Opinion

Fitting Transporter Activities to Cellular Drug Concentrations and Fluxes: Why the Bumblebee Can Fly

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A recent paper in this journal argued that reported expression levels, $k_{cat}$ and $K_m$ for drug transporters could be used to estimate the likelihood that drug fluxes through Caco-2 cells could be accounted for solely by protein transporters. It was in fact concluded that if five such transporters contributed ‘randomly’ they could account for the flux of the most permeable drug tested (verapamil) 35% of the time. However, the values of permeability cited for verapamil were unusually high; this and other drugs have much lower permeabilities. Even for the claimed permeabilities, we found that a single ‘random’ transporter could account for the flux 42% of the time, and that two transporters can achieve $10^{-6}$ cm·s$^{-1}$ 90% of the time. Parameter optimisation methods show that even a single transporter can account for Caco-2 drug uptake of the most permeable drug. Overall, the proposal that ‘phospholipid bilayer diffusion (of drugs) is negligible’ is not disproved by the calculations of ‘likely’ transporter-based fluxes.

Pre-eminence of Transporter-Mediated Drug Uptake

For cases in which a drug must interact with one or more intracellular targets, and for all oral drugs, it is necessary for drugs to cross at least one biomembrane. There is an increasing recognition that to cross intact biological membranes drugs must or do hitchhike on transporters that are normally involved with intermediary metabolism (e.g. [1–12]). It is therefore of interest to understand how the use of specific influx and efflux transporters translates into particular transmembrane fluxes and intracellular concentrations (and hence the biological effects of drugs and other solutes). A recent example [13] brings the issue into sharp focus, where removing (genetically) just a single transporter decreased the toxicity (and presumably accumulation) of the drug YM155 (sepantronium bromide) by several hundred-fold. The implication of such data is that any ‘background’ rate involving phospholipid bilayer diffusion must be rather less than 1%, or (as we have put it elsewhere [9,10]) ‘phospholipid bilayer diffusion is negligible’. Another recent example (see Figure 2 in ref [14]) shows that metformin uptake can be accounted for entirely by four transporters. Indeed, this essential lack of permeability in the absence of suitable transporters readily accounts for the failure of drugs to penetrate to the sites where they are required. Anti-tuberculosis drugs provide another important and (for patients) damaging example [15,16].

The nonlinear nature of many biochemical kinetics, and the complex behaviour of even simple biochemical pathways, means that it is hard to ‘guess’ what might happen without seeking to model it first (e.g. [17–19]). Thus, a recent article in this journal [20] (and its subsequent

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supplementary information [21] sought to carry out just such a modelling study, based on a series of stated assumptions. The authors [20] also drew a major conclusion that (we consider) was at some variance with the data presented. The two main purposes of the present paper are (i) to go through their data and main argument, and, (ii) because natural evolution has at least one selection step, to study what happens when instead of making assumptions solely about forward modelling, one simply fits the observables to appropriate models and their parameters (Figure 1).

A Note on the Word ‘Passive’ and Why One Should Use More Explicit Alternatives

Despite our clear previous explanation of this term [9], Matsson and colleagues [20] (and many other workers) continue to use the word ‘passive’ to mean two entirely different things (Figure 2). The first usage involves a thermodynamic statement only, and is best referred to as ‘equilibrative’ (‘passive’ transport is thermodynamically equilibrative; the ‘active’ version requires an input of free energy and is then concentrative). We would stress that, as such, the word ‘passive’ has nothing of itself to say about a mechanism of how a drug crosses a membrane. However, ‘passive’ transport is also far too often taken to mean ‘transport via bilayer lipidal’ diffusion, a perfectly acceptable intent provided this is made explicit, but one that is then best served by calling it ‘bilayer lipidal diffusion’ directly. Carrier-mediated diffusion may be active or passive in the thermodynamic sense (and, for those purposes, is best referred to as either concentrative or equilibrative). A very well-established term for the latter (carrier-mediated equilibrative transport) is ‘facilitated diffusion’, while the term ‘active transport’ is perfectly adequate for concentrative transporter-mediated solute influx (or efflux). All of this therefore entirely avoids the ambiguity common with the use of the term ‘passive’. We reiterate strongly that much trouble would be avoided if the word ‘passive’ were dropped completely from all debates about transmembrane drug uptake mechanisms. Conflating the two by showing its truth for one meaning (thermodynamic) but then claiming that this thereby shows the other meaning of bilayer lipidal is at best unscientific. (Zheng and colleagues [22] illustrate this with an example in which bilayer transport was not even measured directly as a dependent variable, and for a drug whose uptake is stereoselective and hence necessarily transporter-mediated.)
A less ambiguous terminology of transport reactions that avoids use of the word ‘passive’

**Mechanistic aspect of transport**

| Purely lipoidal | Transporter-mediated |
|-----------------|----------------------|
| May be driven by e.g., a pH gradient or membrane potential | Active transport |

**Thermodynamic aspect**

| Concentrative | Equilibrative |
|---------------|--------------|
| Simple diffusion | Facilitated diffusion |

Fig. 2. Two Orthogonal Aspects of Cellular Uptake in which the Word ‘Passive’ Is Sometimes (and Unhelpfully) Used to Describe (and, in the Worst Cases, Confute) Two Completely Different Concepts. The first is a thermodynamic usage meaning ‘equilibrative’, for which the antonym is ‘active’ or better ‘concentrative’. The second usage is intended to be a mechanistic usage, and is sometimes taken to mean ‘via bilayer lipoidal bilayer diffusion’, in which case it is best to state this. Carrier-mediated but equilibrative diffusion is historically referred to as ‘facilitated diffusion’. Needless to say, showing that transport is equilibrative (or ‘passive’) does not explain whether its uptake is transporter-mediated or otherwise. To avoid any such ambiguity, we suggest strongly that all workers simply avoid the word passive entirely, and replace it with words that describe precisely and explicitly which of the two meanings (thermodynamic vs mechanistic) is intended.

