Why most transporter mutations that cause antibiotic resistance are to efflux pumps rather than to import transporters

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Keywords

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49 ## Abstract

50

51 Genotypic microbial resistance to antibiotics with intracellular targets commonly arises from
52 mutations that increase the activities of transporters (pumps) that cause the efflux of intracellular
53 antibiotics. *A priori* it is not obvious why this is so much more common than are mutations that
54 simply inhibit the activity of uptake transporters for the antibiotics. We analyse quantitatively a
55 mathematical model consisting of one generic equilibrative transporter and one generic
56 concentrative uptake transporter (representing any number of each), together with one generic
57 efflux transporter. The initial conditions are designed to give an internal concentration of the
58 antibiotic that is three times the minimum inhibitory concentration (MIC). The effect of varying the
59 activity of each transporter type 100-fold is dramatically asymmetric, in that lowering the activities
60 of individual uptake transporters has comparatively little effect on internal concentrations of the
61 antibiotic. By contrast, increasing the activity of the efflux transporter lowers the internal antibiotic
62 concentration to levels far below the MIC. Essentially, these phenomena occur because inhibiting
63 individual influx transporters allows others to 'take up the slack', whereas increasing the activity of
64 the generic efflux transporter cannot easily be compensated. The findings imply strongly that
65 inhibiting efflux transporters is a much better approach for fighting antimicrobial resistance than is
66 stimulating import transporters. This has obvious implications for the development of strategies to
67 combat the development of microbial resistance to antibiotics and possibly also cancer
68 therapeutics in human.

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Introduction

In order to understand genotypic antimicrobial resistance and how to combat it, a starting point should be an understanding of the main kinds of mutation that can cause it. For present purposes, we assume that the molecular targets of the antibiotic are intracellular (and indeed when the microbes themselves are inside host cells, their access presents its own problems [1]). Broadly, these mutations are of then of three kinds [2-4]: (i) mutations in or overproduction of one or more targets of the antibiotic (e.g. DNA gyrase and topoisomerase IV for ciprofloxacin [5]), (ii) mutations that lead to inactivation of the antibiotic (e.g. of chloramphenicol [6] and aminoglycosides [7]), or (iii) mutations that affect the ability of the antibiotic to be transported to a compartment containing its sites of action in the target microbe.

To enter the target microbe, antibiotics (as do other drugs, e.g. [8-14]) require transporters. (In Gram-negatives, outer-membrane proteins may also play a role [15-17].) The precise identities of these uptake transporters are in general not well understood, because mutations tend to lead only to partial resistance. However, they have been identified for antibiotics such as aminoglycosides [18], chloramphenicol [19], cycloserine [20] and fosfomycin [21, 22]. In addition, bacteria have also evolved a variety of efflux pumps that serve to remove such antibiotics (see later, and also many other substances [23, 24]) from the cells. Thus, mutations that affect transporter activity can in principle involve uptake transporters, efflux transporters, or upstream regulators of their activity. Our focus is on this collective class, viz. transporters. In particular, consistent with the difficulty of identifying transporters for their uptake, we note that the very great bulk of transporter-mediated resistance is mediated via (multi-drug) efflux rather than influx transporters (e.g. [25-45]). The focus of this article is to enquire as to the reasons why this might be so.

To this end, we create a very simple and generic model (Fig 1), consisting of two types of influx and one type of efflux transporter. For the influx transporters, one is a generic equilibrative transporter and one is concentrative for uptake, i.e. it has the capability of raising the concentration of the drug of interest to a higher level inside than outside. Such transporters necessarily require a source of free energy; in prokaryotes this is mainly ATP [46, 47]. The effluxer is also taken to be ATP-

driven. We assume that a drug (antibiotic) has been added at 3x the minimum inhibitory concentration (MIC), which for our purposes is taken to be 1 concentration unit in the case of the wild type, but that the drug does not itself alter the expression levels of the transporters (cf. [48]).

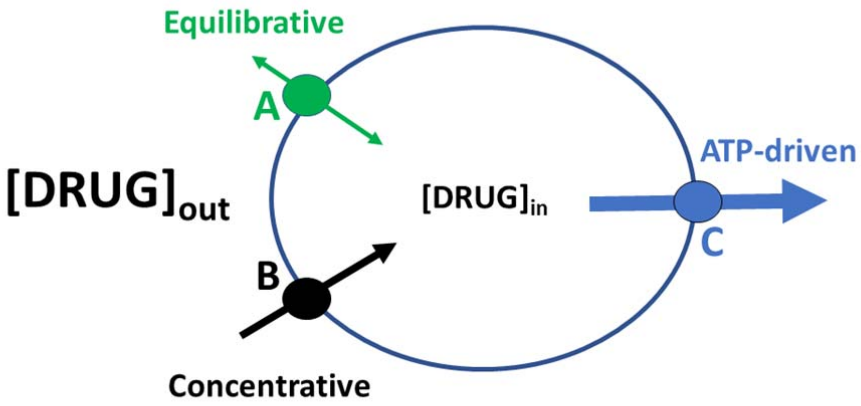

Fig 1. The generic model in which we have a suite of (A) equilibrative and (B) concentrative influx transporters, together with a generic ATP-driven efflux transporter.

