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# Pharmaceutical profiling in drug discovery

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**Drug discovery research organizations are building capability for measuring an ensemble of crucial 'drug-like' properties. These structure–property relationship (SPR) data complement current SAR information. This pharmaceutical profiling strategy enables research teams to better plan and interpret discovery experiments, be alerted to potential 'show stoppers', improve property liabilities, and select the best candidates for advancement. High throughput property assays for physicochemical properties – solubility, permeability, lipophilicity, stability, and p$K_a$ – *in vitro* ADME – metabolism, transporters, protein binding and CYP inhibition – and *in vivo* PK/exposure provide a wealth of data for teams to make informed decisions.**

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▼ Modern drug discovery research requires the continual application of strategies to increase efficiency, implement new technologies and increase candidate quality. Current strategies view discovery in terms of four stages: 'hit' selection, lead selection, lead optimization and development selection (Fig. 1). All organizations make crucial choices on the activities, tools and advancement criteria for each stage. Activities focus on:

- Chemical synthesis: to explore pharmacophore chemical space
- Biological testing: to measure activity and selectivity

Together, these form the successful SAR strategy, the correlation of structure and biological activity.

Pharmacokinetics (PK) and toxicology traditionally had a minor role during discovery. When studies revealed that poor properties cause development attrition [1], organizations implemented rigorous testing during candidate selection to ensure that compounds with poor properties did not advance. Implementation of this strategy revealed another need: series SAR was improved during early stages but the candidates were later rejected for inadequate PK or safety. These failures consumed resources, time and enthusiasm that could be expended on other series.

## Drug-like properties

Studies of drug databases showed that successful drugs tend to have 'drug-like properties'. Drug-likeness, when viewed at the *in vivo* level, is thought of in terms of PK and safety. These complex i*n vivo* properties result from an interaction of physicochemical and structural properties, such as solubility, permeability and stability, which are studied *in vitro*. These properties are, in turn, dictated by fundamental molecular properties, such as molecular weight, hydrogen bonding and polarity, which are studied *in silico*. As a result of the importance of properties, a new strategy emerged: testing the 'drug-like' properties of compounds during early discovery using high-throughput property methods *in silico*, *in vitro* and *in vivo* (often termed 'pharmaceutical profiling'). By studying the properties of drug candidates during discovery, increased efficiency and success should result.

Viable pharmaceutical products require both activity and drug-like properties [2]. 'Holistic' evaluation of each candidate considers both activity and properties, to determine modifications to the structural framework that will optimize overall performance (Table 1). van de Waterbeemd [3] discussed 'property-based design'. The resulting 'structure-property relationships' (SPR) complement SAR. Lipinski [4] discussed the close relationship between properties and potency: poorer solubility and permeability can be tolerated for highly potent candidates, but more favorable properties are necessary for lower potency compounds.

Most organizations acquire data on key properties of compounds shortly after synthesis or selection from libraries [2,5–8]. The strategy of applying property information during discovery

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has lead to several productive tactics (Box 1). In many cases, properties are measured to optimize PK. Properties that affect human absorption (e.g. permeability, solubility) and metabolism (e.g. stability) are used. Compounds that are highly active *in vitro* might not be therapeutic *in vivo* if they have insufficient PK properties. A less potent compound might provide better therapeutic effect if its properties permit enhanced *in vivo* exposure. Simple models [9–11] and physiologically based software for the prediction of intestinal absorption, such as iDEA (http://www.lionbioscience.com) and GastroPlus (http://www.simulations-plus.com) have been developed for human PK predictions.

An early tactic for using property information was to kill any losers early and cheaply. However, under the SAR strategy, compound synthesis must occur for research and patent purposes. Also, compounds with poor properties might be the only available starting-point for a target [12,13]. There has, therefore, been a shift to an optimization approach, where resources are directed toward improving structures for increased activity, selectivity and properties, despite their current performance.

