# Day 1 Agenda

Keynote Session: Overview of challenges in discovery of Gram-negative antibacterials

- Lynn Silver, LL Silver Consulting
- Hiroshi Nikaido, University of California Berkeley

Session 1: Barriers to compound penetration and efflux avoidance
MODERATOR: Richard Lee, St. Jude Children's Research Hospital

- John Finn, former Trius Therapeutics
- Wright Nichols, former AstraZeneca
- Hiroshi Nikaido, University of California Berkeley
- Lynn Silver, LL Silver Consulting

Session 2: Case studies: Finding ways to overcome barriers to compound penetration and efflux avoidance
MODERATOR: Carl Balibar, Merck

- Fred Cohen, Achaogen
- Erin Duffy, Melinta Therapeutics
- Ruben Tommasi, Entasis Therapeutics

# Day 1 Agenda

Session 3: Enabling technologies to measure compound permeability and accumulation

MODERATOR: Alita Miller, Entasis Therapeutics

- Kyu Rhee, Weill Cornell Medical College
- Derek Tan, Memorial Sloan Kettering Cancer Center
- Helen Zgurskaya, University of Oklahoma

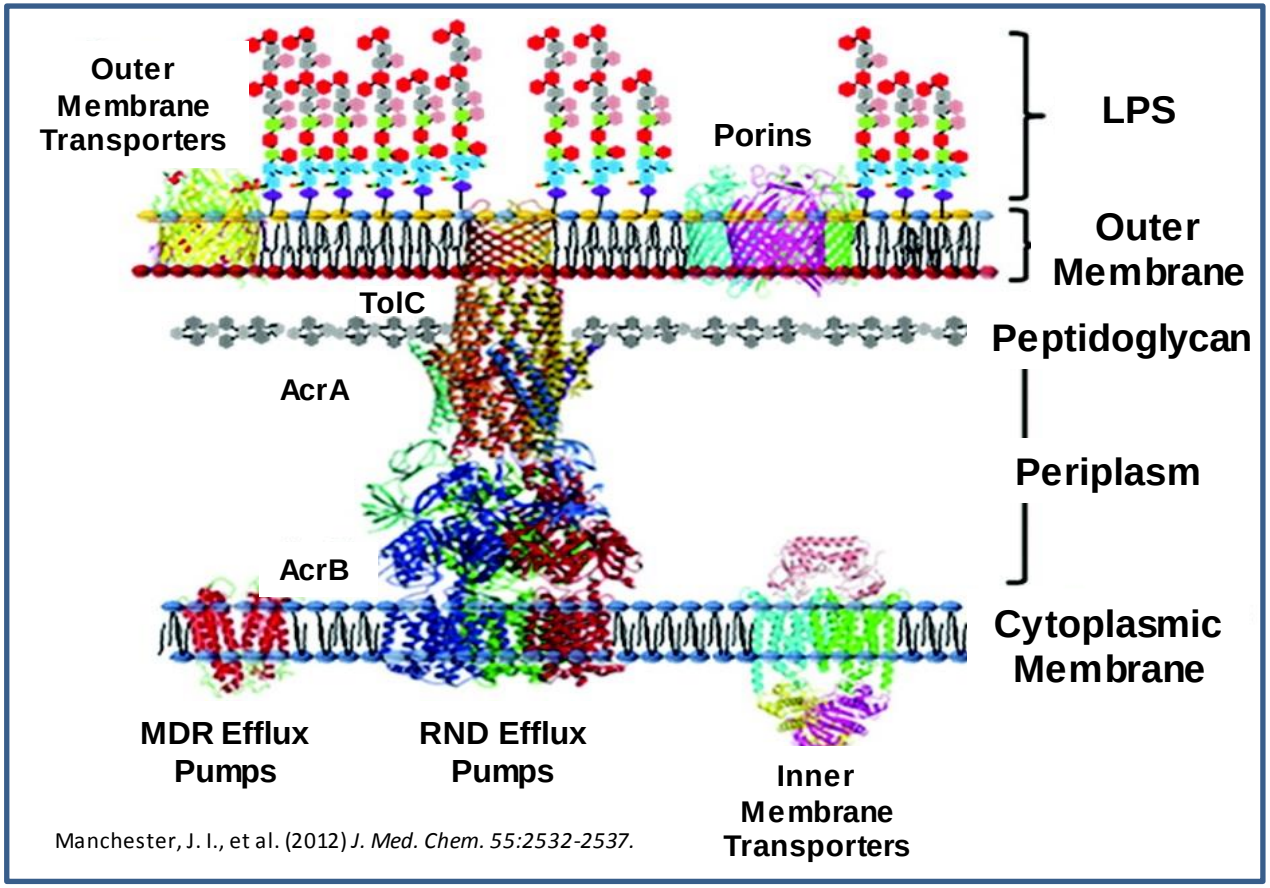
Session 4: Establishing physicochemical guidelines for compound entry & efflux

MODERATOR: Troy Lister, Spero Therapeutics

- Heinz Moser, Novartis
- Lynn Silver, LL Silver Consulting
- Mathias Winterhalter, Jacobs University Bremen, Innovative Medicines Initiative Translocation project (presentation not included)

# Keynote Session:
# Lynn Silver, LL Silver Consulting

# Overview of challenges in discovery of Gram-negative antibacterials




*Lynn Silver, PhD*

*LL Silver Consulting, LLC*

# Challenges…

- It’s hard enough to discover ANY developable novel antibacterials
- Let alone anti-GN agents

- Although note that it’s pretty easy to kill bacteria with toxic stuff
- First point: Selectivity is paramount

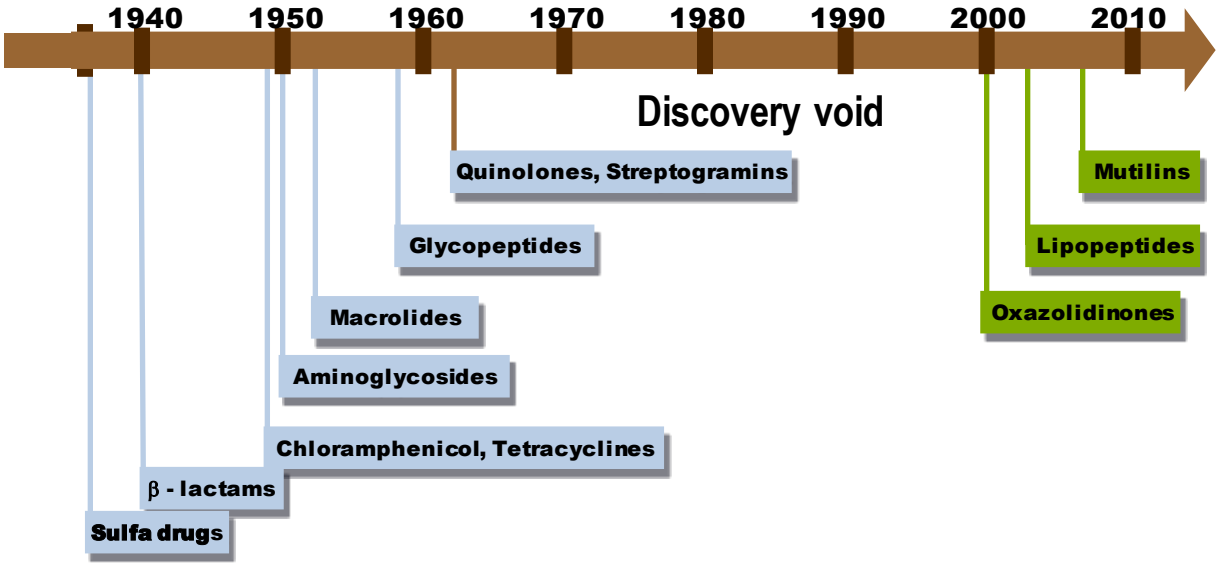
# Empiricism vs Rationalism

- We’re scientists
- We’re rational
- But most antibiotics (antibacterials) have been discovered empirically

- And rational approaches haven’t worked so well…yet

# The “Innovation gap” in novel classes

## Obscures the “Discovery void”



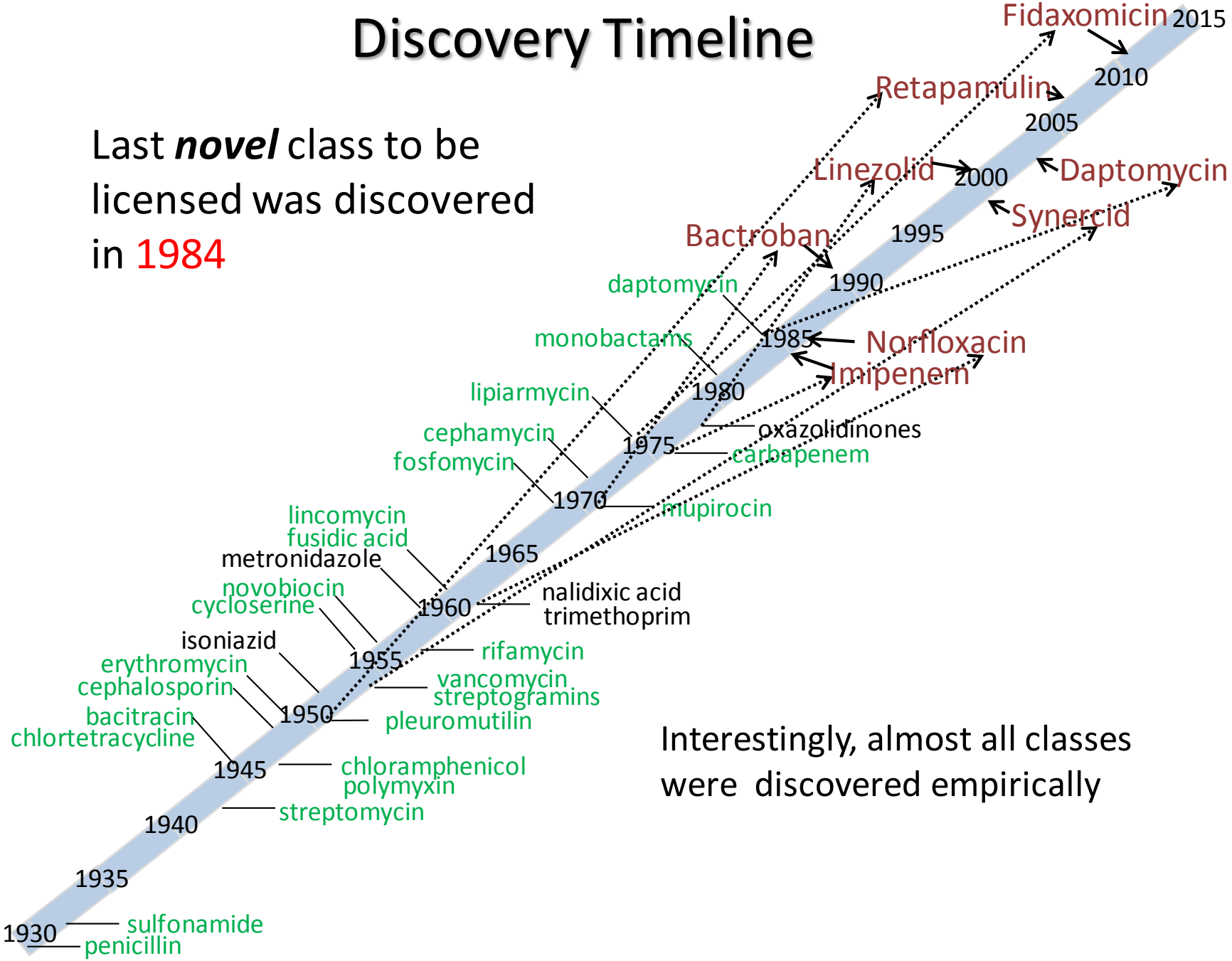

**Between 1962 and 2000, no major classes of antibiotics were introduced**

**No registered classes of antibiotics were discovered after 1984**

Fischbach and Walsh, 2009

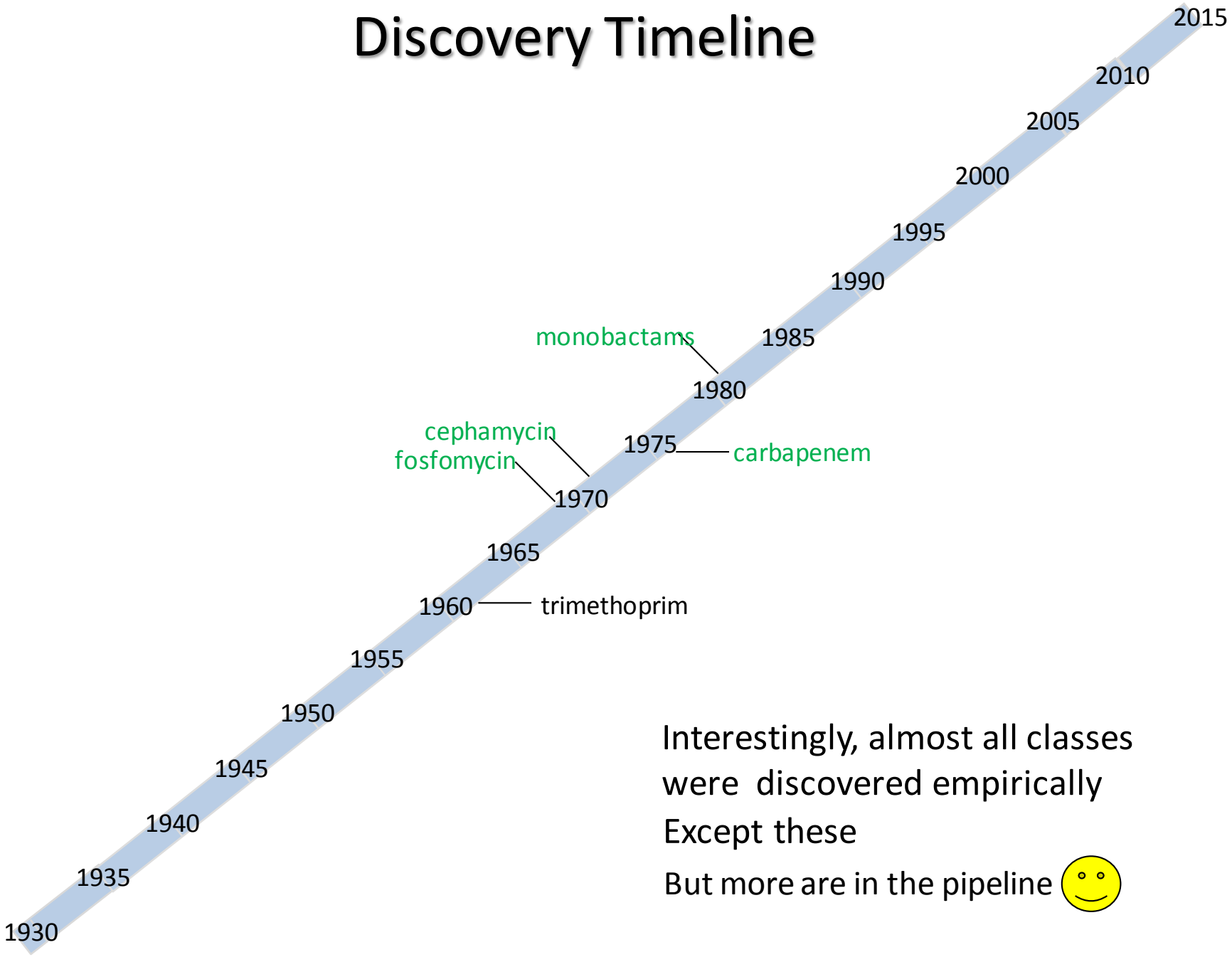
# Discovery Timeline

Last ***novel*** class to be licensed was discovered in 1984




Interestingly, almost all classes were discovered empirically

# Discovery Timeline




Interestingly, almost all classes were discovered empirically

Except these

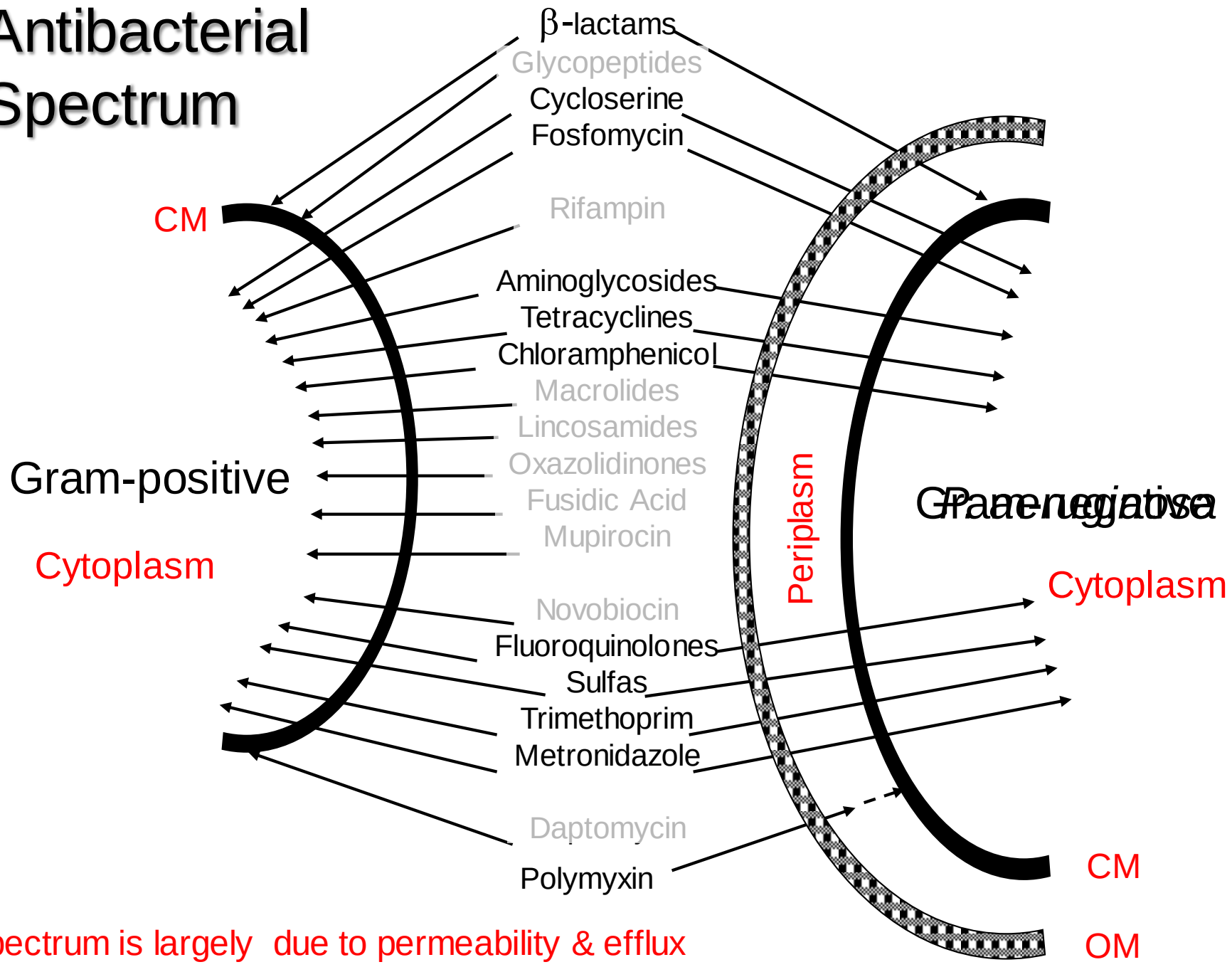
But more are in the pipeline

# One problem is targets

- Single-enzyme targets are generally subject to rapid resistance development
- Choosing “multi-targets” and/or avoiding resistance-prone targets is paramount

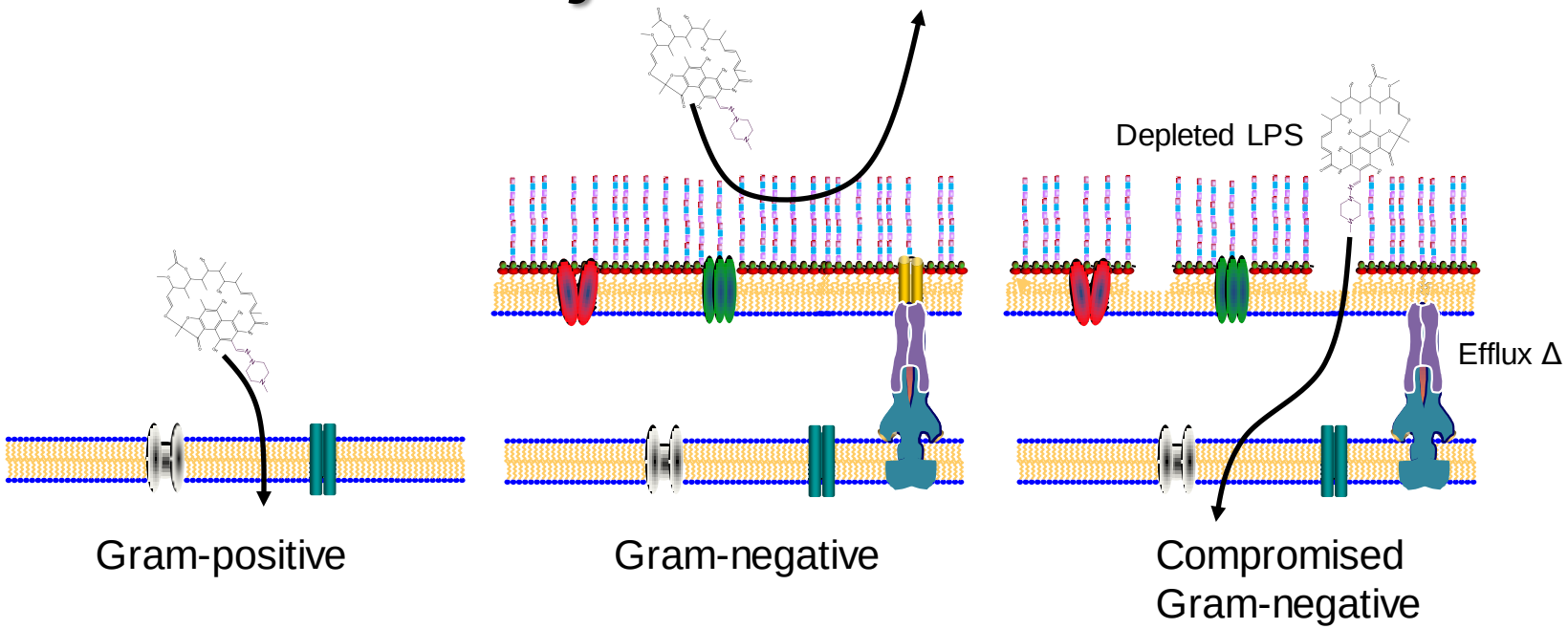
- Where are the targets located?
  - Often in the cytoplasm

# Antibacterial Spectrum



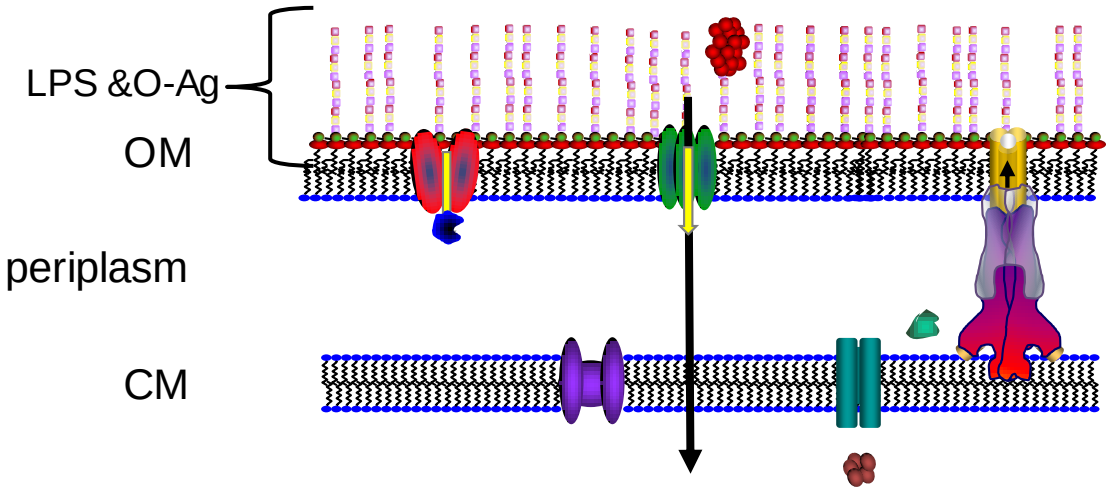

Spectrum is largely due to permeability & efflux

# But it's not just OM and Efflux




- Since the major permeability difference between GN and GP the OM…
- And OM-permeable and effluxΔ GNs are sensitive to many GP drugs
- Some assume finding ways of crossing the OM and avoiding efflux will allow GN entry
- But novel compounds (such as cytoplasmic enzyme inhibitors) need qualities that also permeate the CM.

# GN barriers (simplistic view)



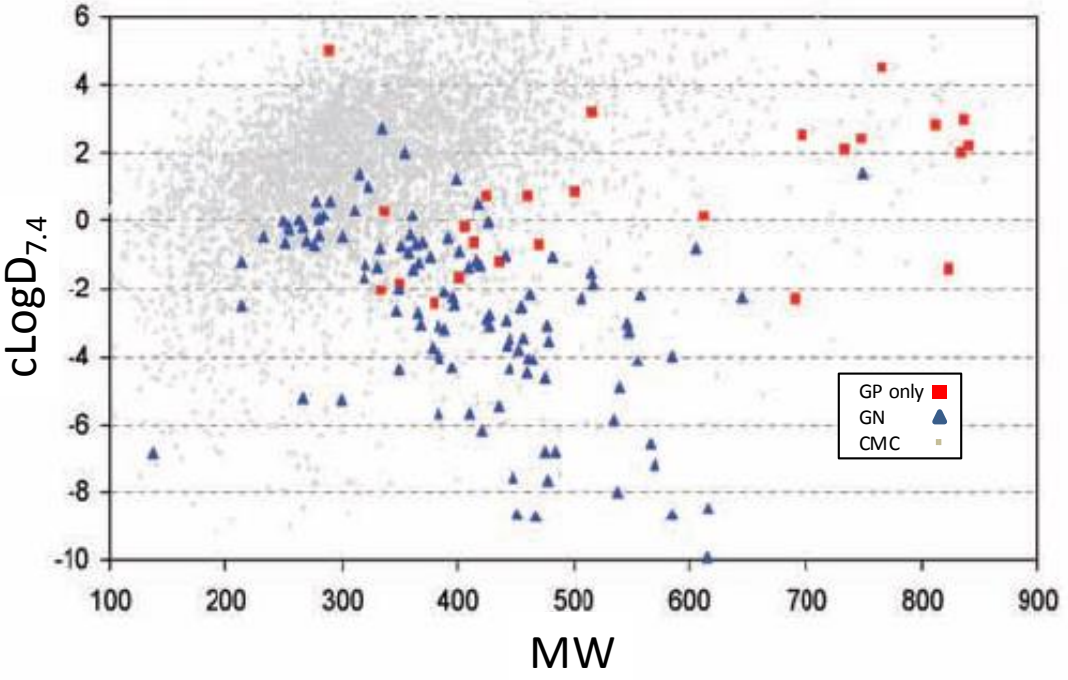

- OM excludes hydrophobic and hydrophilic compounds.
- Penetration of hydrophilic compounds through OM is via:
  - general porins [<600 MW, prefer hydrophilic, charged]
  - facilitated diffusion of specific hydrophilic solutes [OprD, Tsx]
- But hydrophilic and highly charged molecules entering the periplasm
  - penetrate the CM slowly or not at all
  - unless actively transported [or via PMF]
- Molecules that do enter can be effluxed
- **What molecules can accumulate in the GN cytoplasm?**

# How to get compounds into the cytoplasm of GNs

- Proposals for studying and overcoming the barriers to Gram-negative entry focus on
  - Processes of periplasmic entry and residence
  - Substrate characteristics of porins, pumps and permeases
  - This will benefit periplasmic targeted compounds
- But compounds designed to get to the periplasm will be unlikely to get to the cytoplasm since
  - sieving properties of OM and CM are more or less orthogonal
  - (effluxability may correlate with CM diffusibility)
- Dependence on transporters is resistance-prone
- Is there a Gestalt approach to solve the simultaneous equations of entry through both membranes and efflux avoidance?

# In addition to characterizing barriers, characterize compounds

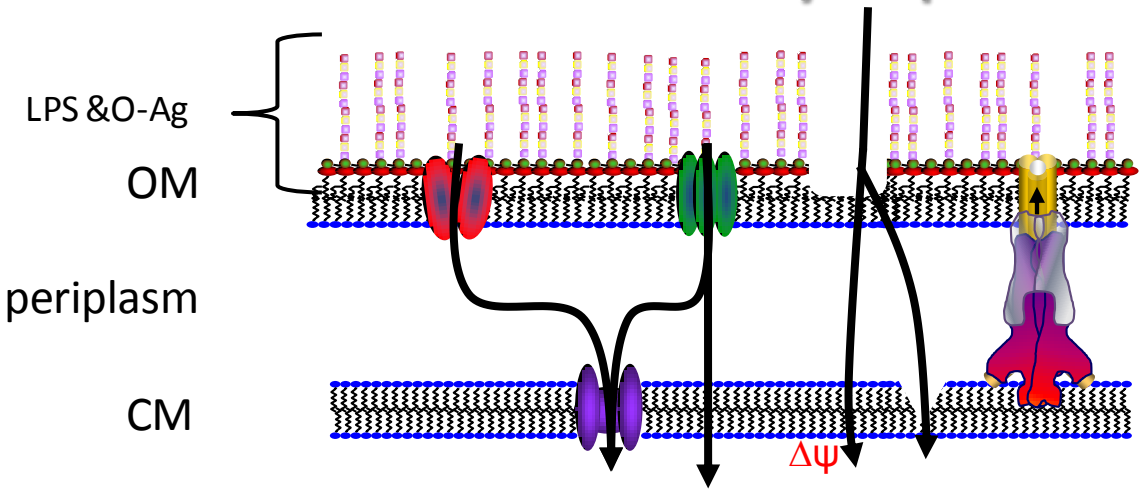
- Can we develop rules for entry by studying existing compounds?
- In 2008, O'Shea and Moser analyzed physicochemical characteristics of registered antibacterials making the distinction between GN and GP actives and noted general physicochemical differences between them.




- Now focus on compounds getting into the cytoplasm
- And how they get there

Adapted from O'Shea, R. O. and Moser, H. E. (2008) *J. Med. Chem.* ***51:2871-2878.***

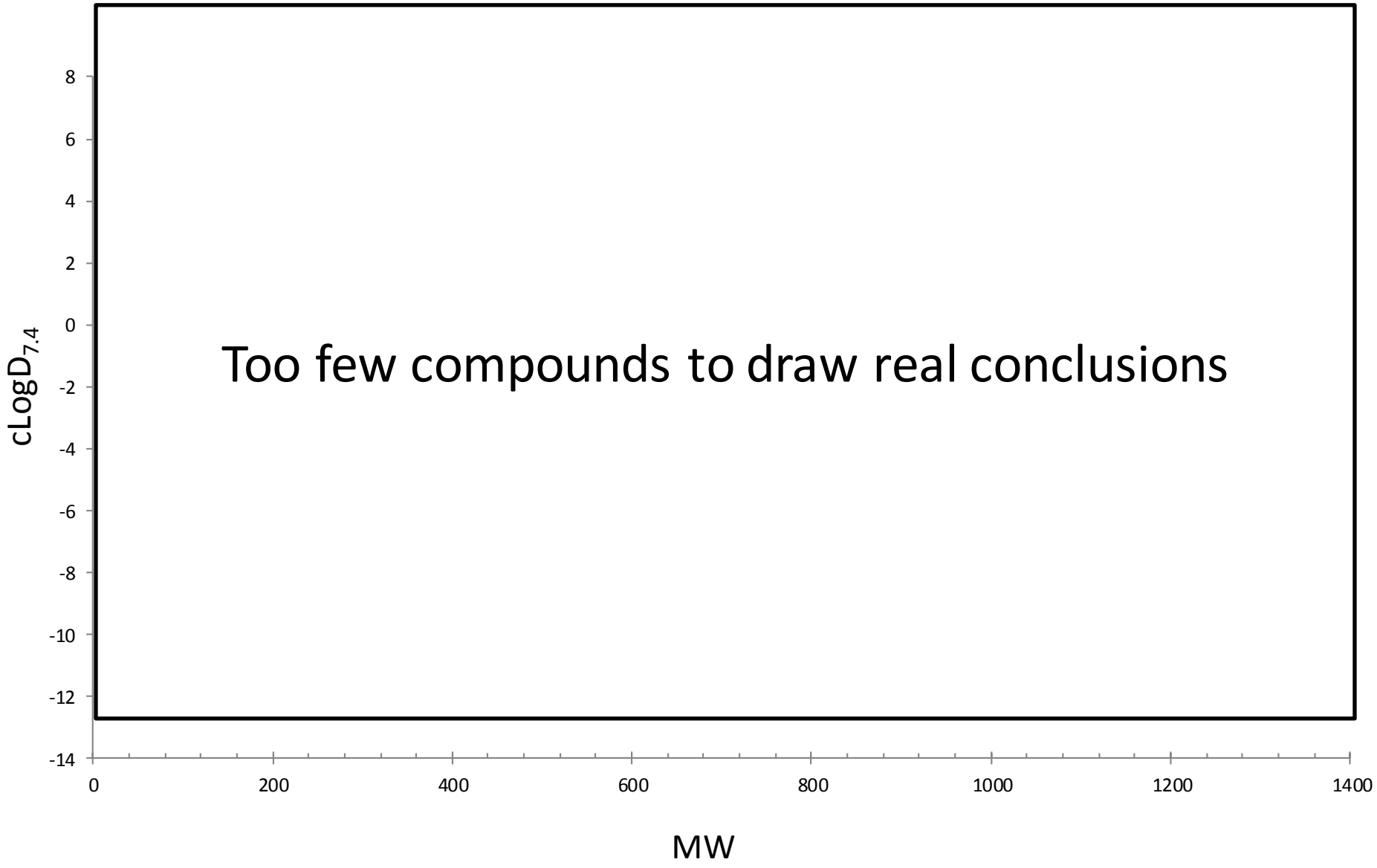
# Routes to the cytoplasm




- Diffusion (no transporters)
  - Hydrophilic molecules: Cross OM rapidly via porins, may avoid efflux –poor CM passage
  - Lipophilic molecules: Cross OM slowly, can be effluxed – good CM passage
- Active transport
  - Hydrophilic molecules cross OM via porins, CM via transporters [ATP or PMF driven]
- Self-promoted uptake [SPU] through OM
  - Cationic molecules, avoid efflux; CM passage via Δψ or polycations may disrupt CM
- Trojan horse
  - Piggyback on active or facilitated transport; must avoid rapid resistance

[• OM permeabilizers and EPIs as adjuncts ]

  - Combine with CM-transiting molecules [properties of GP drugs]


Too few compounds to draw real conclusions
cLogD7.4
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
0
200
400
600
800
1000
1200
1400
MW

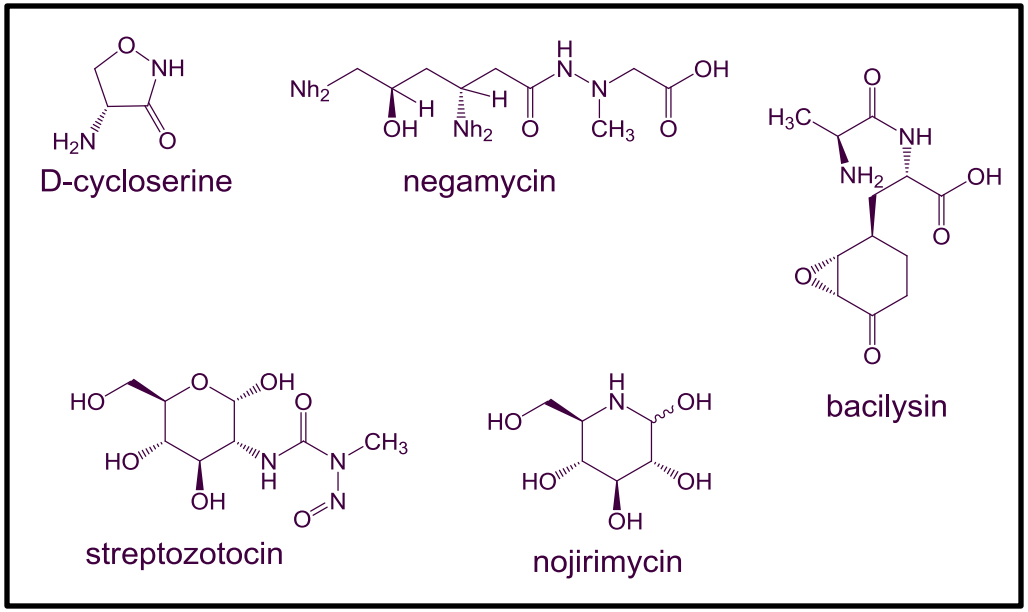
# What to do?

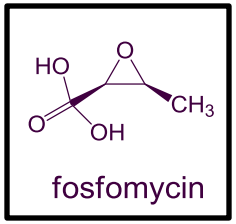
- Survey molecules for entry into G- cytoplasm
  - Use activity-independent measurement of cytoplasmic accumulation
  - Study knowns first, then large diverse chemical library
  - Focus on compounds not actively transported
  - Formulate hypotheses/rules correlating physicochemistry with cytoplasmic entrance
  - Synthesize new chemicals to test hypotheses
  - Make large "Gram-negative" chemical library following "rules"
- Extend studies of CM diffusion of ionic species
- Explore self-promoted uptake
- Study many GN species
- Do permeabilizers and Trojan horses work?

