Strategy of Utilizing *In Vitro* and *In Vivo* ADME Tools for Lead Optimization and Drug Candidate Selection

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Abstract: The high-throughput screening in drug discovery for absorption, distribution, metabolism and excretion (ADME) properties has become the norm in the industry. Only a few years ago it was ADME properties that were attributed to more failure of drugs than efficacy or safety in the clinic trials. With the realization of new techniques and refinement of existing techniques better projections for the pharmacokinetic properties of compounds in humans are being made, shifting the drug failure attributes more to the safety and efficacy properties of drug candidates. There are a tremendous number of tools available to discovery scientists to screen compounds for optimization of ADME properties and selection of better candidates. However, the use of these tools has generally been to characterize these compounds rather than to select among them. This report discusses applications of the available ADME tools to better understand the clinical implication of these properties, and to optimize these properties. It also provides tracts for timing of studies with respect to the stage of the compound during discovery, by means of a discovery assay by stage (DABS) paradigm. The DABS provide the team with a rationale for the types of studies to be done during hit-to-lead, early and late lead optimization stages of discovery, as well as outlining the deliverables (objectives) at those stages. DABS has proven to be optimal for efficient utilization of resources and helped the discovery team to track the progress of compounds and projects.

Keywords: ADME, lead optimization, discovery assay by stage, high throughput screening, drug-drug interactions, transporters.

INTRODUCTION

Combinatorial chemistry and high throughput absorption, distribution, metabolism and excretion (ADME) screening have been added to the drug discovery process and have increased the number of compounds processed during this stage. However, the number of new products approved annually has largely been unchanged over the past two decades. Innovations in discovery do not appear to be enhancing the success of pharmaceutical companies. The safety and clinical efficacy continue to determine what is approved for the market. During the last decade, clinical development times have demonstrated a trend to shorter periods, particularly for life threatening diseases like cancer and HIV. However, a recent survey by the Tufts University Center for the Study of Drug Development found that the combined clinical development and approval times for new small molecule drugs took an average of almost seven years [1].

The process of discovering new drugs has evolved significantly during the last two decades and now embraces the optimization of the ADME properties. Today, pharmaceutical companies conduct more studies to understand the roles of enzymes and transporters in drug disposition and drug-drug interactions, to assess drug absorption, distribution, metabolism and elimination, and to predict the pharmacokinetics and other ADME properties in man, during lead optimization and candidate selection. Although this evolution has provided many *in vitro* tools which many companies employ during high throughput screening (HTS) and lead optimization, we believe that *in vivo* PK optimization provides a more integrated means for optimizing ADME properties during drug design. However, *in vitro* methods are certainly important to the understanding of the specific factors contributing to drug disposition and, when relevant to the *in vivo* pharmacokinetics, can be a highly efficient means for ADME optimization. This article will discuss some of the *in vitro* and *in vivo* tools and strategies employable during drug discovery for lead optimization and candidate selection. It should be noted that this article reflects the opinions and practices of the authors and may not reflect the practices at other pharmaceutical companies.

STRATEGY

The ADME strategies for drug discovery and development will vary based on the clinical goals of a program. Consequently, the therapeutic area, the severity and rarity of the disease, the duration of drug treatment, and the ADME properties of competitive products are factors that should be considered in developing an adequate ADME profile during lead optimization and candidate selection. The primary goal is to provide a competitive product based on efficacy and adequate safety, avoiding PK-based failures in the clinic. We believe that drug target potency and selectivity should be the principal objective for the drug design strategy prior to lead optimization; optimal activity and selectivity are the most important early goals for the chemistry of drug

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design. Thus, moving the classes of molecules with greatest selectivity in pharmacological properties to lead optimization is a critical first step in the design strategy for new drugs. The emphasis on structure-phamacological activity by medicinal chemists prior to lead optimization provides a more efficient means for compound optimization than optimizing pharmacological and ADME properties in parallel.

