene amplification in CIP-resistant cells

FISH analysis of CIP-R cells

Mrp4 and Dnaj3 co-amplified in CIP-resistant cells





and in moxifloxacin-exposed cells ?

TaqMan Low Density Array of ABC transporters

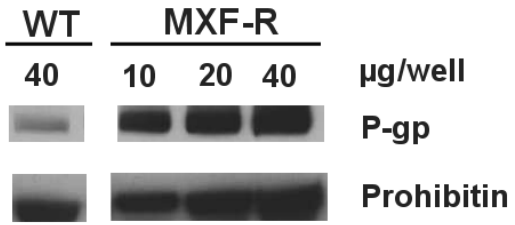
gene	Cell line	
	ciprofloxacin-resistant	moxifloxacin-resistant
Abca1	-1.53[a]	-3.51
Abca2	-1.13	-1.12
Abca3	1.10	-1.56
Abca4	nd	nd
Abca5	-1.85	2.31
Abca6	nd	nd
Abca7	-1.36	-1.20
Abca8a	nd	nd
Abca8b	(1.39)	76.20
Abca9	(-17.45)	(-10.48)
Abca13	1.66	2.02
Abca14	nd	nd
Abca15	nd	nd
Abcb1a*	(-1.38)	76.35
	(-1.89)	84.42
Abcb1b	1.03	1.72
Abcb2	1.10	4.30
Abcb3	-1.35	3.17
Abcb4	1.01	2.48
Abcb6	-1.12	-1.22
Abcb8	1.00	-1.25
Abcb9	6.08	9.83
Abcb10	1.40	1.43
Abcb11	(1.15)	(1.19)

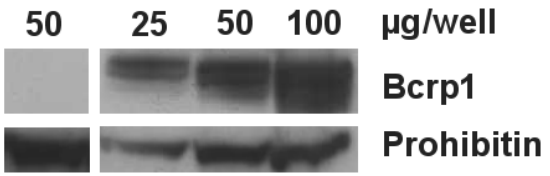
gene	Cell line	
	ciprofloxacin-resistant	moxifloxacin-resistant
Abcc1	1.08	-1.26
Abcc2*	(1.44)	(-1.26)
	(9.25)	(5.09)
Abcc3	-1.15	1.09
Abcc4	14.59	-1.82
Abcc5	-1.26	1.29
Abcc6	nd	nd
Abcc7	nd	nd
Abcc8	(1.22)	(7.74)
Abcc9	nd	nd
Abcc10	1.01	-1.40
Abcc12	nd	nd
Abcd1	1.35	2.22
Abcd2	1.10	1.79
Abcd3	-1.04	-1.51
Abcd4	-1.43	-2.03
Abce1	-1.04	-1.00
Abcf2	-1.01	1.21
Abcf3	-1.08	1.11
Abcg1	-1.93	-4.92
Abcg2*	(1.12)	108.41
	(1.17)	99.47
Abcg3	nd	nd
Abcg4	(1.62)	(-1.07)
Abcg5	nd	nd
Abcg8	nd	nd

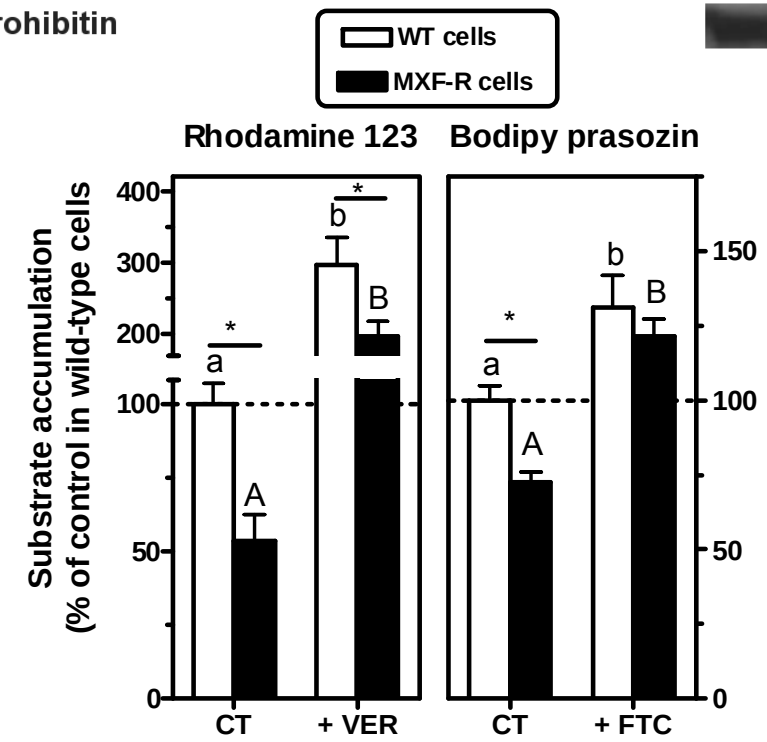
Vallet et al., FEBS-ABC meeting. (2010)

and in moxifloxacin-exposed cells ?