A key emerging application of property data is for more informed discovery decisions. Many discovery experiments are affected by properties such as solubility or permeability [12,13]. Property information gives insights for the diagnosis of root causes of complex processes (e.g. solubility, permeability and stability affect *in vitro* assays as well as bioavailability). Statistical tools can be used to correlate properties and activity, and to learn which properties can be enhanced to improve activity [2,14]. Property information can aid in the planning of experimental conditions, synthetic modifications and formulations for *in vivo* experiments. Research teams can be alerted to major faults, to save resource investment in 'show stoppers'. If a team focuses solely on SAR, structural series with poor properties can result (e.g. SAR will often favor compounds with high lipophilicity because this will enhance target protein binding; however, such compounds could have increased liabilities for solubility, bioavailability or metabolic stability). Property information can also be used to select compounds for expensive and time-consuming biological models, or to prioritize compounds for further study. Poulain [15] included property considerations in hit-to-lead medicinal chemistry by starting with a screening library that fit the 'rule-of-5' [4] and profiling properties for subsequent synthetic analogs. With limited resources and compressed discovery time-lines, research teams need all the information they can get for informed decisions.

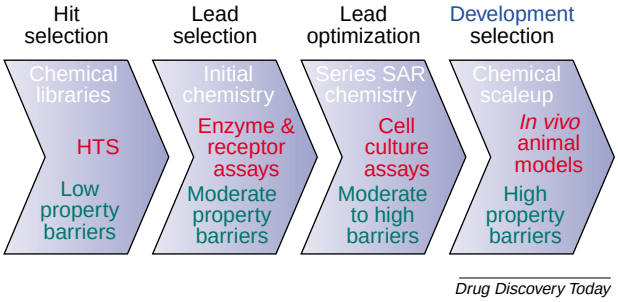


**Figure 1.** Drug discovery stages and their associated chemical synthesis (white) and biological testing (red) activities.

There are two ways to improve *in vivo* activity. The SAR approach modifies the structure for improved target binding. The SPR approach modifies the structure for improved properties that enhance delivery to the target. Structural modifications for improved SPR include molecular size, hydrogen bonding, polarity, ionizability and blockade of unstable positions. In the past, selection and optimization were driven by activity, but parallel property studies add a new discovery dimension (Fig. 2). In some cases, structural modifications that improve properties might diminish activity or another property [10]. Structural modifications should start with substructures that have minimal effect on SAR.

**Table 1. Parallel structure–activity relationships (SAR) and structure–property relationships (SPR) strategies both have *in vitro* and *in vivo* assay tactics**

| SAR | SPR |
|---|---|
| ***In vitro* assays** | ***In vitro* assay** |
| HTS | Integrity |
| Enzyme/receptor assays | Solubility |
| Cell-based assays | Permeability |
| | Lipophilicity |
| | p$K_a$ |
| | Stability |
| | Metabolite screening |
| | Transporters |
| | CYP450 inhibition |
| | Cell exposure |
| | Plasma–protein binding |
| ***In vivo* assays** | ***In vivo* assays** |
| Animal model | PK/exposure |

**Box 1. Opportunities for applying pharmaceutical profiling information**

- Prioritize compounds using enhanced data
- Interpret activity and property results
- Diagnose complex processes by individual properties
- Correlate properties with activities for optimization
- Better experiments knowing compound characteristics
- Alert research teams to 'show stoppers'
- Select compounds for expensive *in vivo* studies
- Predict *in vivo* ADME
- Guide structure modifications for properties
- Compare prodrugs
- Advance candidates likely to succeed

## Pharmaceutical profiling assays

### *Criteria for pharmaceutical profiling assays*

Property assays in pharmaceutical development are highly validated, accurate and precise for the in-depth study of individual compounds. However, the requirements in discovery are different. Discovery deals with large numbers of compounds (hundreds to thousands), small sample size (mg) and short time-lines (days to weeks). Thus, discovery assays must be high throughput, conservative in sample use, inexpensive and rapid. It is also important to evaluate the relevance of assay conditions. Many property assays seem uncomplicated and easy to implement; however, experimental conditions can greatly affect results and assays should be carefully developed. Methods should be developed and validated for correlation to high quality data. The assay predictability should be understood by the scientist using the information for proper interpretation. Tools are often developed using a diverse structural set, but are usually most consistent within a compound series.

