The impact of early ADME profiling on drug discovery and development strategy

The increased costs in the discovery and development of new drugs, due in part to the high attrition rate of drug candidates in development, has led to a new strategy to introduce early, parallel evaluation of efficacy and biopharmaceutical properties of drug candidates. Investigation of terminated projects revealed that the primary cause for drug failure in the development phase was the poor pharmacokinetic and ADMET (Absorption, Distribution, Metabolism, Discretion and Toxicity) properties rather than unsatisfactory efficacy. In addition, the applications of parallel synthesis and combinatory chemistry to expedite lead finding and lead optimisation processes has shifted the chemical libraries towards poorer biopharmaceutical properties. Establishments of high throughput and fast ADMET profiling assays allow for the prioritisation of leads or drug candidates by their biopharmaceutical properties in parallel with optimisation of their efficacy at early discovery phases. This is expected to not only improve the overall quality of drug candidates and therefore the probability of their success, but also shorten the drug discovery and development process. In this article, we review the early ADME profiling approach, their timing in relation to the entire drug discovery and development process and the latest technologies of the selected assays will be reviewed.

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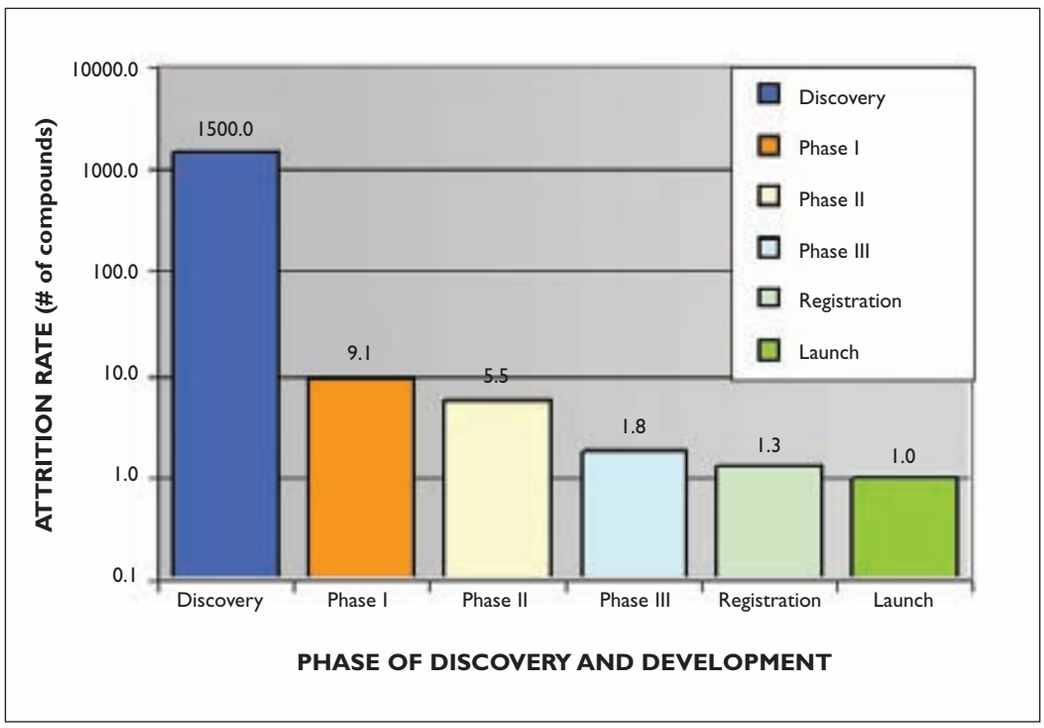
Discovery and development of a new drug is a long, labour-demanding process. Recent studies[1] revealed that the average time to discover, develop and approve a new drug in the United States has steadily increased from 8.1 years in the 1960s to 14.2 years by the 1990s. The actual time may be even longer as the above calculation considered the starting point from chemical synthesis, thereby not including the time for target identification and lead finding/selection process, which in general takes a minimum of two more years. Typically, the whole process is fragmented into 'Discovery', 'Development' and 'Registration' phases.

The 'Discovery' phase, routinely three to four years, involves identification of new therapeutic targets, lead finding and prioritisation, lead optimisation and nomination of new chemical entities (NCEs). In the 'Development' phase, the drug candidates are

Figure 1
Attrition rate of drug candidates, as denoted by the number of compounds that would be required to generate a new drug at the different phases of discovery and development[8,15]



subject to preclinical testing in animals (also known as 'Phase I') for about two years. In addition, the preliminary clinical trials in man and the subsequent full clinical trials (known as Phase II and III) will take a much longer time (~8.6 years)[1]. According to DiMasi, the 'Registration' phase averaged around 1.8 years in the late 1990s. Each pharmaceutical firm has their own collection of drug candidates which are being allocated to different phases of the discovery and development process, often referred to as the 'pipeline'. Discovery scientists primarily focus on 'hunting' for drug candidates, whereas in Development one is trying to 'promote' NCEs as novel and safer commercial medicines.

Discovery and development of a new drug are also extremely costly. Despite the drastic upsurge in R&D expenditures (by a factor of 20-50), the output of pharmaceuticals (number of new drugs launched per year) remains virtually flat from 1963 to 1999[2-3]. Concurrently, the productivity of the pharmaceutical industry, as measured by NCE output per dollar, declined continuously during the past decades[4]. As a result, the average cost to discover and develop a new drug, taking into account the drug candidates dropped along the way, soared to $800-$900 million in 2003[2-3,5], in comparison to that of $138 million in 1979 and $318 million in 1991[2]. A separate analysis estimated the cost at $1.3-1.6 billion in 2005[6]. Despite the recent strong performance in earnings[7], major pharma firms still have the urgency to improve the efficiency and effectiveness of the drug discovery and development strategy in order to appeal to the investors and to secure continuing growth. In addition to the increasingly tight regulatory hurdles, big pharmaceutical firms also suffer from their failing discovery of NCEs. Commonly, it is attributed to:

- The selection of improper targets in early phases that lack proof of concept in man.
- High attrition rate during development phases due to poor pharmacokinetics.
- Poor toxicological and safety-related pharmacological properties.
- Elongated discovery and development time course.

Attempts to address these challenges embrace:

- Pursuit of physiologically viable targets using genomics and proteomics strategies.
- Initiation of combinatorial chemistry techniques which escalate production of NCEs entering the pipeline.