P-gp and Bcrp are functional



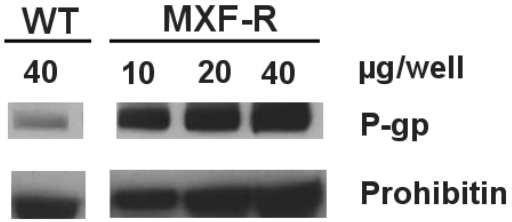


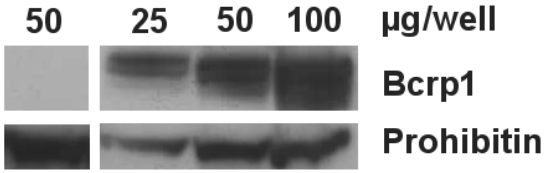


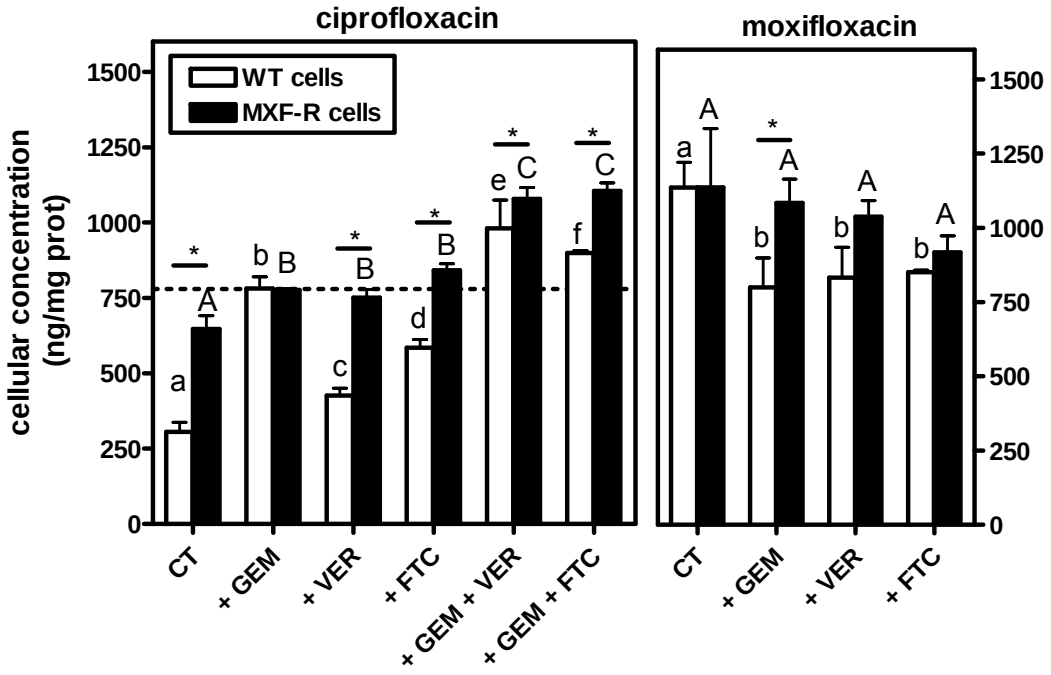
Vallet et al., FEBS-ABC meeting. (2010)

and in moxifloxacin-exposed cells ?

