Predictive compound accumulation rules yield a broad-spectrum antibiotic

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https://www.nature.com/articles/nature22308#methods

Most small molecules are unable to rapidly traverse the outer membrane of Gram-negative bacteria and accumulate inside these cells, making the discovery of much-needed drugs against these pathogens challenging. Current understanding of the physicochemical properties that dictate small-molecule accumulation in Gram-negative bacteria is largely based on retrospective analyses of antibacterial agents, which suggest that polarity and molecular weight are key factors. Here we assess the ability of over 180 diverse compounds to accumulate in *Escherichia coli*. Computational analysis of the results reveals major differences from the retrospective studies, namely that the small molecules that are most likely to accumulate contain an amine, are amphiphilic and rigid, and have low globularity. These guidelines were then applied to convert deoxynybomycin, a natural product that is active only against Gram-positive organisms, into an antibiotic with activity against a diverse panel of multi-drug-resistant Gram-negative pathogens. We anticipate that these findings will aid in the discovery and development of antibiotics against Gram-negative bacteria.

Drug-resistant bacteria are a major public health concern[1–3], and Gram-negative bacteria are particularly troubling as they are insensitive to many commonly used antibiotics[2,4–8]. Exacerbating this problem is the fact that a new class of antibiotics that are active against Gram-negative bacteria has not been introduced into the clinic since the quinolones in 1968[4]. This void in discovery is not due to a lack of effort; as one example, in 2007 GlaxoSmithKline reported screening around 500,000 synthetic compounds for activity against *Escherichia coli*, but no tractable hits were identified[1].

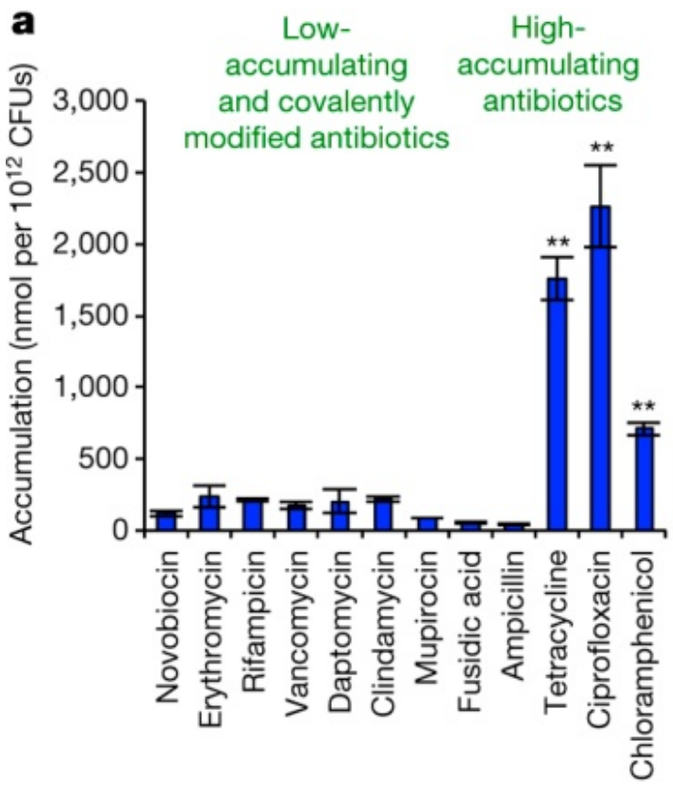
Gram-negative bacteria have two cellular membranes, and the lipopolysaccharide-coated outer membrane is very challenging for small molecules to cross[5,8,9]. Compounds that are able to traverse this outer membrane typically do so through narrow β-barrel proteins called porins, channels that are lined with charged amino acids[10]. Once inside the cell, small molecules are susceptible to efflux pumps[5,6,8]; thus, to accumulate in Gram-negative bacteria to a level that is sufficient for activity, small molecules typically must traverse porins faster than they are pumped out.