# Exploit Natural Products

- Source of the majority of antibacterial agents.
- Evolved for the task.
- Do natural products enter GNs well?
  - Many use permeases and illicit entry
    - Which may make them subject to rapid resistance
    - But this should be revisited
- Important to continue to explore NPs
  - Unculturables
  - Genome mining
  - Hypersensitive screening

# Transported compounds that might be able to enter by diffusion




| | MW | ClogD7.4 |
|---|---|---|
| **bacilysin** | 270 | -4.49 |
| **negamycin** | 248 | -5.87 |
| **streptozotocin** | 265 | -1.45 |
| **nojirimycin** | 179 | -2.37 |
| **D-cycloserine** | 102 | -1.85 |
| | | |
| **fosfomycin** | 138 | -5.99 |

# Proposal

- Approach the GN entry problem by studying both barriers to entry and characteristics of compounds that accumulate in the cytoplasm
- Require activity-independent measure of accumulation in cytoplasm
- Test whether physicochemical and/or structural descriptors correlate with routes of entry into the cytoplasm.
- If rules can be deduced, make GN-specific libraries

# Keynote Session:
# Hiroshi Nikaido,
# University of California Berkeley

# Can we predict the permeation rates of drugs across the outer membrane?

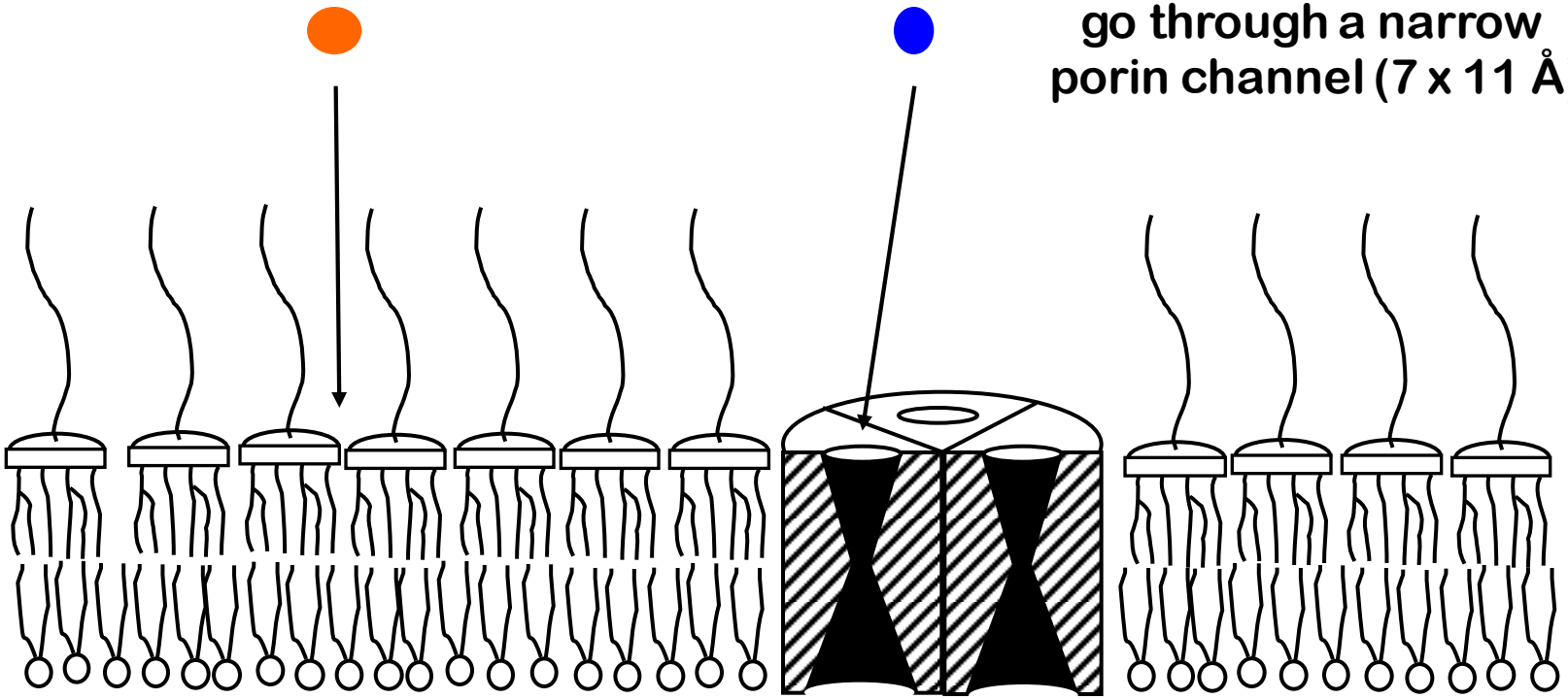
Hiroshi Nikaido University of California, Berkeley

nhiroshi@berkeley.edu

# Outer Membrane is an Effective Permeability Barrier

Hydrophobic agents must penetrate through a highly impermeable asymmetric bilayer

Hydrophilic agents must go through a narrow porin channel (7 x 11 Å)

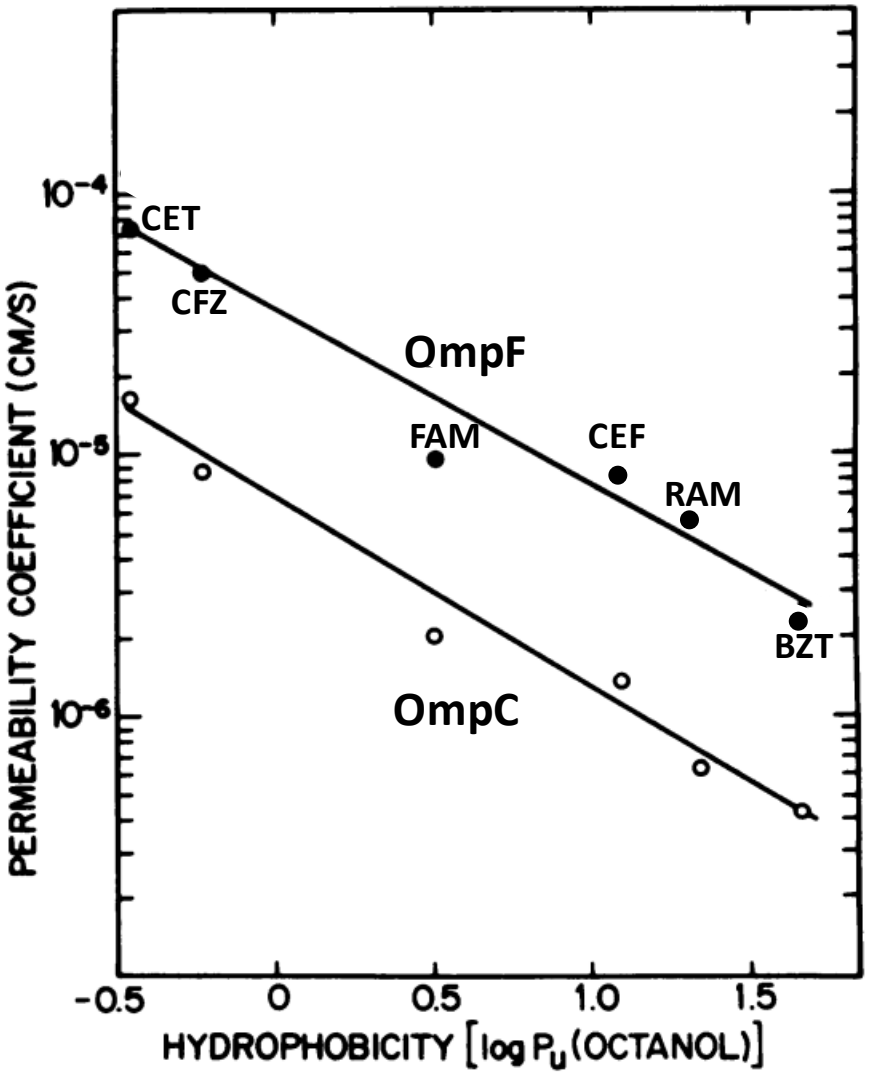


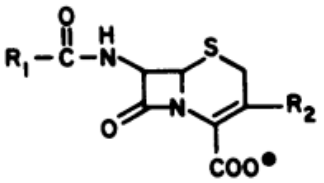
Outline of Presentation

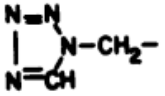
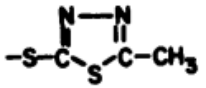
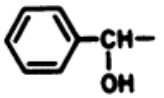
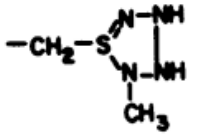
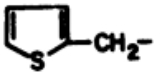
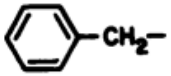
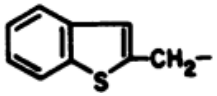
1. *E. coli* Outer Membrane: Permeation through Porin channels
   1.1.1 β-Lactams
   1.1.2 Other compounds (Quinolones, aminoglycosides, tetracycline)
2. Relatives of *E. coli*, e.g. *Enterobacter cloacae, Klebsiella pneumoniae*
3. Non-fermenters (*Pseudomonas aeruginosa*, *Acinetobacter baumannii*)
4. Entry through the Asymmetric Bilayer Region
5. Endogenous, Constitutive, RND-type Efflux Pumps

1.1.1 β-lactams through OmpF porin channels of *E. coli*

**Cephalosporin Permeation Across OM Can Be Determined Precisely in Intact Cells By Combining It With Subsequent Hydrolysis in Periplasm: A Strong Effect of Hydrophobicity**



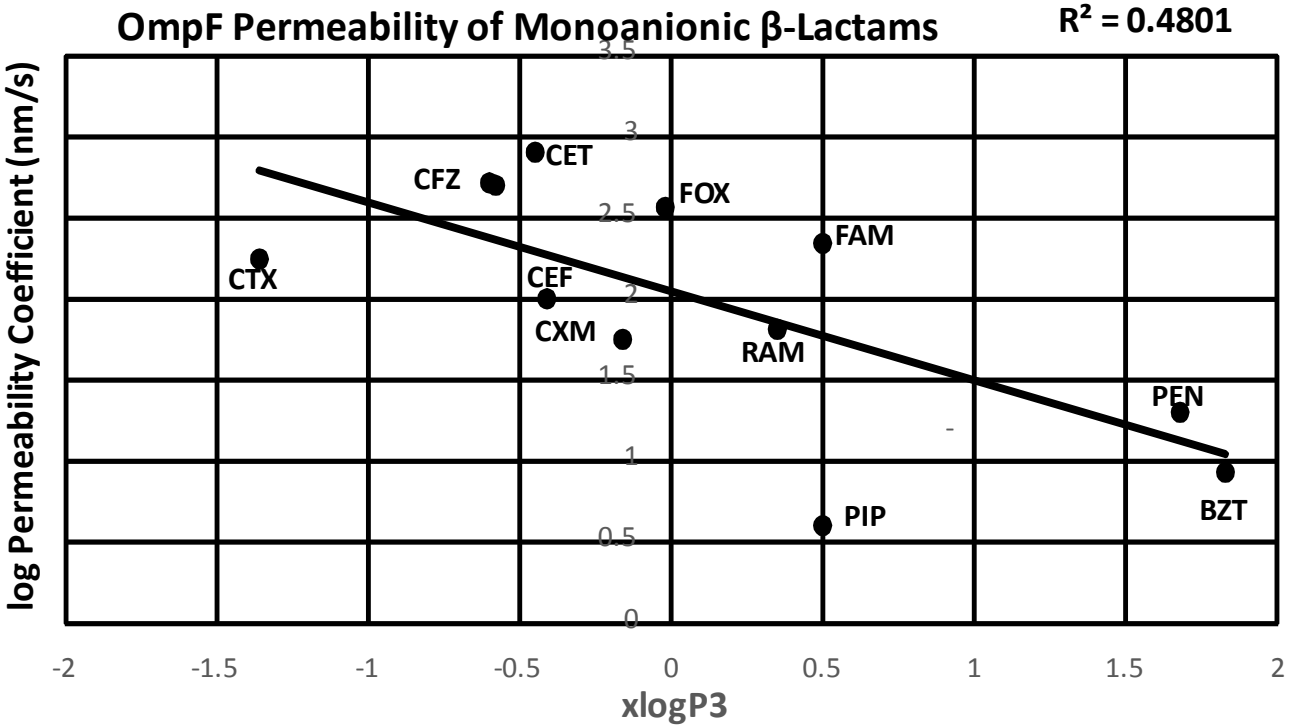




| | $R_1$ | $R_2$ |
|---|---|---|
| CEPHACETRILE | N≡C–$CH_2$– | –$CH_2OCOCH_3$ |
| CEFAZOLIN |  |  |
| CEFAMANDOLE |  |  |
| CEPHALOTHIN |  | –$CH_2OCOCH_3$ |
| CEPHALORAM |  | –$CH_2OCOCH_3$ |
| BENZOTHIENYL-CEPHALOSPORIN |  | –$CH_2OCOCH_3$ |

Nikaido, Foulds, and Rosenberg J. Bacteriol. 153: 232 (1983)

In 1983, the presence of drug efflux systems was not known in bacteria. However,

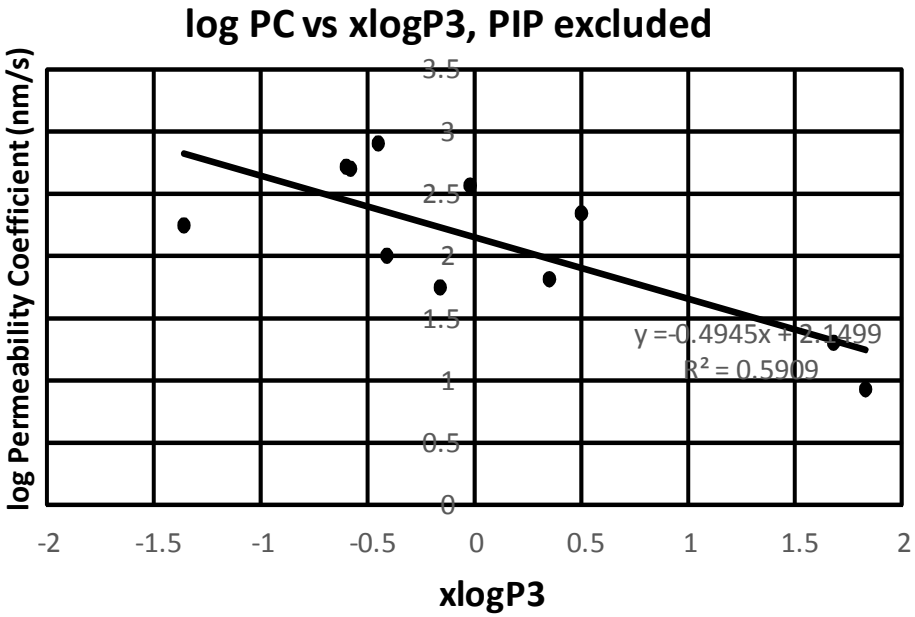
1. In 2009 we determined the efflux kinetics of cephalosporins in E. coli. (Nagano and Nikaido, PNAS 106:5454-5458). Because the $K_{0.5}$ values for the efflux of most cephalosporins are quite high (cephalothin (90 µM) and cephamandole (20 µM)), we can show that efflux made very little difference in our 1983 data.
2. We also tested more compounds by proteoliposome swelling assay, which is not affected by efflux, of course. (Yoshimura and Nikaido, AAC 27: 84 (1985))

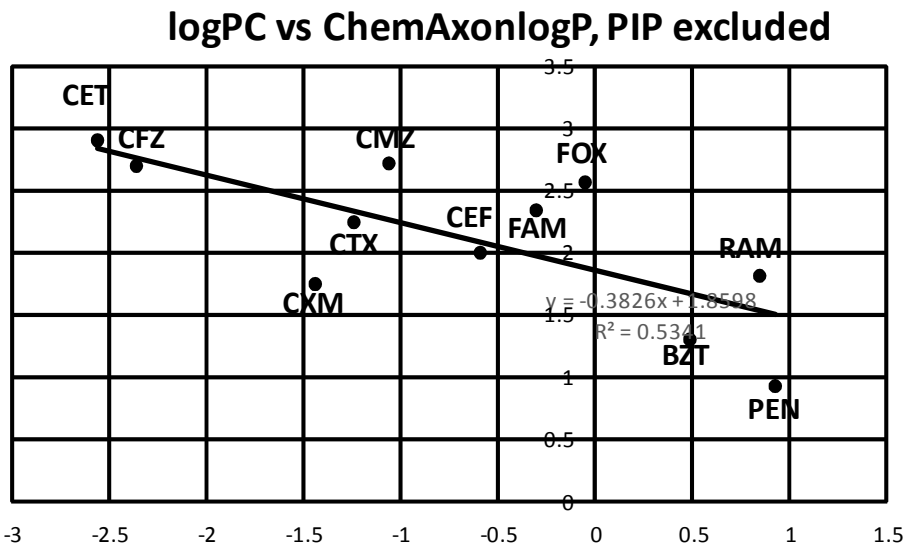



Plotting the data against xlogP3, however, produced a horrible fit.

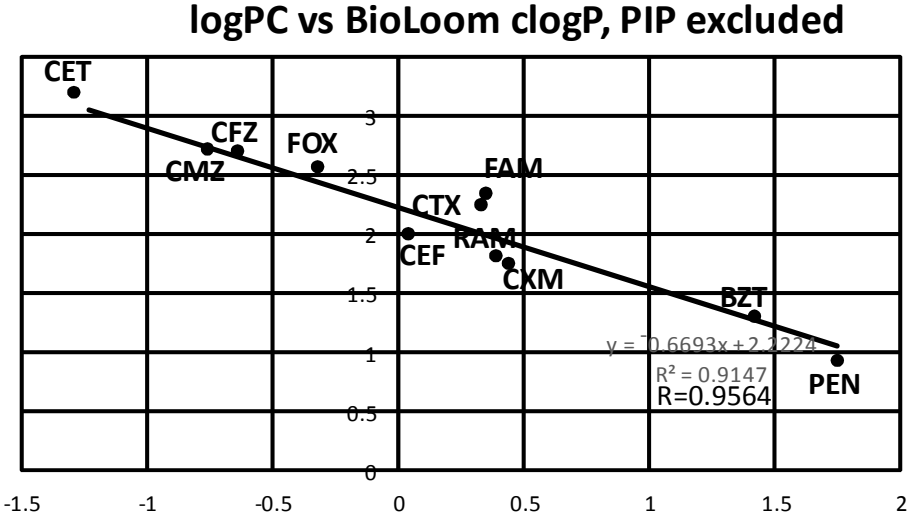
**(To avoid negative exponents of 10, PC is henceforth always shown in nm/s, rather than the usual cm/s)**

# How logP values are calculated is CRUCIAL



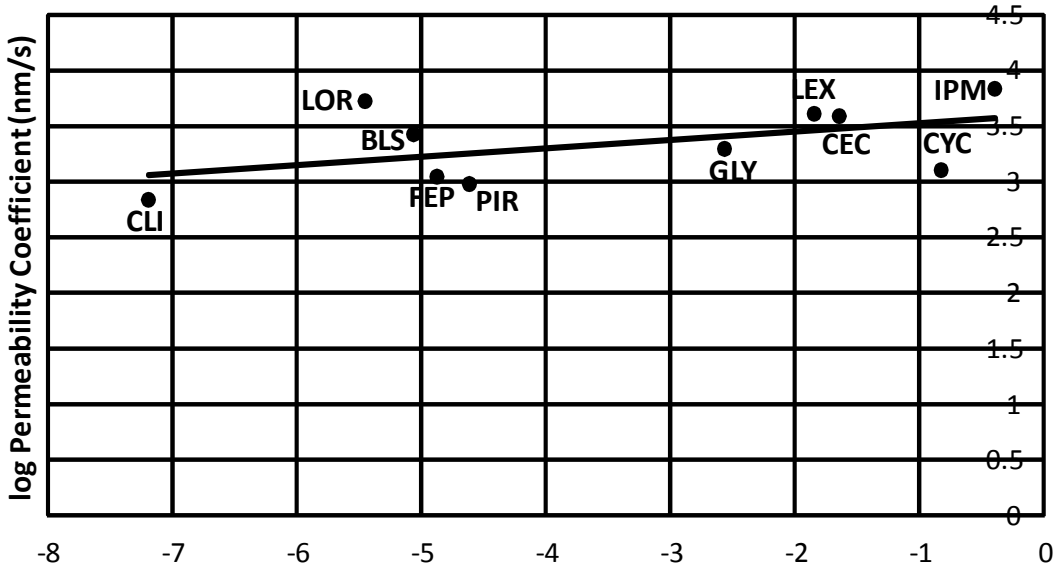




The near perfect correlation with clogP on the right also shows that the permeability cannot be determined by "specific" interactions between the drug and the channel.



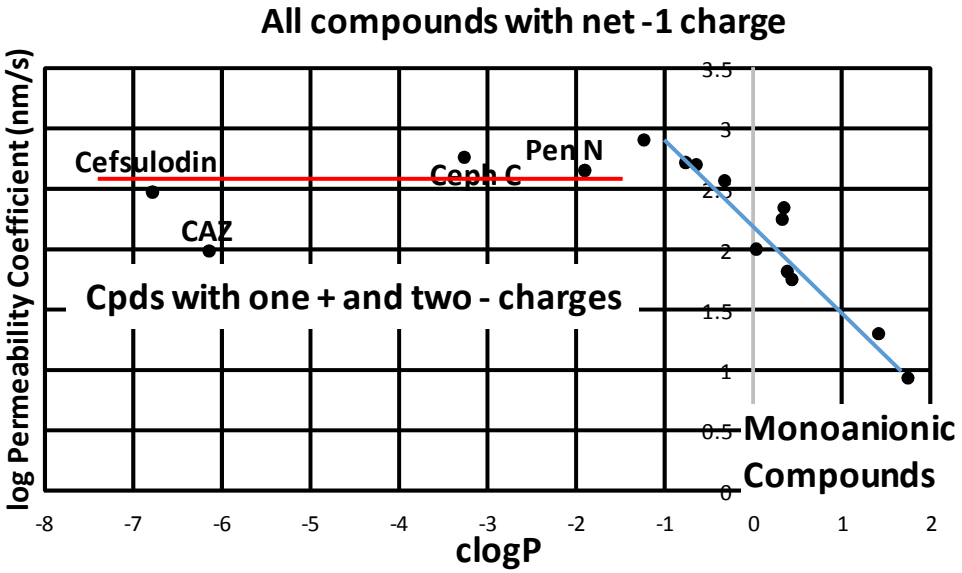
# What about zwitterionic compounds?

Zwitterionic Compounds




**Source:**
**Yoshimura & Nikaido, AAC 27: 84 (1985)**
**Nikaido et al. AAC 34: 337 (1990)**

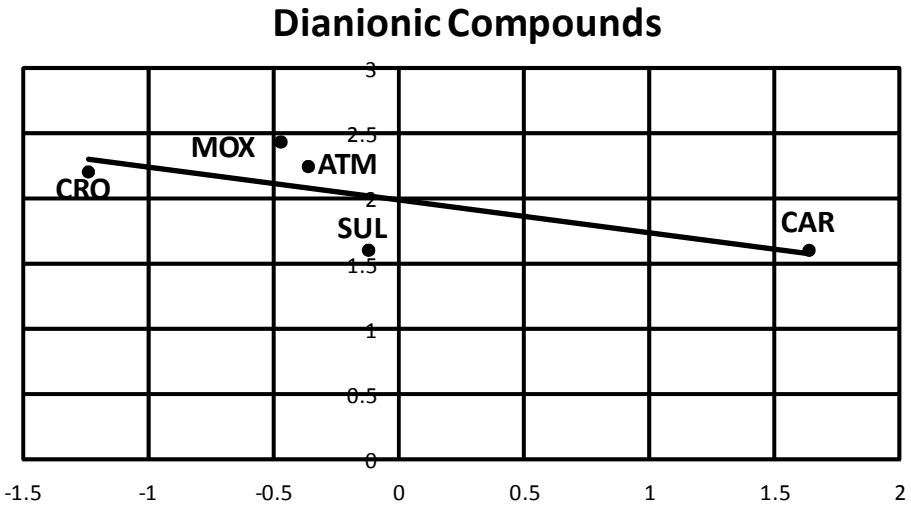
**No negative effect of hydrophobicity for zwitterionic compounds?? The situation becomes clear when we examine compounds with one positive and two negative charges.**

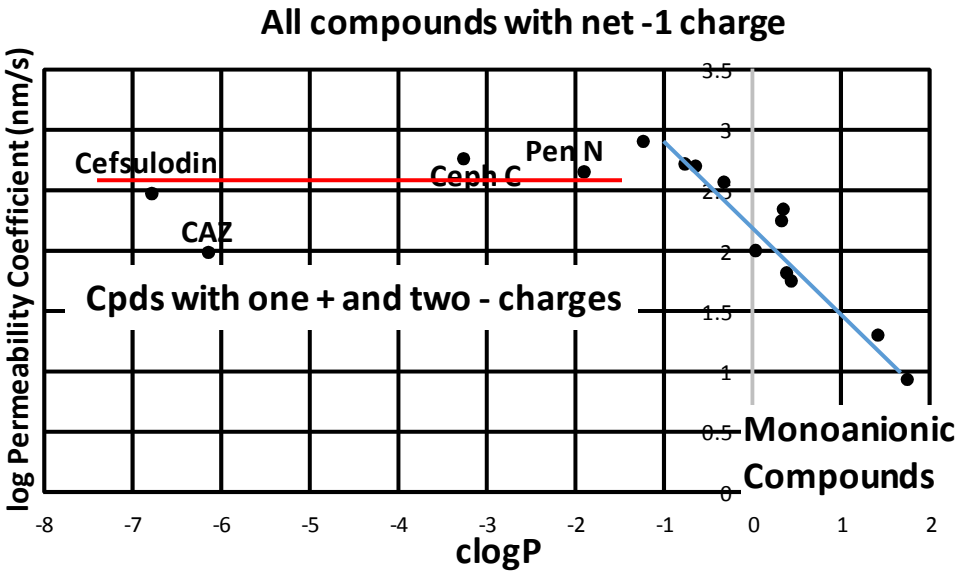



**Zwitterionic Compounds are Simply Too HYDROPHILIC!**

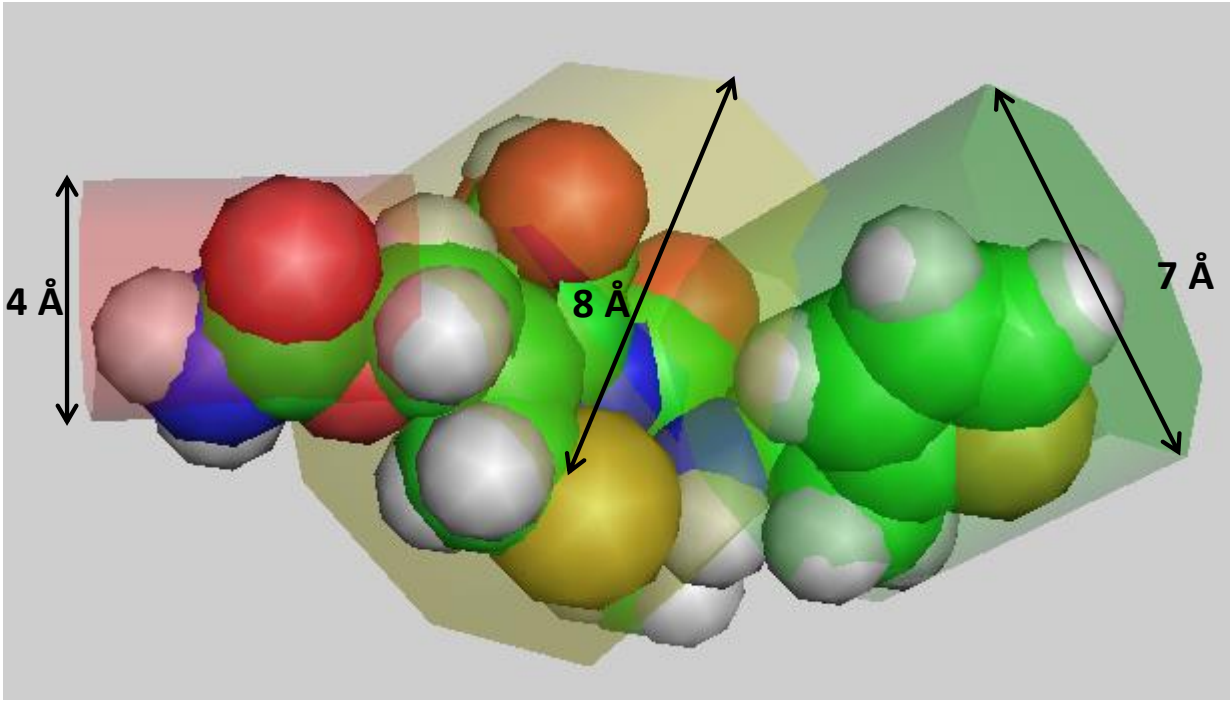
What about DIANIONIC compounds?

They are only modestly less permeable (logPC mostly between 2.5 and 2.0) than the hydrophilic compounds with -1 net charge (between 3.0 and 2.5)



Why does not the MW influence the permeation rates of cephalosporins?




**Cephalosporins may be thought of as a connection of three cylinders. The central part containing the nucleus is the widest (about 8 Å), although it is thin. Thus cephalosporins can pass through the narrowest part of OmpF channel (7 x 11 Å). In compounds with higher molecular weights, additional atoms are present in two outside cylinders.**
**The zwitterionic cephaloridine diffuses somewhat (1.7 x) faster than the disaccharide lactose with the diameter of about 8 Å). (Nikaido and Rosenberg, 1981; 1983)**

**Does the SPECIFIC interaction of drugs with the channel determine the diffusion rate?**

**For example, the influential review by Pagès, James, and Winterhalter (Nat Rev Microbiol 2008) argues that the diffusion rate can be calculated by the simple formula $J = [k_{on}/(2 + K\,\Delta c] \bullet \Delta c$ where $k_{on}$ is the association rate constant for the specific binding site.**

**This is still quite controversial.**

**1. This theory does not explain how simple sugars such as arabinose diffuses extremely rapidly through porin channels.**

**2. This theory comes from a blind application of what has been done with LamB (a channel SPECIFIC only to maltodextrins and relatives) to essentially NONSPECIFIC PORINS. Specific channels bind ligands with $K_D$ between 0.2 (FadL) to 60 (maltopentaose for LamB) μM. In a striking contrast, $K_D$ for AMP in OmpF is about 1 M! With such a large difference, "quantity changes into quality". SPECIFIC CHANNELS are NOT PORINS!**

OM Permeability Prediction.

1. If SPHERICAL, measure radius of gyration in VMD ("measure rgyr").
   If CYLINDRICAL, measure radius of the largest cylinder.

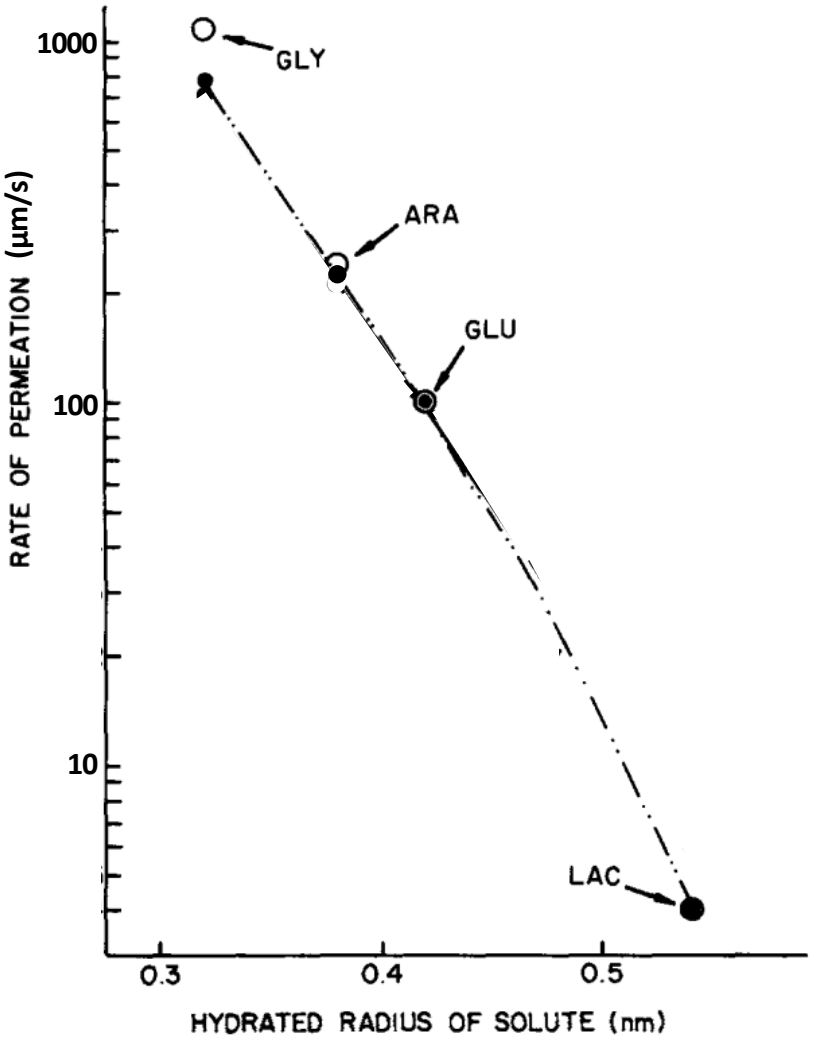
2A. If these are larger than 6 Å, consider diffusion through bilayer (discussed later)

2B. For compounds with the hydrated radius of around 5Å, go to 2C.

2C. If clogP (BioLoom) is <-1.0, the base logPC≈3 for zwitterions. For cpds with -1 net charge, subtract 0.5. For dianionic cpds, subtract 0.5 again. For each net positive charge, add 0.5.

2D. If clogP >-1.0, logPC decreases by 0.67 for an 1.0 increase of clogP.

3. For smaller compounds, the predicted logPC increases according to the figure on the right.



## Test of the Prediction Scheme

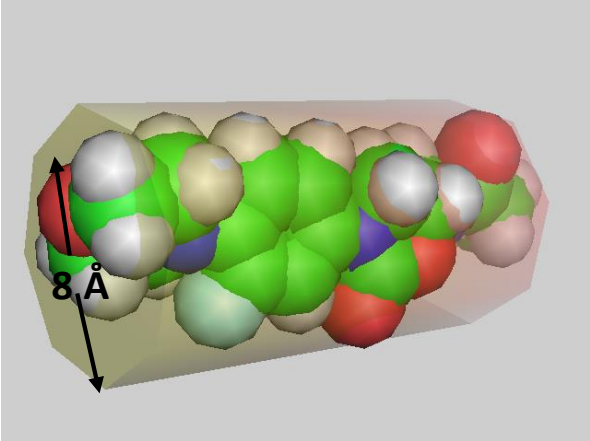
Azlocillin was not included in our analysis.
Its clogP is 1.56, from which we predict the Permeability Coefficient of 16 nm/s.
At its MIC of 16 µg/ml or 35 nmol/ml,
Influx rate predicted = PC*A*Δc where A is the surface area for 1 mg (dry weight) *E. coli*.
This results in the rate of 16 x l0-7 x 132 x 35 = <u>0.007 nmol/s/mg</u>.

When azlocillin efflux was measured by Lim and Nikaido (2010), it was found to follow a sigmoidal curve with the $V_{max}$ of 0.4 nmol/mg/s, $K_{0.5}$ of 1 µM, and the Hill coefficient of 4.
At its MIC, the periplasmic concentration should be enough to inhibit the most sensitive PBP (in this case PBP3). IC50 for azlocillin is 0.15 µg/ml, or 0.3 µM (Lei & Li, Acta Pharmacol Sin 10:177, 1989). At this concentration, the efflux rate is <u>0.003 nmol/s/mg</u>.

This is a very good agreement, especially when we consider that more than IC50 is probably needed to get a complete inhibition of growth.

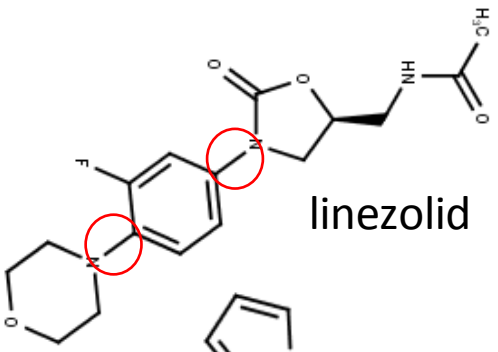
**Linezolid: An apparent exception that proves the rule?**

Linezolid is a rigid straight cylinder with the radius of only 4Å. And it is not that hydrophobic (clogP =0.42).

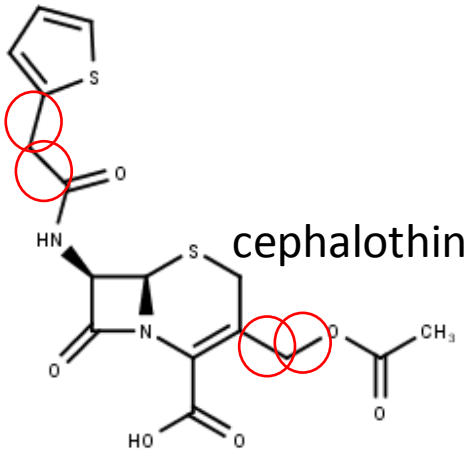



So our prediction scheme would predict a reasonably fast permeation with a PC of 80 nm/s, or half-equilibration time of about 3 seconds. But its gram-negative MIC values are very high, and the LC/MS study of Zhou et al. (Anal Chem 2015) showed the half-equilibration time in a ΔtolC strain to be around 15 min.

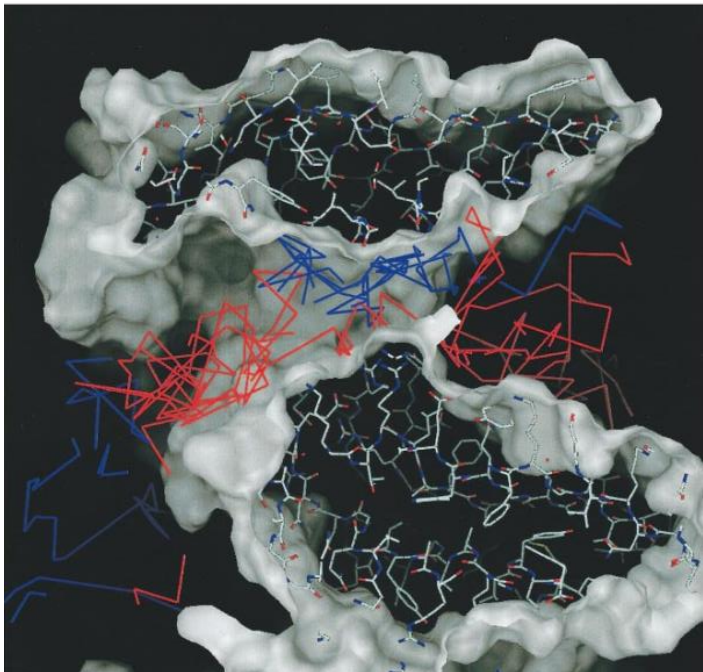
**OmpF channel is not a straight cylinder, and large ligands must be flexible, as in cephalosporins, to pass through the channel. A RIGID cylinder like linezolid has little chance for permeation.**



linezolid



cephalothin

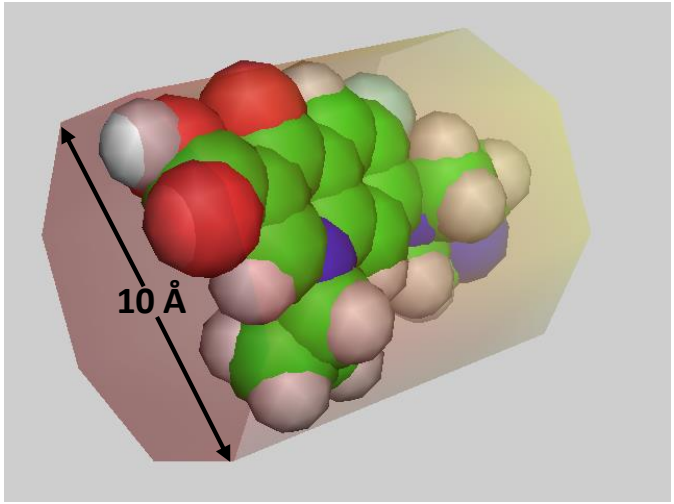


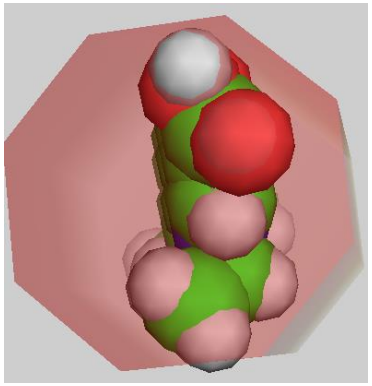
**Predicted Permeation Rates of A Few Common Agents**

## Ciprofloxacin

pKa= 6.3
clogP= -0.47

Predicted logPC is close to that of cefoxitin, i.e. 2.5



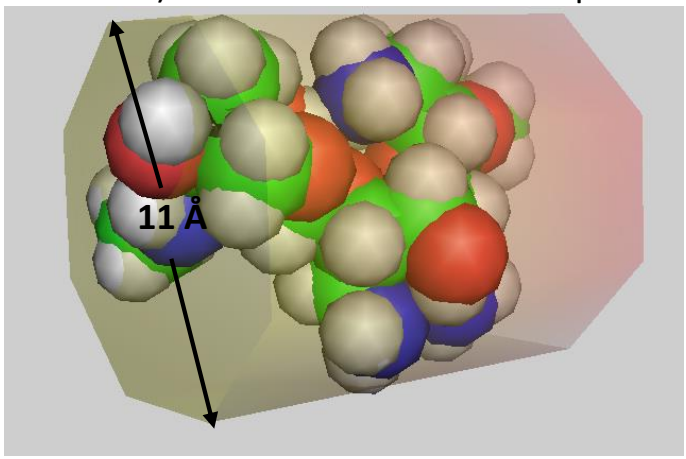



$t_{1/2}$ measured by Mortimer & Piddock (1991) (with norfloxacin) was about 10 s. This corresponds to logPC of 1.3. In the fluorescence assay using norfloxacin (Cama et al., JACS 2015), permeation rate of 10 molecules/s/OmpF trimer (at the gradient of 1 mM) was obtained. This corresponds to logPC of 1.6 and $t_{1/2}$ of about 5 s.