P-gp and Bcrp are functional
but do not play a major role in FQ accumulation







Vallet et al., FEBS-ABC meeting. (2010)

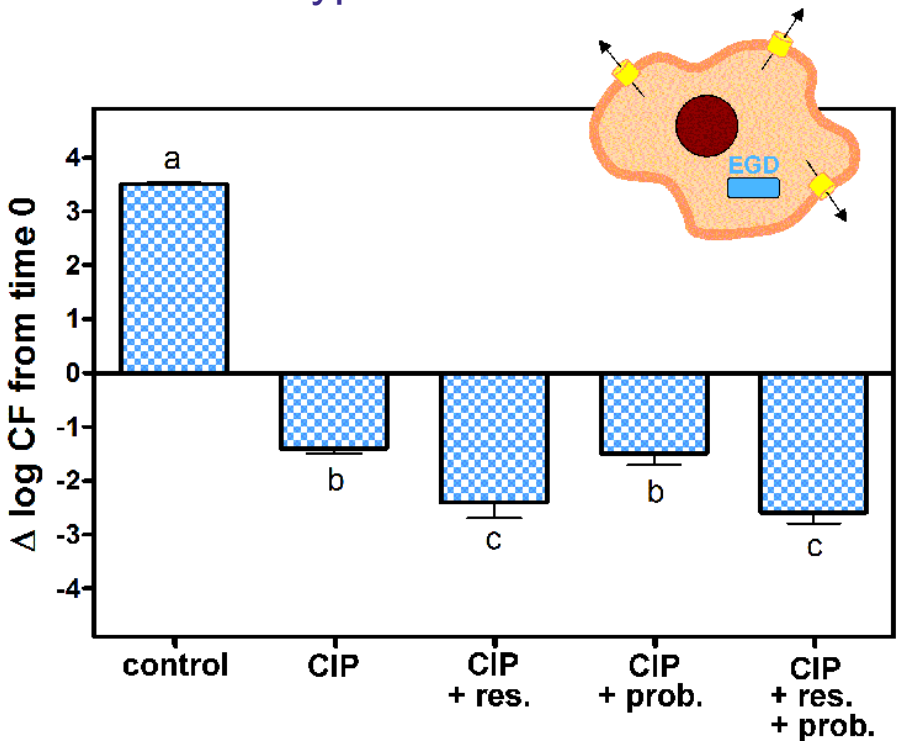
Increased efflux ….
Consequences for activity against intracellular bacteria and Cooperation between procaryotic and eucaryotic transporters

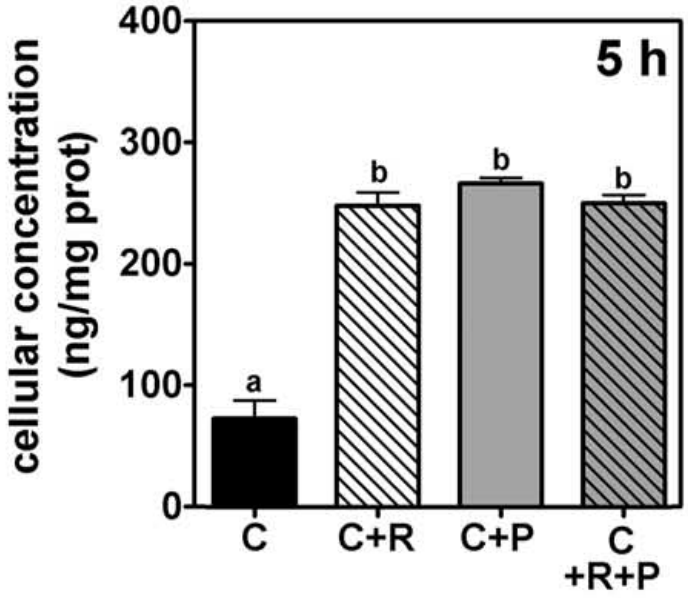
Les aventures de Tintin, Hergé, Belgium

Increased efflux in ciprofloxacin-resistant cells : consequence for antibiotic activity