**Fluxes across Caco-2 Cell Membranes Explicable Via Transporter Reactions**

Matsson and colleagues [20] proposed, as a model, the well-known Caco-2 cell system, and sought to estimate how ‘likely’ it was, given the known expression profiles and $k_{cat}$ values of a subset of transporters, whether or not they could reasonably be expected to account for the fluxes observed in the case of two drugs (propranolol and verapamil) with unusually high permeabilities. At first glance, this is an interesting idea. Note that Caco-2 cells are thought (from transcriptomics or proteomics measurements) to express several hundred (e.g. [23–25]) of the ca 450 catalogued SLC transporters, although (i) there is considerable variation in this between laboratories [26], (ii) it is not known how reliable the expression profiling data are [26], and (iii) it is recognised that ‘unknown’ transporters might be present. Thus, some of the authors of [20] already published that there is an enormous expression level of an ‘HPT1’ human peptide transporter [26,27] (indeed it is the highest expressed transporter in Caco-2 cells in each of the 10 laboratories participating in [26]), but such a transporter seems to make no appearance at all in [20]. Thus, in the absence of any knowledge, nor of the inclusion of such highly expressed transporters, these estimates are always likely to be underestimates. We entirely appreciate the complexities of biological systems, and hence, the difficulty of reproducing the behaviour of even the well-established Caco-2 system. However, to give an indication of the variance observable within and between laboratories, Box 1 shows some of the data from precisely such a comparison [26]. Obviously the variance between laboratories for the three drugs atenolol, metoprolol and talinolol is at least an order of magnitude (sometimes more), with their median values for A → B being ca 0.5, 45 and $1.34 \cdot 10^{-6}$ cm·s$^{-1}$.

Regarding the choice of drugs, Matsson and colleagues [20] state “Classical examples include propranolol and verapamil. These have permeability coefficients across Caco-2 intestinal epithelial cell monolayers (the most commonly used cellular barrier for permeability studies)
in the range 200–1000 - 10^{-6} cm s^{-1} [28,29].” Actually the rate published for R- or S-verapamil in [28] was ~100 - 10^{-6} cm s^{-1}, and even decreased as concentrations exceeded 100 µM, presumably because of substrate inhibition, with a similar value in [29]. Some of the authors of Matsson et al. [20] in their reference 19 [30] published a value of 155 - 10^{-6} cm s^{-1}, that for propranolol in Artursson and Karlsson [31] was 41.9 - 10^{-6} cm s^{-1}, in Carmenisch et al. [32]
41.7 · 10⁻⁶ cm·s⁻¹, van Breemen and Li [33] gave 50 · 10⁻⁶ cm·s⁻¹, while that for propranolol in Figure 3 of [29] was ~700 · 10⁻⁶ cm·s⁻¹, but no matter. Corti and colleagues [34] (their Table 2) give 41.9, 10⁶ cm·s⁻¹ for propranolol and 15.8 · 10⁻⁶ cm·s⁻¹ for verapamil. This said, the ‘observable’ rates stated in Figure 3A(i) of [20] as 1310 · 10⁻⁶ cm⁻¹ for verapamil and 230.10⁻⁶ cm⁻¹ for propranolol come from Table 3 of a paper by Avdeef [29] (P. Matsson, personal communication), and are obviously at some variance with these other numbers. (They are based on a very rapid stirring – 700 rpm – that does not occur adjacent to natural epithelia.) Anyway, although these high values are close to being complete outliers (Table 1), we shall take the larger numbers as given, and the question arises as to whether typical fluxes of individual carriers can come close to being able to achieve these overall values of \( P_{\text{app}} \).

The authors [20] (and most of the data have subsequently been made available as Supplementary Information [21]), took random samples of individual transporters whose \( k_{\text{cat}} \) values (for just 18 transporters using unstated substrates), \( K_m \) and expression levels were drawn from a random distribution of a known subset. Note the wide variation for each one – in Figure 2B of [20] the \( k_{\text{cat}} \) value for VMAT2 varied 200-fold). They found [20] that that the observed rates for verapamil and propranolol at 50 \( \mu \)M were reached in 7% and 18% of cases, and that if it is was assumed that five transporters might be involved equally then this would be found for 35% of cases for verapamil (and presumably a significantly greater percentage for propranolol, though that was not stated). Presumably these drugs were chosen because of their high fluxes, albeit that their uptake shows enantioselectivity (e.g. [35,36]) and thus must be transporter-mediated, so this is very far from making this an ‘unlikely’ event. Thus, even though we consider this to be entirely the wrong strategy, this seems to us to be a rather positive endorsement of the fact that most flux is perfectly capable of going via transporters even for drugs that were apparently chosen to have the highest total rates. Matsson et al. [20] also comment that “marketed drugs target between one and eight distinct proteins (5th to 95th percentile range [37]).” Actually, on average each marketed drug has six known targets [38], so we may assume this is something of an underestimate. In the case of verapamil, it is transported by multiple isoforms of SLC22 [39–41] among others yet uncharacterised [42,43], as is propranolol [44,45], so the calculations presented by Matsson and colleagues are necessarily likely to underestimate the transporter-mediated fluxes. As we have said before [6,9], absence of evidence is not evidence of absence. It is also worth commenting that, in the absence of other knowledge, the absolute transcript level alone can be an adequate surrogate for predicting fluxes in genome-wide studies [46].

