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Pharmacophore-based discovery of ligands for drug transporters

Cheng Chang¹, Sean Ekins^{1,2}, Praveen Bahadduri¹, and Peter W. Swaan¹

¹ Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, MD 21201 and

² ACT LLC, 1 Penn Plaza-36th Floor, New York, NY 10119

Abstract

The ability to identify ligands for drug transporters is an important step in drug discovery and development. It can both improve accurate profiling of lead pharmacokinetic properties and assist in the discovery of new chemical entities targeting transporters. *In silico* approaches, especially pharmacophore-based database screening methods have great potential in improving the throughput of current transporter ligand identification assays, leading to a higher hit rate by focusing *in vitro* testing to the most promising hits. In this review, the potential of different *in silico* methods in transporter ligand identification studies are compared and summarized with an emphasis on pharmacophore modeling. Various implementations of pharmacophore model generation, database compilation and flexible screening algorithms are also introduced. Recent successful utilization of database searching with pharmacophores to identify novel ligands for the pharmaceutically significant transporters hPepT1, P-gp, BCRP, MRP1 and DAT are reviewed and challenges encountered with current approaches are discussed.

Keywords

Pharmacophore; QSAR; transporter; ligand; substrate; inhibitor; database screening; ADME

1. Introduction

1.1 Identifying ligands for transporters

Advancement in the accurate prediction of lead pharmacokinetic properties such as absorption, distribution, metabolism and excretion (ADME) may significantly impact the intended goals of the FDA Critical Path Initiative to enable faster, less expensive and more predictable drug development. As has been emphasized in recent reviews [1–3], optimizing lead pharmacokinetic properties early in the drug discovery phase has great potential in lowering the attrition rate in the later (more expensive) development stages. Most pharmacokinetic prediction algorithms attempt to model passive diffusion mechanisms when treating membrane permeation of drugs. As a result, the involvement of transporters in drug disposition complicates the prediction process. Therefore, accurate pharmacokinetic property profiling requires different calculation methods for leads that interact with transporters and those that do not. Thus, the ability to identify ligands (i.e. substrates and inhibitors) for clinically significant transporters is essential to improve lead profiling.

For example, the recent phase II failure of CI-1034, an endothelin-A receptor antagonist for the treatment of pulmonary hypertension, was attributed to its inhibition of the major hepatic organic anion transporter polypeptides (OATPs), which lead to its observed clinical

hepatotoxicity [4]. In fact, screening of lead-transporter interactions has been suggested as routine pre-clinical procedure [4]. Transporters can also play a positive role in drug development. For example, L-valyl esterification of antiviral agent acyclovir rendered valacyclovir substrates of hPEPT1, thus leading to a markedly higher oral bioavailability compared to the parent drug, acyclovir [5]. Although this mechanism was not clarified until after its development, more rational approaches utilizing valyl esterification to improve drug pharmacokinetic properties are underway [6]. Pravastatin is a prominent example to illustrate the major role of transporters in determining its pharmacokinetic behavior. As a substrate of both OATP and MRP2, pravastatin has prolonged exposure to the liver (its target organ) through efficient enterohepatic circulation [7–9]. In general, the achievement of such favorable pharmacokinetic properties as in the case of pravastatin through utilization of transporters is purely serendipitous and we still await a more rational approach in drug discovery. However, the use of transporters as pharmacokinetic drug targets has led to the establishment of small biopharmaceutical companies. For example, XenoPort, Inc. (Palo Alto, CA), was founded in 1999 solely for the purpose of rationally utilizing the body's intrinsic transport systems to improve drug pharmacokinetic properties. Considered an innovator in the industry, we can expect other companies to follow suit and focus more efforts on transporter proteins as therapeutic targets.

1.2 *In silico* approaches

The ever increasing computational power and the continuously improving sophistication of modeling algorithms have enabled computers to penetrate virtually all stages of pharmaceutical research and development [10]. The various *in silico* modeling approaches and techniques that can be applied to modeling drug transporters has been reviewed recently [11]. Briefly, the study of transporter-drug interactions can be approached from two distinct perspectives: either from the perspective of the transporter protein or the transported ligand, thus differentiating the methodologies into target-based or ligand-based methods. The target-based approaches directly calculate the three-dimensional structure of transporters, often based on homology to available crystal structures of genetically and functionally related transporter proteins [12]. The ligand-based methods analyze transport or inhibition activities of a set of ligands (substrates and inhibitors) and derive transporter-ligand interaction models without prior knowledge of transporter structure. Most commonly utilized ligand-based methods are quantitative structure activity relationship (QSAR) and pharmacophore modeling. Because of the limited number of available transporter templates, target-based approaches are –currently– not as widely applied as ligand-based approaches [11]. Interestingly, a trend has recently emerged in which the algorithms from target- and ligand-based approaches are integrated to gain additional structural insights and to further validate the respective individual methods; for example, a combinatorial approach to studying cytochrome P450s [13] and the androgen receptor [14] combined homology modeling, docking and QSAR approaches. The resulting models are synergistic in many aspects.

