

Transport across membranes: Multiple drug resistance, mechanisms and new tools

How do antibiotics play with multidrug transporters in eucaryotic cells ?

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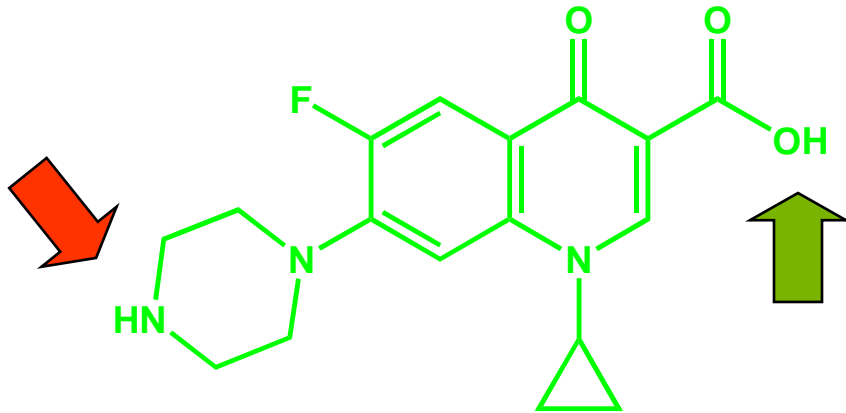
**Are antibiotics
substrates for MDR
transporters
in eucaryotic cells
?**



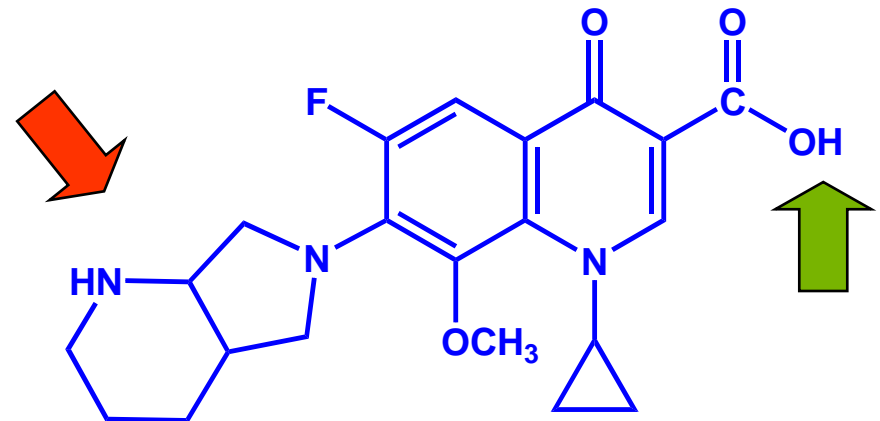
*Die Bremer
Stadtmusikanten*

Fluoroquinolone antibiotics

ciprofloxacin

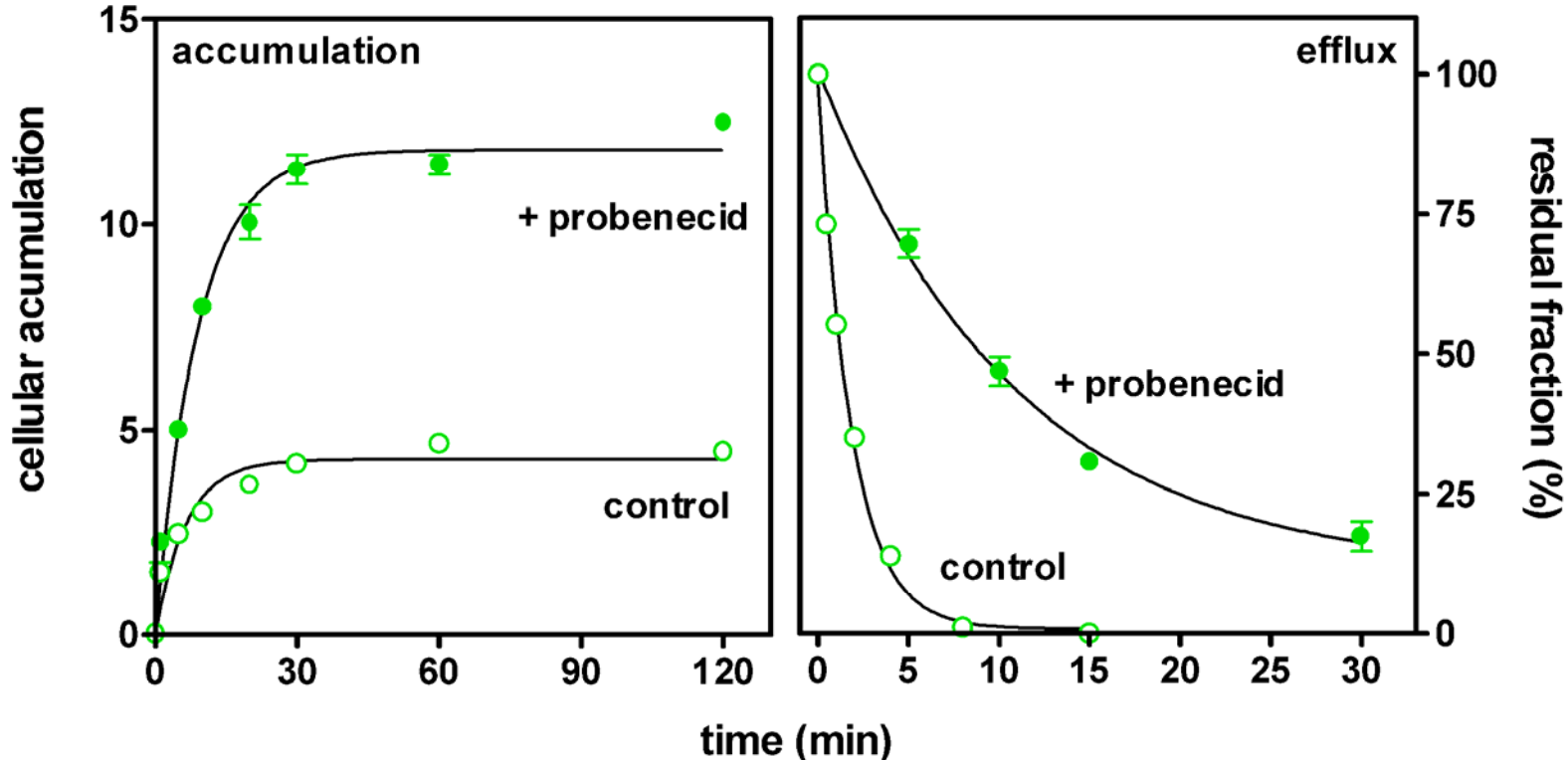


moxifloxacin



Kinetics of accumulation and efflux for ciprofloxacin

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

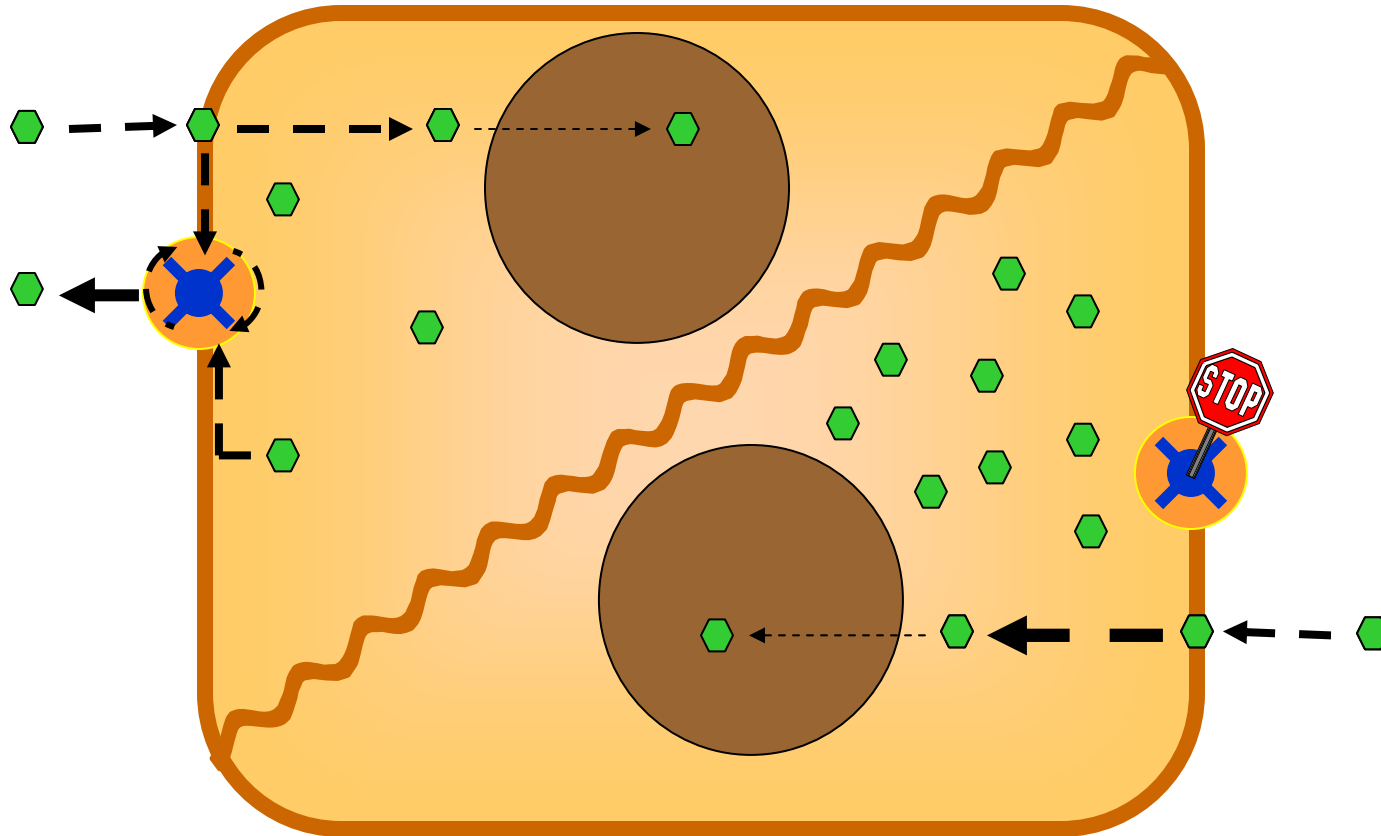


extracell. conc. 17 mg/L; probenecid 5 mM

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

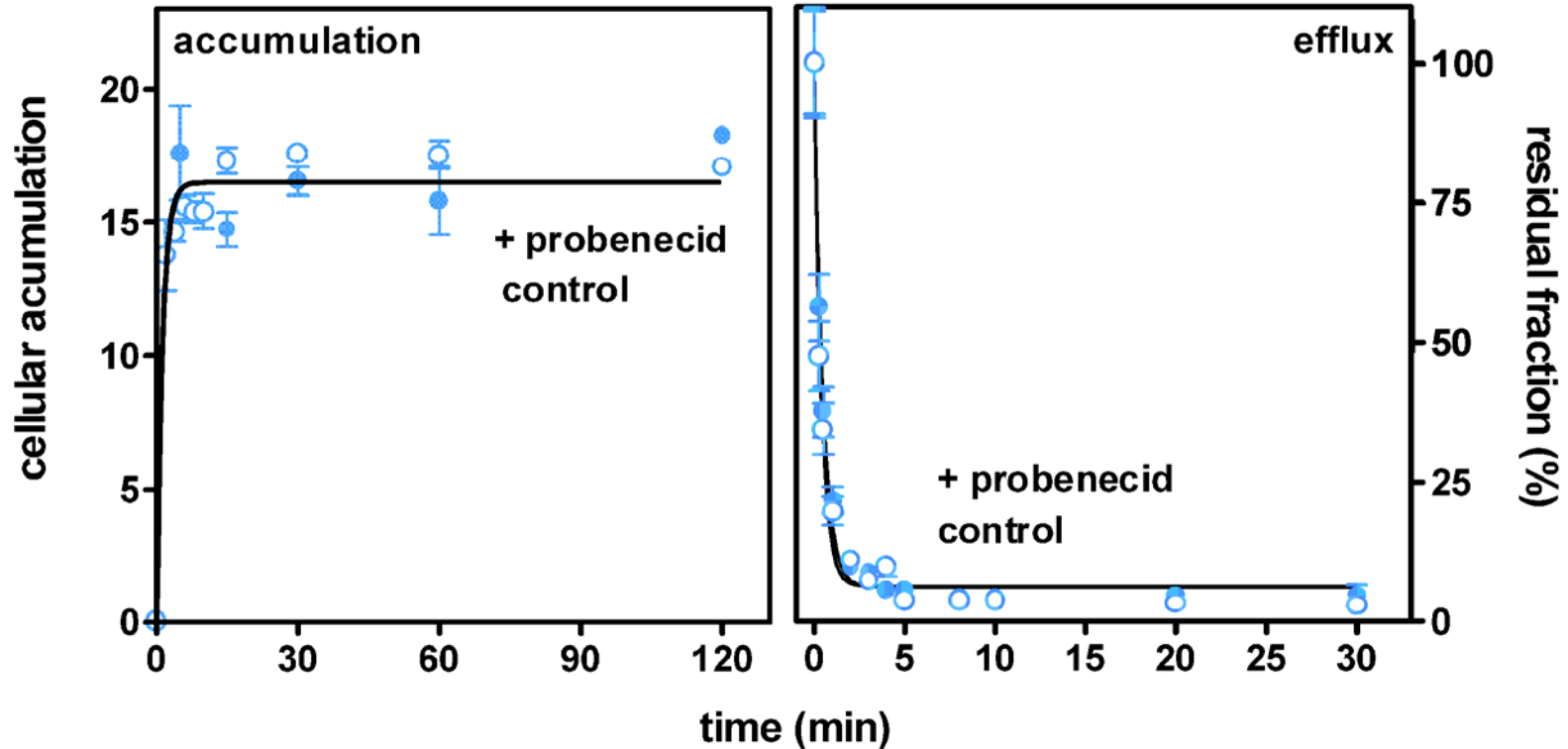
Ciprofloxacin, classical model

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



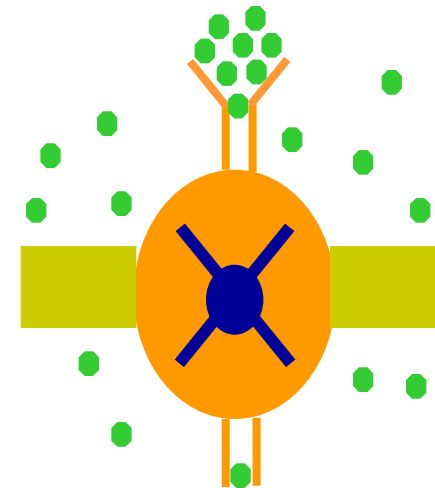
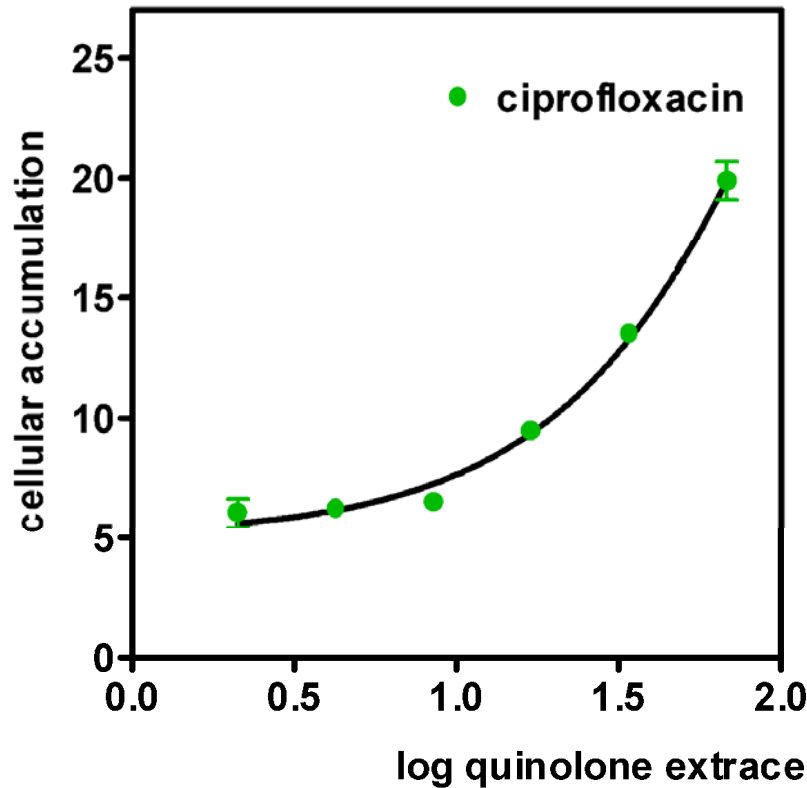
Kinetics of accumulation and efflux for moxifloxacin

