

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

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## 45 **Keywords**

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48 AMR – antimicrobial resistance – efflux pumps – transporters – antibiotics

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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## 72 **Introduction**

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

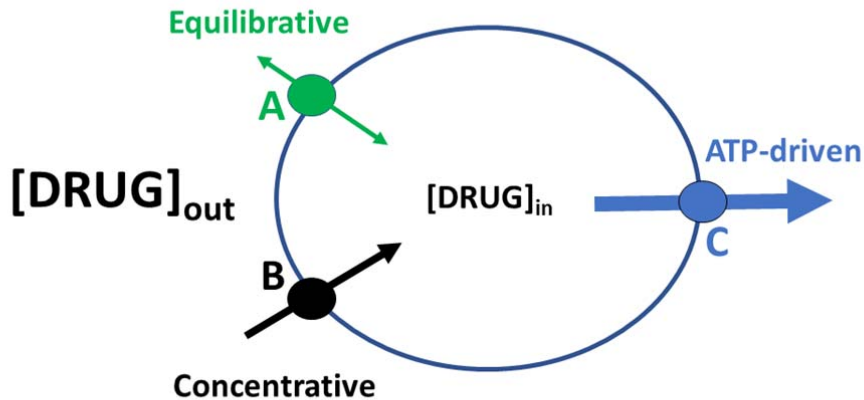
82

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

95

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

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



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

106

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

118

## 119 **Materials and methods**

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

124

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

132

133 The model was set up to mimic typical assays, and parameters were set to values that are  
134 comparable to what is found in the literature as follows. Total volume of the assay is 150  $\mu\text{l}$  (from  
135<sup>61</sup>). Each assay is estimated to have  $10^6$  cells, with an average volume of  $4 \times 10^{-15}$  l per cell<sup>62</sup>  
136 (grown in rich media). Estimates of the proportion of volume taken by the periplasm are around  
137 30%<sup>63</sup>. Thus, the total cell volume in the assay is estimated at  $4 \times 10^{-9}$  l and the cytoplasmic volume  
138 at  $2.8 \times 10^{-9}$  l. For the inner membrane surface area we adopt the average value in the range  
139 considered by Wong and Amir<sup>64</sup>  $34.5 \mu\text{m}^2$  ( $3.45 \times 10^{-7} \text{ cm}^2$ ), which corresponds to a total  
140 surface area of  $0.345 \text{ cm}^2$  (*i.e.* for all  $10^6$  cells); note that Thanassi *et al.* provide an  
141 estimate 3-fold lower ( $0.103 \text{ cm}^2$ )<sup>65</sup>.

142

143 Kinetic parameters for the efflux pump (*C*) come from Nagano and Nikaido for AcrB (part  
144 of *acrAB/tolC*) with nitrocefin<sup>66</sup>; they cite a  $K_m$  of 5  $\mu\text{M}$ ,  $k_{cat}$  of  $10 \text{ s}^{-1}$  and a  $V_{max}$  of  $2.35 \times 10^{-11}$   
145  $\text{mol/s}/10^9$  cells, which implies a total of  $2.35 \times 10^{-12}$  mol of transporter. Considering that our  
146 simulation contains  $10^6$  cells, the adjusted amount of transporter is then  $2.35 \times 10^{-15}$  mol

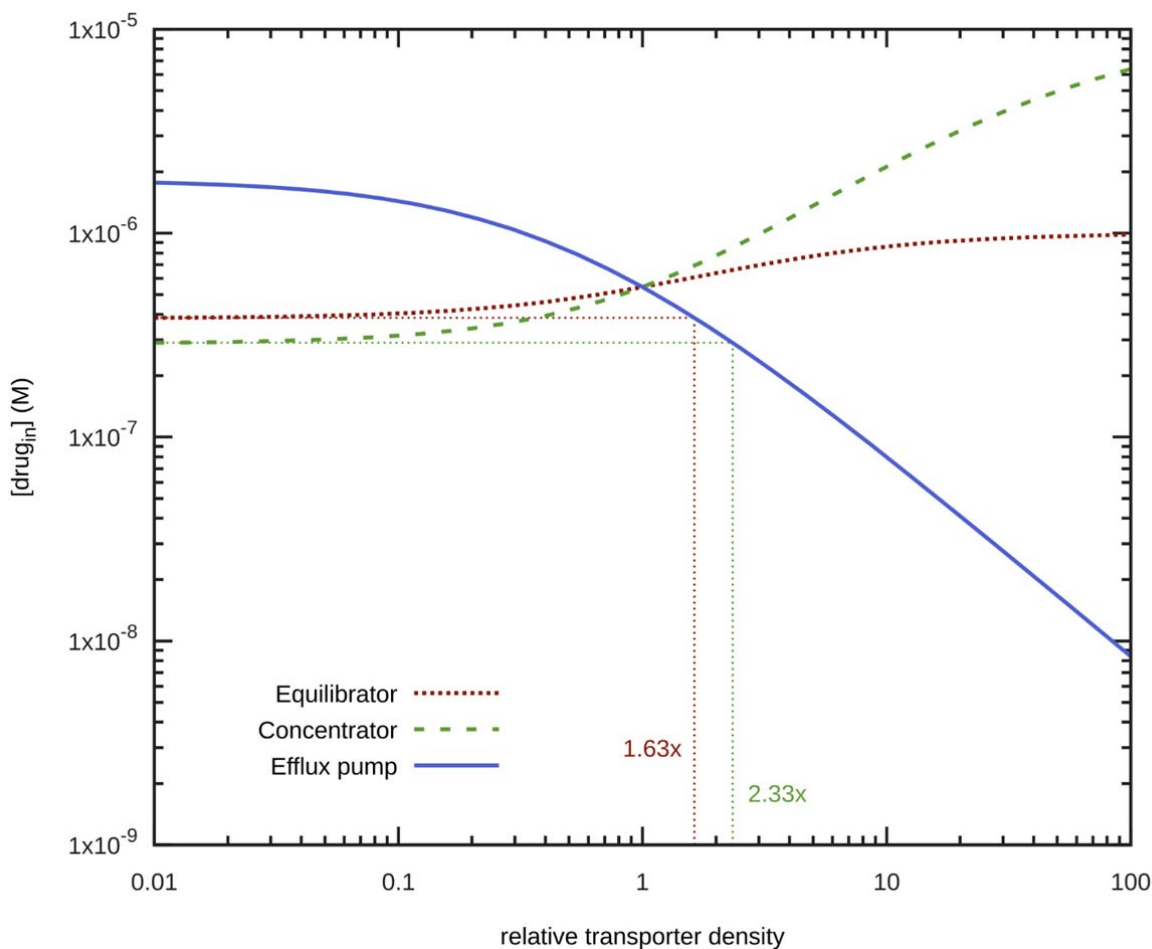
147 (considering the surface area estimated above, this corresponds to a surface density of  $6.8 \times 10^{-15}$   
148 mol/cm<sup>2</sup>) with a  $V_{max}$  of  $2.35 \times 10^{-14}$  mol/s, assuming the same  $k_{cat}$  as for nitrocefin. For  $K_m$   
149 we chose a higher value (500  $\mu$ M).