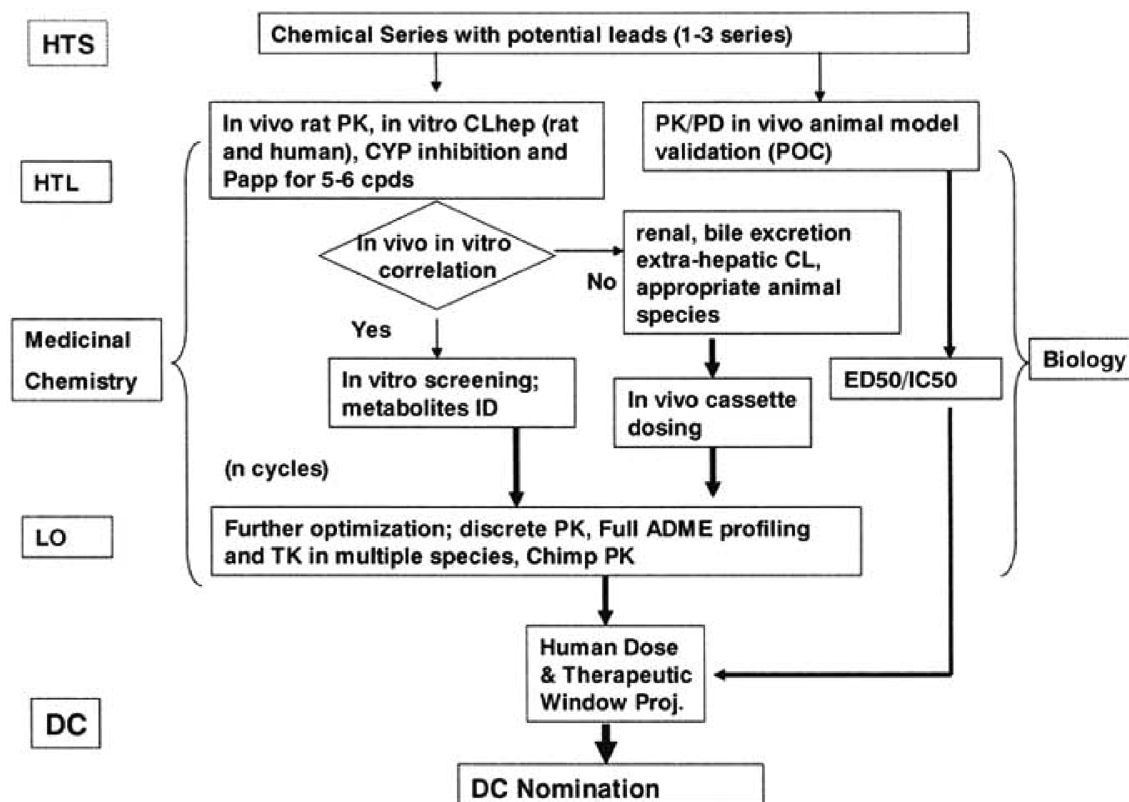
The authors have designed a strategy for ADME assays to be performed at hit-to-lead (HTL), early lead optimization (Early LO) and late lead optimization (Late LO) stages, which delineates the types of studies that sould be performed and the deliverables at each stage Fig. (**1**). This has helped the company optimize efficiently its resources , without hampering the discovery and development of desirable leads. Similarly, the company has also developed a DABS for safety assessment which is described elsewhere [2]. After the chemical scaffolds have been identified through high throughput screens, each series moves to HTL where the ADME staff gets involved in the identification of any pharmacokinetic issues with the series, identification of *in vitro* or *in vivo* tools for rapid screening of compounds, and at the same time assisting with the proof-of concept (POC) studies with pharmacology Fig. (**2**). Once the target is validated by *in vivo* studies the project moves to the Early LO stage, and screening tools are then utilized in the Early LO stage to optimize the PK properties of compounds. At the same time, the potential for drug-drug interactions (DDIs) are evaluated. Support is provided to pharmacology for building PK/PD relationships in the disease model. Once a lead is identified, the project enters into the Late LO stage. It is at this stage extensive efforts are spent on studies that lead to human PK, dose and therapeutic window projections in conjunction with the safety assessment data. There may be one or more compounds at this stage, and comparative ADME properties performed help select the best candidate for nomination for development. The studies encompassed at this stage also include predictions of human DDIs, identification of the most relevant animals species to humans to assess safety, based on the metabolic similarity and exposure in the species. Late LO stage is then followed by Development Candidate (DC) selection. Throughout the LO stages, the communication with cross-functional team is maintain at a high level, and is considered a key for the progression of the right molecule and the allocation of appropriate resources in a timely fashion.