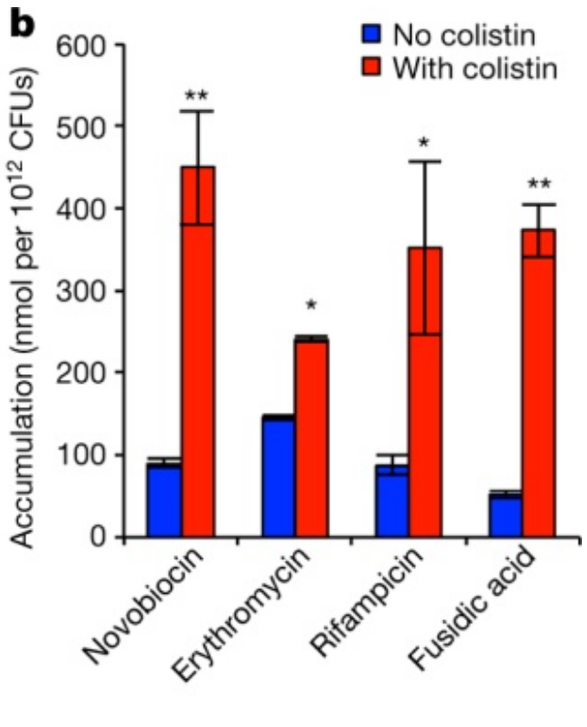
Central to the problem of discovering antibiotics that are effective against Gram-negative bacteria is a limited understanding of the physicochemical properties that enable small-molecule accumulation in Gram-negative bacteria, with current knowledge based largely on retrospective analyses of known antibiotics[11,12]. In 2008, O'Shea and Moser reported that antibiotics that are effective against Gram-negative pathogens almost always have a molecular weight of less than 600 Da and tend to be very polar, as measured by $ClogD_{7.4}$ (the predicted octanol/water distribution coefficient at pH 7.4). These observations are consistent with porin architecture[11] and have been reinforced by other retrospective studies[12]. However, there are antibiotics that meet these polarity and size criteria but are inactive against Gram-negative species, suggesting that these properties do not fully encompass the determinants from these small data sets (10–20 compounds and all within a single structural class). Perhaps most importantly, the canonical view about the importance of $ClogD_{7.4}$ and molecular weight for Gram-negative activity has not led to general strategies to convert Gram-positive-only compounds into broad-spectrum antibiotics. The seminal observation over 50 years ago that derivatizing penicillin G into ampicillin results in broad-spectrum activity[17] has not been generalizable, and important classes of antibiotics have coverage only against Gram-positive organisms despite intensive derivatization efforts. A systematic analysis of the accumulation of an unbiased and structurally diverse set of small molecules in Gram-negative bacteria has not been previously reported. Towards this end, we first assembled a diverse set of 100 compounds and quantified their capacity to accumulate in *E. coli*. As there are many variables affecting small-molecule accumulation (for example, multiple porins, efflux pumps and varying lipopolysaccharides)[5,6,8], compounds were assessed in accumulation assays using whole cells, instead of model systems. From these experiments, subsequent structure–activity relationship (SAR) studies, and computational analyses, we developed predictive guidelines for small-molecule accumulation in Gram-negative bacteria.

Accumulation studies

The accumulation method was adapted from known protocols[13,14,18] and liquid chromatography with tandem mass spectroscopy (LC–MS/MS) was used to quantify accumulation of each compound. The assay method was evaluated with antibiotics that have known high (tetracycline, ciprofloxacin and chloramphenicol) and low (novobiocin, erythromycin, rifampicin, vancomycin, daptomycin, clindamycin, mupirocin and fusidic acid) levels of accumulation. Ampicillin was also used as a 'low-accumulation' control as it is rapidly covalently appended to penicillin-binding proteins, preventing measurement by

Study aimed at working out the properties of molecules that can enter Gram-negative bacteria