neither accumulation nor efflux affected
by probenecid



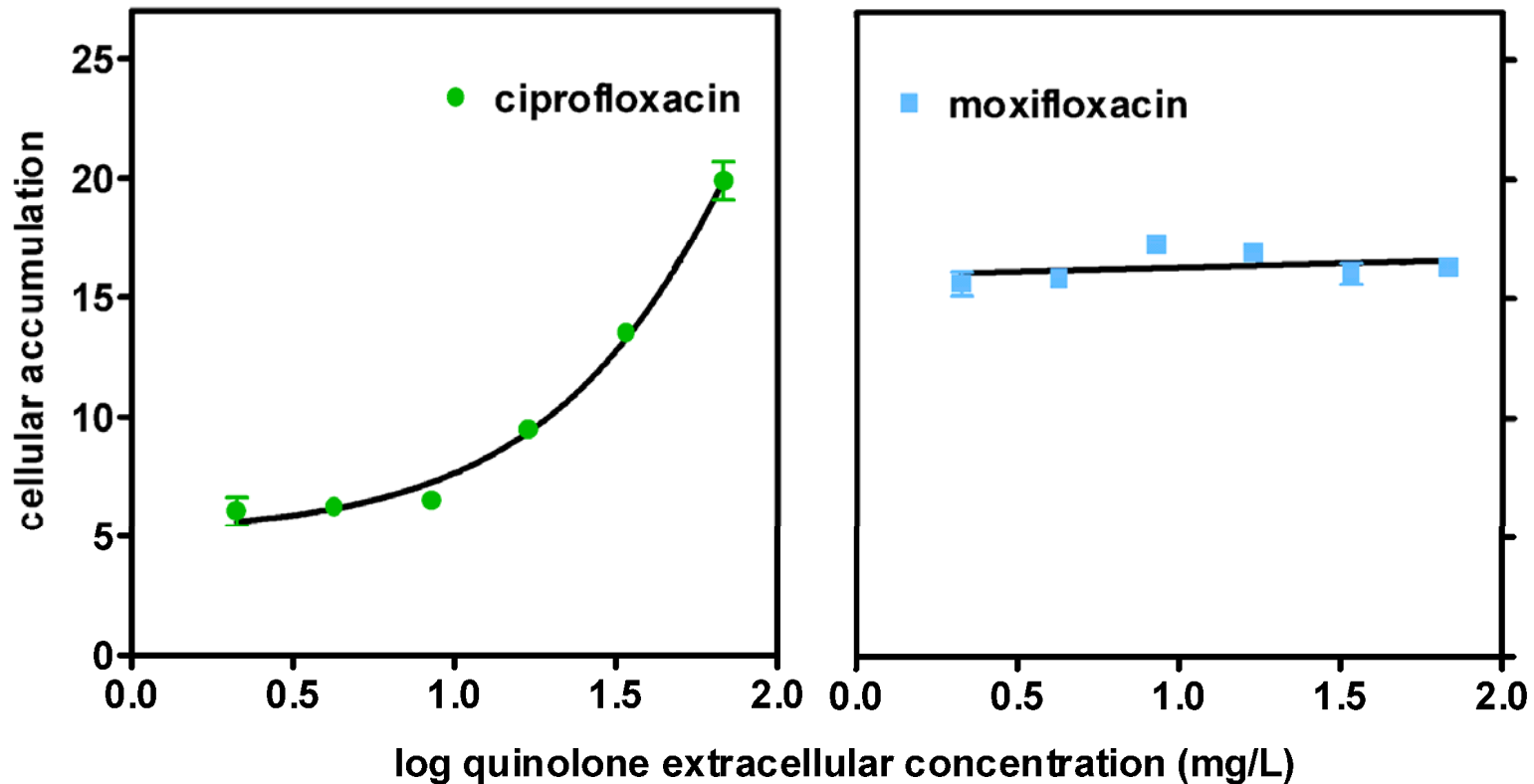
Quinolones as inhibitors of ciprofloxacin efflux

- ciprofloxacin efflux inhibited by ciprofloxacin



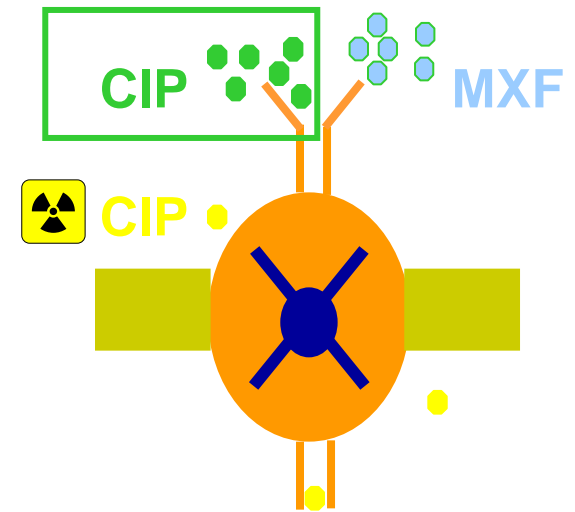
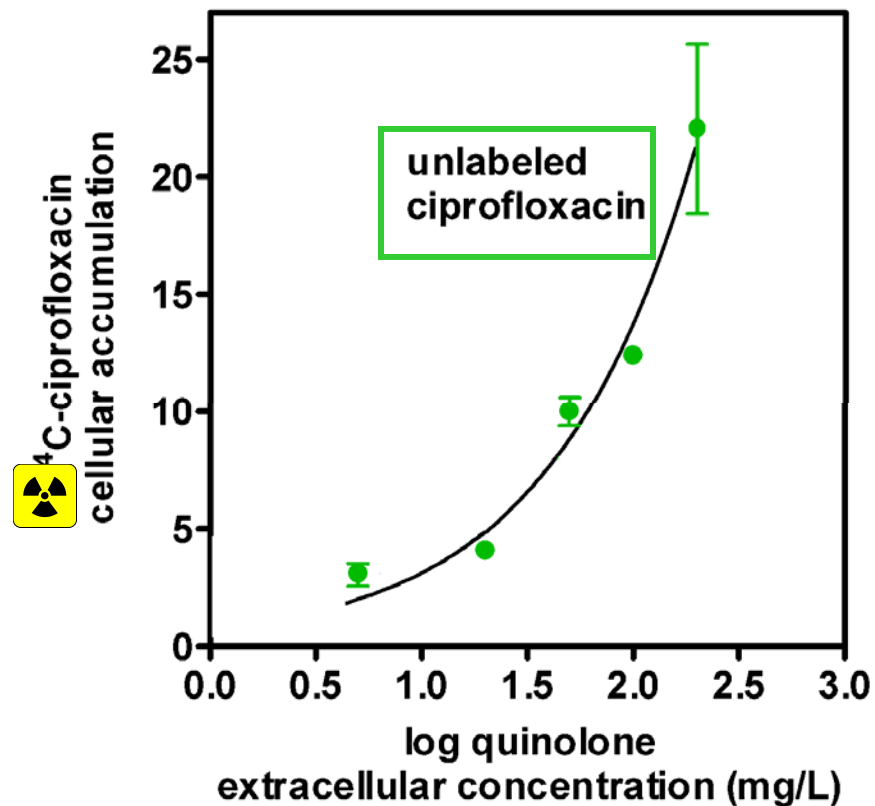
Quinolones as inhibitors of ciprofloxacin efflux

- ciprofloxacin efflux inhibited by ciprofloxacin
- moxifloxacin not affected



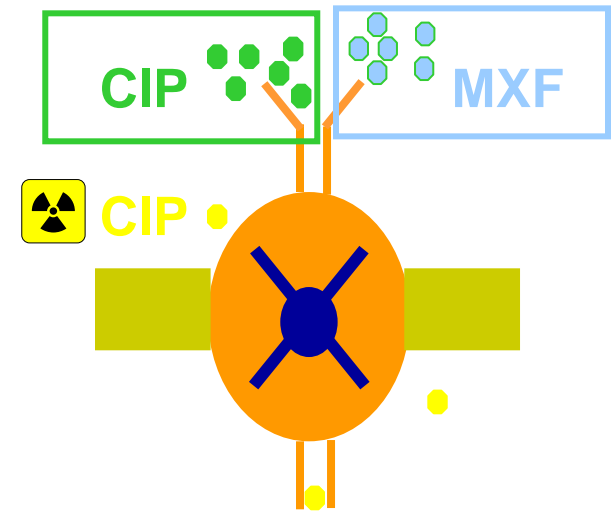
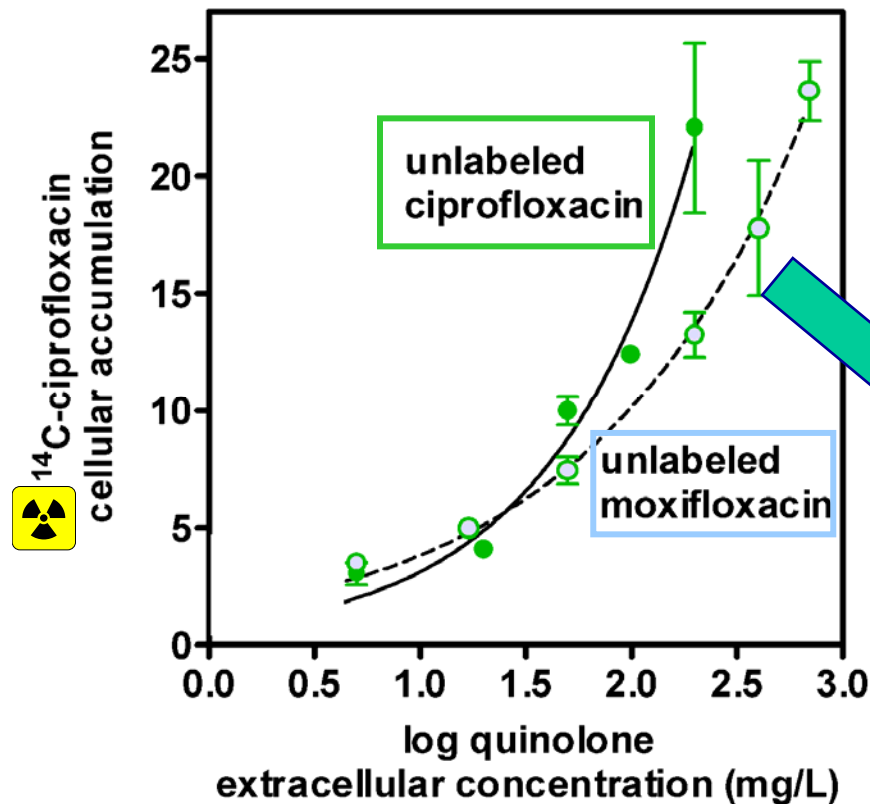
Quinolones as inhibitors of ciprofloxacin efflux

- ciprofloxacin efflux inhibited by ciprofloxacin
moxifloxacin



Quinolones as inhibitors of ciprofloxacin efflux

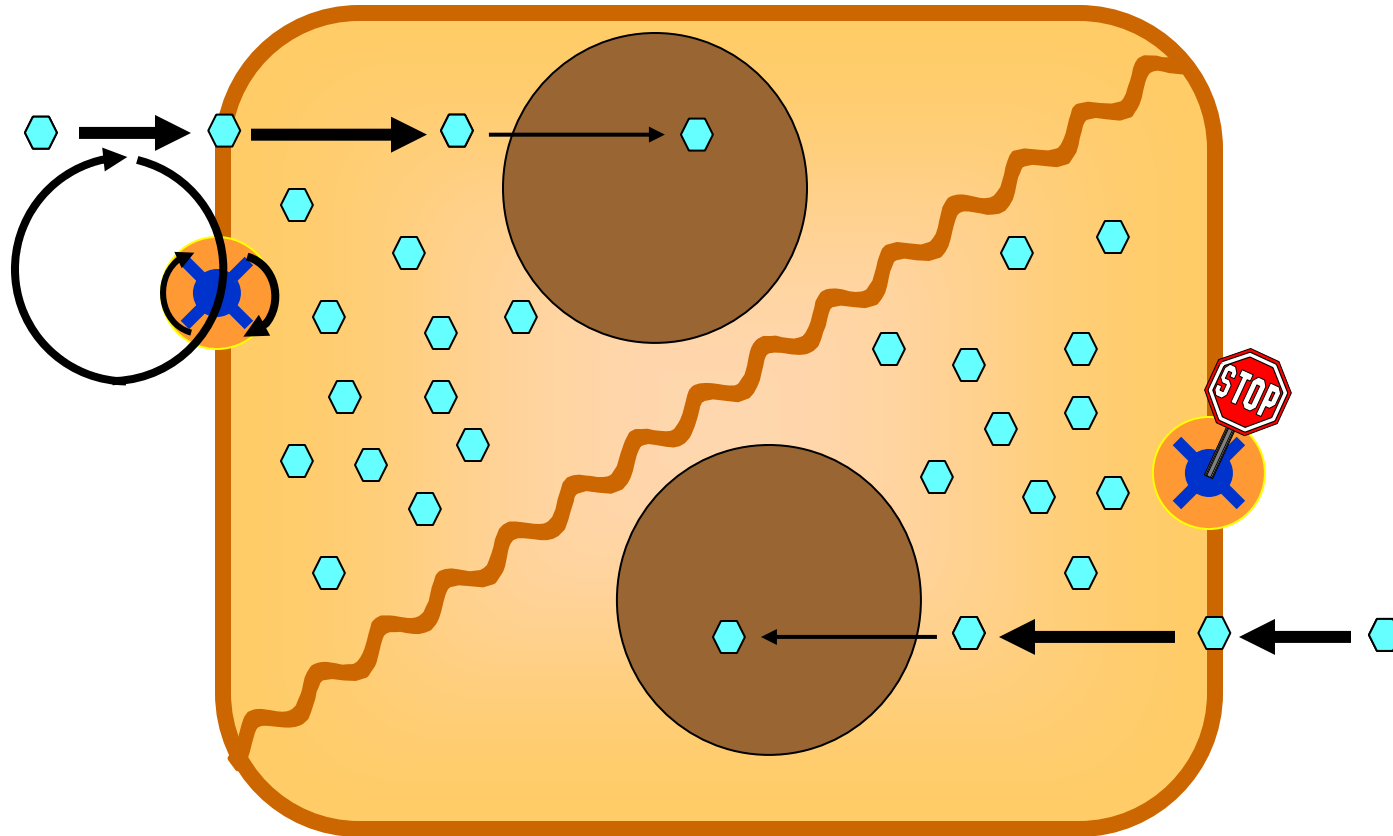
- ciprofloxacin efflux inhibited by ciprofloxacin
moxifloxacin



moxifloxacin also able to interact with the transporter !

