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What would be the observable consequences if phospholipid bilayer diffusion of drugs into cells is negligible?

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For drug transport across (i.e., through) an intact biological membrane, two main routes are possible: drugs may cross (i) through the phospholipid bilayer portion of the membrane, and/or (ii) via proteinaceous pores or transporters. Perhaps surprisingly, there is in fact no direct scientific evidence that the first of these takes place at any significant rate because, in the experiments performed to date, it has neither been varied as an independent variable nor measured directly as a dependent variable. Using a standard hypothetico-deductive framework, I assess the intellectual and observable consequences of assuming that, for drugs, phospholipid bilayer diffusion is negligible – 'PBIN' – (i.e., may be neglected, relative to transporter-mediated transmembrane fluxes). Predictions and postdictions of the PBIN hypothesis are not refuted by available experimental evidence.

Introduction

It is easy in science (and other fields) to take the 'textbook' or 'standard' view of a system or a process as a given, without necessarily bothering to look into the actual experimental evidence (if any) on which it might have been based. However, the history of science is full of examples in which a theory or scientific viewpoint, once widely believed, was supplanted by one that had better explanatory and predictive power (i.e., of both existing and novel data) [1,2], albeit often in the face of considerable rearguard action [3]. Sometimes this change in thinking was driven by the acquisition of new evidence, but in some cases the main driver was simply the reinterpretation of existing evidence – which newer ideas must also necessarily explain.

This widespread acceptance of a particular view seems to have come to pass in the field of cellular drug uptake, where it is commonly believed that (leaving aside endocytosis) most drugs can and do enter intact cells by passing fully through whatever unhindered phospholipid bilayer portions might exist, and – because biomembranes have lipophilic interiors – at a rate that correlates (in some fashion) with log D (see Glossary) or log P. I note, of course, that correlations of two dependent variables show nothing, except that they exist in the systems stated (while one may be causal of the other, both might instead be effects of a separate cause, or indeed entirely unrelated to each other mechanistically). However, I know of no paper in which phospholipid bilayer transport in intact biological cells has ever been varied as an independent variable (and without changing any relevant transporter-mediated uptake), nor of any in which the actual passage of a drug diffusing through the bilayer has ever been measured directly. It therefore follows that the concrete, data-driven evidence that we have that this takes place *in vivo* is, in fact, precisely zero.

What has of course been done many times is that the transfer of drugs across biological membranes has been measured, and it has been assumed or stated that this occurred via the bilayer. Obviously, assuming or stating something as a mechanism when it has not actually been measured directly can be rather hazardous, and indeed does not count as experimental evidence for it at all. I contrast the situation with experiments in which the activity of genetically encoded protein transporters has been varied independently (Figure 1).

Glossary

BDDCS: the Biopharmaceutics Drug Disposition Classification System, a 2×2 matrix that can be used to classify drugs into four main classes depending on whether their solubility and/or extent of metabolism is 'high' or 'low'; see [54].

KNIME: the Konstanz Information Miner (http://www.knime.org), a freely available workflow environment for creating and running cheminformatics and related workflows.

log D: the logarithm of the distribution coefficient, D. D is the ratio of the sum of the concentrations of all forms of a compound (ionised plus non-ionised) in each of two phases, typically 1-octanol and an aqueous buffer, whose pH must be specified.

log P: the logarithm of the partition coefficient, P. P is a measure of the hydrophobicity of a molecule; log P is the logarithm (base 10) of the ratio of the concentration of a solute molecule in an organic solvent, usually 1-octanol, to that of the non-ionised form of the same molecule in water.