However, natural (Darwinian) evolution has a selection step in it, and it is precisely this that accounts for the fact that complex organisms evolve, however ‘unlikely’ or ‘implausible’ that may be [47–49]. Thus, from our perspective, the correct strategy is to start with the data and find the parameter values that can fit it for one or more transporters, and how often such a fit can be obtained [17,50]. This was performed 1000 times, and on each occasion, with just a single transporter, we could, within the bounds of the parameters given by Matsson and colleagues, achieve a flux of 1310 · 10⁻⁶ cm·s⁻¹ on every single occasion. We therefore did not repeat the analysis with more than one transporter. The data are given in Figure 3. Two features are of note. First, and fairly obviously, is the fact that a given \( V_{\text{max}} \) can be obtained from varying the coupled values of \( k_{\text{cat}} \) and transporter concentration. Secondly, although they represent different aspects of enzyme action [51], the values for \( V_{\text{max}} \) and \( K_m \) are not actually completely independent of each other under selection. This is in fact related to the Haldane relationship discussed below.

**Permeabilities of Other Drugs**

A table of various substances’ permeability coefficients in Caco-2 cells is given in Table 1 of [30] (and stated to have been redrawn in Figure 2A of [52], though the former has 23 and the latter 31 data points). (Note that Bergström and colleagues [30] also avoided unstirred layer effects, albeit that they anyway have equal (ir)relevance to measurements of fluxes and the transporter kinetics...
Table 1. A Comparison of the Values of Caco-2 Permeability Chosen for Verapamil and Propranolol by [20] (and Taken from [29]) with Those Given in Various Other Papers

| Compound   | $10^6 \times \text{Caco-2} \ P_{\text{app}} \; \text{cm s}^{-1}$ | Reference |
|------------|---------------------------------------------------------------|-----------|
| verapamil  |                                                                 |           |
|            | 1310                                                          | [20,29]   |
|            | 155                                                           | [30]      |
|            | 15.8                                                          | [34]      |
|            | 26.3                                                          | [32]      |
|            | 9.8                                                           | [82]      |
|            | 45.7                                                          | [83]      |
|            | 12.4                                                          | [84]      |
|            | 152                                                           | [85]      |
|            | 62.4                                                          | [86]      |
|            | 69.4                                                          | [87]      |
|            | 22                                                            | [88]      |
|            | 22–24                                                         | [89]      |
|            | 9                                                             | [90]      |
|            | 25                                                            | [91]      |
|            | 22                                                            | [92]      |
| propranolol|                                                                 |           |
|            | 230                                                           | [20,29]   |
|            | 41.9                                                          | [34]      |
|            | 50                                                            | [33]      |
|            | 27.5                                                          | [60]      |
|            | 29.2                                                          | [64]      |
|            | 25.8                                                          | [64]      |
|            | 44.6                                                          | [64]      |
|            | 39.8                                                          | [64]      |
|            | 57                                                            | [64]      |
|            | 59.7                                                          | [64]      |
|            | 30.1                                                          | [81]      |
|            | 41.7                                                          | [32]      |
|            | 17.5                                                          | [82]      |
|            | 26.3                                                          | [63]      |
|            | 39.8                                                          | [83]      |
|            | 12.9                                                          | [84]      |
|            | 27                                                            | [93]      |
|            | 8–16                                                          | [35]      |
|            | 35.3                                                          | [94]      |
|            | 21.8                                                          | [62]      |
|            | 27.5                                                          | [87]      |
|            | 11.1–27.7                                                     | [95]      |
|            | 16                                                            | [88]      |
|            | 21–36                                                         | [89]      |
|            | 8.2                                                           | [96]      |
Caco-2 permeability values for various drugs

Figure 4. Some Values of Caco-2 Permeability of Various Drugs and Their Relative Independence from Log P.

Data are replotted from Table 1 of [30].

with which they are supposed to be comparing.) We have plotted out those data (Figure 4), from which at least three conclusions are evident: (i) the \( P_{app} \) for very few of the compounds exceeds even \( 100 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \), and of the only two that exceed \( 200 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \), one (ethinyl estradiol) is a steroid that is heavily metabolised to its sulphate and is transported by the sterol transporter SLC51 [53,54] and (as the sulphate) by a series of anion transporters [55–57], while the other (phenazopyridine) is a rarely used local anaesthetic (and adenine analogue) that, in fact, is seen as poorly transported/metabolised (class IV) in the BDDCS system [58]; (ii) there is no discernibly linear relationship between permeability and the log of the octanol:water partition coefficient (see also [1,9]) (that we have purposely plotted on the ordinate to highlight the fact that it is not an independent variable), (iii) as previously pointed out [4,6,9] almost all of them do have known transporters. While the contributions of paracellular and efflux transporters is not known (and verapamil is a well known P-gp inhibitor, e.g. [59]), similar conclusions on the normally rather lower values for Caco-2 permeability may be drawn from the compilations of Artursson & Karlsson [31] (20 drugs, highest permeability \( 54.5 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \)), Corti et al. [34] (21 drugs, highest permeability \( 83 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \)), Yee [60] (~26 drugs, highest permeability \( 71 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \)), Carmenisch et al. [32] (~25 drugs, highest permeability \( 61.7 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \)), Pade & Stavchansky [61] (9 drugs, highest permeability \( 45.5 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \)), Yazdianian et al. [62] (51 drugs, highest permeability \( 36.6 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \)), Hou et al. [63] (77 drugs, highest permeability \( 52.5 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \)), Uchida et al. [64] (8 drugs, highest permeability \( 55.3 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \)), and Lozoya-Agullo et al. (2015) [65] (15 drugs, highest permeability \( 41.8 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \)). The median permeability of the drugs listed in the cited references is less than \( 20 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \), which is considerably lower than the kinds of numbers given above and highlighted in [20].