Moxifloxacin, 'futile-cycle' model

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



**Can we make
eucaryotic cells
« resistant » to
antibiotics ?**

**A way to further
characterize
efflux transporters**

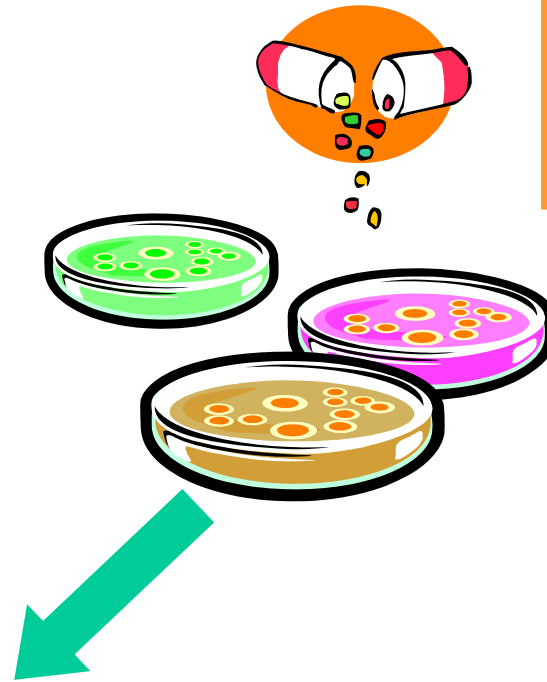


*Die Bremer
Stadtmusikanten*

Over-expression of efflux pumps as mechanism of resistance

How to get resistant cells ?

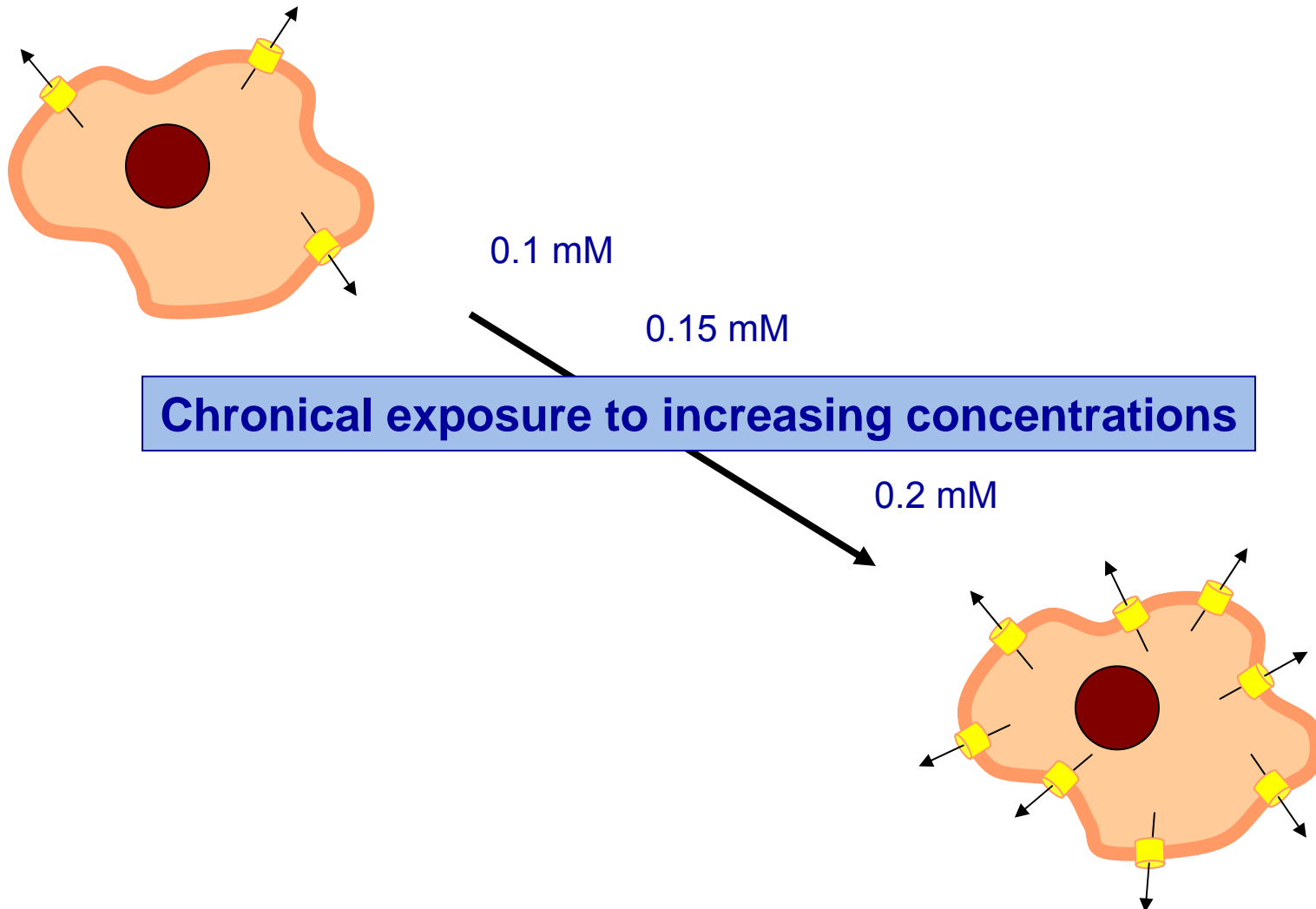
- step-wise increase in AB concentration
- several passages at each step



time and drug consuming!

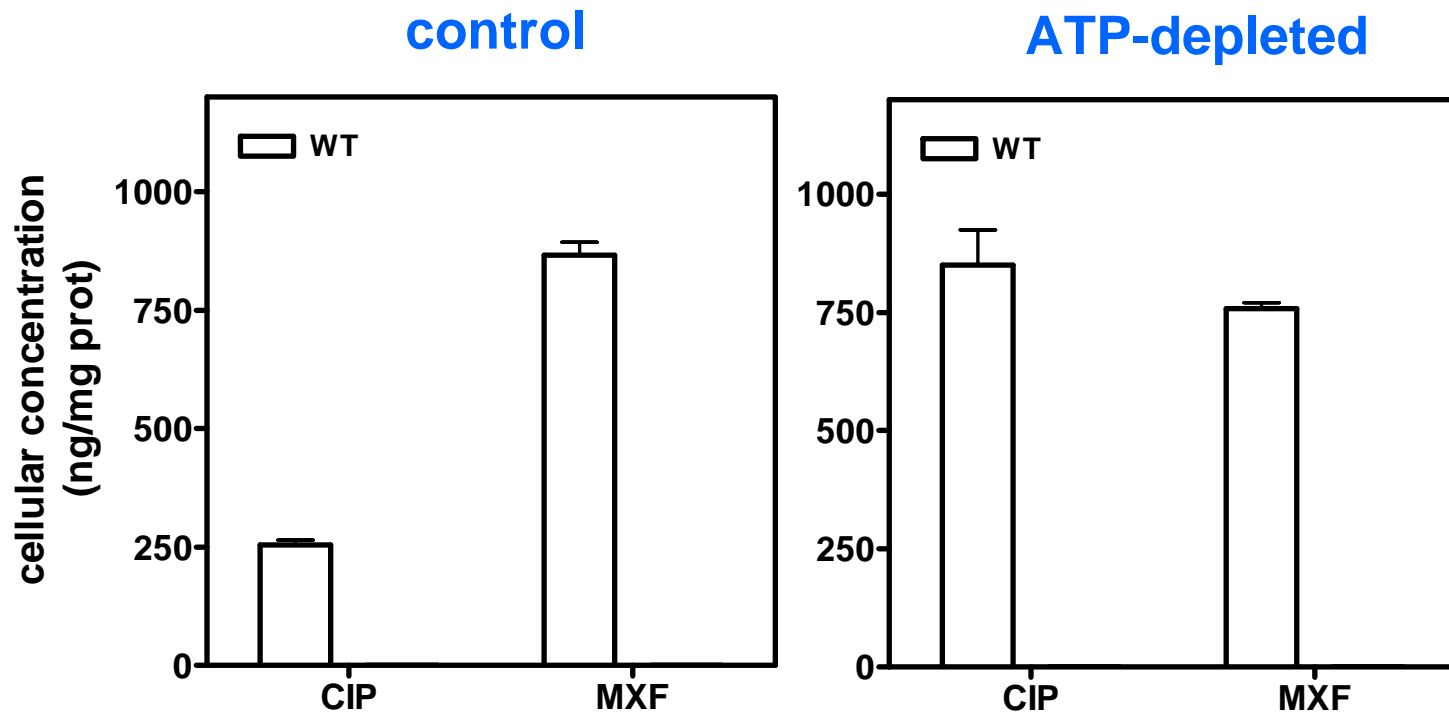
multifactorial multidrug resistance

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



Ciprofloxacin « resistant » cells: phenotypic analysis

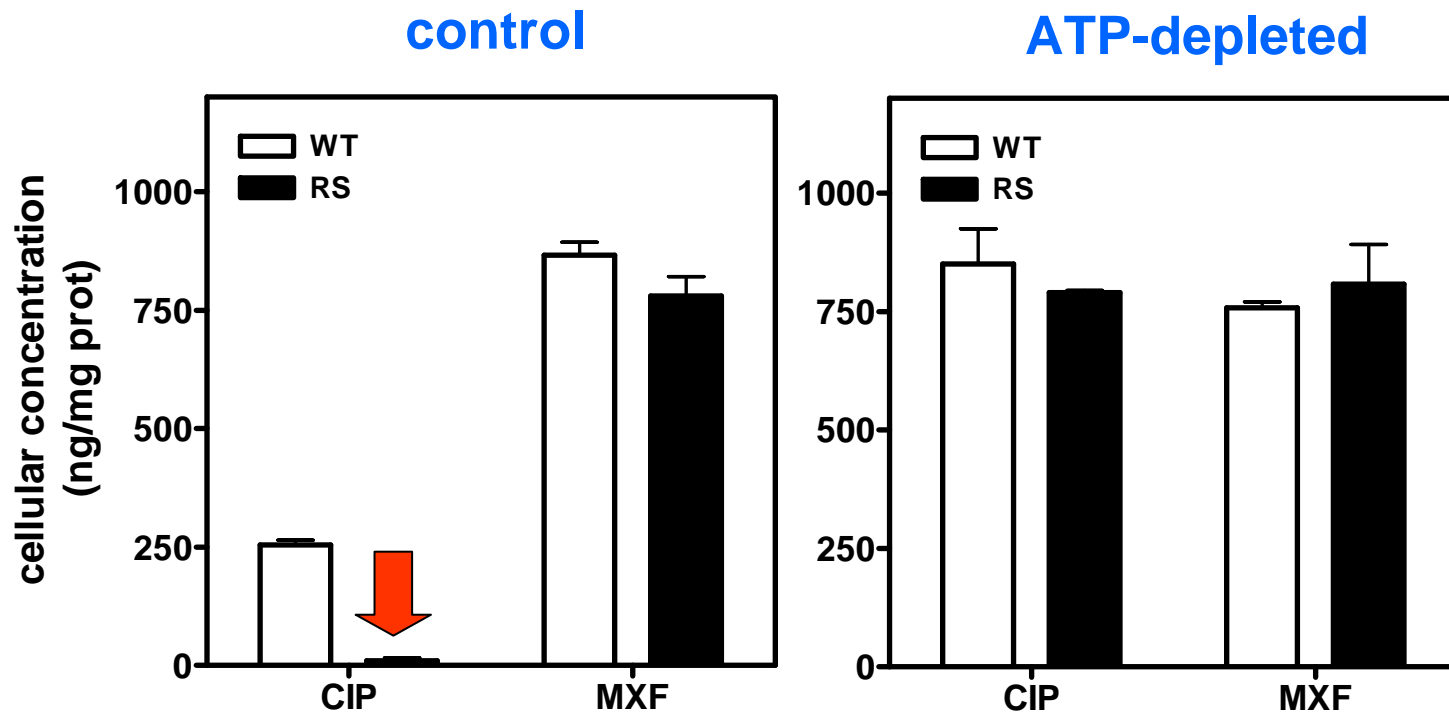
ATP-dependent reduction in cell accumulation of CIP; MXF non affected



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

Ciprofloxacin « resistant » cells: phenotypic analysis

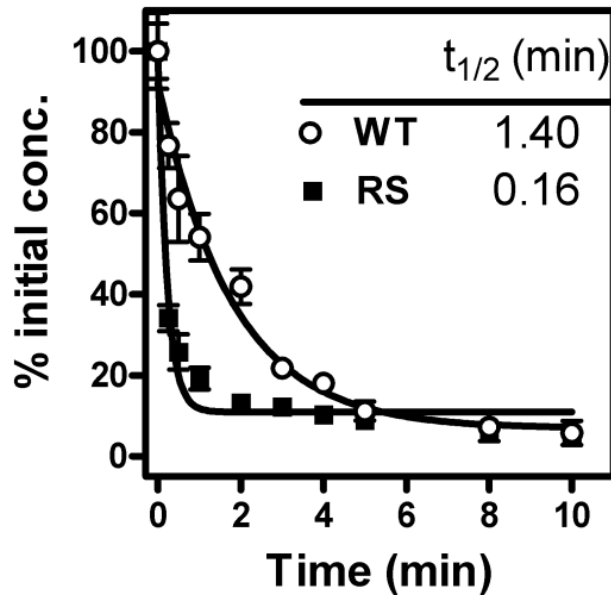
ATP-dependent reduction in cell accumulation of CIP; MXF non affected



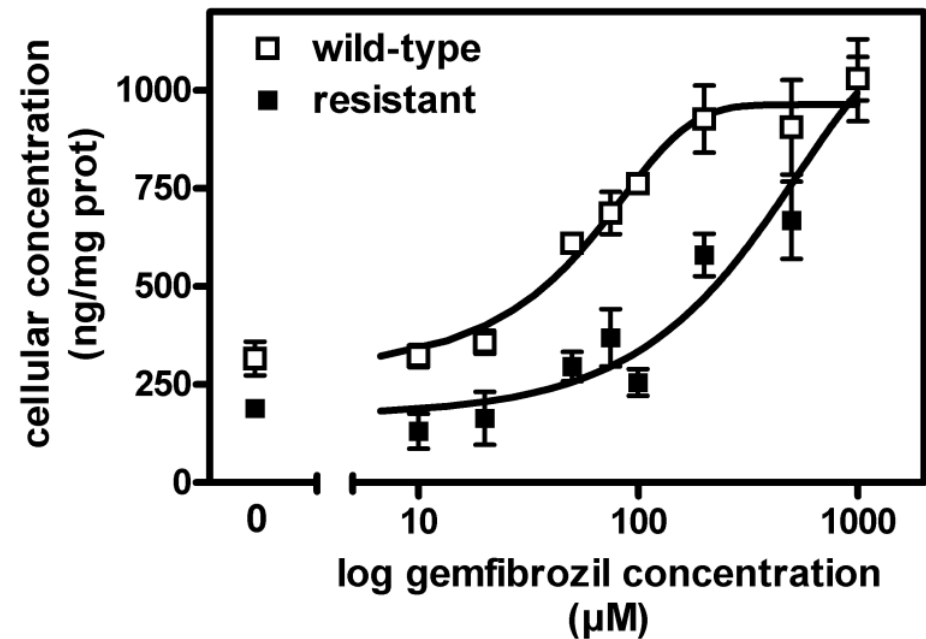
Michot et al., *Antimicrob. Ag. Chemother.* (2006) 50:1689-1695