However, the genomics approach was counteracted by a large number of novel but risky targets which attributed to a 50% rise in the failure rate over the clinically validated approaches[8]. The downside of combinatorial chemistry is to shift the discovery compound libraries towards large,

'greasy' and biologically inactive molecules which can rarely survive in the development phase. Indeed, Lipinski's recent drugability analysis of NCE collections from Pfizer and Merck led to the findings that new NCEs tend to have higher molecular weight and higher LogP and in turn, poorer solubility and permeability[9]. Therefore, eliminating compounds with the worst ADMET properties as early as possible has become an attractive approach[10-14]. Alternatively, expert advice concerning ADME properties could guide chemists to structure-activity relationship (SAR) based modifications to optimise for 'drug like properties' (eg, good absorption, high bioavailability with metabolic stability, required distribution).

What is behind the high attrition rate?

Even when a candidate reaches the development 'pipeline', this will not guarantee the launch of a commercial drug. This milestone indicates nothing else but a probability for success, as many development candidates will not pass through the preclinical and clinical testing. Companies reserving a large number of candidates with a higher probability for success in development phases are viewed as possessing 'strong pipelines'. Attrition rate analyses of NCEs in the development phases of 10 big pharma companies in the US and Europe over 1991-2000 led to interesting findings[8]. Albeit distinct among therapeutic areas, the average success rate for drug candidates entering development Phase I will only be around 11% (**Figure 1**). In other words, the vast majority of the compounds that are being worked on in the development phases will end up in the 'waste bin', with no return on the expenditure for the investment. As the development process advances, the potential for the surviving candidates to become a drug gradually escalates, in proportion to the growing expenditure for development per target. A couple of comparable studies led to similar conclusions[14-15].

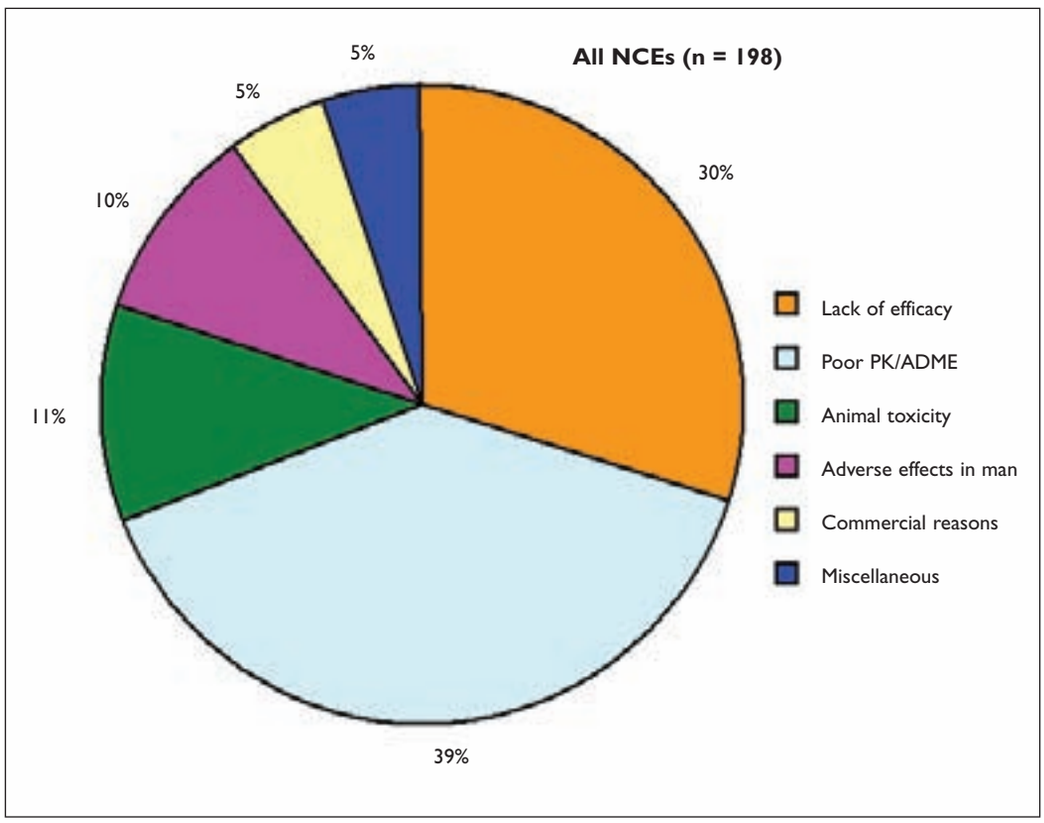
Drug candidates might fail during development because of numerous reasons. Kennedy analysed the causes by which 198 NCEs failed in clinical development[15] and found that the most prominent cause of the failures was associated with poor pharmacokinetic (PK) and ADME properties (**Figure 2**). Although lack of efficacy was still one of the main reasons for terminations, the unsatisfactory PK/ADME, toxicology and adverse effects accounted for up to two-thirds of the total failures. A separate analysis[8] also led to a similar conclusion, particularly true for the early 1990s. However, Kolo & Landis's report revealed that even with the latest improvement in PK/Bioavailability aspects, the total loss of drug candidates in development due to ADME (PK/bioavailability, formulation), toxicology and pharmacology (safety) remains near 50%.

Where did the traditional drug discovery and development process go wrong?

In principle, most of the above issues might have been foreseen in early discovery by using ADME, toxicological and pharmacological profiling tools. However, it takes time and effort to reform the mindset of scientists and managers in drug discovery and development. Traditionally, optimisation of efficacy was strongly associated with discovery and drugability with development, applied sequentially in their own 'kingdoms'. Specifically, the majority of the project teams in discovery concentrated on the improvement of *in vitro* efficacy during lead selection and optimisation. In the end, the champion NCEs, always those with most potent inhibition or binding properties to the *in vitro* target in test tubes/microplates, were 'pushed over the wall' to the development phase, as if the world leading universities picked their best candidates simply by the criteria of nothing else but the academic record of the applicants. From the discovery point of view, the goal was achieved with the identification of active candidates relative to the therapeutic target.

However, from the development point of view, excellent *in vitro* efficacy would not always translate into *in vivo* potency. First, full *in vitro* characterisations of the drug candidate including pre-formulation assessment, biopharmaceutics, toxicology and safety pharmacology give a fair prognostic picture for *in vivo* performance. As a next step, preclinical experiments provide confirmation of pharmacodynamic (PD)/pharmacokinetic (PK) behaviour of the compound in selected animal species. To complete the evaluation, pharmaceutical development has to assess the developability of the candidates as potential commercial drugs (eg, optimisation of drug delivery by various formulations, etc). Although these processes are scheduled way before the more challenging and costly clinical trials, many NCEs display signs of poor 'drugability'. For example, some NCEs could neither dissolve in aqueous media nor permeate across the gastrointestinal membrane to reach the concentration at the required therapeutic level, often referred to as 'brick dust' by development colleagues. Fast (metabolically unstable) or ultra-slow (potential accumulation) metabolism, toxic and adverse effects are not necessarily obvious from animal experiments executed at single dose most of the time. As a consequence, these matters are frequently misinterpreted during the assessment

Figure 2
Analysis of the reasons for failure of 198 drug candidates in clinical development[15]



of the *in vivo* efficacy, which (without mechanistic data) imposes additional challenges during the development process.