Although the availability of only a few templates for accurate homology modeling poses a challenge, additional difficulty is encountered when identifying the ligand binding domains (LBD) of transporters. This can be mainly ascribed to the dynamic nature of the ligand translocation process: whereas receptor binding can be described simply by ligand recognition and binding, affinity for a transporter protein is more complex. In essence, the translocation process can be broken down into several distinct events: ligand recognition, binding of co-transported ions, conformational changes, shuttling of the ligand across different binding domains and, eventually, translocation and release on the other side of the membrane. Importantly, multiple binding sites may exist and ligand binding to these domains may be temporary and, therefore, diffuse. With the increasing number of crystal structures for

membrane proteins, especially two of the major facilitator transporters [15,16], we should expect such integrated studies for transporters in the future.

Two methods are available for the computational design or identification of novel ligands for transporters, the *de novo* and virtual screening approaches. In the *de novo* approach, a quantitative model (a 3D-QSAR model or a binding pocket with scoring functions to predict the affinity of different docked molecules) is first generated which guides subsequent chemical synthesis of more potent lead compounds. This is generally followed by *in vitro* testing to provide feedback for improving the quantitative model which, in turn, provides more accurate synthesis suggestions. The virtual screening approach takes advantage of the enormous number of readily available compounds from current commercial vendors of molecule databases. Criteria describing transport requirements such as pharmacophore models can be used to screen these databases as a means to rapidly select molecules with potential affinity for the target transporter (Figure 1). The returned hits are subsequently purchased from the vendor and tested *in vitro* for their affinity. Thus, the expensive and time-consuming chemical synthesis step required in the *de novo* approach is avoided by selecting from the commercially available compounds. As a result, virtual screening is more tolerable to the false-positives in model predictions compared to the chemical synthesis method [17], and subsequently has become more frequently utilized for the identification of novel transporter ligands (described below). Virtual screening is one of the many data mining methods to analyze the increasingly available data derived from genomics, proteomics and many other –omics studies. For a detailed discussion of drug delivery-related data mining techniques, we would kindly refer the reader to the review by Ekins *et al.* in this volume.

The aforementioned transporter models include pharmacophores, QSAR models and binding pocket models with scoring functions to rank-order docked structures. 3D-QSAR models require the accurate alignment of ligands for reliable activity prediction, which limits their application in automated database screening. However, it is likely that some transporters can tolerate flexibility and the possession of multiple binding sites would further indicate a degree of promiscuity [18] that could enable different conformations of the same molecule to bind. On the other hand, 2D-QSAR models are easy to implement for screening databases, but lack the easily interpretable 3D feature information useful for the design of new highly active drugs. Another popular approach that has gained significant interest recently is the flexible docking method, which is more accurate in ligand identification if a binding pocket structure is available. However, this method has only found limited application to transporter studies due to the intrinsic difficulties of deriving transporter structures *and* identifying their binding pockets. Thus, most researchers utilize pharmacophore-based database screening method to identify transporter ligands [19–22]. Accordingly, in this review our discussion on the discovery and identification of transporter ligands is focused on the application of pharmacophore models.

2. Pharmacophore-based database screening

When first introduced in the early 1900s by Paul Ehrlich, a “pharmacophore” referred to a “molecular framework that carries (phoros) the essential features responsible for a drug’s (pharmacon) biological activity” [23]. With the increased knowledge of three-dimensional molecular structures, this concept expanded to also include the requirement of spatial arrangement of essential molecular “features”, e.g. steric and electrostatic characteristics or hydrogen-bonding capabilities [24]. The pharmacophore model can be derived either from a receptor binding site (direct method), or from a set of active ligands (indirect method). To the best of our knowledge, there are no reports on protein-based pharmacophore models for transporters mainly due to the aforementioned obstacles related to determining transporter binding site structures. Hence, our discussion will be focused on the indirect methods. Before

the introduction of automated or semiautomated computer programs, pharmacophore models were routinely sketched manually by medicinal chemists with chemical intuition when analyzing structure-activity relationship data. Published as the first article in the first issue of the Journal of Computer-Aided Molecular Design in 1987, the work by Mayer and colleagues [25] heralded the era of computer-assisted pharmacophore generation. The semi-automated method proposed in this work influenced the implementation of most current programs, directly or indirectly [26]. Since then, different pharmacophore perception algorithms have been described, including ALADDIN [27], DISCO [28], GERM [29], COMPASS [30], GASP [31], Catalyst Hiphop [32], [33], SCAMPI [34], Catalyst Hypogen [35], CLEW GAMMA [36], PARM [37] and DANTE [38]. Detailed discussion and comparison of these programs has recently been published [26,39]. Some important aspects relevant to database screening are discussed below.