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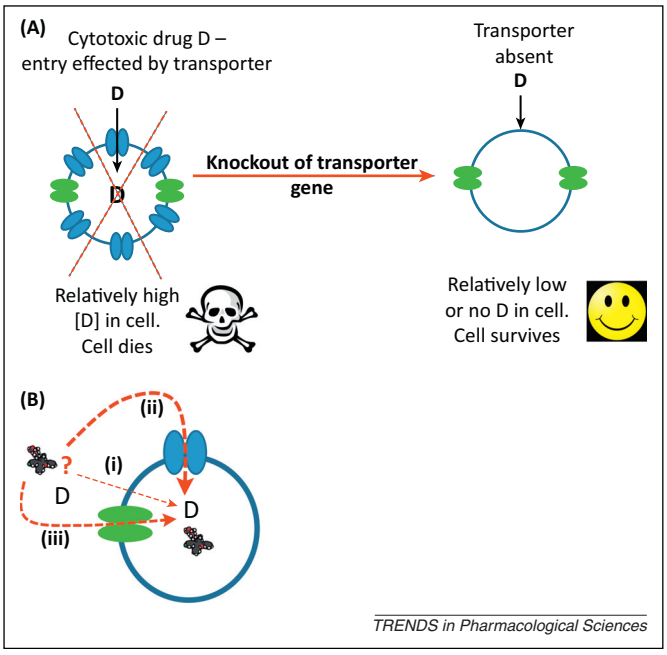

Figure 1. Varying the mechanistic basis for drug uptake as an independent variable causes predictable and measurable consequences. **(A)** This is straightforwardly done for proteinaceous transporters, for instance by knocking out the relevant genes, as illustrated for the case of a cytotoxic drug whose entry to the cell is via a transporter coloured in blue. Other types of transporters (in green) exist but are not used by the drug. Precisely these types of experiments have been carried out for antimetabolites (e.g., [15]) and candidate anticancer drugs [22], leading to straightforward and accurate inferences about the role of such transporters in the uptake of the target drug(s). The uptake (and effectiveness) of many other drugs has also been found to covary with the expression levels of particular transporters (e.g., gemcitabine and ENT1 [46]). **(B)** By simply observing the extracellular disappearance or intracellular uptake of a drug, one may seek to infer that the transport of the externally added drug entering a cell occurs (i) via its transport through the bilayer. Obviously, other interpretations of the mechanisms of this drug transport are possible, however, such as transport through the blue (ii) or green (iii) transporters (or both). Thus, no logically correct inference is possible if neither bilayer diffusion nor the presence or activity of relevant transporters are varied in known ways, nor measured directly. We are aware of no experiments in which phospholipid bilayer transport has been varied independently in this way, and under circumstances where any changes in competing transporter-mediated uptake have also been monitored (figures not drawn to scale).

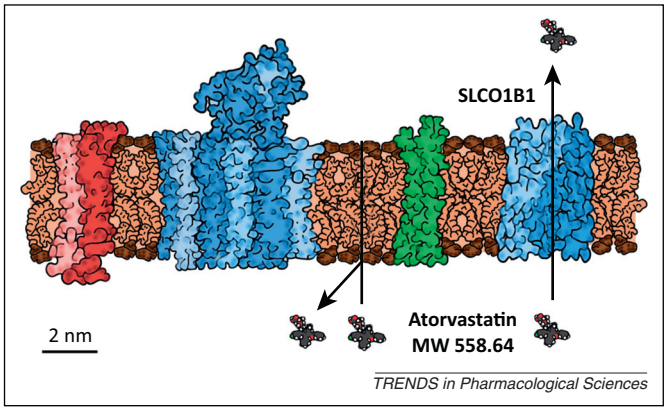

Figure 2. A typical biomembrane, drawn approximately to scale. The illustration uses common protein:phospholipid mass ratios, and shows a typical drug (atorvastatin), also drawn to scale. Phospholipids are in brown, proteins in other colours. The question arises as to whether any of the drug passes through those phospholipid bilayer regions that remain unaffected by the presence of the proteins. According to this Opinion, it is much easier to account for many types of observations if one recognises that in fact the overwhelming bulk of transmembrane drug transport occurs via proteinaceous pores and transporters (that are involved in intermediary metabolism) and not through phospholipid bilayers. Abbreviation: SLCO1B1, solute carrier organic anion transporter family, member 1B1.

