Transport across membranes

Summer School, Jacobs University (Bremen), July 2007

Efflux pumps inhibitors:

the long journey from procaryotes to eucaryotes

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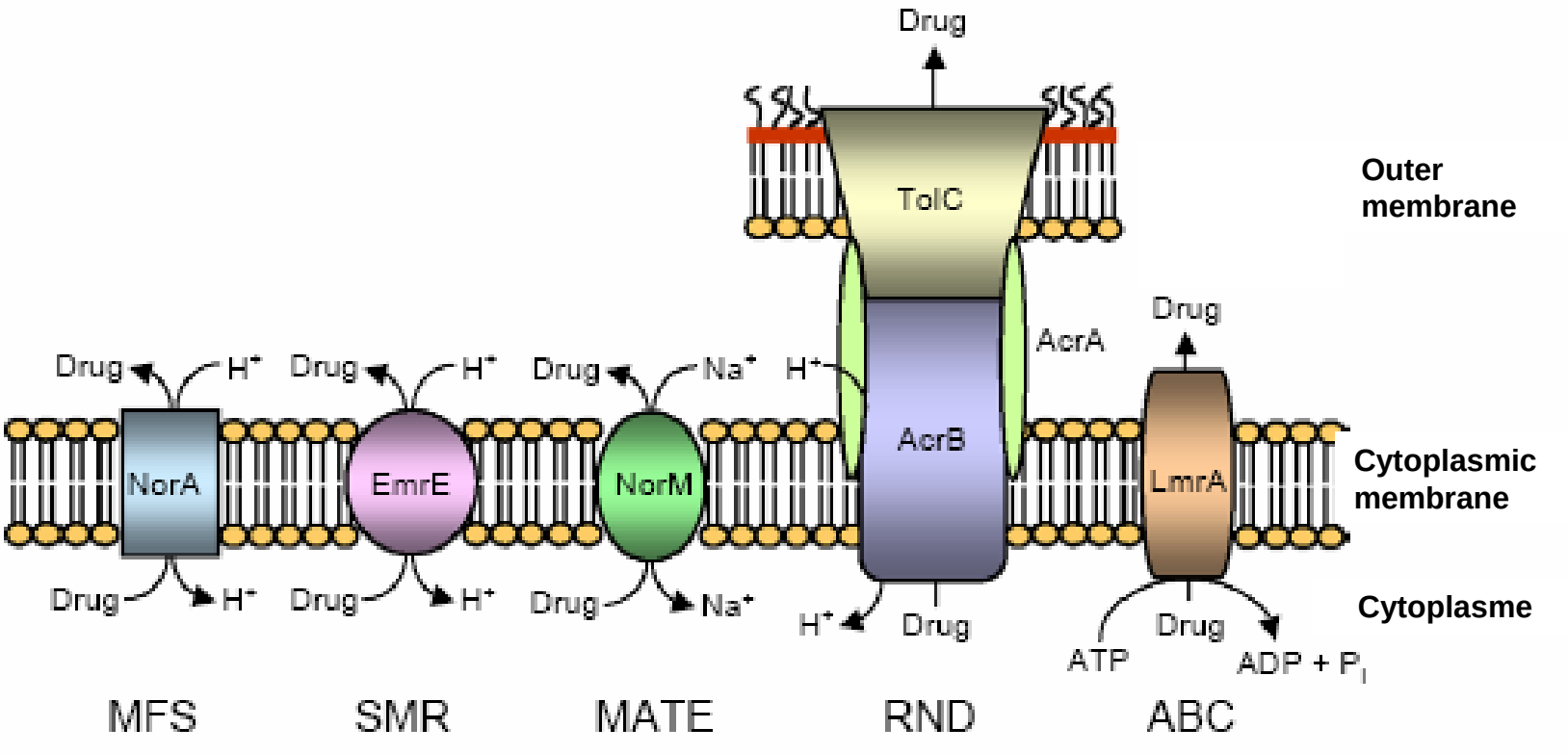
- **Efflux pumps and their clinical relevance**
- **Efflux pump inhibitor's properties**
- **EPI DISCOVERY**
  - ✓ **By rational design**
  - ✓ **By screening**
    - **- bacterial EPI**
    - **- eucaryotic EPI**