2.1 Pharmacophore “features”

Pharmacophore models used to be represented by specific atoms and chemical groups such as a phenyl group or a carbonyl group. The introduction of functional features such as hydrogen bond acceptors, hydrogen bond donors, hydrophobes, positive/negative charges and ring aromatics in 1990 [26] expanded pharmacophore model coverage and enabled the discovery of more diverse ligands for different targets. These feature definitions have become standard libraries and were widely incorporated into different programs with minor modifications. One caution in implementing these functional features is that they are not mutually exclusive. For example, an amine group can be classified as either hydrogen bond acceptor or hydrogen bond donor. Therefore, the inclusion of structure-activity relationship data and further clarification of the role of these multi-feature groups in model building will improve overall model accuracy [26]. Since structural complementarity also plays an important role in ligand recognition, the inclusion of shape factors provides another effective implementation in pharmacophore modeling is. Shape considerations can be quantified either as excluded volumes where the regions cannot be occupied by potential hits or as molecular surfaces that require to be filled. As an example, the so-called “Shrink-Wrap” method has been proposed and implemented in DANTE as a way to incorporate shape information into pharmacophore modeling [40].

2.2 Databases and screening algorithms

Four different types of database formats were designed to facilitate flexible search using pharmacophore models. Chem-X (Chemical Design, Ltd.) and UNITY (Tripos, Inc.) generate and store single conformation 3D structures. This implementation leads to faster database generation and less storage space requirements. The compromise is an extended screening time since multiple conformations have to be generated on-the-fly. Catalyst (Accelrys Software, Inc.) and MACCS-3D (MDL Information Systems, Inc.) calculate and store multiple conformations for each structure during the database generation process. This feature ensures faster screening at the costs of longer database generation times and larger storage space requirements. Thus, multi-conformer databases are advantageous for frequent screening usage while single-conformer databases are suitable for infrequent screening. In addition, NCI has also provided a preliminary multi-conformer database for Catalyst pharmacophores screening queries (<http://cactus.nci.nih.gov/ncidb2/>).

Design of algorithms to allow 3D pharmacophore queries to screen 3D databases is another essential step to ensure correct retrieval of hits from molecule databases. Gund first suggested that the graph theory-based 2D structure searching method could be modified to perform 3D structure searching in 1977 [41]. Several screening algorithms were subsequently developed [27,42,43]. One important improvement in searching algorithm development is the retrieval of partially mapped hits which can greatly increase the hit list diversity. Many current programs allow hits to miss one or more pharmacophore feature by default. The quality of the

pharmacophore model as well as screening algorithm can be assessed using the Güner and Henry (GH) method by screening a database with compounds of known activities towards the target [44].

2.3 Hit list pruning

It is quite common to retrieve large hit lists due to implementation of flexible screening methods. Downstream filters may be required to reduce the hit list to a manageable size containing the most promising hits for *in vitro* testing. Usually the first filter applied is to cluster hits according to their similarities or even molecular weight. This allows resources to be focused on the representative compound from each structure classes. Drug-likeness, which is usually expressed as molecular or physicochemical properties that contribute to favorable ADME profiles such as Lipinski's "rule of five" [45], is also an important indicator when selecting hits for *in vitro* testing. Additional filters tailored to a protein or project could also be added. Chemical intuition is also an invaluable skill when making the final decision among the candidate molecules returned. Finally, commercial availability could be another limiting factor in deciding which compounds are tested.

2.4 In vitro assays

Pharmacophore-based database screening results can be further validated when combined with *in vitro* testing of drug candidate activity. *In vitro* experimental models identified to date are descriptive of drug absorption *in vivo* and are invaluable tools in the discovery of new chemical entities [46–48]. A number of *in vitro* tools are available for studying drug-transporter interactions. The choice of an *in vitro* tool for assessment of hits obtained by pharmacophore searches depends on the overall objective of the study; this may vary from determination of affinity for the transporter of interest for a large set of drug candidates (high throughput screening) to the determination of kinetic parameters or directionality of transport or tissue specificity of a specific transporter. The available methods can be broadly divided in the popular whole cell-based techniques or the less popular non-cell based methods. Cell-based assays utilize fully differentiated monolayers of mammalian cell lines to conduct a range of studies involving the determination of K_i or IC_{50} values and Michaelis-Menten type kinetic parameters in a simple accumulation assay [19,49–51], to the determination of permeability as well as affinity of drug towards a transporter in a transport assay [46,48,52–56]. Drug efflux proteins like P-gp, BCRP, MRP1, MRP2, etc., have become important determinants in the ADME/tox properties of drug candidates. In order to study clinically relevant efflux transporters bidirectional transport assays help determine if the selected drug candidates are substrates, non substrates or inhibitors for a particular efflux transporter [53,57–59]. The transport activity is quantitated by using either radiolabeled substrate or with direct measurement of drug concentration detected with HPLC or LC-MS/MS or other sensitive instrumental methods [53].