## Gentamicin

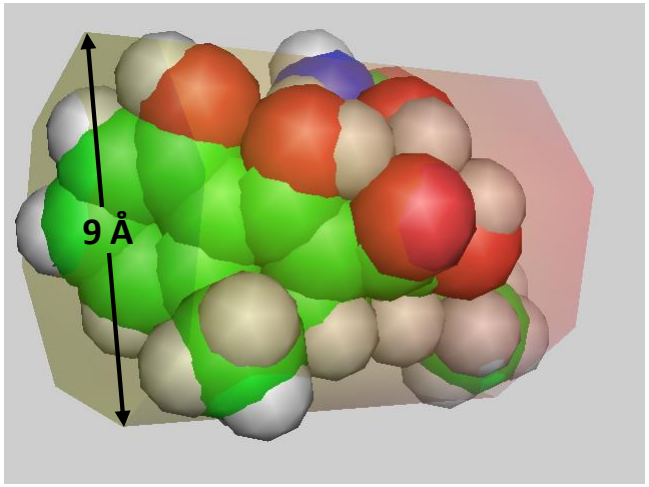
3 or 4 + charges
clogP=-2.4

Predicted logPC is >3.5.

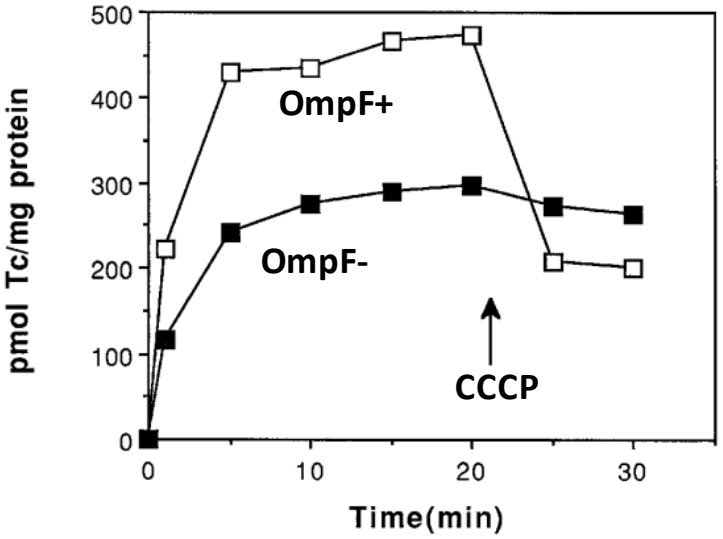



Liposome swelling data by Nakae & Nakae (1982) is often cited, but the results are meaningless as they did not know that charged compounds cannot be used in simple swelling assays. However, the permeability is likely to be very high, as multiple positive charges should pull aminoglycosides into periplasm following the interior-negative Donnan potential.

# Influx of Tetracycline




Tetracycline is hydrophilic (clogP = -2.46), and seems to be barely able to diffuse through OmpF.
Although it has several proton-releasing groups, use of microscopic dissociation constants tells us that up to 7% of Tc exists as an uncharged species at neutral pH (Nikaido & Thanassi, AAC 1993).

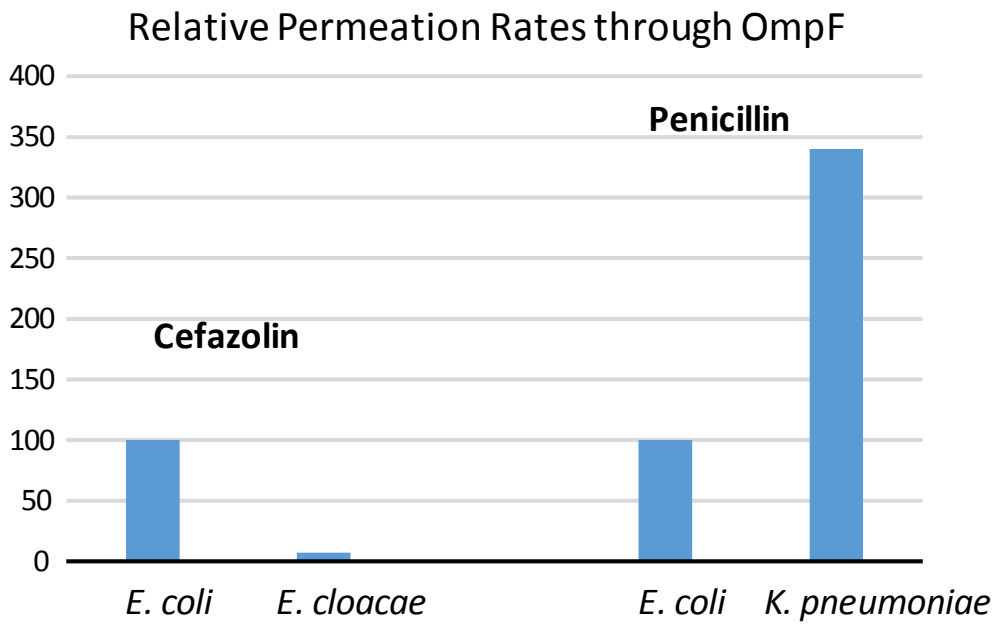



Thanassi, Suh & Nikaido (1995)

Indeed Tc accumulation in *E. coli* is largely dependent on porin.
(It also seems to diffuse with significant rate through the OM bilayer, but this is likely caused by its permeabilization caused by the absence of the major OM protein OmpF.)
It seems to be very rapid, with $t_{1/2}$ of less than 30 sec.

Simulation by solving two simultaneous differential equations suggested the PC across the outer membrane of $10^{-5}$ cm/s, or 100 nm/s, although some of our assumptions are now known to be incorrect. This is only one order of magnitude lower than that predicted from our "rule".

# There are often huge differences in OmpF channel size in different species

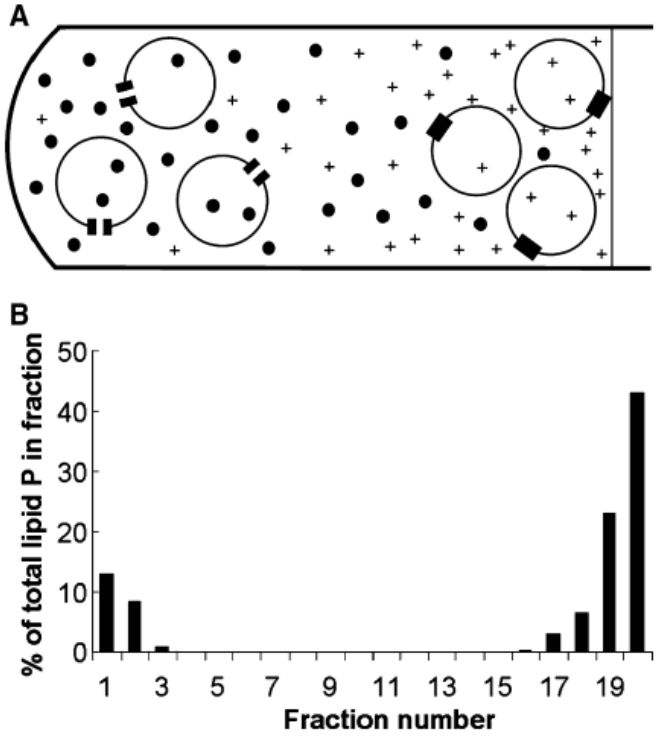



(Vu & Nikaido, AAC 27:398, 1985) (Sugawara et al. J. Bacteriol. 198:3200, 2016)

This very important aspect of porin physiology needs MUCH more study.
**We especially need crystal structures of OmpFs from *E. cloacae* and *K. pneumoniae*.**

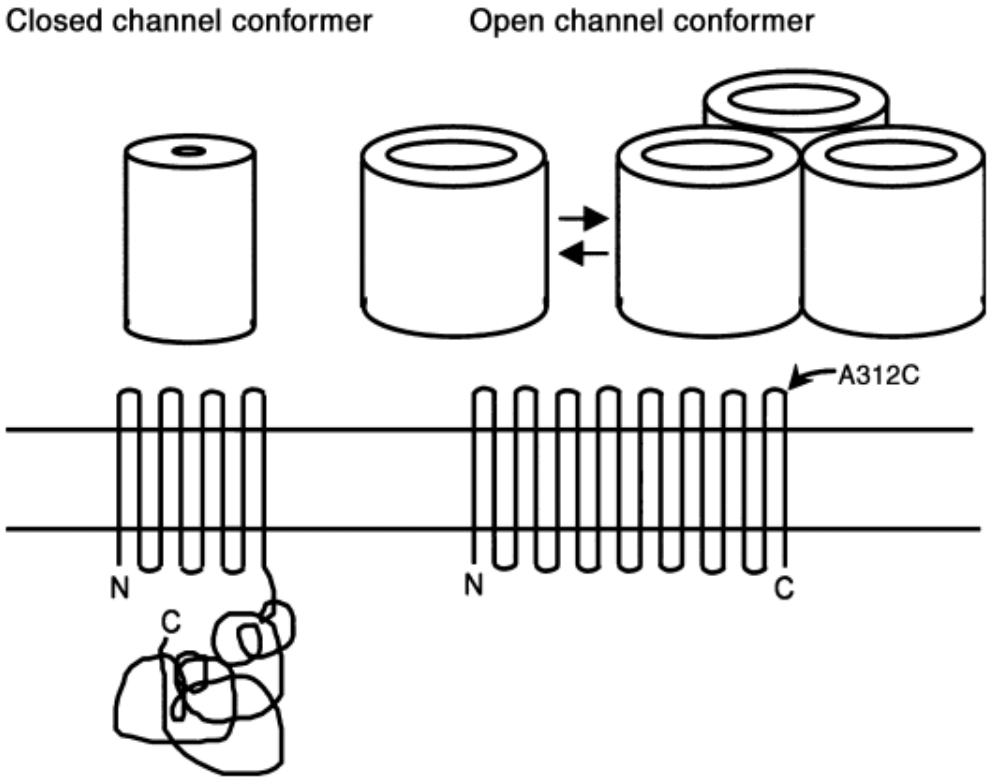
## Slow Porins in Non-Fermenters (*P. aeruginosa*, *A. baumannii*)

The nonspecific porin OprF or OmpA produces LARGE channels, yet allow only SLOW permeation.




Only a portion of OmpA population produces stable, open channels

Sugawara & Nikaido, J. Biol. Chem. (1994)

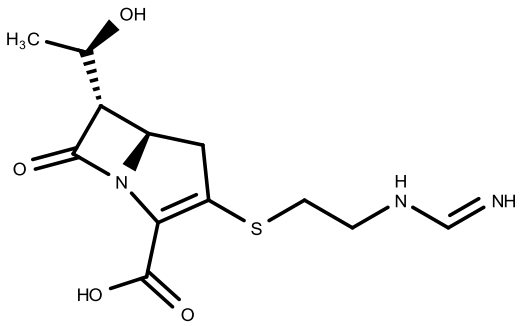



Only a small portion of OprF/OmpA folds as a one-domain, open-channel conformer.
The tendency to fold as an open-channel protein can be altered by point mutations in the protein.

Sugawara, Kojima & Nikaido, FEBS J 2012

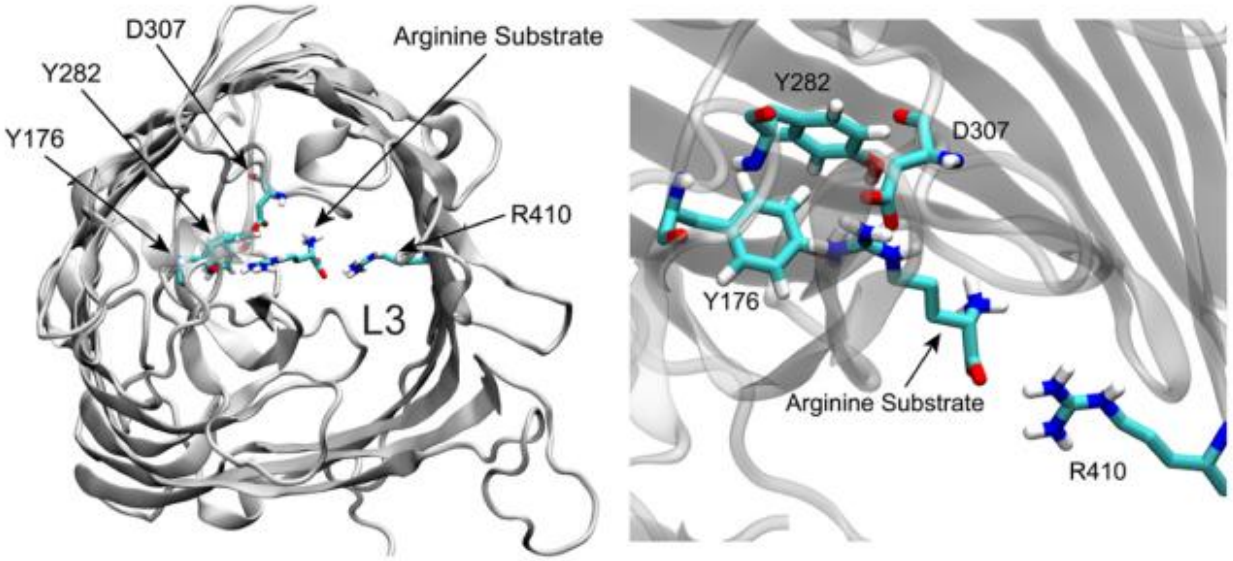
Because the NonSpecific Porin OprF is so inefficient, drugs that are unusually active against *P. aeruginosa* often traverse OM through SPECIFIC channels.

A Classical Example is IMIPENEM, which diffuses through OprD, a basic AA channel




Trias & Nikaido, AAC 1990
Trias & Nikaido, J. Biol. Chem. 1990

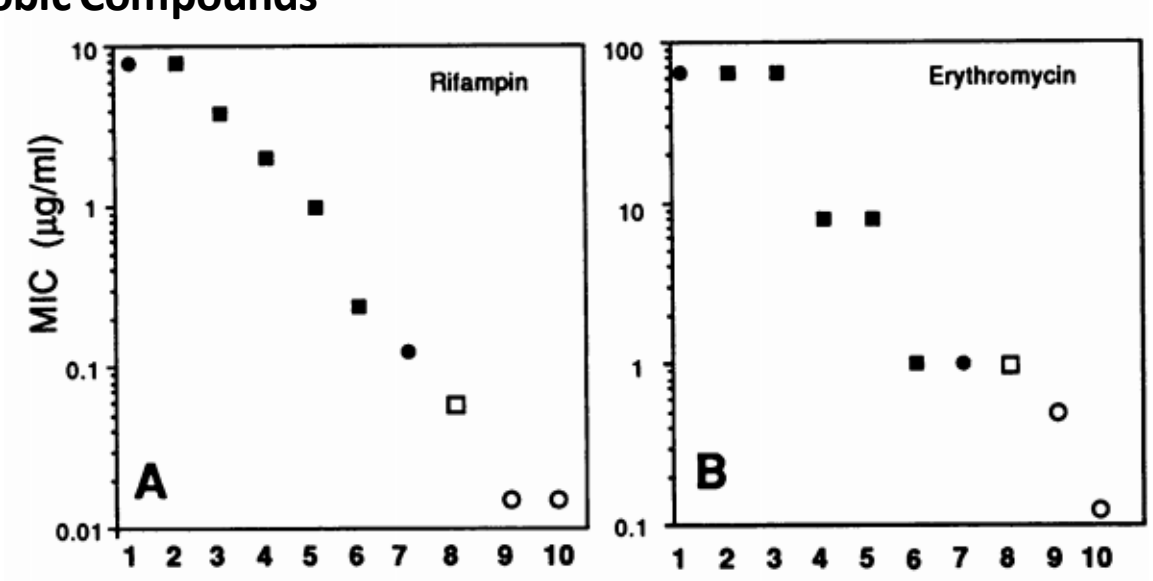
The crystal structure of OprD (OccD1) shows that Arginine binds to the specific binding site (Y176, Y282, D307)

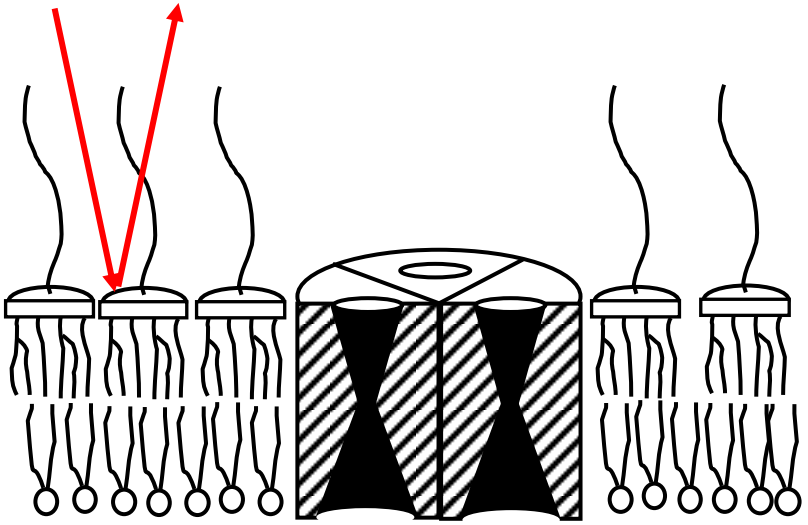



Eren et al. J. Biol. Chem. 2013

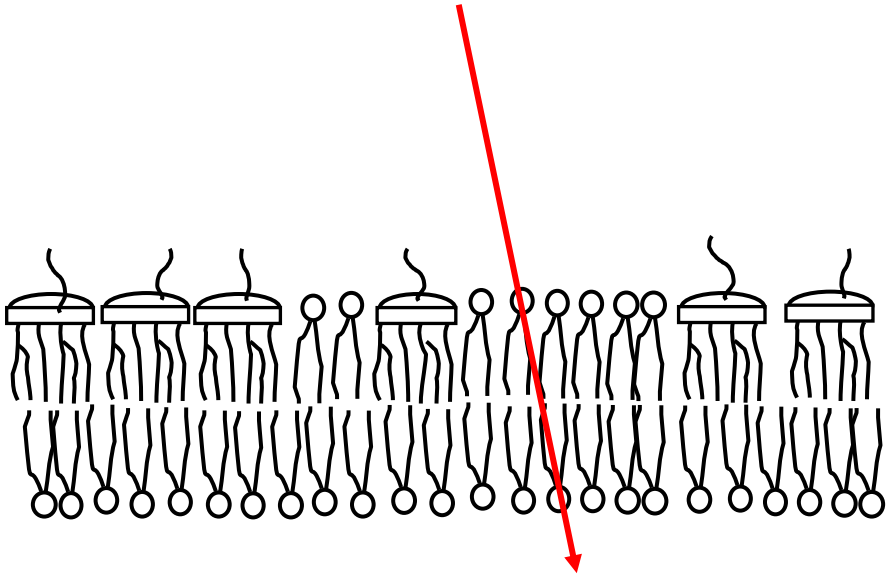
# OM Diffusion of Large, Hydrophobic Compounds

These compounds are likely to traverse OM mostly through its asymmetric bilayer region, as suggested by the vastly increased susceptibility of deep rough and lpx LPS mutants (Vaara, AAC 1993).



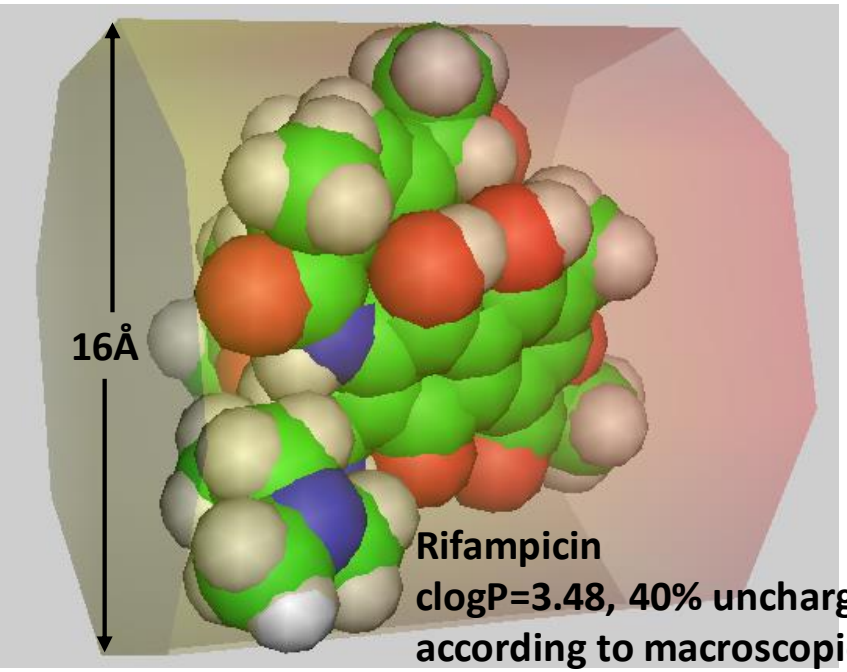



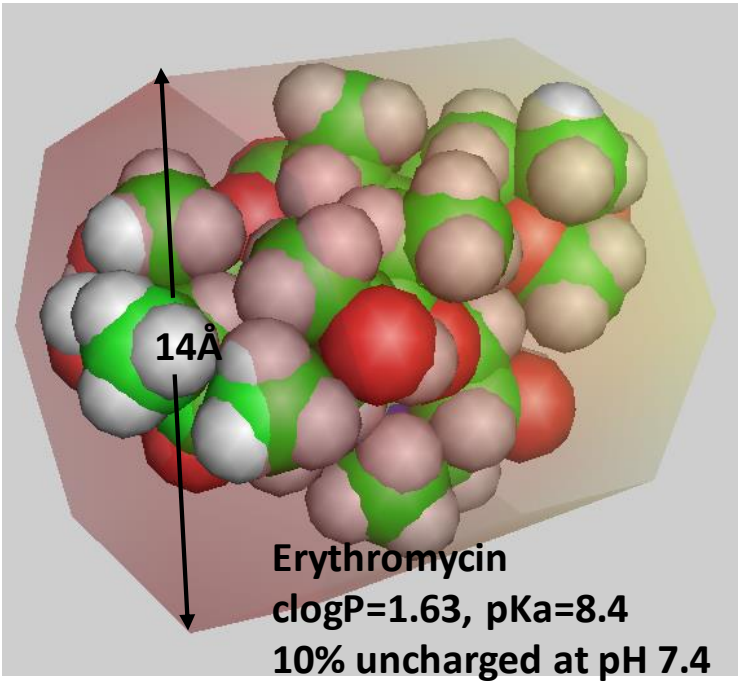
**Normal OM Bilayer, an Effective Barrier**



**Deep Rough OM Bilayer, an Ineffective Barrier**

The models show that they are indeed too large for OmpF channel.




How can we estimate the rate of their diffusion through OM bilayer?

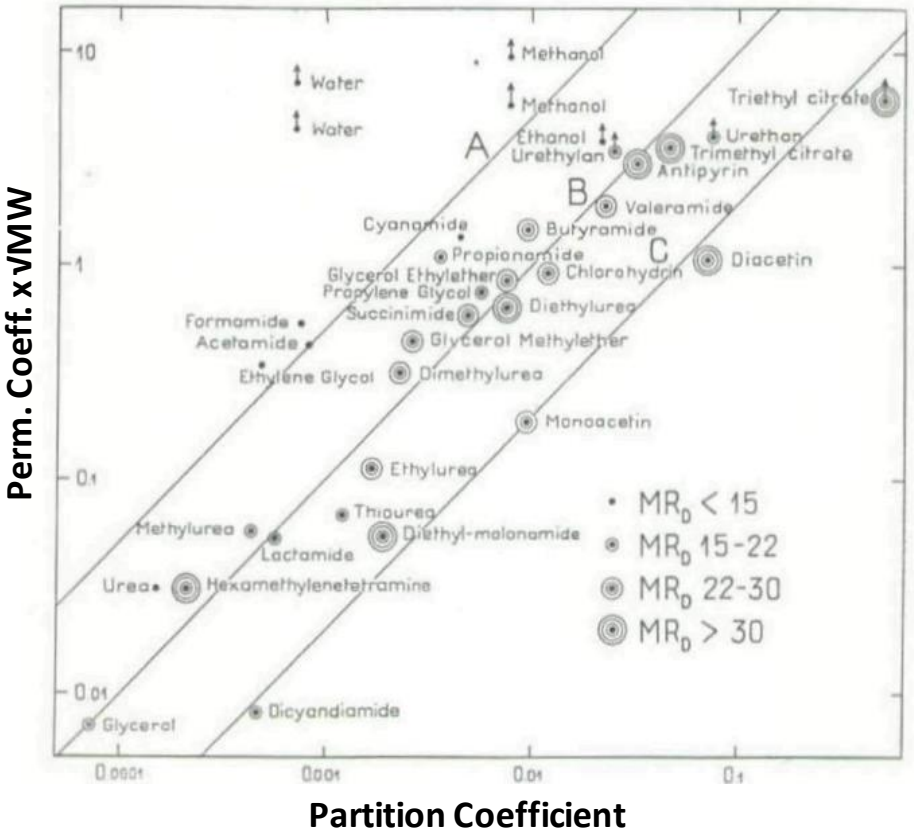
First, calculate the fraction of uncharged species.

With compounds with potential multiple charges, the usual (macroscopic) pKa values may give misleading numbers here.

For example, with tetracycline, use of these values predict that only 0.0001 % is in the uncharged form at pH 7.4. In contrast, use of the proper MICROSCOPIC pKa values shows that 7.1 % is uncharged. (Nikaido & Thanassi, AAC 1993). Unfortunately, programs that calculates microscopic constants (Marvin, SPARC) produce wrong results.

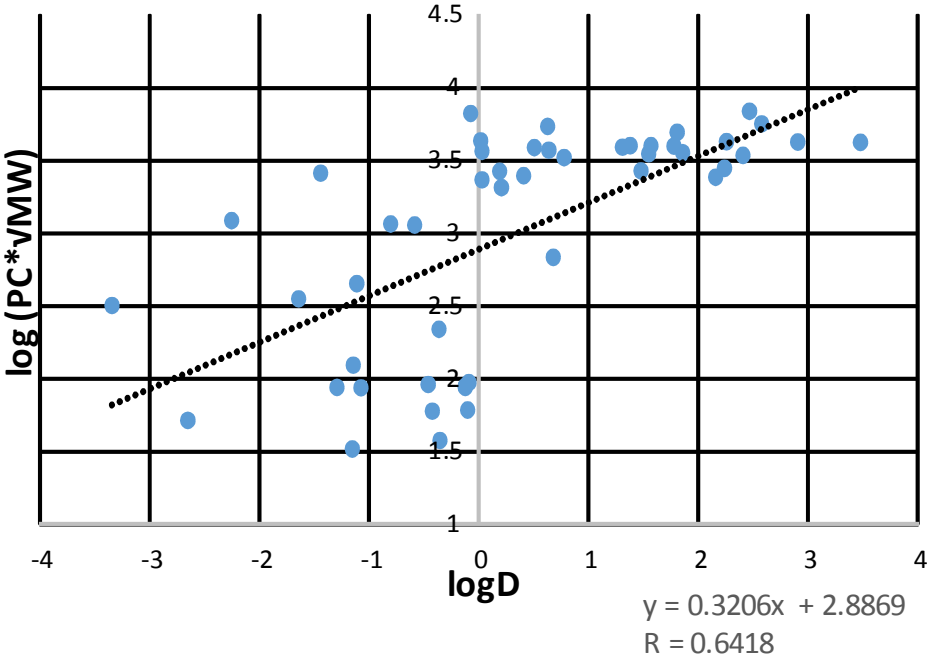
# Effect of Hydrophobicity in Drug Permeation Across Conventional Lipid Bilayer Membranes

Old study of Collander (1949), using *Chara* (algal) cells, showed that permeation rate across membrane bilayer is proportional to the partition coefficient, if correction for size is made.




**PC*√MW seems to reach a maximum around 10 (cm/hr) or 30,000 (nm/s)**

The correlation was quite poor in a study with Caco cells, with ~40 drugs (Yazdanian et al. Pharmaceut. Res. 1998)

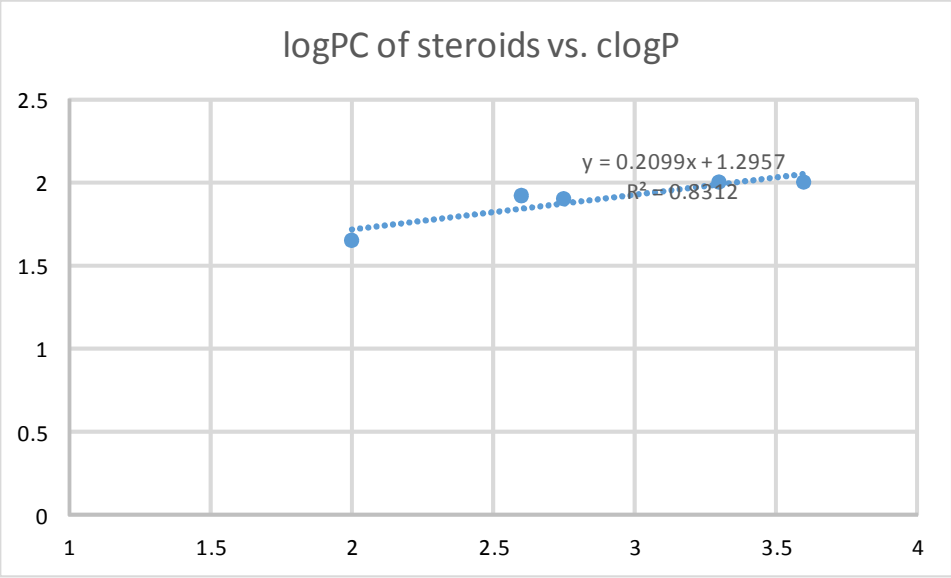



(A better fit can be obtained by using six (!) parameters (Kurkarni, Han, & Hopfinger 2002))

**The permeability again seems to reach a maximum When logD>0.**

## Plésiat and Nikaido (Mol Microbiol 1992) found that diffusion across the asymmetric bilayer of OM was about 60 times slower than across the usual phospholipid bilayer.

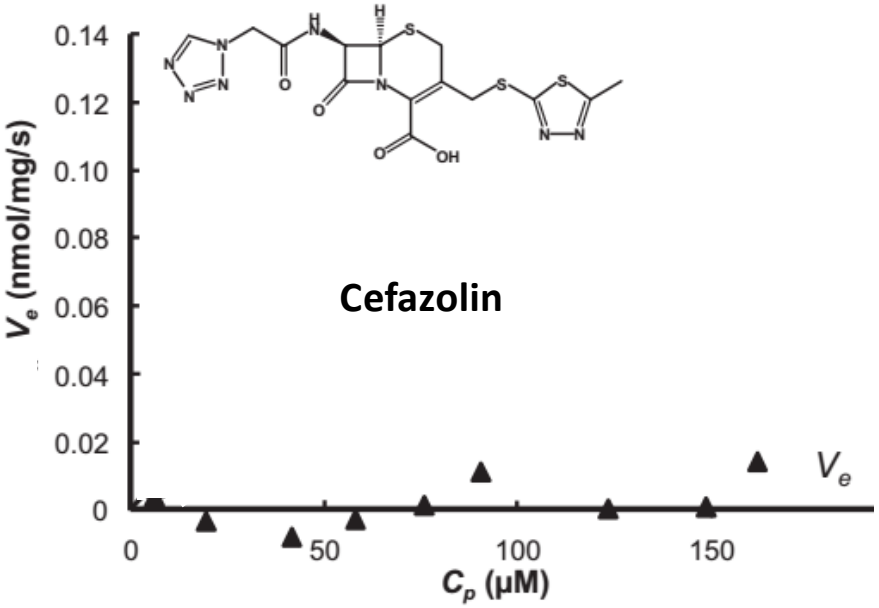
We used steroids, with clogP between 2 and 3.5. For these compounds, Collander's data show that PC*√MW across the conventional phospholipid bilayer membrane reaches 3 x $10^4$. Since this study was done at 20°C, we estimate that at 37°C this will increase to about $10^5$, thus log PC to around 3.8. Across the OM, logPC was about 2, which corresponds to 60-times reduction from the phospholipid permeability.

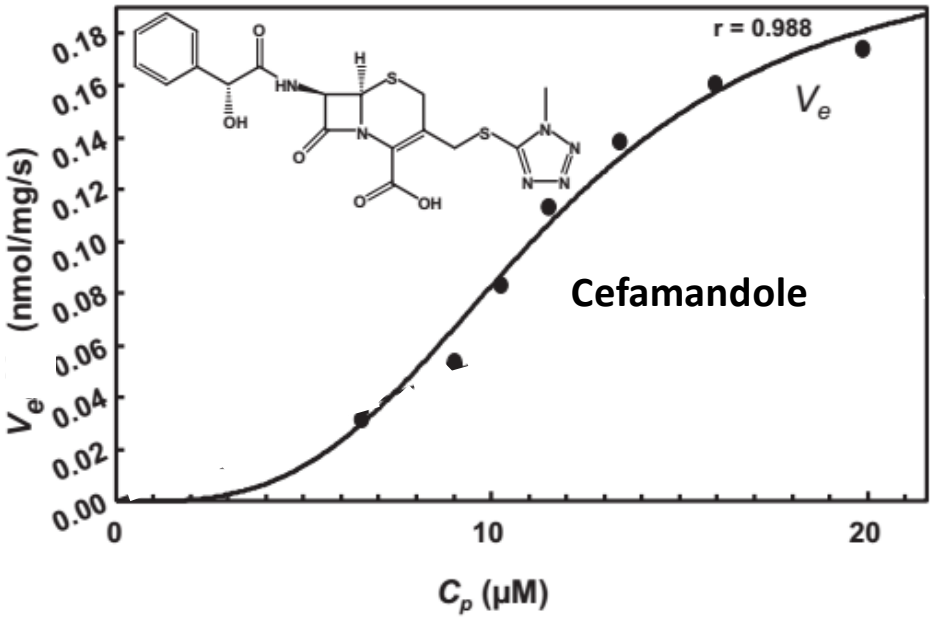



**So, for large, hydrophobic compounds with clogP >0, one would predict log (PC*√MW) of 2.7. For erythromycin and rifampin (taking into account the fraction of uncharged species), permeability coefficients of 2 and 7 nm/s, respectively, are calculated. This is not negligible and comparable to the permeability of hydrophobic lactams, such as penicillin (10 nm/s). But this is much slower than the zwitterionic cephalosporins (around 1,000 nm/s) or the rapidly diffusing monoanionic cephalosporins (around 300 nm/s). This also explains why deletion of AcrAB-TolC efflux pump makes *E. coli* susceptible to these drugs.**

**Can We Design Agents That Are Not Pumped Out by RND Pumps?**

We have measured the efflux parameters of β-lactams via AcrAB-TolC pump (Nagano & Nikaido, PNAS 2009; Lim & Nikaido AAC 2010).




As seen, compared with a reasonable substrate Cefamandole, the very hydrophilic Cefazolin shows no evidence for efflux.
Also in the docking/MD simulation studies (Vargiu et al., PNAS 2012), completely hydrophilic compounds such as kanamycin and glucose showed no evidence for binding to AcrB.