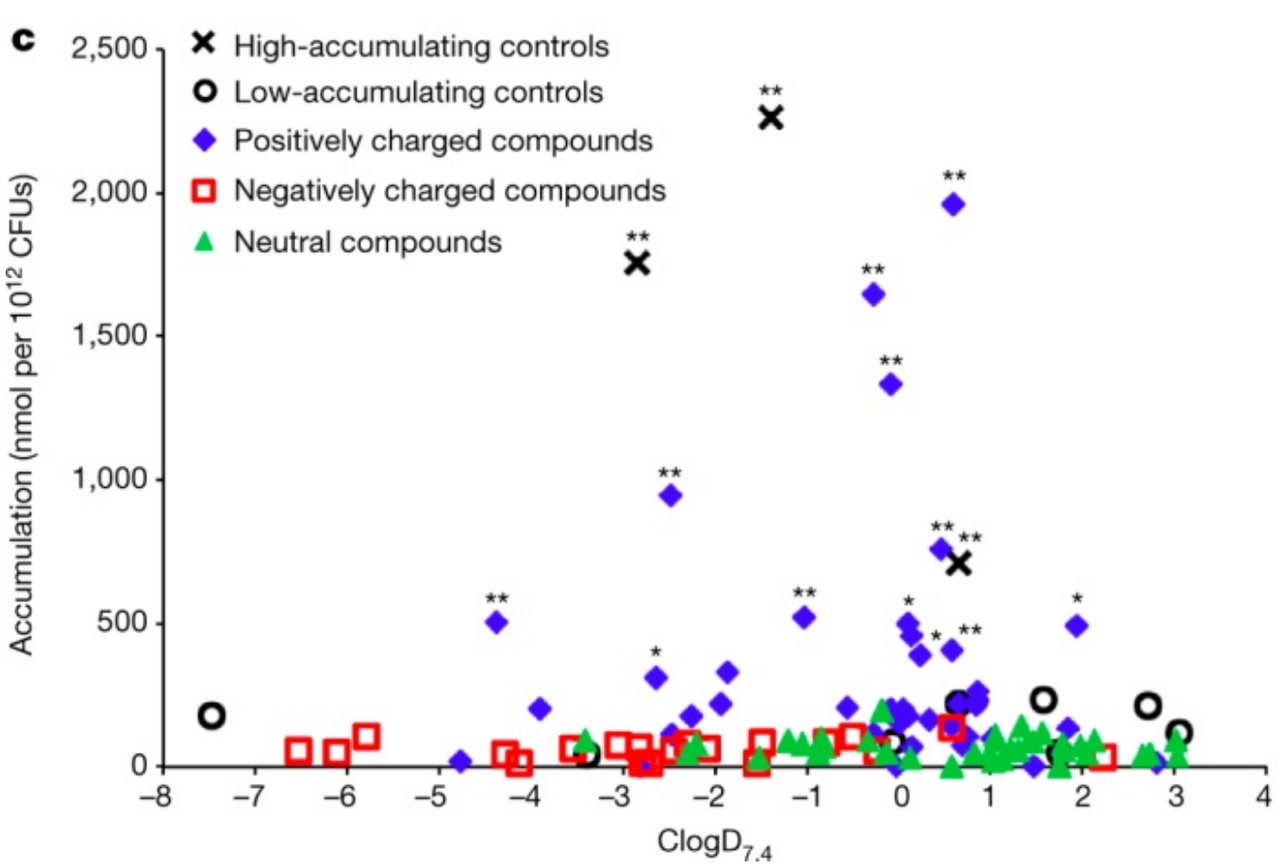




Mass-spec method used to quantify the amount of test molecule that has entered cell.

Basically - treat cells with compound, incubate briefly, wash cells, lyse, extract, mass-spec quantify compound.

Colistin permeabilises outer membrane.



