Implications of the Dominant Role of Transporters in Drug Uptake by Cells

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Abstract: Drug entry into cells was previously believed to be *via* diffusion through the lipid bilayer of the cell membrane, with the contribution to uptake by transporter proteins being of only marginal importance. Now, however, drug uptake is understood to be mainly transporter-mediated. This suggests that uptake transporters may be a major determinant of idiosyncratic drug response and a site at which drug-drug interactions occur. Accurately modelling drug pharmacokinetics is a problem of Systems Biology and requires knowledge of both the transporters with which a drug interacts and where those transporters are expressed in the body. Current physiology-based pharmacokinetic models mostly attempt to model drug disposition from the biophysical properties of the drug, drug uptake by diffusion being thereby an implicit assumption. It is clear that the incorporation of transporter proteins and their drug interactions into such models will greatly improve them. We discuss methods by which tissue localisations and transporter interactions can be determined. We propose a yeast-based transporter expression system for the initial screening of drugs for their cognate transporters. Finally, the central importance of computational modelling of transporter substrate preferences by structure-activity relationships is discussed.

Keywords. Systems Biology, drug transporter, drug uptake, pharmacokinetic modelling, *Saccharomyces*, cheminformatics

INTRODUCTION

The primary route of drug uptake into cells has historically been considered to be by diffusion through the lipid portion of the cellular membrane. This notion persists in the pharmaceutical industry where it is a major consideration in drug discovery and development, as passive drug absorption through the lipid bilayer is thought to be a major determinant of bioavailablity. In recent work [1] we summarised evidence supporting the view that drug uptake is in fact mainly due to transport proteins embedded in the relevant membranes, as illustrated in Fig. (**1**).

After rehearsing the arguments supporting this view we consider further its ramifications, the types of information required to exploit it in a drug discovery setting, and particularly the experimental means by which we might acquire such information. Our experimental focus is on the discovery of transporter substrates in appropriate strains of the yeast *Saccharomyces cerevisiae* as we believe this to be an ideal system in which to study transporters. The advantages of yeast include expression and post-translational modification machinery that is highly similar to mammalian systems (yeast is also eukaryotic), highly developed molecular biology tools for genetic modification, including a genome-wide homozygous 'knockout' library of non-essential (and in heterozygous diploids even of essential) genes, plus very well characterised genome, proteome and metabolome information. This provides an ideal background for the development and validation of uptake transporter assays. We also consider the central importance of computational modelling in both Systems Biology simulations of pharmacokinetics and transporter structure-activity relationships. This leads us to conclude that a co-ordinated, cross-disciplinary effort is required to derive transporter-aware pharmacodynamic and pharmacokinetic models.

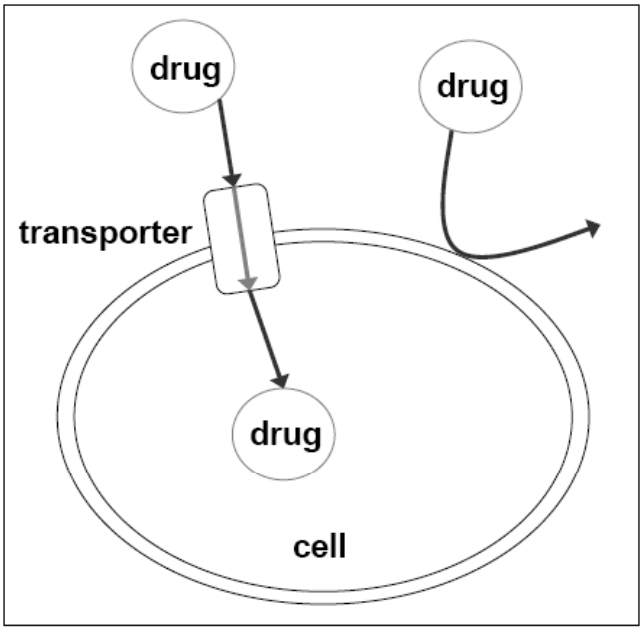

Fig. (1). Uptake of drugs across biological membrane is predominantly *via* transporters and not by diffusion through the lipid.

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DRUGS CROSS MEMBRANES VIA TRANSPORTERS

The notion that lipids provide the major route for solutes to cross membranes can largely be traced back to extrapolating observations made in artificial membrane systems, containing only lipids, to natural biological membranes. Such artificial membranes are essentially lipid bilayers between aqueous phases. A drug can be added to one phase and its appearance detected in the other to measure the transport across the bilayer. Given the relative simplicity of artificial membranes in comparison to real biological membranes, particularly considering the high protein complement of the latter, it is clear that these are fundamentally different types of membrane; any inference of a common mechanism is therefore logically untenable. However, artificial membranes are not entirely without utility for predicting drug transport across membranes (although close inspection of recent results reveals that artificial membrane results might not be quite as useful as claimed, see figures 2-3 in [1]). To understand how this fits with the posited dominant role of transporters, consider the forces governing movement between aqueous and lipid environments. These are predominantly lipophilicity and the capacity to make and break hydrogen bonds. Essentially these forces also govern movement between solvent and protein binding sites, which are also hydrophobic environments. Lipinski's rule of five [2] describing likely drug bioavailability is also based on these forces, and Lipinski *et al*. explicitly declare transporter-mediated uptake to be exceptions to the rule, implying they regard transport as normally a process of diffusion through the lipid. Yet, through recognising that the forces required to interact with lipids are similar to those needed to bind to proteins, we come to understand that these empirically-derived models of transport do not imply the prevalence of either mechanism and are capable of describing both in a general way. We also note the use of transient aqueous pores as a means of transport across artificial lipid bilayers [3,4].

Correlations over many orders of magnitude between lipophilicity and the membrane partitioning and cellular uptake of certain drugs reinforced the lipid-dominant view as it was not considered that drug-protein interactions could achieve such correlations over the same wide range. Such interactions are known, however, including some (e.g. those involving narcotic agents) for which there is direct structural evidence [5].

The concentration of drugs in certain tissues beyond that which can be explained by the stoichiometry of internal binding sites (even considering gratuitous binding), especially when coupled to the widely-known presence of efflux transporters that largely act against such accumulation, absolutely requires an active uptake process. Perhaps the key argument supporting a dominant role for transporter-mediated drug uptake is the large number of drugs already known to be transported by identified proteins into the cell. Individual reports, spread disparately across the scientific literature and usually considered as exceptions to the lipid-dominant view of uptake, actually number in the hundreds (in earlier work [1] we identified 393 drug-transporter relationships from the literature, though given the strict evidence required this is clearly a considerable underestimate) - such 'exceptions' are numerous indeed.

The arguments above promote the view that drugs predominantly cross biological membranes into cells *via* protein transporters, whose specificity is initially unknown, and that probably normally act to transport metabolites (a contributing factor in the metabolite-likeness of many drugs [6]). For more detail we direct the reader to our earlier work [1]. We must now consider processes that have previously been thought to be predominantly lipid-mediated as transporter-mediated, and this view has important implications for understanding drug pharmacokinetics.

OVERVIEW OF UPTAKE TRANSPORTERS

Although membrane proteins are abundant (analysis of genome sequences suggests that around one quarter of human genes encode proteins that localise to membranes [7,8]) and of great clinical significance, our knowledge of their structures and functions lags considerably behind our understanding of soluble proteins which, generally speaking, are much easier to express and to study. Given their involvement in sensing, signalling, transport, secretion, anchoring, and other major processes, it is clear that to fully understand how biology operates we must better understand membrane proteins. In the context of drug discovery, transporters are a major determinant of drug disposition.