# Session 1: Barriers to compound penetration and efflux avoidance

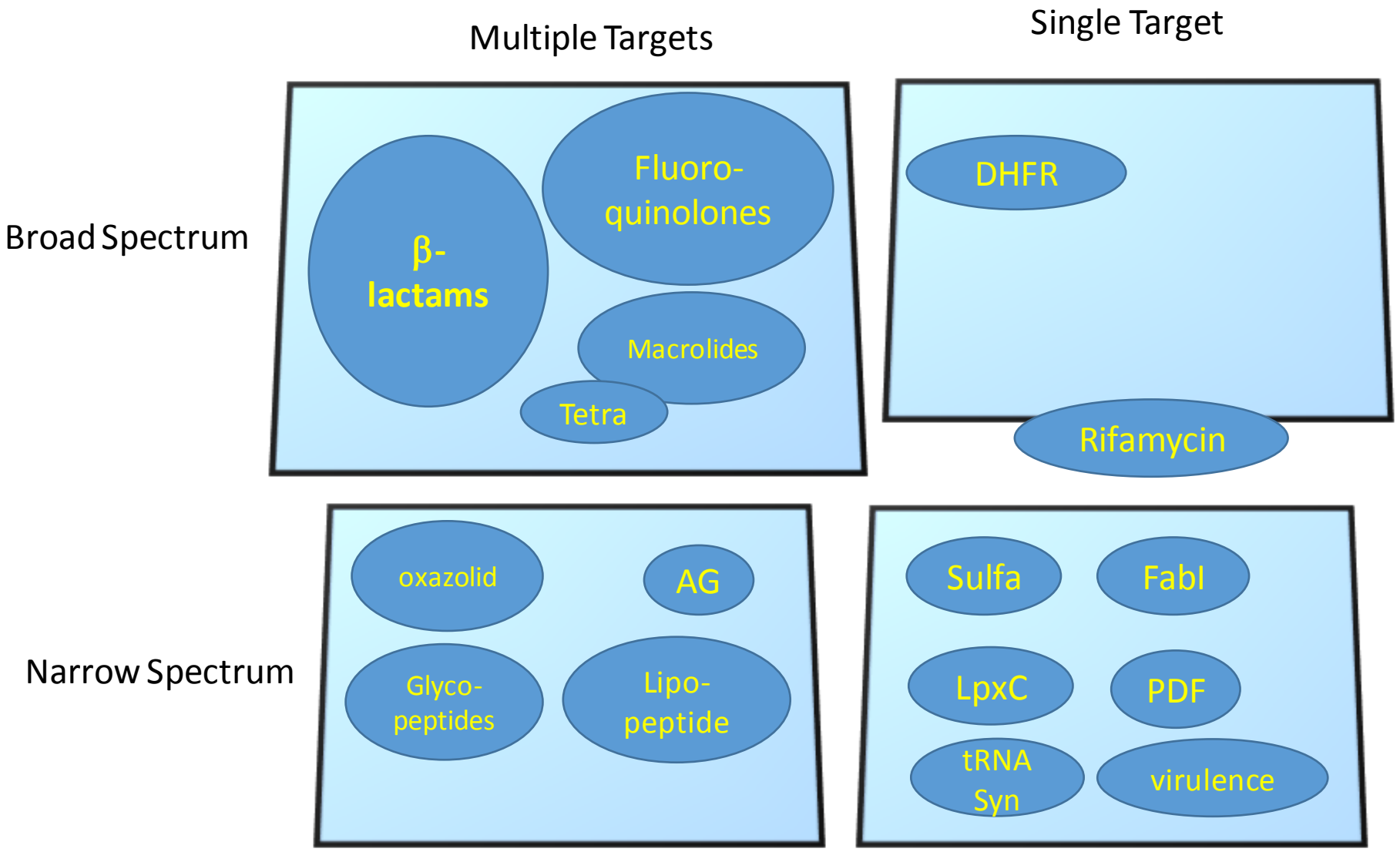
# Observations and Comments from a SBDD Perspective

John Finn

- The Trius TriBE program focus was to design in Gram-negative activity by using the concepts of Silver and Nikaido
  - Dual-targeting
    - GyrB/ParE
  - Fluoroquinolone-like compounds
    - Highly potent, small molecular weight (high ligand efficiency)
    - Charged molecules (especially diamines)

- The TriBE program progress was made with many small steps
  - Compounds were built almost an atom at a time
  - Many iterations of SBDD
  - Avoid the traps of bias towards what you have

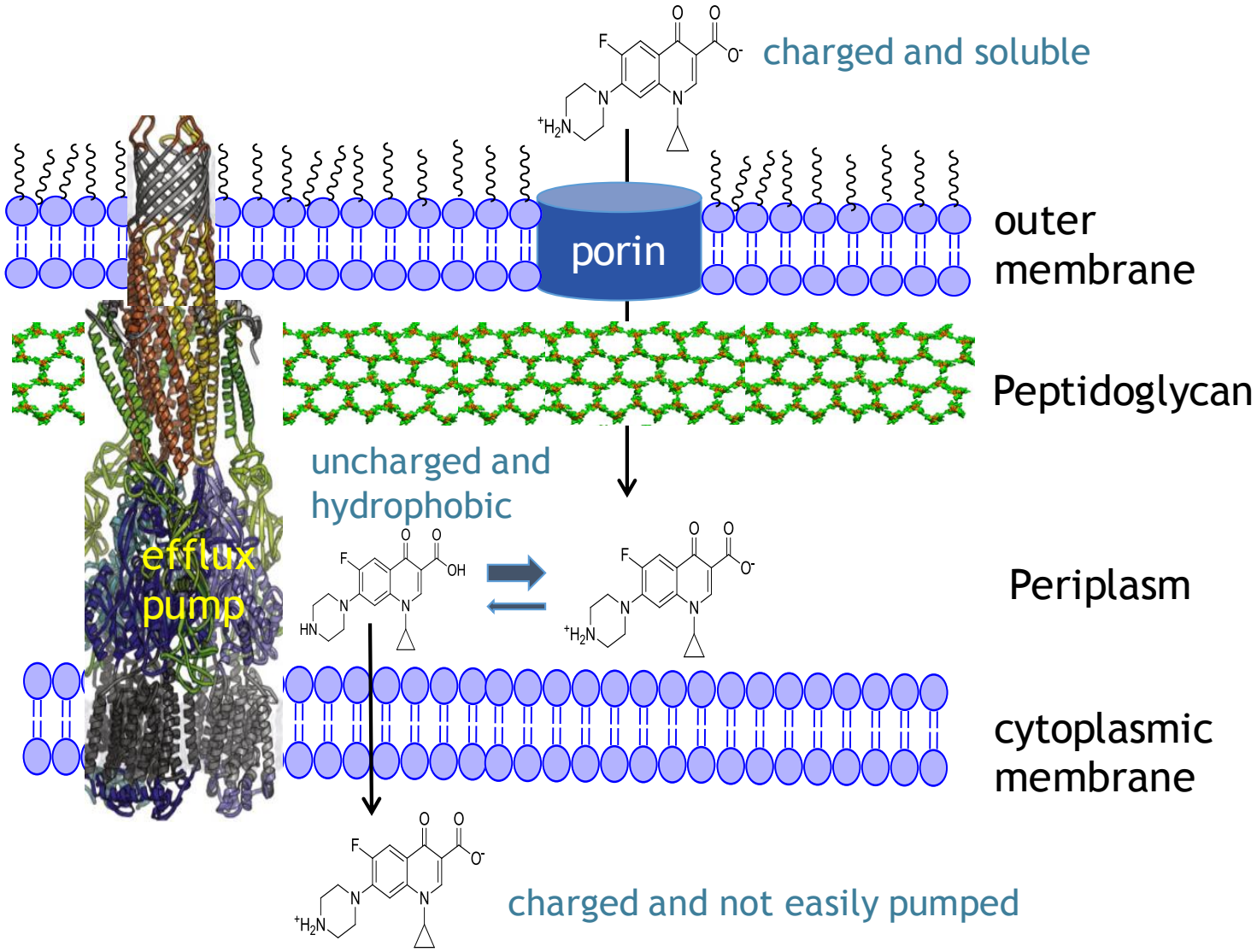
# "Benefits of multi-targeting" Lynn Silver *Nat. Reviews Drug Dis.* **2007**, *6*, 41



# Potential Paths to Enter a Gram-negative

- Front Door
  - Porins
  - It works for fluoroquinolones and tetracyclines
- Back Door
  - Active uptake via a Trojan Horse strategy
  - Fear of resistance
- Bust open a new door
  - Self promoted uptake like the aminoglycosides
  - Fear of toxicity

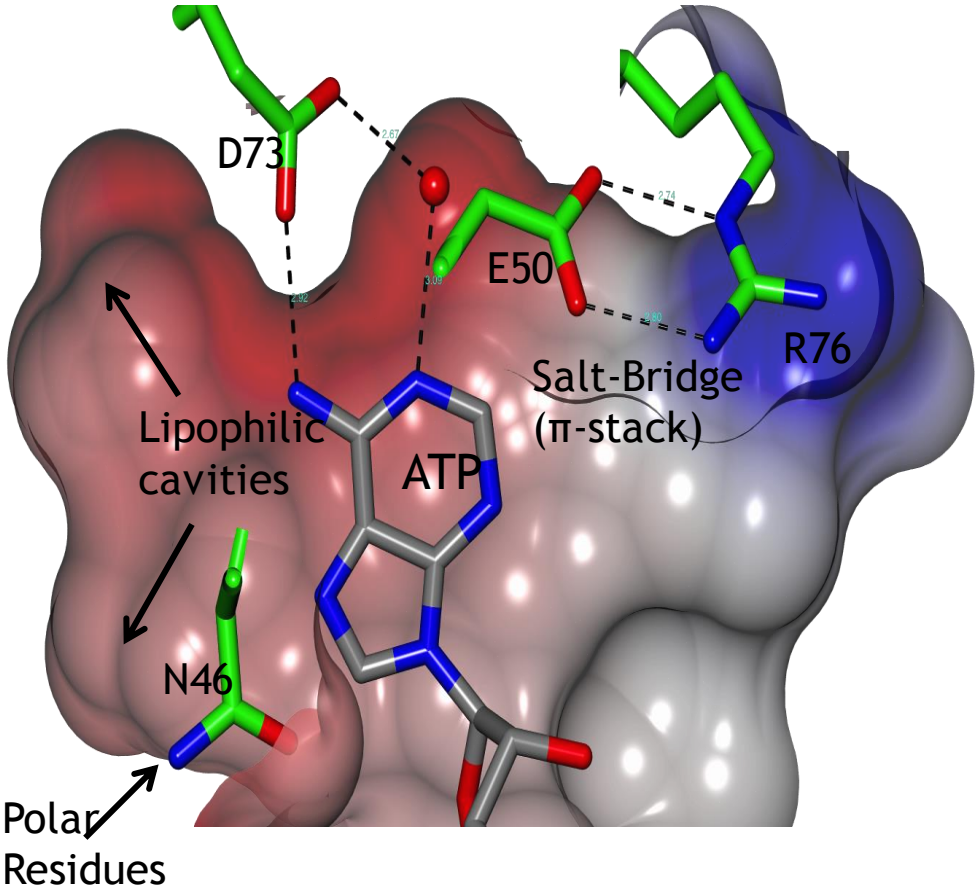
# Gram-Negative Design: “Chameleon Strategy”




Ref: Nikaido and Thanassi *AAC* **1993**, *37*, 1393

# GyrB/ParE Active Sites Include Polar Binding Sites

## GyrB-Adenine Binding Pocket



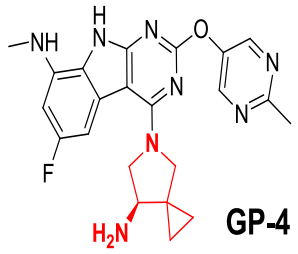

Key Features

- Highly conserved: spectrum and dual targeting
- Unique pocket: selectivity
- Deep pockets: potency
- Balance of interactions: Antibacterial spectrum /drug properties

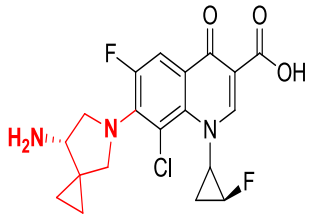
Binding Site includes polar residues that have not been extensively exploited in drug discovery to increase solubility / explore charged molecules

# TriBE Discovery and Optimization

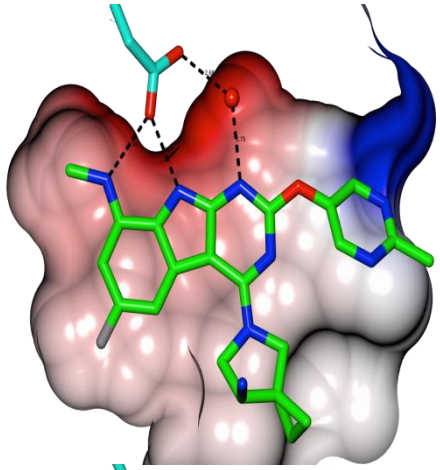
High potency, dual-targeting, broad-spectrum, plus drug properties



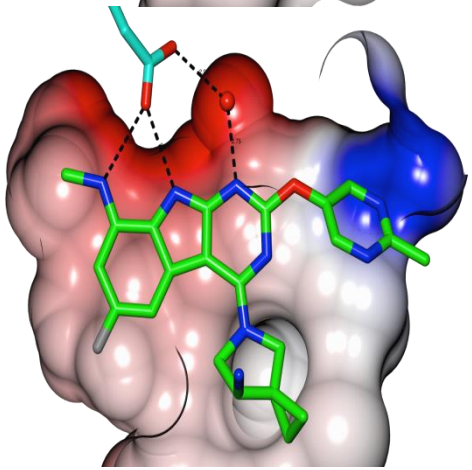

GP-4



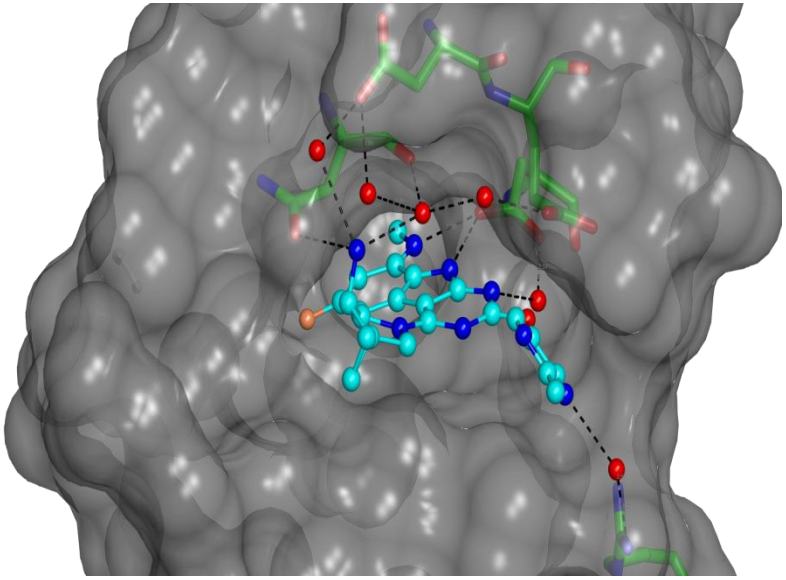

Sitafloxacin



*E. coli* GyrB
$K_i$ = <20 pM



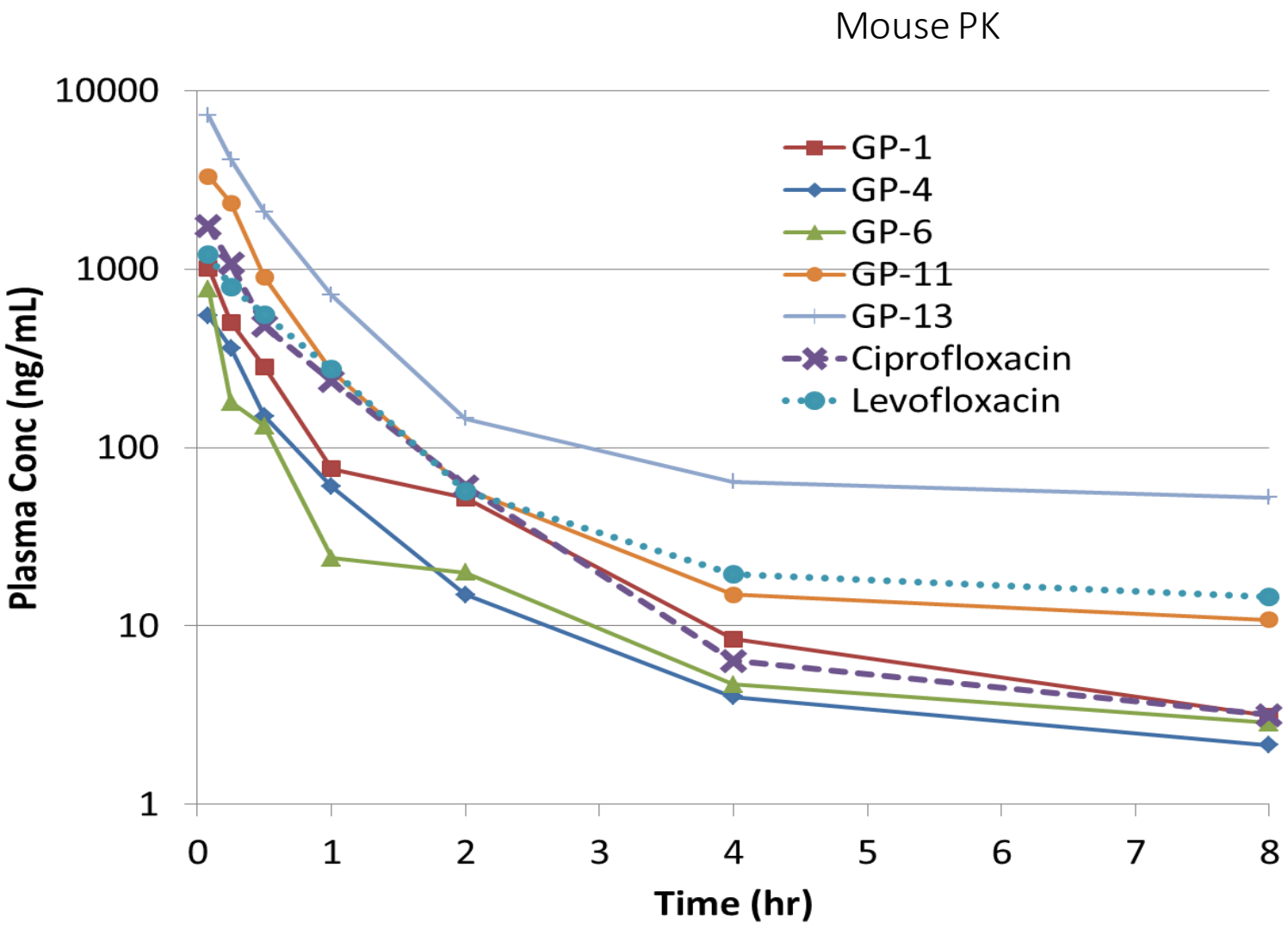
*E. coli* ParE
$K_i$ = <20 pM



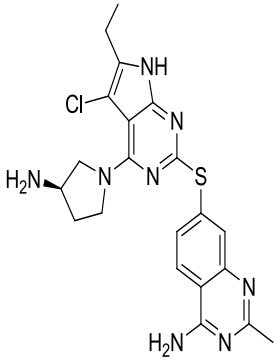
*E. coli* MIC 0.25 μg/mL
**no effect of serum on MIC**

- Total of 7 Hydrogen bonds
  - 3 to protein, 4 to water network

# TriBE compounds have similar properties to fluoroquinolones

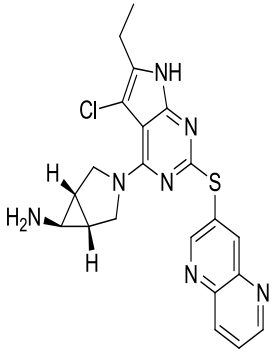


# Issue 1: Confirm single MOA
# Off target activity is common!



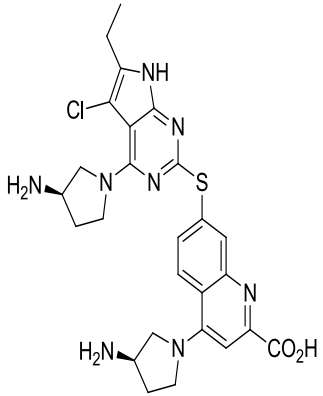
**717**
MW 455
Basic amine and a weakly basic salt bridge

*E. coli* MIC 16 μg/mL



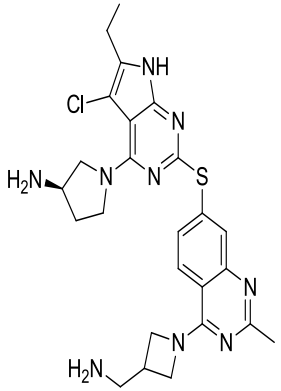
**800**
MW 438
Plus 1 Charge

*E. coli* MIC 4 μg/mL



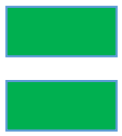
**825**
MW 553
Zwitterionic Net Plus 1

*E. coli* MIC 8 μg/mL



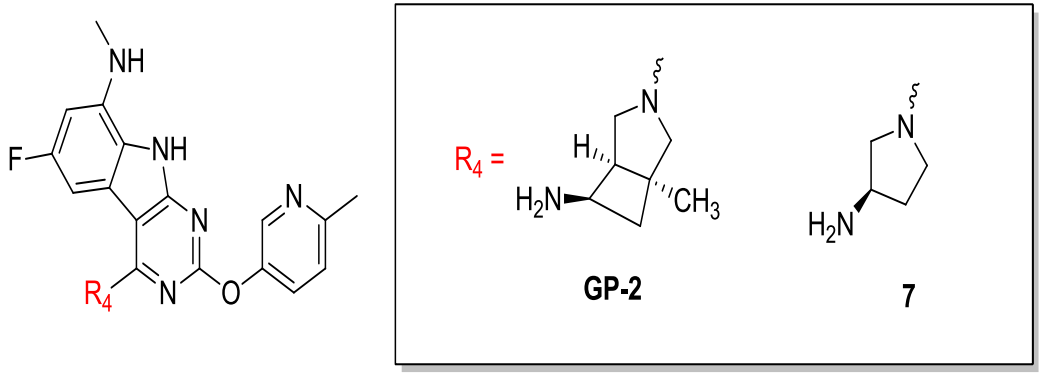
**922**
MW 524
Dication plus weak base

*E. coli* MIC 4 μg/mL



# Issue 2: Potency
# Are your compounds smarter than Cipro?

*P. aeruginosa* efflux pumps SAR




| | Cipro | GP-2 | 7 |
|---|---|---|---|
| PAO397<br>*P. aeruginosa* five pump deletion strain | MIC 0.008 μg/mL | MIC 0.03 μg/mL | MIC 0.06 μg/mL |
| PAO1<br>*P. aeruginosa* wild type strain | MIC 0.13 μg/mL | MIC 1 μg/mL | MIC 32 μg/mL |
| Fold change | 16x | 32x | 512x |

# Unanswered Questions

- Can the activity be improved by better compound properties?
  - Better entry / better pump avoidance
- Almost an ideal case where many modifications can be made in solvent accessible region thereby retaining the enzymatic potency
  - We seemed to reach a peak level of activity that is hard to beat but easy to lose
  - But activity is always better on the imp strain
  - Focus is more on better PK, properties (e.g. solubility, protein binding) and safety
- It would be useful to measure porin entry and understand efflux SAR

# Antibacterial profile of an early lead

| Antibacterial Potency MIC (μg/mL) | |
|---|---|
| *E. coli (wt)* | 4 |
| *E. coli + serum** | 2 |
| *E. coli (ΔtolC)*** | 0.5 |
| *E. coli (imp)**** | 1 |
| *K. pneumoniae MDR*[a] | 32 |
| *K. pneumoniae WT* | <0.5 |

Serum* = 20% mouse serum
ΔtolC** = pump knock-out
Imp*** = permeability mutant
*K. pneumoniae* strains used :MDR ATCC 700603
WT ATCC 10031

# Recommendations/Make Antibacterial Drugs Great Again!

- Improved microbiological tools
  - Isogenic strains of pump knockouts for: *A. baumannii* and *K. pneumoniae*
  - Porin permeability assays: P. aeruginosa, *A. baumannii* and *K. pneumoniae*

- Mechanism of action assay service to make the technology more widely used
  - Macromolecular synthesis

- Focus (exclusively) on SBDD approaches
  - Screening is very low probability (the potency issue!)
  - Creating an antibacterial screening library is predicted to be a waste of resources
    - Money is better spent on supporting independent projects with good rationales

- Narrow focus of Gram-negative of projects to those with realistic chance
  - **Must answer the question of potency compared to fluoroquinolones**
  - Clear plan to achieve activity on targets located in the cytoplasm
    - Strong rationale to build compound properties compatible with G- activity
  - Shift focus to targets that are located in the periplasm or outer membrane

# Kinetic Modeling of Gram-Negative Permeability

Wright Nichols

Consultant Microbiologist

Cambridge, MA

February 6 2017

NIAID & The Pew Charitable Trusts: Challenges in the Discovery of Gram-negative antibacterials: the entry & efflux problem. Feb 6-7 2017, Rockville, MD

# Three Interesting Questions

1. **How can I know whether my new compound penetrates to the cytoplasm, irrespective of growth inhibition?**

2. **What's more important: outer or cytoplasmic membrane permeability?**

3. **What's more important: diffusion in or pumping out?**

# A Minimum (Envelope) Permeability Coefficient

**When growth just balances influx:**
**a cell envelope permeability coefficient >$10^{-8}$ cm.$s^{-1}$ approx. indicates that the compound can passively reach the cytoplasm at a reasonable rate**

## Examples of Lipid Bilayer Permeability Coefficients[1]

| Solute | *P* (cm.$s^{-1}$) | Gm-ve ($c_{cyt}/c_{ext})_{t\rightarrow\infty}$ |
|---|---|---|
| 2'-deoxyadenosine | $9.40 \times 10^{-7}$ | 0.996 |
| erythromycin | $2.12 \times 10^{-8}$ | 0.838 |
| tryptophan, pH 6.0 | $4.10 \times 10^{-10}$ | 0.0912 |
| $Na^+$ | $1.20 \times 10^{-14}$ | $2.94 \times 10^{-6}$ |

[1]For sources see: Nichols WW. 2012. Permeability of Bacteria to Antibacterial Agents. In Antibiotic Drug Discovery and Development Volume II (T.J. Dougherty & M.J. Pucci, eds). Springer Publishing Company. pp 849–879.
Nichols WW. 2016. Modeling the kinetics of the permeation of antibacterial agents into growing bacteria and its interplay with efflux. Submitted.

# Permeability of Two Layers

**Example:**

$$\frac{1}{P} = \frac{1}{10^{-6}} + \frac{1}{10^{-8}}$$

**Outer membrane is 100-times more permeable**

**Compound crosses the cytoplasmic membrane with the Chemistry threshold coefficient**

$$P = 0.99 \times 10^{-8}$$

**If the layers differ widely in permeability, the overall coefficient is slightly lower than the lowest coefficient of the contributing layers**


Brodin et al. 2012. Passive diffusion of drug substances: the concepts of flux and permeability. In: Steffansen et al (eds.) Molecular Biopharmaceutics. Pharmaceutical Press, London (2010). pp 135-152.

Nichols WW. 2016. Modeling the kinetics of the permeation of antibacterial agents into growing bacteria and its interplay with efflux. Submitted.

# Influx Balanced against Efflux

## Inferences from kinetic analysis

**The efflux coefficient acts reciprocally against the permeability coefficient for the membrane containing the efflux pump**

$$\left(\frac{c_{\text{cytoplsm}}}{c_{\text{external}}}\right)_{t\to\infty} = \frac{1}{1+\frac{k}{P}}$$

**Multiple efflux pumps in one membrane: *additive kinetics***

**Cytoplasmic and outer membrane pumps: *additive and multiplicative kinetics***


Nichols WW. 2012. Permeability of Bacteria to Antibacterial Agents. In Antibiotic Drug Discovery and Development Volume II (T.J. Dougherty & M.J. Pucci, eds). Springer Publishing Company. pp 849–879.

Palmer M. 2003. Efflux of cytoplasmically acting antibiotics from Gram-negative bacteria: periplasmic substrate capture by multicomponent efflux pumps inferred from their cooperative action with single-component transporters. J Bacteriol 185:5287–5289.

Nichols WW. 2016. Modeling the kinetics of the permeation of antibacterial agents into growing bacteria and its interplay with efflux. Submitted.

# Conclusions from Kinetic Modeling

- [Ignoring efflux] The cytoplasmic concentration of a solute in a bacterial cell should reach that of the external medium in a reasonably short time if its envelope permeability coefficient is higher than ~$10^{-8}$ $cm.s^{-1}$

- The permeability coefficient must be >$10^{-8}$ $cm.s^{-1}$ for both the outer and cytoplasmic membranes
  - a lower value for either one would be limiting

- Pump arrangements
  - for two pumps in one membrane, efflux pump efficiencies add together
  - Gram-negative envelope: when there is an efflux pump in each membrane, their efficiencies both add and multiply

# Influx and Efflux of Drugs Across IM

Hiroshi Nikaido

University of California, Berkeley

# Influx into Bacterial Cytosol is Usually Quite Fast

Example: Tetracycline

7.1% of the drug is in uncharged form, on the basis of microscopic pKa values (Nikaido and Thanassi, 1993).

Since its clogP is -2.46 (i.e. P=0.0035), Collander data says PC*√MW should be around 0.35, or the PC 0.017. However, the unit of PC in Collander is cm/hr. So, it will be around 50 nm/s or 0.5 x $10^{-5}$ cm/s. Because only 7% of the drug is in uncharged form, the actual PC should be ~ 3.5 x $10^{-7}$ cm/s.

Because the half-equilibration time, $t_{1/2}$ (in second) is

$$t_{1/2} = \ln 2 * (V/A) * (1/PC)$$

in *E. coli* cells (V=0.004 $cm^3$/mg, A=132 $cm^2$/mg) it will be around 1 min.

In contrast, in animal cells, e.g. hepatocytes, the term (V/A) will be nearly four orders of magnitude larger, and the permeation of drugs such as this becomes a very slow process, unless it is facilitated by carriers.

*E. coli* IM is full of "singlet" Efflux Pumps

These pumps are presumably important in exporting drugs into periplasm so that they can be exported out of the cell by RND tripartite efflux machinery, such as AcrAB-TolC.

Their significance can be seen in the extremely sensitive assay data of Nichols et al. Phenotyic Landscape of A Bacterial Cell, Cell 143: 1097 (2010), which can be accessed and analyzed at ecoliwiki.net/tools/chemgen. Among about 15 MFS pumps suspected of function in drug efflux, deletion of Bcr, YcaE, YdhC, YfcJ, YgsS, YidY (MdtL), or YjiO (MdtM) was found to increase the susceptibility of *E. coli* to tetracycline at least at one of the four concentrations used (0.25, 0.5, 0.75, and 1.0 µg/ml). (SMR family pumps are only involved in the efflux of cationic substrates, so not relevant here). Interestingly, Nishino's 2001 paper using *ΔacrAB* strain overexpressing many of these MFS pumps found no increased resistance to tetracycline, except Bcr and MdfA.

## How Do We Measure the Efflux Parameters of Singlet Pumps?

1. For precise determination of kinetic parameters, measurement of periplasmic drug concentration is essential. Develop a sensor protein (similar to TetR, used in cytosol by A. Sigler et al. (Eur. J. Biochem. 2000)) but expressed in periplasm?

2. If time-curves of drug accumulation can be obtained in *ΔacrAB* cells expressing only one relevant singlet pump, numerical solution of the differential equations?

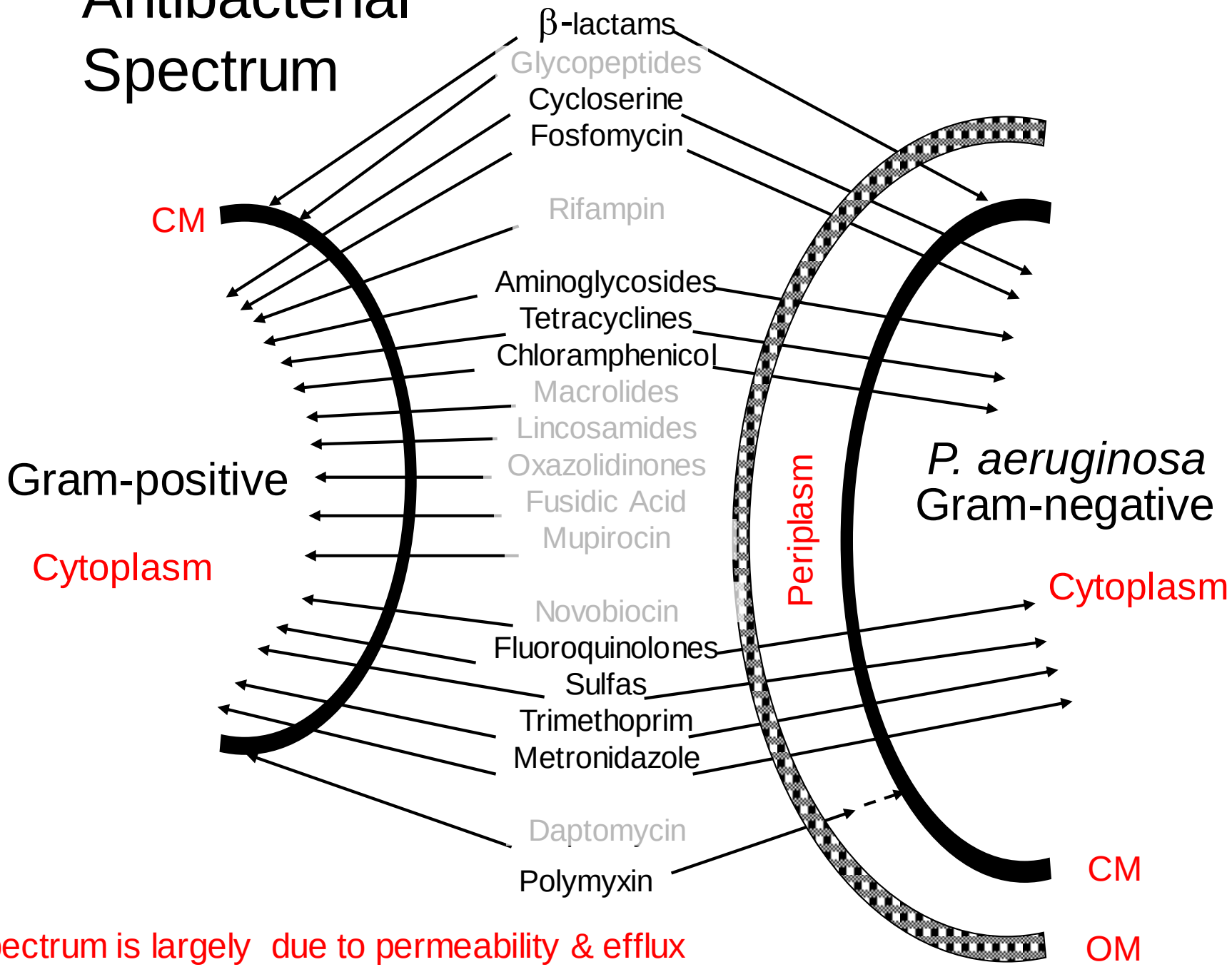
3. In *ΔacrAB* cells expressing only one singlet pump, its activity may be measured
   (a) by increases in oxygen consumption (detected e. g. by Seahorse bioanalyzer), or
   (b) by direct assay of the proton flux (detected by pH meter under anaerobic conditions, a la I. C. West (1970)).

4. More effort is needed in this direction.
   LC-MS detection of drugs (Zhou et al., Anal Chem 2015; Davis et al. ACS Chem Biol 2014)?
   Use of microfluidics to overcome the problems of fast kinetics?

# Session 1: Barriers to compound penetration and efflux avoidance

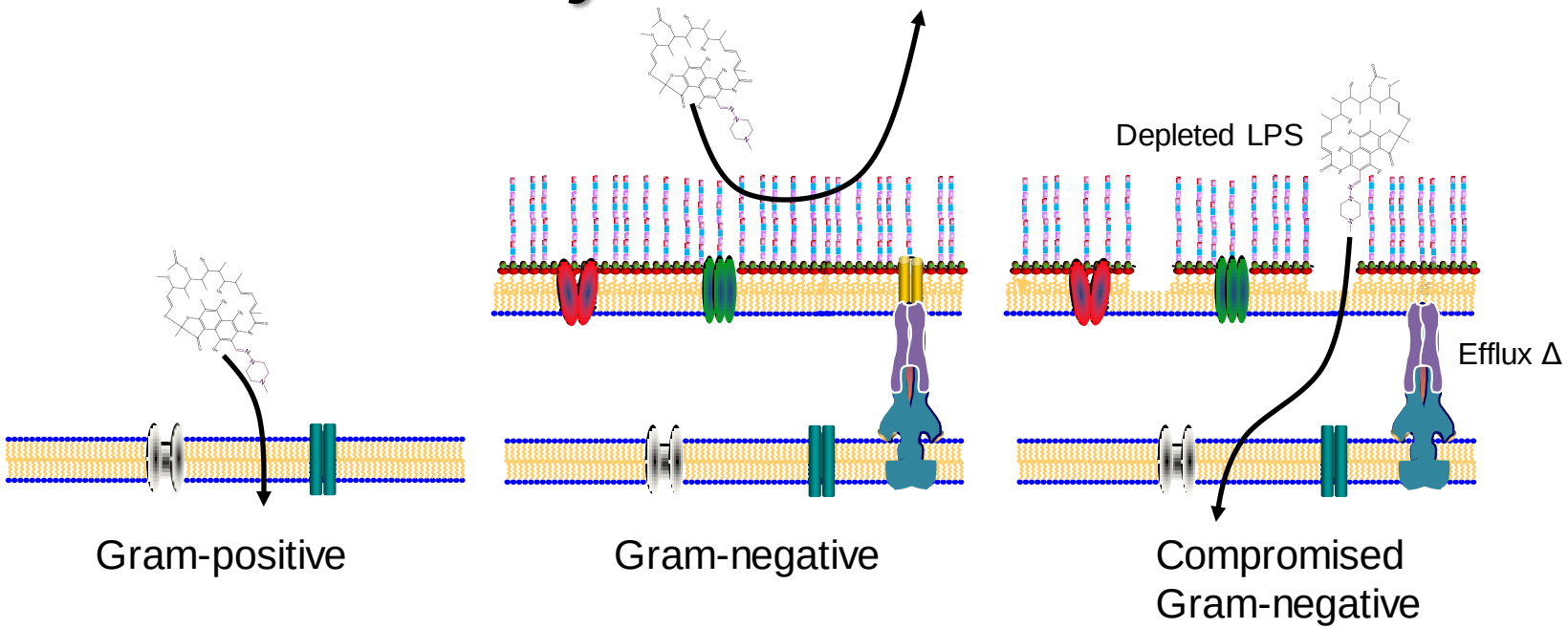
*Lynn Silver, PhD*
*LL Silver Consulting, LLC*

# Antibacterial Spectrum



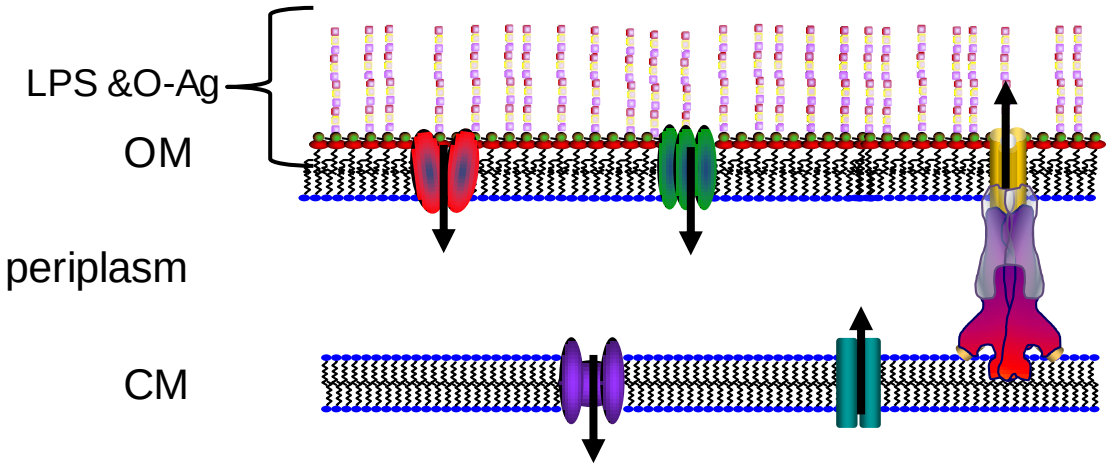

Spectrum is largely due to permeability & efflux

# But it's not just OM and Efflux



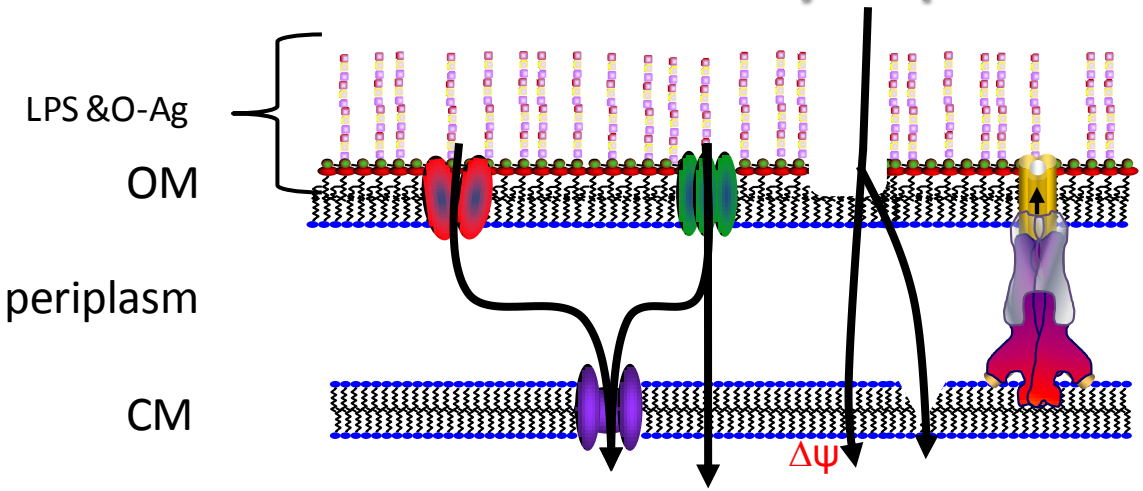

- Since the major permeability difference between GN and GP the OM…
- And OM-permeable and effluxΔ GNs are sensitive to many GP drugs
- Some assume finding ways of crossing the OM and avoiding efflux will allow GN entry
- But novel compounds (such as cytoplasmic enzyme inhibitors) need qualities that also permeate the CM.