Efflux pumps



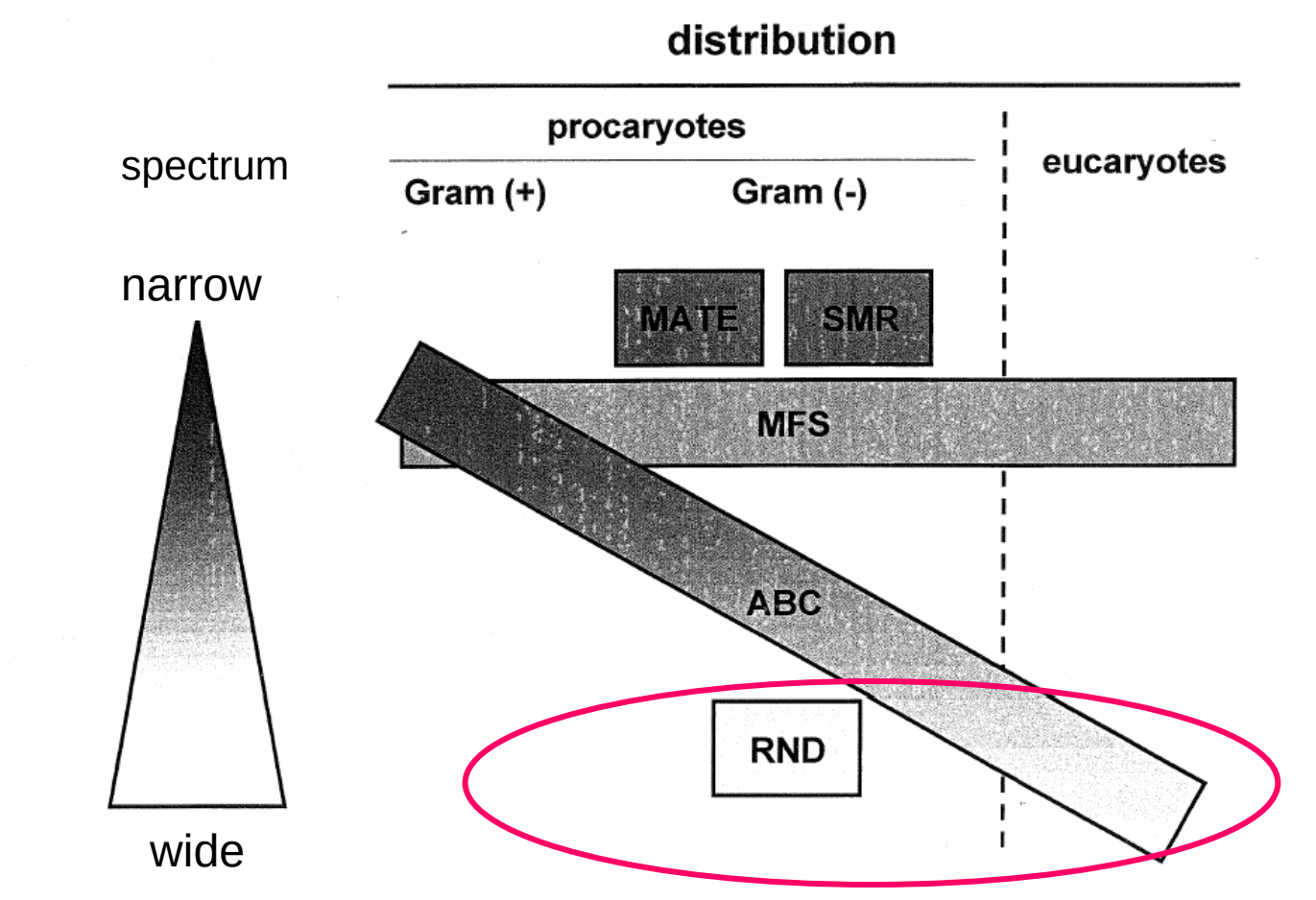
⇨ **UBIQUITOUS**

Eucaryotes: ABC-pump mediated resistance

Bacteria: mainly RND (Gram -) and MFS (Gram +) pumps

Kumar *et al. Adv. Drug Deliv. Rev*. 2005, **57**: 1486

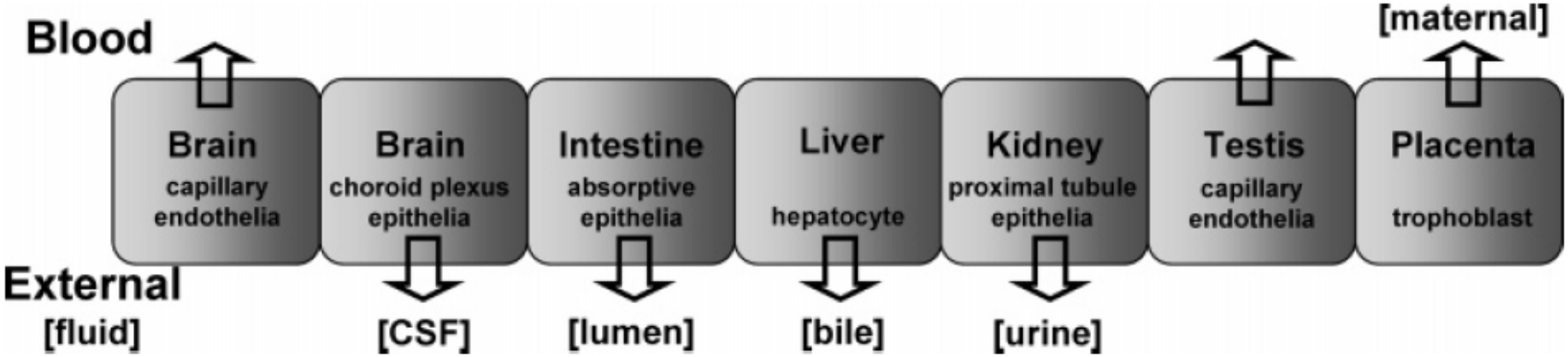
Efflux pumps spectra



Mesaros *et al., La lettre de l'Infectiologue* 2005, **4**: 117

Efflux pumps clinical relevance

Physiological function = protective role



P-gp membrane localisation

Raub T. J., *Mol. Pharm.* 2006, **3**: 3

Efflux pumps clinical relevance

Physiological function = protective role

But efflux pumps are able to expel a wide range of molecules, including a lot of drugs

Bacteria: Antibiotics, detergents, dyes, bile salts….

Eucaryotes: Anti-cancer drugs (P-gp, MRP, BCRP…)
Antifongic drugs (CDR1, *Candida albica*)
Antiparasital drugs (Pgh1, *Plasmodium falciparum*)
Antibiotics…

Efflux pumps clinical relevance

Bacteria:

- ↗ of the MCI of antibiotics (within the same class, or broad range of ATB)
- Cause intrinsic resistance (*Pseudomonas*,…)
- Several pumps can be expressed at the same time

Eucaryotes:

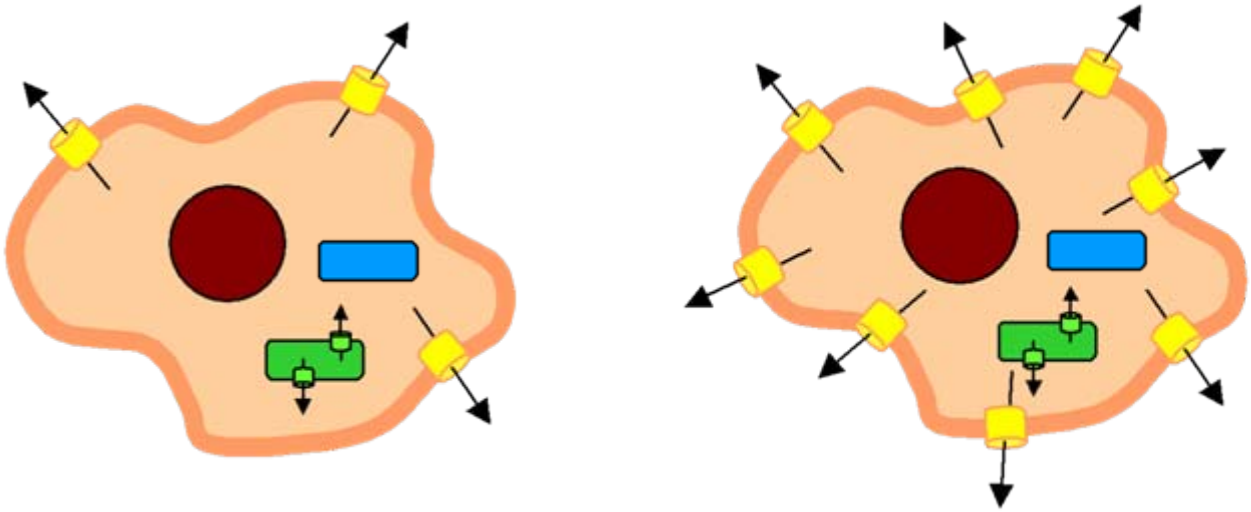
- Related to therapeutic failures (40% of tumors develop resistance to anti-cancer drugs)
- Related to negative prognosis or poor outcome for chemotherapy
- Modulate drugs pharmacokinetic: ↘ bioavailability, ↗ excretion

Efflux pumps clinical relevance

For both bacteria and eucaryotes:

- Efflux pumps can confer MDR resistance
- They can add themselves to other resistance mechanisms

Intracellular infection



⇨**Efflux pumps can be seen as a « new » target:**
Efflux pump inhibitors (EPI), by blocking the pumps, will restore drugs activity

Efflux pump inhibitors

An Efflux Pump Inhibitor will:

- restore the activity of the drug in resistant cells (intrinsic or acquired resistance)
- be devoid of effect in wild type cells
- decrease the frequency of apparition of resistant mutants

Efflux pump inhibitors

An Efflux Pump Inhibitor will:

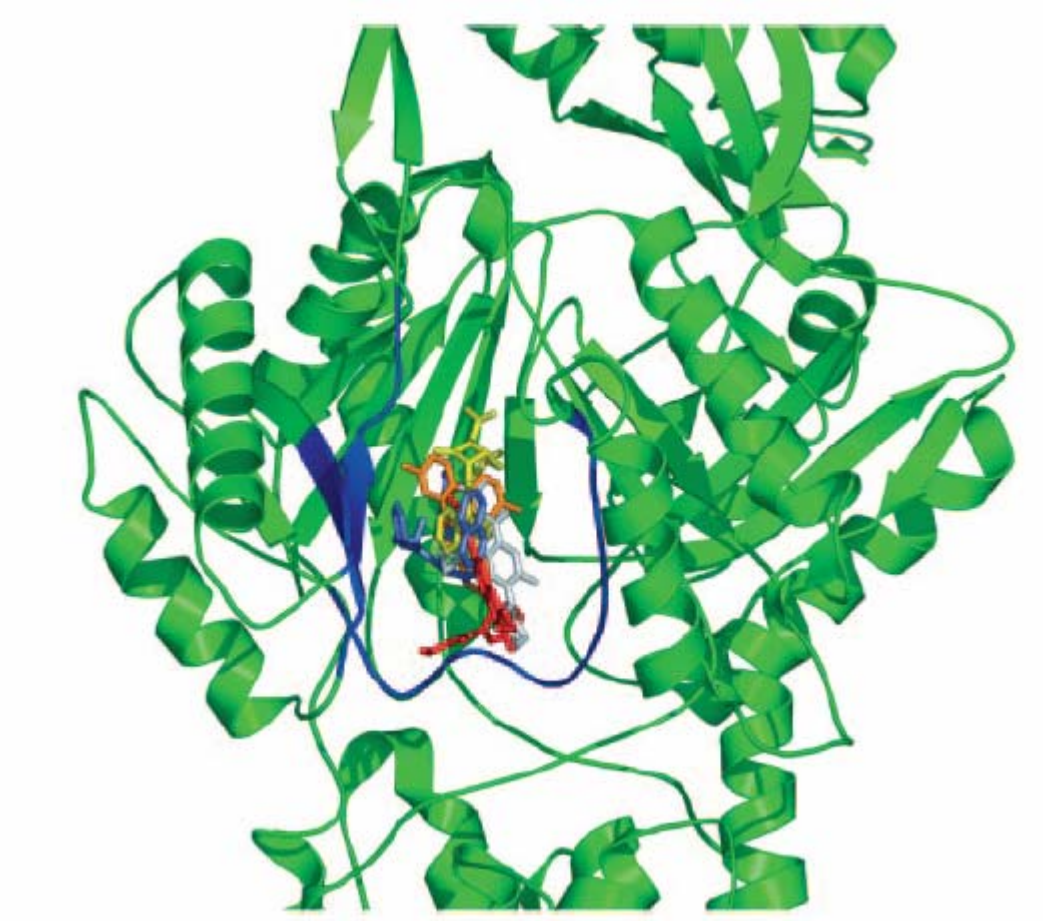
- restore the activity of the drug in resistant cells (intrinsic or acquired resistance)
- be devoid of effect in wild type cells
- decrease the frequency of apparition of resistant mutants

An EPI would act by:

- binding to the pump with an increased affinity (competitive inhibition)
- impairing access to the binding site for the drug (non competitive inhibition)
- *dissipating the energy source used by the pump (not clinically relevant)*

MDR pumps binding sites

AcrB periplasmic drug binding pocket

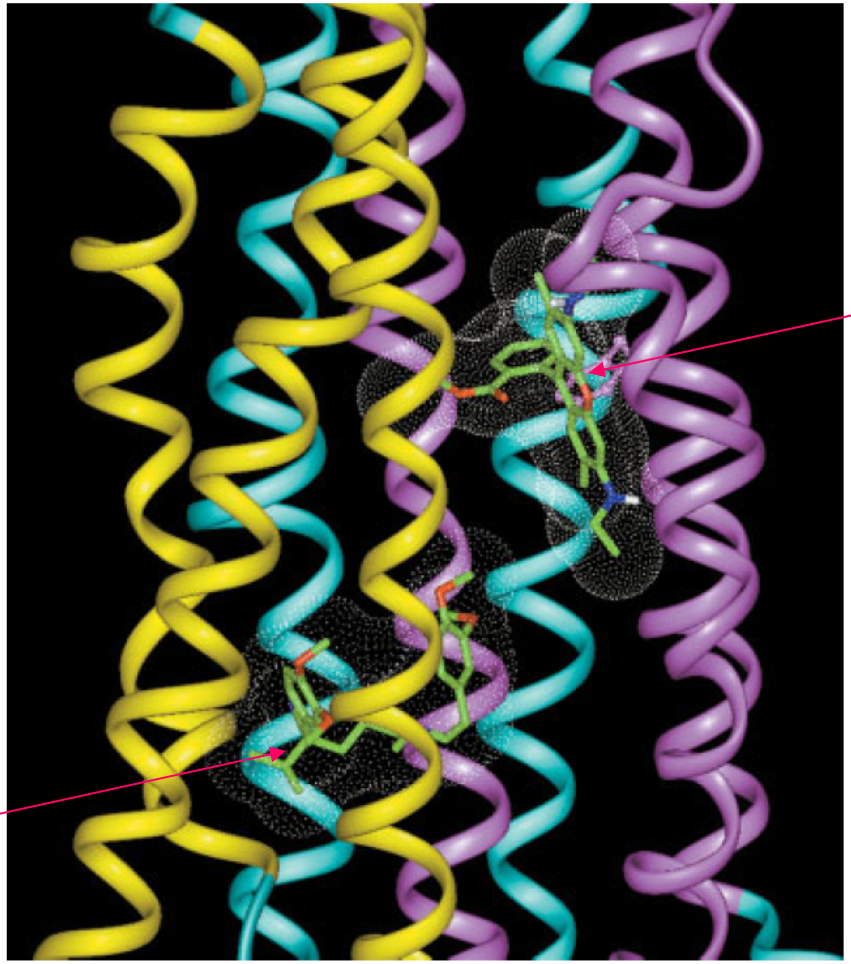


Yu et al., *J. Bacteriol.* 2006, **187**: 6804

MDR pumps binding sites

Modeling of the binding of two P-gp substrates

Longitudinal view
across the membrane



Vandevuer et al., *Proteins* 2006, **63** : 466

EPIs criteria

To be used *in vivo*, an EPI should:

- be stable and not toxic

- be selective of a family of pumps:
    - eukaryotic pumps: selective of one sub-family (P-gp, MRP, BCRP)
    - bacterial pumps: a wide inhibitor (Gram +/Gram -) would be advantageous, devoided of activity against human pumps

- not cause side effects by perturbing efflux pumps physiological role

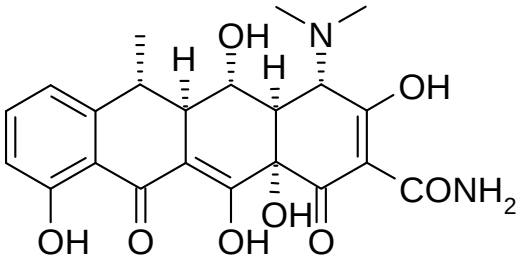
- be co-administrable with the drug

Otherwise, EPIs can be used *in vitro* as tools to detect the presence of efflux pumps (diagnostic) and to study them (affinity, binding sites…)….

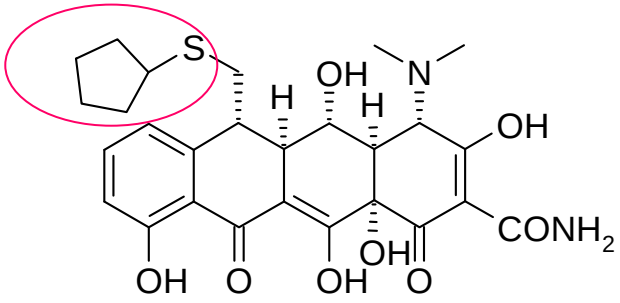
EFFLUX PUMP INHIBITORS DISCOVERY

Rational design

Case of specific pumps: Tetracycline pumps



Doxycycline



13-cyclopentylthio-tetracycline

Chemical modification of the substrate:

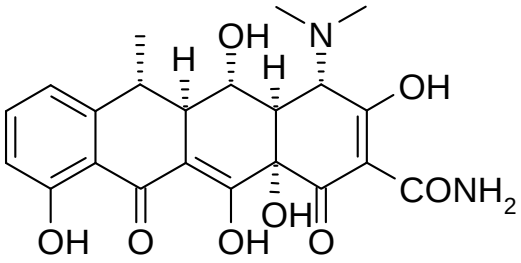
⇨ 6-fold increase of the affinity of 13-CPTC for Tet(B)