As described in Box 2 and the supplementary information, we have also used COPASI to model this system using 10,000 values of \( K_m \), \( K_m \), and protein expression drawn from the best-fit log-normal distribution given in the supplementary data [21] of [20]. A number of points follow from this Figure: (i) there is a tendency for a particular transporter to dominate, i.e. there is a law of diminishing returns, (ii) in our hands, we could achieve the ‘target’ flux of \( 1310 \times 10^{-6} \text{ cm}^{-1} \text{s}^{-1} \) for
**Box 2. Materials and Methods and Relevant Calculations**

As described in the supplementary material of Matsson et al. [20], an apparent permeability, \( P_{\text{app}} \), can be calculated from the flux of a drug passing through one or several transporters. First the steady state flux, \( J_e \), at which the drug passes through each transporter is calculated using the Henri-Michaelis-Menten equation, given: the concentration of the drug (\( \left[ D \right] \)), the concentration of the transporter (\( \left[ T \right] \)), the area (\( A \)), the area density of proteins in the membrane (\( \rho \)), the turnover number \( k_{\text{cat}} \), and the Michaelis constant \( K_m \). The sum of the steady-state fluxes \( J_{\text{tot}} \) through all the transporters is then the total steady-state flux of drug entry:

\[
J_{\text{tot}} = \sum J_e = \sum \frac{\left[ T \right]_0 \times A \times \rho \times k_{\text{cat}} \times \left[ D \right]}{K_m + \left[ D \right]},
\]

That steady-state flux of drug entry would correspond to a certain apparent permeability \( P_{\text{app}} \) through the following equation:

\[
P_{\text{app}} = \frac{J_{\text{tot}}}{A \times \rho} \times \frac{1000}{A \times \rho},
\]

where \( A \) is the area of the membrane in the permeability assay, taken as 0.33 cm² (Matsson, personal communication).

The factor 1000 converts the concentration of the drug (\( \left[ D \right] \)) from pmol·L⁻¹ to pmol·cm⁻². We constructed a kinetic model in the software COPASI [97] version 4.15 that incorporates Eqs (1) and (2) and supply this model as supplementary data in the SBML format [88]. We then used this model to: (a) find many sets of parameter values that lead to rates of entry through a single transporter equivalent to the permeability of verapamil, and (b) generate 10,000 models with those parameters sampled randomly from appropriate distributions (see supplementary data) with 1, 2 and 5 transporters. We provide COPASI native files for both (a) and (b) in the supplement.

It is, as usual, necessary that all numbers entered in Eqs (1) and (2) be in compatible (i.e. self-consistent) units. Thus to make this process more transparent, we converted all data in the supplementary material of Matsson et al. [20] to compatible units as follows:

- transporter concentrations (\( \left[ T \right]_0 \)): pmol·mg⁻¹ total protein;
- drug concentrations (\( \left[ D \right] \)): pmol·L⁻¹;
- area (\( A \)): cm²;
- protein area density: mg·cm⁻²;
- turnover numbers (\( k_{\text{cat}} \)): s⁻¹;
- Michaelis constant (\( K_m \)): pmol·L⁻¹;
- fluxes (\( J_e \) and \( J_t \)): pmol·s⁻¹;
- apparent permeability (\( P_{\text{app}} \)): cm·s⁻¹.

Verapamil with just a single transporter on more than 12% of the occasions (Figure 5), and for 2, 3, 4 and 5 transporters the percentage successes were 23%, 35%, 45% and 54% (the latter marked on the Figure), (ii) for propranolol the success with 5 transporters was 80% and, for a more typical value for \( P_{\text{app}} \) of 1.0·10⁻⁶ cm·s⁻¹, we could achieve this in 90% of simulations for 5 transporters (Figure 5). (An entirely separate simulation in R – not shown – led to the same conclusion.)

Given that entirely reasonable expectations of transporter expression profiles can thus easily account for the fluxes of even the most rapidly permeable drugs, and even more so for the vast majority of other less permeable drugs, we see no need to invoke bilayer lipoidal permeation at all. In many cases, the transporters involved in Caco-2 transport are entirely well established and leave no room for bilayer lipoidal diffusion. Of course the fact that most drugs have nothing like those large permeabilities means that it is even easier to explain their permeabilities even in terms of ‘random’ expression levels, \( K_m \) and \( k_{\text{cat}} \) values (Figure 5).

**Explicability of a Solely Transporter-Mediated Flux of Some Other Drugs**

We noted above the fact [13] that much more than 99% of the transport of sepantronium bromide (YM155) could be shown to pass through a single transporter (SLC35F2), and have stressed [9] that a straightforward way of estimating this is to vary the expression levels of known transporter enzymes. Thus, Chu and colleagues [66] varied the expression level of the PepT1 (SLC15A1) transporter in Caco-2 cells and looked at the effect of this on the transport of cepheixin. We have replotted those data in Figure 6, where it is obvious that, within experimental error, the background rate in the absence of SLC15A1 is indistinguishable from zero. To
interpret this, we can do little better than quote the original: “In Caco-2/hPEPT1 cells, an excellent correlation was observed between cephalaxin uptake and hPEPT1 expression ($R^2 = 0.96, P < 0.005$). This demonstrates that cephalaxin uptake is directly proportional to hPEPT1 expression” [66].

So, to be clear, even with the most extreme assumptions (most permeable drugs, not recognising all the transporters and their multiple isoforms, no selection for $k_{cat}$, independence from each other of individual transporter expression profiles, $k_{cat}$ and $K_m$, etc.) most of the time one can in fact easily account for $P_{app}$, simply on the basis of the arguments and data presented [20], for a fully transporter-mediated transport of drugs. There is consequently no need to invoke lipid bilayer diffusion at all.