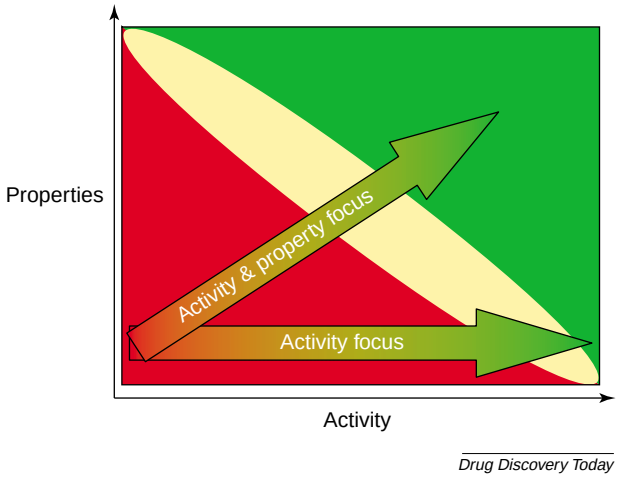


**Figure 2.** Discovery research previously focused on activity optimization. The most active candidates were advanced. Unfortunately, a significant percentage failed as a result of poor properties. An emerging strategy is to optimize both activity and properties during discovery, moving compound series out of the red area, where either activity or properties are inadequate, to the green area where candidates have the best opportunity to succeed. This requires the capability of property profiling throughout discovery.

### *Overview of tools for property assessment*

*Rules and filters* Like the 'rule-of-5' [4], rotatable bonds [16] and polar surface area [17] are easy to use, provide immediate feedback [12] and are effective.

*In silico models* These are easy and inexpensive to use [18]. Predictions from structure or measured properties are performed. These tools predict properties of virtual compounds and libraries when planning for chemical synthesis and of compounds with insufficient material for analysis. Over-weighting compounds from the structural class in the model improves series predictions. *In silico* models are constantly improving. Clark [18], and other contributors to the special edition of *Advanced Drug Delivery Reviews,* extensively discuss the capabilities and limitations of *in silico* models.

*High throughput assays* These assays (50–1000 per day) provide rapid measurement of compound properties early in the compound lifetime. Little material is used and large numbers of compounds are assayed. Moderate quality control (QC) should be performed. The use of highly predictive *in silico* methods for some properties (e.g. Log D, p$K_a$) and high-throughput assays for others, efficiently provides information for hit selection through lead optimization.

*In-depth analyses* These provide detailed data of high confidence for decisions in candidate selection during later discovery stages. Large quantities of material, longer time-lines and careful QC are required. Only a limited number of compounds can be studied.

### *Barrier-assay model*

There are many more assays that could be implemented in discovery than resources available. In selecting assays, it is important that they relate to defined needs, so discovery scientists can directly apply the information. Assay selection is aided by the model in Figure 3. A compound encounters many barriers on its path to the therapeutic target. The barrier might be physical (membrane), physicochemical (solubility, pH) or biochemical (metabolism). Each barrier attenuates the amount of compound reaching the therapeutic target. The assays selected for pharmaceutical profiling should provide information on how the compound performs at a barrier. In this way, the medicinal chemist can envision how a structure can be modified to improve properties that optimize transmission through a barrier. A diagram of the

complexity of the *in vivo* multiple barriers is shown in Figure 4, along with profiling assays that provide information on compound performance at these barriers.

In the following section are a series of assays that reflect this 'barrier-assay' model. These assays can be applied individually to understand performance at a particular barrier, or multiple assays can be performed to diagnose complex *in vivo* processes (e.g. bioavailability) or to apply statistical analysis [2,14].

## High throughput physicochemical profiling

### *Integrity*

It is easy to overlook the quality of compounds used in discovery. Compounds might have degraded in HTS screening plates or storage vials. Purchased compounds might not have undergone proper QC. A measured biological activity or property could be caused by an impurity. Thus, integrity can be considered a barrier to SAR and requires verification. Integrity profiling is typically performed using LC–UV–MS technology [19]. This rapid analysis uses HPLC with a wide solvent polarity gradient to separate diverse sample components, UV detection estimates their relative amount, and MS confirms their identity through their molecular weight. Criteria are used for acceptance of the material (e.g. >80% purity, MW consistent with structure). Integrity profiling can be initiated as soon as hits from HTS are selected and continued as new compounds are synthesized.