Ciprofloxacin « resistant » cells: phenotypic analysis

↗ efflux rate



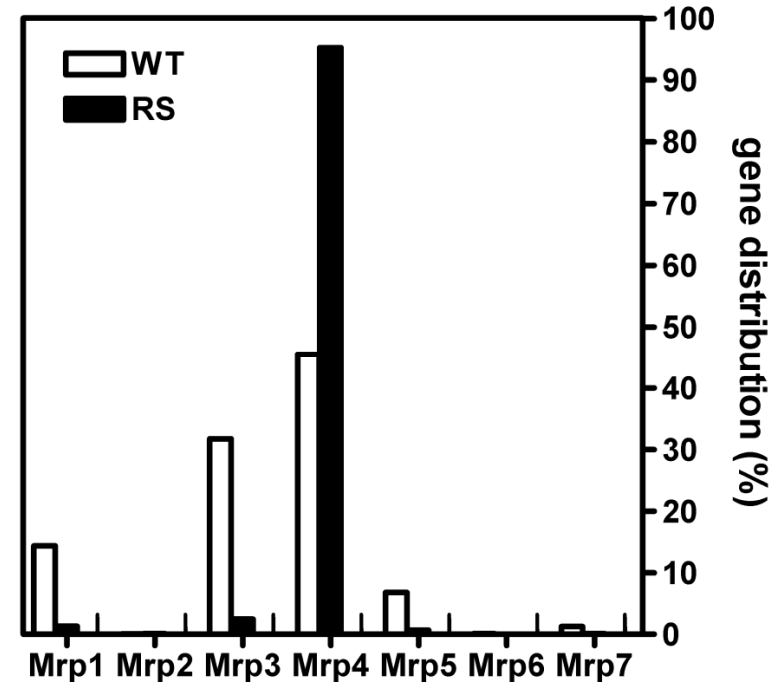
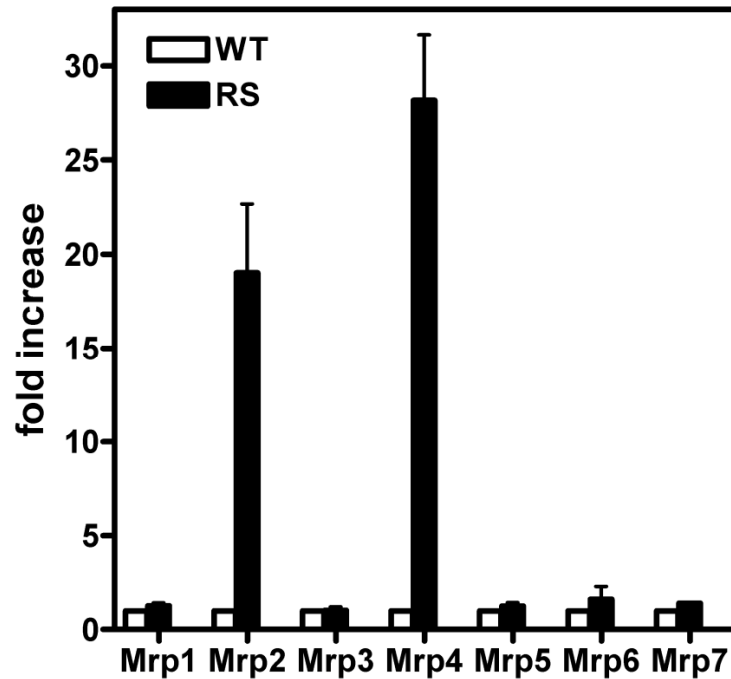
↗ IC_{50} gemfibrozil



Ciprofloxacin « resistant » cells: genotypic analysis

ARNm levels (Real-Time PCR)

↗ expression Mrp2 and Mrp4, but Mrp4 predominates



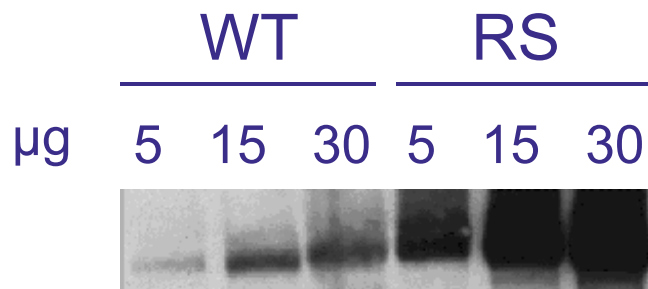
Ciprofloxacin « resistant » cells: proteomic analysis

detection of the proteins by

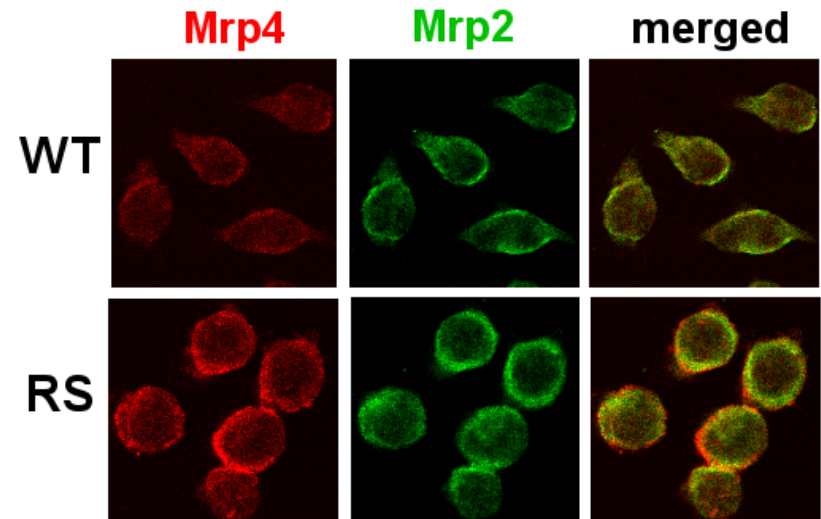
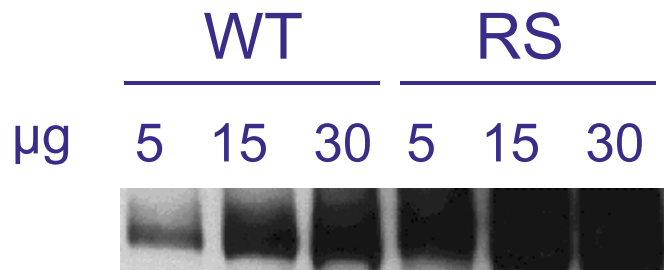
Western-Blot of membrane fraction

Confocal microscopy

Mrp2



Mrp4

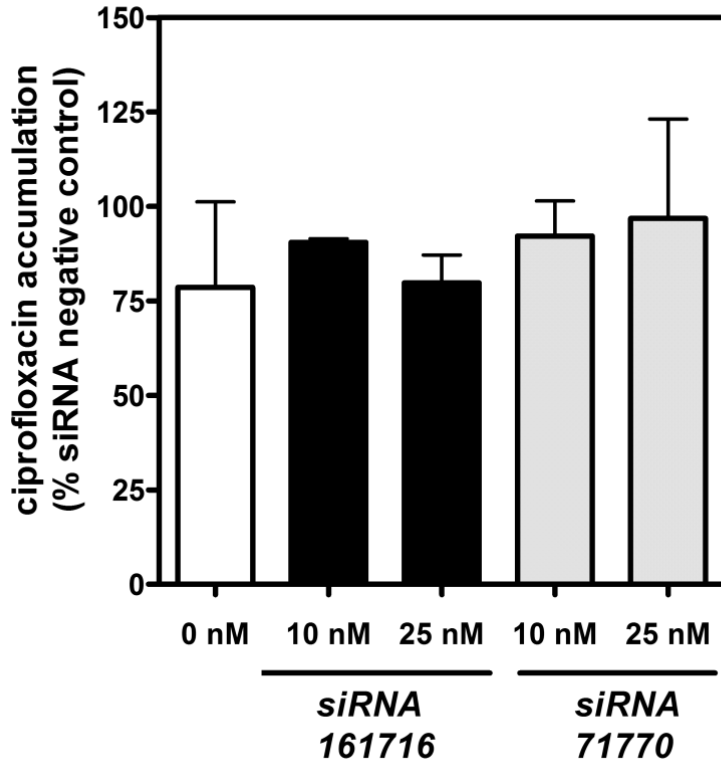


Marquez et al., *Antimicrob. Ag. Chemother.* (2009) 53:2410-6

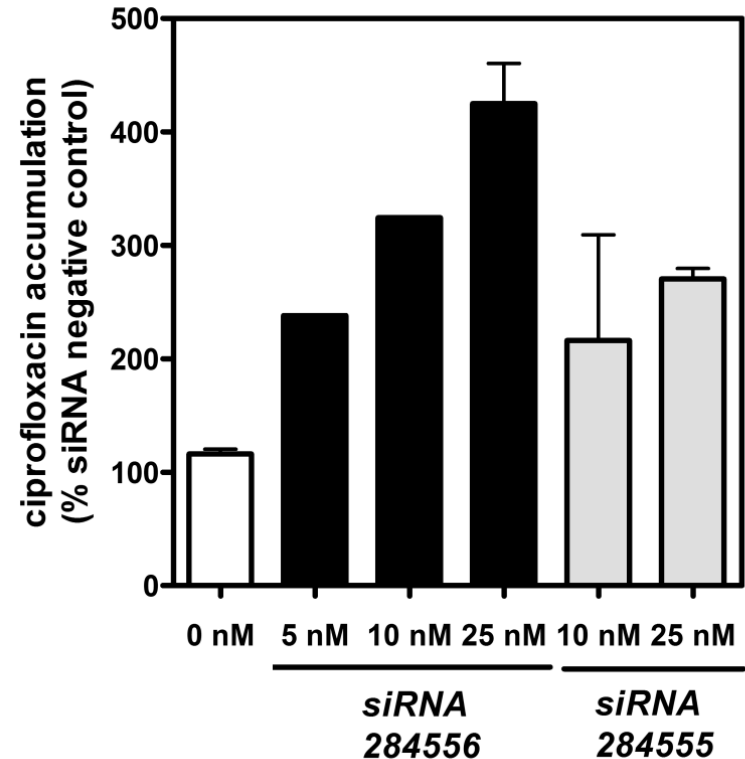
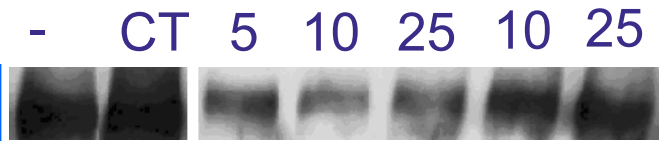
Ciprofloxacin « resistant » cells : which transporter ?

Specific extinction of gene expression by siRNA

Mrp2



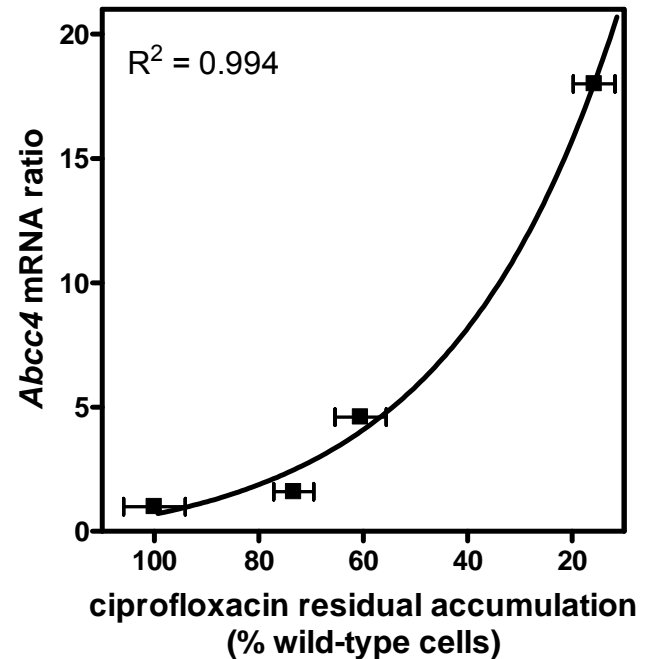
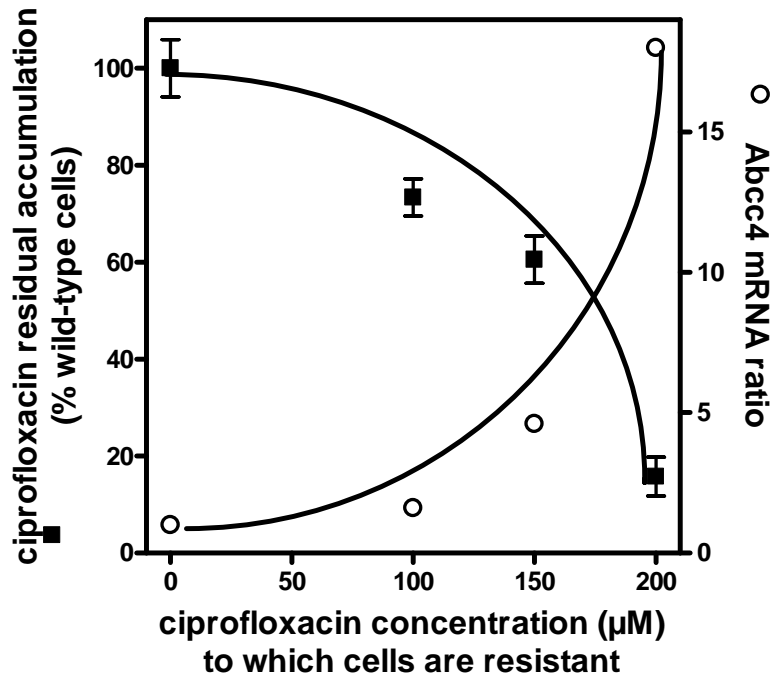
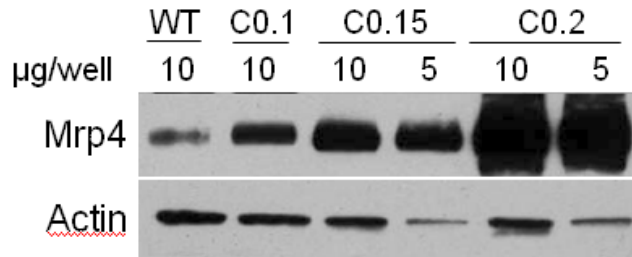
Mrp4