**Two Irrelevancies on which We Have Nothing Discriminating to Say**

Matsson et al. [20] also make much of two other features: (i) a statement (no actual data are shown) that transport rates are ‘linear’ with substrate concentrations over wide ranges, and that this supposedly cannot be explained by combinations of transporters, and (ii) that equality of transport rates in two directions is hard for transporter-only theories to explain. Regarding (i), we have previously pointed out [6,9] that, especially in the absence of any knowledge of the transporters involved nor their detailed enzyme kinetics, linearity or its lack is not a criterion of anything (similarly, on the other side, we do not seek to claim that saturation ‘proves’ transporter involvement). Regarding (ii) we have also previously pointed out [6] that, for equilibrative transporters performing facilitated diffusion, this is a simple thermodynamic consequence of the Haldane relation (of enzyme kinetics, that can be read in any suitable textbook.
such as [67–69]). Specifically, the Haldane relation states that \( (V_{m,f} \times K_{m,f})/(V_{m,d} \times K_{m,d}) = K_{eq} \).

Not only do transporters explain this bidirectional equivalence of fluxes straightforwardly but it is a necessary fact for enzymes or transporters where \( K_{eq} = 1 \). Put another way, for a given external substrate concentration, instantaneous fluxes can differ between the two directions in a Caco-2 set-up even when \( K_{eq} = 1 \) (i.e. transport is equilibrative), simply because \( K_{m} \) and \( V_{max} \) values can be whatever they are, subject to the constraint of the Haldane relationship. Matsson et al. [20] state “equilibrative transporters (which mediate substrate flux along concentration gradients; (their) Box 1) can – under certain circumstances – give rise to direction-independent rates. Thus, near-unity flux ratios do not unambiguously exclude transporter involvement”. Indeed they do not, as when measurements are performed properly (a recent example of near-unity ratios is [70]) they directly reflect the Haldane relationship. Possibly a failure to understand this principle follows from the conflation of two meanings of the word ‘passive’, but we do hope that this particular line of reasoning can be cast properly in the context of the Haldane relationship, which is where it belongs.

**What Criteria Should One Use to Assess the Role of Transporters in Drug Uptake?**

We have previously set down why some criteria raised in this debate about the mechanisms of transmembrane drug transport are simply non-discriminatory. We gave two above and others elsewhere [9]. These are not therefore of interest. Much more important is a general strategy used throughout modern molecular genetics to determine the involvement of a gene (product) in a process. This is to vary the expression of the gene product as an independent variable (whether as a knockdown or via a regulatable promoter such as tetO [71]), and to observe the effects of that on the dependent process of interest (such as uptake transport). We already gave many hundreds of examples [1]. Similar comments apply to the role of the Henle-Koch postulates in microbiology (e.g. [72,73]).
However, Mattson et al. state “At first glance, the transporters only model may appear impossible (or at least extremely daunting) to test: to exhaustively confirm the hypothesis, one would need to identify the missing carriers for all transported drug molecules”. Not at all, and it is no more daunting than seeking the genes (and their products) that are responsible for any biological process of interest. Certainly the first step in any systems biology model is qualitative – to identify the players [7,8,18,19]. However, when one has identified them, it is easy to assess their contributions, and we gave examples above (such as that for cephalaxin in Figure 6). Indeed Matsson et al. [20] later comment “One avenue to identify such novel (sic) drug transporters would be the use of genome-wide single-gene knockout libraries in model organisms like Saccharomyces cerevisiae, CRISPR–Cas9 knock-out libraries in human cells, or human haploid genetic screens. Oddly enough this is precisely what we have previously stressed [9], and what we [11] already did (though these papers were not cited by Matsson et al. [20]). Others have adopted a similar and highly effective strategy (e.g. [13]) showing extremely clearly that when the pertinent transporters are removed the background uptake (or toxicity of a cytotoxic drug) is negligible. What we now need are QSAR models for each of the main transporter families, to incorporate into the digitally available human metabolic network [8,74,75].

Other Evidence That Protein Carrier-Mediated Transport Is the Dominant Means of Transmembrane Uptake of Pharmaceutical Drugs
As we have stressed before (e.g. [6,9,10,76]), and we do not repeat the references here, there is considerable evidence for a requirement for transporters for the transmembrane transport of even very small and often hydrophobic molecules. These include alkanes, fatty acids, gases such as CO₂, O₂ and NO, ammonia, glycerol and so on, so the bilayer lipid permeability in real biological membranes must necessarily be very small. This also provides a ready explanation for a variety of features that are not easily explained (at least without extra ad hoc hypotheses) by a view that has it that much or most of the cellular uptake of pharmaceutical drugs occurs through the phospholipid bilayer. Indeed, given that the effect of changing lipids in biophysical terms is not seen as that great, any heterogeneity of uptake between cells, tissues and organisms is most simply explained in terms of the varying expression of the relevant transporters [4,6,9,10]. Imaging mass spectrometry (e.g. [77–79]) is beginning to provide outstanding data on the very considerably extent of heterogeneity of drug transport and distribution, while the human proteome atlas [80] and comparable transcriptome data [81] show the equivalent heterogeneity of transporters and other proteins.

Concluding Remarks
In conclusion (and see also the Outstanding Questions box), the test proposed [20] to see if a random selection from a nominally known distribution of properties of known transporters is a nice idea. Despite the opposite interpretation taken [20], however, the forward modelling data do indeed show that transporters can easily account for the uptake of even the most permeable drugs, even when their permeabilities are given as being several times greater than those of other comparable measurements. This is even more the case for all the other drugs that naturally have considerably lower experimental permeabilities. Parameter estimation data based on selection show it even more clearly. In a similar vein, and famously (if apocryphally), it was suggested that physics-based calculations implied that the bumblebee could not fly. Happily the bumblebees were selected by evolution so that they could, just as transporters were selected to be able to sustain the necessary transport fluxes.

Note Added in Proof
A recent major review stresses the importance of the issues discussed in [99].