150

151

## 152 Results

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



155

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

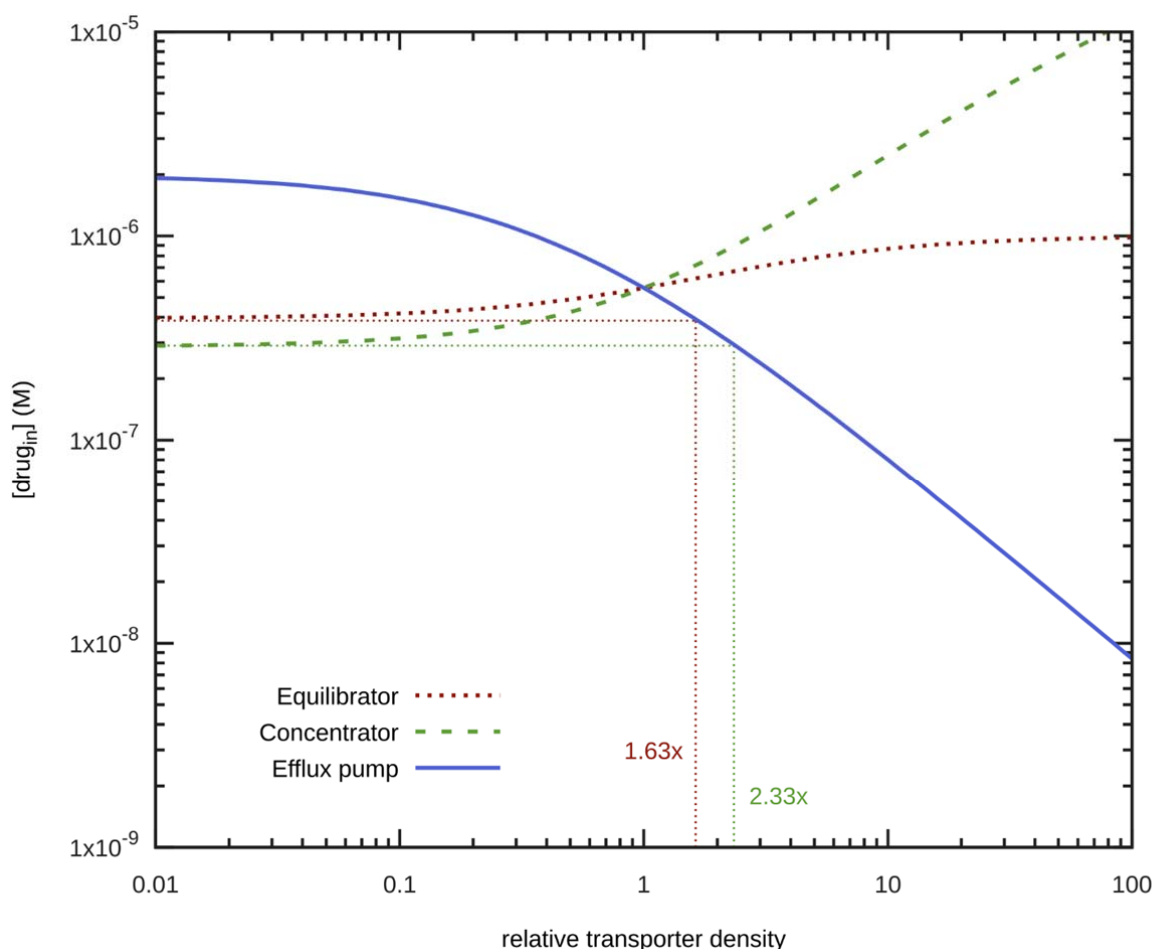
159

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.

164

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).

168



169

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.



173

174

## 175 **Discussion**

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

188

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

196

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

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211

## 212 **Conflict of interest statement**

213 The authors declare that they have no conflicts of interest.

214

## 215 **Legends to figures**

216 (above)

## 217 **Supplementary information**

218 A zip file containing the COPASI model and results files.

219

## 220 **Author contribution statement**

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

223

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