⇨ Competitive inhibitor → Useful in addition to tetracyclines

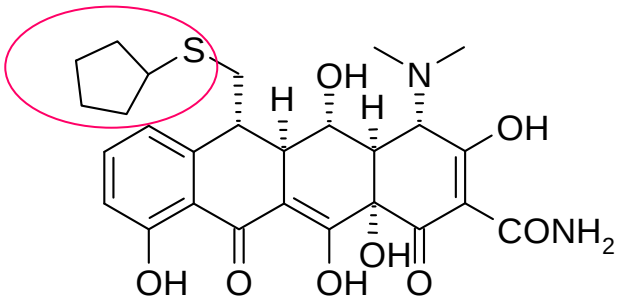
Nelson and Levy, *Antimicrob. Agents Chemother.* 1999, **43**: 1719

Rational design

Case of specific pumps: Tetracycline pumps



Doxycycline



13-cyclopentylthio-tetracycline

Different classes of Tet efflux pumps

Other resistance mechanisms (ribosomal protection)

Development of new tetracyclines not recognised by these pumps

↳**by-pass the efflux pump**

Rational design

Case of specific pumps: Tetracycline pumps

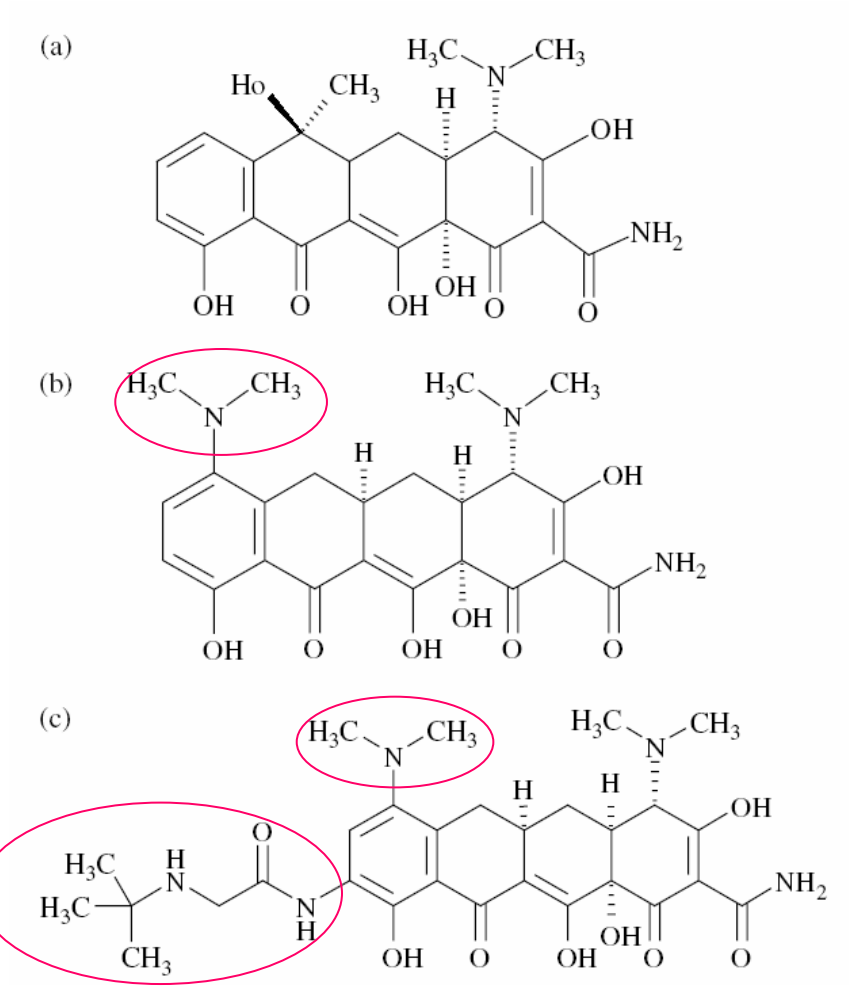
↳By-passing efflux pumps

Tetracycline

Minocycline

Substitution that impairs efflux

Tigecycline

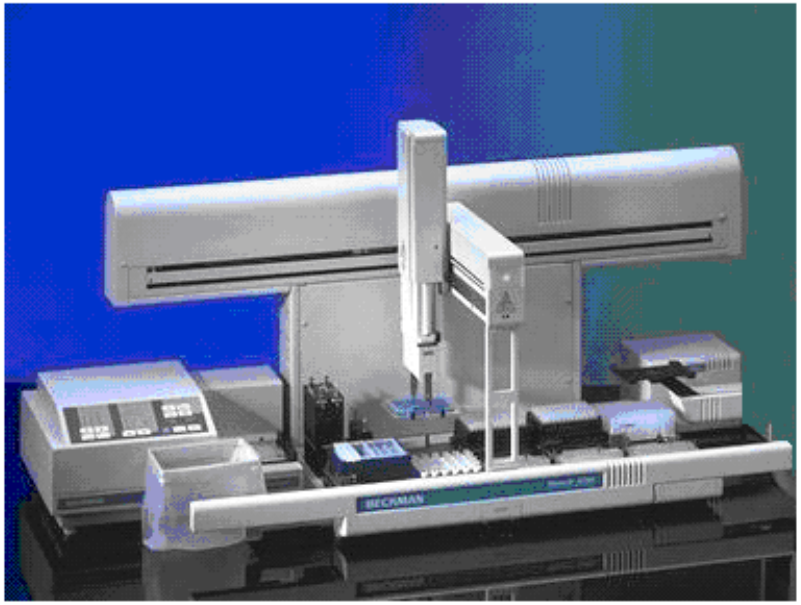


Nelson and Levy, *Antimicrob. Agents Chemother.* 1999, **43**: 1719

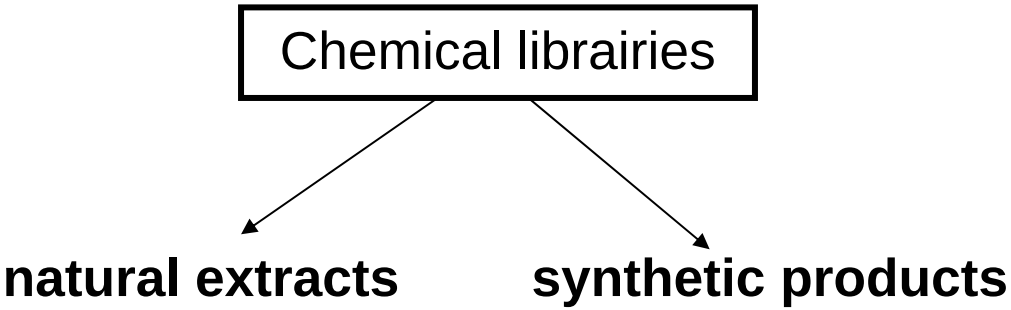
Screening for EPI

Chemical librairies

natural extracts **synthetic products**



Screening for EPI



Screen:

- Against bacterial strains over-expressing efflux pumps (*S. aureus* NorA, *P. aeruginosa* MexAB-OprM…) or against cancer cells

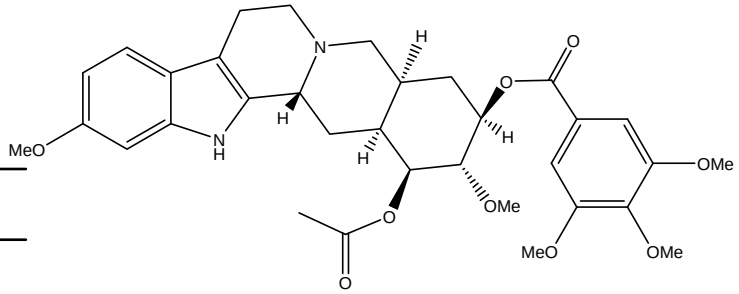
- First screen: ↗ potency of the drug?

- Second screen: ↗ intracellular accumulation of the drug?