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

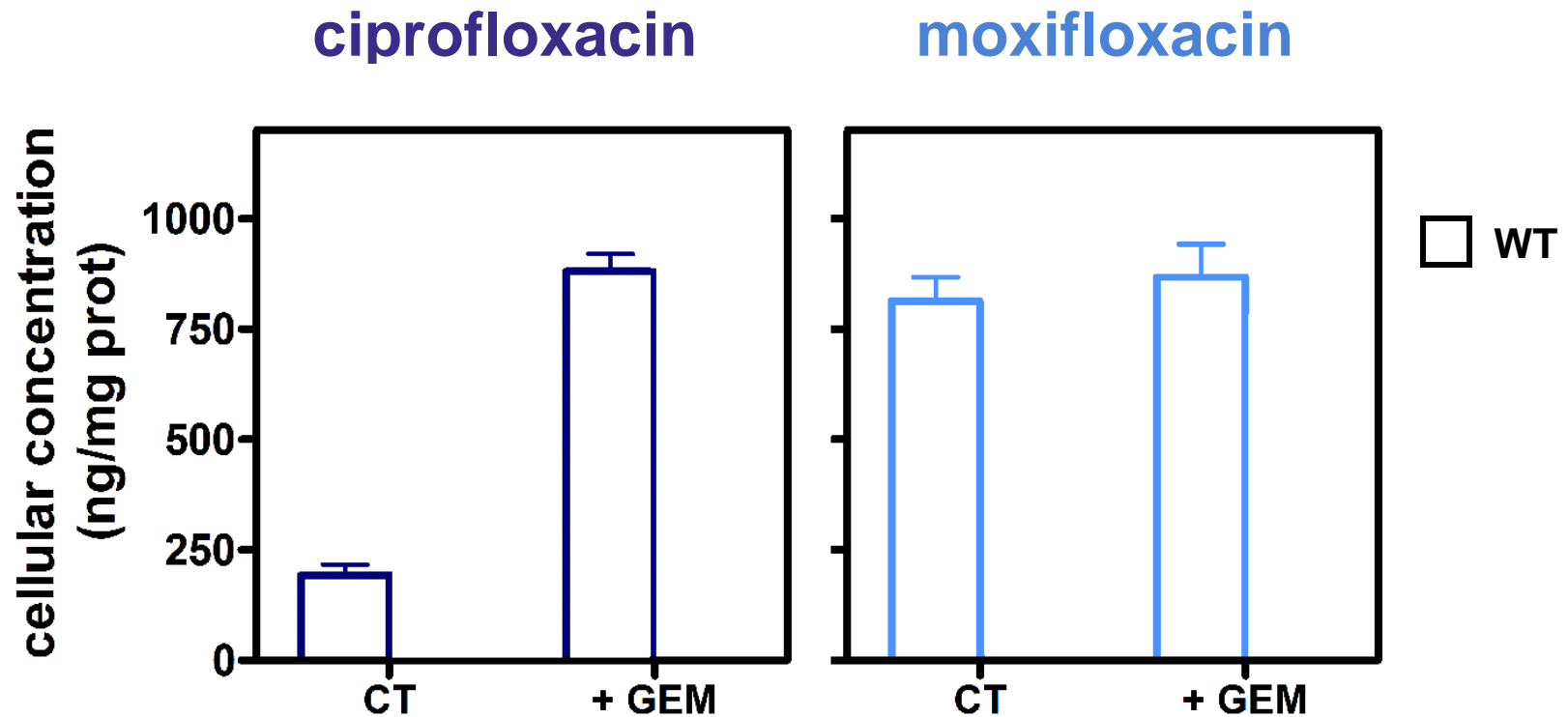
Acquisition of resistance is a stepwise process

Accumulation and Mrp4 expression during selection of resistance



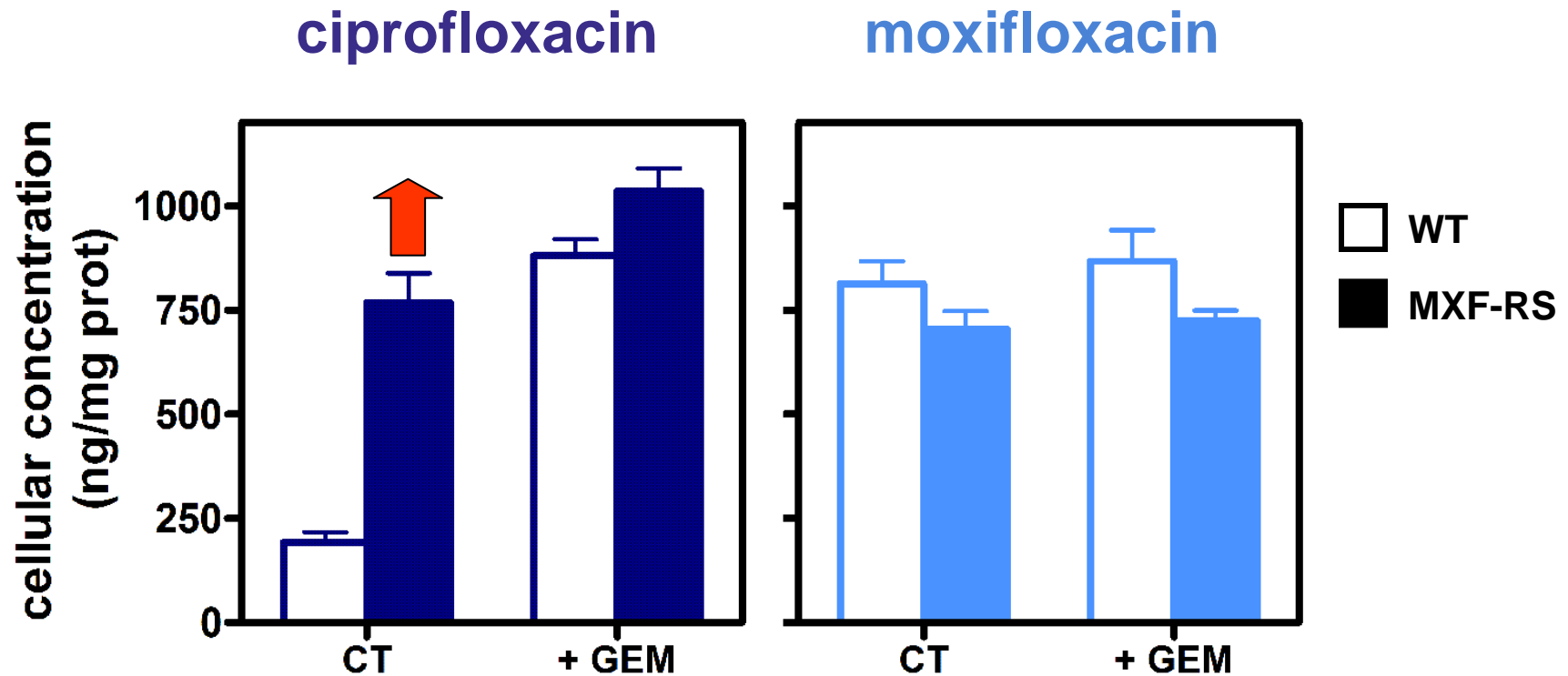
Can we select for moxifloxacin « resistant » cells ?

FQ accumulation and gemfibrozil effect



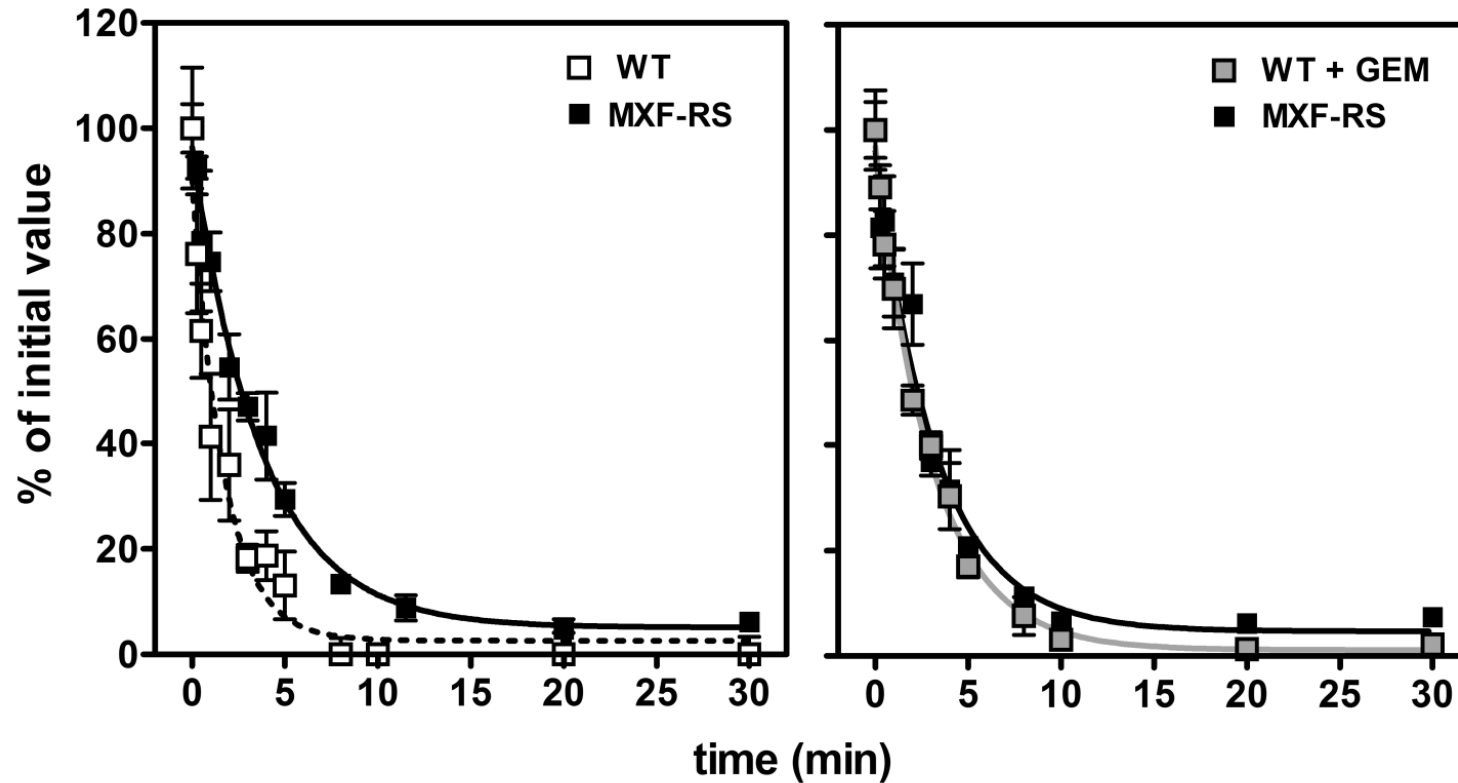
Can we select for moxifloxacin « resistant » cells ?

FQ accumulation and gemfibrozil effect



Can we select for moxifloxacin « resistant » cells ?

Ciprofloxacin efflux

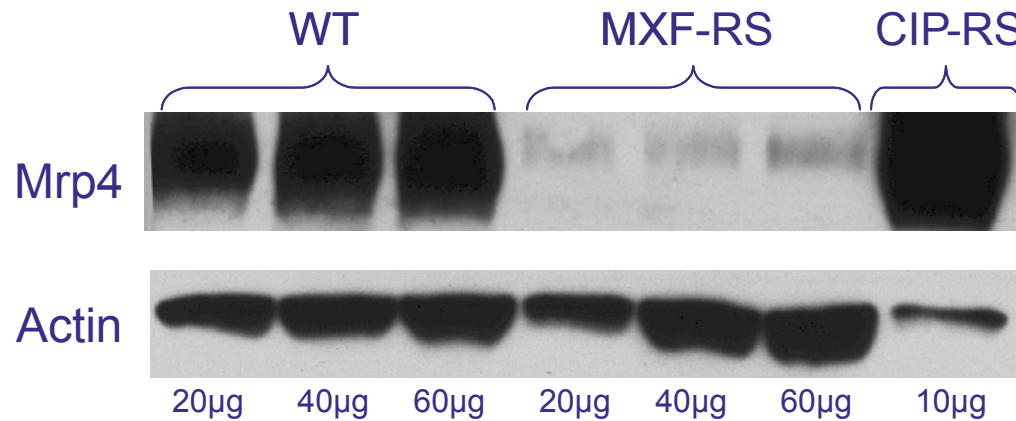


$$T_{1/2} \text{ WT} \gg T_{1/2} \text{ WT+GEM} = T_{1/2} \text{ MXF-RS}$$

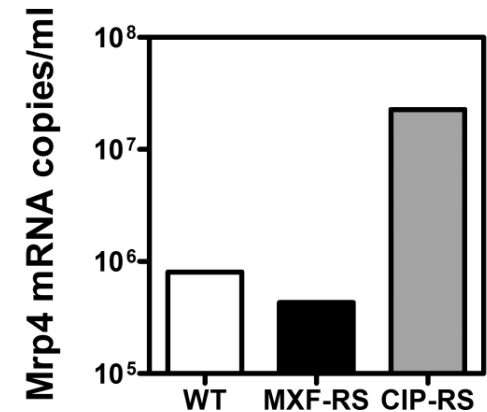
Moxifloxacin-exposed cells are « anti » resistant!

Mrp4 expression

Western-Blot



ARNm (Real Time PCR)

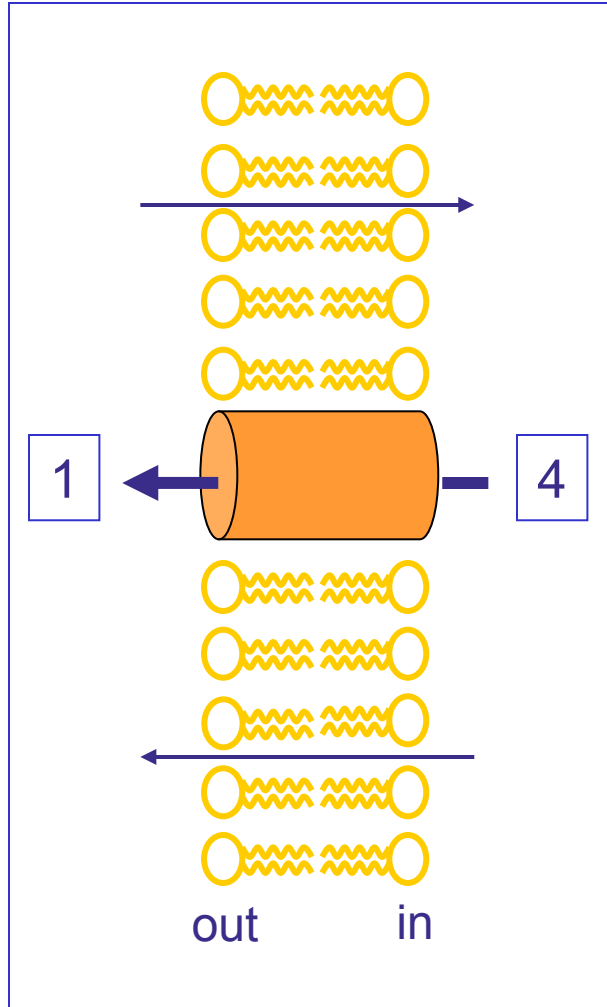


Vallet et al., FEBS-ABC meeting. (2010)