ADME PROPERTIES

The ideal ADME properties for an oral drug are good bioavailability, blood clearance and volume of distribution that are anticipated to give half-life commensurate with a convenient dosing regimen and low potential for drug-drug interactions and metabolism-dependent toxicities in humans. All these properties are *in vivo* properties and so they should be assessed as early in the discovery process as practical. Often, the need for rapid *in vitro* ADME screening is driven by the desire to use ADME screens as an element in the selection process of high throughput screens of chemical libraries. *In vitro* screens for absorption in cell based systems such as Caco-2 or in constructed systems such as PAMPA, metabolic clearance and CYP inhibition data with liver microsomes or with recombinant enzymes are often employed to provide ADME data for screening large chemical libraries. We believe, however, that it is advantageous not to screen compounds with *in vitro* ADME assays prior to *in vivo* pharmacokinetic assessments because the number of false negatives that have been observed with the Caco-2 system and the infidelity of metabolic stability in predicting *in vivo* half-life. The authors believe that HTS assays should be focused on identifying potential leads by

HTL	Early LO	Late LO
Assays 1. *In vivo* rat PK 2. In vitro hepatic clearance across species 3. Caco-2 permeability 4. *In vitro-in vivo* correlations 5. Plasma/target tissue exposures in pharmacological models 6. Support to enhance exposures for POC in model systems	**Assays** 1. *In vivo* PK optimization: Cassette/Discrete PK screen in appropriate species 2. Metabolic profiles 3. Use of *in vitro* screen to overcome PK liabilities 4. *In vitro* CYP involvement and inhibition 5. Transporters' involvement/inhibition 6. Plasma protein binding 7. PK/PD relationship in pharmacology models	**Assays** 1. *In vivo* PK in higher species (including Chimp) as needed 2. Modeling human PK and dose projections 3. Toxicokinetics 4. ID metabolites in Tox species 5. Validation of bioanalytical methods 6. Initiation of bioanalytical method development for metabolites of pharmacological or toxicological interest
Deliverable 1. Identification of PK issues 2. Identification of relevant in vitro or *in vivo* tools for screening 3. Exposure-based POC model testing	**Deliverable** 1. Optimization of animal PK profile for lead 2. DDI potentials for the leads 3. PK/PD relationship in the pharmacology model	**Deliverable** 1. Full ADME profiles in animals 2. Projections for human PK and dosage 3. DDIs at projected doses 4. Identification of safety assessment species 5. Projections for safety margins

Fig. (1). ADME Discovery Assay By Stage.



HTS: high throughput screening
HTL: hit to lead
LO: lead optimization
DC: drug candidate

Fig. (2). Iterative pathways from hit-to-lead to lead optimization.

intrinsic properties that can be extended to the intact organism such as pharmacological activity and specificity.

Routinely, the initial screening strategy involves studying a set of about six compounds from a chemical series for pharmacokinetics in the rat, clearance with animal and human liver microsomes, permeability determination with the Caco-2 system. The collective data provide *in vitro* and *in vivo* correlation of hepatic clearance and plasma clearance in rats, based on similarity in rank orders of compounds. It also identifies species that best represent clearance in humans. If the rat is found not to provide a good correlation between *in vitro* and *in vivo* clearance, we move to dogs or monkeys for finding a species most representative of *in vitro* human clearance. This is done for each series in the program. Once established, the selected animal species is used for the PK screening of compounds in that series, using an IV cassette dose format. A lack of an *in vitro*-*in vivo* correlation triggers correlation screening using S9 fraction. If the *in vivo* clearance of compounds turns out to be near or higher than the hepatic blood flow, the cause of the high clearance is determined by identifying major metabolites that could help in designing molecules with better metabolic stability, determining the RBC partitioning, and the plasma stability. At this point an *in vitro* metabolic screen can be instituted. At the lead optimization stage, hepatic, gut and/or pulmonary first-pass effect are determined, as needed, to evaluate the degree and extrahepatic nature of the clearance. Renal and biliary excretion measurement of the compound help in keeping things in perspective to determine if alterations in structure would lead to any beneficial effect in terms of decreasing metabolic clearance. Caco-2 studies identify any issues with permeability of compounds and efflux pump substrate selectivity that could hinder compound delivery to the target tissue. If permeability is identified as a major issue then PAMPA membrane system is used as a primary screen (and Caco-2 as a secondary screen).