Measure of how lipophilic (fat loving) the compound is

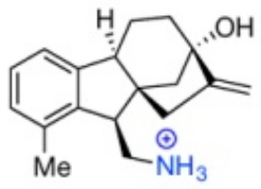
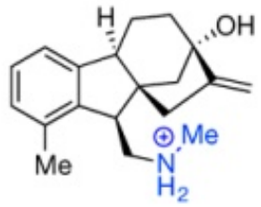
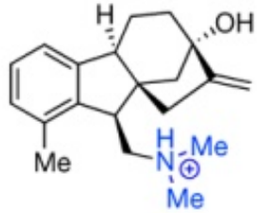
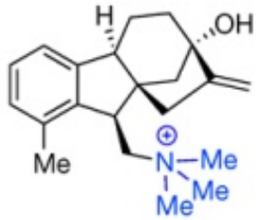
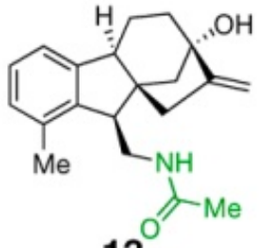
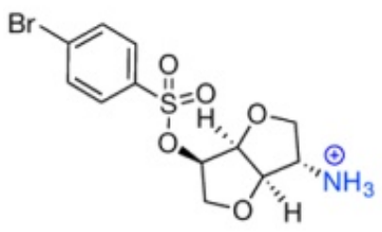
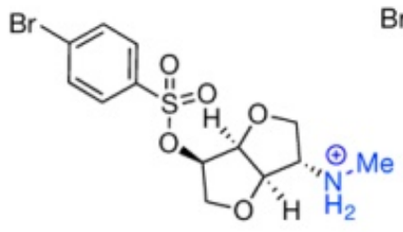
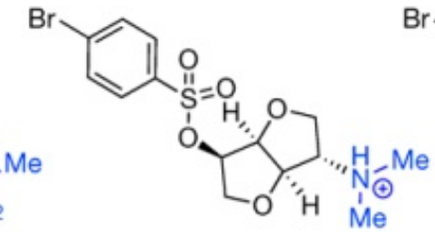
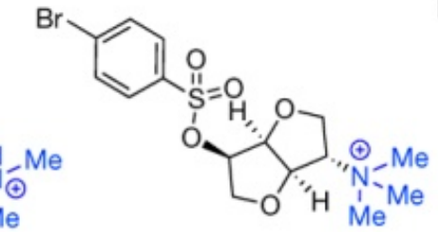
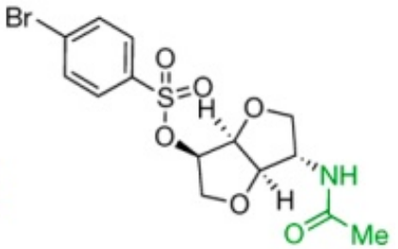
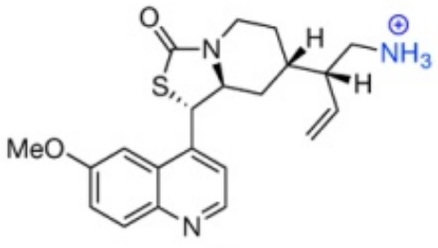
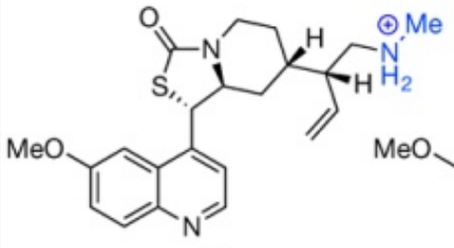
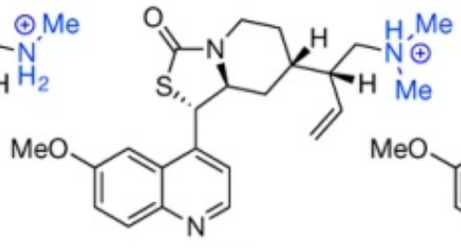
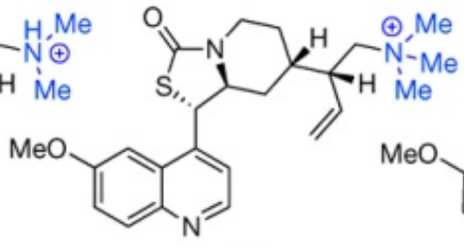
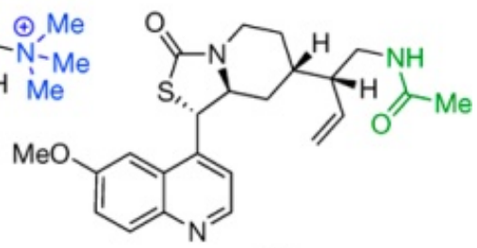
Measured for a number of known antibiotics.