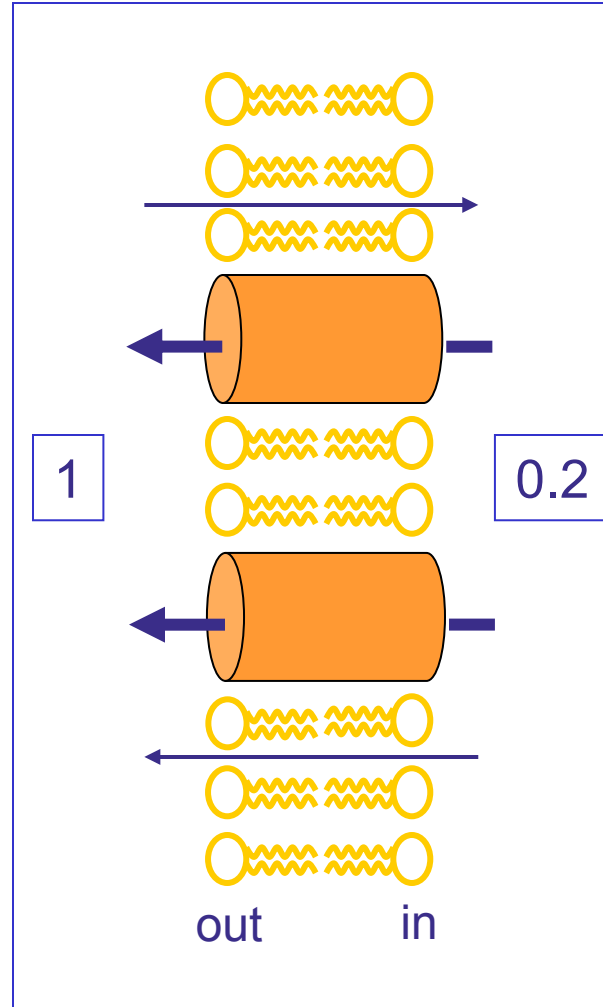
Fluoroquinolone transport: model

ciprofloxacin

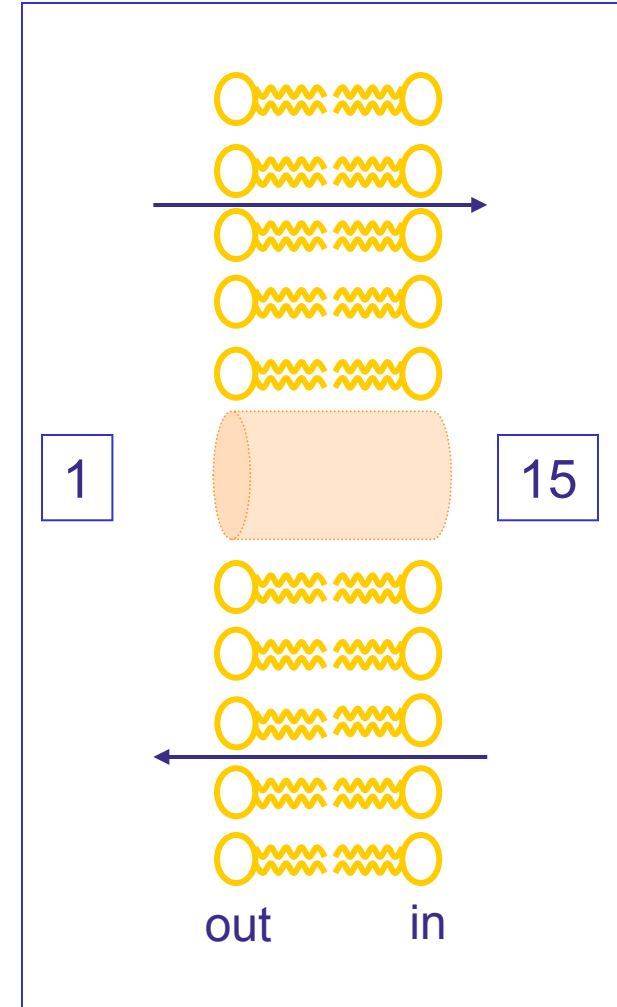
WT



CIP-RS



MXF-RS





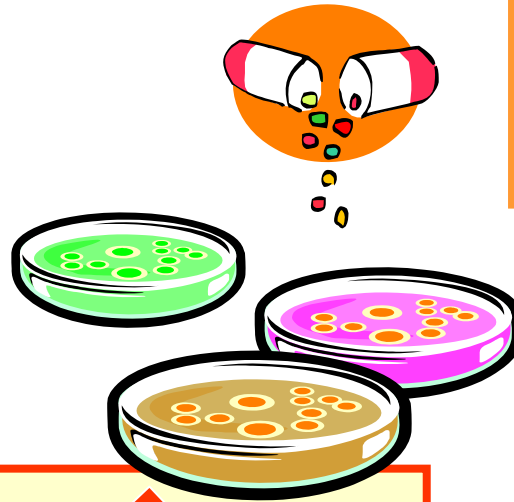
*Die Bremer
Stadtmusikanten*

**Efflux ...
is it enough for
resistance ?**

Efflux pumps ... and what next ?

How to get resistant cells ?

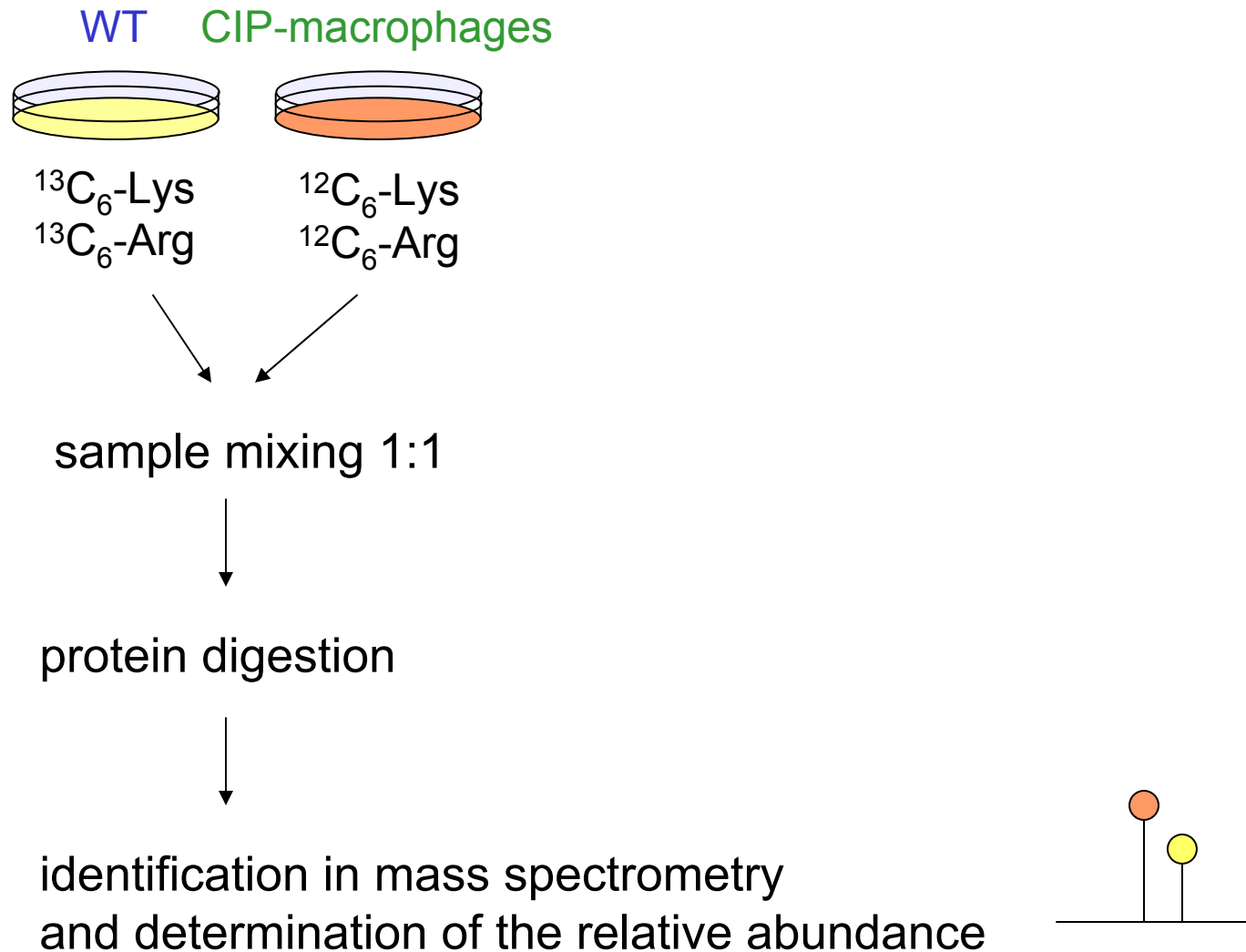
- step-wise increase in AB concentration
- several passages at each step



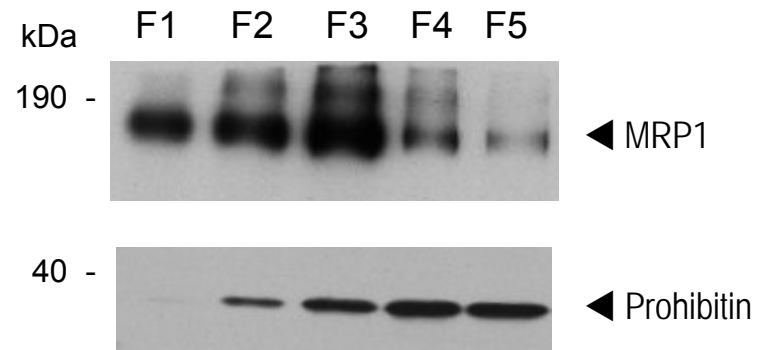
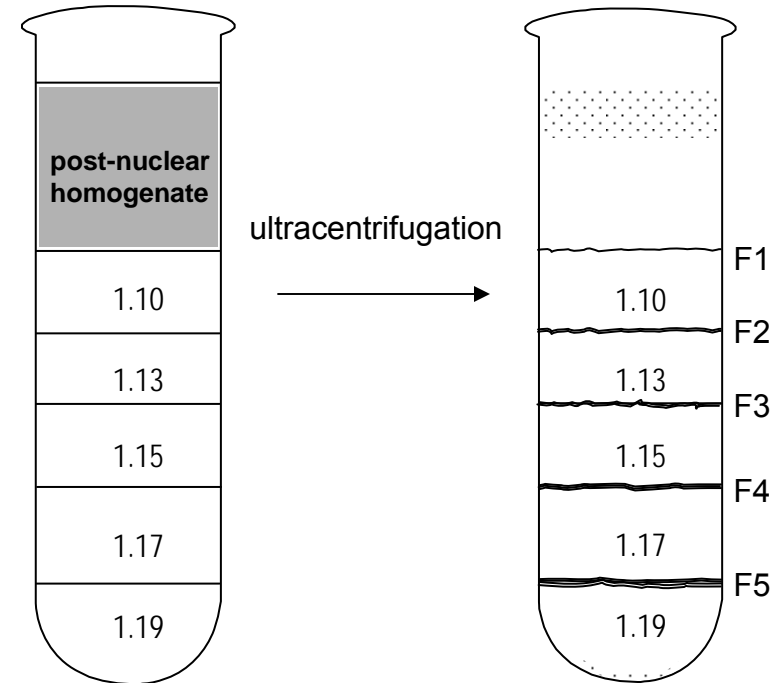
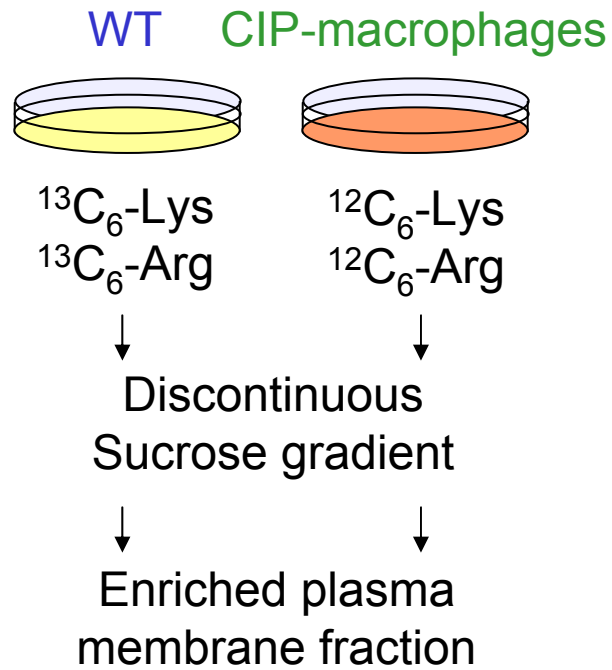
time and drug
consuming!

multifactorial multidrug resistance

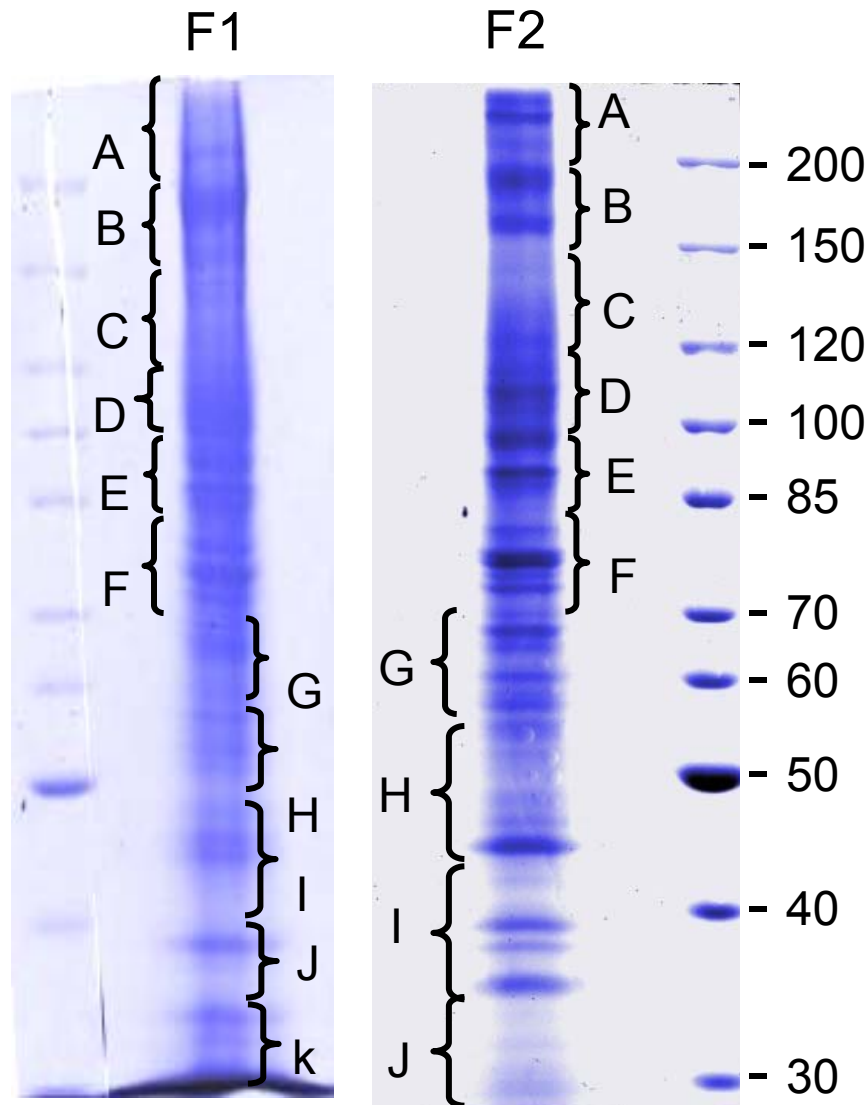
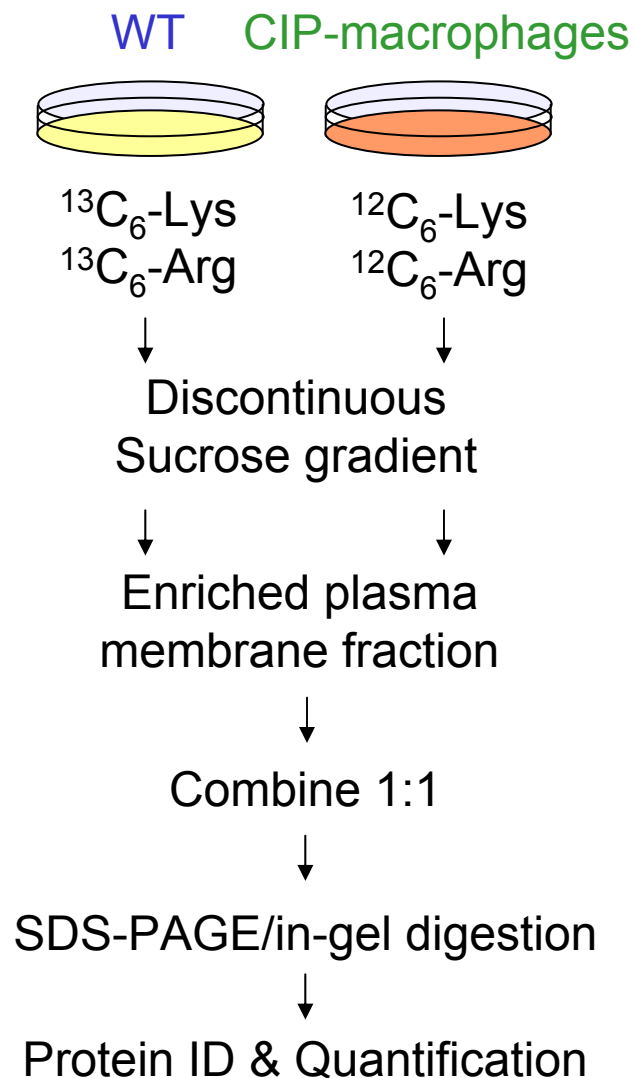
Stable Isotope Labeling Aminoacid in Culture