In parallel to PK optimization in humans, we are also involved in studying PK in the pharmacology model for the disease. For early Proof of Concept studies for new targets, to maintain sufficient exposure of compound to animals use of alternative dosing routes, like IP, SC or use of osmotic pumps is explored. Other ways to enhance exposure to target tissues utilize metabolic inhibitors, e.g. 1-aminobenzot-riazole [3, 4]; or transport inhibitors, e.g. verapamil, GF120918, MK571, LY335975, Ko143 [5]. Same inhibitors are also tested in cell based assays for determining target-based activities. It is critical to understand the reason for lack of desired exposure *in vivo*. Typically, a balance of lipophilicity and hydrophilicity is required for permeability for a compound, and a cut off of LogP of 5 can be considered safe. There also are alert features built in the data bases to label and exclude compounds based on the

Lipinski's rule of 5 [6] from further studies. Bioavailability prediction models are routinely being used based on that rule [7]. Other ADMET in silico models are also considered [8]. Considering that Pgp substrates are generally lipophilic in nature, modification of structure to enhance hydrophilicity by attaching polar groups is suggested, to reduce the Pgp selectivity. Also Pgp substrate selectivity has to be weighed in appropriately by the scientists in conjunction with the *in vivo* PK data in animals. For example, if a compound is highly permeable and is a Pgp substrate, and if at therapeutically low doses the absorption *in vivo* occurs rapidly in a narrow section of the upper GI tract, absorption of the compound will likely not be affected by Pgp. Likewise, if the therapeutic dose is relatively large, the concentration in the gut likely would be high enough to saturate the transporter to have significant effect on absorption. However, if the target tissue is extravascular, e.g. brain, then the tissue uptake may be affected by Pgp. The *mdr1a/1b* knockout mouse models have also been successfully used to understand Pgp related issues *in vivo*. The new efflux pump BCRP, also present in Caco-2 cells, has recently gained recognition and importance in drug clearance and drug-drug interactions. Other drug transporters, e.g. OCT, OATPs, MRPs, are looked at when we have extensive biliary secretion that could lead to drug-drug interactions in the clinic. These studies come into play around Development Compound nomination stage.

Distribution into target tissues is routinely measured in our labs using non-labeled compounds, by the usual cut and grind method, especially for CNS or tumor target. But early stage tritium labeling has become so feasible that for selected compounds, especially to resolve or clear any issues, whole body autoradiography is being done with successful results for drug candidates entering preclinical development. The technique provides the ability of distinguishing radioactivity in the fluid and tissue space, e.g. distribution in CNS vs. CSF [9]. Other imaging techniques used in drug discovery and development, e.g. PET, MRI, etc., are described elsewhere [10]. The use of imaging techniques can significantly decrease the workload relative to the tissue excision and histology, speeding the evaluation of lead molecules.

STRUCTURAL ALERTS

Direct examination of the chemical structure of the new entity provides an assessment of certain physicochemical and safety properties. Structures containing, for example, furan, thiophene and methylene dioxide ring systems, and aromatic nitro and amine groups should be evaluated strenuously for the formation of reactive intermediates. Potentials for the formation of quinine imines and methides from the parent structures also should be looked at. Formation of reactive, electrophilic species from such compounds is easily screened using simple glutathione conjugation systems. Acyl glucuronide are other class of active metabolites that have long been implicated as potential source of toxicity for some drugs. In general, they have the potential to form covalent bonds to proteins, disrupting cell function and may lead to idiosyncratic drug reactions. In the event of acyl glucuronide formation its stability data is established in relation to other drugs, including zomepirac which was withdrawn from the market for toxicity issues. In the recent years the importance of formation of acyl-CoA adducts also has been realized, as they may also lead to generation of reactive metabolites capable of binding irreversibly to proteins [11]. Likewise, UGT isoform involvement is established or relevant studies conducted to discern if bilirubin conjugation (UGT1A1) will be affected significantly to cause, e.g. hyperbilirubinemia. For that purpose commercially available, expressed UGT isoforms are utilized. For *in vivo* studies, Gunn rats, which are deficient in UDP glucuronyltransferase activity, are used as a model for Crigler-Najjar Type 1 disease and Gilbert Syndrome. In the event of hyperbilirubinemia, other mechanisms by which this effect could occur also need to be evaluated. These involve interaction with MRP2 as well as MRP1 and 3, and OATP2 which are involved in the efflux of bilirubin glucuronides, and uptake of bilirubin by hepatocytes, respectively. Similarly, GY/TR^- and EHBR rats as models of MRP2 deficiency (Dubin-Johnson Syndrome) are utilized for mechanistic evaluations.