Ciprofloxacin and *Listeria*

Wild-type cells and bacteria





Lismond et al., Antimicrob. Ag. Chemother. (2008) 52:3040-46

Increased efflux in bacteria

MIC of *Listeria* strains and effect of reserpine

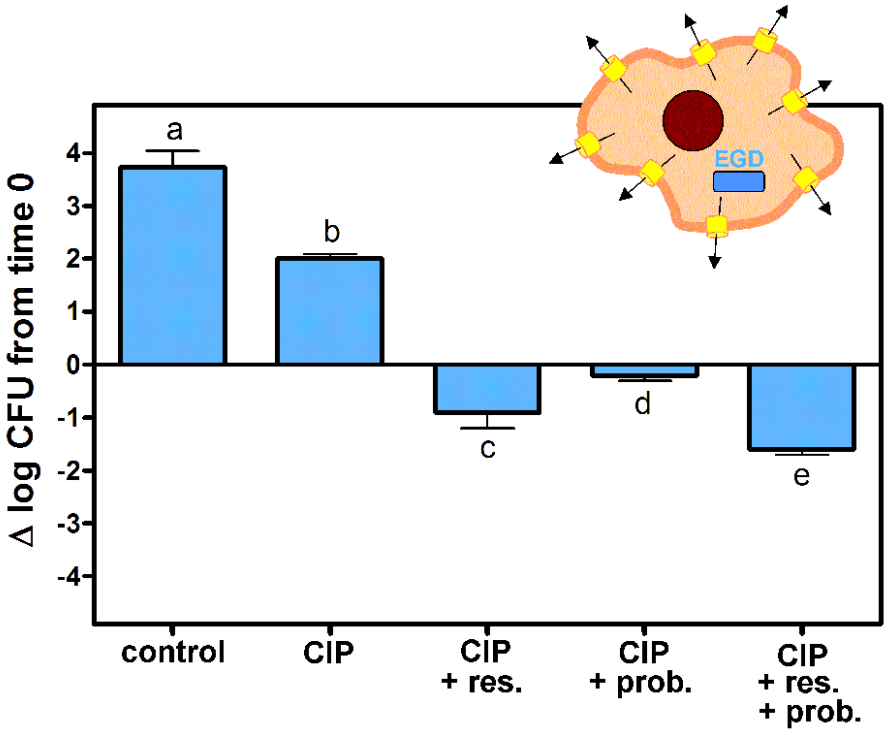
quinolone	MIC (mg/L)			
	EGD		CLIP	
	Res. (-)	Res. (+)	Res. (-)	Res. (+)
CIP	1.2	1.0	**5.0**	1.0
MXF	0.6	0.6	0.5	0.25

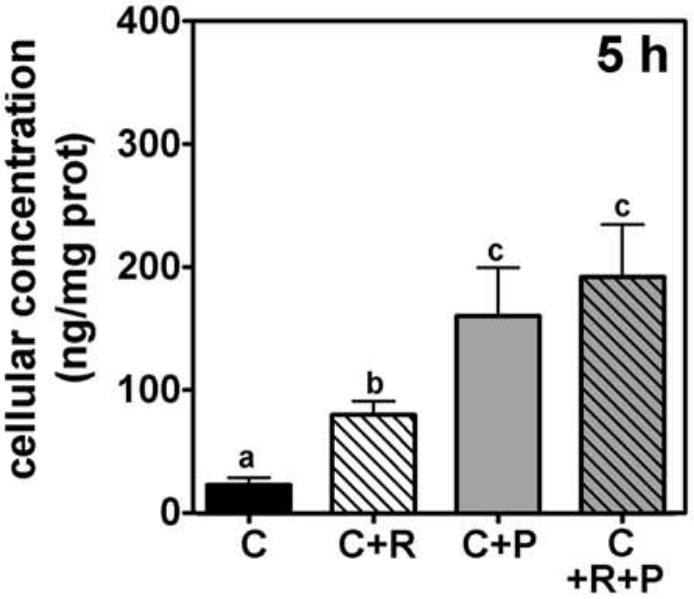
Lismond et al., Antimicrob. Ag. Chemother. (2008) 52:3040-46

Increased efflux in ciprofloxacin-resistant cells : consequence for antibiotic activity