No wonder, that the late discovery of poor drug-like properties and adverse side-effects is heart-breaking for both development teams, often referred to as 'teams with licences to kill', and drug discovery teams. Under such a condition, the question is whether to continue the development of the NCE and try to optimise it with great hardship, or just kill the compound and start from scratch. However, the latter decision, (to throw the NCEs back over the wall) is always hard to make, as this means potential delays to the product launch, loss in exclusive patent protection and deterioration of competitive position against other pharmaceutical firms that are working on the same therapeutic target. The matter is made worse by the discovery teams which beg their development colleagues to rescue their 'babies'. Unfortunately, there is very little that development teams can do to salvage NCEs with inadequate drug-like properties and adverse side-effect profiles. For instance, development of proper formulations may help address drug delivery issues of poorly soluble compounds. However, such improvement can also be very costly and time-consuming and miracles may not always take place. Some notorious GPCR antagonists were classic examples for these efforts. Frequently, the clinical dose format may have to be approved by marketing teams and/or patients. Eventually, such projects may not survive after struggling for years and end up with a big expenditure and disappointed scientists.

How to improve our drug discovery and development process?

A new trend in the modern drug discovery and development process is emerging to evaluate therapeutic and drug-like features of NCEs together as early as possible. This requires that two major elements are in place:

1) A new mentality to break up the wall between drug discovery and development. Only interdisciplinary and translational teams can drive the projects effectively toward the same goal, the clinical launch of the medicine. It is imperative to migrate from sequentially assessing efficacy and drugability to a parallel process in the new drug discovery and development strategy (**Figure 3**). Promotion of drug candidates at each phase of the long discovery and development process will rely not only on a particular parameter but their overall performance and the

potential to become a commercial drug, similar to the admission of students to the top universities to foster the winners of the future requires all-around young talent as the more favourable choice over students with solely an outstanding academic record.

2) The new strategy for parallel profiling of efficacy and drugability of NCEs in early discovery raised a new, important question as most of the tools to appraise drugability and side-effects such as absorption, metabolism, distribution, excretion, toxicity and pharmacology were only available in the development phase. These assays were usually costly, labour-intensive, low throughput and required a great deal of materials, thereby not feasible for early drug discovery phases. Recently, major efforts have been made to develop and implement high-throughput (HT), miniaturised, fast profiling assays with good predictivity for *in vivo* drugability, at reasonable cost for usage during early drug discovery. These assays can assist directly the selection of early hits or leads and can be implemented for the optimisation cycle of chemical synthesis.

What is feasible to profile in early discovery?

First, one needs to be realistic and to implement profiling assays with the balance of two major criteria: overall importance of the matter addressed by the assay (the impact) and availability of high quality assays (the quality and throughput). Therefore assays are selected based on their predictive value towards major reasons for failures in later development phases. Most of these assays address instant hurdles in early discovery such as solubility and metabolism with clinical consequences. **Table 1** outlines the most important assays recommended for implementation in early drug discovery to tackle the above issues.

Furthermore, the recognition that a single assay usually has very limited prognostic values, lead to the implementation of the 'assay suite' which addresses various elements of the complex clinical performance of drugs, collectively (eg, solubility and cell permeability of NCEs affect hERG inhibition). These grant project teams with greater power to solve the puzzles from divergent approaches.

Establishment of such a large number of profiling assays in early discovery is not trivial. First, most of the ADME assays listed above were primarily adopted from assays that have been employed in development laboratories. Such assays, albeit predictive, commonly tend to be time-consuming, labour-intensive, not cost-effective, require a large amount of sample material, and therefore are impractical for direct application in early discovery. Adaptation of these assays is not always straight-forward. New approaches and methods have been continuously invented to assist early drug discovery. For example, special sandwich-type devices consisting of donor and receiver chambers partitioned by either artificial membrane or cellular monolayer in micro-plate format are used to simulate the membrane permeation course of drug candidates. Most of the pharmaceutical labs have dedicated resources and time to develop and integrate automated assay workstations as it is not always easy to acquire an off-the-shelf system to work for a particular customised profiling assay.

Furthermore, it is important to work out a viable strategy with development colleagues to assure that the new profiling assays are developed and implemented in discovery labs to create 'neither gap nor redundancy' with the existing assays in development labs. It is commonly a good practice to utilise the expertise in discovery for fast screening of drugability of NCEs and leave the small fraction of problematic compounds to the development teams.

Finally, the profiling assays in support of early drug discovery should have a good balance between quality (predictivity and reproducibility), quantity (turn-around time, throughput) and cost (capital, operating, manpower and compound materials)[16]. Within each organisation, the assay strategy is primarily defined by specific requirements and the impact on the drug discovery process. For instance, maintenance of a cell-based permeability assay, such as a full Caco-2 assay, for examination of transport mechanism during late discovery and early development, is very costly. To offer a fast and cost-effective screening tool for the assessment of permeation of candidates during the early drug discovery phase, permeability could be estimated using artificial membranes, simple cell monolayer, such MDCK or fast-cultured Caco-2 models (eg 3-7 day). The throughput may be further enhanced and the cost will be greatly reduced in conjunction with a proper assay configuration (reducing number of replicates and time/data points) and analytical platform (reducing analytical time, pooling analytical samples and parallel LC/UV or LC/MS)[17].

Adequate ADME profiling assay suite for early drug discovery

Although each major pharmaceutical firm has its own favourite approach to implement the profiling assay suite, the underlying principle shares commonality. As projects advance from lead finding (LF) to lead optimisation (LO) and eventually to the development phase, the required throughput (number of

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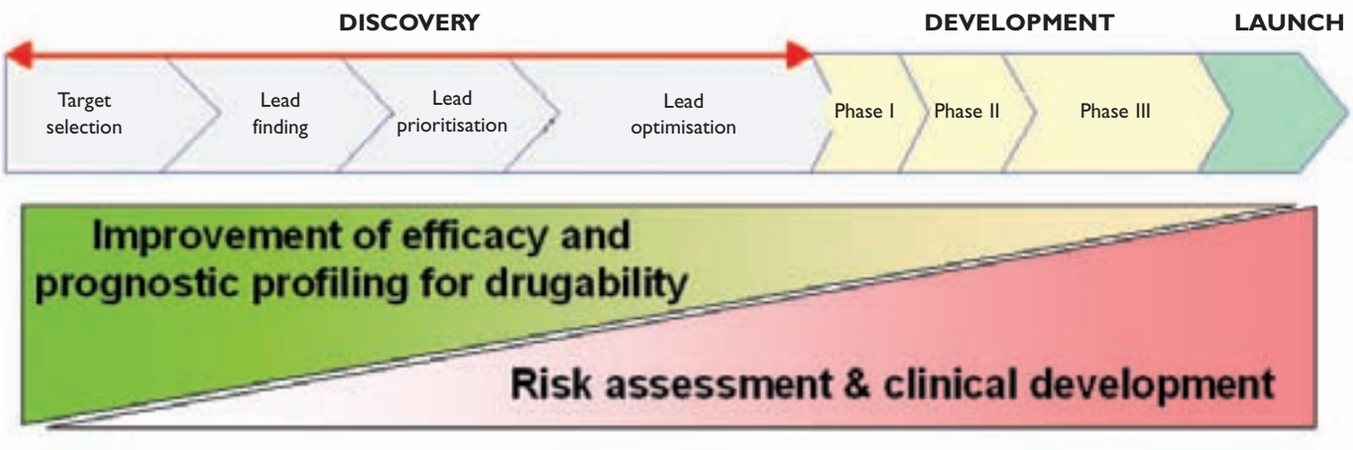