Transporters are intrinsic transmembrane proteins that mediate the passage of solutes by passive or active mechanisms. Passive mechanisms utilise the solute's favourable electrochemical potential to drive movement, while active processes can be directly or indirectly coupled to an energy source (typically ion gradients or consumption of ATP) to drive movement of solutes with unfavourable gradients. Direct coupling to an energy source is referred to as primary active transport, while secondary active transport couples movement against the solute's electrochemical gradient to the transport of a second solute with a favourable gradient which is actively maintained by a separate system. A detailed classification of transporter function, the Transport Classification Database (TCDB), has been developed and described elsewhere [9-12]; other major online resources describing transporters are listed in Table (**1**).

Of primary interest in transporter-mediated drug uptake are the Solute Carriers (SLCs). The more than 300 SLCs known in humans are organised by function into nearly 50 families. They mediate facilitated diffusion or secondary active transport, but not primary active processes. Most are found in the cell membrane, but members of SLC25 are specific to the mitochondrial membrane [19]. Comprehensive reviews of each major SLC family can be found via the 'SLC Tables' URL given in Table (**1**) and in the review of Hediger *et al*. [13].

Certain SLCs have very specific functions and (consequently) a narrow substrate specificity which, while not precluding a role in drug uptake, minimises their potential involvement. In contrast other SLCs can mediate the uptake of a wide range of substrates as they have evolved to facilitate processes such as nutrient uptake or detoxification that necessarily require broad specificity. A clear example of

Table 1. Major Computational Resources Describing Transporters

Name	Reference	URL	Description
TCDB	[12]	http://www.tcdb.org	The major classification of transporters
SLC Tables	[13]	http://www.bioparadigms.org/slc/intro.htm	Overview of solute carriers (SLCs)
TransportDB	[14, 15]	http://www.membranetransport.org	Database of predicted transporters with cross-species comparisons
HTDB	[16]	http://lab.digibench.net/transporter	Membrane transporters in humans
YTPdB	[17]	http://rsat.ulb.ac.be/ytpdb/	Membrane transporters in yeast (*S. cerevisiae*)
TP-Search	[18]	http://www.tp-search.jp	Known drug-transporter interactions

this is the peptide transporter protein PEPT1 (encoded by human gene SLC15A1), a proton-dependent oligopeptide transporter (POT; TCDB:2.A.17) family member that primarily transports nutrients across the brush border membrane of the intestines and is thought to provide the major pathway for the absorption of dietary nitrogen. (PEPT1 is also expressed in the kidney, liver and pancreas.) The substrate specificity of PEPT1 includes all 400 naturally-occurring dipeptides and potentially all tripeptides, but it can also transport xenobiotic molecules such as β-lactam antibiotics, ACE inhibitors [20], antiviral prodrugs modified with valine [21,22], and other non-native substrates. The structure-activity relationships have been well charac-terised [23,24]. The usefully broad substrate specificity that evolved to allow uptake of many nutrients also provides for the uptake of many diverse drugs. By no means is the behaviour of PEPT1 anomalous; many other SLC transporters show similarly broad substrate specificity and this provides a route of uptake for many different drugs. Of particular importance are transporters from SLC families 21 (organic anion transporting), 22 (organic anion/cation/zwitterion transporting) and 28 (nucleoside transporting), which are already known to transport many drugs. More detail on drug-transporter relationships culled from the literature can be found in the supplementary material of Dobson and Kell [1].

IMPLICATIONS OF TRANSPORTER-MEDIATED UPTAKE

A drug in development can be discarded for many reasons, such as lack of efficacy, adverse effects, toxicity, or poor pharmacokinetics - the processes of drug absorption, distribution about the body, metabolism by processing enzymes, and excretion from the system (collectively referred to as ADME). Drug metabolism aside, transporter-mediated uptake is a major component of these processes. Identifying likely ADME, efficacy and toxicity failures early in the development process can dramatically reduce the costs of drug discovery but, as the factors determining pharmacokinetics are many and complex, such failures have proven particularly difficult to predict. To a considerable extent, existing ADME prediction methods assume that the processes of absorption, distribution and excretion, which are largely determined by movement into and out of tissues, are governed by the biophysics of diffusion across membranes. The role of transporters, while known in principle, is often considered of marginal influence. That these distribution processes are in fact predominantly carrier-mediated leads to the realisation that drug absorption, distribution and clearance are largely determined by the substrate preferences and tissue distributions of transporters, and this information is required to fully model pharmacokinetics. Failure to be transported to a target site by a transporter can underlie lack of efficacy, while excess concentrations can cause toxicity. Thus transporter molecules are involved in all three of the major means of compound attrition in drug discovery.

Table 2. The Implications of a Dominant Role for Transporter-Mediated Drug Uptake

The Implications of a Dominant Role for Transporter-Mediated Drug Uptake
Understanding drug pharmacokinetics requires a more sophisticated model of uptake that takes much greater account of the role of transporter proteins.
Transporter variants contribute to differential drug response, including by ethnicity, gender and age.
A better understanding of the differences between human and model organism transporters will enhance the utility of animals as pharmacokinetic models.
Drug-drug, drug-nutrient, and drug-gut flora interactions will occur at transporters.
A combination of better characterisation of transporter substrate preferences and tissue expression will provide better models of drug absorption, distribution (including targeting or avoiding specific tissues), and excretion.
High-throughput systems for characterising transporter substrate preferences are needed to assess the many transporters involved and their variant forms.
Chemoinformatic models based on the above are needed to predict likely drug-transporter interactions and allow the pharmacokinetic profiles of drugs to be accounted for early in the drug development pipeline.

PHARMACOKINETIC MODELLING AND SYSTEMS BIOLOGY

Physiology-based pharmacokinetic (PBPK) models are already widely-used to understand drug disposition [25]. The basic approach is to represent organs as compartments connected by the blood, and partitioning between the blood and organs is calculated by equations built upon experimentally-determined and predicted properties. These can be considered as properties describing the organism, including organ volumes and surface areas, rate of blood flow and pH, and properties of or about the drug, such as lipophilicity, bioavailability, volume of distribution, organ/blood partition coefficients (which, to a limited degree, might reflect the kinetic parameters of transport and binding processes). These latter properties may also be predicted from structure by computational models [26]. These parameters are, by and large, sensible and relevant to drug disposition and, while PBPK models can be of some use in modelling drug disposition, they are currently insufficient to capture (implicitly) the role of drug transporters, which can exhibit considerable specificity (including to stereoisomers whose biophysical properties are essentially identical).

The methods of PBPK modelling are similar, in some ways, to those of Systems Biology. The latter is a relatively recent development within molecular biology that favours integrative approaches over reductionist ones, and closely combines high-throughput experiments with mathematical modelling. Thus Systems Biology shifts the focus of biological research from individual molecules to the role of the network (see [27], or for an engineer's perspective [28]). Bruggeman and Westerhoff [29] describe two main approaches to Systems Biology, the so-called top-down and bottom-up approaches.

In the top-down method, the system's responses to perturbations (such as by drugs, gene knockouts, environmental changes, *etc*.) are characterised by high-throughput analyses at the levels of metabolites or their fluxes, transcripts and/or proteins. These analyses should be as comprehensive as possible and, therefore, the different levels of analysis are referred to as the metabolome, fluxome, transcriptome, or proteome (by analogy with the genome). Recently, major inroads have been made into determining the metabolome (defined as the native small molecule complement) of the human system [30]. Through the use of genome sequence and literature analyses, many of the reactions and interactions between these small molecules have been integrated to produce genome-scale reconstructions of human metabolism [31-34]. Essentially, relations between system components are inferred by common responses. This approach is attractive because, in theory at least, it does not require a detailed model of processes that determine response as the lack of detail is countered by the richness of the experimental data. Practically speaking, the information content of even very high numbers of independent perturbations is insufficient to fully 'reverse engineer' the structure of the underlying network in any great detail (the system cannot be fully identified); nevertheless, much can be learned from this approach.