Intuitively, lowering the internal concentration of the drug by blocking the concentrative one only works if the equilibrative ones are collectively slower than an individual concentrator, and this is unlikely if there are several. Similarly, trying to lower the internal concentration by blocking one of the equilibrative ones would just let the concentrative one(s) 'pick up the slack'. This already suggests the general reason why a partial inhibition of uptake activity might have comparatively little effect. Of course if we start with the drug at a level above its MIC it is clear that increasing the effluxer activity can serve to bring to a level below the MIC (and that lowering any starting efflux activity would increase antibiotic sensitivity). We now wish to assess these intuitions by putting some concrete numbers on these fluxes. In systems biology [49-53], this is commonly done by casting the enzymatic rate equations into the form of ordinary differential equations, and this is what we do here.

Materials and methods

As previously [54], all simulations were performed using COPASI, here version 4.27, with the LSODA integrator [55-57] (http://copasi.org/), which reads and writes SBML-compliant models [58-60]. It contains a full suite of enzyme rate equations, and admits automated parameter sweeps. Model files including the precise parameters are included as supplementary data.

The simulations were carried out with a differential equation-based model with three compartments (Fig 1), viz. the intracellular space, the inner membrane, and the extracellular space (including the periplasmic volume). Three different transporters are considered: transporter *A* is an equilibrator that allows transport in both directions (*Keq* = 1), *B* is a concentrative influx transporter; even though allowing transport in both directions, it favors transport into the cell (modelled by setting *Keq* = 10 or *Keq* = 100). *C* is an efflux pump that only transports the drug from the cytoplasm to the outside.

The model was set up to mimic typical assays, and parameters were set to values that are comparable to what is found in the literature as follows. Total volume of the assay is 150 µl (from [61]). Each assay is estimated to have 10^6 cells, with an average volume of 4×10^{-15} l per cell [62] (grown in rich media). Estimates of the proportion of volume taken by the periplasm are around 30% [63]. Thus, the total cell volume in the assay is estimated at 4×10^{-9} l and the cytoplasmic volume at 2.8×10^{-9} l. For the inner membrane surface area we adopt the average value in the range considered by Wong and Amir [64] 34.5 µm^2 (3.45×10^{-7} cm^2), which corresponds to a total surface area of 0.345 cm^2 (*i.e.* for all 10^6 cells); note that Thanassi *et al.* provide an estimate 3-fold lower (0.103 cm^2) [65].

Kinetic parameters for the efflux pump (*C*) come from Nagano and Nikaido for AcrB (part of acrAB/tolC) with nitrocefin [66]; they cite a K_m of 5 µM, k_{cat} of 10 s^{-1} and a V_{max} of 2.35×10^{-11} mol/s/10^9 cells, which implies a total of 2.35×10^{-12} mol of transporter. Considering that our simulation contains 10^6 cells, the adjusted amount of transporter is then 2.35×10^{-15} mol

(considering the surface area estimated above, this corresponds to a surface density of 6.8×10^{-15} mol/cm^2) with a V_{max} of 2.35×10^{-14} mol/s, assuming the same k_{cat} as for nitrocefin. For K_m we chose a higher value (500 μM).

Results

Fig 2 shows our 'baseline simulation, in which a steady-state intracellular level of the drug similar to that outside is obtained by balancing the three main fluxes.