A broad choice of mammalian cells is available to conduct the *in vitro* screening. The whole cell based assays either use wild-type mammalian cells that express drug transporters or stably transfected cell line that overexpress the desired drug transporter under investigation. The most commonly used wild type cell lines are Caco-2 (P-gp and PEPT1) [53,60,61], SKPT-0193 Cl. 2 (PEPT2) [62,63], MDCK, or drug-selected MCF7 (BCRP) [64] cell lines. Caco-2 cells, when grown on semipermeable polycarbonate membrane differentiate into a highly functional epithelial barrier similar to intestinal columnar epithelium and is a valuable transport model system for the transporters like P-gp and PEPT1 expressed in small intestinal epithelium [46, 47]. Stably transfected mammalian cell lines overexpressing drug transporter of interest are commonly used as *in vitro* assay tool. Chinese hamster ovary cell line stably transfected with human small peptide transporter PEPT1 has been used to screen drug candidates selected by pharmacophore screening [19, 65]. MDCK-II cells stably transfected with MDR1 gene have

proven to be a good cell model for evaluating the affinity of drug candidates towards P-gp [66]. MDCK-MDR1 and Caco2 cell models have been used extensively to study drug efflux characteristics. The MDCK-MDR1 cell model offers an important time advantage (5–6 day model) over Caco-2 cells that require 21–28 days to develop into fully differentiated monolayers with functional tight junctions. The Calcein-AM assay has also been handy to test large number of drug candidates in a high throughput screen setting [53,67]. However, the limited sensitivity and relatively high concentrations required of the drug under investigation severely limits the applicability of this assay for drugs with low solubility or compounds with limited availability.

The non-cell based assay uses isolated membrane fractions that express transporter proteins and usually involves non-radioactive based colorimetric or fluorimetric detection of the drug activity quantitation. The non cell based assays are popular with studies involving P-gp, utilizing ATPase activation during drug transport by P-gp. Other examples of non cell based drug assays include parallel artificial membrane permeation assays (PAMPA)[68], inside-out membrane vesicles [69,70], and immobilized artificial membranes (IAM) [71,72].

3. Successful database searching applications in studying transporters

3.1 hPEPT1

The human intestinal peptide transporter (hPEPT1) is a proton-coupled, low-affinity, active, oligopeptide transport system with broad substrate specificity which enables transport of its natural substrates di- and tri-peptides occurring in food products [73,74], a broad range of peptide-like pharmaceutically relevant compounds, such as β -lactam antibiotics [75] and angiotensin-converting enzyme (ACE) inhibitors [76–78]. The transporter has been recognized as an important intermediate in the oral bioavailability of peptidomimetic compounds including the aforementioned valacyclovir [5,49].

Recent work has used the molecular structures of well known, relatively high affinity substrates from three different drug classes, Gly-Sar (dipeptide), bestatin (peptidomimetic) and enalapril (ACE inhibitor), to generate a Catalyst HIPHOP model for hPEPT1 [19]. The resulting pharmacophore consisted of two hydrophobic features, a hydrogen bond donor, hydrogen bond acceptor, and a negative ionizable feature which would indicate these are important features for interaction with this transporter. The pharmacophore was used to search the Comprehensive Medicinal Chemistry (CMC) database of over 8,000 “drug-like” molecules in an attempt to identify other hPEPT1 ligands. 145 virtual hits (~1.8 % of the total database) mapped to the pharmacophore features. Seven (0.09 % of the total database) of the best scoring molecules with drug-like molecular weight (i.e. MW < 500) were selected and purchased for *in vitro* testing. Three molecules, fluvastatin, aspartame and repaglinide, mapped to the pharmacophore features and were also experimentally verified as hPEPT1 inhibitors. The role of the transporter in the oral bioavailability of these three compounds had not been previously noted and this may explain to some extent the relatively high bioavailability and/or the variable pharmacokinetic and pharmacodynamic profile for these compounds. A further computational data mining exercise was performed with the pharmacophore by searching a database of over 500 commonly prescribed drugs which were created based on a clinician’s reference [79]. Sixty five molecules were selected using the pharmacophore; of these, 27 were known substrates or inhibitors for the transporter based on published observations. This pharmacophore-based database screening approach represents an efficient approach for identifying the role of peptide transport in absorption of new chemical entities prior to *in vitro* testing of these compounds.