Ciprofloxacin and *Listeria*

cells and overproducing
efflux pumps for ciprofloxacin



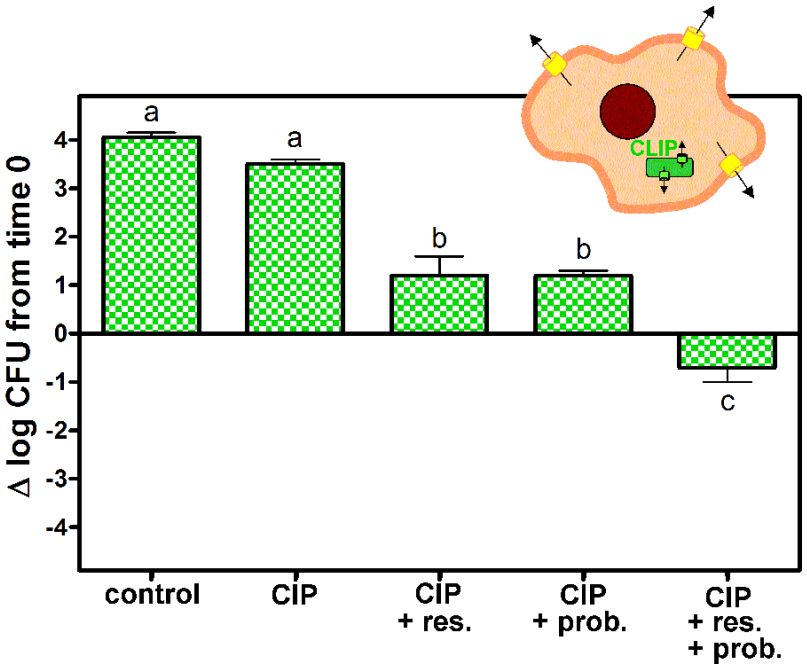


Lismond et al., Antimicrob. Ag. Chemother. (2008) 52:3040-46

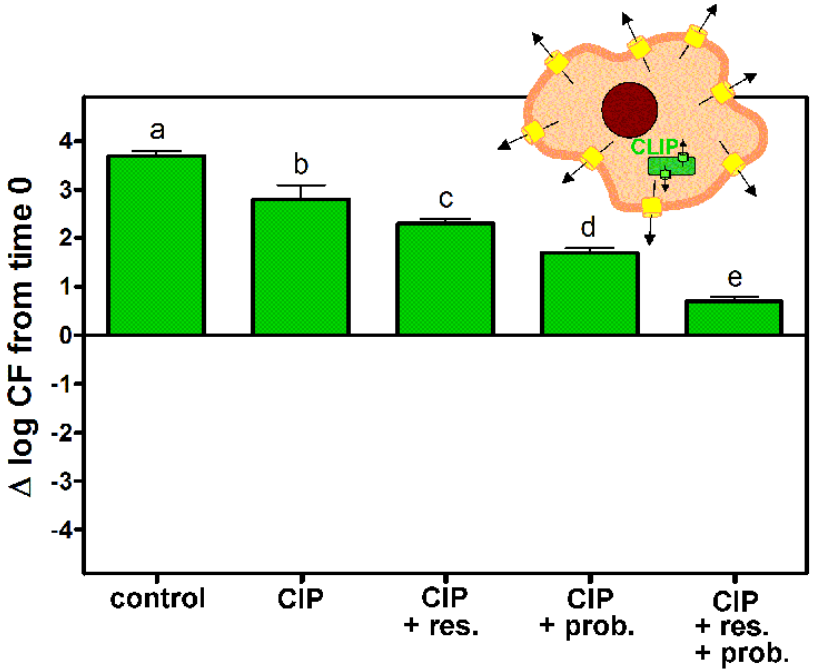
Increased efflux in ciprofloxacin-resistant cells : consequence for antibiotic activity