# GN barriers




- OM excludes hydrophobic and hydrophilic compounds.
- Penetration of hydrophilic compounds through OM is via:
  - general porins [<600 MW, prefer hydrophilic, charged]
  - facilitated diffusion of specific hydrophilic solutes [OprD, Tsx]
- But hydrophilic and highly charged molecules entering the periplasm
  - penetrate the CM slowly or not at all
  - unless actively transported [or via PMF]
- Molecules that do enter can be effluxed
- **What molecules can accumulate in the GN cytoplasm?**

# Routes to the cytoplasm




- Diffusion (no transporters)
  - Hydrophilic molecules: Cross OM rapidly via porins, may avoid efflux –poor CM passage
  - Lipophilic molecules: Cross OM slowly, can be effluxed – good CM passage
- Active transport
  - Hydrophilic molecules cross OM via porins, CM via transporters [ATP or PMF driven]
- Self-promoted uptake [SPU] through OM
  - Cationic molecules, avoid efflux; CM passage via Δψ or polycations may disrupt CM
- Trojan horse
  - Piggyback on active or facilitated transport; must avoid rapid resistance

[• OM permeabilizers and EPIs as adjuncts ]

  - Combine with CM-transiting molecules [properties of GP drugs]

# Session 2: Case studies: Finding ways to overcome barriers to compound penetration and efflux avoidance

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# Achaogen Approach to Understanding Permeability

**Frederick Cohen**
On behalf of the Research Team

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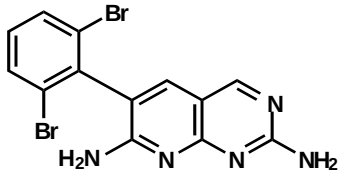
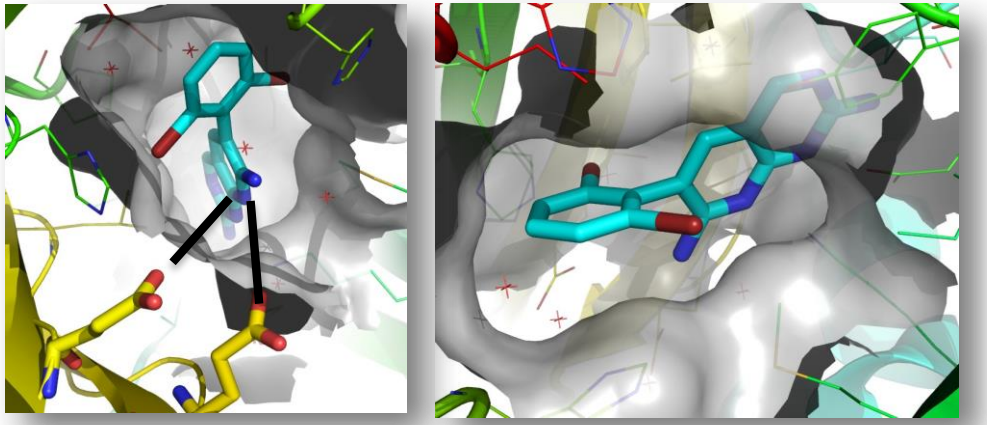
# Project Strategy for AccC




A1

| Strain | MIC (µg/mL) |
|---|---|
| *E. coli* | 32 |
| *E. Coli ΔtolC* | 1 |
| *K. pneumoniae* | 64 |
| *A. baumannii* | 128 |
| *P. aeruginosa* | >256 |
| *S. aureus* | 256 |
| $pK_a$ | 3.2 |
| mwt | 395 |
| cLogD | 3.5 |
| charge (pH 7.4) | 0 |

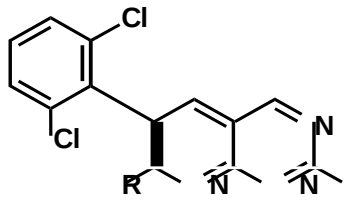


- Gram(–) barriers are preventing entry
  - Hypothesized that this was due to poor physiochemical properties
- Strategy: Use structure- and property-based design to discover new inhibitors optimized for Gram(–) entry while maintaining target potency
- Chance to *prospectively* apply property rules
  - Primarily focused on adding charge and reducing LogD



Miller, J. R., et al (2009). A class of selective antibacterials derived from a protein kinase inhibitor pharmacophore. *PNAS*, *106*(6), 1737–42

# Amine Substitution Improves Cellular Entry

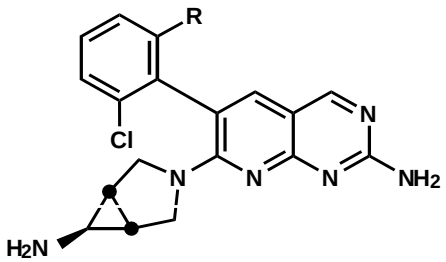


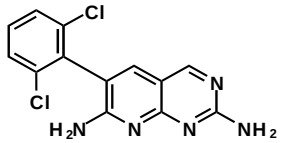

| | | A805 | A008 | A993 | A981 |
|---|---|---|---|---|---|
| MIC, (μg/mL) | *E. coli* KD65 | 64 | 32 | 16 | 2 |
| | *E. coli* KD65 (Δ*tolC)* (shift) | 0.5 (128) | 0.25 (128) | 0.5 (32) | 0.25 (8) |
| | *E. coli* KD65 +PMBN (shift) | 1 (64) | 2 (64) | 4 (4) | 0.25 (8) |
| *Pa*AccC $IC_{50}$ (nM) | | 320 | 140 | 64 | 33 |
| $cpK_a$ | | 3.3 | 5.0 | 10.5 | 8.1 |
| mwt | | 307 | 390 | 389 | 387 |
| cLogD (pH 7.4) | | 2.8 | 3.5 | –0.1 | 2.7 |
| Charge (pH 7.4) | | 0 | 0 | 1 | 1 |
| $MIC_{(WT\ E.\ coli)}$:$IC_{50}$ ratio | | 660 | 580 | 900 | 300 |

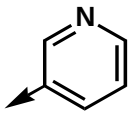
Best amine has only an 8-fold shift due to efflux or the outer membrane;

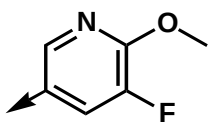
Tuning pKa is also important.

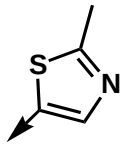
# Combining Modifications on Both Vectors




| | | A805 | A990 | A881 | A886 |
|---|---|---|---|---|---|
| MIC, (µg/mL) | *E. coli* KD65 | 64 | 2 | 1 | 0.5 |
| | *E. coli* KD65 (Δ*tolC)* (Fold) | 0.5 (128) | 0.063 (32) | 0.016 (64) | 0.008 (64) |
| | *E. coli* KD65 +PMBN (Fold) | 1 (64) | 0.25 (8) | 0.06 (16) | 0.03 (16) |
| | *P. aeruginosa* (Δ*mex*)[1] | 16 | 1 | 2 | 1 |
| *Pa*AccC $IC_{50}$ (nM) | | 320 | ≤ 15 | ≤ 15 | ≤ 15 |
| $cpK_a$ | | 3.2 | 8.1 | 8.1 | 8.1 |
| mwt | | 395 | 430 | 478 | 450 |
| cLogD (pH 7.4) | | 3.5 | 2.5 | 3.0 | 3.6 |
| Charge (pH 7.4) | | 0 | 1 | 1 | 1 |

[1]The *P. aeruginosa* APAE006 strain contains targeted knockouts of efflux pumps MexAB-OprM, MexCD-OprJ, and MexEF-OprN, and the efflux pump components MexXY are expected to be compromised by the absence of OprM.

A886 has MIC90s (n=20) of 1 and 4 mg/mL against clinical isolates of *E. coli* and *KPN*

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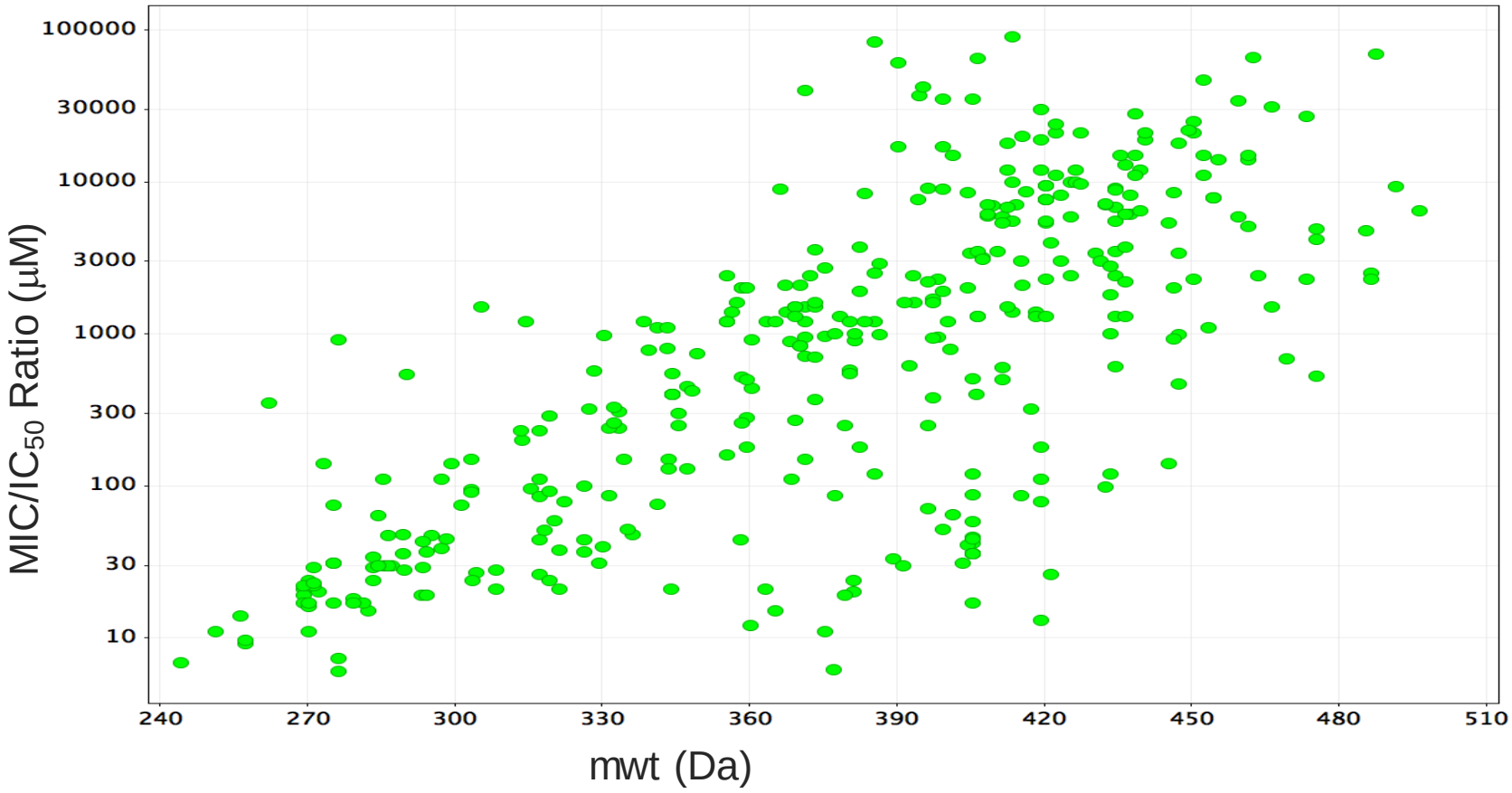
# Efficiency Data for LpxC Inhibitors




-MIC = geomean of MIC against 5 clinical isolates of *P. aeruginosa*;

-$IC_{50}$ = Inhibitory concentration against LpxC from *P. aeruginosa*.

-For this series of inhibitors against *P. aeruginosa*, molecular 'size' is the best predictor of overall permeability

-Instead of binning compounds by 'compartment of action' analyze how well compounds reach site

# Achaogen Approach to Optimizing Permeability

- Use matched pairs of strains and conditions to assess the contribution of each barrier
  - *E. coli* wt; *E. coli* Δ*tolC* or Δ*acrAB*; ± PMBN
  - *P. aeruginosa* wt or Δ*mex* ± PMBN
  - Requires large MIC panels, typically 15–20 strains/conditions for a primary panel
- Generate on-target potency for assessment of overall permeability
  - Requires robust biochemistry
- Don't be afraid to make inactive molecules to test specific hypotheses
- Drivers of permeability are likely to specific to each strain/chemical scaffold combination

- Biotin carboxylase program deprioritized due to large mutation liability in *P. aeruginosa*. This will likely be the case for any Single-Target:

**1 gene →1 protein ⊢ inhibitor**

  - This could be the subject of another workshop.

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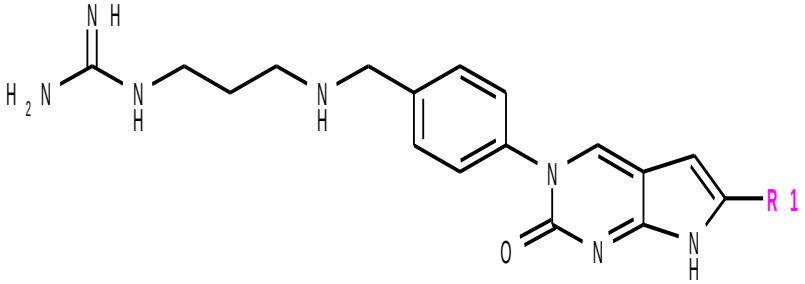
Erin Duffy

Melinta THERAPEUTICS
The Antibiotics Company

# De novo Design of the Pyrrolocytosines: Exploring the Role of Efflux in Driving Broad-Spectrum Activity

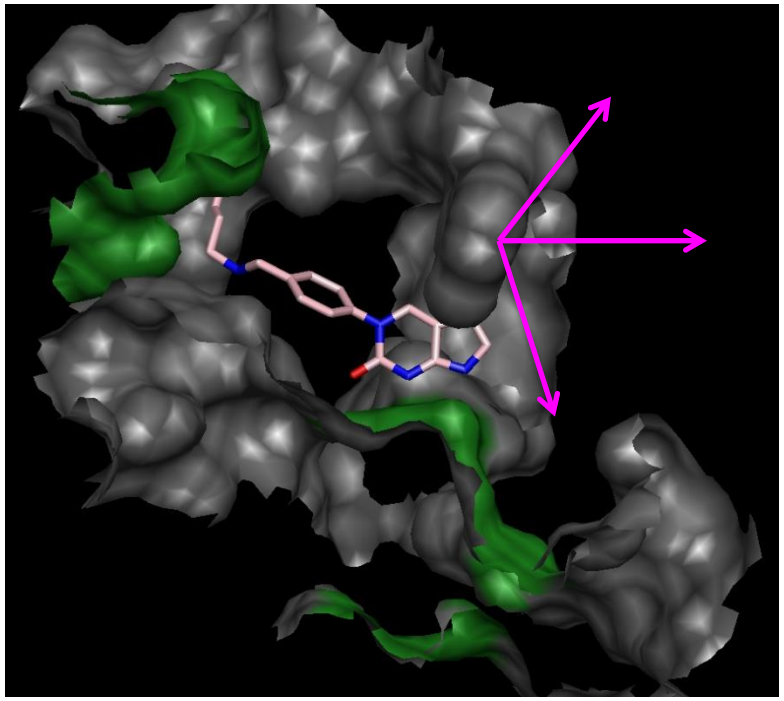


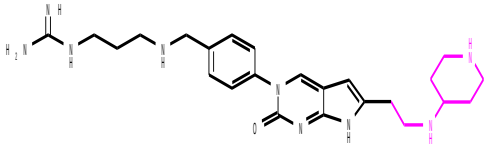

Panels used in this study:

1705xxx are clinical isolates, collected in the US between 2005-2007, by Eurofins

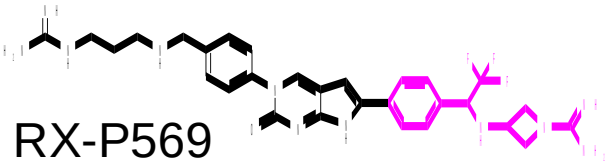
*P. aeruginosa* panel courtesy of Professor Herbert Schweizer

# Essential to our strategy is the ability to explore chemistry in target "open space" to drive Gram-negative activity

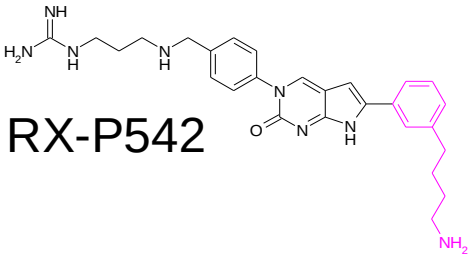




RX-P106



RX-P569




RX-P542

# Each exploration delivered some promise for Gram-negative coverage, but efflux limits broad-spectrum potential

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| Bacterial Strain | Phenotype | RX-P2 | P106 | P569 | **RX-P542** |
|---|---|---|---|---|---|
| *E. faecium* A6349 | VanA, Lin-R (G2576U) | 128 | 16 | 4 | **4** |
| *S. aureus* 11540 | MRSA (USA300) | 16 | 2 | <0.25 | **0.5** |
| *E. coli* 1705863 | | >128 | 2 | 2 | **2** |
| *E. coli* 1705878 | ESBL, MDR | >128 | 2 | 2 | **1** |
| *K. pneumoniae* 1705966 | | >128 | 4 | <0.25 | **<0.25** |
| *K. pneumoniae* 1705949 | KPC, MDR | >128 | 4 | 4 | **8** |
| *P. aeruginosa* 1705886 | | >128 | >128 | 64 | **32** |
| *P. aeruginosa* 1705904 | MDR | >128 | >128 | >128 | **64** |
| *A. baumannii* 1705943 | | >128 | 2 | 0.5 | **0.5** |
| *A. baumannii* 1705936 | MDR | >128 | >128 | 128 | **64** |
| "Efflux" | | | | | |
| *P. aeruginosa* PAO1 | parent | >128 | >128 | 64 | **64** |
| *P. aeruginosa* PAO750 | Δ(mexAB-oprM) Δ(mexCD-oprJ) Δ(mexEF-oprN) Δ(mexJKL) Δ(mexXY) OpmH+ Δ-opmH362 Δ-psc | >128 | 4 | <0.25 | **<0.25** |

# A computational, clustering approach to finding chemistries that influence efflux

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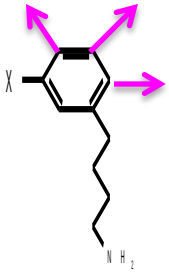


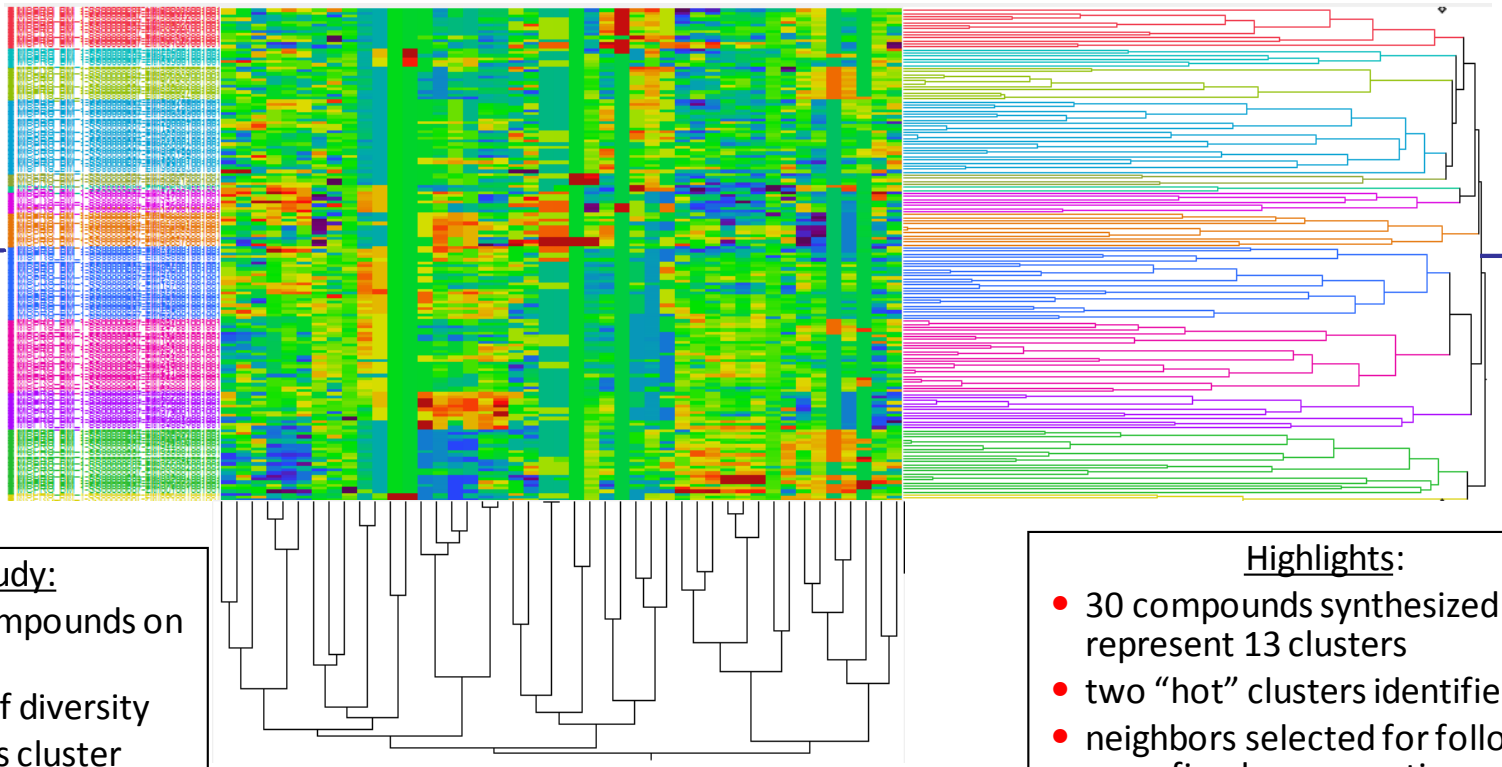

**Case Study:**

- 162 virtual compounds on RX-P542
- three points of diversity
- color indicates cluster membership

**Molecular Landscape:**

- 45 molecular properties
  - binding properties
  - physico-chemical properties
- grouped into 7 clusters
- "heat map" = range for every property (red-green-blue)

**Highlights:**

- 30 compounds synthesized to represent 13 clusters
- two "hot" clusters identified
- neighbors selected for follow-up – confirm key properties
- one "very hot" cluster offers broadest spectrum
- additional new virtual compounds doped in for increased tuning
- 103 made in total; 30 confirmed broadest-spectrum

# Exemplars from two clusters suggest efflux can be minimized

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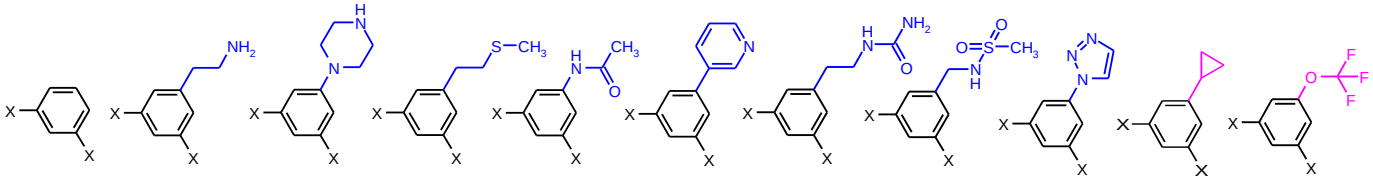

| Bacterial Strain | Phenotype | P542 | P658 | P606 | P708 | P741 | P762 | P696 | P689 | P756 | **P715** | **P605** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *E. faecium* A6349 | VanA, Lin-R (G2576U) | 4 | 4 | 4 | 1 | 2 | 4 | 2 | 4 | 4 | **8** | **4** |
| *S. aureus* 11540 | MRSA (USA300) | 0.5 | 1 | 2 | 1 | 2 | 0.5 | 2 | 2 | 0.5 | **0.5** | **1** |
| *E. coli* 1705878 | ESBL, MDR | 1 | 8 | 16 | 16 | 64 | 32 | 32 | 128 | 32 | **8** | **1** |
| *K. pneumoniae* 1705949 | KPC, MDR | <0.25 | 4 | 64 | 64 | 128 | >128 | 64 | 128 | 128 | **1** | **1** |
| *P. aeruginosa* 1705904 | MDR | 64 | >128 | >128 | 64 | >128 | >128 | >128 | >128 | >128 | **32** | **16** |
| *A. baumannii* 1705936 | MDR | 64 | >128 | >128 | 64 | >128 | 64 | 64 | >128 | >128 | **32** | **16** |
| "Efflux" | | | | | | | | | | | | |
| *P. aeruginosa* PAO1 | parent | 64 | >128 | >128 | 64 | >128 | 128 | 64 | >128 | >128 | **16** | **8** |
| *P. aeruginosa* PAO750 | Δ(mexA-oprM) Δ(mexCD-oprJ) Δ(mexEF-oprN) Δ(mexJKL) Δ(mexXY) OpmH+ Δ-opmH362 Δ-psc | <0.25 | 1 | 4 | <0.25 | 2 | 0.5 | 2 | 1 | 1 | **1** | **0.25** |

# Making neighbors in those clusters delivers compounds with broad-spectrum activity

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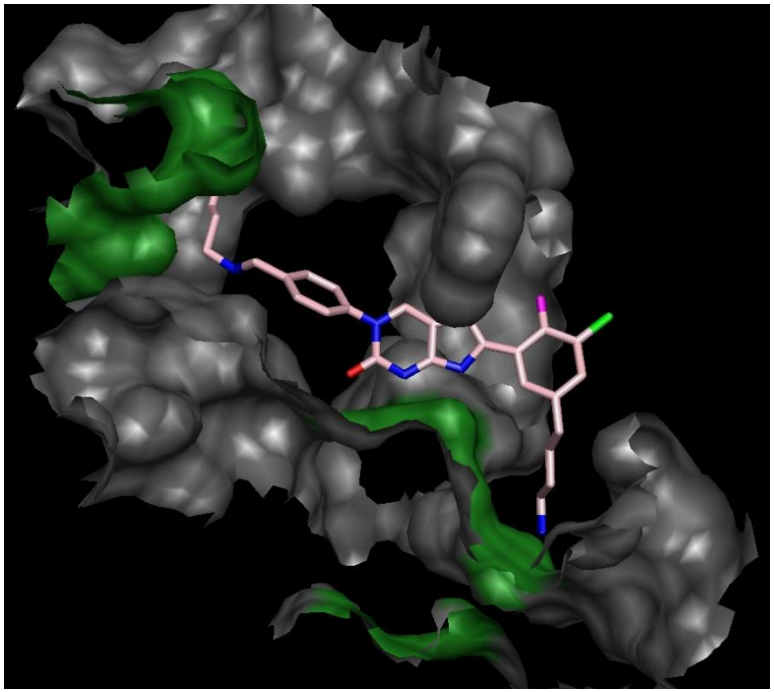
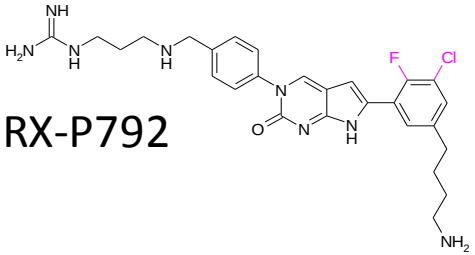
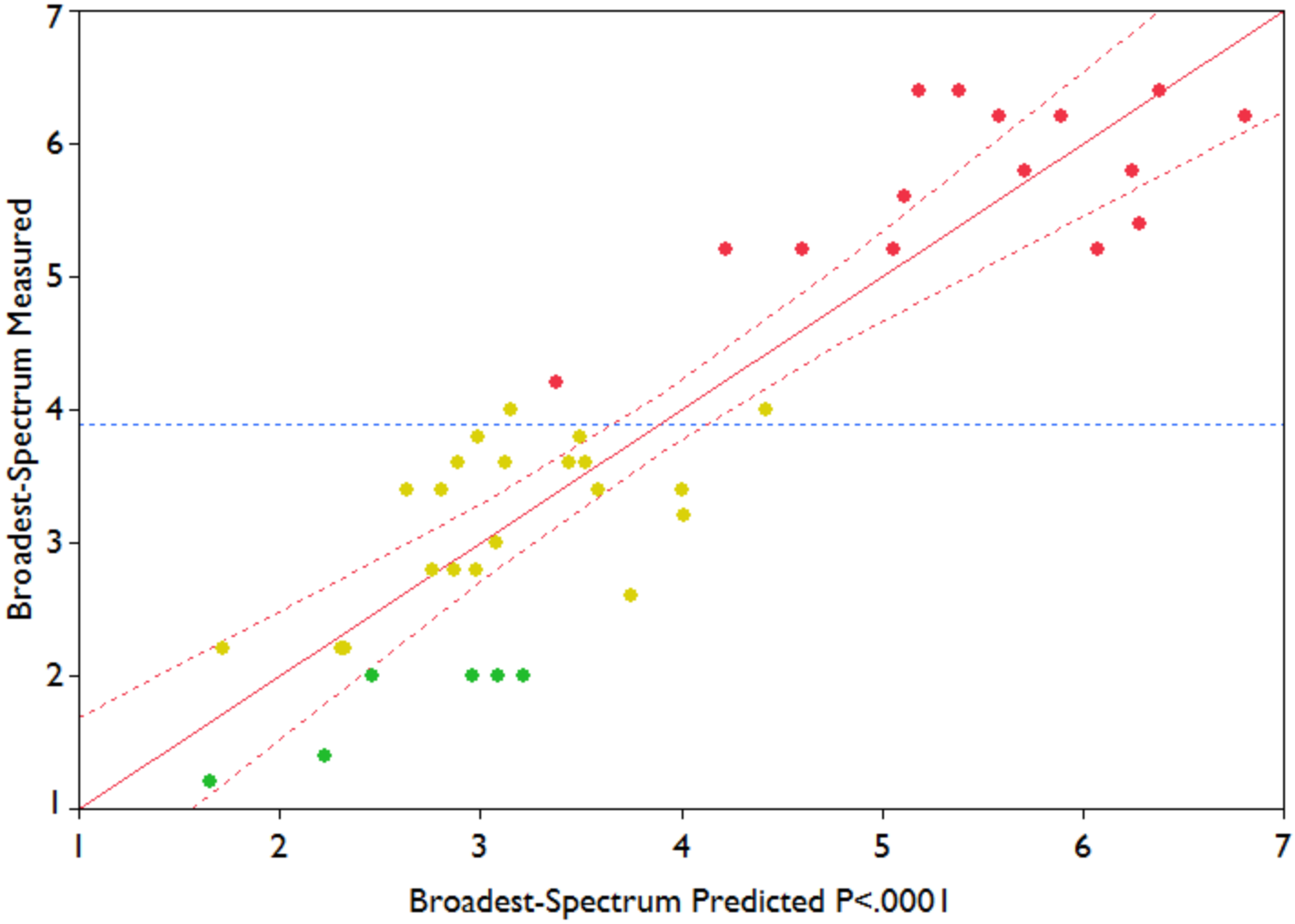





RX-P792

| Bacterial Strain | Phenotype | RX-P542 | RX-P792 |
|---|---|---|---|
| *E. faecium* A6349 | VanA, Lin-R (G2576U) | 4 | 0.5 |
| *S. aureus* 11540 | MRSA (USA300) | 0.5 | ≤0.25 |
| *E. coli* 1705863 | | 2 | ≤0.25 |
| *E. coli* 1705878 | ESBL, MDR | 1 | 0.5 |
| *K. pneumoniae* 1705966 | | 8 | ≤0.25 |
| *K. pneumoniae* 1705949 | KPC, MDR | <0.25 | ≤0.25 |
| *P. aeruginosa* 1705886 | | 32 | 2 |
| *P. aeruginosa* 1705904 | MDR | 64 | 4 |
| *A. baumannii* 1705943 | | 0.5 | ≤0.25 |
| *A. baumannii* 1705936 | MDR | 64 | 2 |
| "Efflux" | | | |
| *P. aeruginosa* PAO1 | parent | 64 | 2 |
| *P. aeruginosa* PAO750 | Δ(mexA-oprM) Δ(mexCD-oprJ) Δ(mexEF-oprN) Δ(mexJKL) Δ(mexXY) OpmH+ Δ-opmH362 Δ-psc | <0.25 | 0.25 |

# A reasonable correlation can be drawn with three molecular properties




Dipole moment, acceptor hydrogen bonds and total aromatic solvent-accessible surface area

# Reducing the gap between parent and efflux-deficient strains correlates with activity against MDR strains

| | MIC (µg/mL) against *P. aeruginosa* 1705904 (MDR) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| RX- | P766 | P873 | P870 | P869 | P770 | P716 | P875 | P792 | P759 | P777 | P776 |
| | 4 | 4 | 8 | 16 | 8 | 16 | 16 | 8 | 16 | 128 | 128 |

| ΔMIC | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 128 | | | | | | | | | | 1 | 1 |
| 64 | | | | | | | | | 1 | 4 | 4 |
| 32 | | | | | | 1 | 1 | 1 | 4 | | |
| 16 | | | 1 | 1 | 1 | 4 | 4 | 5 | 4 | 3 | 4 |
| 8 | 4 | 5 | 5 | 5 | 8 | 4 | 4 | 3 | | 1 | |
| 4 | 5 | 4 | 3 | 3 | | | | | | | |
| 2 | | | | | | | | | | | |
| 1 | | | | | | | | | | | |
| 0 | | | | | | | | | | | |

| Strain | Description |
|---|---|
| PAO200 | Δ(mexAB-oprM) |
| PAO238 | Δ(mexAB-oprM) Δ(mexCD-oprJ) |
| PAO255 | Δ(mexAB-oprM) Δ(mexEF-oprN) |
| PAO280 | Δ(mexAB-oprM) Δ(mexXY) |
| PAO314 | Δ(mexAB-oprM) Δ(mexCD-oprJ) Δ(mexJKL) |
| PAO325 | Δ(mexAB-oprM) Δ(mexCD-oprJ) Δ(mexJKL) Δ(mexXY) |
| PAO397 | Δ(mexAB-oprM) Δ(mexCD-oprJ) Δ(mexEF-oprN) Δ(mexJKL) Δ(mexXY) ΔopmH |
| PAO509 | Δ(mexAB-oprM) Δ(mexCD-oprJ) Δ(mexEF-oprN) Δ(mexJK) Δ(mexXY) |
| PAO1095 | Δ(mexAB-oprM) Δ(mexCD-oprJ) Δ(mexEF-oprN) Δ(mexJK) Δ(mexXY) Δ(triABC) |

ΔMIC is from parent (PAO1); panel is from H. Schweizer

# This leads to a characteristic, “flat” pattern of activity across resistant Pseudomonads

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| Strain (MICs in µg/mL) | Ciprofloxacin | Tobramycin | Tigecycline | Pip/Tazo | Cefepime | Ertapenem | Colistin | **RX-P792** |
|---|---|---|---|---|---|---|---|---|
| P. aeruginosa 1705886 | 0.125 | 0.5 | 8 | 8 | 2 | 16 | 2 | **2** |
| P. aeruginosa 1705888 | 0.125 | 0.5 | 8 | 128 | 32 | 128 | 1 | **2** |
| P. aeruginosa 1705911 | 0.125 | 0.5 | 8 | 128 | 16 | 32 | 1 | **2** |
| P. aeruginosa 1705890 | 0.25 | 0.5 | 8 | 128 | 64 | 16 | 2 | **2** |
| P. aeruginosa 1705896 | 0.25 | 0.5 | 8 | 4 | 4 | 16 | 8 | **4** |
| P. aeruginosa 1705906 | 16 | 0.5 | 8 | 128 | 32 | 128 | 1 | **2** |
| P. aeruginosa 1705898 | 32 | 0.5 | 32 | 128 | 32 | 128 | 2 | **4** |
| P. aeruginosa 1705899 | 64 | 0.5 | 16 | 128 | 16 | 8 | 1 | **4** |
| P. aeruginosa 1705907 | 0.125 | 1 | 16 | 16 | 2 | 4 | 1 | **4** |
| P. aeruginosa 1705909 | 0.125 | 1 | 16 | 1 | 8 | 0.25 | 1 | **2** |
| P. aeruginosa 1705892 | 0.25 | 1 | 16 | 128 | 16 | 128 | 1 | **4** |
| P. aeruginosa 1705893 | 0.25 | 1 | 16 | 64 | 16 | 128 | 2 | **4** |
| P. aeruginosa 1705908 | 0.25 | 1 | 16 | 8 | 8 | 2 | 1 | **4** |
| P. aeruginosa 1705913 | 0.25 | 1 | 16 | 8 | 4 | 16 | 1 | **4** |
| P. aeruginosa 1705891 | 0.5 | 1 | 8 | 128 | 32 | 32 | 1 | **4** |
| P. aeruginosa 1705915 | 0.5 | 1 | 32 | 1 | 16 | 0.25 | 0.5 | **4** |
| P. aeruginosa 1705889 | 2 | 1 | 8 | 128 | 16 | 64 | 1 | **4** |
| P. aeruginosa 1705902 | 32 | 1 | 16 | 128 | 32 | 32 | 2 | **4** |
| P. aeruginosa 1705895 | 32 | 2 | 32 | 32 | 16 | 128 | 1 | **4** |
| P. aeruginosa 1705903 | 32 | 2 | 16 | 128 | 64 | 32 | 1 | **4** |
| P. aeruginosa 1705897 | 64 | 2 | 32 | 128 | 64 | 128 | 1 | **4** |
| P. aeruginosa 1705887 | 1 | 4 | 32 | 128 | 32 | 128 | 0.5 | **4** |
| P. aeruginosa 1705912 | 32 | 8 | 2 | 128 | 32 | 64 | 1 | **1** |
| P. aeruginosa 1705900 | 64 | 16 | 4 | 128 | 32 | 128 | 1 | **1** |
| P. aeruginosa 1705901 | 16 | 128 | 16 | 128 | 128 | 128 | 2 | **4** |
| P. aeruginosa 1705905 | 32 | 128 | 32 | 128 | 64 | 128 | 8 | **4** |
| P. aeruginosa 1705910 | 32 | 128 | 16 | 128 | 128 | 128 | 1 | **4** |
| P. aeruginosa 1705904 | 128 | 128 | 32 | 128 | 128 | 128 | 8 | **4** |

# Session 3: Enabling technologies to measure compound permeability and accumulation

# The Holy Grail of Compound Uptake Assays would be:

- Robust (sensitive, reproducible)
- Involve direct detection of compounds (w/o need for pre-labelling)
- Kinetic
- Quantitative
- Whole cell-based, including relevant strains
- Capable of informing sub-cellular localization
- High throughput
- Cost-effective

# Traditional methods & their limitations

- **Direct detection**
  - Radiometry, Fluorometry, Spectroscopy
    - Usually low throughput*
    - challenges associated with non-specific binding or other assay-dependent influence on results
    - specific compound localization undefined
- **Indirect detection**
  - Electrophysiology, Liposome swelling
    - low throughput, technically challenging
  - Differential MICs of engineered strains
    - Relies on inherent antibacterial activity which may differ due to differences in target potency, metabolism, other parameters of compounds under study