In a separate strand of activity, researchers have studied the transport of drugs across protein-free lipid (or other hydrophobic) bilayers or membrane structures. On the assumption that cells also contain similar phospholipid bilayers, and that these are not modified materially by the presence of (what is, by mass, usually considerably more) protein (Figure 2), they have been tempted to extrapolate such *in vitro* findings to biological membranes *in vivo*. This again lacks real logic because the properties of biological membranes do differ in many ways from those of these artificial ones (not least by the presence of aquaporins [4]).

Few studies have systematically sought to understand how drug uptake and cellular lipid composition may covary; however, if one believes (as I do not) that most (or a significant part) of the uptake flux of drugs in intact cell membranes occurs via phospholipid bilayers, such lipid variation is not to be invoked as a major mechanism of variable uptake anyway because, for a significant number of drugs, the majority of the difference in ('background') rates between, for example, MDCK and Caco-2 cells does not vary more than twofold [5].

Thus, starting in 2008 [6], my colleagues and I have been developing the idea that the phospholipid bilayer portion of intact biological membranes is not in fact naturally significantly permeable to drugs and common xenobiotics because evolution simply selected against that (and note of course that biomembranes are osmotically active). Arguably it is precisely the 'laminated' hydrophilic–hydrophobic–hydrophilic structure of phospholipid bilayers that stops real, undamaged biomembranes from being leaky to small molecules (in either direction). Leaving aside endocytosis, paracellular transport, and so on, then, and concentrating on cases where a drug genuinely traverses a cellular (or indeed intracellular) membrane, how drugs do cross membranes is to be seen as being via the many proteinaceous transporters (e.g., [7,8]) (Figure 2) encoded by the relevant genome. Many of those for small molecules are recorded in the metabolic networks reconstructed and curated, for example for yeast [9] and humans [10,11]. Of course, these transporters are taken to be there not specifically for the benefits of pharmaceuticals companies but for the purposes of intermediary metabolism. To this end we have summarised the evidence for the dominance of transporter-mediated uptake in several review and experimental articles [5,6,12–19].

However, such reviews are necessarily retrospective. By contrast, if one starts (as a hypothesis [20,21]) by accepting that the phospholipid bilayer diffusion of drugs across intact biological membranes is negligible (PBIN), a great many interesting and thus prospective consequences follow, that I explore below. I shall point at some of the relevant evidence, but readers will wish to judge for themselves the extent to which the observations (including their own) have yet been made or not, and whether the PBIN view thus has useful predictive (or postdictive) power. I group the consequences into three classes; some consequences really relate to more than one class.

Predicted consequences of the PBIN view

Consequences of a low or negligible natural permeability to drugs of phospholipid bilayers in intact biomembranes

Most tissues (lacking transporters) will not in fact take up drugs in significant amounts unless suitable proteinaceous transporters are present. A particularly clearcut example has recently been provided by Superti-Furga and colleagues [22], who used a near-haploid human cell line [23,24] in a very elegant manner to show that the candidate (and rule-of-5-compliant) anticancer drug sepantronium bromide (or YM155) simply does not enter mammalian cells unless a particular transporter (SLC35F2: solute carrier family 35, member F2) is present. There is neither a detectable effluxer nor any significant background transport (i.e., this demonstrates PBIN in a very clear way). Indeed, it is worth stressing that if background (lipoidal bilayer-dependent) rates of uptake were not negligible this type of experiment [15] could not and would not work.

Because the expression profiles of transporters vary strongly between different tissues in an organism (e.g., http://www.proteinatlas.org), there will be an extreme heterogeneity of uptake of specific drugs into the cells and tissues of that organism (as in [25]). This might be observed, for instance, by mass spectral imaging, or by other spectroscopic techniques that can effect spatially distinct chemical imaging, and has been (e.g., [26–28]).