Ciprofloxacin and *Listeria*

bacteria overproducing efflux pumps for ciprofloxacin



bacteria and cells overproducing efflux pumps for ciprofloxacin

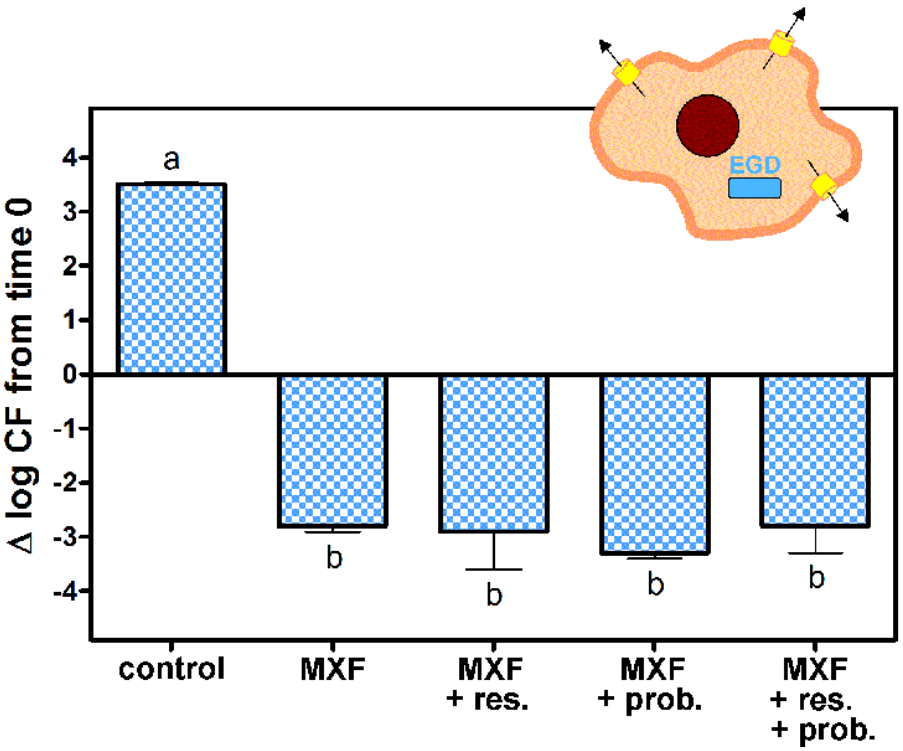


Lismond et al., Antimicrob. Ag. Chemother. (2008) 52:3040-46

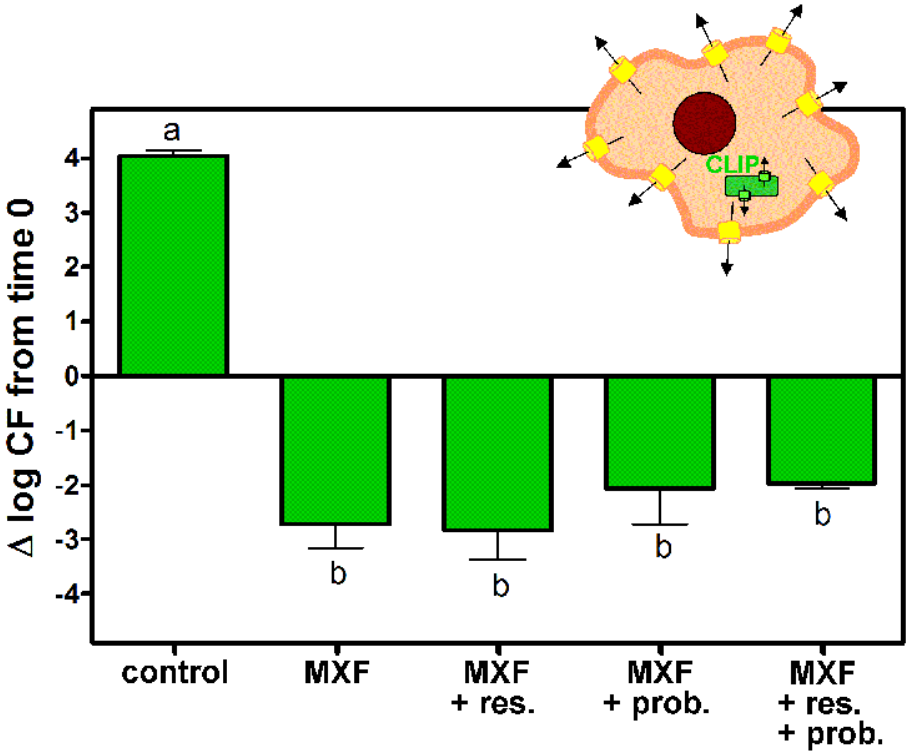
Increased efflux in ciprofloxacin-resistant cells : consequence for antibiotic activity

Moxifloxacin and *Listeria*

Wild-type cells and bacteria



cells and bacteria overproducing efflux pumps for ciprofloxacin



Lismond et al., Antimicrob. Ag. Chemother. (2008) 52:3040-46

Conclusions

Toulouse: fontaines

Food for thought ...

- in procaryotic cells, active efflux truly contributes to resistance

  → need of appropriate diagnostic tools
  → selection of antibiotics that are poor substrates

- in eucaryotic cells, active efflux can modify
  - pharmacokinetics (cellular accumulation; barrier effects)
  - pharmacodynamics (intracellular activity)

  → selection of antibiotics that are poor substrates
  → combinations with efflux pumps inhibitors ?

same ?

- expression of efflux pumps can be modified by exposure to drugs

  → reduced susceptibility during treatment
  → cell metabolism modifications

Coworkers …

- **L. Piddock and M. Garvey**

PatA/PatB

Antimicrobial Agents Research Group,
University of Birmingham, UK

- **B. Devreese and M. Aerts**

Proteomics

Laboratory for Protein Biochemistry and Biomolecular Engineering,
Ghent University, Belgium

- **E. Jacquet and N. Nhiri**

TaqMan Low Density Array

Institut de Chimie des Substances Naturelles,
CNRS, Gif sur Yvette, France

- **P. Courvalin**

Resistant strains

Unité des Agents antibactériens,
Institut Pasteur, Paris, France

Efflux in our team …

UCL Université catholique de Louvain

www.facm.ucl.ac.be

Let’s dream to the next pump we will discover …

Toulouse: première pompe à gaz naturel pour véhicules