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**Supplemental Information**

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**Resources**

1. http://en.wikipedia.org/wiki/Bumblebee#Misconception_about_flight
2. www.copassi.org

**References**

1. Dobson, P.D. and Kell, D.B. (2006) Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? Nat. Rev. Drug Discov. 7, 205–220
2. Dobson, P. et al. (2009) Implications of the dominant role of cellular transporters in drug uptake. Curr. Top. Med. Chem. 9, 163–184
3. Giacomini, K.M. et al. (2010) Membrane transporters in drug development. Nat. Rev. Drug Discov. 9, 215–236
4. Kell, D.B. et al. (2011) Pharmaceutical drug transport: the issues and the implications that it is essentially carrier-mediated only. Drug Discov. Today 16, 704–714
5. Giacomini, K.M. and Huang, S.M. (2013) Transporters in drug development and clinical pharmacology. Clin. Pharmacol. Ther. 94, 3–7
6. Kell, D.B. et al. (2013) The promiscuous binding of pharmaceutical drugs and their transporter-mediated uptake into cells: what we need to know and how we can do so. Drug Discov. Today 18, 218–223
7. Kell, D.B. (2013) Finding novel pharmaceuticals in the systems biology era using multiple effective drug targets, phenotypic screening, and knowledge of transporters: where drug discovery went wrong and how to fix it. FEBS J. 280, 5957–5980
8. Kell, D.B. and Goodacre, R. (2014) Metabolomics and systems pharmacology: why and how to model the human metabolic network for drug discovery. Drug Discov. Today 19, 171–182
9. Kell, D.B. and Oliver, S.G. (2014) How drugs get into cells: tested and testable predictions to help discriminate between transporter-mediated uptake and lipoidal bilayer diffusion. Front. Pharmacol. 5, 211
10. Kell, D.B. (2015) What would be the observable consequences if phospholipid bilayer diffusion of drugs into cells is negligible? Trends Pharmacol. Sci. 36, 15–21
11. Lanthaler, K. et al. (2011) Genome-wide assessment of the carriers involved in the cellular uptake of drugs: a model system in yeast. BMC Biol. 9, 70
12. Nigam, S.K. (2015) What do drug transporters really do? Nat. Rev. Drug Discov. 14, 29–44
13. Winter, G.E. et al. (2014) The solute carrier SLC35F2 enables YM155-mediated DNA damage toxicity. Nat. Chem. Biol. 10, 768–773
14. Han, T.K. et al. (2015) Four cation-selective transporters contribute to apical uptake and accumulation of metformin in Caco-2 cell monolayers. J. Pharmacol. Exp. Ther. 352, 519–528
15. Kjellsson, M.C. et al. (2012) Pharmacokinetic evaluation of the penetration of antituberculosis agents in rabbit pulmonary lesions. Antimicrob. Agents Chemother. 56, 446–457
16. Dartois, V. (2014) The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. Nat. Rev. Microbiol. 12, 159–167
17. Mendes, P. and Kell, D.B. (1998) Non-linear optimization of biochemical pathways: applications to metabolic engineering and parameter estimation. Bioinformatics 14, 869–883
18. Kell, D.B. (2006) Metabolomics, modelling and machine learning in systems biology: towards an understanding of the languages of cells. The 2005 Theodor Bücher lecture. FEBS J. 273, 973-984
19. Kell, D.B. (2008) Systems biology, metabolic modelling and metabolomics in drug discovery and development. Drug Discov. Today 11, 1085–1092
20. Matsson, P. et al. (2015) Quantifying the impact of transporters on cellular drug permeability. Trends Pharmacol. Sci. 36, 255–262
21. Matsson, P. et al. (2015) Supplementary Information: addendum to ‘Quantifying the impact of transporters on cellular drug permeability’. Trends Pharmacol. Sci. 36, [http://dx.doi.org/10.1016/j.tips.2015.02.009](http://dx.doi.org/10.1016/j.tips.2015.02.009)
22. Zheng, Y. et al. (2015) pH dependent but not P-gp dependent bidirectional transport study of S-propranolol: the importance of passive diffusion. Pharm. Res. 32, 2516–2526
23. Sun, O. et al. (2002) Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. Pharm. Res. 19, 1400–1416
24. Anderle, P. et al. (2004) Intestinal membrane transport of drugs and nutrients: genomics of membrane transporters using expression microarrays. Eur. J. Pharm. Sci. 21, 17–24
25. Landowski, C.P. et al. (2004) Transporter and ion channel gene expression after Caco-2 cell differentiation using 2 different microarray technologies. AAPS J. 6, e21
26. Hayashi, R. et al. (2008) Comparison of drug transporter gene expression and functionality in Caco-2 cells from 10 different laboratories. Eur. J. Pharm. Sci. 36, 383–396
27. Ahln, G. et al. (2009) Endogenous gene and protein expression of drug-transporting proteins in cell lines routinely used in drug discovery programs. Drug Metab. Dispos. 37, 2275–2283
28. Engman, H. et al. (2003) Enantioselective transport and CYP3A4-mediated metabolism of R/S-verapamil in Caco-2 cell monolayers. Eur. J. Pharm. Sci. 19, 57–65
29. Avdeef, A. et al. (2005) Caco-2 permeability of weakly basic drugs predicted with the double-sink PAMPA pKa(flu) method. Eur. J. Pharm. Sci. 24, 333–349
30. Bergström, C.A.S. et al. (2003) Absorption classification of oral drugs based on molecular surface properties. J. Med. Chem. 46, 558–570
31. Artursson, P. and Karlsson, J. (1991) Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelium (Caco-2) cells. Biochem. Biophys. Res. Commun. 175, 890–895
32. Camenisch, G. et al. (1998) Estimation of permeability by passive diffusion through Caco-2 cell monolayers using the drugs’ lipophility and molecular weight. Eur. J. Pharm. Sci. 6, 317–324
33. van Breezem, R.B. and Li, Y. (2005) Caco-2 cell permeability assays: to measure drug absorption. Expert Opin. Drug Metab. Toxicol. 1, 175–185
34. Corti, G. et al. (2006) Development and evaluation of an in vitro method for prediction of human drug absorption - II. Demonstration of the method suitability. Eur. J. Pharm. Sci. 27, 354–362
35. Wang, Y. et al. (2010) Stereoselective transport and uptake of propranolol across human intestinal Caco-2 cell monolayers. Crit. Rev. 22, 361–368
36. Mori, Y. et al. (2001) Stereoselective pharmacokinetics and pharmacodynamics of verapamil and norverapamil in rabbits. Biopharm. Biodeg. 24, 806–810