*MS possible exception

Weill Cornell Medicine

# Bacterial membrane permeation: By the masses for the masses

Kyu Rhee MD PhD

Department of Medicine and Microbiology & Immunology

10.20.16

Capsule

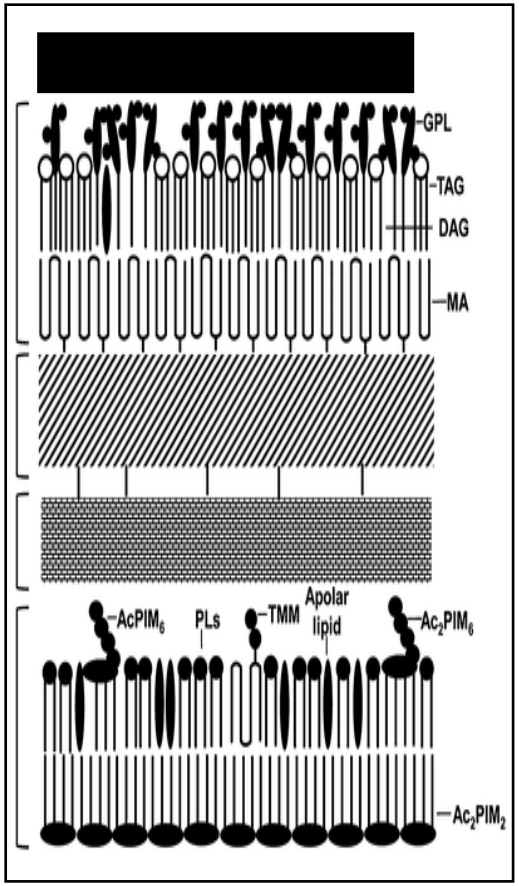
Mycomembrane or Outer membrane OM

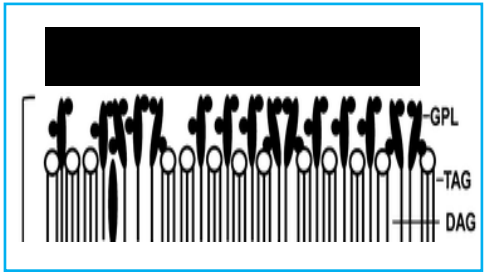
Periplasmic space

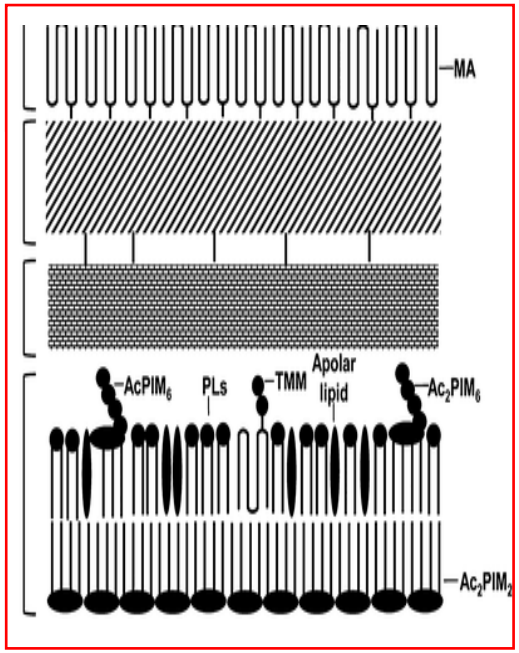
Arabinogalactan AG

Peptidoglycan PG

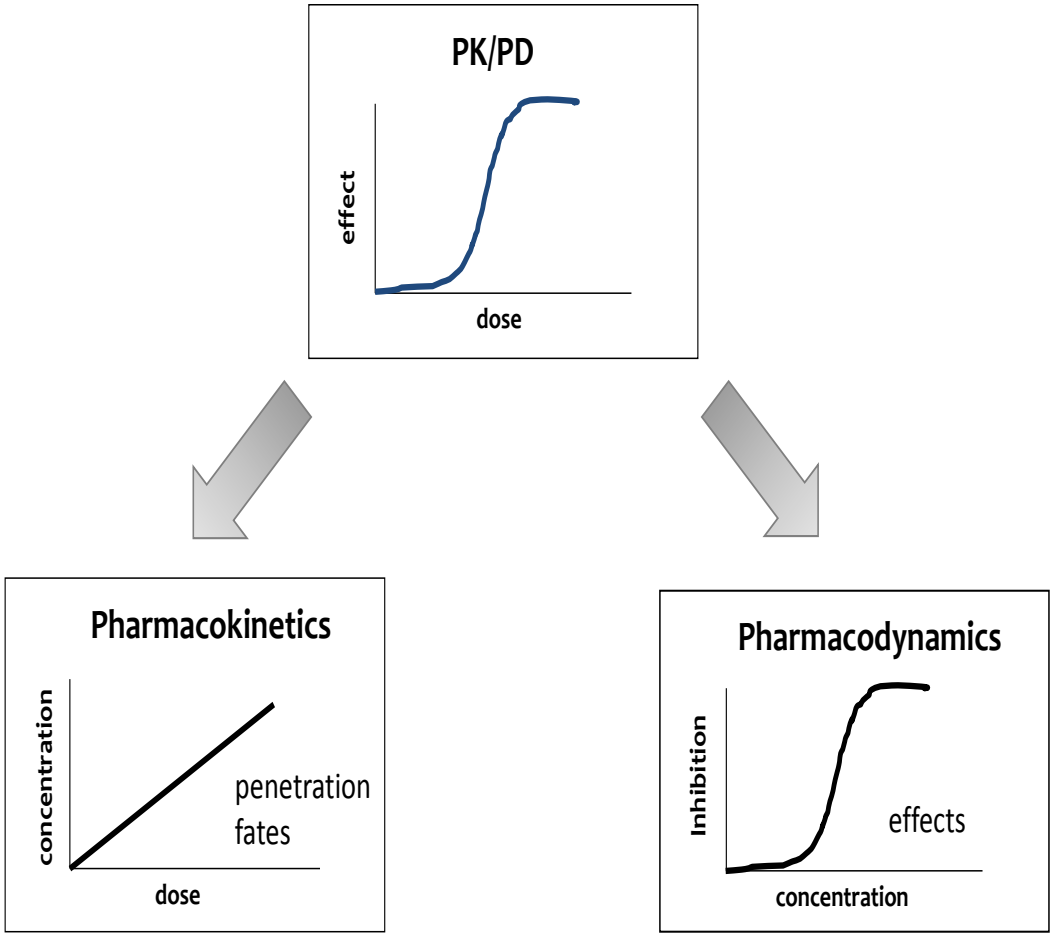
Plasma membrane or Inner membrane IM



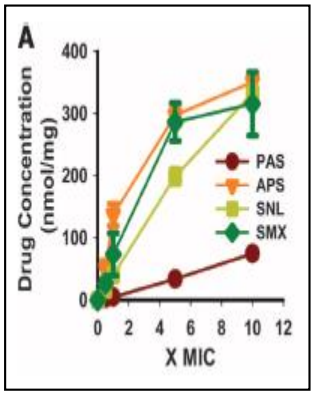



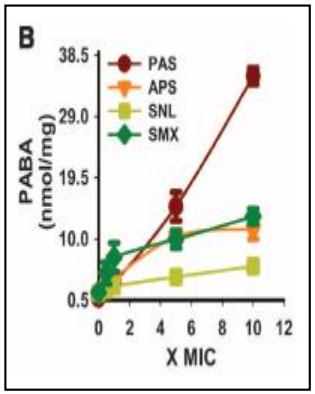



Drug Activity = PK + PD

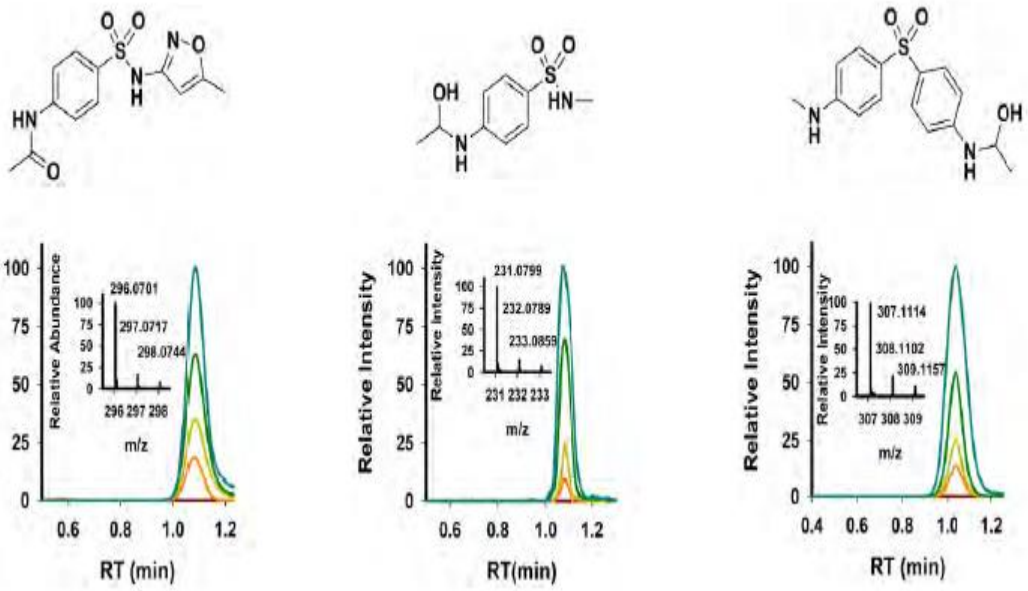

PK/PD
effect
dose
Pharmacokinetics
concentration
penetration
fates
dose
Pharmacodynamics
Inhibition
effects
concentration

$$\text{SAR} = \delta(\text{PK} + \text{PD})$$




***Penetration Inhibition***

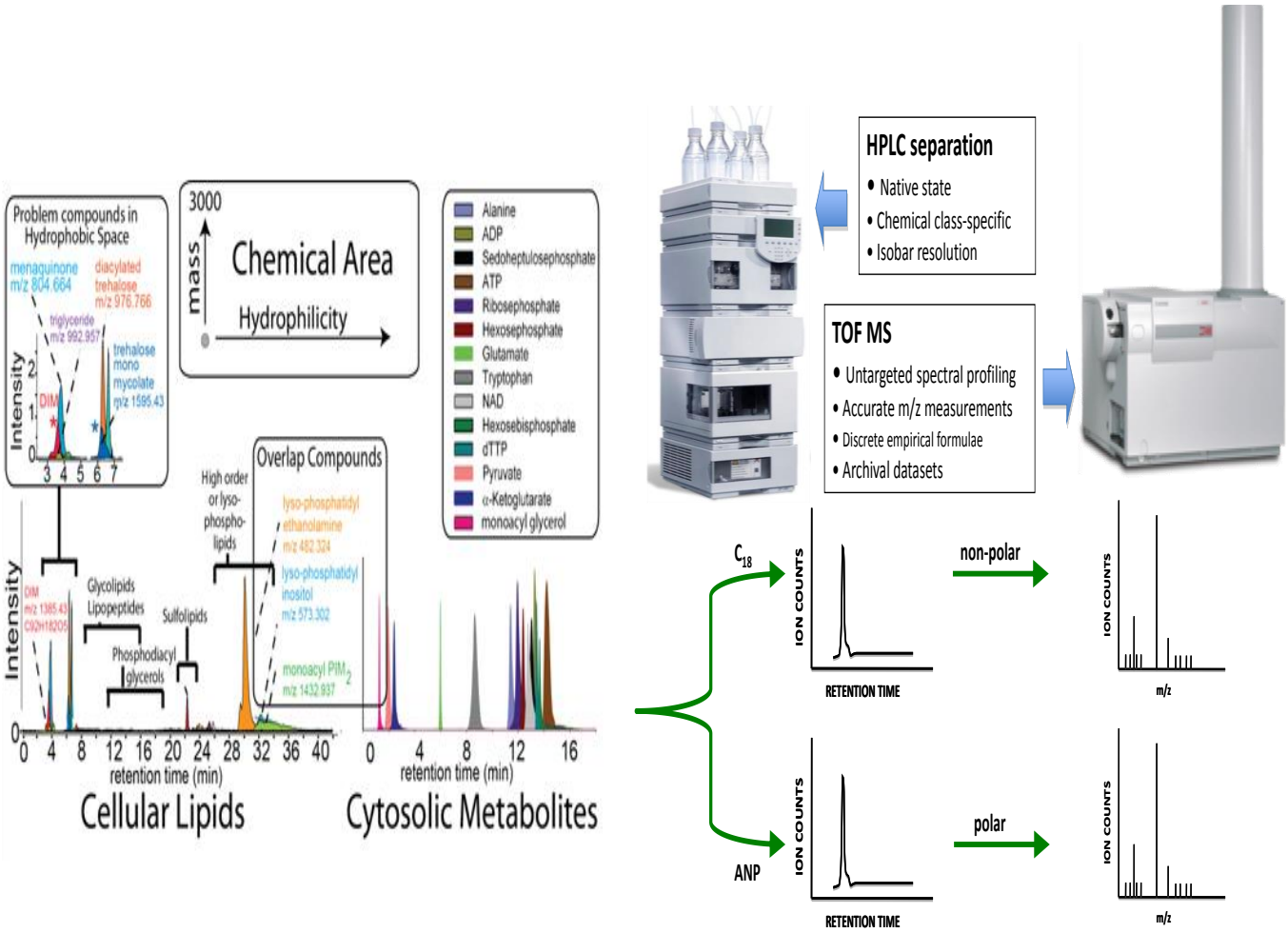


PK = *target exposure* + target binding

Target exp$\sum$ [(penetration –efflux) +/-$^{(*)}$

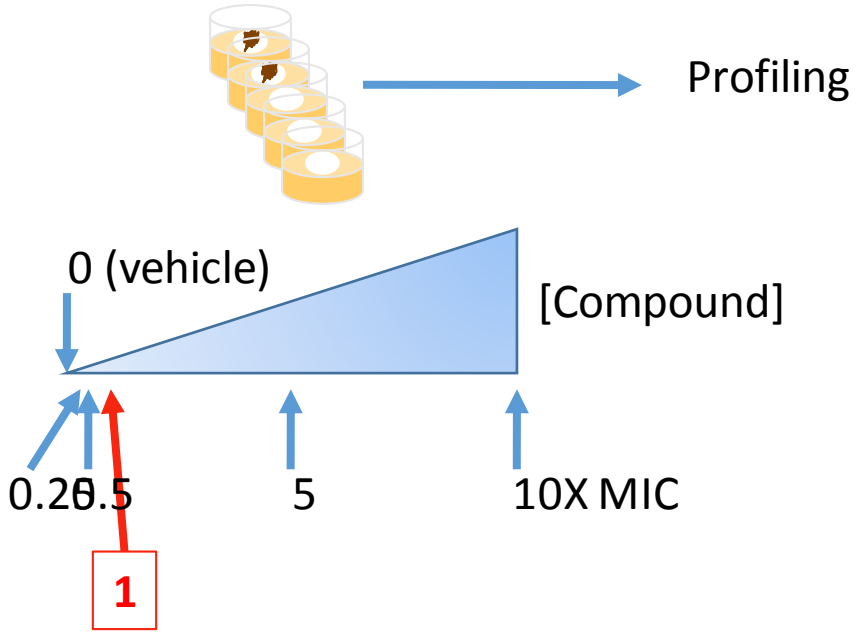
*(activation/retention/degradat

# Technologic platform: Sensitive, unbiased, multiplex profiling

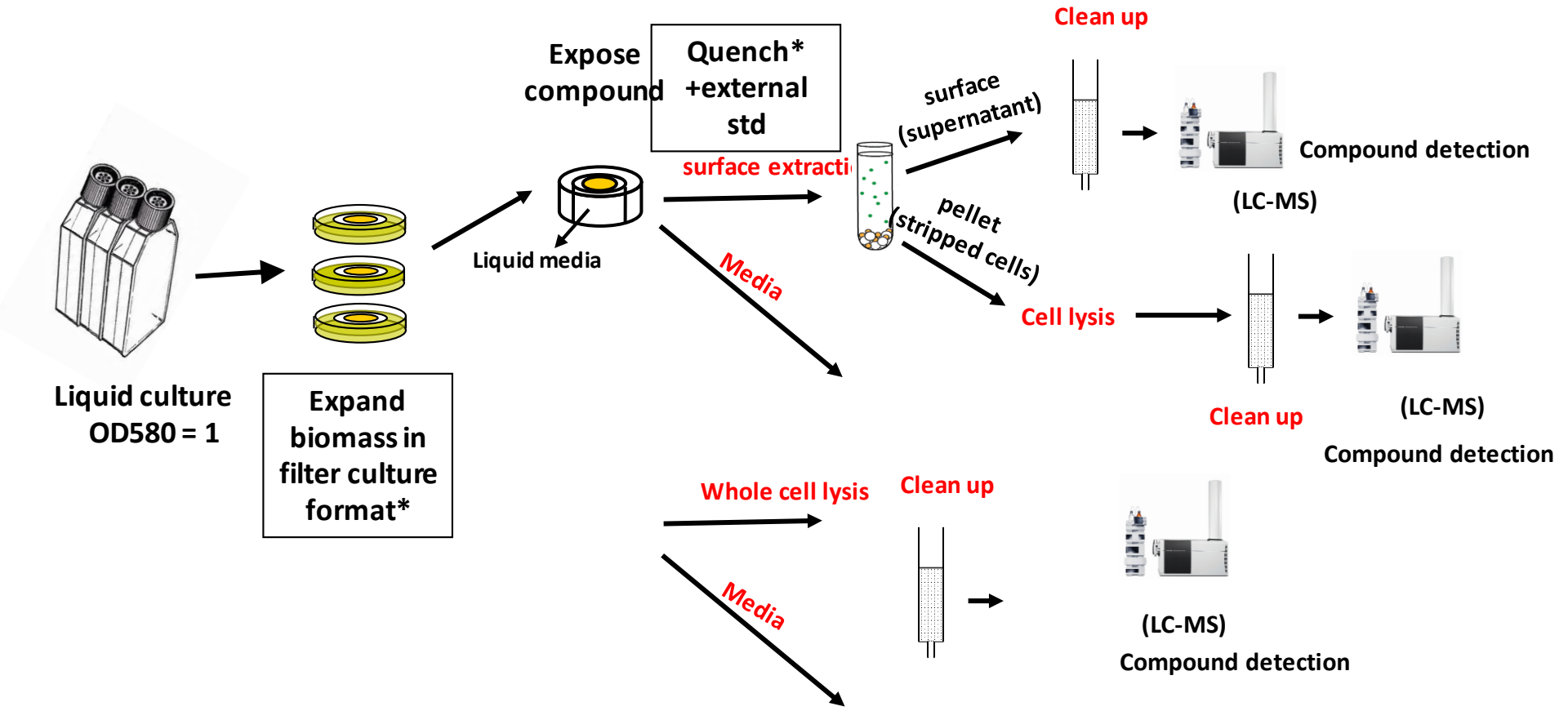


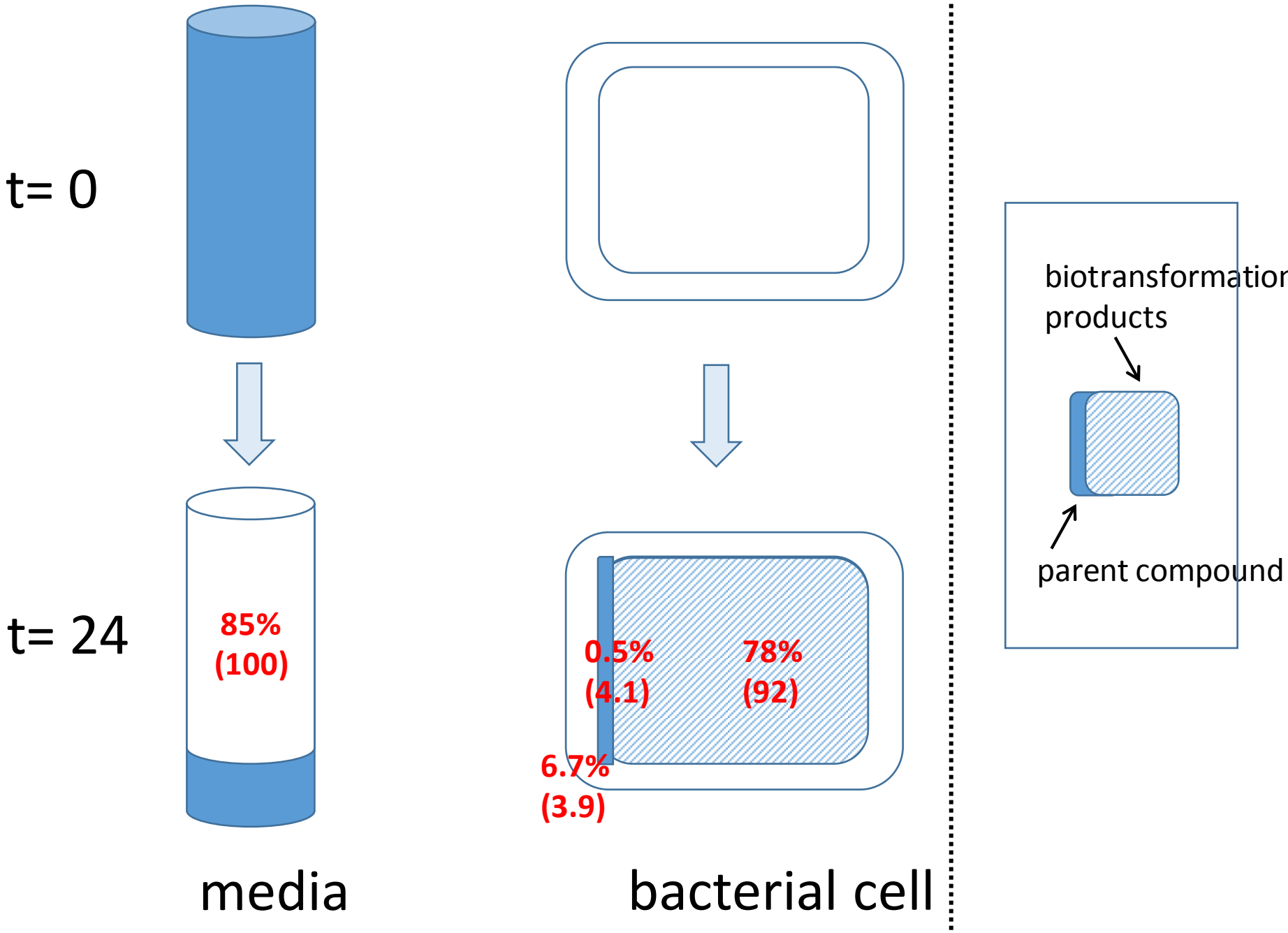
Expose
Quench
Recover
Analyze

# MIC assay and biocalibration curves




Dose exposure = concentration x time


Liquid culture
OD580 = 1
Expand biomass in filter culture format*
Liquid media
Expose compound
Quench* +external std
surface extracti
Media
surface (supernatant)
pellet (stripped cells)
Clean up
Compound detection
(LC-MS)
Cell lysis
Clean up
(LC-MS)
Compound detection
Whole cell lysis
Media
Clean up
(LC-MS)
Compound detection


t= 0
t= 24
85%
(100)
0.5%
(4.1)
78%
(92)
6.7%
(3.9)
media
bacterial cell
biotransformation
products
parent compound

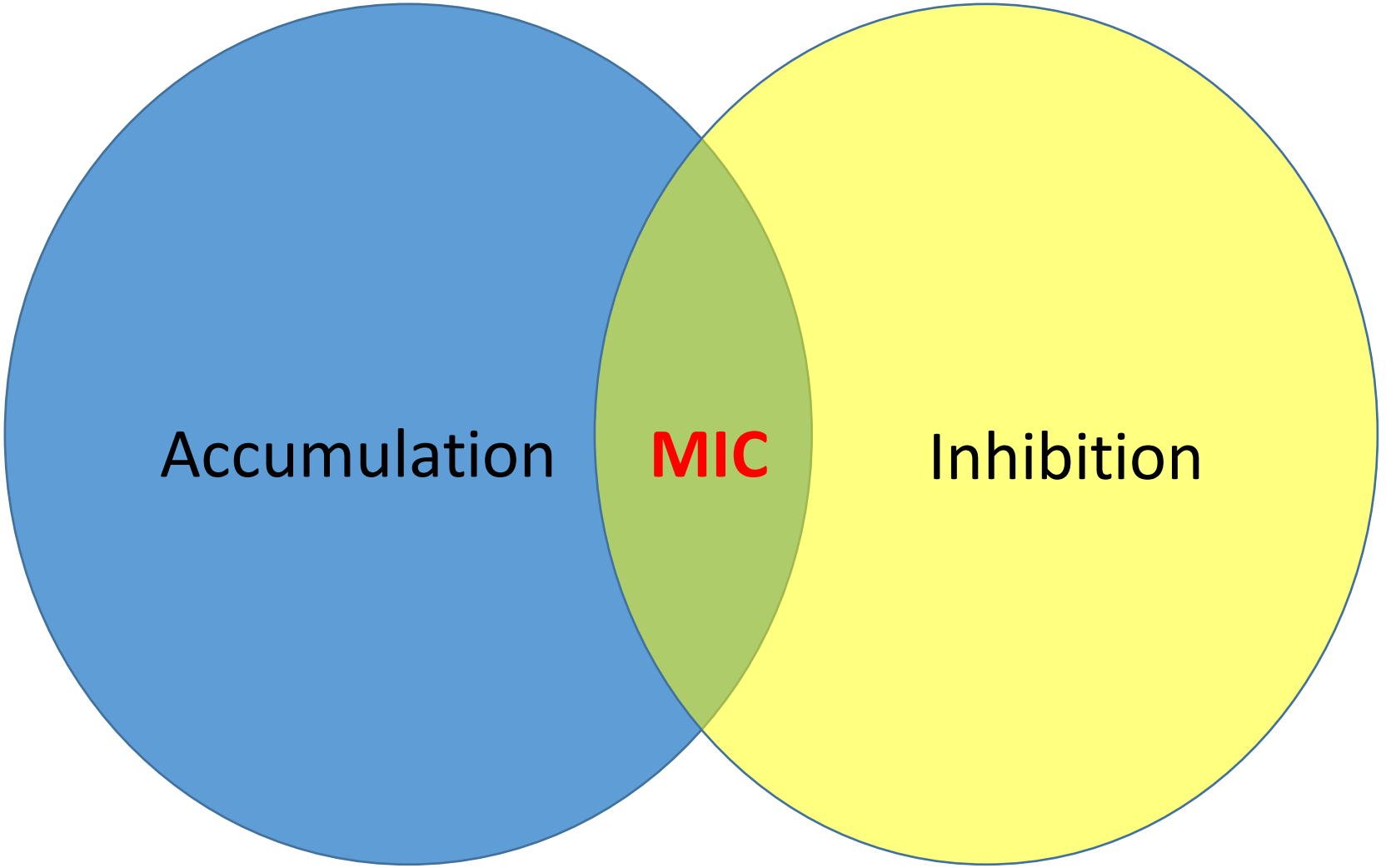
# MS-based mass balance analysis

**Advantages**

- Sensitive
- High throughput
- Native analysis
  - Compound
  - Biological barrier
- Molecular resolution
- Linked MOA profile

**Disadvantages**

- Ionizability
- Endpoint measurements
- Relative quantitation
  - Otherwise requires time-intensive standardization


Accumulation
MIC
Inhibition

# Toward a General Platform for Structure- and Activity-Independent Quantitation of Small-Molecule Permeability in Bacteria

**Derek S. Tan**

Chemical Biology Program
Memorial Sloan Kettering Cancer Center
and Tri-Institutional Research Program
New York, New York

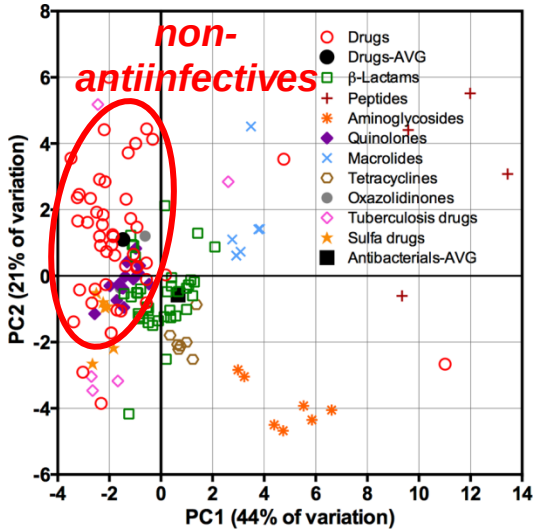
# Bacterial Permeability of Small Molecules

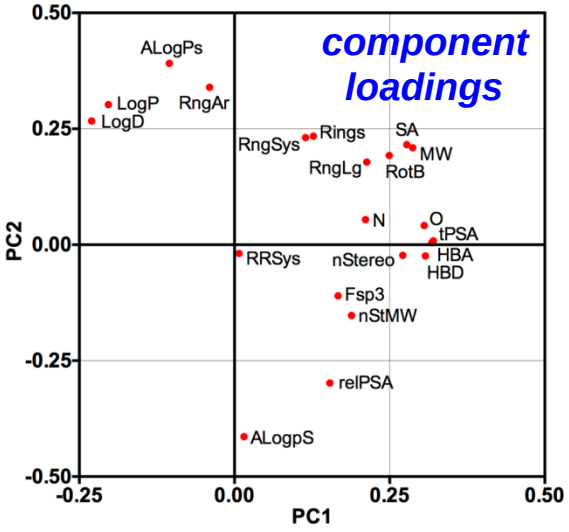
*Permeability is poorly understood and a major obstacle to rational antibiotic discovery*

- **Principal component analysis of 21 structural and physicochemical properties**

| | Parameter | Description | Source |
|---|---|---|---|
| Size & Hydrogen Bonding | MW | molecular weight | Instant JChem |
| | SA | surface area | Instant JChem |
| | N | number of nitrogens | Instant JChem |
| | O | number of oxygens | Instant JChem |
| | HBD | number of hydrogen bond donors | Instant JChem |
| | HBA | number of hydrogen bond acceptors | Instant JChem |
| Hydrophobicity & Polarity | LogD | calc *n*-octanol/water partition coefficient (pH 7.4) | Instant JChem |
| | LogP | calc *n*-octanol/water partition coefficient | Instant JChem |
| | ALogPs | calc *n*-octanol/water partition coefficient (Tetko) | http://www.vcclab.org |
| | ALogpS | calc aqueous solubility (Tetko) | http://www.vcclab.org |
| | tPSA | topological polar surface area | Instant JChem |
| | relPSA | tPSA ÷ SA | Instant JChem |

| | Parameter | Description | Source |
|---|---|---|---|
| 3D Structure | nStereo | number of chiral atoms | Instant JChem |
| | nStMW | nStereo ÷ MW (stereochemical density) | Instant JChem |
| | Fsp3 | number of sp3 carbons ÷ number of carbons | Instant JChem |
| | RotB | # rotatable bonds | Instant JChem |
| Ring Content | Rings | number of rings | Instant JChem |
| | RngAr | number of aromatic rings | Instant JChem |
| | RngLg | number of atoms in largest ring | Instant JChem |
| | RngSys | number of ring systems | Instant JChem |
| | RRSys | Rings ÷ RngSys (ring complexity) | Instant JChem |


| | Parameter | PC1 | PC2 |
|---|---|---|---|
| Size & Hydrogen Bonding | MW | **0.2870** | 0.2093 |
| | SA | 0.2776 | 0.2159 |
| | N | 0.2111 | 0.0543 |
| | O | **0.3055** | 0.0414 |
| | HBD | **0.3075** | -0.0243 |
| | HBA | **0.3178** | 0.0044 |
| Hydrophobicity & Polarity | LogD | -0.2301 | 0.2668 |
| | LogP | -0.2032 | **0.3021** |
| | ALogP | -0.1048 | **0.3910** |
| | ALogS | 0.0153 | **-0.4139** |
| | tPSA | **0.3204** | 0.0084 |
| | relPSA | 0.1535 | **-0.2983** |
| 3D Structure | nStereo | 0.2712 | -0.0228 |
| | nStMW | 0.1886 | -0.1526 |
| | Fsp3 | 0.1668 | -0.1100 |
| | RotB | 0.2493 | 0.1926 |
| Ring Content | Rings | 0.1271 | 0.2343 |
| | RngAr | -0.0402 | **0.3396** |
| | RngLg | 0.2131 | 0.1781 |
| | RngSys | 0.1147 | 0.2310 |
| | RRSys | 0.0074 | -0.0188 |

***Antibiotics have distinct structural and physicochemical properties compared to non-antiinfectives***


- Davis, T. D.; Gerry, C. J.; Tan, D. S. "General platform for systematic quantitative evaluation of small-molecule permeability in bacteria." *ACS Chem. Biol.* **2014**, *9*, 2535–2544
- *Review:* Lewis, K. "Platforms for antibiotic discovery." *Nat. Rev. Drug Discov.* **2013**, *12*, 371–387.

# LC-MS/MS Analysis of Compound Accumulation in Bacteria

*Structure & activity-independent quantitation of permeability of diverse molecules*

**Incubate bacteria with compound (± efflux pump inhibitors)**

→ **Centrifuge** → **Supernatant**

→ **CFU determination**

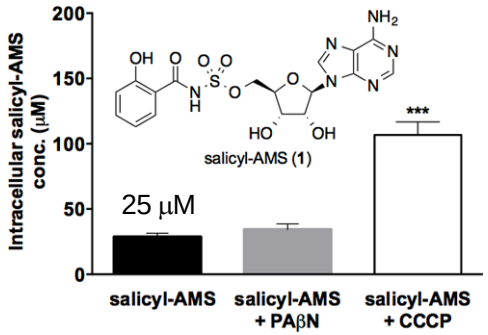
→ **Wash (4x PBS) & centrifuge** → **Washes**

→ **Lyse (sonication) & centrifuge** → **Lysate** → **Intracellular**

**LC-MS/MS analysis vs. internal standard**

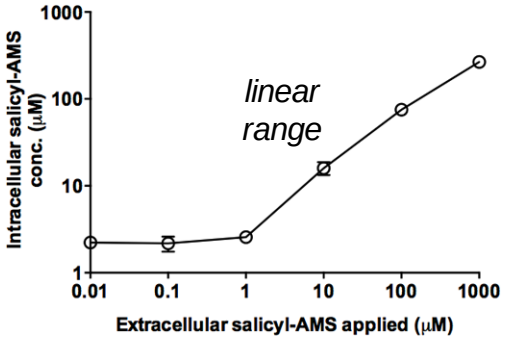
*linear range of detection: 0.0025–100 μM (PBS)*

## • Salicyl-AMS accum/efflux



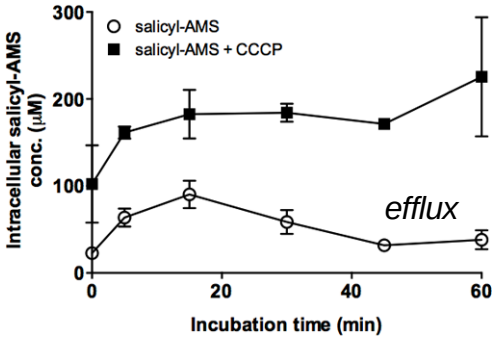

*100 μM extracellular*
*30 min, tryptic soy broth*

## • Concentration effects



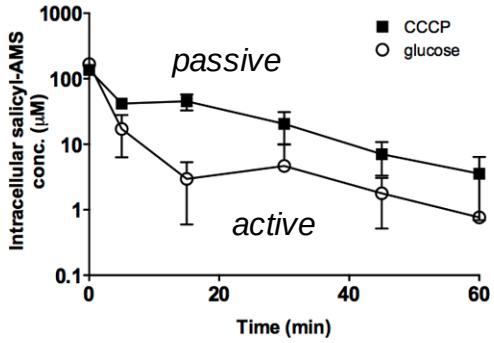

*0.01–1000 μM extracellular*
*20 min, PBS*

## • Accumulation kinetics




*100 μM extracellular*
*0–60 min, PBS*

## • Efflux kinetics

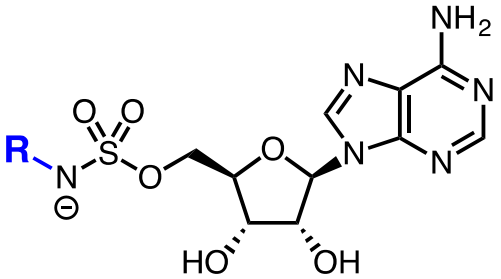



*100 μM extracellular*
*15 min pre-load, PBS + CCCP*


- Davis, T. D.; Gerry, C. J.; Tan, D. S. "General platform for systematic quantitative evaluation of small-molecule permeability in bacteria." *ACS Chem. Biol.* **2014**, *9*, 2535–2544.
- *LC-MS/MS quantitation:* Cai, H.; Rose, K.; Liang, L. H.; Dunham, S.; Stover, C. *Anal. Biochem.* **2009**, *385*, 321−325.

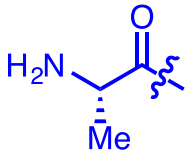
# Permeability of a Panel of Diverse Acyl-AMS Congeners

*LogP alone is insufficient to explain observed permeability trends*

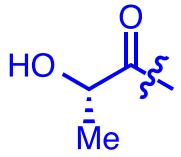



R =

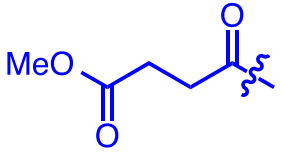

**H-AMS**
*MW 346*
*ALogPs –1.26*




**Ala-AMS**
*MW 417*
*ALogPs – 1.36*

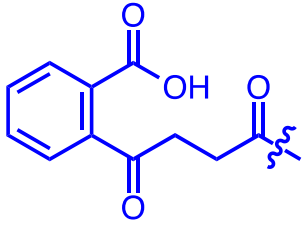



**Lac-AMS**
*MW 418*
*ALogPs –1.23*

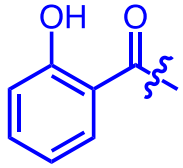



**Me-suc-AMS**
*MW 460*
*ALogPs –0.97*

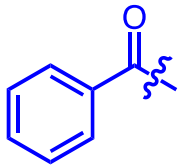

**Anthra-AMS**
*MW 465*
*ALogPs –0.70*



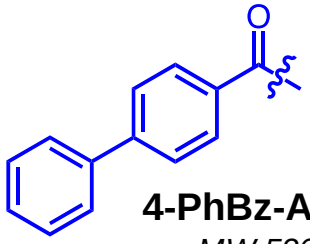

**OSB-AMS**
*MW 550*
*AlogPs –0.49*



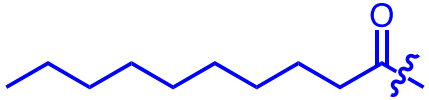

**Sal-AMS**
*MW 466*
*ALogPs –0.44*



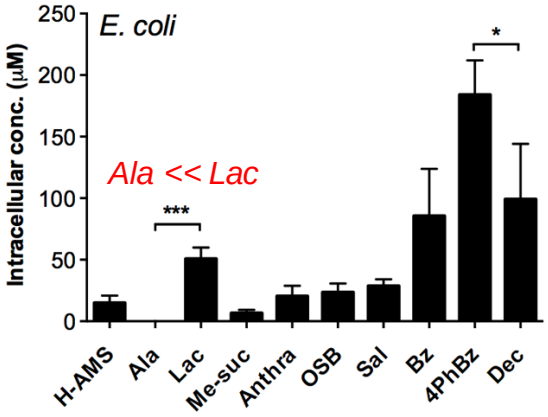

**Bz-AMS**
*MW 450*
*ALogPs –0.35*

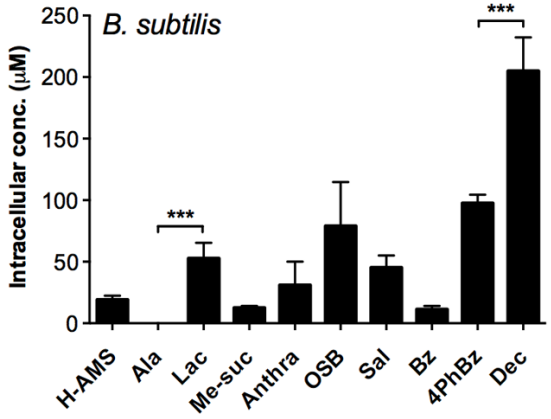


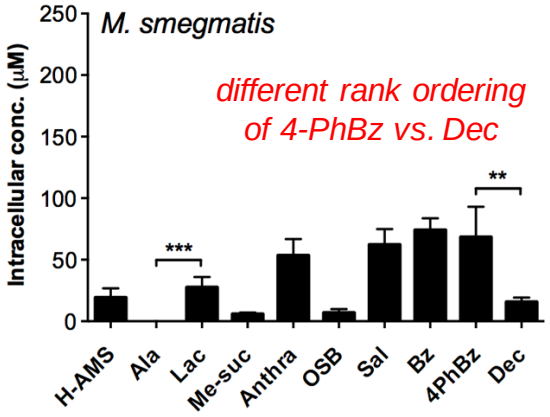

**4-PhBz-AMS**
*MW 526*
*ALogPs +1.25*




**Dec-AMS**
*MW 500*
*ALogPs +1.37*




*100 μM, 30 min*
*rich media*


• Davis, T. D.; Gerry, C. J.; Tan, D. S. "General platform for systematic quantitative evaluation of small-molecule permeability in bacteria." *ACS Chem. Biol.* **2014**, *9*, 2535–2544.