### *Solubility*

Solubility is a barrier that limits the concentration of a compound in the activity test solution, as well as in the intestine, and limits bioavailability. Solubility dictates when a formulation or salt form is needed for animal experiments. Poor solubility compromises other property assays [12,13]. For discovery, it is beneficial to measure 'kinetic' solubility in which a compound DMSO solution is added to aqueous buffer, because this is how discovery activity experiments are conducted.

Several high throughput assays for solubility have been described: turbidimetry [4], nephalometry [20] and direct UV [21]. In direct UV, a 20 mg $ml^{-1}$ DMSO solution is added to buffer in a 96-well plate. The final DMSO concentration is kept at or below 0.5%, because DMSO enhances solubility. The solution equilibrates for 18 h, is filtered to remove precipitate and the concentration is measured using an UV plate reader. Lipinski [4] noted that solubility is not likely to limit fraction absorbed for an orally administered drug with a dose of 1 mg $kg^{-1}$, if the solubility is greater than 65 µg $ml^{-1}$, but it is likely to limit absorption if the solubility is less than 10 µg $ml^{-1}$. Solubility measurements at several pHs are encouraged because pH can dramatically affect solubility throughout the intestine.

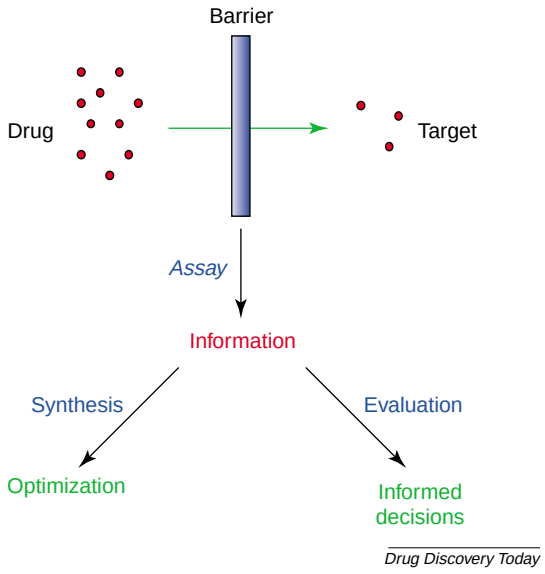


**Figure 3.** Barrier-assay model for pharmaceutical profiling. Barriers attenuate the concentration of compound reaching the therapeutic target. Pharmaceutical profiling assays provide data to assist the understanding of compound performance at a barrier.

### *Lipophilicity*

Many drug research parameters are affected by lipophilicity, the tendency of a compound to partition into non-polar versus aqueous environments. Increasing lipophilicity of a compound series generally increases permeability, protein binding and volume of distribution, and decreases solubility and renal extraction [3]. The common measure of lipophilicity is Log P (log of partition coefficient between octanol and water). The term Log P is used when all the solute is in the neutral state and Log D is used when the pH causes part or all of the solute to be ionized. Literature values for many compounds were compiled by Hansch [22]. For higher throughput, the 'shake flask' method can be scaled down to 96 deep-well plates [23]. Reversed phase HPLC [24,25] and *in silico* methods (e.g. ProLog D) are effective over wide lipophilicity and structural ranges and are efficient for early discovery.

### *Physicochemical stability*

Discovery activity assays (e.g. HTS, enzyme/receptor, cellular, animal) are inaccurate or variable when the compound is chemically converted in the assay buffer or dosing solution. Other destabilizing conditions for discovery compounds include pH from 2–8 in the intestine, laboratory light exposure and storage degradation [26]. Stability assays can be conducted in 96-well plates under various conditions and LC–MS is used to measure the remaining compound versus control [27].