- And then confirmation of an interaction between the efflux pump and the EPI (interference in photoaffinity labelling, co-crystallisation…)

Bacterial EPI

Reserpine (indole alkaloid)



Organism	Pump	Family	Substrates
B. subtilis	Bmr	MFS	FQ
S. aureus	NorA	MFS	FQ
MRSA	Tet(K)	MFS	Tetracycline
S. pneumoniae	PmrA PatA and PatB	MFS ABC	NOR FQ
L. monocytogenes	Lde	MFS	FQ

⇨ Inhibition of Gram + efflux pumps, mainly involved in FQ efflux

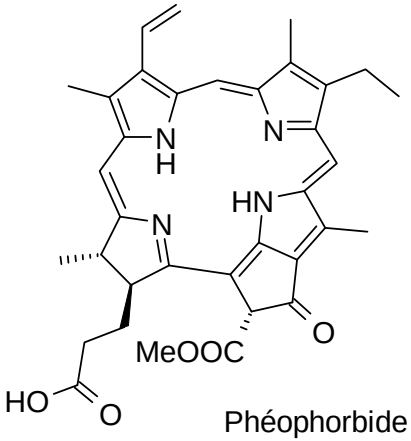
⇨ As resistance to reserpine has been reported, this suggests a direct binding to the pump (at least to Bmr*)

Neurotoxic at the concentrations required to inhibit pumps *in vivo*
→ reserpine can not be used in humans

*Klyachko *et al., J. Bacteriol.* 1997, **179**: 2189

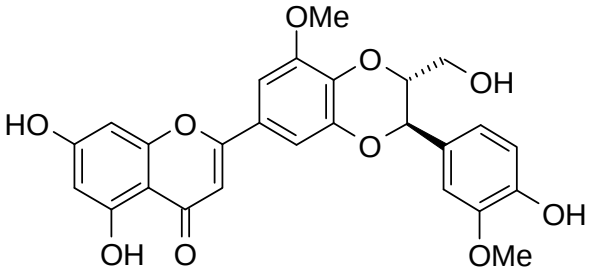
Bacterial EPI

Screening of natural compouds



Phéophorbide A

porphyrin



5'-Méthoxyhydnocarpin D

flavonolignan

⇨ NorA pump inhibitors (MFS family), isolated from *Berberis* plants

⇨ Restore berberine activity, a weak antibiotic produced by the plant

⇘ **Plants may have evolve so that they produce weak antibiotic associated with EPI**

Stermitz *et al*., *Proc. Natl. Acad. Sci. USA* 2000, **97**: 1433

Bacterial EPI

Screening of natural compouds

- Most identified EPI are provided by plant extracts

- Most are active against Gram + pumps (MFS family), and not against Gram - pumps

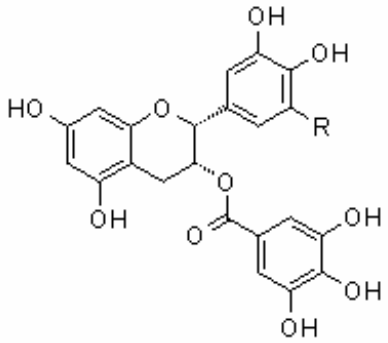
- Some are also active against eukaryotic pumps

⇨ All these molecules have a **large size and are lipophilic** (alkaloids, flavonolignans, flavones/isoflavones, catechin gallates, diterpenes….)

⇨**Starting point for further lead optimization**

⇨**Need to prove the EPI/pump interaction**

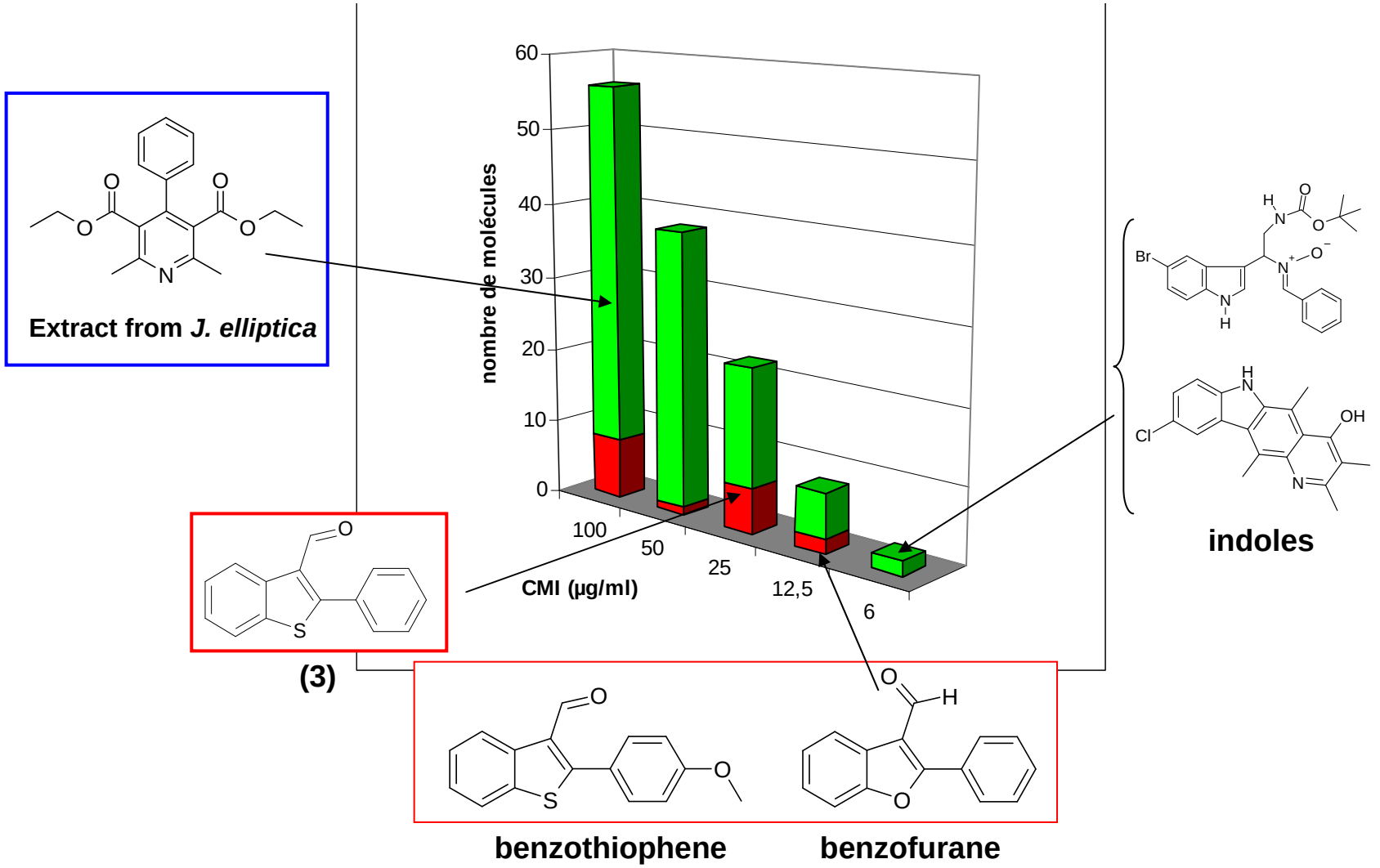
⇨**Check for *in vivo* toxicity…**



Epicatéchine-gallate (R = H)
Epigallocatéchine-gallate (R = OH)