Synthesise 100 natural product-like compounds and measure accumulation

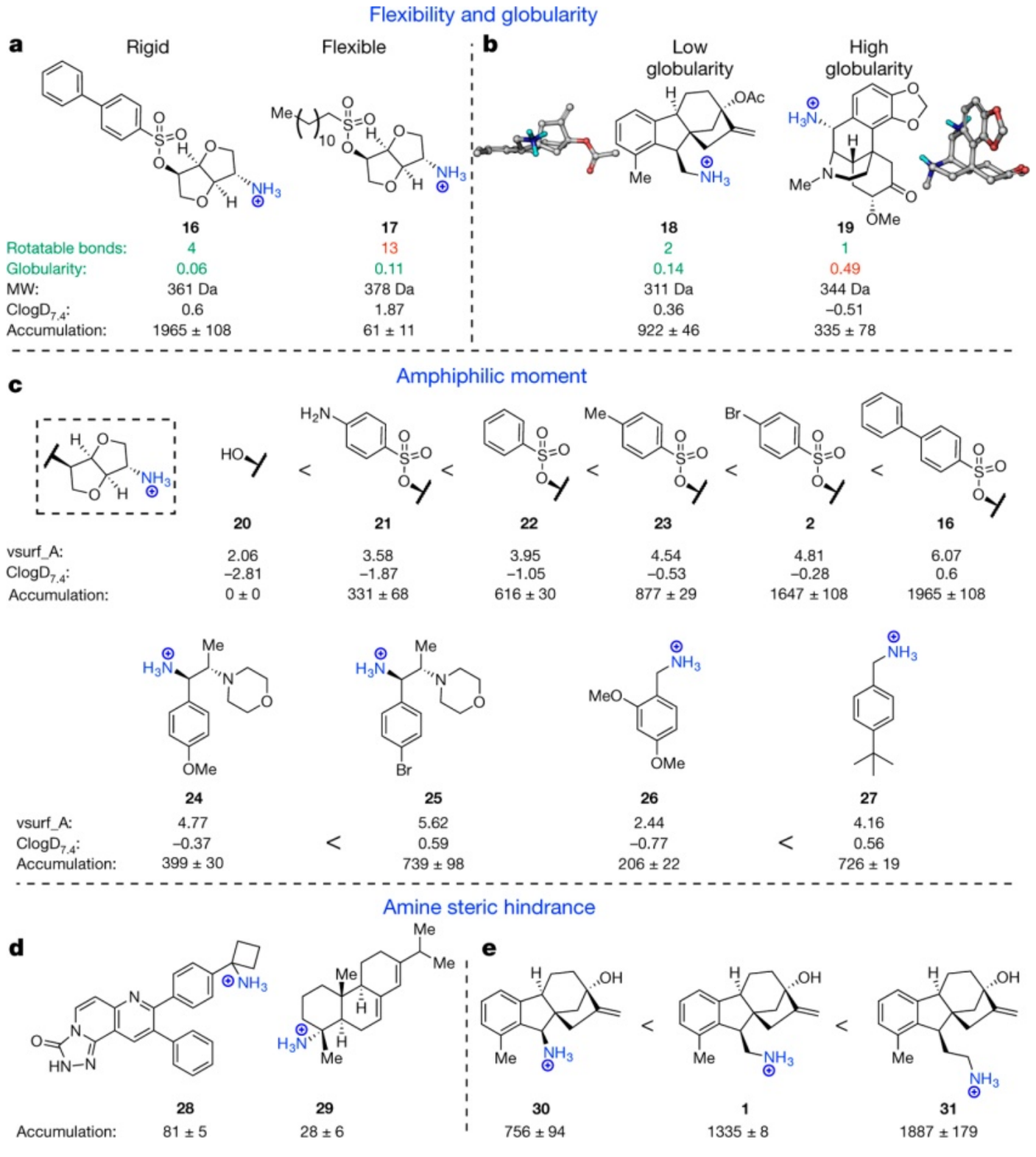
charge - not lipophilicity appears to be critical.