At the other extreme one can adopt a bottom-up approach in which one attempts to deduce higher biological processes that contribute to the observed response from the reactions and interactions of molecular components that underpin them. This requires the representation of all system components and the interactions between them. The utility of such an approach is clear if one considers the analogous problem of smashing a radio and attempting to put the pieces back together [35]. The pile of electronic components is insufficient to reconstruct the radio; one also needs to know the wiring diagram connecting them to make the radio work. This is also the case with biological systems; knowing the molecular components alone is not enough as the reactions and interactions that exist between them are required to replicate system behaviour. This places great demands on the knowledge required to build a complete model, including not only reactions but also kinetic parameters (or reasonable estimates thereof). In practice one soon reaches a situation where arguably the most sensible strategy is to combine the best possible bottom-up model that knowledge permits and inferences from top-down methods in a 'middle-out' approach [36].

Physiological modelling can be considered a cousin (or, perhaps more correctly, an uncle) of Systems Biology, favouring a more 'top-down' strategy as experimentally-determined disposition is modelled as a function of the system and drug parameters described earlier. This relies on these parameters being sufficiently informative as to capture implicitly the underlying determinants of the observed disposition. It seems clear that the involvement of transporters inevitably requires more than a small handful of physicochemical and experimental terms to represent the full complexity of carrier-mediated uptake and efflux, and to account for these transporters a bottom-up approach is likely to be more successful. That said, a multi-scale, tissue-level model generated by the bottom-up approach is probably still some way off. A more immediately useful approach, and one already adopted to a limited extent for some transporters [37], is to supplement existing PBPK models with transporter reactions and localisations to create a middle-out model. Most usefully, such models will take advantage of Systems Biology markup languages such as SBML [38,39] and CellML [40] (the two may yet converge [41]). Ultimately, this middle-out approach will also require those other reactions that alter transporter function, such as regulatory processes and post-translational modifications; but, even without these, it will likely offer a considerable advance on current PBPK models.

EFFECTS OF TRANSPORTER PROTEIN VARIANTS

To understand absorption, distribution and excretion requires knowledge of the particular transporter variants an individual possesses. Such variants can be due to single nucleotide polymorphisms (SNPs), alternative splicing, and other mechanisms, giving rise to different substrate specificities, reaction kinetics, tissue expression profiles and regulation. Modelling uptake as a general biophysical process that is broadly similar in all humans can only ever result in general pharmacokinetic models, but differential drug responses are determined by the genetic and environmental circumstances of the drug's recipient, including variants of the transporters responsible for drug uptake.

Pharmacogenetics describes the influence of genetic variants on drug disposition (and other processes) and is a well-established field [42-44]. Particularly for drug-metabolising enzymes and efflux transporters, the role of genetic variants in differential pharmacokinetic response is well known [45,46]. Studies have already identified some of the important variants of uptake transporters and the topic has been reviewed extensively, both generally [47-50] and more specifically. Specific reviews cover the influence of transporter polymorphisms in cancer therapy [51,52], on drugs grouped by therapeutic application (diabetes [53], psychiatric disorders [54,55], cholesterol-lowering by statins [56-62]), in gastrointestinal disorders [63], along the intestinal tract [64], and for transporter families already known to be involved in drug uptake [65-69]. Studies have also highlighted how polymorphisms in non-coding regions might lead to differential expression [70]. A rich source of pharmacogenetic information is available through Pharm-GKB [71]. Further pharmacogenetic information can be found in DrugBank [72,73] and the Drug ADME Associated Protein Database [74].

As ethnic groups have developed, certain genetic variants have become associated with them. Along with cultural practices (particularly drug-diet interactions) these can cause some of the differences in drug disposition sometimes associated with ethnicity. Uptake transporter variants causing differential disposition of simvastatin and its active metabolite have been found for SLCO1B1 [75]. These variants were among many found to differ between African and Caucasian populations, possibly leading to differential response. Likewise, SLC22A16 variants have been linked to the differential response to doxorubicin of Asian breast cancer patients [76].

If transporter variants can alter pharmacokinetics within a species then such variation can also happen across species boundaries, and potentially to a much greater extent. Animal models, particularly mice, rats, dogs and monkeys, are often used in ADME studies. While the shortcomings of using animals as a model of humans are well known, their utility could be enhanced by a better understanding of the differences between animal and human ADME determinants, including uptake transporters. Some of these differences have already been identified. Ho *et al.* [59] found that NTCP, the major bile acid uptake transporter in hepatocytes, is capable of transporting rosuvastatin in humans but not in rats, in spite of the fact that the two proteins have very high sequence similarity. Nozaki *et al.* [77] found that delayed plasma elimination of methotrexate due to simultaneous use of non-steroidal anti-inflammatory drugs (NSAIDs), mediated by interactions at OAT3 (SLC22A8) and several efflux transporters, is observed in human kidney slices but not in those of rats. A further interaction at OAT3 between famotidine and probenecid is not seen in rats and this is thought to be due to differences in OAT3 activity as well as differences in expression of OCT1 [78]. The monkey model is thought to represent human-like OAT activity better and exhibits comparable OCT expression, suggesting that monkeys are a better *in vivo* model for predicting human famotidine clearance [79]. Better characterisation of transporters from model organisms will allow a greater understanding of the extent of their utility as pharmacokinetic models of humans.

As well as inter-individual differences, transporter expression profiles are known to differ within the individual as a function of developmental state. In mice and rats, age-related differences in kidney function [80] are attributed to variable expression of Oat1-3 [81] (though not equivalently even across the two rodents). The organic cation transporters of mice were also found to alter expression with developmental state, with adult expression levels of certain mOcts not reached until three weeks after birth [82] and gender-specific expression not apparent until day 30. Developmental and gender differences were also found for mOatps [83]. These studies also identify that expression differs by gender, so uptake transporters will be a factor in pharmacokinetics differences between males and females [84-87]. Regulation of rOct2, but not rOct1 or rOct3, is via the androgen response-mediated transcriptional pathway [88].

DRUG INTERACTIONS AT TRANSPORTERS

Interactions between co-administered drugs, or between drugs and dietary components, can alter pharmacokinetics considerably. The drug interaction phenomenon is still relatively poorly understood and difficult to study given the number of molecular entities and possible combinations thereof. For any new drug, evaluating the potential for interactions is a key part of the development and approval process. Interactions at drug-metabolising enzymes are the subject of intensive study and increasingly this is also true for efflux transporters, but less so for uptake transporters [89]. That said, there is already much known about uptake transporter-mediated drug interactions [90-92], but accepting the importance of transporters leads to recognising that much greater emphasis should be given to their possible role.

When drugs are likely to be co-administered, the ability to screen transporters in the laboratory prior to administration can flag potential interactions. Understanding that co-administration of fludarabine and imatinib was likely in the treatment of chronic myeloid leukaemia, and knowing that fludarabine is absorbed by the equilibrative nucleoside transporters (ENT1 and ENT2), Woodahl *et al.* [93] identified a transporter-mediated interaction in isolated lymphocytes. Similarly, Kitamura *et al.* [94] speculated that a drug interaction might occur at OATP1B1 between the statins that are known substrates and the drug SMP-797 (an acyl-CoA cholesterol acyltransferase inhibitor), which was likely to be co-administered. Although screens in hepatocytes and transfected oocytes did not detect any interaction, this demonstrates the utility of transporter screens in exploring transporter-mediated interactions prior to administration.

The widespread use of statins and their transport by organic anion transporters (SLC family 21) makes them highly likely to be involved in drug interactions. Already, many statin-interacting organic anion transporter substrates are known [95-100], including not only drugs but also components of various fruit juices and extracts [101]. Given that many transporters plausibly evolved to handle exogenous substances derived from the diet, drug-diet

interactions [102] must be considered more likely now that the major role of transporters is understood. Information on dietary components can be found within HMDB (http:// www.hmdb.ca) [30], and FDA-approved food additives within FooDB (http://www.foodbs.org/foodb), although there are too many possible dietary components from the variety of foodstuffs that can be consumed to screen physically.