Figure 3
New strategy for drug discovery and development where the optimisation of efficacy and drugability will be performed in parallel. The new strategy will require profiling of NCEs occurring in early discovery, allowing for only all-around optimised candidates to be promoted to the subsequent development phase

candidates to be considered) drastically declines and the predictivity of the profiling assay (the content of the information) proportionally escalates (**Figure 4**). During phases prior to or during HTS stages (eg target identification, etc.) the potential drug-like features are commonly addressed by *in silico* tools such as 'rule of five' and polar surface area (PSA), etc. Due to their simplicity, high throughput and low cost, the *in silico* tools are particularly appropriate for the design of novel chemical scaffolds or new combinatorial libraries. For parameters which are dependent primarily on chemical structure of NCEs such as ionisation constant (pKa) or PSA, the prediction results are generally reasonable. The downside of the *in silico* approach, however, appears as the inauspicious predictivity for assays involving complicated processes or mechanisms (than just molecular configuration) such as solubility and permeability.

By the end of HTS or lead prioritisation (LP), *in vitro* profiling assays should be available to address drugability. One approach can segregate the *in vitro* suite into a 'primary' suite and a 'secondary' suite, as shown in **Table 2**. *In vivo* assays may be addressed in a separate suite. The most inventive part of this process involves the continuous optimisation of the 'spider graph' (**Figure 4**) along with the enhancement of efficacy and introduction of discrete, obligatory profiling filters at the designated phase transition milestones. During the cycles of chemical synthesis in LO phase, one should always avoid gaining the efficacy and activity at the expense of deteriorating drugability.

Solubility and solubilisation

Solubility is a critical factor as drug substances have to be dissolved before they can be absorbed. Solubility and rate of dissolution are the crucial players in the famous Biopharmaceutical Classification System (BCS)[18], as absorption of passively transported drugs across the gastrointestinal (GI) tract is the combined product of both permeability and solubility according to Fick's first law[19]. Data from *in vitro* assays such as HTS activity or membrane permeability assays could be misinterpreted without considering solubility. Dosing poorly soluble compounds in pharmacological animal testing is also very risky as it commonly fails in deriving a correlation between dose and *in vivo* efficacy. Under these circumstances, it is problematic to differentiate the issues between solubility and efficacy. On the other hand, solubility data may help one to understand other PK or PD mechanisms. For instance, highly soluble, low molecular weight compounds (MW <250) can be transported via the paracellular route across the GI membrane.

The challenge to reliably estimate solubility of drug substances should not be underestimated. In reality it is very difficult to precisely quantify or predict the aqueous solubility due to the complicated solubilisation process and solid phase chemistry of drug candidates. A variety of approaches are employed (eg *in silico*, kinetic, equilibrium solubility) which should be utilised in tiers to deal with the solubility and dissolution issues occurring at the different phases of drug discovery and development.

– Thermodynamic or equilibrium solubility has been considered as the 'gold standard' for solubility determination. In the conventional approach known as the 'saturation shake-flask' method, solid compounds are agitated for a long period of time (24-72 hours) to ensure that an equilibrium is achieved between an aqueous solution and the solid material under specific conditions (buffer, pH, temperature, etc.). The saturated solutions are

then separated from the residual solids using the filtration or centrifugation techniques. The supernatant or clear saturated solutions are then quantified using high performance chromatographic column (HPLC) or LC-MS (mass spectrometry), in comparison to the standard curves created from solutions of the test compounds with known concentration (typically in methanol or other organic solvents). The thermodynamic solubility is viewed as being accurate and reliable over a reasonably large solubility range (eg 1-50,000mg/L) provided that enough material is available. In addition, it also reflects the packing information of the compound in solid phase (polymorph) and thus is well-accepted by pharmaceutical industry and FDA. The drawbacks, however, are that the method is quite time-consuming, labour-intensive and sometimes requires a lot of sample material. Therefore, it is generally not feasible for high throughput screening of aqueous solubility in early drug discovery.

Comparable to the 'shake-flask' method, a thermodynamic or equilibrium solubility pH profile can be derived using the potentiometric titration approach[20]. Specifically, the intrinsic (for neutral species only) and equilibrium (solubility of all species at a pH) solubility data are determined using Noyes-Whitney titration over the range of pH 1-12. In principle, when a test compound precipitates from aqueous solution due to changes of pH, the titration curve typically deviates markedly from its respective curve obtained in aqueous titration. Careful data analysis using sophisticated software will lead to a solubility pH profile in the equilibrium mode and the data were found to correlate well with the published data from the 'shake-flask' method[21]. This approach provides a comprehensive profiling of equilibrium solubility, along with the intrinsic solubility, in a broad pH range. In addition, the data collection is in the automated mode, which becomes very useful for late discovery and development where a complete characterisation is necessary. The downside of the assay is the low throughput (1-2 samples per day). In addition, the quality of the data relies highly on the accuracy of the pKa data determined in aqueous solution, the experience of operators, and sometimes the nature of the test compounds (number of pKa's and solubility, etc). Thus, whereas it is an ideal tool for solubility characterisation for late discovery, it is not practical for HT solubility screening in early discovery.