3.2 Dopamine transporter

The dopamine transporter (DAT) has been implicated as the monoamine transporter that is most closely associated with the reinforcing effects of cocaine [80,81]. The binding of cocaine blocks the reuptake of dopamine by DAT, thus potentiating the dopaminergic effect [80]. Substantial efforts have focused on the identification of DAT inhibitors as a potential treatment for cocaine abuse [82,83]. However, many of these cocaine analogs demonstrate a cocaine-like behavioral profile in animal models of drug abuse [84]. Hence, there is great interest in the identification of novel DAT inhibitors. Pharmacophore-based database screening is capable of picking leads with completely different backbones and has been used in DAT inhibitor searching studies.

In the first such attempt, Wang and colleagues generated a pharmacophore model based on accumulated data on cocaine and its analogues and screened the NCI 3D-database [22]. The model identified the nitrogen atom, carbonyl group and the aromatic ring center as crucial binding determinants for DAT and returned 4094 hits from the NCI database. The total number of compounds was filtered to 385 by using molecular weight and structural diversity criteria. The first round of the [³H]-mazindol reuptake assay verified the impressive hit rate of 63% by categorizing 44 compounds out of 70 tested as DAT inhibitors. Among the 44 positive hits, 13 novel DAT inhibitors were further characterized in the [³H]-DA reuptake assay. The most promising compound was selected and subject to modifications to further improve its activity and selectivity. The structure-activity relationship study led to the discovery of a potent DAT inhibitor which partially mimics the effect of cocaine in rat. This pioneering study represents an efficient drug discovery paradigm; novel leads are first rapidly identified through virtual screening and inexpensively verified by in vitro testing followed by further structural modification. Subsequent SAR studies have been more focused on the optimization of the lead. The same group later updated their DAT pharmacophore with more experimental data [21]. The improved model contains one tertiary amine and two phenyl groups. Upon screening the ACD database, 3,4-disubstituted pyrrolidines were discovered and verified as novel DAT inhibitors [21]. More recently, additional experimental evidence prompted the same group to modify the pharmacophore model, replacing the tertiary amine with a hydrogen bond acceptor sp² N atom in a ring system [20]. This model was used to screen the NCI database again and suggested substituted pyridines as DAT inhibitors. In fact, sub microMolar inhibitors were later verified with the DA reuptake assay [20]. By continually updating their pharmacophore models that consist of specific structural groups or atoms and utilizing the models to screen different databases, Wang and colleagues have successfully discovered three classes of potent DAT inhibitors. However, more diverse hits should be expected when chemical functions (e.g. hydrogen bond donors, hydrogen bond acceptors, hydrophobic features) are used to construct the pharmacophore model.

In the meantime, Hoffman and colleagues have adopted a different approach to screen the NCI database for the identification of novel DAT inhibitors [85]. In order to incorporate 70 structurally diverse DAT inhibitors, which poses significant difficulties in pharmacophore modeling, the authors devised a 2D-QSAR model using genetic algorithm-partial least squares (GA-PLS). Although the descriptors used to generate the 2D-QSAR model are not amiable to interpretation, the model itself is highly efficient in database screening. Initial screening of the NCI database returned 25 hits, which upon high-throughput pharmacological analysis, yielded five micromolar-range inhibitors (Table 1). As shown in Table 1, the identified lead compounds display diverse structures, which increases the chance that some of them will pass all the stages of drug development processes.

3.3 P-gp

The efflux transporter, P-gp, is a large, membrane-bound protein normally expressed at many physiological barriers, including the intestinal epithelium, canalicular domain of hepatocytes, brush border of proximal tubule cells and capillary endothelial cells in the CNS [86]. P-gp results in reduced oral drug absorption and enhanced renal and biliary excretion of substrate drugs and its expression at the BBB is a key factor in limiting the CNS entry of many drugs. Thus, it plays a significant role in determining drug disposition. P-gp also represents an attractive target for multidrug resistance reversal agents because co-administration of P-gp inhibitors has been observed to increase the oral bioavailability of P-gp substrate drugs [87] and several compounds are in clinical studies [88].

The significance of P-gp has attracted many research efforts and resulted in a large number of experimental datasets characterizing its function, which has enabled extensive computational modeling [69,70,89,90]. Several pharmacophore models of inhibitors have been constructed and validated with molecules excluded from model building, suggesting these models are certainly adequate for ranking new molecules and interactions with P-gp in early drug discovery, yet their application in lead optimization may still be limited with narrow structural series [69,70].