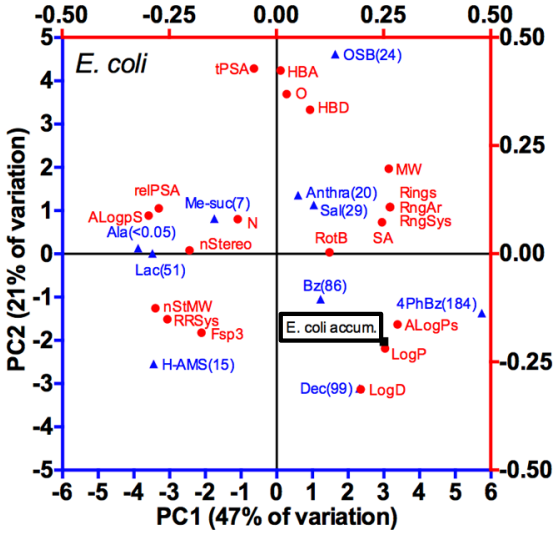
# Cheminformatic Analysis of Permeability of Acyl-AMS Panel

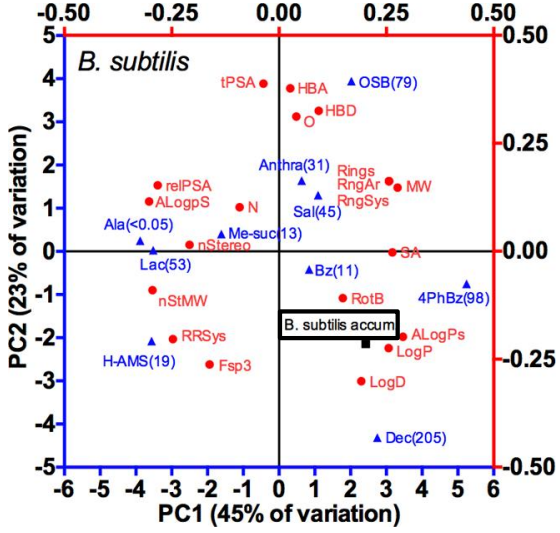
*Complex and non-obvious correlations between structure and permeability*

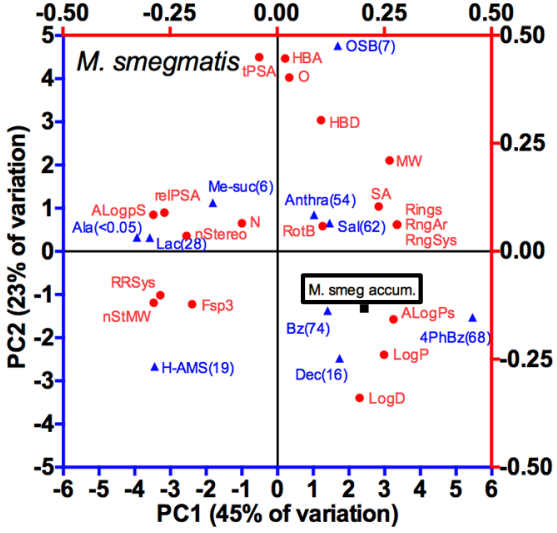
- **Principal Component Analysis:** visual indications of properties that correlate with permeability

All strains: hydrophobicity

*B. subtilis*: rotatable bonds, surface area




- **Pearson pairwise correlation coefficients:** quantitative correlations between properties and permeability

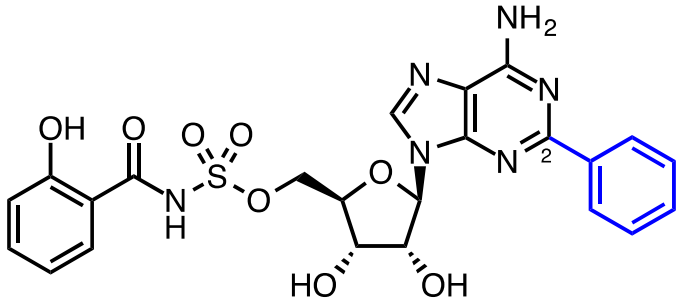
| | Parameter | *E. coli* | *B. subtilis* | *M. smeg* |
|---|---|---|---|---|
| Size & Hydrogen Bonding | MW | 0.46 | 0.55 | 0.16 |
| | SA | 0.52 | **0.78** | 0.01 |
| | N | -0.38 | -0.34 | -0.12 |
| | O | -0.26 | 0.01 | -0.32 |
| | HBD | -0.33 | -0.04 | 0.19 |
| | HBA | -0.36 | -0.05 | -0.30 |
| Hydrophobicity & Polarity | LogD | **0.70** | 0.52 | 0.60 |
| | LogP | **0.71** | **0.67** | 0.48 |
| | ALogPs | **0.84** | **0.83** | 0.35 |
| | ALogpS | **-0.84** | **-0.76** | -0.35 |
| | tPSA | -0.56 | -0.21 | -0.43 |
| | relPSA | **-0.76** | **-0.80** | -0.27 |
| 3-D Structure | nStereo | -0.24 | -0.25 | -0.36 |
| | nStMW | -0.43 | -0.48 | -0.34 |
| | Fsp3 | -0.27 | 0.31 | **-0.74** |
| | RotB | 0.20 | **0.79** | -0.41 |
| Ring Content | Rings | 0.61 | 0.08 | **0.75** |
| | RngAr | 0.61 | 0.08 | **0.75** |
| | RngSys | 0.61 | 0.08 | **0.75** |
| | RRSys | -0.49 | -0.02 | **-0.76** |

**red** = positive correlation
**blue** = negative correlation
**bold** = $p < 0.05$ (*t*-test)

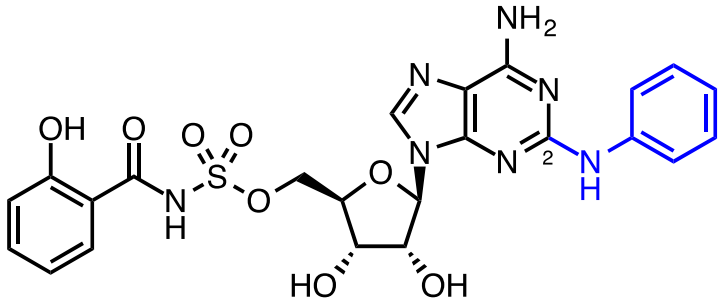

• Davis, T. D.; Gerry, C. J.; Tan, D. S. "General platform for systematic quantitative evaluation of small-molecule permeability in bacteria." *ACS Chem. Biol.* **2014**, *9*, 2535–2544.

# Testing Predictions Based on Cheminformatic Analysis

*Designed analogues accumulate to higher levels in E. coli as predicted*



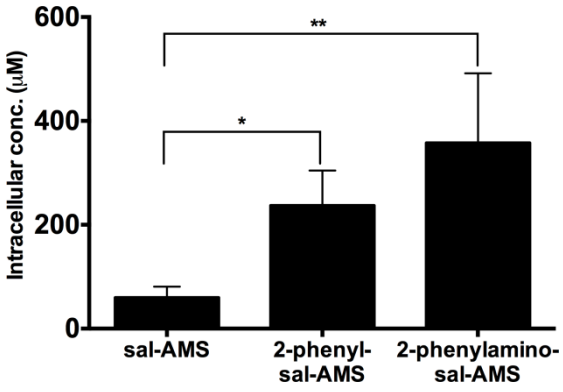
**salicyl-(2-phenyl-AMS)**



**salicyl-(2-phenylamino-AMS)**

| | sal-AMS (1) | salicyl-(2-phenyl-AMS) | salicyl-(2-phenylamino-AMS) |
|---|---|---|---|
| MW | 466 | 543 | 558 |
| SA | 563 | 671 | 688 |
| N | 6 | 6 | 7 |
| O | 8 | 8 | 8 |
| HBD | 5 | 5 | 6 |
| HBA | 12 | 12 | 13 |
| LogD | -1.43 | 0.75 | 0.69 |
| LogP | -2.11 | 1.88 | 1.52 |
| ALogPs | -0.44 | 1.58 | 1.81 |
| ALogpS | -2.21 | -3.06 | -3.16 |
| tPSA | 212 | 212 | 224 |
| relPSA | 38 | 32 | 33 |
| nStereo | 4 | 4 | 4 |
| nStMW ‡ | 8.6 | 7.4 | 7.2 |
| Fsp3 | 0.29 | 0.22 | 0.22 |
| RotB | 5 | 6 | 7 |
| Rings | 4 | 5 | 5 |
| RngAr | 3 | 4 | 4 |
| RngLg | 6 | 6 | 6 |
| RngSys | 3 | 4 | 4 |
| RRSys | 1.33 | 1.25 | 1.25 |

‡ For clarity, values shown are [nStMW x 1000]




μM: 60 237 358

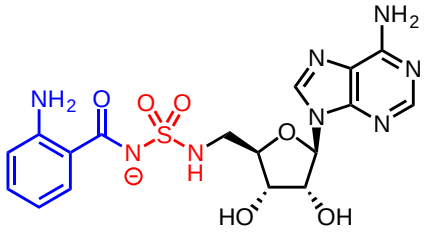
***E. coli*, 100 μM salicyl-AMS**
**37 ° C, 30 min, LB**

***E. coli* accumulation correlates with size, hydrophobicity, aromatic ring content**

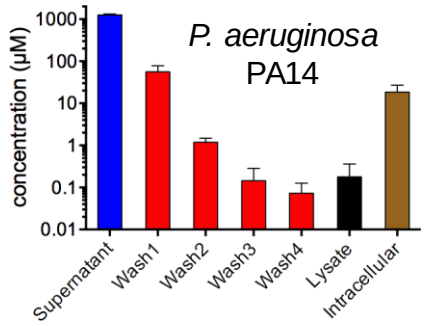

• Davis, T. D.; Gerry, C. J.; Tan, D. S. "General platform for systematic quantitative evaluation of small-molecule permeability in bacteria." *ACS Chem. Biol.* **2014**, *9*, 2535–2544.

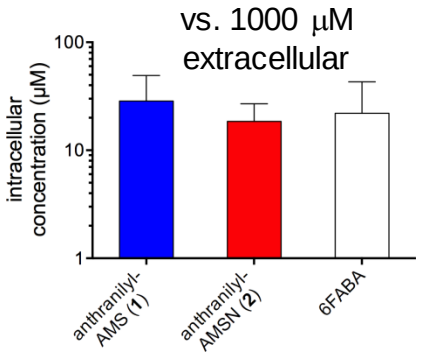
# Future Directions

*Increasing throughput, expanding strains, developing robust cheminformatic models*




**anthranilyl-AMSN**




***n = 8 with removal of outliers (Grubbs' test)***

- **Near-Term Goals**
  - Evaluate 100–1,000 compounds in single chemotype
  - Develop predictive cheminformatic models
  - Assess robustness of models experimentally
- **Increasing Throughput**
  - evaluate other cell recovery protocols
  - streamline incubation protocol
  - multiplex compounds
  - leverage automated instrumentation
- **Expanding Strains**
  - evaluate wt vs. pump knockout vs. permeability mutant strains
  - evaluate approaches to differentiating subcellular compartments
  - expand to other Gram-negative pathogens (*e.g.*, *P. aeruginosa*)
- **Developing Robust Cheminformatic Models**
  - investigate machine learning approaches
  - investigate non-linear modeling approaches
  - identify motifs with idiosynchratic transport mechanisms


• Ji, C.; Sharma, I.; Pratihar, D.; Hudson, L. L.; Maura, D.; Guney, T.; Rahme, L. G.; Pesci, E. C.; Coleman, J. P.; Tan, D. S.* *ACS Chem. Biol.* **2016**, *11*, 3061–3067.

# Acknowledgments

***www.dstan.org***

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Chemical Biology

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A partnership with the Sloan Kettering Institute

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**Prof. James Coleman (East Carolina)**
**Prof. Everett Pesci (East Carolina)**
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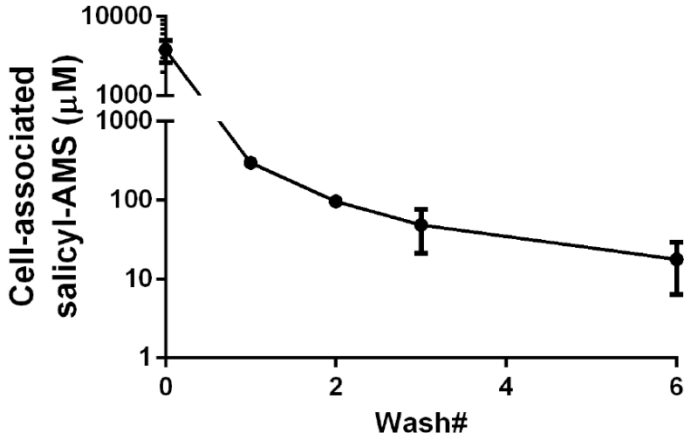
***Tri-Institutional Stem Cell Initiative***
***Starr Cancer Consortium***

# LC-MS/MS Analysis of Compound Accumulation in Bacteria

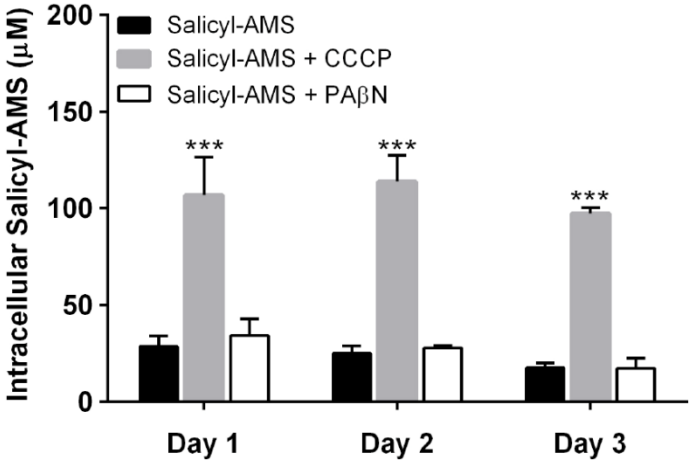
*Optimization and reproducibility of compound recovery protocol*

- **Four washes sufficient to remove extracellular compound (Fig. S18)**




***B. subtilis*, 1,000 μM salicyl-AMS**
**30 ° C, 1 h, LB**

- **Protocol has low day-to-day variability (Fig. S19)**

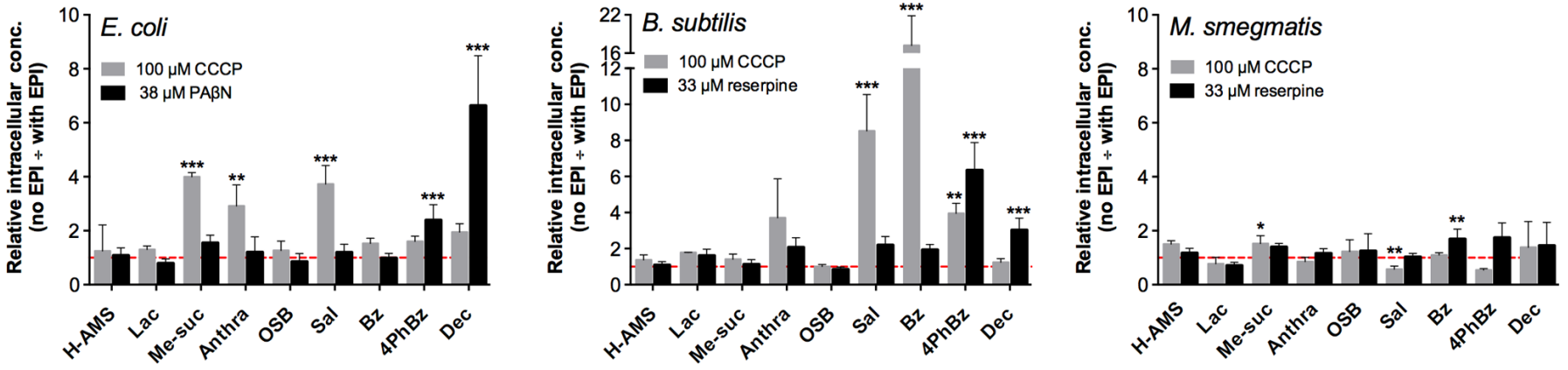



***E. coli*, 100 μM salicyl-AMS**
**37 ° C, 30 min, tryptic soy broth**
**± 100 μM CCCP *OR* 20 μg/mL PAβN**


• Davis, T. D.; Gerry, C. J.; Tan, D. S. "General platform for systematic quantitative evaluation of small-molecule permeability in bacteria." *ACS Chem. Biol.* **2014**, *9*, 2535–2544.

# Cheminformatic Analysis of Efflux of Acyl-AMS Panel

*Larger analyses are required to identify robust correlations*




| | Parameter | *E. coli* CCCP | *E. coli* PAβN | *B. subtilis* CCCP | *B. subtilis* reserpine | *M. smegmatis* CCCP | *M. smegmatis* reserpine |
|---|---|---|---|---|---|---|---|
| Size & Hydrogen Bonding | MW | 0.06 | 0.30 | -0.05 | 0.42 | -0.27 | 0.41 |
| | SA | 0.02 | 0.64 | -0.15 | 0.44 | -0.07 | 0.44 |
| | N | 0.26 | -0.13 | -0.05 | -0.04 | -0.19 | -0.15 |
| | O | 0.24 | -0.25 | -0.23 | -0.36 | 0.07 | -0.20 |
| | HBD | 0.32 | -0.31 | -0.01 | -0.24 | -0.33 | -0.33 |
| | HBA | 0.15 | -0.30 | -0.20 | -0.34 | -0.12 | -0.30 |
| Hydrophobicity & Polarity | LogD | 0.09 | 0.64 | 0.27 | **0.74** | -0.29 | 0.48 |
| | LogP | -0.13 | **0.67** | 0.16 | **0.71** | -0.20 | 0.59 |
| | ALogP | -0.13 | **0.78** | 0.03 | **0.80** | -0.21 | 0.64 |
| | ALogS | 0.18 | -0.64 | -0.02 | **-0.81** | 0.26 | **-0.68** |
| | tPSA | 0.21 | -0.40 | -0.31 | -0.48 | 0.00 | -0.40 |
| | relPSA | 0.01 | **-0.77** | -0.06 | -0.67 | 0.12 | -0.65 |
| 3-D Structure | nStereo | -0.30 | -0.22 | -0.19 | -0.14 | -0.27 | **-0.67** |
| | nStMW | -0.27 | -0.34 | -0.11 | -0.39 | 0.11 | -0.62 |
| | Fsp3 | -0.04 | 0.57 | -0.50 | -0.32 | 0.64 | -0.26 |
| | RotB | 0.03 | **0.77** | -0.39 | 0.05 | 0.43 | 0.27 |
| Ring Content | Rings | -0.06 | -0.20 | 0.38 | 0.66 | **-0.69** | 0.48 |
| | RingAr | -0.06 | -0.20 | 0.38 | 0.66 | **-0.69** | 0.48 |
| | RngSys | -0.06 | -0.20 | 0.38 | 0.66 | **-0.69** | 0.48 |
| | RRSys | 0.02 | 0.27 | -0.46 | -0.51 | 0.66 | -0.41 |

- **Significant differences between bacterial strains**
- *E. coli* CCCP: no statistically significant correlations
- *E. coli* PAβN: + hydrophobicity, + rotatable bonds – polarity
- *B. subtilis* CCCP: + ring content
- *B. subtilis* reserpine: + hydrophobicity, + ring content – polarity
- *M. smegmatis* CCCP: – ring content
- *M. smegmatis* reserpine: + ring content, + hydrophobicity – polarity, – 3D structure


- Davis, T. D.; Gerry, C. J.; Tan, D. S. "General platform for systematic quantitative evaluation of small-molecule permeability in bacteria." *ACS Chem. Biol.* **2014**, *9*, 2535–2544.

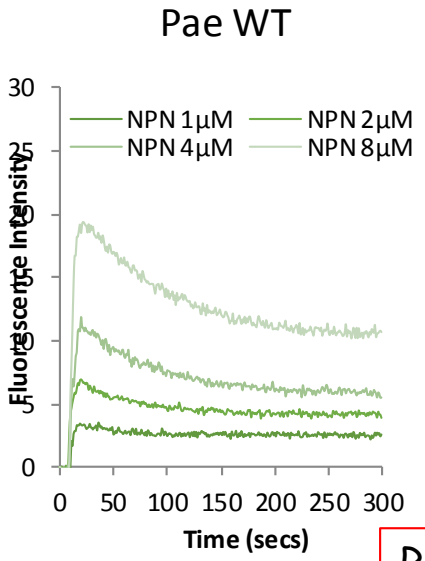

Helen Zgurskaya

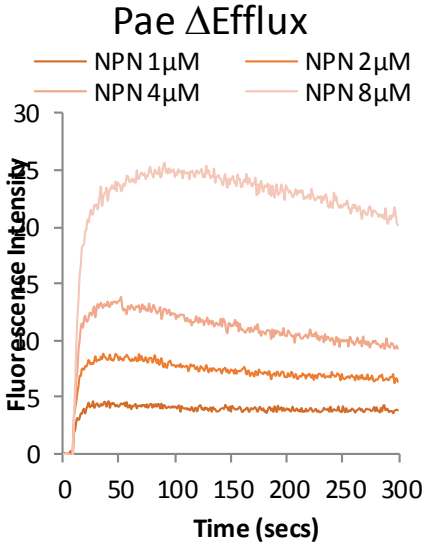
Department of Chemistry and Biochemistry

University of Oklahoma

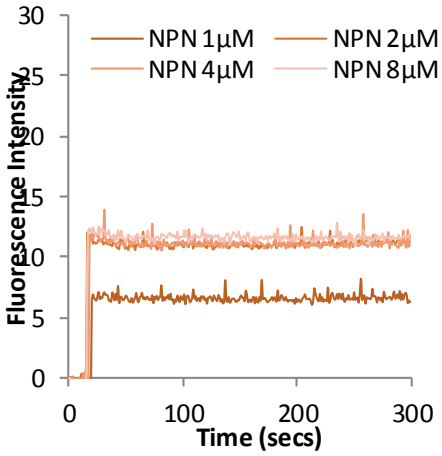
# Kinetics of drug uptake is determined by assay conditions

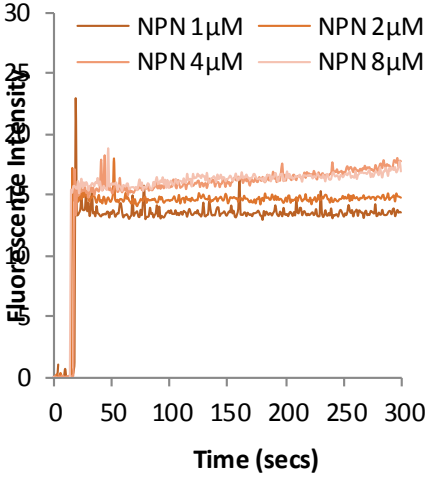
37°C → ice → RT




RT → RT → RT

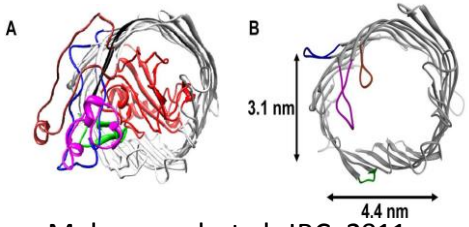


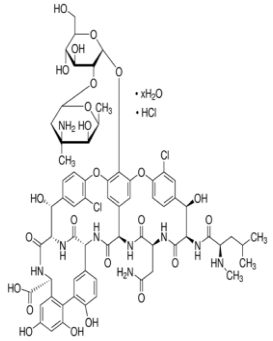

Cold shock permeabilizes the OM; too complicated kinetics is often an artefact of experimental conditions

# Hyperporination permeabilizes outer membranes of different species in controlled manner

EcPore



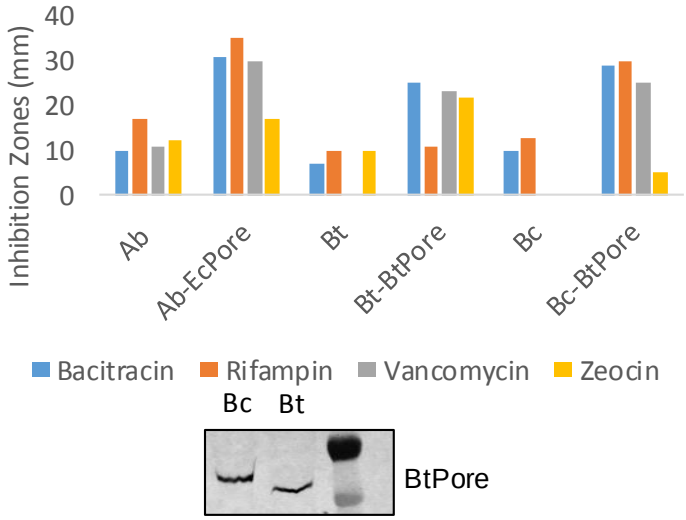

Mohammad et al. JBC, 2011



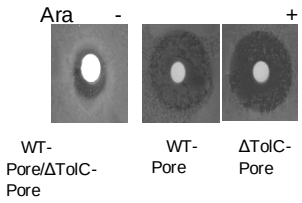
Vancomycin
MW 1485.7 Daltons

BtPore

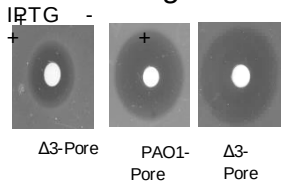
*A. baumannii*
*B. cepacia*
*B. thailandensis*

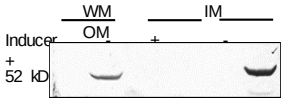


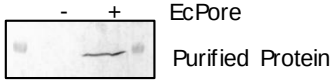

*E. coli*




*P. aeruginosa*







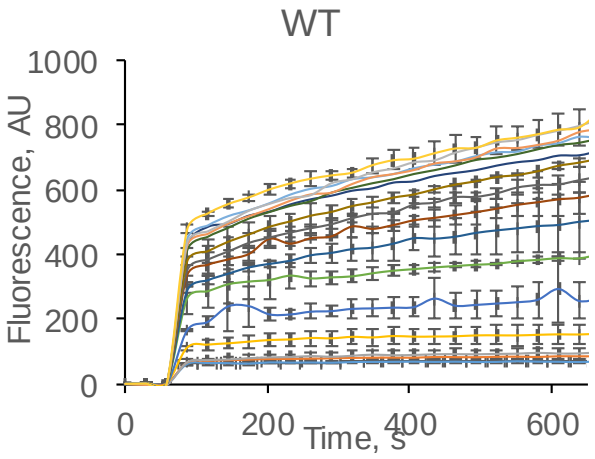
Krishnamoorthy et al., 2016, AAC

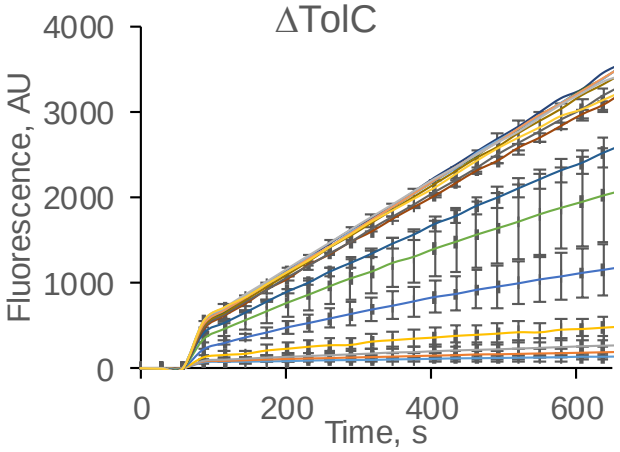
Krishnamoorthy et al., submitted

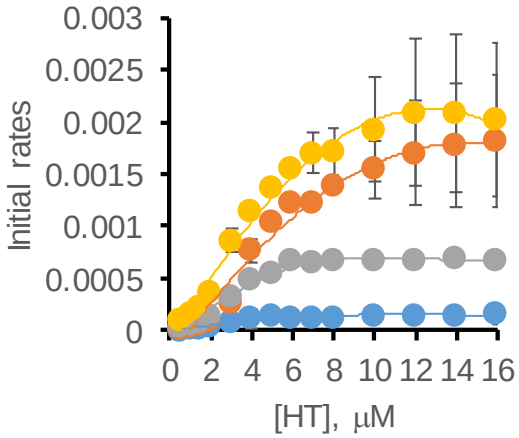
Under preparation

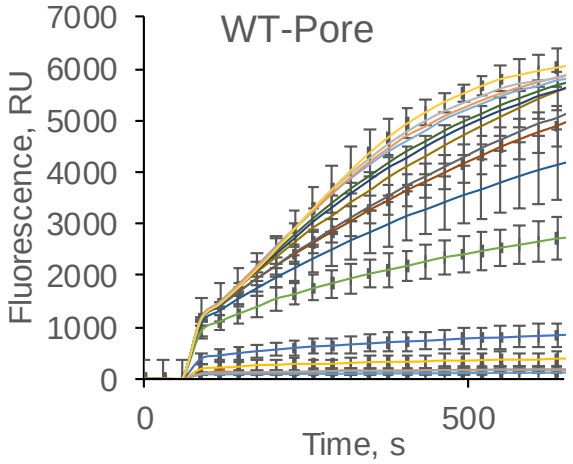
The “Pore” is not selective and does not discriminate based on hydrophilicity, charge and mass up to 2000 Da

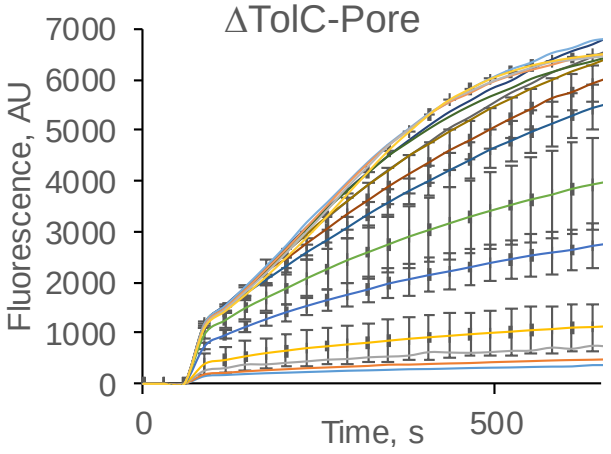
# High-density kinetic data are needed for mechanistic insights and modeling: continuous assays, including microfluidics

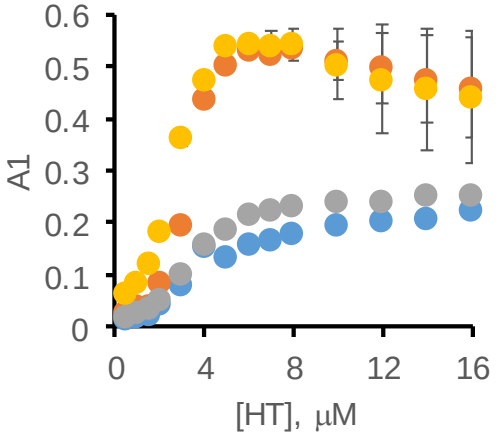


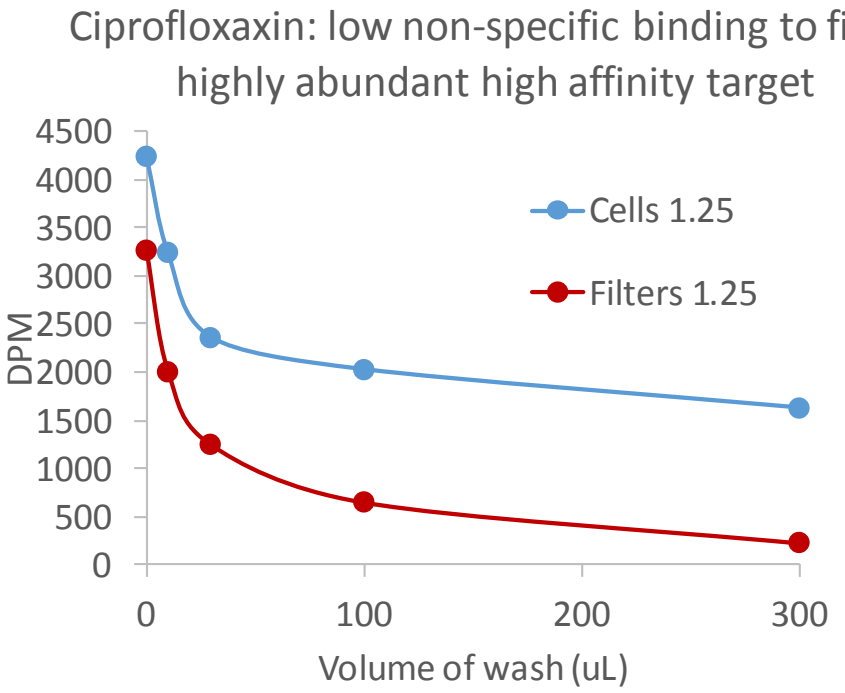



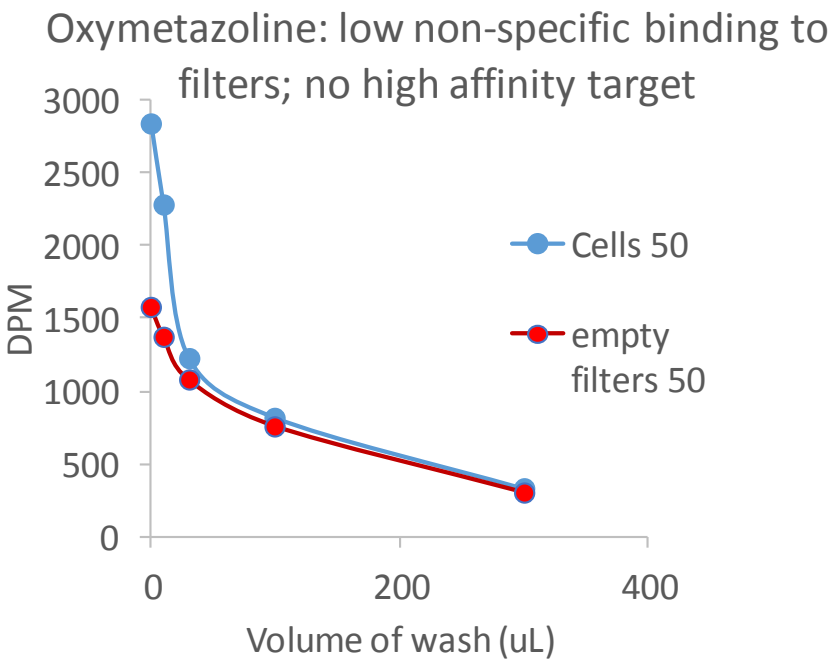




For most uptake data, either initial rates (slow kinetics) or steady-states (fast kinetics) could be extracted

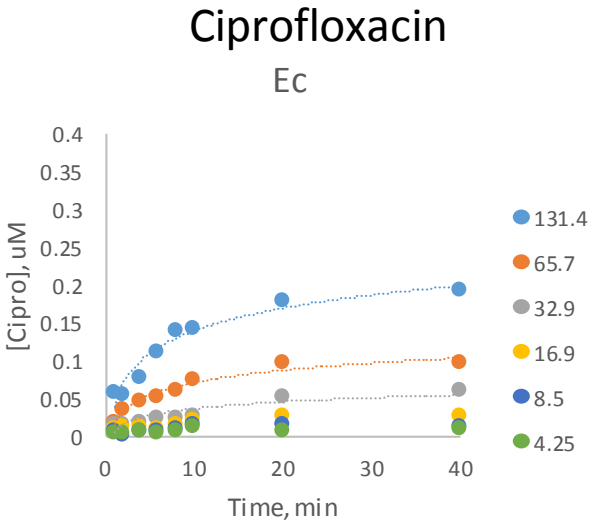
# Traditional filter (discontinuous) assays are sensitive to non-specific binding and drug affinity to intracellular targets

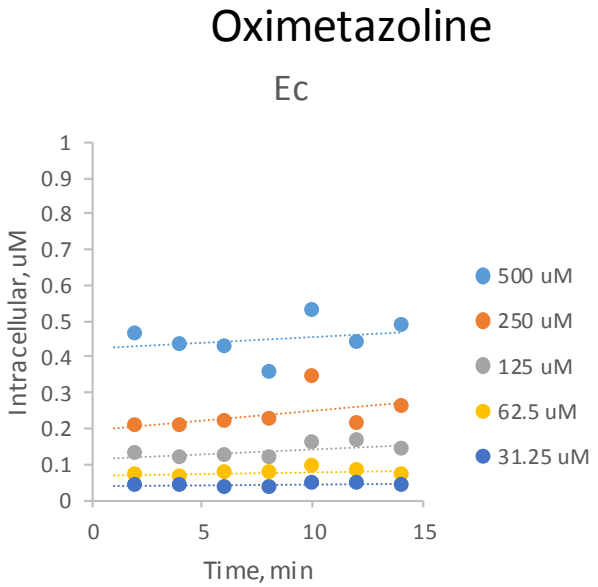


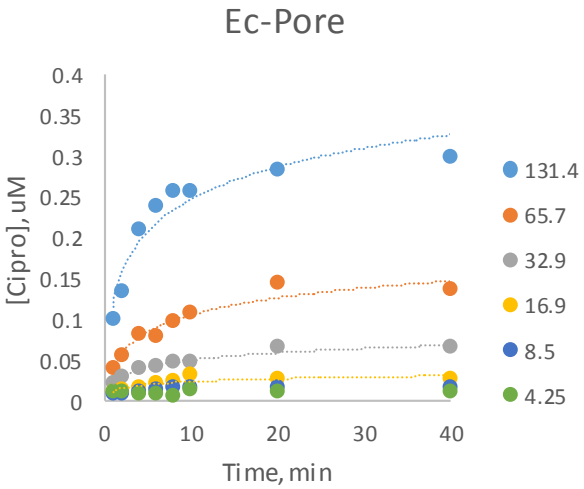



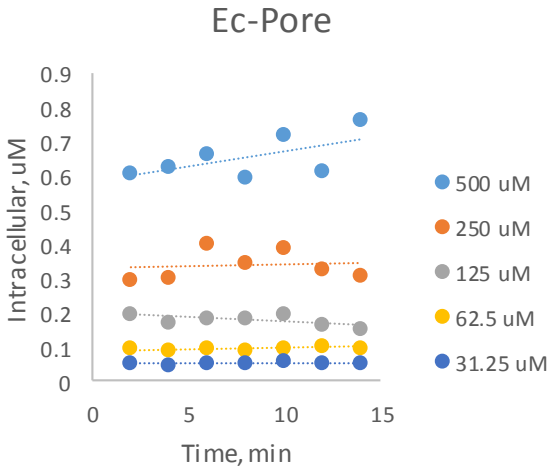

There is no a washing protocol that generates the same S/N ratio for two different compounds. Additional complications arise from: 1) binding to plastic or glass surfaces (negative rates); 2) precipitation from solution during incubations (negative rates); 3) binding to LPS (high noise); 4) covalent complexes etc