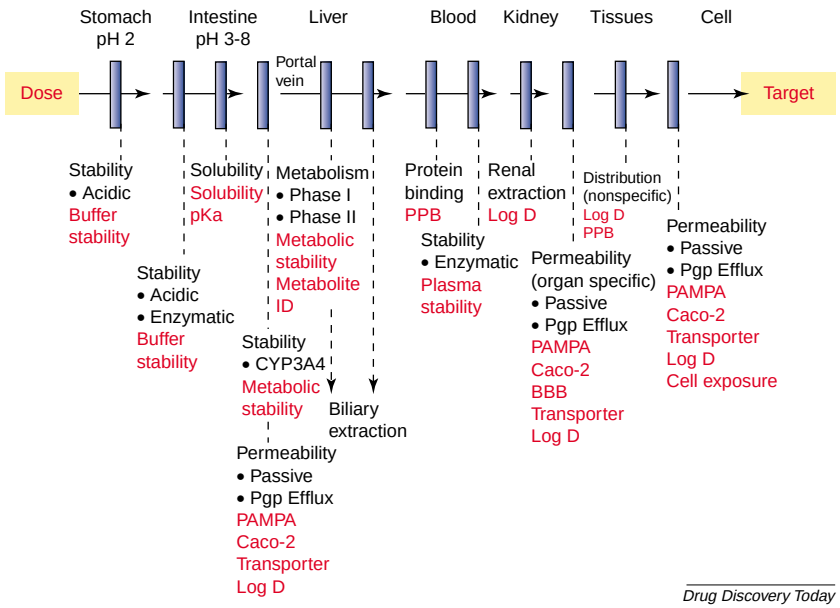


**Figure 4.** Schematic of some of the barriers encountered by drugs between dosing and the therapeutic target. Examples of pharmaceutical profiling assays that provide information about these barriers are shown in red.

### *p*K$_a$

Ionized compound is more soluble in water than the neutral form, but less permeable. Ionization is determined by the p$K_a$ and aqueous pH; thus, p$K_a$ has a major effect on ADME. Bases equilibrate between the neutral and protonated forms; amines have p$K_a$s around 10–11 and weaker bases have lower p$K_a$s; acids equilibrate between the neutral and deprotonated forms. Carboxylic acids have p$K_a$s around 3–5 and weaker acids (e.g. phenols) have higher p$K_a$s. A useful rule is that equal concentrations of neutral and charged species are present in solution when pH equals p$K_a$ for monoacids and monobases. Some compounds have multiple p$K_a$s.

This property is measured in high-throughput mode using capillary electrophoresis [28] and spectral gradient analysis (SGA) [29]. The SGA method creates a pH gradient over a 2 minute period and the UV absorbance of the compound throughout the gradient is used to calculate p$K_a$. Chemists can modify the ionizable groups on a molecule to change solubility and permeability.

## High throughput *in vitro* ADME profiling

### *Metabolic stability*

Many discovery compounds exhibit low bioavailability because of high rates of metabolism. These are 'phase I' cytochrome P450 oxidations (e.g. hydroxylation, dealkylation) or 'phase II' conjugations (e.g. glucuronidation). Metabolism reduces the circulating drug concentration and increases elimination. Plasma and cells also contain enzymes that convert drugs (e.g. esterase). Metabolic stability assays are conducted with liver microsomes [30; Li, D. unpublished data], S9 fraction [10,26], hepatocytes [31] and plasma [32]. Microsomes contain the cytochrome P450 oxidizing enzymes (CYP) and some phase II conjugating enzymes (e.g. UDP-glucuronosyltransferases). The S9 fraction is a cruder preparation than microsomes and contains the microsomal enzymes, as well as additional metabolizing enzymes. Hepatocytes contain all the liver metabolizing enzymes that are found *in vivo*. Microsomes are easiest and cheapest to use, but increased detail can be obtained with the S9 fraction and hepatocytes. Plasma contains other types of enzymes found in blood, such as esterases and amidases. Stability incubations are automated using laboratory robots.

The metabolic stability assay requires high sensitivity (<1 μM) and selectivity (from incubation matrix interferences) that can only be achieved using LC–MS–MS. Janizewski [33] described an integrated instrument with a throughput of 30 seconds per sample, which is likely to be the prototype for future applications.

Metabolic stability results are greatly affected by DMSO concentration, sample concentration and microsomal preparation (Li, D. unpublished data). The data can be misleading with improper methodology.

It is important to remember that metabolism rates and mechanisms vary among animal species. Thus, rodent metabolism studies are most useful in early discovery to assist interpretation of rodent pharmacology studies, provide alerts of liabilities and assist structural modification. Human metabolism studies become more important as the project moves toward development and require increased safety precautions.