Such heterogeneity can lead to distributions of drugs in tissues that differ widely, despite the same gross pharmacokinetics/pharmacodynamics; a tissue in which two thirds of the cells take up no drug and one third take up three times as much still takes up the same total amount of drug, but may display both lack of efficacy (in the two-thirds) and toxicity (in the one-third) [19]; these two causes account for the bulk of present-day attrition in drug-discovery programs [29,30]. One cannot properly use ensemble (average) measurements to disguise functional cellular heterogeneity [31,32].

If phospholipid bilayer diffusion is normally negligible, there will be tissues (largely lacking in transporters that may be expressed elsewhere) which, despite the relative functional similarity of their phospholipids, are very poor at taking up drugs: the blood–brain [33], blood–testis [34], and blood–retina [35] barriers exhibit these properties. Of course, drug efflux transporters can also contribute to this type of phenomenon [36], but poor uptake transport follows directly from PBIN.

Thus, PBIN accounts easily for all types of variation in the uptake of drugs as cellular circumstances are varied because, if something like a cell or tissue type is changed, so too are the expression levels (or activities) of the relevant transporters in those cells of the organism (and the heterogeneity is enormous, and can be measured; e.g., http://www.proteinatlas.org). By contrast, there is little obvious tissue-specificity of the permeability properties of phospholipid bilayers, nor therefore any major means by which those who believe it to be important can expect it to vary so much between different cells or tissues in an organism. In a similar vein, PBIN provides a natural and simple explanation for the variation of uptake of the same drug between individuals whose transporter activities may vary as a result of single-nucleotide polymorphisms or other genetic differences (pharmacogenomics) [37]. PBIN also provides a natural and simple explanation for the variation (which can be huge [38,39]) of uptake of the same drug between different species (of organism). Furthermore, PBIN provides a natural and simple explanation for any variation of uptake of the same drug as an organism changes its physiology, for example diurnally [40], via nutritional changes, or exercising, in disease, or as it ages, etc.

A straightforward prediction is that molecular dynamics (MD) simulations of 'real' biomembranes, set up with natural protein:phospholipid ratios of 2:1 or 3:1, will show that drugs do not cross them at significant rates via the phospholipid bilayer portion (but may do so overwhelmingly via the relevant transporters if they are present in the same membrane simulation). Such MD simulations of transporters embedded in realistic membranes are indeed beginning to appear (e.g., [41–43]).

Consequences of the fact that individual drugs must and do use specific and identifiable transporters

Proteinaceous transporters will be discovered for all xenobiotics that cross biomembranes; a survey of TransPortal [44] or DrugBank [45] indicates that at least one transporter is already known for most drugs. Furthermore, there will be clear relations (*sensu lato*) between the extent of drug uptake and the expression levels of particular transporters in different cells or tissues; the fluorinated anti- (pancreatic) cancer nucleoside gemcitabine provides one example (e.g., [46,47]).

Removing or decreasing the activity of a particular transporter by genetic or other means will change in a predictable way the extent of uptake of drugs that are its substrates [15,22]. If removing all relevant transporter activity leads to negligible uptake then one may clearly infer that phospholipid bilayer diffusion is negligible. Similarly, increasing the activity of a particular transporter by genetic or other means will change in a predictable way the extent of uptake of drugs that are its substrates [48]. This and the previous paragraph probably represent the most important strategies by which one may usefully assess the relevance of specific transporters in the cellular uptake of a named drug.

Finally, drugs (and metabolites and nutrients) that share the same transporter will compete with each other for it [49,50], and the covariation of their uptake between tissues will tend in part to follow the covariation of transporter expression.