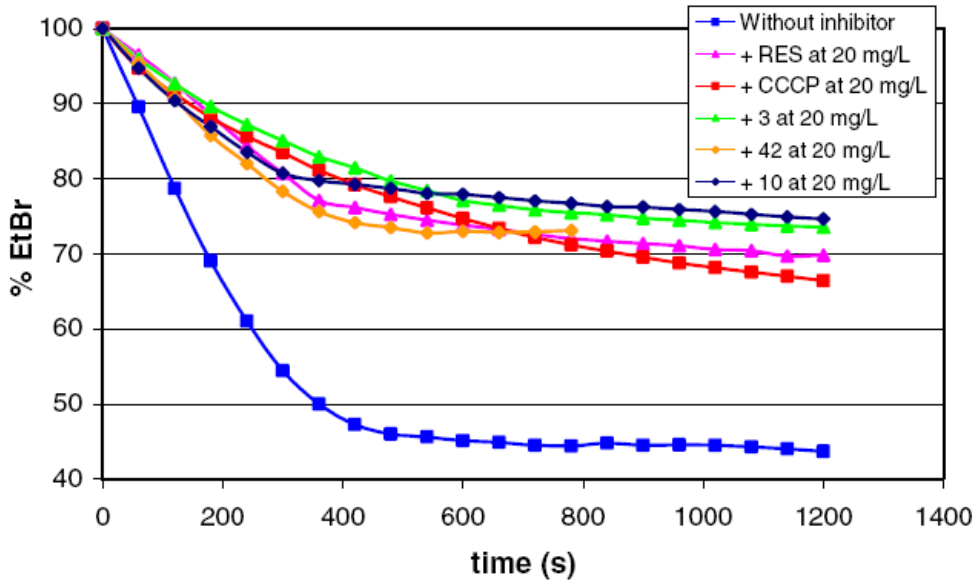
Results from a screen against NorA

Among ~ 4 000 molecules ⇨ 180 were able to restaure CIP activity



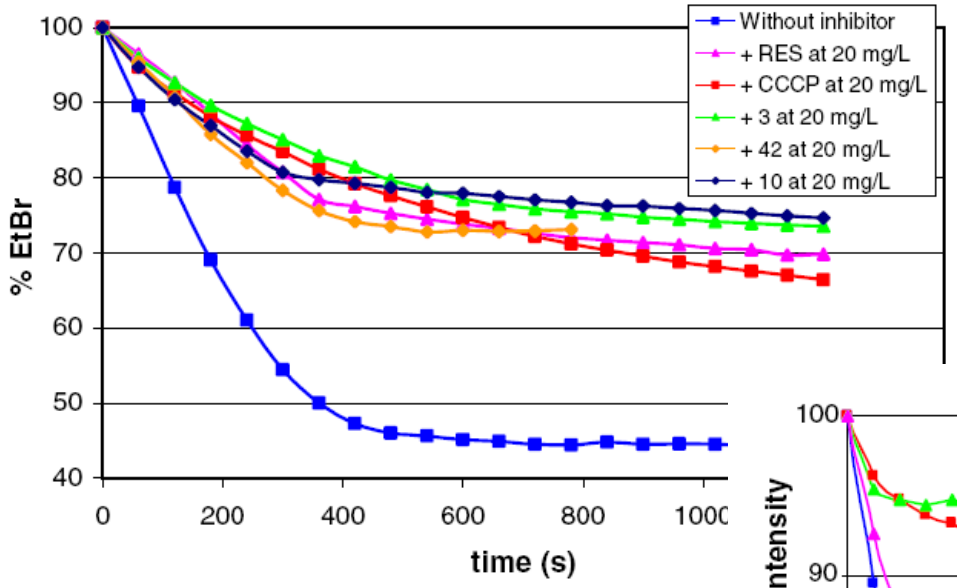
Distribution of actives molecules according to their MIC against *S. aureus* NorA in presence of CIP at ¼ of its MIC

Results from a screen against NorA

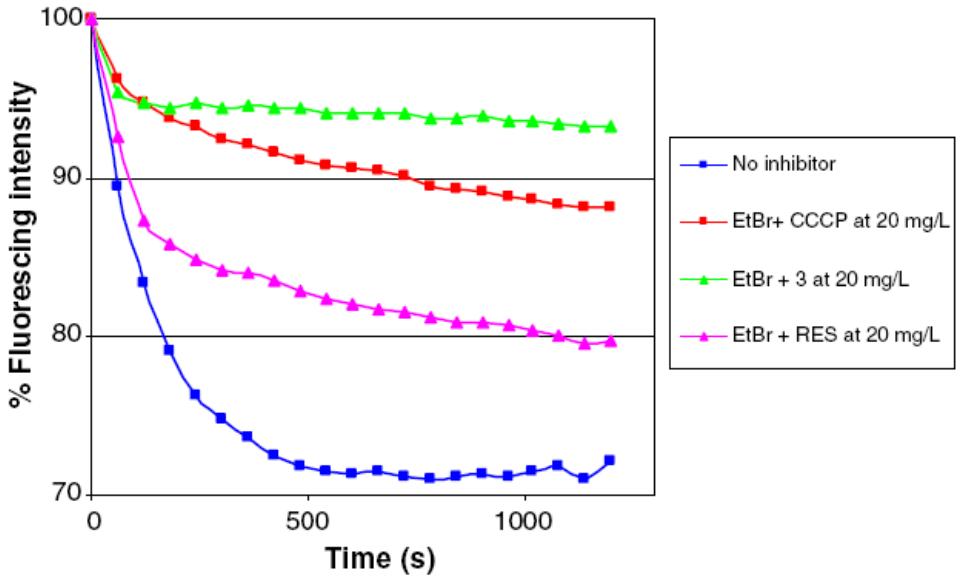


EtBr efflux from *S. aureus* NorA

Results from a screen against NorA



EtBr efflux from *S. aureus* NorA



EtBr efflux from a *B. subtilis* over-expressing NorA

Fournier dit Chabert et al., *Bioorg. Med. Chem.* 2007, **15**: 4482

ABC families within eucaryotes

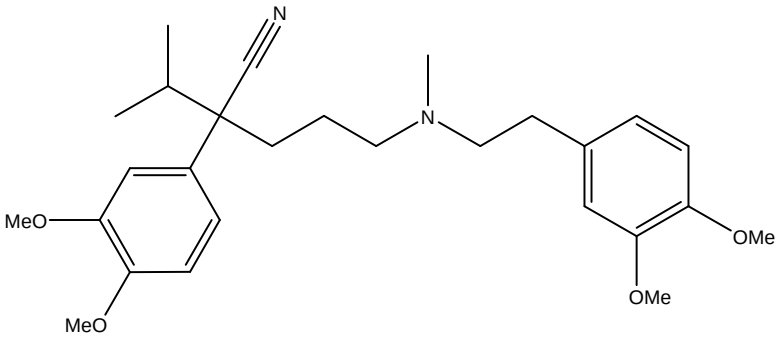
TABLE 1 List of human ABC genes, chromosomal location, and function

Symbol	Alias	Location	Function
ABCA1	ABC1	9q31.1	Cholesterol efflux onto HDL
ABCA2	ABC2	9q34	Drug resistance
ABCA3	ABC3	16p13.3	Phosphatidyl choline efflux
ABCA4	ABCR	1p22.1-p21	N-retinylidiene-PE efflux
ABCA5		17q24	
ABCA6		17q24	
ABCA7		19p13.3	
ABCA8		17q24	
ABCA9		17q24	
ABCA10		17q24	
ABCA12		2q34	
ABCA13		7p11-q11	
ABCB1	PGY1, MDR	7p21	Multidrug resistance
ABCB2	TAP1	6p21	Peptide transport
ABCB3	TAP2	6p21	Peptide transport
ABCB4	PGY3	7q21.1	PC transport
ABCB5		7p14	
ABCB6	MTABC3	2q36	Iron transport
ABCB7	ABC7	Xq12-q13	Fe/S cluster transport
ABCB8	MABC1	7q36	
ABCB9		12q24	
ABCB10	MTABC2	1q42	
ABCB11	SPGP	2q24	Bile salt transport
ABCC1	MRP1	16p13.1	Drug resistance
ABCC2	MRP2	10q24	Organic anion efflux
ABCC3	MRP3	17q21.3	Drug resistance
ABCC4	MRP4	13q32	Nucleoside transport
ABCC5	MRP5	3q27	Nucleoside transport
ABCC6	MRP6	16p13.1	
CFTR	ABCC7	7q31.2	Chloride ion channel
ABCC8	SUR	11p15.1	Sulfonylurea receptor
ABCC9	SUR2	12p12.1	Potassium channel regulation
ABCC10	MRP7	6p21	
ABCC11		16q11-q12	
ABCC12		16q11-q12	
ABCD1	ALD	Xq28	VLCFA transport regulation
ABCD2	ALDL1, ALDR	12q11-q12	
ABCD3	PXMP1,PMP70	1p22-p21	
ABCD4	PMP69, P70R	14q24.3	
ABCE1	OABP, RNS4I	4q31	Elongation factor complex
ABCF1	ABC50	6p21.33	
ABCF2		7q36	
ABCF3		3q25	
ABCG1	ABC8, White	21q22.3	Cholesterol transport
ABCG2	ABCP, MXR, BCRP	4q22	Toxin efflux, drug resistance
ABCG4	White2	11q23	Cholesterol transport
ABCG5	White3	2p21	Sterol transport
ABCG8		2p21	Sterol transport