### *Major metabolite screening*

When significant instability is found, identifying the conversion products can benefit the design of more stable structural analogs. Also, metabolites can be synthesized and tested for activity and toxicity. Thus, metabolite studies should be performed during lead optimization synthesis. LC–MS–MS provides rapid structure elucidation [34,35] by using the fragmentation of the parent compound as a template to rapidly interpret the structures of metabolites, and requires minimal sample (10–100 ng). When MS does not

provide sufficient structural detail, LC–NMR [36] is used, but this requires 1–100 μg of sample.

### *Permeability*

Compound activity at intracellular targets or good absorption after oral dosing requires lipid membrane permeation. Permeability *in vivo* is a complex phenomenon, involving several possible mechanisms: passive diffusion, paracellular, active transport and efflux. Artursson [37] and Mandagere [10] have estimated that the predominant mechanism of gastrointestinal (GI) absorption for most commercial drugs is passive diffusion. Therefore, compound selection and optimization for passive diffusion is an effective approach.

Several assays exist for permeability. Early methods used liposomes or immobilized artificial membrane (IAM) chromatography [38]. Cellular models for permeability have been widely implemented using Caco-2 [39,40] and MDCK (Madin-Darby canine kidney) cells [41]. Monolayers of cells are grown on porous filters and test compounds in buffer are placed on the apical (A) side of the monolayer. The rate of compound appearance on the basolateral (B) side is measured using HPLC or LC–MS to calculate permeation rate. Cellular models incorporate several mechanisms of permeability. Diagnosis of each mechanism for structural optimization requires studies under multiple sets of conditions [e.g. A→B, B→A, with P-glycoprotein (Pgp) inhibitors]. Expression of transporters varies with laboratory or passage and must be monitored. Lipinski [12] noted that Caco-2 experiments are most appropriate at 100 μM for predicting GI absorption of a 1 mg $kg^{-1}$-dosed drug, where transporter saturation is more likely. Also 1 μM is most appropriate for predicting CNS penetration. However, Caco-2 is commonly performed at 10 μM. Inaccurate results are also generated for compounds that are not fully soluble at the intended assay concentration. Higher throughput 96-well versions of Caco-2 have been reported [42].

Parallel artificial membrane permeability assay (PAMPA) is receiving considerable attention [43,44]. Instead of a cellular monolayer, an artificial membrane is created using lipid in organic diluent. An UV plate reader is used for rapid and inexpensive quantitation. PAMPA is higher throughput than Caco-2 and requires ~5% of the resources. An efficient and productive permeability approach is to use PAMPA for high-throughput passive diffusion assessment for all discovery compounds and Caco-2 for the mechanistic study of selected compounds.

Permeability data can provide an early estimation of barriers to GI absorption, cell assay membrane permeation, and to diagnose bioavailability. Permeability through specialized *in vivo* membranes, such as the blood-brain barrier (BBB), can be predicted using specialized cell culture models [45] or PAMPA modified for BBB [46].

### *Active transporters*

Transporters help explain discrepancies between passive diffusion and *in vivo* permeation. Pgp is one of the most studied transporters and is active in the intestine, BBB and drug-resistant cancer cells [47]. Polli [48] discussed application of three assays for Pgp affinity: ATPase, Calcein AM and MDR1-MDCK permeation. Many groups use the Caco-2 cell line for studying Pgp. Pgp screening helps avoid series for which Pgp greatly limits permeability. Some Pgp substrates are also CYP3A4 substrates, resulting in extraction through 'cycling' [3]. Reduced Pgp affinity might improve bioavailability, brain penetration or activity in cancer cells.

Several uptake transporters (e.g. OCT1, OATP1, OATP2, MOAT) and efflux transporters (MRPs) are being studied [49] providing the possibility of permeation-enhancement design to increase absorption via reduced efflux or increased active uptake.

### *CYP450 inhibition*

Several drug products were withdrawn because they inhibit the metabolism of a co-administered drug, resulting in toxicity. Assays were developed to measure the rate of metabolism of a 'probe' compound to its fluorescent metabolite by a particular CYP450 isoenzyme in the presence and absence of test compound [50,51]. If the probe metabolism is inhibited, a reduced amount of the probe metabolite is observed by fluorescence [50] or MS [52] detection. Organizations can monitor CYP450 inhibition at a single concentration (e.g. 3 μM) and then predict $IC_{50}$ values from models [51] or measure a full curve for inhibitory compounds. High inhibition should trigger detailed toxicological evaluation.