Consequences of the fact that specific drugs hitchhike on transporters that mainly have intermediary metabolites as their natural substrates

Because the natural substrates of these transporters are posited to be (and in many cases clearly are) intermediary metabolites, the principle of molecular similarity implies that successful (i.e., marketed) oral drugs, that necessarily cross biomembranes, will bear structural similarities to at least one metabolite (Figure 3), as has been shown [12,18], and this should be useful in drug design. The structural similarities between marketed drugs and

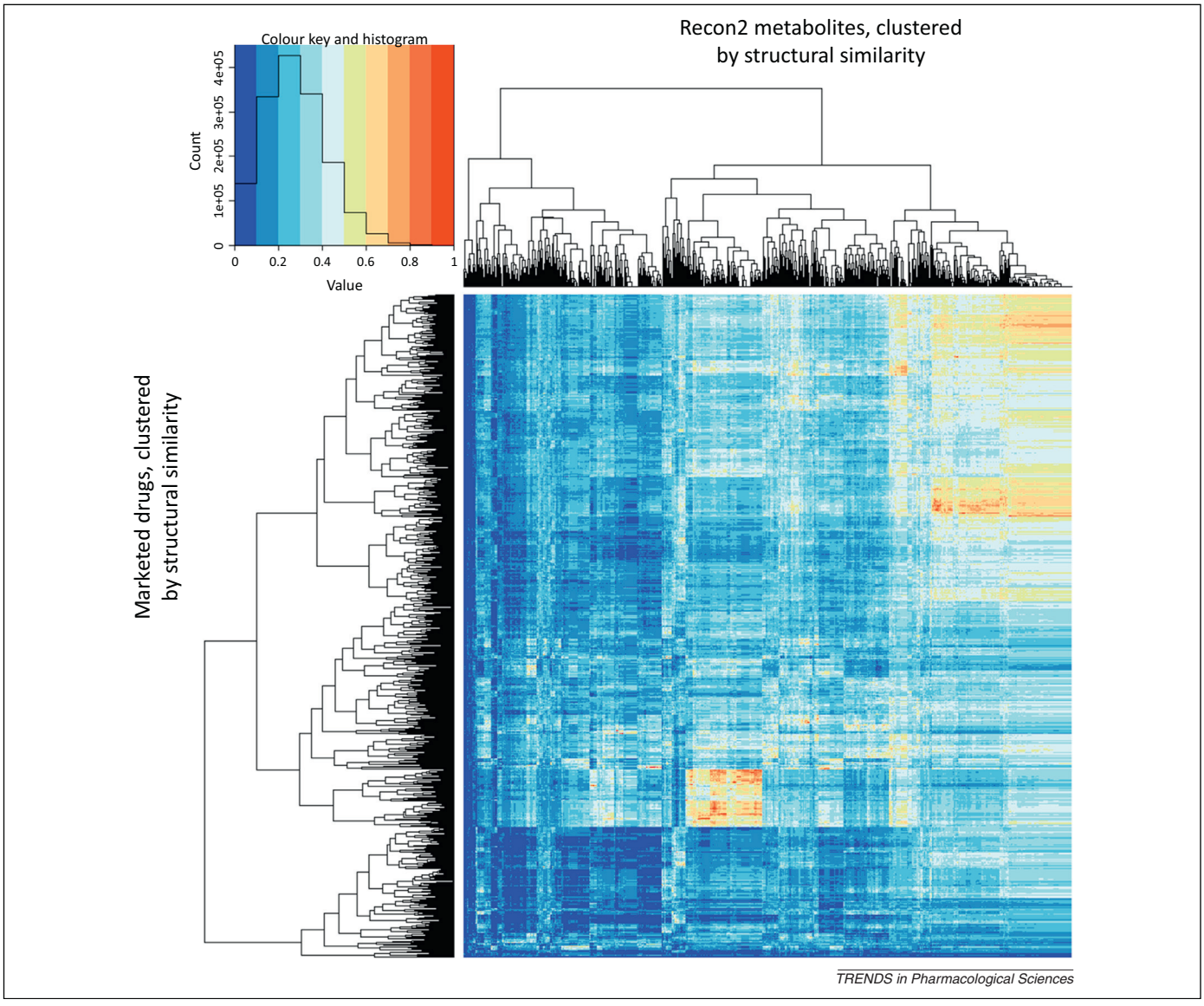

Figure 3. The metabolite-likeness of drugs. A comparison (based on [18]) of the structural similarities of marketed drugs and the majority of the metabolites of the human metabolic network reconstruction Recon2 [10]. Drugs that are also metabolites were removed from the list of drugs. Using the MACCS encoding [61], the similarity of each substance is compared via a KNIME workflow [62] using the Tanimoto similarity coefficient [63,64], whose values are encoded according to the colours indicated. Drugs and metabolites are clustered using an agglomerative clustering algorithm. A full description is given elsewhere [18], but for 90% of all marketed drugs there is at least one metabolite with a Tanimoto (structural) similarity using the MACCS encoding of 0.5 or greater. This could be used as a powerful filter during drug-discovery programmes. Abbreviations: KNIME, Konstanz Information Miner; MACCS, Molecular Access System.

human metabolites will also apply for the subset of natural molecules that undergo transmembrane transport [12]. From a cheminformatics point of view [51], the better descriptors relating drugs to metabolites will be molecular and substructural descriptors rather than bulk biophysical ones such as log P or 'total polar surface area'; these need to be assessed using suitable hold-out sets [52].