ASSAY SELECTED	ISSUES TO BE ADDRESSED
ADME Assays	
Solubility (equilibrium, kinetic solubility)	Oral absorption
Rate of dissolution	Oral absorption
Membrane permeability (PAMPA, cell models, BBB)	Oral absorption and BBB penetration
Active transport	Oral absorption and drug-drug interaction
Ionisation Constant (pKa)	Oral absorption and binding mechanism
Lipophilicity (LogP, LogD)	Oral absorption, cell membrane penetration, distribution
Chemical stability	Chemical integrity in body fluids, tissues and oral absorption
Metabolic clearance	Bioavailability and clearance
CYP450 inhibition	Metabolism and drug-drug interaction
CYP450 induction	Metabolism and drug-drug interaction
Protein/Serum-binding	Clearance, distribution and bioavailability
Metabolite Identification	Metabolic mechanism
Toxicological Assays	
hERG, other cardiac ion channels	QT liability, cardiotoxicity
Genetic toxicology	Teratogenicity, mutagenicity
Target organ/cytotoxicity	Hepatotoxicity, neurotoxicity, nephrotoxicity, haematological side effects, etc
Safety Pharmacological Assays	
Broad scale pharmacology profiling	Adverse effects associated with receptor, channel, enzyme activation/inhibition/modulation

Table 1: Profiling assays that are useful in early discovery

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Table 2: Comparison of '*in vitro* primary suite' and '*in vitro* secondary suite' in ADME profiling

IN VITRO PRIMARY SUITE	*IN VITRO* SECONDARY SUITE
Applicable to most of the LP/LO candidates	Applicable to promising compounds in late discovery
Common issues or interests to discovery	Full characterisation for promotion to development
Rank the compounds and flag the potential issues	Address the warning raised in primary assays
Reasonable throughput and short turn-around time	Limited throughput and longer turnaround time
Minimal sample required (1-2mg for the suite)	More compound material required
User-friendly streamlined compound logistics	Individual request may be offered
Good cross-site validation and data reporting on a global standard	Cross-site validation and global standards remain
Good quality and reasonable predictive values	Improved quality over primary assays

— *In silico* prediction is an alternative approach to estimate water solubility. It provides an opportunity to project solubility in a cost-effective and fast fashion. However, the major challenge for the *in silico* solubility prediction lies in its reliability and predictivity. This is because solvation and solubilisation of drug substances does not only rely on chemical structure but also on a complex interplay between hydrogen-bond acceptor and donor properties, conformational effects and crystal packing energy. For these reasons standard deviations of predicted water solubility from the experimentally determined values (thermodynamic solubility) are still relatively substantial. Many commercial products claiming satisfactory agreement between the computational and experimental data were unable to deliver adequate predictions for drug-like NCEs. This is not surprising as most of the *in silico* tools were trained using non-drug-like chemicals in the non-relevant solubility range (10^{-12}-10^{3}g/L). The new trend will greatly help this problem by establishing collaborations between commercial vendors that develop the new *in silico* tools and pharmaceutical labs that have large collections of thermodynamic solubility data derived from drug-like molecules. In addition, some *in silico* predictions may require other physico-chemical parameters such as partition co-efficient (logP) or melting points (MP) of the tested compounds that may not necessarily be available in the early stage of drug discovery.

— Kinetic solubility, first introduced by Lipinski[22], imparts a fast and economic avenue to estimate the aqueous solubility of early discovery compounds where a higher throughput (50-100 per day) and minimal sample consumption are prerequisites (10s pmol). Although a number of analytical approaches are utilised such as turbidity[22-23], nephelometry[24-25], laser cytometry[26] and a direct-UV method[27], kinetic solubility assays always start with compounds that are pre-dissolved in organic solvents such as DMSO. The introduction of compound stock solutions into designated media in microtiter plates (or vials) allows for monitoring the concentration at which compounds crash out of the solution. Kinetic approaches generally do not necessarily involve a long incubation period with thorough agitation and assays can be accomplished quickly and efficiently. Its predictivity for thermodynamic solubility perks up in comparison to *in silico* tools, in particular for the well-known commercial drugs, most of which have relatively decent physicochemical properties and a homogeneous solid format[28]. The method tends to be problematic for NCEs in the early discovery phase where compounds might be impure, unstable, colourful, and in mixed solid polymorphs[28-29]. The errors of kinetic solubility may originate from:

- Insufficient incubation time (instant readings vs 24-72 hour incubation).
- Presence of DMSO (0.5-5%) in kinetic approach (over-estimates solubility).

- Loss of polymorph information due to pre-dissolution of solids in DMSO.
- Interference by impurities, degradation products or the colour of the samples.
- Different analytical approaches used than the conventional HPLC approach.
- Deviation in robotic liquid handler when automation is involved.

— Development and implementation of HT- and miniaturised equilibrium solubility has become a new trend to derive reliable aqueous solubility of NCEs in early drug discovery[28]. The approach applies novel technologies in a number of key steps (sample handling, incubation, phase separation and quantification, etc) to assure that the assay is miniaturised and the throughput is significantly enhanced without noticeably sacrificing quality[30]. The predictivity of the new approach is validated against not only a few commercial drugs but also a collection of NCEs in the early discovery pipelines with divergent chemistry and possibly unfavourable physicochemical properties.

It is worthwhile to mention that efforts are devoted to adopt the direct-UV approach that is commonly used in the determination of kinetic solubility to derive equilibrium solubility. Typical tactics include minimising (eg 0.5%) the involvement of organic solvents such as DMSO, extending incubation time and developing state-of-the-art analytical algorithms to flag the spectral interference by impurities and degradation products[19]. The method has demonstrated appreciable enhancement in throughput in comparison to the conventional 'shake-flask' method and also exhibited improvement in quality over some kinetic solubility approaches. In comparison to the 'shake-flask' method, the direct UV protocol may have compromised the quality in several key steps to accommodate the required throughput.

Permeability and active transporters

Permeability, referred to as the capability of NCEs to penetrate across the human GI tract, is another key factor governing human oral absorption[31-32]. Ideally, oral absorption of drug substances is measured by quantifying the fraction of the designated drug absorbed through the human GI tract. Although the data derived are considered very reliable and serve as 'gold standards' in the assessment of oral absorption of drug substances, the approach is impractical in early discovery due to the intricate and costly experimental procedures. Alternatively, high throughput *in vitro* permeability assays using either artificial membranes or cell-based models, are becoming the methods of choice in early drug discovery[11,31].

— Parallel artificial membrane permeability assay, known as PAMPA[33], offers a fast and robust tool for screening permeability of NCEs in early discovery phases[33-37]. The method monitors the capability of drug candidates to permeate through a chemical membrane immobilised on a 96-well filter plate. The fraction of NCEs pervaded through the chemical membrane is quantified simply using a UV plate reader (primary) and LC/MS (supplementary for those lacking UV chromophores). As a simple chemical model, however, it can only estimate permeability for compounds with a passive transcellular diffusion mechanism and small molecular weights (eg <500). Preferably it may serve as a pre-screening tool for permeability ranking when multiple *in vitro* models are introduced to address GI permeability.