Less direct interactions can occur when transporter activity or expression is altered by a drug even though the transporter itself is not the site of interaction. Caffeine and other phosphodiesterase inhibitors cause a reduction in the uptake of glycyl-sarcosine by the peptide transporter PEPT1, which is reported to be a consequence of their inhibitory effects on a second SLC transporter, NHE3 (encoded by SLCA9A3) [103], a Na^+/H^+ exchanger that maintains the proton gradient on which the peptide transporter depends [104]. Inhibition of NHE3 activity causes a reduction of PEPT1-mediated uptake. Similarly, the glutamate transporter is up-regulated by amitriptyline [105]. Hirai *et al.* [106] report that PPARα agonists up-regulate seven transporters, including five SLCs, and down-regulate MRP1 in mouse intestine and liver, suggesting the potential for interactions with the many substrates of these transporters. These indirect interactions can be useful if well understood and managed carefully. For example, there is benefit in co-administering pemetrexed and gemcitabine to bladder cancer cells *in vitro* and this is probably due (at least in part) to pemetrexed increasing the expression of the nucleoside transporter ENT1, for which gemcitabine is a substrate [107]. These sorts of interactions will be difficult to model without a bottom-up approach.

DESIGNING DRUGS TO TARGET TRANSPORTERS: PRODRUGS

Many potential drugs fail as they have only limited bioavailability (the fraction of administered drug that reaches the systemic circulation). One strategy to address this is to devise a modified form, a prodrug [108-111], with properties more amenable to absorption, possibly at the cost of much reduced activity, that is converted to a more active form by drug metabolising enzymes *in vivo*. Prior to understanding the involvement of transporters in absorption, the process of 'improving' poorly absorbed drugs was largely restricted to altering lipophilicity and other properties to move the drug towards Lipinski space [2]. Understanding that transporters are responsible for most drug uptake, even for drugs following Lipinski's rule of five (which probably covers drug-transporter as well as drug-lipid interactions), suggests that this strategy is successful as it enhances the ability to interact with uptake transporters. The approach is, however, limited by the fact that the picture of uptake is very general and does not describe a particular drug-transporter interaction. Designing prodrugs to target a specific uptake transporter, also taking into account the transporter's tissue localisation, can result in much improved and tissue-directed uptake [112]. This approach has already been successfully used to piggyback drugs through bile acid transporters [113, 114], where coupling bile acids via valine to acyclovir enhanced uptake through the sodium-dependent bile acid transporter hASBT and led to a two-fold increase in bioavailability in rats [115]. It has also been used to enhance acyclovir transport by addition of the L-valyl ester to form valaciclovir [116], an ideal hPEPT1 substrate, which is converted to its active form in hepatocytes. Li *et al.* review general and targeted prodrug strategies for nucleoside analogues that usually have poor bioavailablity. This includes a consideration of the types of chemistry that can be used in prodrugs as they should ideally be substrates of drug-metabolising enzymes to produce the active form once absorbed. Alternatively, the prodrug may be a metabolic precursor of more active forms, as is seen with pemetrexed, the folate metabolites of which are transported into cells by the reduced folate carrier and the folate receptor [117].

The targeted prodrug strategy is particularly promising for transport of drugs across the blood brain barrier (BBB) [118,119], where tight junctions negate paracellular transport. Peptide transport across the BBB can be enhanced by glucosylation of drugs (even though this causes a decrease in lipophilicity) to allow targeting to the GLUT1 transporter [120]. It is also speculated that the transport of L-DOPA by LAT1, the Large Amino acid Transporter expressed at the BBB, can be utilised to enhance the uptake of the antiviral phosphonoformate by conjugation with tyrosine [121]. Coupling nipecotic acid to ascorbate allows transport by SVCT2, which correlates with anticonvulsant activity in mice [122,123].

The major implication of the dominant role of transporters in drug uptake is that far greater account of their actions must be taken, including those of their variants that come in many guises and can underpin idiosyncratic drug response. When drugs or nutrients interact with the same transporter, as substrates or inhibitors, there is potential for drug interactions to occur. Understanding how these factors influence ADME is vital to drug development. The extent to which current PBPK models incorporate the influence of uptake transporters is typically much less than is required. However, when represented appropriately in the languages of Systems Biology, they can easily be supplemented with details of transporter function; although, ultimately, a bottom-up approach will be required to take account of the multiple (and often subtle factors) that control transport. By designing drugs towards specific transporters, absorption, tissue distribution and excretion can be controlled, particularly by the targeted prodrug strategy. Overall, many metabolites are quite hydrophilic, and a move towards hydrophilicity by coupling metabolite moieties to candidate drugs may considerably lower their promiscuity, as promiscuity correlates strongly with lipophilicity [124].

We now turn our attention to the nature of the missing transporter information and to how it might be acquired in an efficient manner. The required information consists of essentially two parts: the first is knowledge of the inter-actions of drugs with transporters (plus the kinetics thereof), while the second concerns the tissues in which these transporters are found (at least for initial models, but as these improve they will likely also require detailed expression information at sub-tissue and sub-cellular levels). A third consideration is the interaction of drug metabolites with transporters, but this is not our primary concern here. When

represented appropriately this combination of kinetics and localisation will, to a great extent, explain drug disposition.

TRANSPORTER LOCALISATION

The cellular and anatomical localisations at which transport reactions occur can most simply be inferred by identifying the presence of the enzymes and transporters that mediate them. Much progress into characterising protein localisation has already been made through the application of transcriptomics and proteomics technologies to various cell types and tissues. Major resources are described in Table (**3**). By adding tissue-specific expression data, genome-scale human metabolic reconstructions, which are currently largely agnostic regarding tissue type, can be tailored to represent the metabolism of various tissues [125]. If reconstructions are well annotated with appropriate molecular species identifiers (small molecules might point to ChEBI [126] or PubChem [127], or use InChI [128,129] strings, while proteins might refer to UniProt [130] or Ensembl [131, 132]) it is trivial to cross-reference them with equivalently annotated tissue expression resources, particularly in an integrated workflow environment [133]. Alternatively, if the middle-out approach to modelling is used, it is similarly trivial to add transporter localisation.

There is not a simple relationship between transcript and protein levels [134,135] (mainly because of differential rates of degradation of the relevant species) so quantitative proteomic data are preferable [136]. Many initiatives to determine the proteome profiles of different tissues are in progress and the integration of data from these is facilitated by the development of standards that ensure proper reporting and annotation, as available through the PRIDE database [137,138]. However, the available databases mainly report qualitative, rather than quantitative, results despite experimental methods being available to determine protein levels quantitatively. While traditionally the favourite method of proteomics was the 2D gel, which uses isoelectric focusing and size-based electrophoresis to separate the mixture of isolated proteins, handling difficulties and relatively poor reproducibility (particularly for membrane proteins) make it largely unsuitable for high-throughput screening. Our preference is for mass spectrometry-based approaches [139,140]. Typically the proteome extract is enzymatically digested (e.g. by trypsin) followed by chromatographic separation before injection into the mass spectrometer. The pattern of digestion fragments is compared to computational 'digestion' patterns to identify proteins. Due to differences in the efficiency of ionisation and/or the detectability, the intensity of a peak cannot be directly correlated to the fragment's (and so the parent protein's) absolute concentration in the sample (although progress is being made in predicting this [141]). It is, however, possible to compare the peak intensity of an analyte to an isotopically-labelled version of that analyte at a known concentration (a standard) to assess absolute concentration. Labels currently in use are ICAT (Isotope-Coded Affinity Tags) [142], iTRAQ (Isobaric Tag for Relative and Absolute Quantification) [143], MeCAT (Metal Coded Tags) and GIST (Global Internal Standard Technology) [144], which applies N-terminal label post-digestion. *In vivo* labeling can be achieved by adding stably isotopically labeled (SIL) nutrients to the growth medium (^{13}C-glucose and/or ^{13}C-labeled (heavy) amino acids [145,146]). Label-free quantitation methods make use of mathematical and statistical approaches to quantify proteins [147,148].