We have used a preliminary P-gp substrate pharmacophore to qualitatively analyze whether certain molecules may have a propensity to bind to this transporter. Gleevec maps well to the pharmacophore features (Figure 2A). Recently, several studies have suggested that Gleevec binds P-gp with a K_i value of 18.3 μM , using a calcein-AM efflux assay, indicating it is a substrate as well as a modulator of human P-gp, which may be indicative of Gleevec drug interactions via P-gp [91, 92]. Another compound, curcumin maps well to the pharmacophore (Figure 2B) and has been shown to inhibit P-gp mediated Rhodamine-123 (R-123) efflux, in a dose-dependent manner in primary cultured rat hepatocytes [93], human cervical carcinoma cell line as well as inhibiting MRP1 and MRP2 [94]. These computational methods appear to be promising for predicting the P-gp involvement with Gleevec and curcumin. More recently, several P-gp pharmacophores have been used for database screening and identification of potential P-gp substrates and inhibitors. The first such report appeared in 2003, where Rebitzer and colleagues used a propafenone derivative MDR modelator-based pharmacophore model to screen Derwent World Drug Index. Among the returned 28 hits, 9 were previously described MDR-modulators [95]. A more detailed discussion of the P-gp pharmacophore-based database screening study were later published [96]. However, the experimental verification of the results was not discussed. A more recent publication by the same group utilized a self-organizing map model based on 131 propafenone-type inhibitors to mine over 130,000 compounds in the SPECS database [12]. Co-localization of molecules to the area of the most active compounds was used to restrict the testing to 7 compounds of which 2 were μM inhibitors. Another study has undertaken a comparison of different P-gp pharmacophore models for substrates and inhibitors and applied them to search structurally diverse molecule databases to identify new molecules and their P-gp inhibitor and substrate status [97]. Two inhibitor pharmacophore models and one substrate pharmacophore model were generated in this study. The validity of the models as well as screening methods was first evaluated using a database of 189 known P-gp substrates and non-substrates [98]. After the quantitative validation using GH Scores, all three models were applied to screen a database of over 500 commonly prescribed drugs used previously with hPEPT1 study. From the retrieved hits, seven drugs with previously undocumented P-gp affinities were selected and purchased for *in vitro* testing. Both [^3H]-digoxin transport assay and ATPase activation assay were carried out to characterize each candidate. All seven drugs were either μM inhibitors or substrates of P-gp [97]. This combined *in silico* and *in vitro* approach can be efficiently applied to identify molecules that have affinity

for P-gp which may be clinically relevant and represent further examples of database screening relevant to drug delivery.

3.4 MRP1

The multidrug resistance protein 1 (MRP1) is another efflux transporter that contributes to MDR [99]. MRP1 transports a broad range of substrates, ranging from anticancer drugs like vincristine, mitoxantrone, daunorubicin to organic anionic substrates like the conjugates of glutathione, glucuronide and sulfates [58,100,101]. Due to its increasing significance in MDR, there is a major interest to the discovery of MRP1 inhibitors as MDR reversal agents [102].

Recently we selected five diverse highly potent MRP1 inhibitors that have been developed as MDR reversal agents (LY329146, LY402913, dehydrosilybin, indolopyrimidine and phenoxymethylquinoxalinone II) from a recent review [102] and generated a Catalyst HIPHOP model. The model contains three ring aromatic and three HBA features (Figure 3). The model was applied to screen the literature database of over 500 clinically used drugs and retrieved eight hits (candesartan, eprosartan, fexofenadine, losartan, sulfasalazine, telmisartan, vancomycin and zafirlukast), among these, three drugs have been previously documented to have MRP1 affinity (losartan [103], sulfasalazine [104] and zafirlukast [105]). In addition, the recent work [103] indicates that tetrazole compounds are susceptible to P-gp and MRP1 mediated efflux. This suggests that the tetrazole-containing candesartan could also inhibit MRP1. Our model was generated based on synthesized high affinity MRP1 inhibitors, yet it successfully pulled out diverse commonly used drugs as MRP1 inhibitors. The literature data are relatively recent indicating the ongoing discovery of MRP1 ligands is a new occurrence and deserving of further study. More experimental verification is required to test the selected compounds.

3.5 BCRP

The Breast Cancer Resistance Protein (BCRP) is a second member of the G subfamily of ABC transporter family. Expressed on the apical side at the subcellular level in placenta [106], breast [64], liver hepatocytes and endothelium [107]. BCRP expression confers resistance to cancer cells from mitoxantrone and anthracyclines [64,108,109], camptothecin derived topoisomerase I inhibitors [110], methotrexate [111] and flavoperidol [112]. This half transporter, unlike P-gp and MRP1, has one ATP binding site and consumes one ATP molecule per substrate molecule transferred [113]. The diverse range of drugs that are exported by BCRP has prompted the design of many high affinity inhibitors [114]. These available data enabled our model generation. We recently calculated a pharmacophore model to describe BCRP inhibition requirements. Four BCRP inhibitors (GF120918, Ko143, nelfinavir, nicardipine) were prepared as the training set and a complex HIPHOP pharmacophore containing three HBA and three hydrophobic features was generated (Figure 4). The model was again utilized to search the database of over 500 commercially available drugs. Among the 37 returned hits, 6 were previously identified BCRP ligands (digoxin [57], docetaxel [115], indinavir [116], lopinavir, ritonavir, saquinavir [116] and the training set compound nicardipine). Current *in vitro* testing will elucidate the role of some of the remaining 31 proposed hits for BCRP.