— The Madin-Darby canine kidney (MDCK) cell model is one of the commonly used cell monolayer systems to assess the human intestine barrier[70]. MDCK cell lines can reach full differentiation in three to seven days and are therefore relatively easy for cell culturing and assay maintenance, in comparison to other cell lines such as human colon adenocarcinoma cells (Caco-2). However, MDCK cell lines originate from dog kidney and the expression of transporters is quite different from human intestine[31]. As a result, the MDCK monolayer is commonly used for permeability evaluation of NCEs transported by the passive transcellular diffusion mechanism[38-39] (as does the PAMPA model), rather than for accurately predicting permeability of compounds involving active uptake and efflux mechanisms. Efforts are devoted to extend the permeability estimation using P-glycoprotein (Pgp)-transferred MDCK to account for the contributions of efflux transporters[40].

— The Caco-2 cell permeability model, exhibiting morphological as well as functional similarities to human intestinal enterocytes, has therefore been better received in drug discovery and development than other epithelial cell cultures[39,41]. Caco-2 cells extensively express a variety of transport systems including efflux proteins of not only Pgp but also the dipeptide transporters (PEPT1) normally found in small intestinal enterocytes. The expression of multiple transport systems in Caco-2 cells offers great advantages over simplified transport models as it can investigate the interplay among different transport systems and differentiate the relative contributions from passive and active transport mechanisms to the overall permeability across the human GI tract. Being human in origin, the Caco-2 cells

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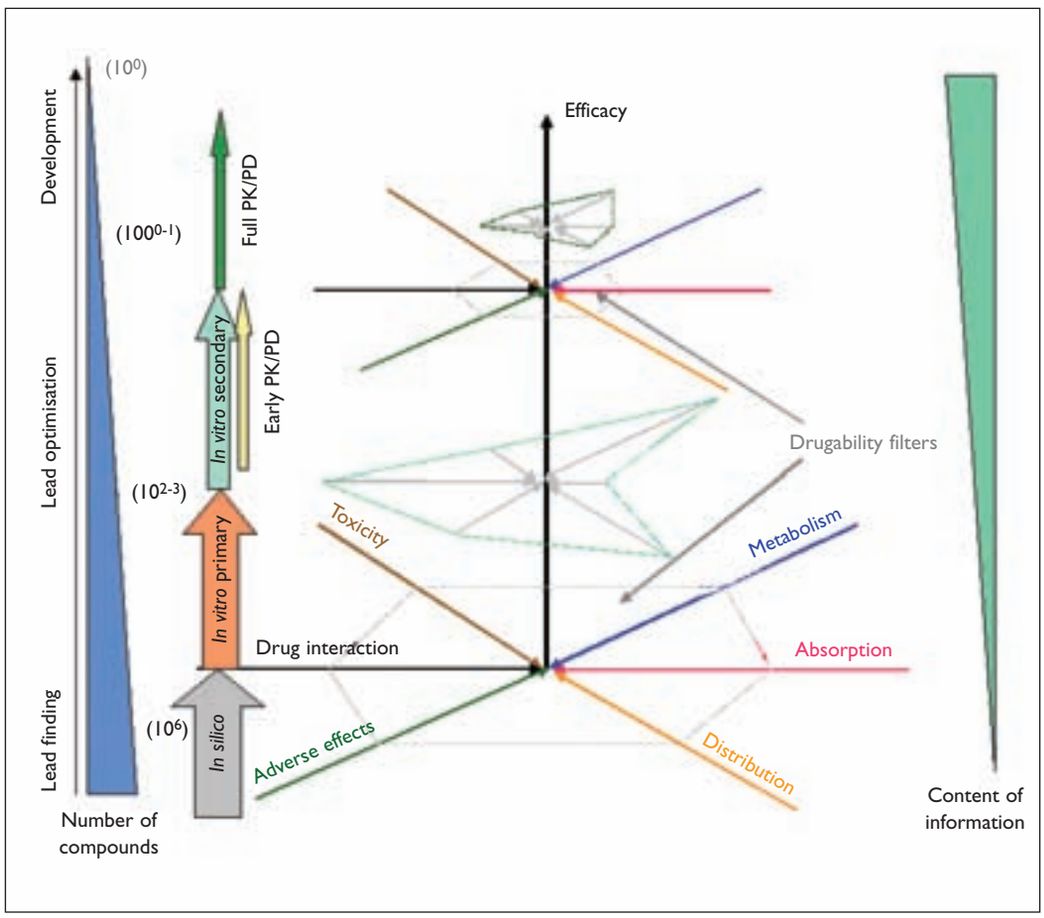

Figure 4
Schematic presentation of an ideal way to establish and utilise comprehensive profiling tools in drug discovery and development. The vertical axis represents the enhancement of *in vitro* and *in vivo* efficacy (increasing from bottom to top). Those perpendicular to the 'efficacy' axis signify the improvement of divergent drugable properties (inward). Discrete drugability filters may be exploited at key phase transition milestones to assure comprehensive profiling data are assessed along with efficacy results

provide information for human transporters in well-differentiated enterocytes, such as tight junction and brush-border enzymes that are found in the human GI tract.

The latest development of the assay involves seeding and culturing Caco-2 cells over 96-well transwell filter inserts consisting of polycarbonate or polyethylene terephthalate (PET). Whereas cell culturing process could be reduced to 3-10 days, it is believed that the full course of 18-21 day cell culturing ensures that all necessary transporters are fully differentiated for the accurate permeability assessment of the compounds with passive or active transport mechanisms[39]. The permeability assay is commonly carried out in the bi-directional mode (apical to basolateral chambers and basolateral to apical compartments) to estimate the permeability and to differentiate the contribution of the active transporters in the permeation process of NCEs. The assay is generally quantified by using LC/MS method. The throughput of the assay can be substantially enhanced by automating the cell culturing, permeability assay and by streamlining the quantification[42].

Overall, the 96-well Caco-2 permeability model demonstrates a good correlation to human oral absorption and offers a comprehensive tool to accurately project the permeability across the GI tract for drug candidates using different transport mechanisms. The method is reliable, shows good predictivity to human absorption and requires minimal material for testing. However, its drawbacks are complicated in cell culturing, the high cost for assay maintenance and potential problems for profiling across a broad pH range.

Ionisation constant (or dissociation constant)

The ionisation of drug candidates and the distribution of the neutral and ionised species under physiological pH drastically govern the ADME properties of NCEs. Ionisation constant, pKa, defined as the concentration at which the neutral species and ionised form are equally distributed, is a useful thermodynamic parameter to monitor the charge state of drug candidates. First, pKa data can help predict ADME properties of NCEs due to the pH gradient of 1.7-8.0 present in the human GI

tract[19]. For instance, solubility and permeability are modulated severely by the pKa value(s) of a drug candidate as the former is favoured by the ionised form whereas the latter is proportional to the concentration of its neutral species in solution. In addition, pKa data can be used for better understanding the binding mechanisms of therapeutic events and also for the optimisation of chemical reactions. A number of ADME properties such as lipophilicity and solubility pH profiles are derived in combination with aqueous pKa data.