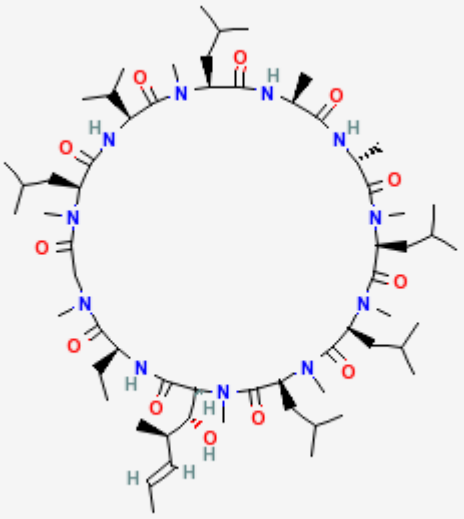
Dean and Annilo, *Annu. Rev. Genomics Hum. Genet.* 2005, **6**:123

P-gp inhibitors

Class	Pharmaceutical code name	USAN name	Chemical class
1st Generation	Verapamil	–	Diphenylalkylamine Ca-channel blocker
	Cyclosporine A	–	Cyclic oligopeptide immunosuppressant
	Tamoxifen	–	Nonsteroidal anti-estrogen



verapamil



cyclosporine A

1st generation: not developped for MDR pumps inhibition, low affinity for pumps, side effects at the concentrations required to inhibit P-gp in vivo

McDevitt *et al., Pharmacol. Ther.* 2007, **113**: 429

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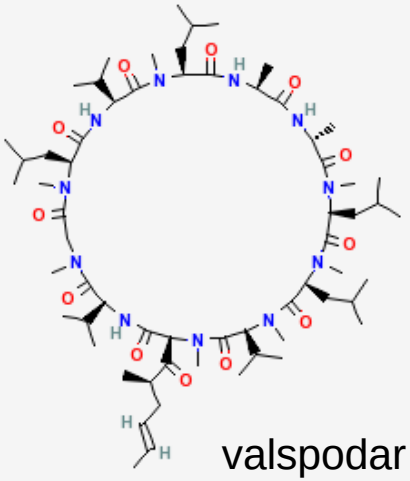
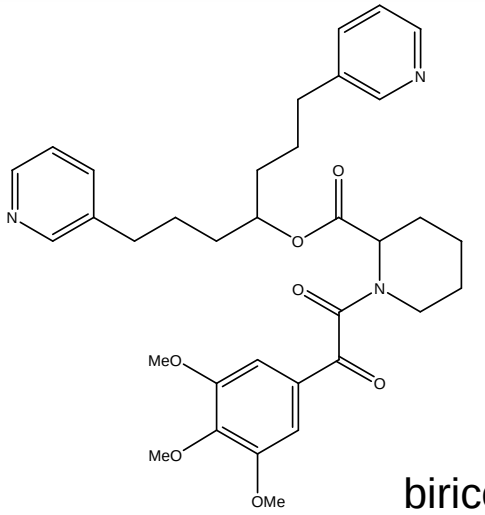
1st Generation	Verapamil	–	Diphenylalkylamine Ca-channel blocker
	Cyclosporine A	–	Cyclic oligopeptide immunosuppressant
	Tamoxifen	–	Nonsteroidal anti-estrogen
2nd Generation	PSC833	Valspodar	Non-immunosuppresive derivative of cyclosporine A
	VX-710	Biricodar	Derivative of FK-506 - macrocyclic antibiotic
	S9788	–	Triazine
	SR33557	–	Indolizin sulfone

QSAR



valspodar



biricodar

2nd generation: more potent, toxicity reduced, but anti-cancer drugs pharmacokinetic impaired (metabolisme & elimination, via interactions with cytochrome P450)

McDevitt *et al., Pharmacol. Ther.* 2007, **113**: 429

P-gp inhibitors

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1st Generation	Verapamil	–	Diphenylalkylamine Ca-channel blocker
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QSAR	Tamoxifen	–	Nonsteroidal anti-estrogen
2nd Generation	PSC833	Valspodar	Non-immunosuppresive derivative of cyclosporine A
	VX-710	Biricodar	Derivative of FK-506 - macrocyclic antibiotic
	S9788	–	Triazine
	SR33557	–	Indolizin sulfone
3rd Generation	GF120918	Elacridar	Acridonecarboximide
	LY335979	Zosuquidar	Cyclopropyldibenzosuberane
Combinatorial chemistry	XR9576	Tariquidar	Anthranilamide
	OC144-093	Ontogen	Diarylimidazole

3rd generation: more specific and powerful, under clinical trials

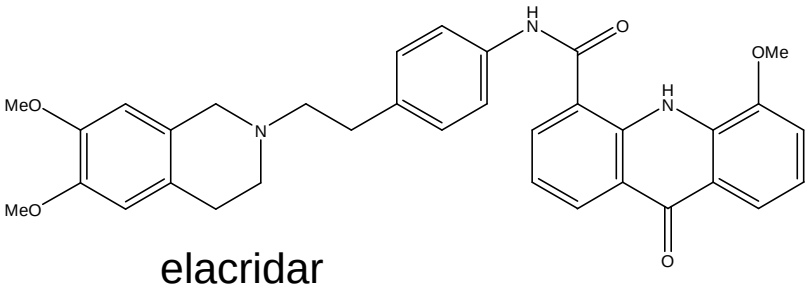
In vivo assays to assess P-gp inhibition:

-Drug efflux assay with CD65+ cells

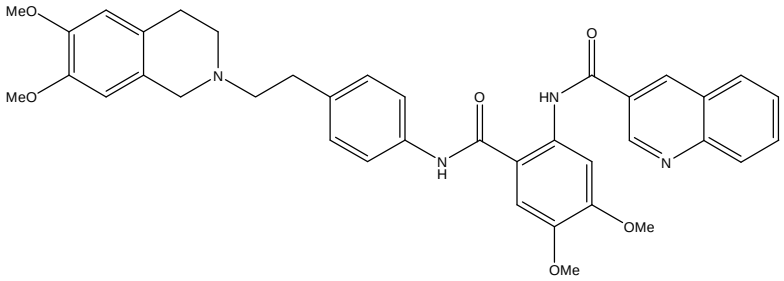
-Use of ^{99}Tc-marker substrates of P-gp