4. Challenges and future perspectives

Based on a recent review [11], 27 homology models for 19 transporters and 38 predictive pharmacophore models from 15 drug transporters have been generated and published to date, yet only a few models (i.e., hPEPT1, P-gp, DAT, BCRP and MRP1) have been applied in the identification of novel ligands using a database searching approach. Currently, the majority of transporter pharmacophore modeling is carried out after chemical synthesis and *in vitro* bioassay work were performed. They are generally successful in the retrospective explanation

of the quantitative structure-activity relationships and their validity is usually corroborated by test set predictions. However, most of these studies do not prospectively guide the identification of novel ligands for transporters mainly due to three reasons.

First, with the application of the virtual screening route, which bypasses the chemical synthesis step required in the quantitative approach, relatively time consuming *in vitro* testing is required to validate model predictions. This normally necessitates a multidisciplinary approach involving coordinated collaborations between computational and experimental scientists. Such free association between academic laboratories would ultimately lead to case reports in the published literature; however, funding restrictions and the relatively few occurrences of program project grants (P01) that focus on this type of collaborative research limits the practical application of synergistic science. Although the pharmaceutical industry successfully applies such an approach, this will rarely result in publications due intellectual property concerns.

Second, unlike the relatively straightforward receptor-based assays, *in vitro* transport experiments are complicated by choosing from the plethora of available cell expression systems, inter-laboratory variability such as growth conditions, passaging techniques, etc. Currently, the Caco-2 human colon carcinoma cell line has been used extensively for permeability and other drug disposition studies. Large differences in affinities have been reported for actively transported substrates from across different research laboratories as well as cells in various stages of their culture (high and low passage numbers) [117]. Also there are reports of inconsistencies in correlation of *in vitro* and *in vivo* permeabilities with carrier mediated drugs suggesting Caco2 transporter expression varies from the expression profile in small intestine [118]. This adds a further hurdle to deriving consistent results. The ability of performing *in vitro* validations under the same or similar conditions as that of the model training data is an important requirement. Recently, a novel approach, meta-pharmacophore modeling, which integrates meta-analysis and pharmacophore modeling has been proposed and successfully applied to study OATPs [119]. In this study, transport activity data derived from different cell lines for both rat Oatp1a1 and human OATP1B1 were compiled and used to derive statistically stable and predictive pharmacophore models. A shape restrained OATP1B1 pharmacophore screening of the prescription drug database returned 33 hits. When applied appropriately, this meta-approach for studying transporters should lead to more tolerable experimental conditions for *in vitro* validation.

Third, there is currently less impetus for identifying novel ligands for transporters as most drug discovery programs target receptors. However, this could change as many of the “common” targets are pursued by the majority of pharmaceutical companies. With the characterization and description of the therapeutic implications of transporters still in its nascent stages, it is likely that novel clinically significant drug targets may be uncovered. Thus, despite the current obstacles, we are expected to see more prospective application of pharmacophore models to transporters in guiding chemical synthesis and bioassays in early stages of drug discovery. More importantly, the critical role of transporters in drug disposition (i.e. absorption, distribution, and excretion) as well as pharmacokinetics and pharmacodynamics necessitates the determination of affinity early in the drug development process. This would avoid late stage compound failures due to predictable properties that could lead to drug-drug interactions and potential other clinical side-effects. We would suggest that in future pharmaceutical companies could use computational models of transporters to actively improve bioavailability and predict pharmacokinetic properties.

In addition to the transporters previously described the following may be amenable to pharmacophore–database searching studies: **1) Organic cation transporters (OCT)** Three human OCT transporters have been identified to date which are involved in the reabsorption and excretion of endogenous compounds like choline and dopamine. A Catalyst