### *Cellular exposure*

Cell-associated drug concentration can correlate with cellular pharmacology. If activity is lower than expected, cellular concentration might be the cause. Low concentration of drug associated with the cell could be a result of efflux, poor passive membrane permeation or intracellular metabolism. When intracellular biochemical conversion is necessary for activity, formation of the active species is monitored. These experiments usually dose cells in culture, followed by washing and LC–MS–MS analysis [53].

### *Plasma protein binding*

Binding of molecules to plasma proteins [e.g. human serum albumin (HAS), $\alpha_1$-acid glycoprotein (α-AGP)] limits their free motion. Plasma protein binding (PPB) reduces the volume of distribution, renal extraction, liver metabolism and tissue penetration. Absorption and half-life increase with PPB. Some effects offset each other with regard to drug concentration, so application of PPB information

for *in vivo* predictions can be confusing for discovery scientists. PPB can be useful for diagnosing complex *in vivo* effects, such as low brain penetration, once *in vivo* experiments have been performed. PPB can be estimated using HSA HPLC chromatography [54], 96-well equilibrium dialysis [55], Biacore®, or 96-well Microcon® ultrafiltration.

## High throughput *in vivo* profiling

### In vivo *exposure*

*In vivo* exposure or PK involves the collection of plasma or tissue samples at certain time points after dosing, followed by measurement of compound concentration. Simple, rapid-throughput sample preparation methods, such as acetonitrile precipitation or solid phase extraction, are used in conjunction with LC–MS–MS. Throughput is accelerated by innovative cassette dosing [56,57] and cassette processing [58] approaches.

There are several ways that *in vivo* exposure studies greatly benefit early discovery. Exposure studies with selected compounds provide an overview of series PK performance. If PK is poor, root causes can be diagnosed, so that structural modifications can be made to improve PK. Less expensive *in vitro* tests can then be used to monitor future compounds in the series [59]. Exposure studies contribute to animal model validation, dosing level selection and the need for formulation can be determined. Exposure in surrogate species is verified before dosing expensive or time-consuming animal models. PK samples from animal activity studies are used to correlate plasma concentration with pharmacology. If no *in vivo* activity is observed, exposure data helps discern if this is because of a lack of sufficient drug concentration in plasma or tissue [60], or because of *in vivo* factors that reduce the expected activity. If *in vivo* activity is observed, exposure data are used for SAR.

## Future directions

Although the profiling of compound pharmaceutical properties has progressed rapidly, some gaps still remain. The accuracy of *in silico* and *in vitro* assays for the prediction of *in vivo* properties should continue to improve (e.g. Caco-2 and PAMPA predictions are ~60–70% correlated to *in vivo* bioavailability). The fundamental basis for establishing advantageous and disadvantageous ranges for assay results needs to be improved (e.g. Lipinski performed extensive database mining to establish solubility ranges). Models and assays should continue to be simpler, faster and cheaper (e.g. PAMPA improved the speed and cost of permeability predictions). Methods of detection that enable higher throughput with specific endpoints need to be developed (e.g. fluorescent probes for CYP450 accelerated inhibition assays). High throughput methods are needed for CYP induction, renal clearance, biliary clearance and key toxicity mechanisms. Medicinal chemists need computational chemists to provide them with SPR information in the same way that they currently provide SAR information to help guide analog series planning. Property prediction software needs to be on the desktop of every medicinal chemist. Continuing education is required for medicinal chemists on isosteres and other methods for improving properties to reinforce the habit of parallel activity and property optimization. The increased use of software tools for multi-dimensional property predictions, such as Spotfire [61] and multivariate statistics [8], should improve *in vivo* predictions.

## Conclusions

Pharmaceutical profiling is an emerging strategy in drug discovery because properties have a major effect on *in vitro* and *in vivo* pharmacology. It is an enhancement of the SAR paradigm. Discovery scientists can understand and control more of the variables that affect their experiments, to achieve increased success. Profiling data assists the diagnosis of compound performance at various barriers, assists prioritization and optimization, and alerts research teams to factors that affect development attrition. Properties can be improved via structural modifications, which is most successful on substructures having minimal SAR effect.

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