One particularly attractive option follows the QconCAT strategy [149]. The proteins of interest from the proteomic mixture are computationally analysed to establish fragments that are both likely to 'fly' in the instrument, lack sites of post-translational modification, and have masses that are characteristic of the protein from which they derive. These signature peptides are concatenated, inserted into a primer and expressed in bacteria growing on labeled media. The concentration of the isolated QconCAT peptide can be determined by standard protein assays. Digestion of the QconCAT gives labeled markers of known concentration, which can be used as a standard for multiple proteins per sample. One might envisage that QconCATs constructed from transporter sequences would be a rapid way of determining transporter levels in a sample quantitatively.

High-throughput generation of protein-specific antibodies and arrays of normal and disease tissues also allow proteomic profiling of tissues. The Human Protein Atlas (http://www.proteinatlas.org/) [150,151] is one of the outcomes of antibody-based proteomics [152].

DISCOVERING DRUG-TRANSPORTER INTERACTIONS

The second major component required for modelling drug disposition is information on the specific interactions between drugs and transporters, including measuring the relevant kinetics. The most common approach is to compare drug transport into cells that differ only by the presence or absence of the transporter of interest, having being genetically manipulated, by gene deletion, or by transient or stable transfection. A particularly favoured host system is the *Xenopus laevis* oocyte which, due to its large size (~1mm in diameter), is relatively easy to microinject with cRNA or cDNA. This approach is also used in cell lines derived from mammals, and experimental protocols are available [161]. Ideally, the expression system allows the coding sequence for the transporter to easily be introduced and the protein to be expressed in its functional form in a real biological membrane, with proper membrane targeting and post-translational modifications.

STUDYING TRANSPORTERS IN YEAST

We focus on the yeast *Saccharomyces cerevisiae* as a system for studying transporter function. There are many reasons for favouring the use of yeast, particularly the ease of manipulation by well-developed molecular biology methods and the fact that it is eukaryotic and so offers some of the same transcript and protein processing mechanisms.

In a comparison of various systems for the production of functional membrane proteins by Junge *et al.* [162], including bacterial, fungal, insect and mammalian cells, yeast compares favourably to insect and mammalian cells (and equivalently to bacteria) in terms of ease of manipulation, robustness and safety, whilst offering elements of the eukaryotic processing machinery that bacterial systems

Table 3. Expression-Profiling Localisation Information Resources

Name	Reference	URL	Description
Human Protein Atlas	[150, 151]	http://www.proteinatlas.org	Immunohistochemical analysis of human proteins in tissues and sub-tissues, plus diseased tissue
BioGPS	[153]	https://biogps.gnf.org	Transcriptomic analysis of human, mouse and rat tissues, organs and cells, plus diseased tissue
dbEST	[154]	http://www.ncbi.nlm.nih.gov/dbEST/	GenBank resource describing ESTs from a range of organisms
HuGEIndex	[155]	http://hugeindex.org	mRNA expression levels of human genes by tissue
TissueInfo	[156]	http://icb.med.cornell.edu/services/tissueinfo/query	EST analysis at tissue levels, based on information from dbEST and predictions.
TiGER	[157]	http://bioinfo.wilmer.jhu.edu/tiger	Tissue-specific gene expression and regulation data
MAPU 2.0	[158, 159]	http://www.mapuproteome.com	A meta-database unifying mass spectrometry-derived proteomic data across tissue and biofluids.
SWISS-2DPAGE	[160]	http://expasy.org/swiss-2dpage	Two-dimensional SDS page database for a variety of tissues

cannot. Yeast grows rapidly on inexpensive and readily available media with doubling times of about 2.5 hours. The molecular biology toolbox to manipulate yeast is well stocked, including many convenient cloning vehicles [163], such as low- and high-copy number circular plasmids, plus linear constructs that readily integrate into the genome for stable transformation. Heterologous genes are usually expressed on 2μm-based plasmids, which maintain high copy numbers but need selection during growth to ensure their stable maintenance [164]. Genes can easily be placed under the control of inducible or repressible promoters (typically *tetO* regulation by the tetracycline analogue, doxycycline [165]). The efficiency and exquisite accuracy of homologous recombination in yeast makes it simple to insert, mutate, or delete genes.

Due to the wealth of existing knowledge on yeast biology and the range of tools available yeast has become a major model organism in Systems Biology. Genome-scale reconstructions [166,167] of the yeast metabolic network are available, detailing known metabolites and the reactions between them, and these have recently been consolidated into a single well-annotated reconstruction using a community-driven approach [168]. Yeast has also been important in the drug discovery setting - Carroll *et al.* [169] highlight the role played by yeast in the discovery of several blockbuster drugs while Simon and Bedalov [170] focus on anticancer drugs. About 30% of the known human disease genes have counterparts in yeast [171]. Osborn and Miller [172] review the successful use of yeast as a screening vehicle of human cDNA libraries to identify human genes *via* complementation in systems as complex as steroid receptor signalling and apoptosis. Zhang *et al*. describe a yeast complementation system for screening such libraries [173]. Surprisingly, successful complementation assays have been carried out with proteins of a sequence similarity as low as 14.5% [174]. For our purposes it is interesting to note that certain studies have focussed upon functional expression of membrane proteins from these libraries, including xenobiotic transporters [175,176].

Historically, yeast is one of the most studied model organisms and this has given rise to a host of tools for its genetic manipulation and molecular biological analysis The first eukaryotic chromosome to be sequenced [177] came from yeast (chromosome III) and yeast was also the first eukaryotic genome to be sequenced in its entirety (sequence and functional annotations are publicly available in the *Saccharomyces* Genome Database (SGD) http://genome-www.stanford.edu/Saccharomyces/ [178]). Other yeast databases include the CYGD at MIPS-GSF (http:// mips.gsf.de/genre/proj/yeast/) and the commercial YPD database at https://portal.biobase-international.com. Building upon this, yeast was the first organism for which whole genome microarrays were available [179]. Other important tools include the yeast 2-hybrid system to discover protein interactions [180], including a split-ubiquitin variant specifically designed to analyse interactions involving membrane proteins [181]), and a library of 3223 TAP-tagged genes [182] that aid protein purification.

Libraries of heterozygous knockouts [183] of almost all protein-coding sequences (5996) and homozygous knockouts of all non-essential genes (4792) were created within the EUROFAN project [184] (http://mips.gsf.de/proj/eurofan/). These libraries possess unique barcodes that identify each deletion mutant, which allows the assessment of their relative rates of growth in competition experiments (e.g. [185-189]) using DNA arrays [190]. They have been successfully exploited as tools for genetic (phenotypic) screens [169,186, 191,192], and in particular existing data regarding the interaction of yeast cells with drugs have pointed up a number of cases in which changes in the activity of specific carriers increase or decrease the sensitivity of cells to xenobiotics [186,193-203] with the clear implication that such carriers effect the entry of these drugs into cells or their exit from them. Genome-wide drug screens exploiting the yeast knockout libraries have been conducted [188,193] as well as synthetic lethality screenings [204] and haploinsufficiency analyses [205]. Strains are readily available commercially via the EUROSCARF consortium

(http://web.uni-frankfurt.de/fb15/mikro/ euroscarf/) and Open Biosystems (http://www. openbiosystems.com/), or *via* the very open yeast research community. Recently Zhu *et al.* [206] introduced a yeast protein chip, though whether membrane proteins are present in their functional form is open to debate [207], although specialist membrane protein arrays have also been developed [208]. Laboratory protocols from the pre-genomic era are available [209].