McDevitt *et al., Pharmacol. Ther.* 2007, **113**: 429

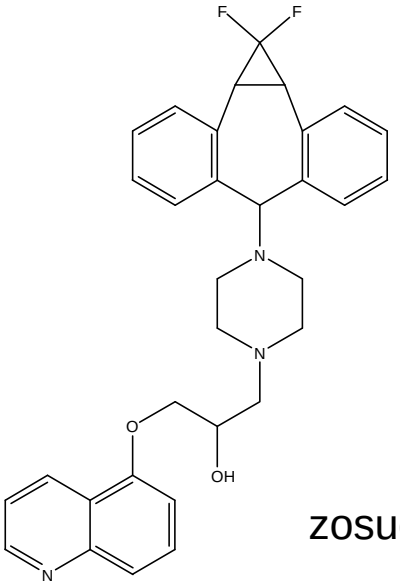
P-gp inhibitors



elacridar



Tariquidar
Non competitive I



zosuquidar

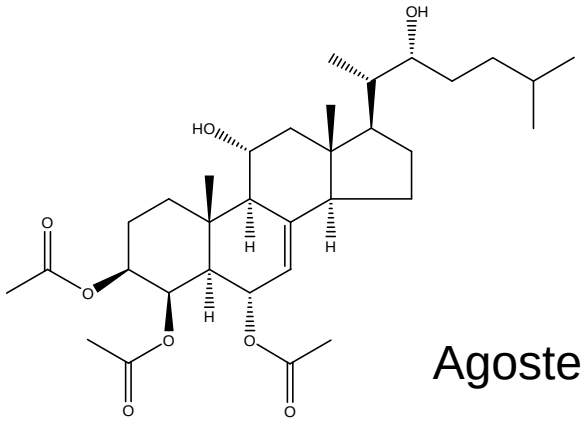
3rd generation

MRP inhibitors

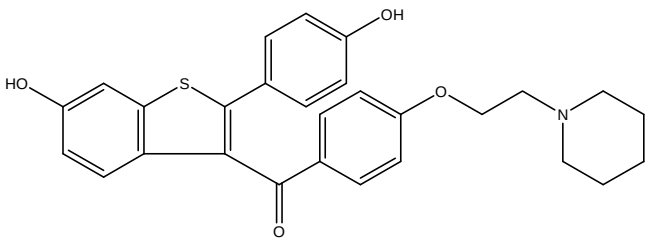
Most P-gp inhibitors are inactive against MRP1 (P-gp substrates are hydrophobic, MRP1 substrates are hydrophilic, conjugated to glutathione)

Known inhibitors:

- Agosterol (competitive inhibitor, binds to the C-terminal half of MRP1)
- Natural flavonoids
- Raloxifene analogs…



Agosterol A



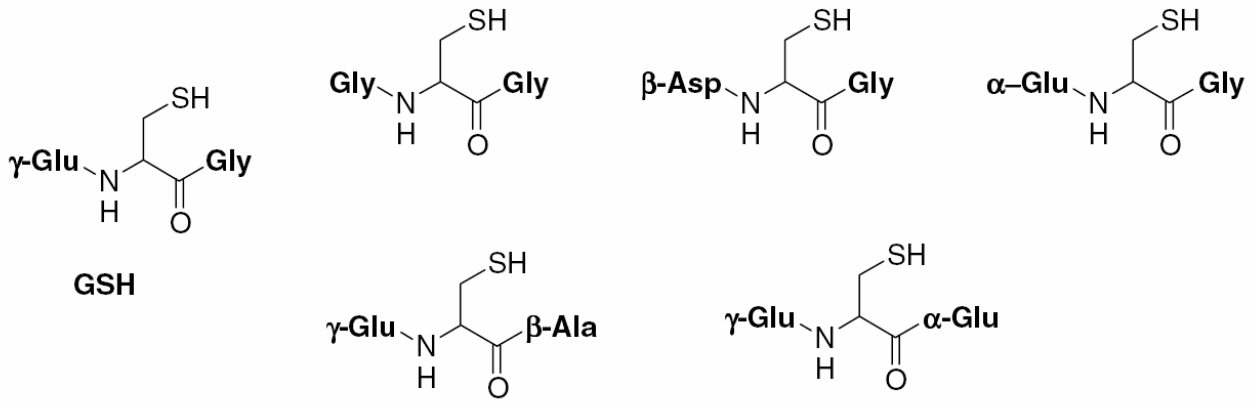
raloxifene

MRP modulators

MRP1 transports drugs either conjugated to glutathione or co-transports them with free glutathione

Glutathione-S-transferase (GST), which catalyses formation of GSH-conjugates, is **a target to modulate MRP1 activity**

Developpment of compounds able to mimic GSH (competitive inhibitors) or GSH-conjugates → PEPTIDOMIMETICS



GSH analogs as possible MRP1 modulators

Selective for MRP1 versus P-gp but may interfere with the physiological role of GSH

Boumendjel *et al., Med. Res. Rev.* 2005, **25**: 453

BCRP inhibitors

Elacridar (GF-120918) is a reference inhibitor for BCRP (also known to inhibit P-gp)

Reserpine

Fumitremorgin (mycotoxin from *Aspergillus fumigatus*) inhibits drug transport and ATPse activity, but neurotoxic $\rightarrow$ derivatives

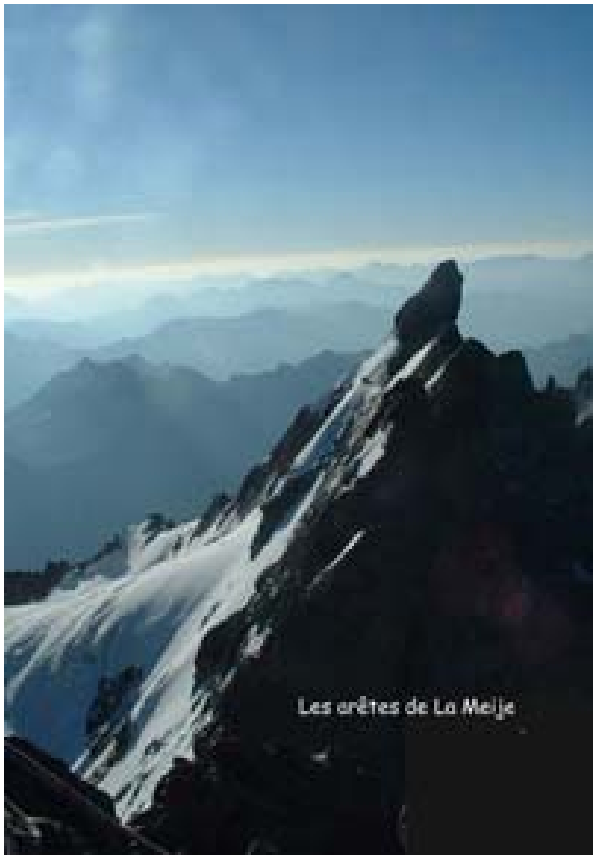
Acridone derivatives

…

EPI discovery:

Successful…

But no clinical EPI yet available!...



Perspectives in EPI development

Different « targets » can be considered:

- The drug binding site
- The NBD of ABC transporters, required for the function of the pump (but highly conserved!)
- Residues involved in communication between several parts of the efflux system (TMD and NBD for ABC pumps, monomers of tripartite pumps…)

But il will remain difficult to get a specific EPI that does not alter the physiological functions of the efflux pump….

Perspectives in EPI development

Others possibilities to tackle efflux resistance:

- Interference with gene expression → Downregulation of MDR transporters (with antisense oligonucleotides, via antagonists of nuclear regulators…)
- Interference with efflux pump assembly (tripartite efflux pumps in Gram- bacteria)

- Bypass MDR efflux by developping drugs which are poor substrates of efflux pumps

  (glycylcyclines vs tetracyclines, ketolides versus macrolides, new fluoroquinolones versus older ones, new anthracyclines…)

Perspectives

For the patient:

- Detect accurately efflux pump(s) over-expression
- Take it into account to propose and adapt an efficient treatment

Developement of new molecules:

- Consider efflux pumps in the early stages of conception of new drugs
- Poursue efforts to develop safe and selectif EPI

This might be accelerated with the structure elucidation of more efflux pumps