Stable Isotope Labeling Aminoacid in Culture



Stable Isotope Labeling Aminoacid in Culture



SILAC: global data

⇒ Identification of 900 proteins with 3 unique peptides

Among proteins detected in both fractions

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

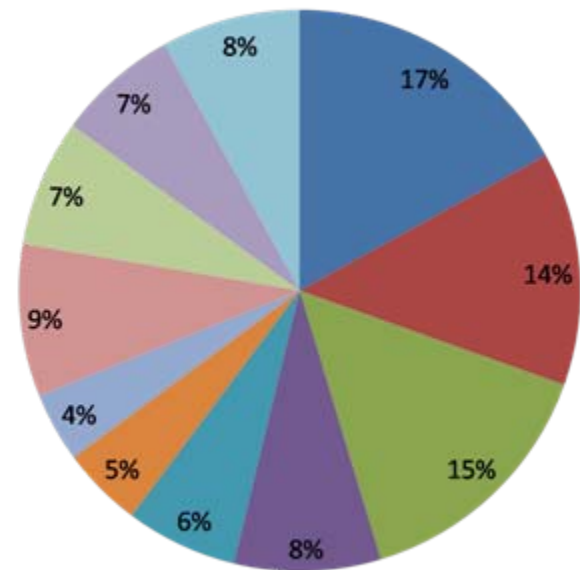
↳ 13 ↘ expression in CIP-macrophages as compared to WT

Among proteins detected in one of the two fractions

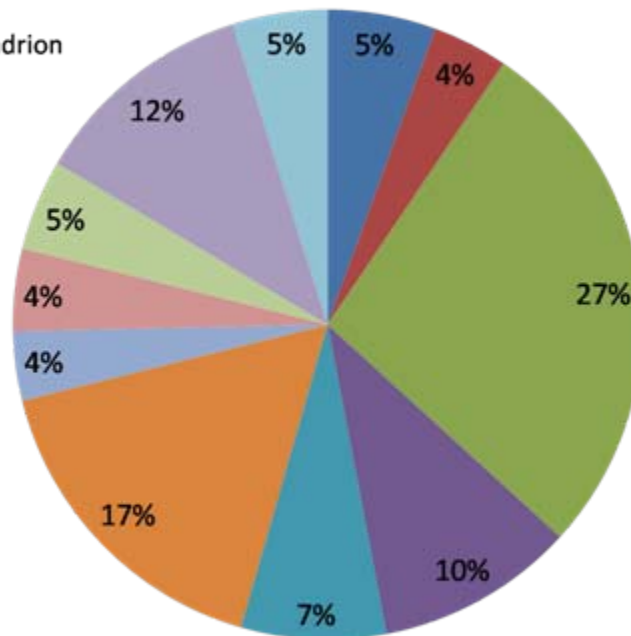
↳ 37/36 ↗ expression in CIP-macrophages as compared to WT

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

Proteins with modified expression in CIP-resistant cells

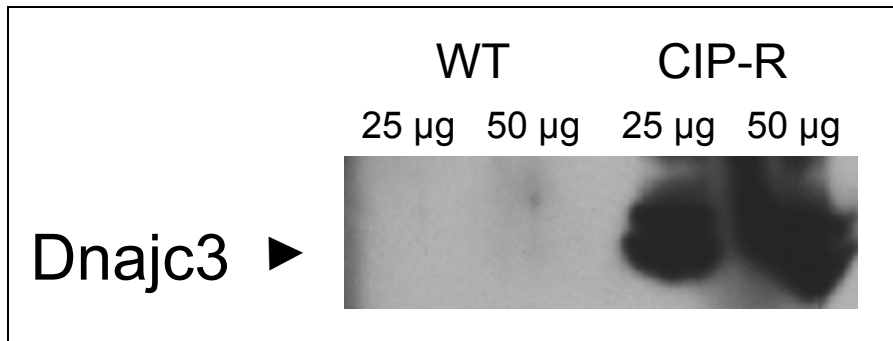


- Plasma membrane
- Unknown membrane protein
- ER/Golgi
- Endosome/Lysosome
- Cytoskeleton
- Ribosome
- Extracellular
- Nucleus
- Mitochondrion
- Cytosol
- Unknown



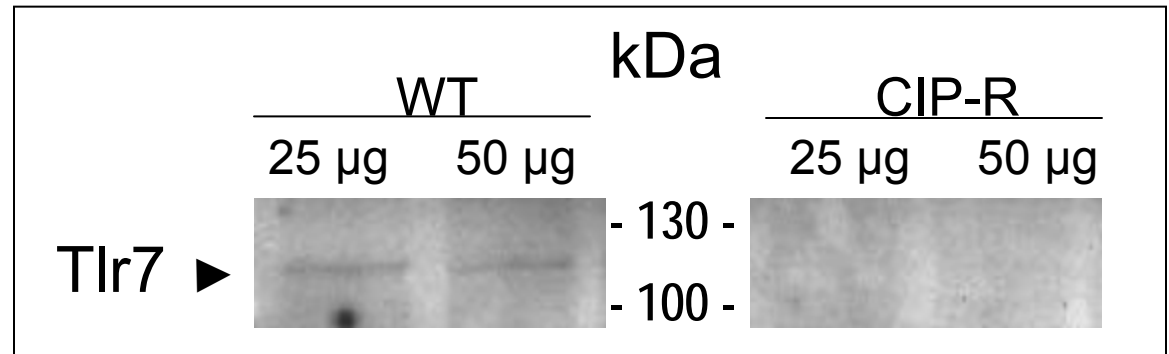
- Cell adhesion and localization
- Immune response
- Transport
- Signal transduction
- Cytoskeleton organization
- Protein metabolism
- Carbohydrate metabolism
- Lipid metabolism
- Nucleid acid metabolism
- Other
- Unknown

Proteins with modified expression in CIP-resistant cells

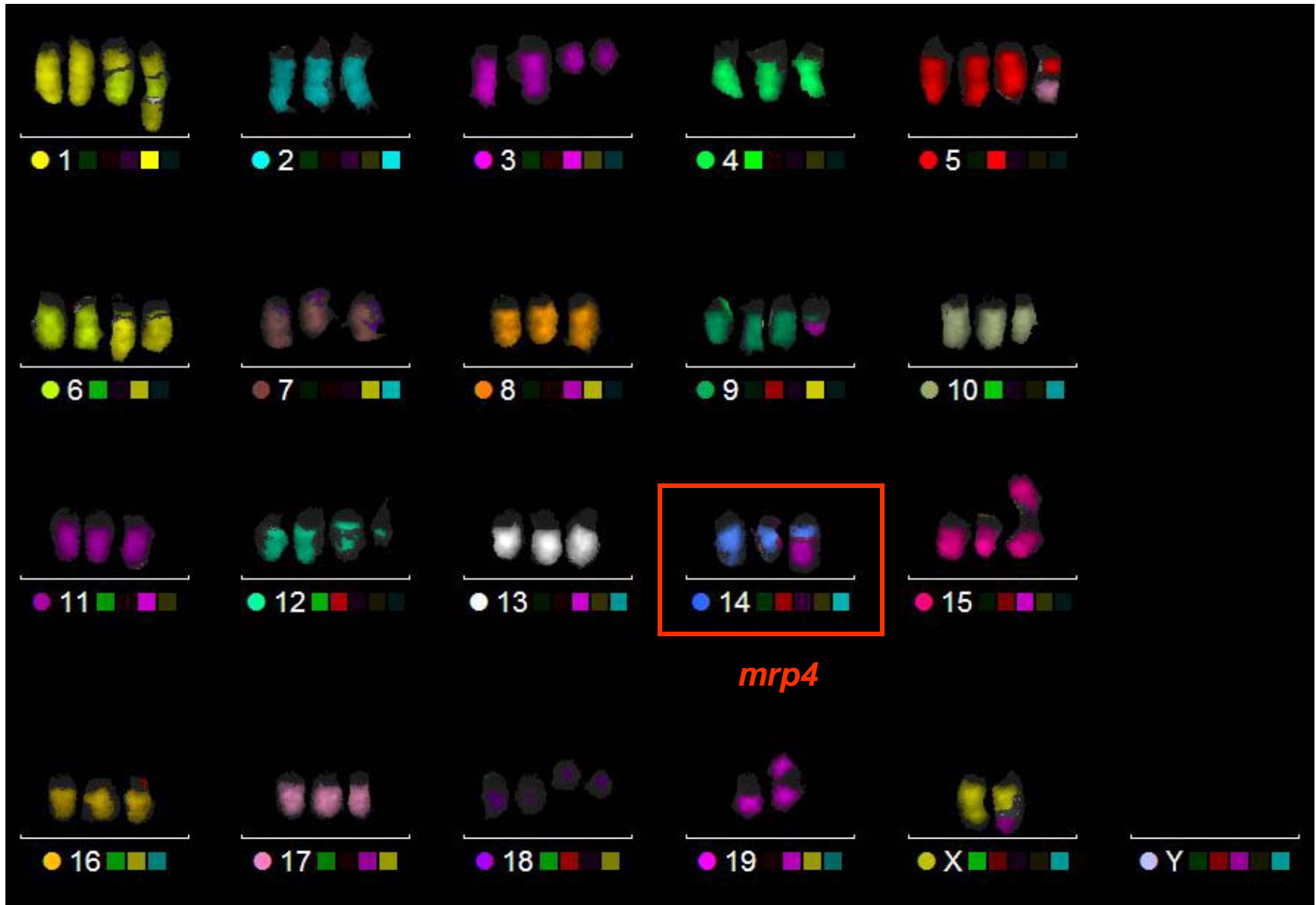


On the same chromosome
as *mrp4* !

Transports
nucleosides/tides
(substrates for Mrp4 !)

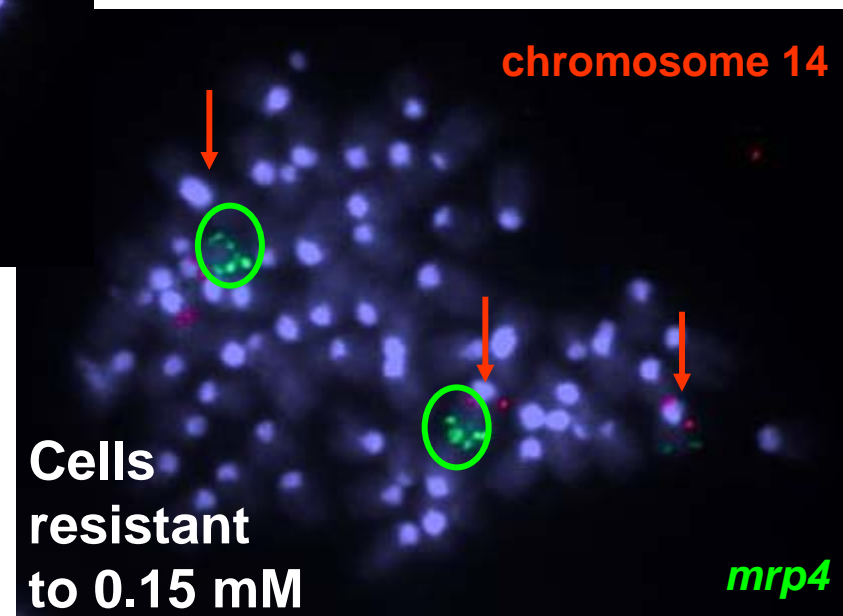
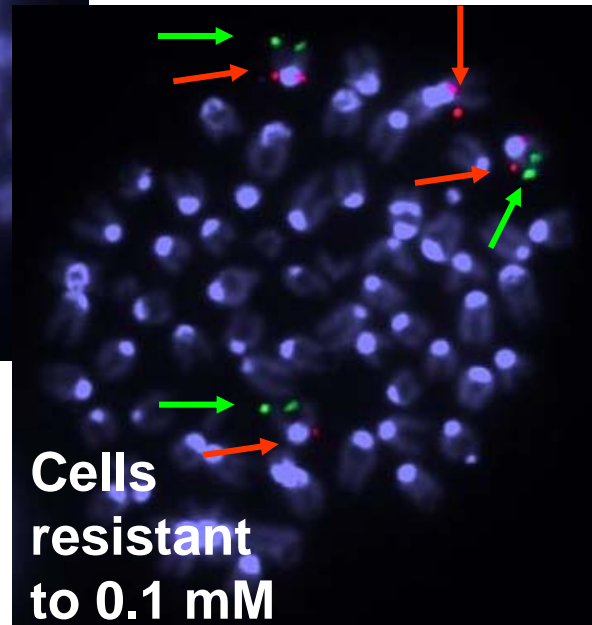
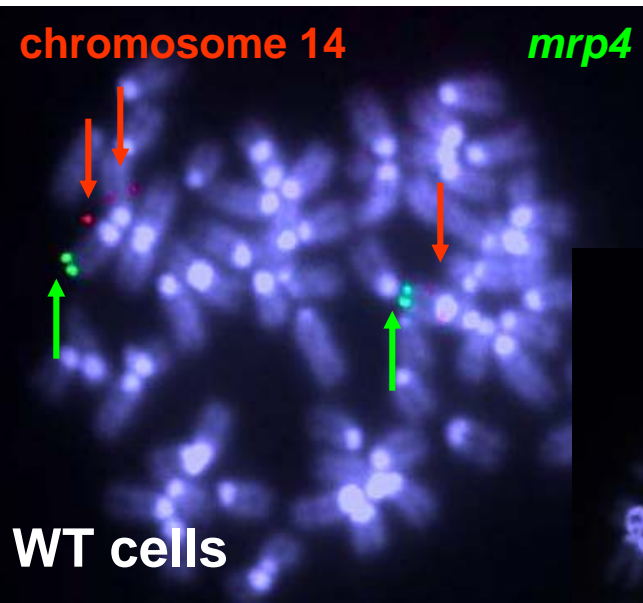


Karyotype of J774 cells



Gene amplification in CIP-resistant cells

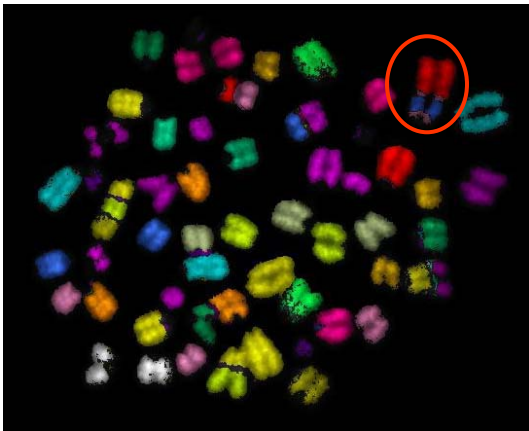
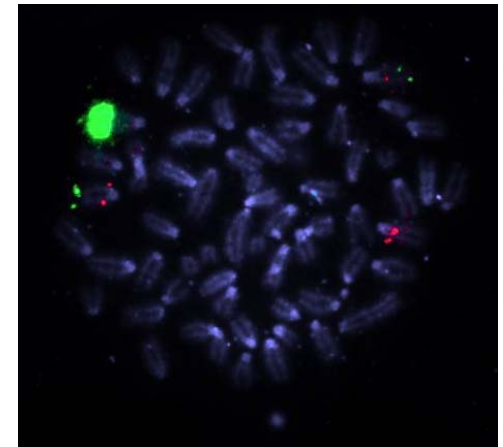
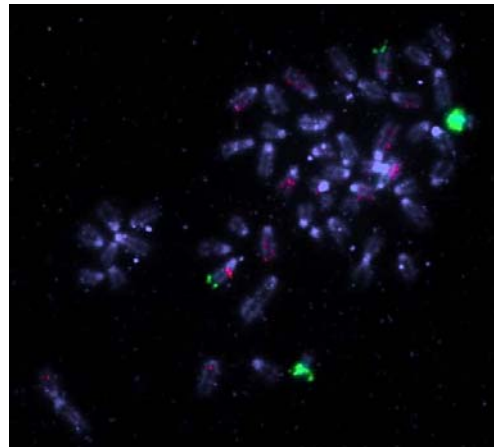
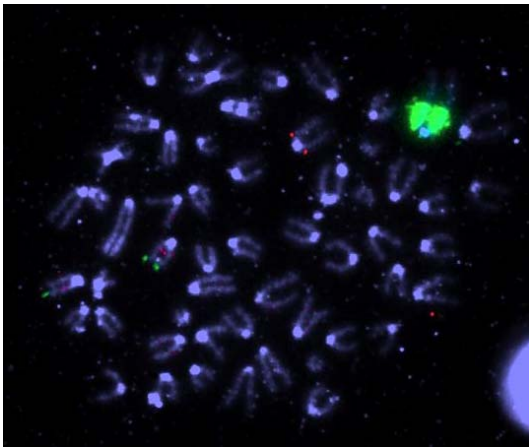
FISH analysis