S. cerevisiae has been used as a convenient expression system for membrane and transport proteins from very early on [175,210-214]. Yeast deletion mutants of various origins have been used previously as background for complementation/functional studies of heterologous mammalian membrane proteins, which are significant in human diseases. For instance, mutations of the mitochondrial ornithine transporter in humans results in hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome. This inherited disorder of the urea cycle results in delayed development, learning disabilities, confusion and ataxia [215]. Morizono *et al.* studied the role of several mutations in the human mitochondrial ornithine transporter, which were expressed in a strain of *S. cerevisiae* lacking its endogenous ornithine transporter YOR130c (ORT1) [216]. Similarly, yeast studies made major contributions to understanding the mechanisms that the multidrug resistance protein MDR1 plays in the resistance of various tumours to anti-tumour drugs [217,218]. Supplementary table (**1**) gives an overview of mammalian membrane proteins which have been successfully expressed in yeast. The table deliberately does not include heterologous membrane proteins expressed in yeast from other fungal species, human parasites or plants.

DETECTING UPTAKE

Detection of cellular uptake by transporters using the sorts of expression systems described above has been approached in many different ways. Uptake differences between wild-type and transformed cells implicate the transporter. An experiment should provide direct proof of uptake by illustrating the presence of the drug on the opposite side of the membrane, but very many drugs are known to interact with transporters by competition with known substrates. Although this is still valuable information it does not demonstrate transport. As well as direct proof of uptake, given the numbers of transporters, variants, drugs and food components that we might wish to screen, broadly applicable screens (ones that can be applied to practically any interesting molecule) are preferred.

Some of the earliest uptake experiments utilised a very simple uptake detection mechanism based on deplasmolysis (protoplast re-swelling due to reversal of water potential upon uptake) to demonstrate the permeability of yeast cells to drugs, although this could not reveal the specific mechanism of uptake [219]. Since then, very many methods have been developed to demonstrate uptake, including a development of these early experiments by detecting the swelling of transfected *Xenopus* oocytes [220]. These tend to focus either upon detecting alterations in the host system due to the drug or direct identification of the drug inside the cell.

If a drug crosses the membrane in a manner linked to the transporter, as determined by reference to the control, and it then induces detectable changes in the host this illustrates that uptake has occurred and implicates the transporter. Useful changes are easily detected and perhaps the simplest is cytotoxicity, but this is limited to drugs with cytotoxic effect, which are relatively few. One might also detect morphological changes, and yeast is a particularly tractable system for this as automated mechanisms for determining morphology have been developed for drug screening [221], although this assumes that phenotypic change requires uptake, which may not be the case (for example, changes in the cell wall could alter morphology). The metabolomic fingerprint or footprint is a particularly rich source of information that can be detected by mass spectrometry [222,223]. Using system changes to detect transporter activity depends on the drug eliciting an effect. Many transporter substrates may have little or no effect on the host system and this limits the approach. Moreover, as the magnitude of the effect depends upon drug action at the targets as well as uptake by the transporter, such responses are not well suited to quantifying transport.

Most uptake assays focus upon detecting the drug inside the cell. Analytical chemistry or enzyme-linked assays can be used, or a drug may be naturally fluorescent (9-amino-acridine fluorescence was used to probe interactions with the yeast thiamine carrier [224]). However, in the general drug discovery environment a drug-specific uptake assay will usually not be available, and so general systems that can be applied to track virtually any molecule are preferable. Radiolabelled drugs are widely used in transporter activity assays, uptake being determined by scintillation counting on extracted cell contents [225-232]. However, it is often difficult to source radiolabelled compounds. Another way of labelling is to conjugate the drug to a fluorescent moiety so uptake can be followed by confocal microscopy, as demonstrated in a study on the reduced folate carrier of fluorescein-methotrexate [233-235]. However, the attachment of fluorescent moieties can potentially alter the nature of the drug-transporter interaction. By contrast, it can sometimes be preferable not to use the native drug but a close analogue thereof, as is the case when the substrate is rapidly metabolised (for example, 2-deoxy-D-glucose served as an analogue in a study of glucose transport in yeast [236]). Other strategies to negate problems of metabolism or rapid efflux have been developed. In the entrance counter-flow assay the cell or vesicle is loaded with an unlabelled version of the substrate that competes at the metabolising enzymes or efflux transporter to limit processing of the labelled substrate [237].

Analytical methods such as HPLC, GC, MS, or any combination can be used to determine the concentration/time profile of a drug in the footprint, or exometabolome, (the metabolome outside the cell) and/or in the fingerprint (the internal metabolome). Identification and quantification of the drug can be achieved if retention times and/or fragmentation patterns are established using the drug as an internal standard [238-240]. If the mechanism of breakdown (and, therefore, its products) are unknown, stable isotopic labelling with subsequent MS analysis is a valuable tool to determine these processes [240-243]. If a radioactive label is used, disappearance from the medium and accumulation within the

cell can directly be measured by scintillation counting and is independent of the metabolic fate of the drug.

Our preference would be for a mass spectrometry-based fingerprinting approach, preceded by two higher throughput assays based on cytotoxicity and DIMS analyses of the footprint, illustrated in Fig. (**2**). The footprint [244,245] is essentially the metabolic profile of the extracellular fluid. It is preferable as it is technically simpler because there is no extraction step involved; only very small sample volumes are required (20 µl, compared to 25 ml from 20-30 mg biomass for fingerprinting). This means that footprinting growth can be run on a 96-well plate and therefore easily combined with the preceding cytotoxicity assay. Loss of the drug from the footprint does not prove uptake; the drug could, for example, have been broken down or been digested, or be interacting with the transporter without being taken up. However, the cytotoxicity and footprinting assays do triage out cheaply drugs that show no interaction. To prove uptake one needs to demonstrate the presence of the drug in the metabolic fingerprint, which is the metabolome of the inside of the cell. Using the pure drug's fragmentation pattern as a standard, its presence in the footprint can be identified by GC-MS or LC-MS. By sampling at intervals and plotting peak area against time, the approach can also be used to determine uptake kinetics. This system benefits from being applicable to most drugs without labelling (the molecular fingerprint of the standard is used to detect and quantify uptake).

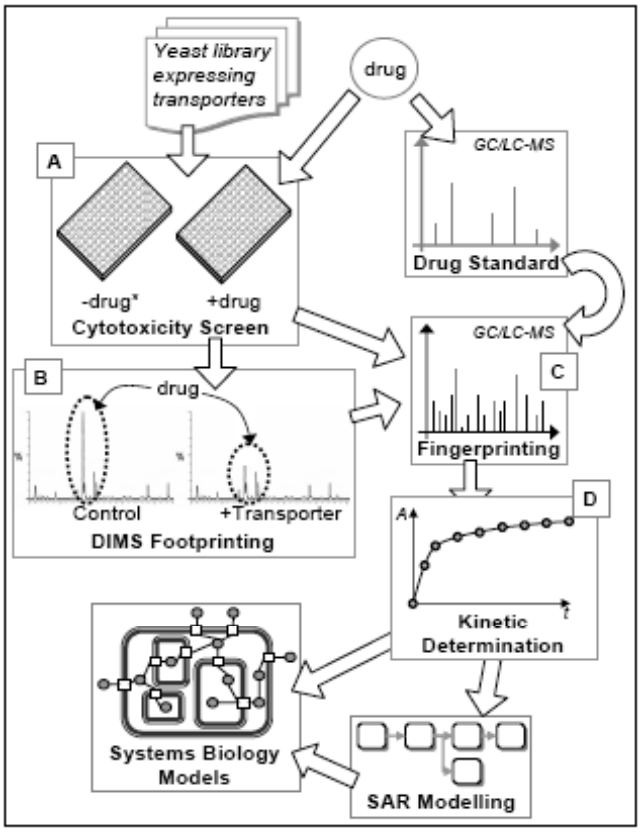

Fig. (2). Workflow for discovering drug uptake by mass spectrometry. Cytotoxicity in step A is a simple screen for cell entry (assuming that cytotoxicity implies uptake). Cytotoxicity at step A leads to step C, otherwise to step B. (*Control determines the growth of transporter-expressing cells without drug, so this step is not required for every screen). Step B is a direct infusion mass spectrometry analysis of the yeast footprint. If the drug remains in the footprint it does not enter the cells (caution is required with efflux). Diminished drug levels in the footprint do not imply uptake, but some interaction is occurring. Step C assesses the fingerprint (internal content) by GC/LC-MS. The presence of drug in the fingerprint can be detected by comparison to the standard, and area under identifying peaks as a function of time used to determine kinetics in step D.