If bilayer diffusion is both important and is claimed to be correlated with log P or log D, then log P or log D should be predictive of cellular uptake. By contrast, because of the molecular specificity of at least some transporters, PBIN predicts that there will be only a very weak or limited correlation between (i) the ability of drugs to cross membranes or (as a surrogate) to be metabolised, and (ii) their bulk log D or log P values; examples (often using tabulated rather than plotted data) include [53,54]. Figure 4 shows data replotted from the tables in [54], which would lead one to suppose that the cellular permeability of drugs is largely independent of the distribution coefficient (log D) of those drugs between buffer (pH 7.4) and octan-1-ol.

Although many enzymes are highly promiscuous [14], and transporters are enzymes, there will be identifiable quantitative structure–activity relationships (QSARs) for each transporter that – when learned – will have high predictive power for untested drugs (independently of biophysical indicators such as log D or log P). Such QSAR and pharmacophore models are beginning to appear [55].

Non-discriminating experiments

I have concentrated on experiments and data that seem to pertain to the predictions of PBIN. However, I am mindful that there is a danger of researchers proposing experiments that, however superficially attractive, are not in fact discriminatory of the two main mechanisms under discussion

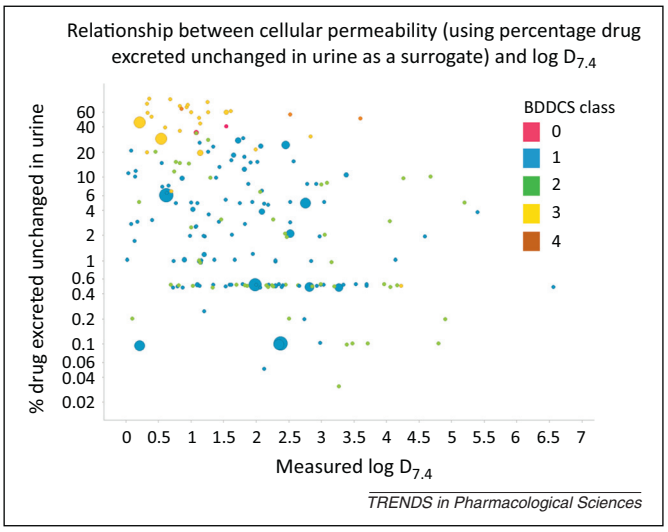

Figure 4. The cellular permeability of drugs versus log D. There is no obvious relationship between the cellular permeability of various drugs (assessed via the percentage unchanged in the urine, given that the metabolism of drugs requires their cellular uptake, and a low value therefore reflects a high permeability) and the measured distribution coefficient at pH 7.4. All data are from [54] (similar but more extensive data show the same when the abscissa is the log P as calculated and displayed in the same table from [54], not shown). The BDDCS class is encoded by the colour as marked, and the aqueous solubility by the size of the symbol.