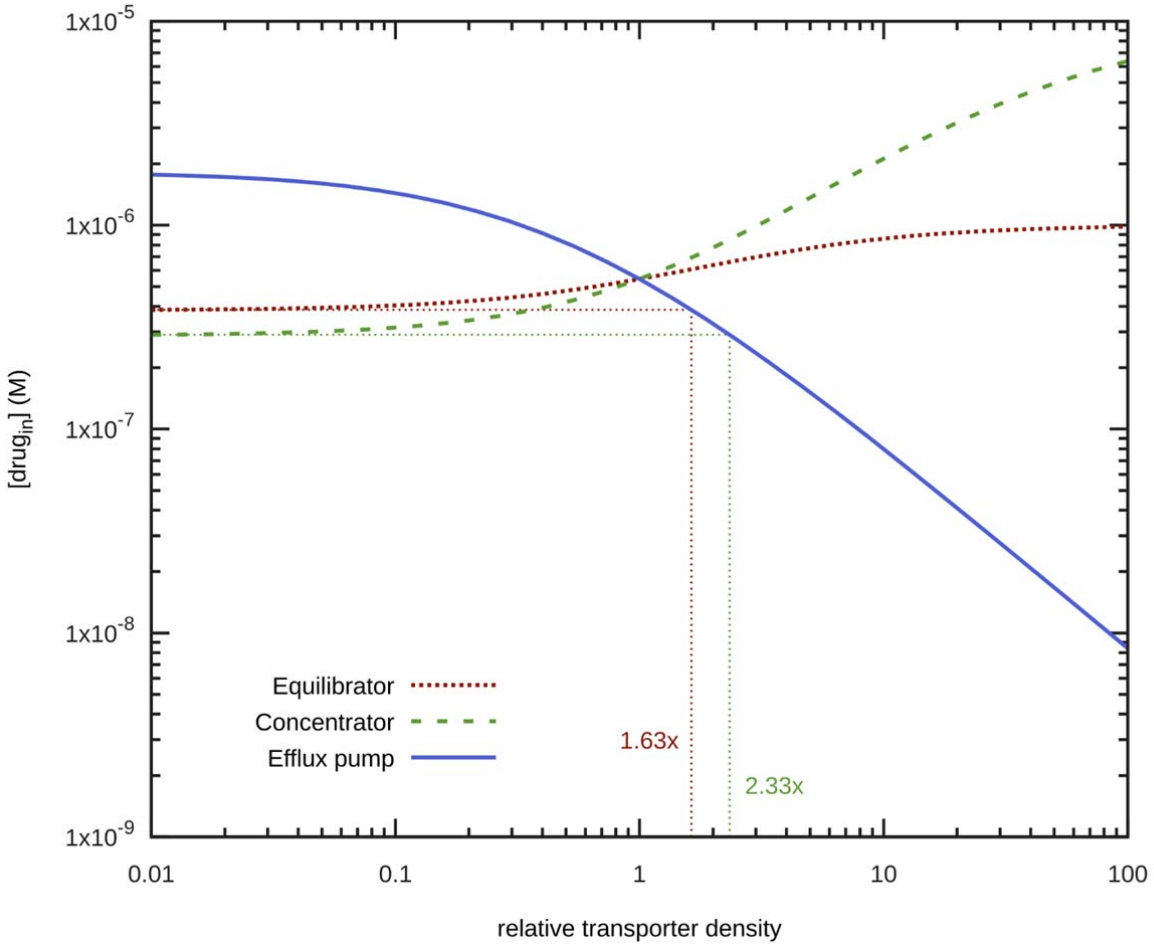

Fig 2 Effect of varying the relative rates of the three generic transporters individually on the normalized accumulation of an antibiotic. Parameters as in Methods and the supplementary files, with K_{eq} for transporter B set at 10.

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160 It is clear that there is a very strong asymmetry; decreasing the individual activities of the
161 equilibrative or concentrative transporters even 100-fold has only a 1.63- or 2.33-fold effect on the
162 steady-state intracellular concentration of the drug, while increasing the effluxer activity by the
163 same amount lowers the intracellular concentration fifty-fold.

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165 Changing the (maximal) degree to which the concentrator concentrates (viz 100-fold rather than
166 10-fold) also has no material effect on the results when individual transporter activities are lowered,
167 and only a marginal effect when the activity of the concentrator is raised (Fig 3, top right).