TRANSPORTER STRUCTURE-ACTIVITY RELATIONSHIPS (SARs)

The later a failure occurs in the drug development pipeline the more expensive it is. Therefore, the goal is to characterise compounds as early on in the process as possible. Virtual library design describes the use of chemoinformatics methods to focus on the parts of chemical space where lead-like and drug-like [246] compounds are found. Similarly, early knowledge of a drug's likely pharmacokinetic properties allows prioritisation of resources. While high-throughput transporter screens allow ligands (substrates and inhibitors) to be identified rapidly, the time and financial cost of even highly efficient screening programmes means that only a relatively small fraction of interesting compounds can be assessed. This means that experimental screens cannot inform drug development until a relatively late stage. To access Systems Biology pharmacokinetic simulations as early as possible in the pipeline computational modelling can be used to predict drug-transporter interactions [247].

If transporter structures were available, SARs could be addressed by computationally docking putative drugs to three-dimensional protein structures; but, of course, these are notoriously difficult to obtain for membrane proteins. Some bioinformatics attempts have been made to predict transporter structure [248], but with only a handful of relevant templates [249,250] homology modelling is of limited utility, while *de novo* prediction seems even less feasible. An alternative approach is to build models that can link changes in ligand structure to measured uptake activity, such that the likely activity of a new ligand can be predicted. To provide deep mechanistic insight, one might endeavour to determine the pharmacophore; the molecular framework that determines drug interactions and so activity [251,252]. This describes the molecular properties and configurations required to interact with the transporter [253]. Many pharmacophore identification strategies have been developed and benchmarked [254]. Pharmacophores are already reasonably well established for some uptake transporters (hOCT1 and hOCT2 [255]; hCNT1, hCNT2 and hENT1 [256]; hASBT [257,258]; hPEPT1 [23,24,259]). Putative drugs related to the pharmacophore are likely to interact, but this prediction is usually qualitative and to determine kinetics further screening is required. To extend the utility of predictors, quantitative models of binding affinity can be constructed. For example, Bailey *et al.* [260] developed a linear combination of weighted terms derived from the PEPT1 pharmacophore to predict approximate affinity for the transporter.

Rather than pursuing the pharmacophore, structure activity relationships (SAR) can be constructed by methods such as Comparative Molecular Field Analysis (CoMFA) [261,262] and various extensions thereof. The interaction fields (typically electrostatic and steric) around a set of aligned compounds are sampled and used as input to partial least squares models regressing against activity (binding affinity). There is an inherent assumption of equivalent binding modes within such analyses. This can limit CoMFA to compounds drawn from a congeneric series (molecules that are fundamentally the same with only slight variations between them), but the promiscuity of some transporters permits uptake of highly diverse compounds. The terms determining PEPT1 substrate affinity defined by Bailey *et al*. [260] include a charge interaction with the substrate's N-terminus, hydrogen bonding capacity to the first peptide carbonyl group, capacity to interact at a hydrophobic pocket, plus steric considerations and other terms, but it is apparent that known ligands do not have to satisfy all of these terms in order to be a substrate. The relaxed nature of transporter substrate binding may present new challenges for chemo-informatics, where the traditional focus has been on rather more specific binding processes.

The widespread use of computational modelling to identify transporter substrates and inhibitors is confounded by a lack of unifying technology to orchestrate the effort. Systems Biology is able to operate because the community has established protocols and standards [263] to share data but there is no widely-accepted standard for reporting SARs. There are also concerns over the statistical validity of some SAR models due to the limited amount of data on which they are built, non-standard validation methods, and a lack of equivalence between data for the same processes generated in different systems [264]; but, ultimately, these are problems that can be fixed. One then envisages a scenario, depicted in Fig. (**3**) where the yeast-based transporter screens feed into SAR modelling, most usefully in an active learning environment [265]. Putative drugs can then be passed through the predictors. Where quantitative models have been built binding predictions might feed directly into pharmacokinetic models, although it is probably more realistic that qualitatively predicted substrates are then kinetically parameterised by further experiments. The predicted or measured kinetics then feed into the Systems Biology pharmacokinetic model. Note also that within these models it is possible to address concerns over the *in vivo* relevance of kinetics measured in different expression systems by parameter tuning algorithms (e.g. [266,267]), where initial parameter estimates are fed into simulations and iteratively adapted to match experimentally-determined drug distributions. Best of all will be the highly important and necessary development of the 'digital' or 'virtual' human (see e.g. [268-271]) – a computer model of the main aspects of human biochemistry and physiology.

To date, transporter SAR modelling has built upon those compounds that happen to have been screened previously, but a stable platform for determining transport, as offered by the yeast expression system, permits the generation of larger (and better-designed) data sets that lead to more accurate and robust SAR models for the major uptake transporters. For a relatively modest investment SAR models can be built for each transporter. In combination with Systems Biology models this will allow prediction of pharmacokinetics early in the drug pipeline.

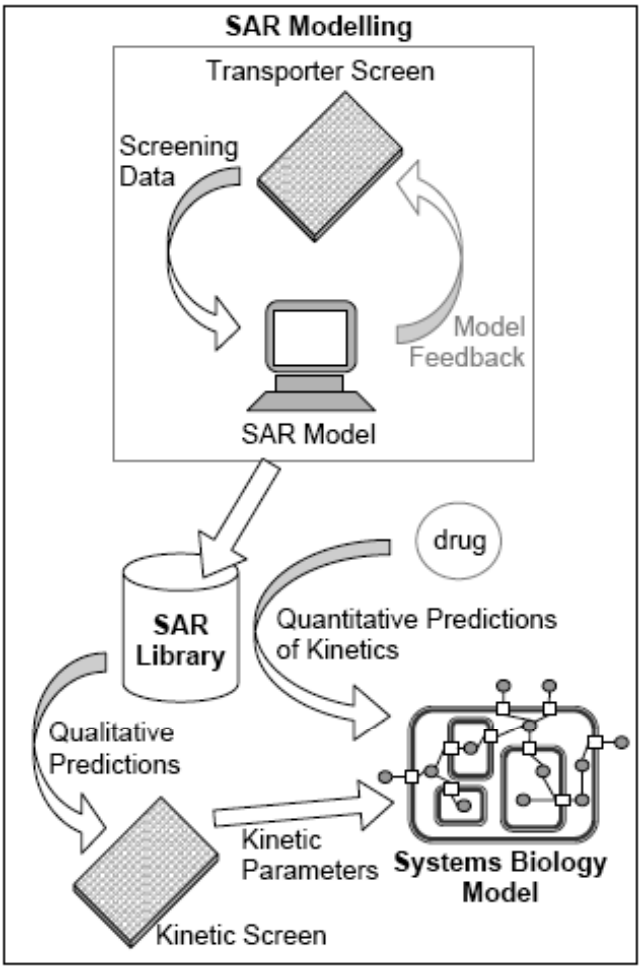

Fig. (3). The experimental challenge presented by drug-transporter interactions can be addressed by building structure-activity relationships that are able to predict whether a putative drug is likely to be a substrate. A useful goal for the drug discovery community is the development of a transporter SAR library that allows interactions to be predicted. This can considerably reduce the load on further transporter screens and so permit earlier application of transporter-aware pharmacokinetics models in drug development.