here. Thus, varying something like temperature to see its effects on drug uptake, especially if one is not measuring all of the activities of the transporter, will be uninterpretable. Similarly, varying lipid composition without assessing the effects of doing that on transporter activities themselves will also not be discriminatory because a substantial literature shows that phospholipids can have profound effects on the activities of membrane proteins (see e.g., [56,57]), as can nutrient molecules (e.g., [58]). Finally, debates around bilayer diffusion tend to obsess about topics such as saturability, promiscuity, stereoselectivity, and the effects of (usually non-existent) inhibitors; the first three in particular are completely non-discriminatory, in contrast to the main class of experiment that we have stressed is worth doing, which is simply to vary the expression and activities of the transporter proteins themselves and to assess the predicted or predictable consequences on drug uptake.

Concluding remarks

Sometimes it is useful to take a step back and, instead of starting with observable data and seeking the hypothesis that best explains them, start with a hypothesis (that did of course originate from data [20]) and assess what might be the predictions or consequences that follow if that hypothesis were to be true. This hypothesis then might be (or indeed might already have been) subjected to experimental assessment to see if the observable data might serve to falsify it.

A theory that explains more observations and has predictive power is normally to be preferred over one that performs less well by those metrics. One example, that I have highlighted before in this context [5,6,14,19], relates to the change in recognition that general anaesthetics, whose potency is typically well correlated with lipophilicity up to a cut-off (that is connected with their solubility), actually cause anaesthesia not by interacting with the phospholipid bilayer but by binding to hydrophobic pockets on target proteins that are in fact the 'cause' (mechanism) of the anaesthesia [59]. In particular, the well-known and strong relationship between the potency of various general anaesthetics and log P, long assumed to be due to membrane partitioning, can be reproduced with the soluble enzyme luciferase [60].

The hypothesis-led approach is the one I have taken here, and the many publications analysed in our various papers on this topic (e.g., [5,6,12–17,19]) provide data and evidence that, perhaps surprisingly, or even shockingly, thus far fail to falsify the hypothesis that, for the transport of pharmaceutical drugs across intact biological membranes, phospholipid bilayer diffusion is negligible. Many opportunities for novel approaches exist (Box 1), however, and I look forward to further tests of these consequences.

Box 1. Outstanding questions

Question	Possible approaches
• How can we measure bilayer-mediated transport or diffusion in real biomembranes **directly**?	• Possibly spectroscopic methods such as NMR or ESR would give signals that differ as drugs pass through the different parts of a phospholipid bilayer versus being transported via a transporter.
• Can we modify phospholipids in a manner that changes their interactions with other lipids, but not those with protein transporters, and that thereby affects drug uptake?	• There may be a role for incorporating phospholipid analogues with 'click chemistry', or other suitably reactive groups, that do not themselves make membranes non-specifically leaky.
• Will we be able to do molecular dynamics (MD) simulations for long enough to observe full transbilayer diffusion at rates comparable to those of transporter-mediated fluxes?	• Computer power has historically increased ~1000-fold every 6 years (though their power consumption has not). Specialised hardware implementations will help. Nevertheless, clever shortcuts will be necessary. MD methods provide an *in silico* approach that may be very useful if definitive 'wet' experiments prove intractable.
• A motivation for addressing drug–metabolite likeness came from a recognition of the importance of drug transporters. While metabolite likeness is a now demonstrable property of most marketed drugs, are drugs closer in structure to the subset of metabolites that are transported across membranes *in vivo*?	• Fairly standard cheminformatics analyses should be able to address these types of question.
• Can the recognition of the role of transporters in cellular drug uptake help in understanding some of the origins of unexpected lack of efficacy and/or toxicity, and thereby help to decrease the appalling late-stage attrition rates still prevailing?	• This probably requires a metabolic network model that incorporates the transporters and their expression profiles, and that also understands the QSARs for each main transporter to assess distributions at a sub-tissue level.
• Can comparative genomics help in understanding species differences in drug transport(ers)?	• This would require a sequence-based approach coupled to knowledge of major differences in drug distributions between different species.

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