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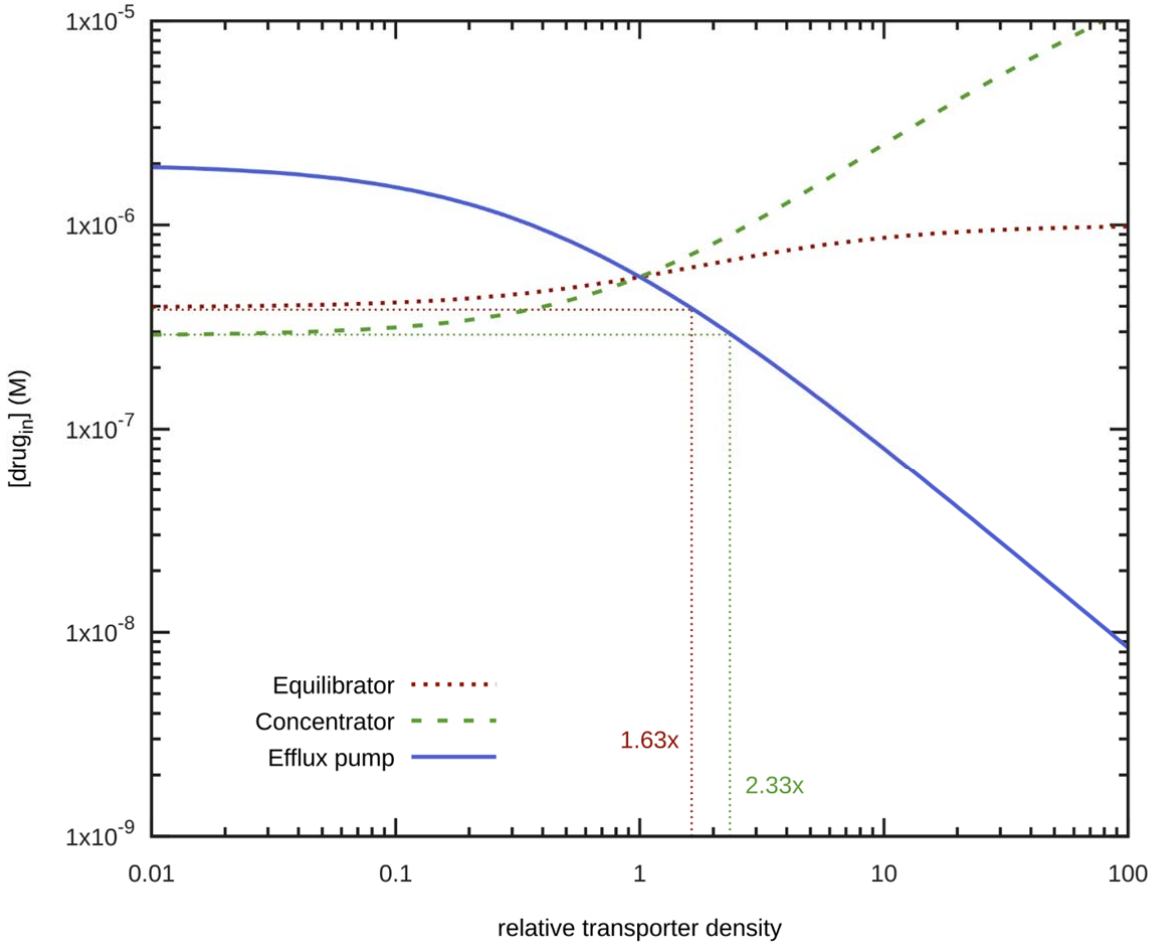

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170 Fig 3. Effect of varying the relative rates of the three generic transporters on the normalized
171 accumulation of accumulation of an antibiotic. Parameters as in Methods and the supplementary
172 files, with K_{eq} for transporter B set at 100.

Discussion

Microbial resistance to antibiotics (AMR) remains a huge problem (e.g. [67-72]). To this end, a major cause is the ability of efflux pumps to create resistance to antibiotics by pumping them out from the cytoplasm of cells (e.g. [25-45]). This is true for cytotoxic substances more generally, including anti-cancer drugs [42, 48]. Many efflux transporters are sufficiently active that even when the drug has relatively tight intracellular binding sites they can effectively remove almost all of it, as is the case with AcrAB/TolC and ethidium bromide [73, 74]. A recent experimental survey of several hundred gene knockouts in *E. coli*, using fluorescent probes as antibiotic surrogates showed that dozens of such efflux transporters could be active and thereby contribute to lowering the steady-state uptake [47]. There is also considerable redundancy and plasticity [75]. Thus, as expected from metabolic control analysis, while there is little effect of single-gene knockouts on fluxes [76], there can be potentially very large effects on the concentrations of intermediary metabolites [77, 78] or, as in our model, the intracellular concentration of an antibiotic of interest,

If there is only a single influx transporter (or one that is overwhelmingly dominant) for a cytotoxic drug of interest, as occasionally happens [13], inhibiting it can lower the toxicity of the drug enormously; in the case of YM155 (sepantronium bromide) this could be by several hundredfold [13]. However, it is possible that mutation of a non-redundant influx transporter might also induce significant metabolic costs, although there are also constraints [79]. Moreover, most cytotoxic drugs can be taken up by multiple transporters [80, 81], and affecting all of them simultaneously is probably not realistic.

The consequences of our simple model are thus clear: in order to inhibit the development of antimicrobial resistance, we need to be able to inhibit the efflux pumps that such bacteria possess and use in abundance. To this end, it is indeed widely considered that inhibitors of efflux pumps might well have a role to play in reducing AMR [42, 82-85]. The present simulations put this thinking on a firm and quantitative footing.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

Legends to figures

(above)

Supplementary information

A zip file containing the COPASI model and results files.

Author contribution statement

EG and GSF originally posed the problem to DBK. DBK defined a suitable system and suggested the idea of modelling it. PM ran all the simulations. All authors contributed to the writing of the ms.

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