SUMMARY

If one considers the number of transporters in the human genome (~1000) and the number of functional variants of these within the population (we are not aware of any reliable figures for transporters) it should be possible to construct an expression library covering most or all uptake transporters. Yet the number of potential transporter substrates and inhibitors (drugs, nutrients, gut microflora metabolites) we might wish to screen against the transporter library is considerably greater. As a result screening all possible transporter-substrate/inhibitor combinations exhaustively is not viable, even if the library only considers transporters that are most likely transport drugs (those that are known to be

promiscuous) or by reference to the abundance of variants in the population. The solution, however, is clear. We should not attempt to screen all possible transport processes, but instead use rigorous experimental designs to identify efficiently the relevant structure-activity relationships in Systems Biology models. Modelling is central to the solution (Fig. (**4**)). What remains is still a mammoth task, requiring molecular and systems biologists, physiologists, chemists, informaticians, and other disciplines, but, as shown by the emerging methods of Systems Biology [168], as well as automated literature analyses [272-275], the key is to coordinate the activities of these disciplines and to share their results.

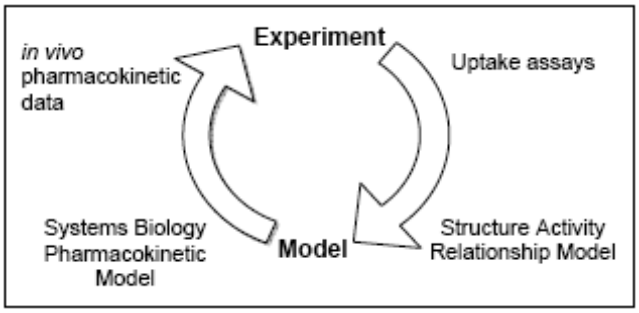

Fig. (4). Exhaustively screening all possible drug-transporter combinations is intractable, and the solution requires a central role for modelling.

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ABBREVIATIONS

SLC	=	Solute Carrier
BBB	=	Blood-Brain Barrier
DIMS	=	Direct Injection Mass Spectrometry
SAR	=	Structure Activity Relationship
CoMFA	=	Comparative Molecular Field Analysis

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Protein	Species	Reference
ACBP*	Bos taurus	[1]
Kidney (MDCK) cell chloride channel*	Canis familiaris	[2]
Na,K-ATPases (beta subunit from kidney)*	Canis familiaris	[3]
Emopamil Binding Protein (EBP)	Cavia porcellus	[4]
Lamin B Receptor (LBR)	Gallus gallus	[5]
D1A dopamine receptor	Homo sapiens	[6]
retina specific ABC transporter*	Homo sapiens	[7]
O-fucosylated epidermal growth factor	Homo sapiens	[8]
ClC2-channel (CLC-voltage gated chloride channels)*	Homo sapiens	[9]
Emopamil Binding Protein (EBP)	Homo sapiens	[4]
Uncoupling Protein 3S (hUCP3S)	Homo sapiens	[10]
UGT2 UDP-Gal transporter*	Homo sapiens	[11]
VpS24	Homo sapiens	[12]
cystic fibrosis transmembrane conductance regulator (CFTR)*	Homo sapiens	[13, 14]
pro-apoptotic Bax	Homo sapiens	[15]
MDR1*	Homo sapiens	[16]
μ-Opioid Receptor	Homo sapiens	[17]
CHIP28 water channel*	Homo sapiens	[18]
neurotensin receptor type 1 (h-NT1-R)	Homo sapiens	[19]
RhCG glycoprotein*	Homo sapiens	[20]
mitochondrial ornithine transporter*	Homo sapiens	[21]
nucleoside transporter (hUGTrel7)*	Homo sapiens	[22]
adenosine receptor (A2a)	Homo sapiens	[23, 24]
Integrin associated protein (CD47)	Homo sapiens	[25]
M1 muscarinic receptor	Homo sapiens	[26]
AQP1*	Homo sapiens	[27]
transferrin receptor (hTfR)	Homo sapiens	[28]
TMEM85	Homo sapiens	[29]
D2S dopamine receptor	Homo sapiens	[30]
fused to beta1, 4-galactosyltransferase	Homo sapiens	[31]
Mitochondrial Ferredoxin	Homo sapiens	[32]
erythroid anion exchanger AE1*	Homo sapiens	[33]
Lamin B	Homo sapiens	[5]
UDP-galactose transporter hUGT1*	Homo sapiens	[34]
UDP-galactose transporter hUGT2*	Homo sapiens	[34]
ARV1*	Homo sapiens	[35]
P450c17	Homo sapiens	[36]
transferrin receptor	Homo sapiens	[37]
Glycosylphosphatidylinositol (GPI)	Homo sapiens	[38]
Ca++-ATPase (hSPCA1)*	Homo sapiens	[39]
(NBMPR-sensitive) equilibrative nucleoside transporter protein (hENT1)*	Homo sapiens	[40]
concentrative nucleoside transporter (hCNT1)*	Homo sapiens	[41]
Rh-glycoproteins (RhAG, RhBG, and RhCG)*	Homo sapiens	[42]
GLUT1*	Homo sapiens	[43]
GLUT4*	Homo sapiens	[43]
UCP2	Homo sapiens	[44]
UCP3	Homo sapiens	[44]
Oxoglutarate Transporter*	Homo sapiens	[45]
UCP3L	Homo sapiens	[45]
5HT5A receptor	Mus musculus	[46]
chromaffin granule Cyt b561 (CGCytb)	Mus musculus	[47]
Murine Golgi CMP-Sialic Acid Transporter*	Mus musculus	[48]
ammonium transporter RhCG*	Mus musculus	[49]
MDR3*	Mus musculus	[50]
MDR3 (P-glycoprotein)*	Mus musculus	[51]
MDR1, MDR2, MDR3 (P-glycoproteins)*	Mus musculus	[52]
Glycosylphosphatidylinositol (GPI)	Mus musculus	[38]
Mammalian stimulating G-protein of the adenylate cyclase complex (Gs-alpha)	Not specified	[53]
MRP1*	Not specified	[54]
SERCA1*	Oryctolagus cuniculus	[55]
SERCA1a Ca++ -ATPase*	Oryctolagus cuniculus	[56]
sarcoplasmic reticulum Ca2+-ATPase*	Oryctolagus cuniculus	[57]
Na,K-ATPases (sheep alpha subunit from kidney)*	Ovis aries	[3]
α (subunit for the gastric H+,K+-ATPase (Hkβ)*	Rattus norvegicus	[58]
NHE2 (Na+/H+ antiporter)*	Rattus norvegicus	[59]
syntaxin 6	Rattus norvegicus	[60]
α and β subunits of the amiloride-sensitive epithelial sodium channel (αβENaC)*	Rattus norvegicus	[61]
VPAC1 receptor	Rattus norvegicus	[62]
type I iodothyronine deiodinase (D1) selenoprotein	Rattus norvegicus	[63]

Sac1	Rattus norvegicus	[64]
17 olfactory receptor	Rattus norvegicus	[65]
AQP3, AQP5, AQP9*	Rattus norvegicus	[27]
Na+/K+ ATPase subunits*	Rattus norvegicus	[66]
metabotropic glutamate receptor (mGluR1α)	Rattus norvegicus	[67]
concentrative nucleoside transporter (rCNT1)*	Rattus norvegicus	[41]
vesicular monoamine transporter (rVMAT1)*	Rattus norvegicus	[68]
UDP-N-acetylglucosamine-alpha-3D-mannosidase-beta-1,2-N-acetylglucosaminyl transferase	Rattus norvegicus	[69]
UCP1	Rattus norvegicus	[45]

Supplementary Table 1. Mammalian membrane proteins that have been expressed in yeast. *Indicates that the expressed protein is a transporter.

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