Most contain primary amines

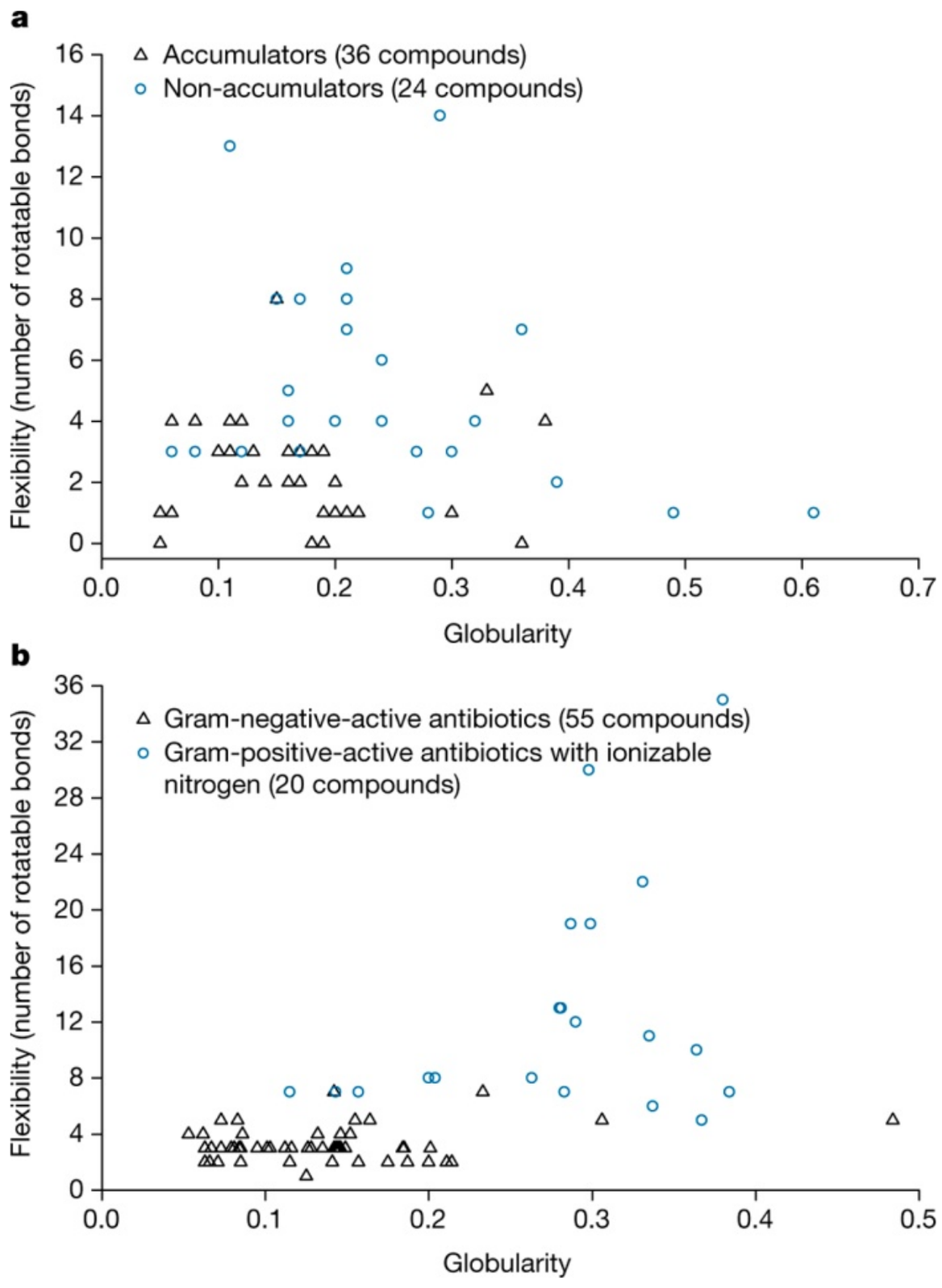
Changing primary amine reduces accumulation

Primary amine	Mono-methyl amine	Di-methyl amine	Tri-methyl amine	Amide
				
1	**4**	**7**	**10**	**13**
Accumulation: 1,335 ± 8	210 ± 29	100 ± 27	30 ± 1	91 ± 4
				
2	**5**	**8**	**11**	**14**
Accumulation: 1,647 ± 108	176 ± 7	7 ± 1	30 ± 1	8 ± 1
				
3	**6**	**9**	**12**	**15**
Accumulation: 458 ± 58	390 ± 24	48 ± 3	44 ± 7	91 ± 9

some primary amide compounds do not accumulate:
Not sole contributing factor - investigate chemical properties of all accumulating compounds



Flexibility and globularity are also important.



Gram negative accumulation rules

On the basis of these analyses, the following guiding principles for compound accumulation in *E. coli* were developed: compounds are most likely to accumulate if

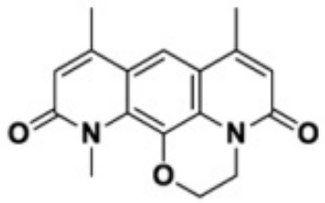
they contain a non-sterically encumbered amine,

they have some non-polar functionality,

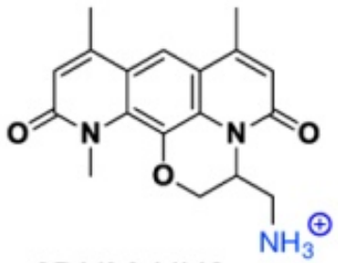
they are rigid

they have low globularity

Applying the rules to convert an anti-gram positive into a broad-spectrum antimicrobial



6DNM
No primary amine
RB = 0; Glob = 0.04
Accum = 298 ± 110



6DNM-NH3
Primary amine
RB = 1; Glob = 0.09
Accum = 1,114 ± 135

	Gram-positive		Gram-negative													
	S. aureus		*E. coli*								*A. baumannii*					
Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
6DNM	1	0.06	>32	>32	>32	>32	>32	>32	>32	>32	16	>32	>32	16	16	>32
6DNM-NH3	0.5	0.03	0.5	0.5	0.5	2	4	8	16	4	4	2	2	2	8	16
Cipro	0.125	32	0.008	0.004	0.008	64	16	>64	64	>64	0.5	32	16	16	64	64