Gene amplification in CIP-resistant cells

mFISH analysis of CIP-R cells

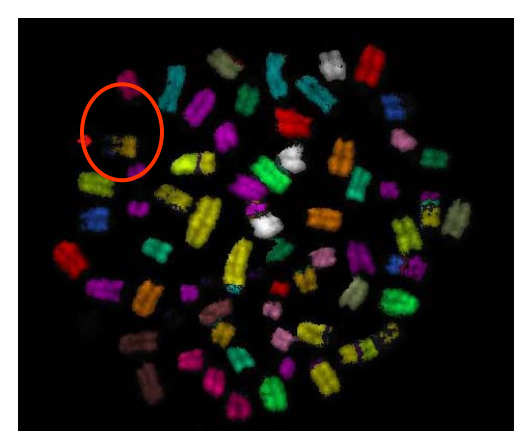
Heterogeneity of cell population !



chromosome 5



chromosome 13

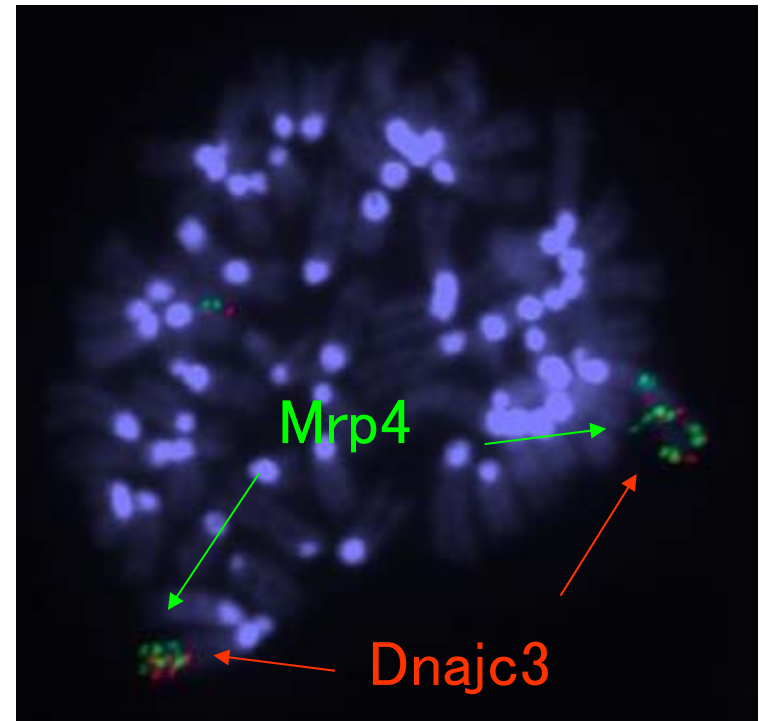
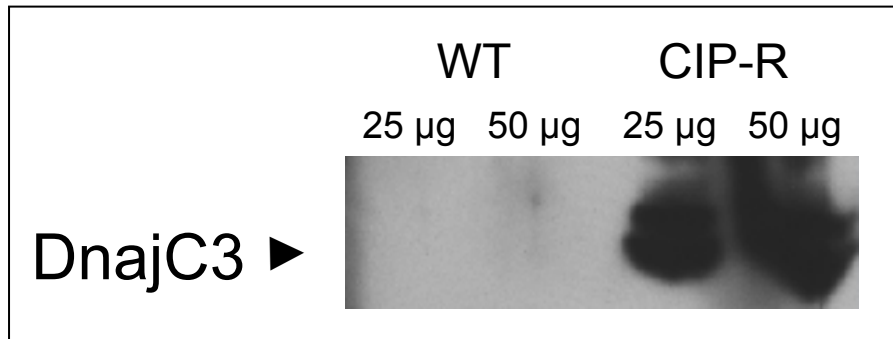


chromosome 16

Gene amplification in CIP-resistant cells

FISH analysis of CIP-R cells

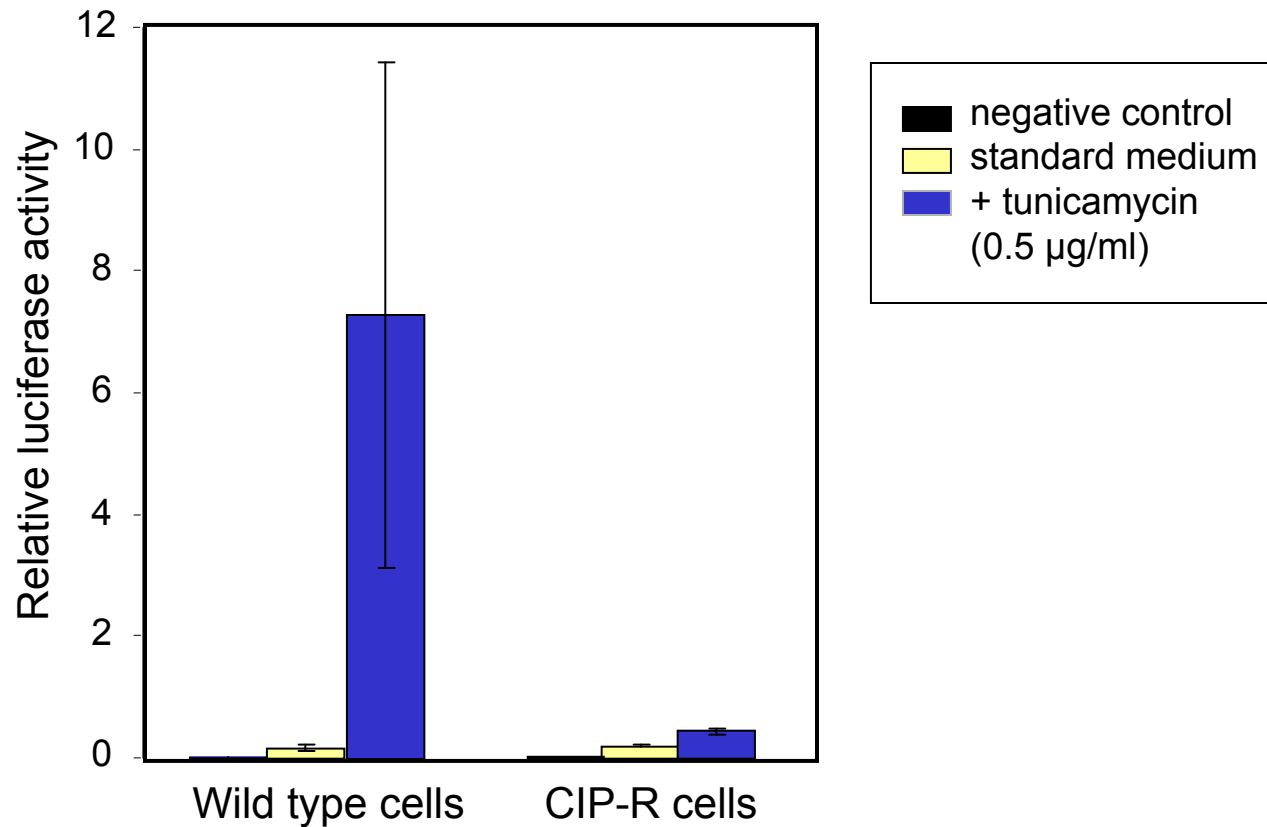
Mrp4 and DnajC3 co-amplified in CIP-resistant cells



Implication of co-amplified genes in resistance

DnajC3 protects CIP-resistant cells from ER stress

Quantification of ER-stress signaling with a Dual Luciferase Assays of cells transfected with Signal ERSE reporter





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and in
moxifloxacin-
resistant cells
?

Expression of ABC transporters

TaqMan Low Density Array of ABC transporters

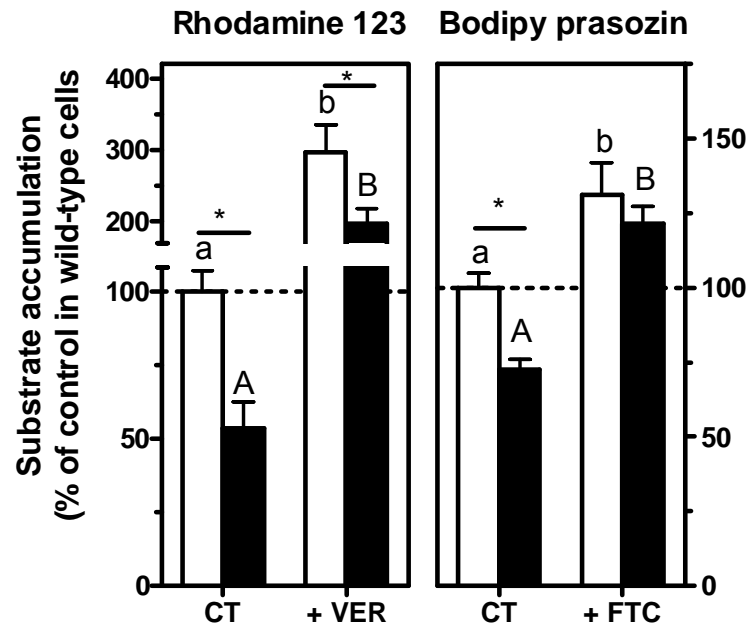
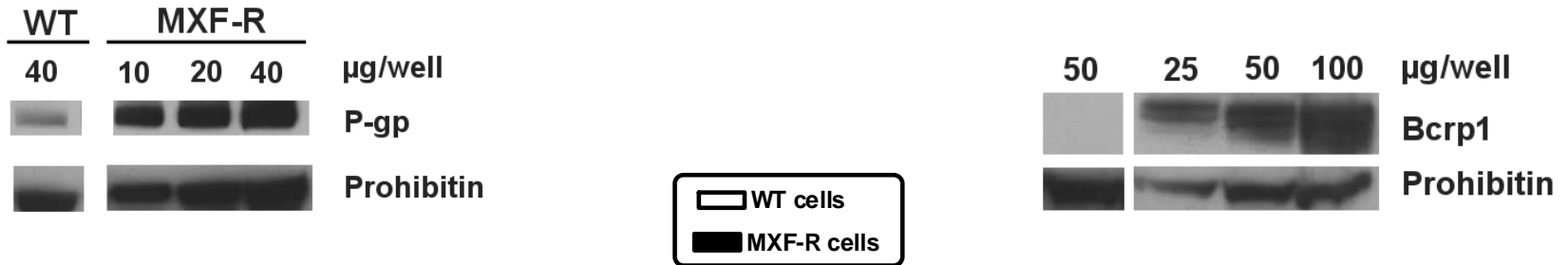
gene	Cell line	
	ciprofloxacin-resistant	moxifloxacin-resistant
Abca1	-1.53 ^a	-3.51
Abca2	-1.13	-1.12
Abca3	1.10	-1.56
Abca4	nd	nd
Abca5	-1.85	2.31
Abca6	nd	nd
Abca7	-1.36	-1.20
Abca8a	nd	nd
Abca8b	(1.39)	76.20
Abca9	(-17.45)	(-10.48)
Abca13	1.66	2.02
Abca14	nd	nd
Abca15	nd	nd
Abcb1a*	(-1.38)	76.35
	(-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)

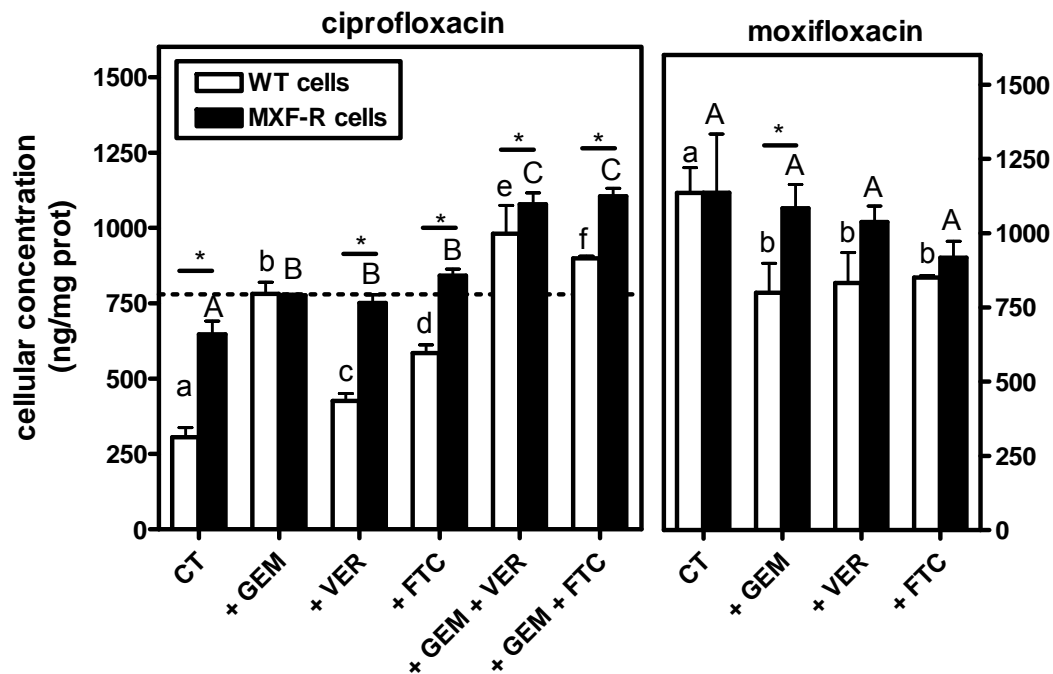
Overexpression of MDR transporters

P-gp and Bcrp are functional



Overexpression of MDR transporters

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



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

Take home message

**Antibiotics are also
substrates for
efflux in eucaryotic
cells**



*Die Bremer
Stadtmusikanten*

Take home message



*Die Bremer
Stadtmusikanten*

**Closely related
molecules behave
very differently with
respect to transport**

Take home message



*Die Bremer
Stadtmusikanten*

**Non substrates
may induce a
'anti-resistant'
phenotype**

Take home message



*Die Bremer
Stadtmusikanten*

**Efflux is only part
of the response to
drug exposure**

Coworkers ...

- **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



- **G. Ameye and H. Antoine-Poirel**

FISH

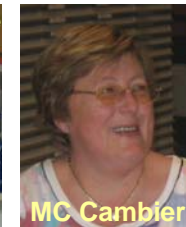
Centre de Génétique,
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Brussels Stadtmusikanten for this story ...



B. Marquez



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