A number of approaches are used to estimate pKa values for NCEs:

- Of many *in silico* tools for pKa prediction, the software developed by Advanced Chemistry Development (ACD/Labs) yields promising pKa data for commercial drugs, in comparison to data collected experimentally. The prediction deteriorates for real NCEs in early discovery phase (LP and LO stages), establishing the necessity for determination of NCEs using *in vitro* assays. This is not surprising as most of the prediction tools are trained by the similar set of commercial drugs. However, it is a useful tool for screening virtual molecules or in the cases where no experimental alternatives are available.
- Potentiometric titration records the pH changes, with a glass electrode, caused by introducing a known volume of titrants to the well-mixed solution of a drug candidate. The method is very reliable to determine pKa and can analyse about 5-10 compounds per day. For low soluble compounds, the method employs co-solvents thereby requiring some experience for data interpretation. In addition, the current size of the titration device still needs a few mg of sample material. Therefore it has been widely accepted in late discovery and early development.
- Capillary electrophoresis (CE) can technically handle a small amount of sample material. Samples are loaded on one end of CE and the migration, or mobility of the compounds is monitored under electric potential. The mobility is highly dependent on the ionisation process and thus pKa data of NCEs can be assessed accordingly[23,38,43]. This tactic demonstrates potential in dealing with some issues in early discovery such as interference of impurities and where only minimal sample material is available. However, the inability to handle poorly soluble compounds that are highly populated in early phase becomes the major hurdle for its applications in early discovery. A new approach utilising an HPLC method appears to address the issues raised when measuring pKa for sparingly soluble compounds[44].
- Spectral Gradient Analyzer (SGA) presents the latest development in the high-throughput determination of pKa[45]. The method establishes a stable and well-defined pH gradient by rapidly mixing acidic and basic buffers, during which drug candidates, predissolved in organic solvent, are introduced at different pH conditions. The spectral changes associated with the variations in ionisation are monitored using an on-line photo-diode-array UV detector to derive pKa values. With usage of co-solvent in the media, the assay works effectively with poorly soluble NCEs. The pKa data measured on SGA correlate very well with those from the potentiometric titration method. The method exhibits a number of advantages in measuring pKa in early discovery such as being high throughput, automatic, reproducible and economic. However, its major drawback is that the success (eg ~70%) greatly relies on the existence of UV chromophores in NCEs and the ionisation-associated UV alterations. In other words, this approach will not work for small molecules such as peptides or other chromophore-containing NCEs where the ionisation occurs at the molecular moiety that is distant from the UV chromophore. The method may require expertise and good understanding of the underlying principle for data processing in particular for compounds containing multiple pKa values that are close to each other in pH. Nonetheless, this tactic can be nicely combined with the potentiometric approach.

Lipophilicity

LogP and LogD are the logarithms of partition co-efficient and apparent partition co-efficient of drug candidates in a lipophilic phase such as octanol and a hydrophilic phase like water. The data are valuable to predict the ADME properties ranging from solubility, permeability to the understanding of transport mechanism. The conventional approach for LogP and LogD determination, the saturation shake-flask method, is to measure the equilibrium distributions of NCEs in octanol and water, not feasible for early discovery. The dual-phase potentiometric titration method involves extracting LogP and LogD by analysing distinctions in the titration curves of pKa in aqueous and octanol media[19]. The method is reliable but only feasible for compounds with measurable pKa data. For those lacking an ionisation centre, HPLC logP technique, also known as eLogP can be applied[46]. Other tactics available to assess LogP and LogD in early stages include liposome chromatography, immobilised artificial membrane (IAM) chromatography and CE approaches (a

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good review given by Avdeef)[19]. The latest development for HT-LogP determination is to utilise technology similar to PAMPA where octanol serves as the immobilised phase in a microtiter filter plate[34].

Chemical integrity and stability

Chemical integrity is valuable for the confirmation of chemical identity, in particular for validated hits derived from high-throughput screening where compounds have been stored in DMSO for a long period of time[11]. For freshly synthesised NCEs in the lead optimisation phase, it is also beneficial to verify that their chemical structure is as designed as well as confirming the purity. This information will be helpful for the proper interpretation of the *in vitro* ADME and efficacy results and the *in vivo* PK/PD data. A typical method to measure it uses LC/MS to verify the identity via the designated molecular weight and the impurity can be roughly quantified using either of the LC/UV, chemical luminescence nitrogen detector (CLND) or evaporative light scattering (ELSD) methods[47].

Chemical stability in general characterises the ability of an NCE to preserve its chemical and physical characteristics under specific conditions. From the drugability point of view, stability for solution and solid phases are both critical to foresee the potential issues of a candidate to become a marketed drug. In early discovery, however, the interest is concentrated in the solution phase as drug candidates in early phase are commonly in amorphous or meta-stable states and will be transferred to the more stable polymorphous forms in the development phase. The stability in solution can be assessed after a long period of incubation or under extreme conditions (eg temperature or pH, etc) and quantified using HPLC[23], CE, thin-layer chromatography (TLC) or LC/MS. It is worthwhile to mention that choosing an adequate analytical approach is pivotal to the quality of the chemical stability assay[48]. With the stability data, one can better understand the *in vitro* ADME and efficacy data and *in vivo* PK results. For example, chemical stability in buffer allows for differentiating the issues of chemical and metabolic stability of NCEs.

Metabolic stability and clearance

Metabolic stability or clearance, particularly hepatic, is recognised as one of the main determinants of drug concentration in blood and has been used effectively to predict bioavailability and toxicokinetics[49]. A metabolically unstable NCE, albeit orally absorbed, might never reach the required therapeutic concentration. On the other hand, a certain degree of instability might be desirable for a prodrug where a metabolite is more active than its parent.

There are a number of tactics to determine metabolic clearance. Whereas comprehensive *in vivo* PK studies in man serve as the ideal source for ADME data including metabolism, bioavailability and clearance, such experiments are not available until a relatively late phase in drug discovery and during clinical trials. This means that lead optimisation might go down a blind alley in terms of ADME features and produce expensive drug candidates with severe liabilities and ultimate threat of termination. Animal models, albeit useful in predicting various aspects of the metabolism and PK issues of NCEs in man, require considerable cost and lack the required throughput necessary in early discovery. Furthermore, they do not always show satisfactory predictivity to human clearance due to the difficulties in deriving proper scaling factors at early stages of drug discovery.

Currently, *in vitro* approaches that are extensively utilised to monitor the metabolic stability as well as to predict the human clearance of drug candidates include the use of recombinant CYP450 enzymes, liver microsomes, S9 fraction (the 9000g supernatant of a liver homogenate), isolated hepatocytes and liver slices[50]. To meet the needs to measure metabolic stability of NCEs in early discovery, the common and reliable approach is to probe the depletion of the test compounds in the presence of liver microsomes using LC/MS and derive the half-life and intrinsic clearance values[51-53]. The *in vitro* data derived also demonstrate a decent correlation with the *in vivo* hepatic clearance values, which are very useful in estimating bioavailability and systemic clearance.