# In the absence of a high-affinity target kinetics of uptake is usually fast

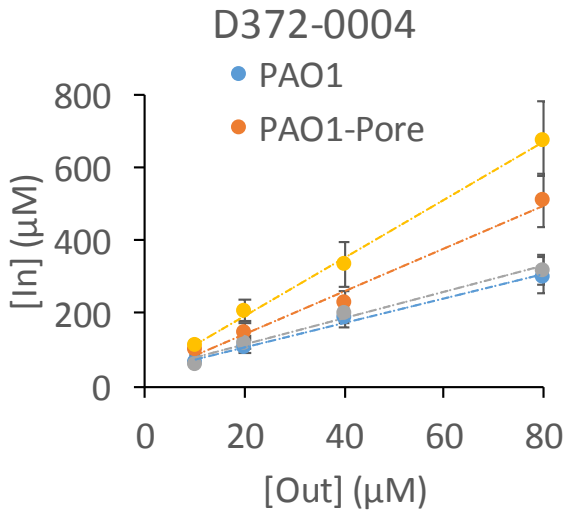


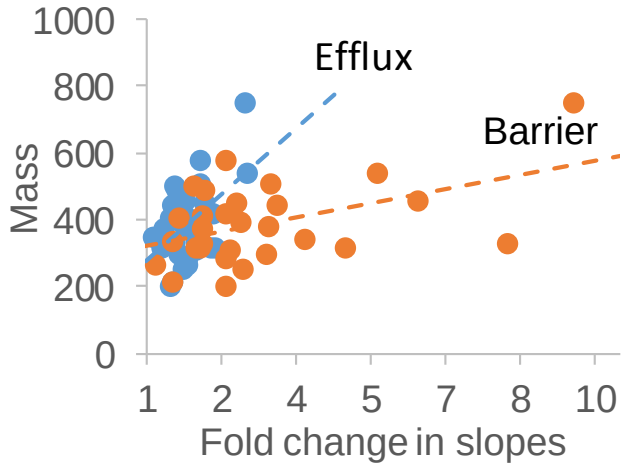



# LC-MS can cover chemical diversity and high-throughput; but kinetic insight is limited; absolute concentrations could be misleading; relative changes in accumulation identify efflux- and OM- specific properties




DTRA/OU P. aeruginosa project:

- 134 Compounds analyzed for detection via LC-MS
- 38 Compounds could not be identified or quantified with the current LC-MS method
- 63 Compounds have been analyzed by LC-MS Kinetic Uptake Experiments
- 7 Compounds cannot be identified in samples with cells
- 44 Compounds have quantifiable data from Kinetic Uptake Experiments
- Four Pae strains (WT, WT-Pore, Delta Efflux, DeltaEfflux-Pore); four concentrations for each compound; two time points= 32 samples x 3 injections per sample= 96 injections per compound + calibration= ~120 injections per compound per experiment = 24 hrs of instrument time

# Session 4: Establishing physicochemical guidelines for compound entry & efflux

The entry and efflux problem

# Challenges in the discovery of Gram-negative antibacterials

**Heinz E. Moser**
**NIBR, GDC Emeryville**
**February 6, 2017**
**Rockville, MD**

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# The Problem

*Historical attrition is well recognized among experts*

- Drug discovery for intracellular targets in mammalian cells is not trivial
  - Requires optimization of multiple parameters in parallel
  - Remains similar for antibiotics
- Additional requirements for Gram-negative antibiotics
  - Additional membrane with fundamentally different architecture and permeability requirements (high polarity)
  - Evolutionary optimized efflux machinery with multiple players and high level of promiscuity
  - Resistance
  - Administration of high doses (safety)

***The chemical space to fulfill these requirements is much more limited***

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# Approaches to improve success

*Multiple parameters have to be addressed, no simple solution*

- Identification of valuable chemical starting points
  - Diversify screening (phenotypic, target-based; implement new technologies and deviate from historical norm)
  - Focus on chemical matter which is expected to increase the chances for success (NPs, lower Mw, more polarity, less aromaticity)
  - Synthetic biology
  - Under-explored hit-finding approaches (e.g. FBDD, DELs)
  - Combine these approaches in smart ways
  - Focus on targets within periplasm (benefits for permeability & safety)
- Improve understanding on permeability and **efflux**
  - Experimental techniques to determine intracellular compound concentration irrespective of biological activity
  - Pragmatic approach for efflux; establish scaffold-dependent SAR

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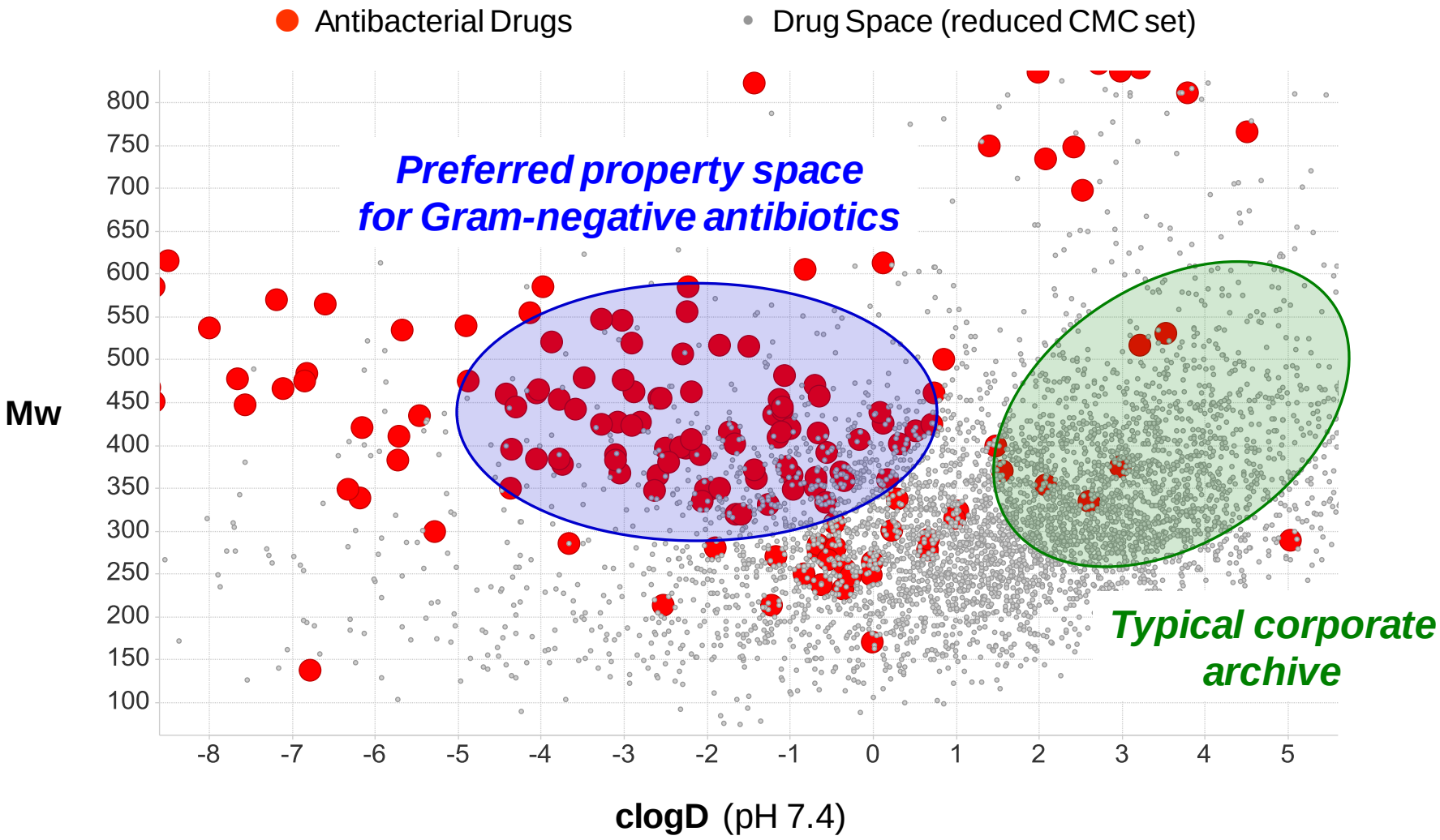
# Property Space of Drugs, Antibiotics, and Archives

*Unique property space for antibiotics, especially for Gram-negative bacteria*

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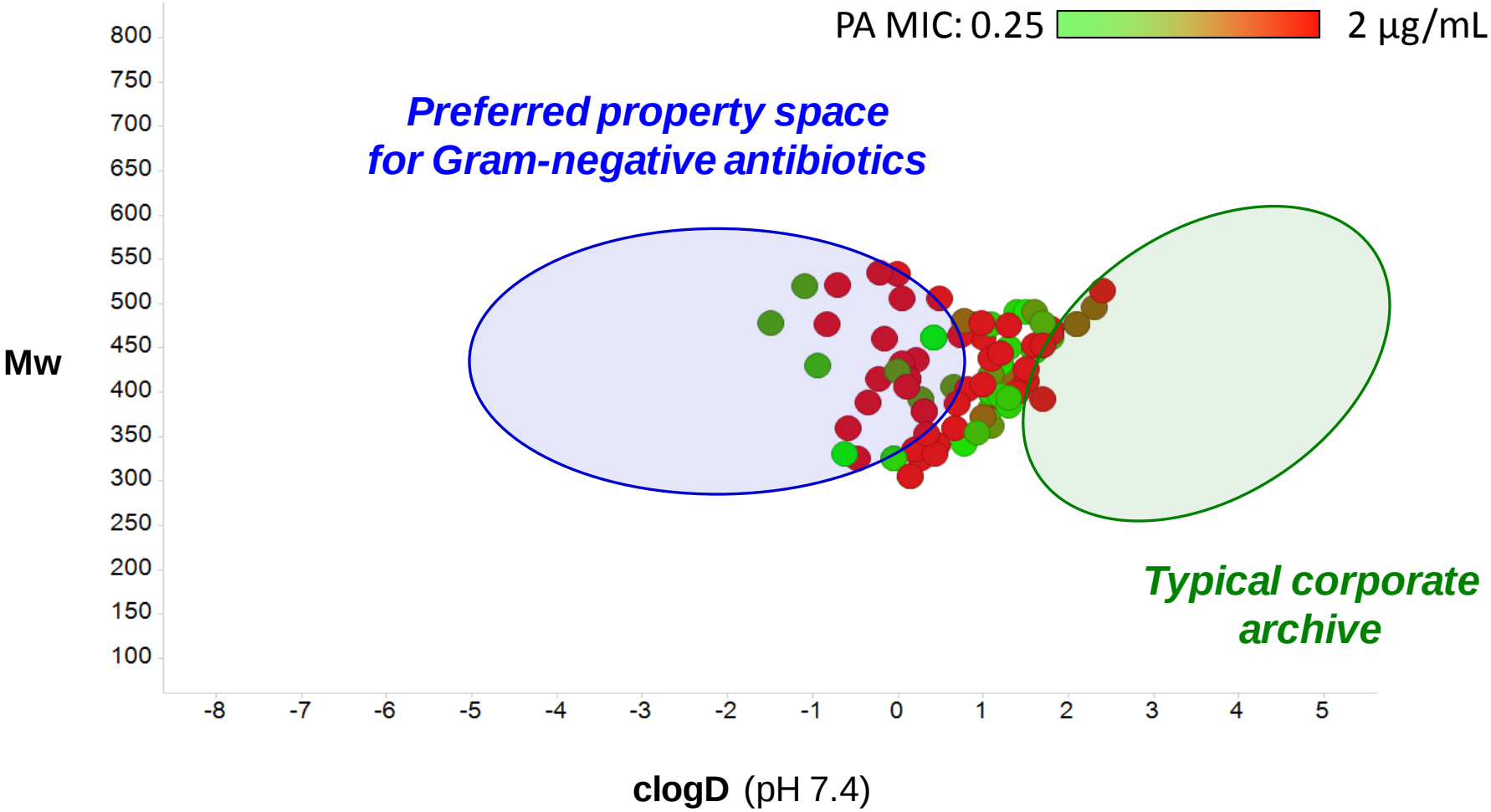
# Property Space of Drugs, Antibiotics, and Archives (I)

*Target 1, localization within cytoplasm*

MIC *P. aeruginosa* ≤8 µg/mL, cytotoxicity ≥100 µM

PA MIC: 0.25 — 2 µg/mL

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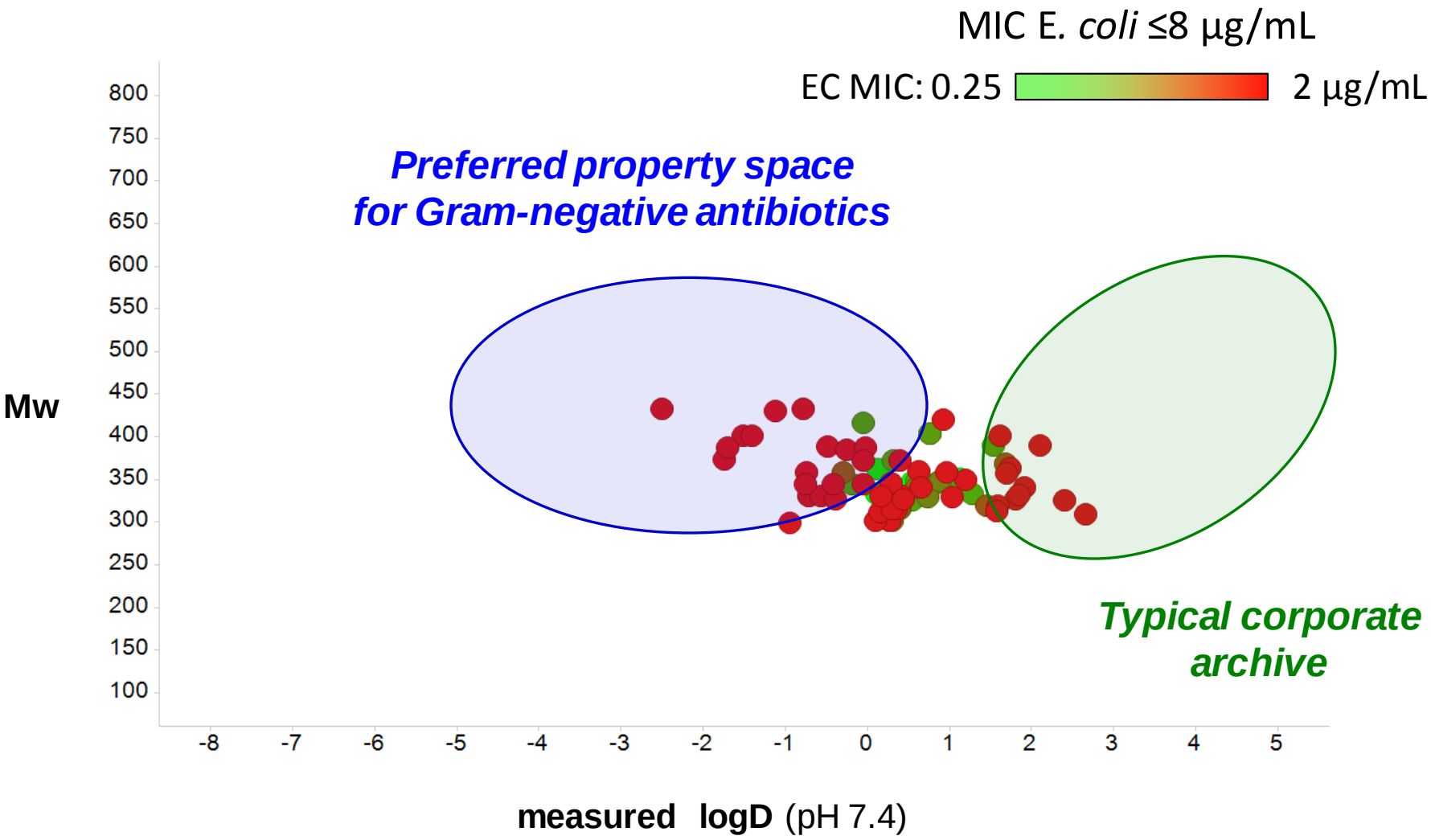
# Property Space of Drugs, Antibiotics, and Archives (II)

Target 2, localization within cytoplasm

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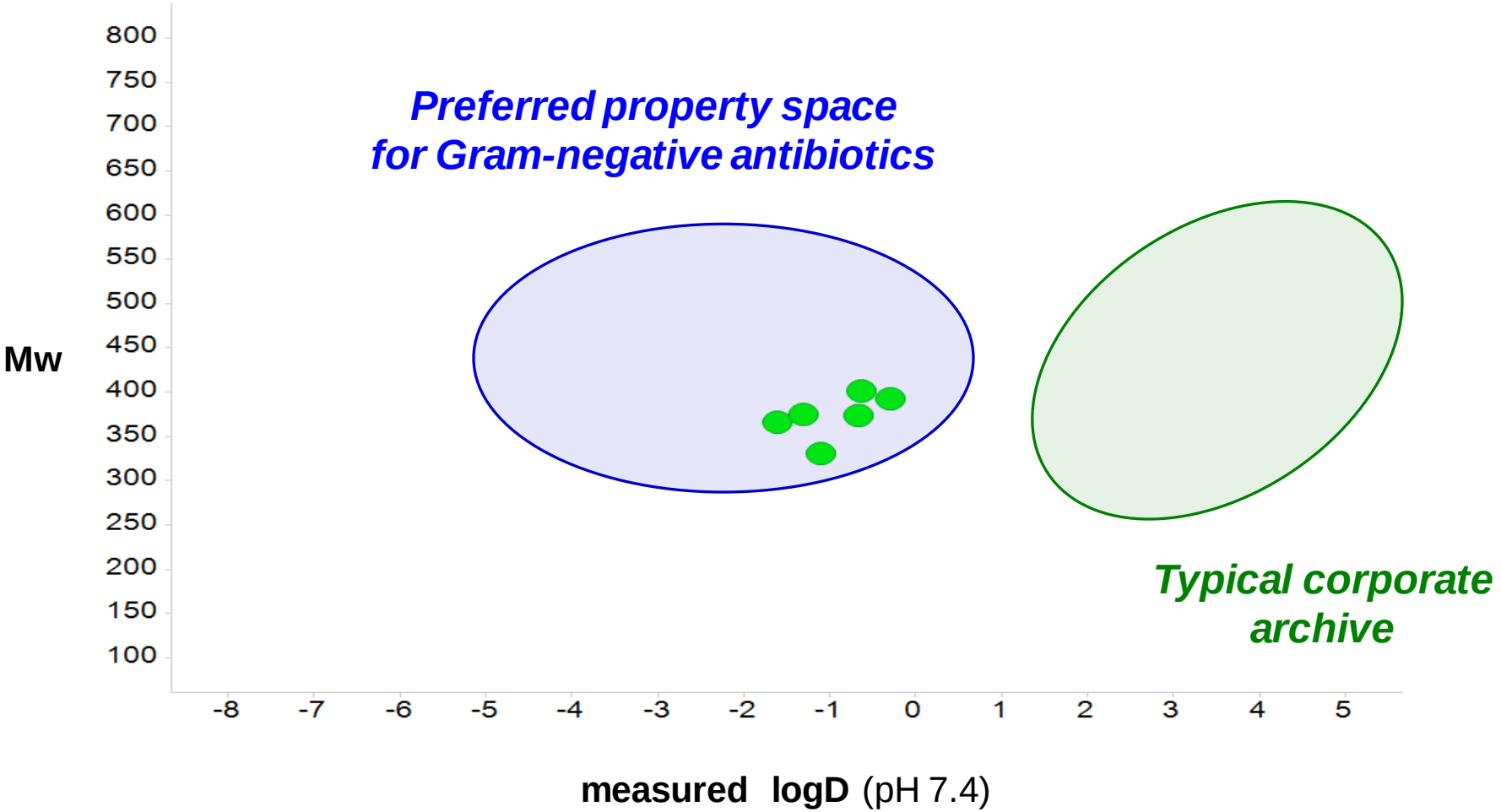
# Property Space of Benchmarking Fluoroquinolones

*Cipro-, Gati-, Levo-, Moxi-, Spar-, and Clinafloxacin (target within cytoplasm)*

FQs: MIC *E. coli* ≤8 µg/mL EC MIC: 0.25 2 µg/mL

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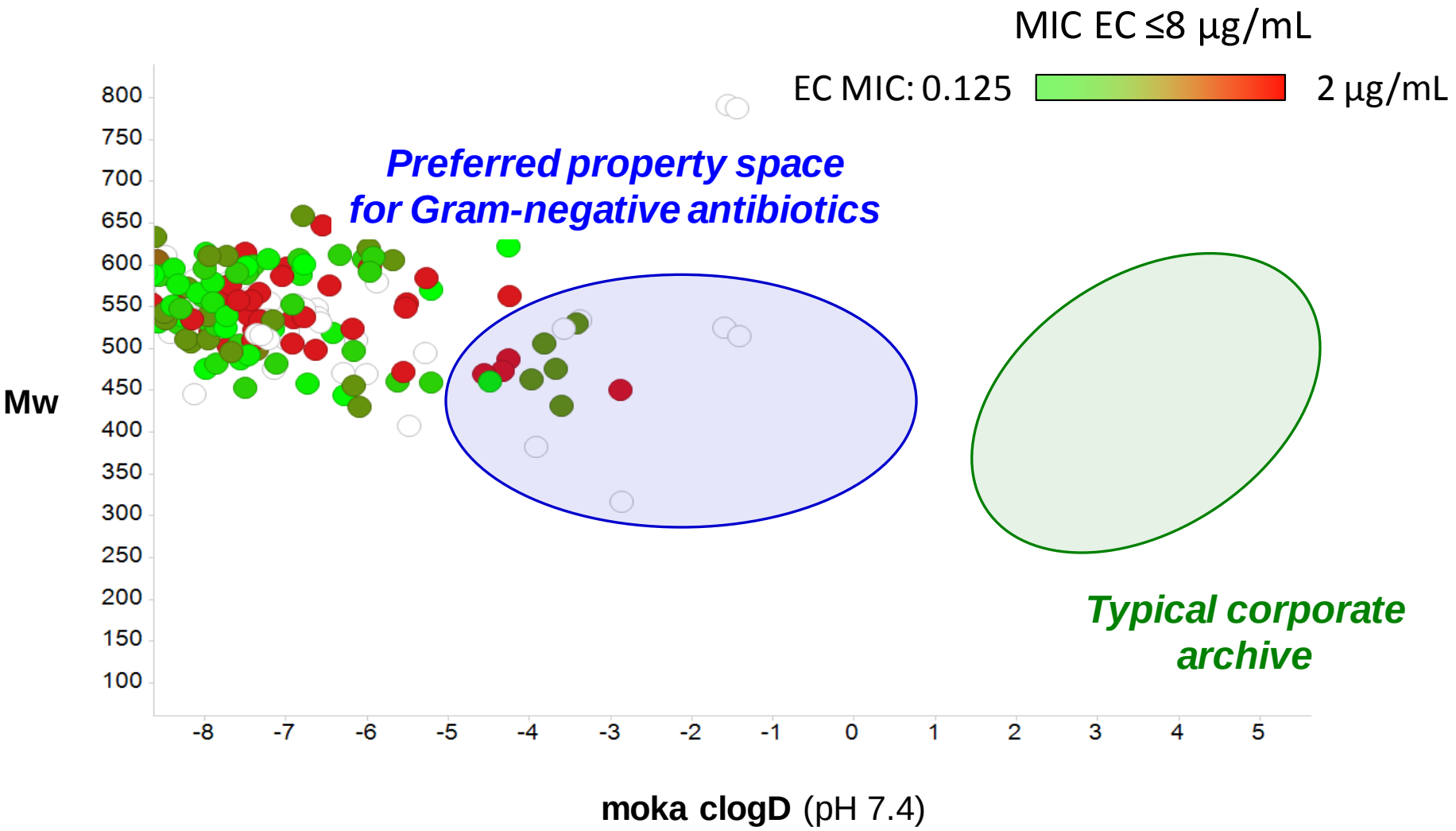
# Property Space of Drugs, Antibiotics, and Archives (III)

Target 3, localization within periplasm

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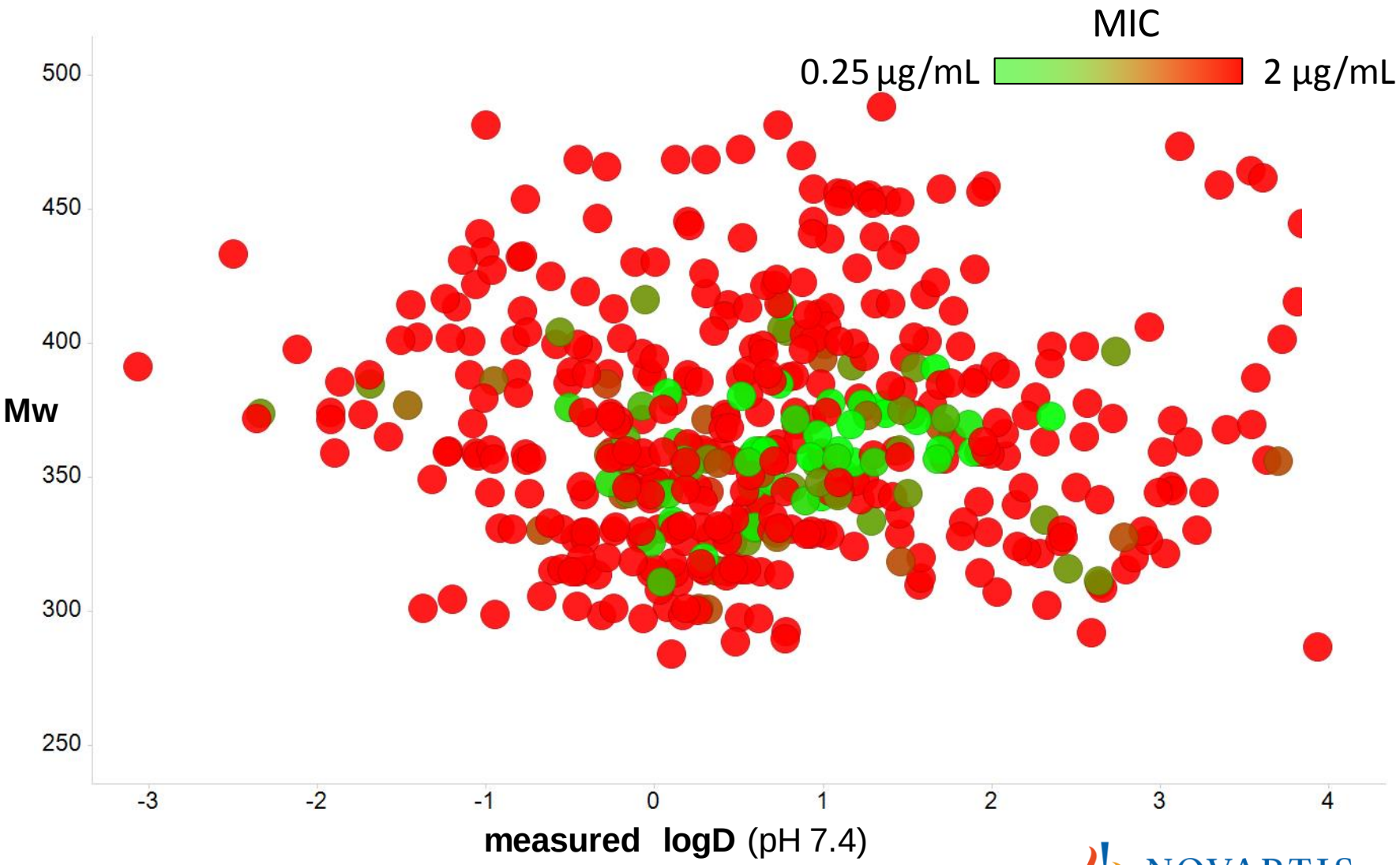
# Property Space of Drugs, Antibiotics, and Archives (IV)

Target within cytoplasm; E. coli wt activity, all compounds

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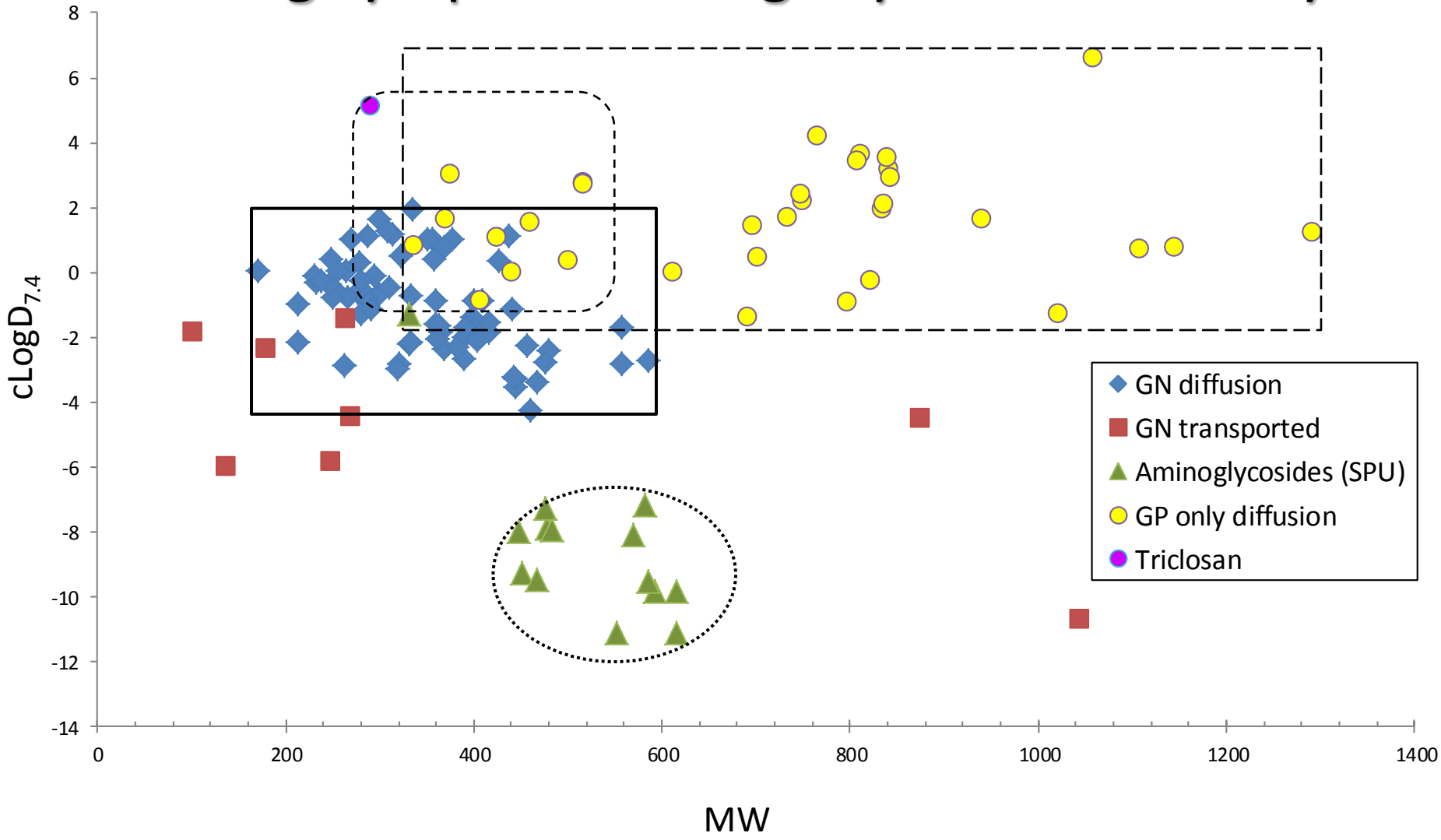
# Session 4: Establishing physicochemical guidelines for compound entry & efflux

*Lynn Silver, PhD*
*LL Silver Consulting, LLC*

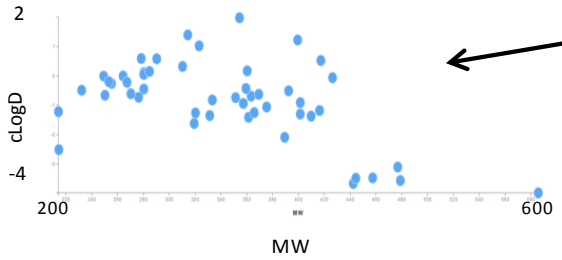
# Binning cytoplasmic drugs by “Route of Entry”



# Binning by route of entry

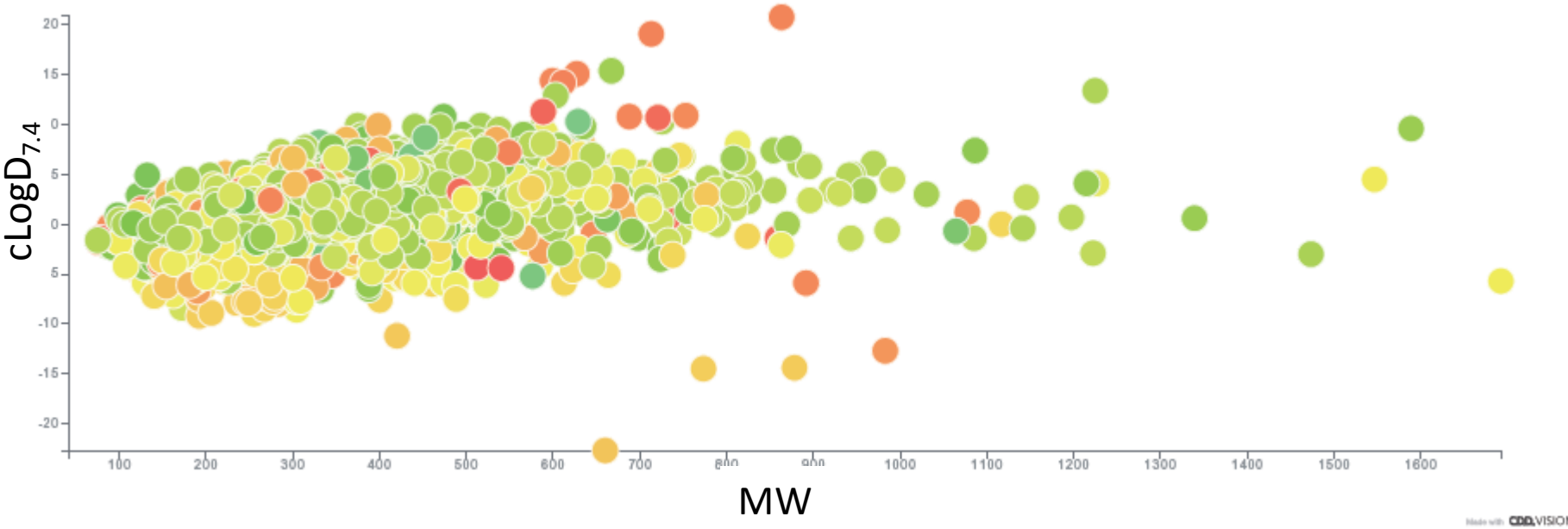
- Require large numbers of compounds to establish “rules” for various routes of entry or efflux-avoidance
- Need diverse compounds that do and do not accumulate in the cytoplasm
- Need method, independent of activity, to measure accumulation of compounds in cytoplasm
- Measure accumulation in genetically defined strains (especially efflux deletions) and with permeability assays to “define” route of entry
- Iteratively derive hypotheses for rules/routes
- What compounds to test?

# CDD ModelBuilding



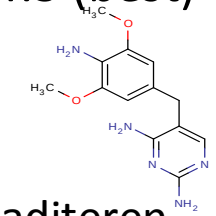

GN cytoplasmic diffused antibacterials
cLogD -4 to 2 MW <600
Used as training set to make a model

Applied to 4604 CMC non-Ab compounds



# Top 100 matches from 4604

- Scores 14.3 (best) to -50.9



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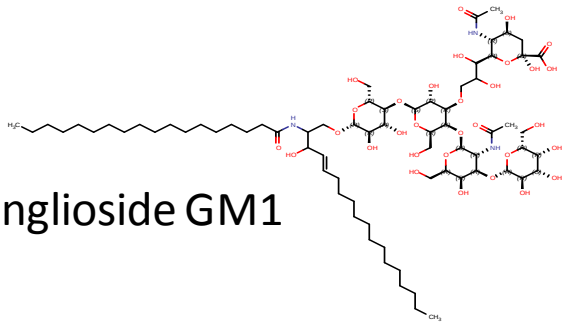
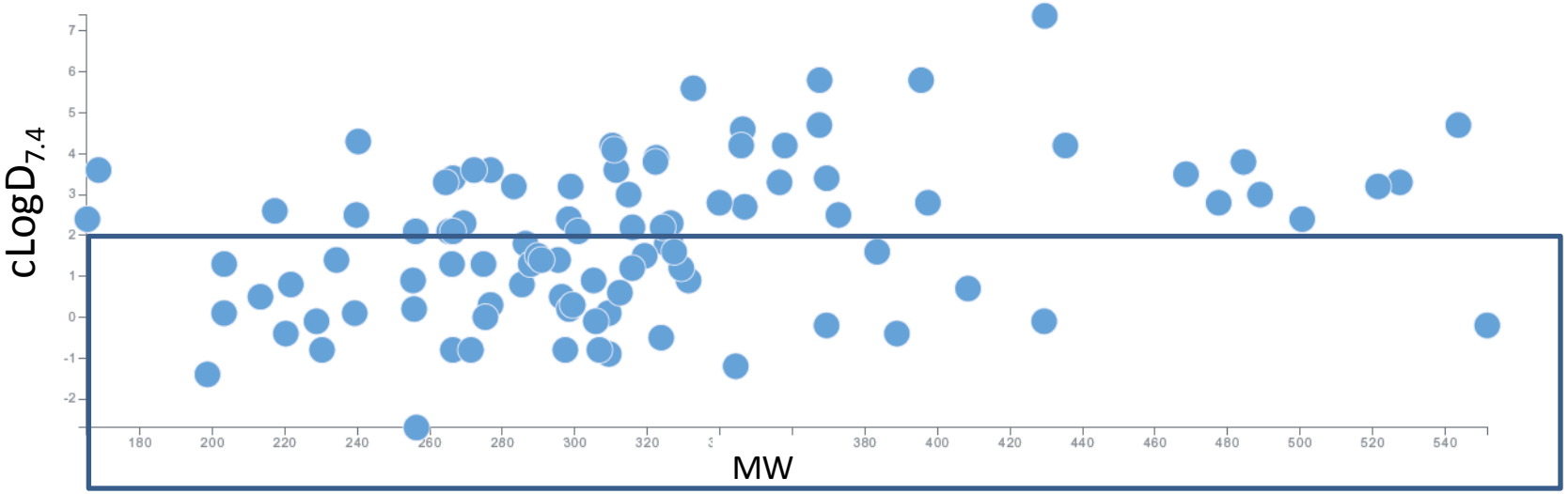


Ganglioside GM1

- Top 100 scores 14.3 to 4.62
- Top 100 include more lipophilic compounds than the training set



# Compounds across scores