37. Rask-Andersen, M. et al. (2011) Trends in the exploitation of novel drug targets. Nat. Rev. Drug Discov. 10, 579–590

38. Mestres, J. et al. (2009) The topology of drug-target interaction networks: implicit dependence on drug properties and target families. Mol. Biosyst. 5, 1001–1017

39. Ohashi, R. et al. (1999) Na+-dependent carnitine transport by organic cation transporter (OCTN2): its pharmacological and toxicological relevance. J. Pharmacol. Exp. Ther. 291, 778–784

40. Yabuuchi, H. et al. (1999) Novel membrane transporter OCTN1 mediates multispecific, bidirectional, and pH-dependent transport of organic cations. J. Pharmacol. Exp. Ther. 289, 768–773

41. Ohashi, R. et al. (2001) Molecular and physiological evidence for multifunctionality of carnitine/orange cation transporter OCTN2. Mol. Pharmacol. 59, 358–366

42. Salomón, J.J. et al. (2014) The verapamil transporter expressed in human hepatic epithelial cells does not interact with beta-receptor agonists. Drug Metab. Pharmacokinet. 29, 101–104

43. Kubo, Y. et al. (2013) Involvement of a novel organic cation transporter in verapamil transport across the inner blood-retinal barrier. Pharm. Res. 30, 847–856

44. Dudley, A.J. et al. (2009) The organic cation transporter OCT2 mediates the uptake of beta-adrenergic antagonists across the apical membrane of renal LLC-PK1 cells. Br. J. Pharmacol. 131, 71–79

45. Kubo, Y. et al. (2013) Propanolol transport across the inner blood-retinal barrier: potential involvement of a novel organic cation transporter. J. Pharmacol. Sci. 102, 3332–3342

46. Lee, D. et al. (2012) Improving metabolic flux predictions using absolute gene expression data. BMC Syst. Biol. 6, 73

47. Kirschner, M.W. and Gerhart, J.C. (2005) The plausibility of life: resolving Darwin’s dilemma, Yale University Press

48. Dawkins, R. (2006) The selfish gene: 30th anniversary edition, Oxford University Press

49. Kent, E. et al. (2013) What can we learn from global sensitivity analysis of biochemical systems? PLoS ONE 8, e79244

50. Keil, D.B. and Knowles, J.D. (2006) The role of modeling in systems biology. In System modeling in cellular biology: from concepts to nuts and bolts (Sgallash, Z. et al., eds), pp. 3–18, MIT Press

51. Curtin, A. et al. (2015) Synthetic biology for the directed evolution of protein biocatalysts: navigating sequence space intelligently. Chem. Soc. Rev. 44, 1172–1239

52. Hubatsch, I. et al. (2007) Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nat. Protoc. 2, 2111–2119

53. Ballatori, N. et al. (2005) OSTA-alpha-OSTbeta: a major basolateral bile acid and steroid transporter in human intestinal, renal, and biliary epithelia. Hepatology 42, 1270–1279

54. Ballatori, N. et al. (2013) The heteromeric organic solute transporter, OSTAalpha-OSTbeta/SLC51: A transporter for steroid-derived molecules. Mol. Aspects Med. 34, 683–692

55. Han, Y.H. et al. (2010) Transporter studies with the 3-O-sulfate conjugate of 17alpha-ethinylestradiol: assessment of human kidney drug transporters. Drug Metab. Dispos. 38, 1064–1071

56. Han, Y.H. et al. (2010) Transporter studies with the 3-O-sulfate conjugate of 17alpha-ethinylestradiol: assessment of human liver drug transporters. Drug Metab. Dispos. 38, 1072–1082

57. Grandvillain, A.S. et al. (2013) New insights into the carrier-mediated transport of estrone-3-sulfate in the Caco-2 cell model. Mol. Pharm. 10, 3285–3295

58. Benet, L.Z. et al. (2011) BDDCS applied to over 900 drugs. AAPS J. 13, S19–547

59. Bansal, T. et al. (2009) Effect of P-glycoprotein inhibitor, verapamil, on oral bioavailability and pharmacokinetics of irinotecan in rats. Eur. J. Pharm. Sci. 38, 580–590

60. Yee, S. (1997) In vitro permeability across Caco-2 cells (column) can predict in vivo (small intestinal) absorption in man–fact or myth. Pharm. Res. 14, 763–766

61. Pade, V. and Stavchansky, S. (1998) Link between drug absorption solubility and permeability measurements in Caco-2 cells. J. Pharm. Sci. 87, 1604–1607

62. Yazdaniann, M. et al. (1998) Correlating partitioning and caco-2 cell permeability of structurally diverse small molecular weight compounds. Pharm. Res. 15, 1490–1494

63. Hox, T.J. et al. (2004) ADME evaluation in drug discovery 5. Correlation of Caco-2 permeation with simple molecular properties. J. Chem. Inf. Comput. Sci. 44, 1585–1600

64. Uchida, M. et al. (2005) A modified fast (4 day) 96-well plate Caco-2 permeability assay. J. Pharmacol. Toxicol. Methods 59, 39–43