pharmacophore model exists for hOCT1 [120], but more robust models are necessary to cover the full spectrum of OCTs in drug disposition. **2) Organic anion transporters (OAT)** The rat kidney organic anion transporter is characterized by its transport of p-aminohippurate, cAMP and cGMP and by its inhibition by various anionic drugs. Yet, despite their clinical relevance, little or no information is available regarding the structure-activity relationships of OATs and they provide an excellent target for pharmacophore-based strategies. **3) Vitamin transporters (SVCT1)** The ascorbate transporter has been shown to be inhibited by some flavonols such as quercetin but less so by the related catechins and may be valuable for prodrug design as a viable means to improve membrane penetration of some drugs by addition of an ascorbic acid analog. **4) Nucleoside transporters (NTs)** Nucleoside transporters are important in mediating transport of nucleosides and nucleoside drugs (e.g., antiviral and anticancer drugs) across cell membranes. These transporters can be classified into two broad categories: the Na⁺-dependent concentrative (energy-dependent) transporters, which physiologically mediate influx of nucleosides, and the Na⁺-independent equilibrative (or facilitative) transporters, which mediate both influx and efflux. Five principal Na⁺-dependent concentrative nucleoside transporters (CNTs) have been identified so far. **5) Sodium taurocholate transporting polypeptide (NTCP)** The transport of bile acids in the liver occurs via the sodium taurocholate transporting polypeptide. Alterations in this activity contribute to cholestasis. **6) The apical sodium-dependent bile acid transporter (ASBT)** The high efficacy of the apical sodium-dependent bile acid transporter combined with its high capacity makes this system an important target for drug delivery purposes, including local drug targeting to the intestine and improving the intestinal absorption of poorly absorbable drugs. ASBT has recently received much attention because of its pivotal role in cholesterol metabolism. Baringhaus and colleagues [121] used Catalyst to develop an enantiospecific pharmacophore that mapped the molecular features essential for ASBT affinity.

As more transporter pharmacophores are developed and published it may be prudent to perform an analysis of the similarities between transporters both across and between families. This may result in a phylogenetic type of analysis by investigating pharmacophores for different transporters, map their distances and correlate this to sequence phylogenetic analysis. Although site-directed mutagenesis data has been generated for a small number of drug transporters this information could be modeled or incorporated to improve future pharmacophores.

Ultimately, the rate limiting step in pharmacophore generation is the collection and analysis of individual published studies that generally study one compound with one transporter. There are several databases of transporter information including the human membrane transporter database (HMTD), Transport DB and TP-Search that can be accessed online. Some of these provide information on molecules associated with particular human transporters which would provide a starting point for future model building. We envisage that some of these databases may also contain pharmacophores in the future or if not they will be collected together in some form of commercially available product.

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Figure 1.

A schematic for pharmacophore-database searching for drug discovery.

Figure 2.
Catalyst P-gp substrate pharmacophore with (A) Gleevec (B) Curcumin mapped to hydrophobic (blue), and H-bond acceptor (green) features.

Figure 3. Pharmacophore model for MRP1 inhibition was mapped to the training set compound LY402913 (A) and the retrieved positive hit sulfasalazine (B) (Yellow, ring aromatic; green, hydrogen bond acceptor; arrow, vector).

Figure 4. Pharmacophore model for BCRP inhibition was mapped to the training set compound GF120918 (A) and the retrieved positive hit docetaxol (B) (Cyan, hydrophobe; green, hydrogen bond acceptor; arrow, vector).

Table 1
List of novel transporter ligands identified through pharmacophore-based database screening.

Ligands	Transporter	Program	Query	Features	Database (size)	Verification	Hits subjects to verification	Ref
P-gp	Catalyst	HypoGen Pharmacophore	4 HYD, 1 HBA	SCUT (576)	Digoxin transport assay; ATPase assay	7	[97]
cholicalCIFerol

misoprostol

nafcillin

repaglinide

salmeterol

telmisartan
P-gp	Catalyst	HypoGen Pharmacophore	2 RA, 1 HYD, 1 HBA, 1 PI	WDI (80,000)	Literature search	28	[95, 96]
AHR-16303B

Terfenadine

Benidipine								

Ligands	Transporter	Program	Query	Features	Database (size)	Verification	Hits subjects to verification	Ref
P-gp	N/M	SOM	N/A	SPECS (134,767)	daunorubicin efflux assay	7	[12]
hPEPTI	Catalyst	HIPHOP Pharmacophore	2 HYD, 1 HBA, 1 HBD, 1 NI	CMC (8,000)	Gly-sar transporter assay	7	[19]

Ligands	Transporter	Program	Query	Features	Database (size)	Verification	Hits subjects to verification	Ref

9111	DAT	GA-PLS-Z (in house)	2D-QSAR	N/A	NCI (237,000)	High-throughput Pharmacological Analysis	25	[85]
16377

64540

289757

354657

3	DAT	QUANTA, Chem-X	Structural Pharmacophore	1 nitrogen atom, 1 carbonyl group, 1 aromatic ring center	NCI (206,876)	Mazindol and DA reuptake assay	70	[22]

Ligands	Transporter	Program	Query	Features	Database (size)	Verification	Hits subjects to verification	Ref
DAT	QUANTA, Chem-X	Structural Pharmacophore	1 tertiary amine, 2 phenyl groups	ACD (215,000)	DA reuptake assay	14	[21]
DAT	QUANTA, Chem-X	Structural Pharmacophore	1 hydrogen bond acceptor sp2 N, 2 phenyl groups	NCI (206,876)	DA reuptake assay	10	[20]

Possibly add MRP1 and BCRP hits...///