The *in vitro* method has the advantage of profiling the metabolism of NCEs in the most relevant species and to predict the human hepatic clearance with relatively high throughput and low sample consumption at the earliest stages of drug discovery. The high quality of the assay also allows for the data to be widely utilised in discovery and development stages, appreciably reducing the requirement of animals and offering both commercial and ethical advantages. However, quantification using LC/MS remains the bottleneck of the assay, in particular when dealing with such a large number of samples and data points, as is also observed with most of the cell or protein-based ADME assays. An alternative approach is to

reduce the time points of the depletion determination (eg a single time point at 20-30 minutes, etc)[11,54]. This modification may be sufficient to offer the metabolic stability rank-ordering in early discovery, provided that comprehensive metabolic profiling will follow in late discovery. Another potential issue of the method involves compounds or their metabolised products that inhibit metabolising enzymes such as CYP450 enzymes. In these cases, it is advisable to collectively interpret the metabolic data along with drug-drug interaction profiling derived from CYP450 inhibition assay.

For NCEs exhibiting high metabolic clearance, it is worthwhile to follow up the metabolism by fully profiling the metabolites, or called metabolite identification. Under those circumstances, the metabolites could be active or inactive to the therapeutic target, which will affect the total efficacy measured. It could get even worse when the metabolites are found toxic, or show adverse effects, which will raise other toxic and safety issues. Therefore the identification of all metabolites will help understand the metabolism mechanism and identify the chemical 'soft spots' of NCEs, which can be utilised to direct the next round of syntheses. A number of methodologies that are used to profile the metabolite identification were reviewed by Watt et al[55].

CYP-450 inhibition: metabolism-related drug-drug interactions

Whereas the drug-drug interactions (DDI) in man are a broad subject covering protein-binding, transport carriers or other pharmaco-dynamic interactions[56], many DDI-related pre-clinical/clinical failures to date have been attributed to CYP450-related hepatic metabolism[57-59]. For instance, a drug candidate that is a potent CYP450 inhibitor may greatly inhibit the metabolism of a co-administered medication, potentially leading to adverse clinical events. Therefore, it is essential to screen compounds in early drug discovery to reveal their major metabolic pathways and to monitor their *in vitro* inhibition of the major CYP450 isoforms in order to predict their *in vivo* effects[60-61].

Despite the many CYP450 isozymes present in man, five major isoforms (3A4, 2C9, 2C19, 2D6, 1A2) account for the metabolism-related DDI issues for greater than 90% of marketed drugs[58,61-62]. Hence, the inhibition of the above five major isoforms should be measured in a high throughput and fully automated fashion. Commonly one tends to probe the metabolism-related drug-drug interactions of drug candidates by monitoring the impact of the test compounds on CYP450 metabolic activity using a known substrate. This is based on the assumption that whenever DDI occurs, regardless of the mechanism(s) involved (eg competitive, non-competitive or uncompetitive), the compound will interfere with the performance of a co-administered drug that is metabolised by the same enzyme[60].

Conventionally, the obtained metabolites are separated and analysed by LC/MS (occasionally by LC/UV)[61, 65-66]. The LC/MS method offers reliable data but its application in early discovery is greatly restricted by the throughput and resources given for method development. For example, in order to accurately quantify the metabolites, one has to ensure that all DDI-induced changes in the metabolism of the designated substrate are completely captured. Analytically it is not trivial as all potential metabolites, instead of just the parental compounds as done in most of ADME assays, will need to be quantified. Therefore, the method is more practical in the late discovery and early development phases.

Recent development of fluorescence-based approaches[61,63-64] demonstrates excellent sensitivity and specificity without metabolite separation and quantification by LC/MS. This novel methodology therefore substantially improves the throughput, turn-around time and cost-effectiveness by parallel monitoring via a 384-well plate-reader. The fully automated method 64 enables us to reliably measure IC_{50} in duplicate over a broad concentration range (0.068-50μM) with a small amount of material consumed (10s pmol). In addition, the NCEs that exhibited DDI issues in the end-point experiments can be automatically cherry-picked for further kinetic studies to monitor the whole inhibitory course and derive insights into the mechanisms. The major concern of the fluorogenic approach is for compounds or their metabolites that are fluorescent at the wavelengths used. However, fluorogenic control experiments are run for all NCEs to raise the flags for compounds or metabolites with potential fluorescent interference. The suspicious compounds can thus be further tested using the LC/MS approach.

Looking ahead

In the last decade, pharmaceutical profiling has made a great leap forward, not only in building up the foundation of comprehensive ADMET diagnostic tools, but also gradually modernising the drug discovery mindset. Nonetheless, this is just the beginning and many unmet needs remain and current assays need to be improved. First,

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while the pharmaceutical profiling paradise is still in the build-up phase, it is imperative to carefully and constantly appraise the strategy and technical platforms. This feedback should be utilised to direct our construction of a full *in silico/in vitro/in vivo*-PK profiling architecture in support of drug discovery and development. In addition, continuous commitments are essential to the development of novel technologies that are crucial to the implementation of early profiling. The technology innovations should be focused on the improvement of quality such as increasing predictivity of the *in silico* and *in vitro* suites to the *in vivo*-PK results. Miniaturisation, automation and cost-effectiveness will become challenges for ADMET profiling. In spite of the considerable expansions in the number of profiling assays in last decade, we are still required to work with the same amount of sample material provided. In addition, the difficulties of sample logistics in handling so many high-throughput profiling assays should never be underestimated. Finally, one should concentrate on knowledge management of the large collection of profiling data. It includes the maintenance of a user-friendly database, exploring comprehensible data mining tools to effectively present the comprehensive ADMET profiling data (such as Spotfire)[71] and developing or evaluating the evocative *in silico* tools (such as GastroPlus of Simulations Plus, Inc, or other models) for the projection of *in vivo* PK/PD properties using collectively the *in vitro* ADMET data[67-69]. This leads to two outcomes:

(1) The co-operative exploitation of all ADMET profiling data, instead of just one or two parameters, will exponentially enhance the power of profiling tools to resolve the puzzles in hunting for better and safer drugs.

(2) The drug discovery and development teams should be trained to make full and adequate uses of the *in silico*, *in vitro* and *in vivo*-PK profiling tiers to prioritise the candidates. Eventually the productivity of pharmaceutical industry will unlikely be improved unless the *in silico-in vitro* correlation (ISIVC) and *in vitro-in vivo* correlation (IVIVC) could be properly established and wisely applied in our own drug discovery and development labs. DDW

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