65. Lozoya-Aguilo, I. et al. (2015) In Situ Perfusion Model in Rat Colon for Drug Absorption Studies: Comparison with Small Intestine and Caco-2 Cell Model. J. Pharm. Sci. 104, 3136–3145

66. Chu, X.Y. et al. (2001) Correlation between epithelial cell permeability of cephalaxin and expression of intestinal oligopeptide transporter. J. Pharmacol. Exp. Ther. 299, 575–582

67. Fenol, A. (1977) Enzyme structure and mechanism. (2nd ed.), W. H. Freeman

68. Keleti, T. (1986) Basic enzyme kinetics, Akadémiai Kiadó

69. Cornish-Bowden, A. (1995) Fundamentals of enzyme kinetics. (2nd ed.), Portland Press

70. Sevin, E. et al. (2013) Accelerated Caco-2 cell permeability model for drug discovery. J. Pharmacol. Toxicol. Methods 68, 334–339

71. Loew, R. et al. (2013) Improved Tet-responsive promoters with minimized background expression. BMC Biotechnol. 10, 61

72. Gradmann, C. (2014) A spirit of scientific rigour: Koch’s postulates in twentieth-century medicine. Microbes Infect. 16, 865–892

73. Potgieter, M. et al. (2015) The dormant blood microbiome in chronic inflammatory diseases. FEBS Microbiol. Rev. http://dx.doi.org/10.1002/1612-686X.12013

74. Thiele, I. et al. (2013) A community-driven global reorganization of human metabolism. Nat. Biotechnol. 31, 419–425

75. Sahico, S. et al. (2014) Membrane transporters in a human genome-scale metabolic knowledgebase and their implications for disease. Front. Physiol. 5, 91

76. Kell, D.B. et al. (2015) Membrane transporter engineering in industrial biotechnology and whole-cell biocatalysts. Trends Biotechnol. 33, 237–246

77. Römpp, A. et al. (2011) Mass spectrometry imaging with high resolution in mass and space (HR2 MSI) for reliable investigation of drug compound distributions on the cellular level. Anal. Bioanal. Chem. 401, 65–73

78. Prideaux, B. and Stoeckli, M. (2012) Mass spectrometry imaging for drug distribution studies. J. Proteomics 75, 4999–5013

79. Prideaux, B. et al. (2015) Mass spectrometry imaging of levofloxacin distribution in TB-infected pulmonary lesions by MALDI-MSI and continuous liquid microjunction surface sampling. Int. J. Mass Spectrom. 377, 699–708

80. Uhlen, M. et al. (2015) Tissue-based map of the human proteome. Science 347, 1260419

81. Melt, M. et al. (2015) The human transcriptome across tissues and individuals. Science 348, 660–665

82. Balm aber, P.V. et al. (2006) Current industrial practices of assessing permeability and P-glycoprotein interaction. AAPS J. 8, E1–E13

83. Goualbes, R. et al. (2011) QSAR-based permeability model for drug-like compounds. Bioorg. Med. Chem. 19, 2615–2624

84. Peng, Y. et al. (2014) Applications of a 7-day Caco-2 cell model in drug discovery and development. Eur. J. Pharm. Sci. 56, 120–130

85. Chung, S.M. et al. (2001) Profound effect of plasma protein binding on the polarized transport of furansamide and verapamil in the Caco-2 model. Pharm. Res. 18, 544–547

86. Faassen, F. et al. (2003) Caco-2 permeability, P-glycoprotein transport ratios and brain penetration of heterocyclic drugs. Int. J. Pharm. 263, 113–122

87. Usansky, H.H. and Sinko, P.J. (2005) Estimating human drug oral absorption kinetics from Caco-2 permeability using an absorption-disposition model: model development and evaluation and derivation of analytical solutions for $V_{tu}$ and $F_{tu}$. J. Pharmacol. Exp. Ther. 314, 391–399
88. Press, B. (2011) Optimization of the Caco-2 permeability assay to screen drug compounds for intestinal absorption and efflux. Methods Mol. Biol. 763, 139–154
89. Skolnik, S. et al. (2010) Towards prediction of in vivo intestinal absorption using a 96-well Caco-2 assay. J. Pharm. Sci. 99, 3246–3265
90. Lin, X. et al. (2011) Attenuation of intestinal absorption by major efflux transporters: quantitative tools and strategies using a Caco-2 model. Drug Metab. Dispos. 39, 265–274
91. Cao, X. et al. (2006) Drug Absorption Principles. In Biopharmaceutics: Applications in Drug Development (Krishna, R. and Yu, L., eds), pp. 75–100, Springer
92. Bansal, T. et al. (2007) Concurrent determination of topotecan and model permeability markers (atenolol, antipyrine, propranolol and furosemide) by reversed phase liquid chromatography: utility in Caco-2 intestinal absorption studies. J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 859, 261–266
93. Walgren, R.A. and Walle, T. (1998) The influence of plasma binding on absorption/exsorption in the Caco-2 model of human intestinal absorption. J. Pharm. Pharmacol. 51, 1037–1040
94. Marino, A.M. et al. (2005) Validation of the 96 well Caco-2 cell culture model for high throughput permeability assessment of discovery compounds. Int. J. Pharm. 297, 235–241
95. Caldwell, G.W. et al. (1998) In vitro permeability of eight beta-blockers through Caco-2 monolayers utilizing liquid chromatography/electrospray ionization mass spectrometry. J. Mass Spectrom. 33, 607–614
96. Hoops, S. et al. (2006) COPASI: a COmplex PAthway Simulator. Bioinformatics 22, 3067–3074
97. Hucka, M. et al. (2003) The systems biology markup language (SBML): a medium for representation and exchange of biochemical network models. Bioinformatics 19, 524–531
98. César-Razquin, A. et al. (2015) A call for systematic research on solute carriers. Cell 162, 478–487