BIOANALYTICAL

Central to the success of all of the above studies is LC-MS/MS which has certainly revolutionized the way we do ADME research, by providing high sensitivity and selectivity for analytes in diversely complex matrices, and short LC run times without requiring much separation work, when ion suppression of coeluting components is not an issue. All that has helped tremendously in high throughput screening of samples generated by cassette dosing in animals. Ion Trap mass spectrometers have helped assigning structure to compounds and metabolites with ease. Fourier Transform Ion Cyclotron Resonance high resolution mass spectrometry has taken us a step deeper into the molecular formulas by looking at the exact masses of parents as well as their metabolites, helping distinguish isobaric ions. Hybrid quadrupole-time-of-flight LC-MS/MS systems are also popular for the characterization of metabolic profiles. LC-NMR and LC-NMR-MS can be used to resolve non-routine issues with unstable metabolites. Incorporation of accelerator mass spectrometry in the discovery arena has also started emerging now [12, 13].

DRUG_DRUG INTERACTIONS

Early toxicokinetic support to safety assessment studies provide valuable information on the *in vivo* induction and inhibition properties of the lead compounds. Alteration in PK on repeat dosing can then trigger studies to explore mechanism of the event and studies to project relevance to humans. Consequently, animal livers from the toxicity testing studies are analyzed for various CYP enzymes' activities including CYP 1A, 3A and 4A [2]. Induction potential of CYP3A4 in human hepatocytes may then be evaluated to get an early assessment on clinical DDIs [14]. These studies provide only an alert if the induction or inhibition is noted in animals or human hepatocytes. Whether these will entail in clinical DDI and adverse labeling has to be assessed based on projected clinical plasma concentrations of the drug and the concomitant drug, dosing duration, therapeutic index of drugs, risks vs. benefits, etc. It should also be noted that *in vitro* metabolic DDI do not always lead to *in vivo* interactions [15].

During lead optimization, it has become customary to determine CYP inhibition potential of lead compounds. Because many drugs are substrates of CYP3A4, the inhibition of this isoform is considered undesirable. The inhibition of CYP2D6, once dreaded, is now considered manageable in most programs, as long as the therapeutic window is not expected to be narrow, by simply treating all patients as CYP2D6 deficient. In contrast, compounds that are cleared only by CYP2D6 are not progressed. It is crucial to determine the contribution of various isozymes, e.g. by relative activity factor method (16), before attempting to evaluate drug-drug interaction potential. A perspective on the conduct of DDI studies became available recently [17]. Recombinant CYP systems are routinely used to identify isoforms involved in oxidative metabolic pathways. Specific inhibitors and monoclonal antibodies are commonly used for that purpose. Antibodies against cytochrome P450 reductase also have been used successfully, e.g. to distinguish between CYP mediated and FMO mediated metabolism. These studies are done before the nomination of the Candidate for development. The selection of compounds that do not rely on one metabolic or transporter pathway are generally preferred to lower the potential for DDIs.

Promising compounds based on their efficacy demonstration in a pharmacology model are carried forward for discrete PK studies in dogs and monkeys. Chimp PK studies have also been employed, especially when there is a poor allometric relationship among the other species. All that helps in performing allometric scaling to predict the PK in humans, taking into account differences in metabolic clearance, where possible. At this stage *in vivo* metabolic profiles are generated for comparison with *in vitro* profiles in humans to identify which animal species best represents human metabolism and thereby providing the rationale for selection of the animal for toxicology studies. The therapeutic window for humans is estimated based on the PK/PD modeling for efficacy and toxicokinetic safety margin. The best efficacy, safety and PK compound from the top candidates is the basis for selecting a compound for preclinical development.

CONCLUSION

The optimizing of the ADME properties of a drug candidate is a complicated process. Utilization of high throughput technologies to eliminated compounds with poor ADME properties at HTS stage has been fruitful in speeding up the candidate selection from a vast number of compounds, but it carries the potential of throwing away potent chemical leads. It is absolutely critical to design studies strategically and to evaluate all data in perspective, keeping the ultimate goal of safety, and efficacy to the patient. A useful ADME process, coined as DABS, was developed at this company to streamline the studies, through a nested sequence of assays that preserve relevance to *in vivo* animal PK during Early LO and relevance to human PK during Late LO. DABS are valuable to track the project and monitor the progression of leads toward nomination for development. Overall, we find that it is cost effective and efficient to identify the ADME issues by comparing *in vitro* and *in vivo* results for a limited number of compounds at the hit-to-lead stage and then selecting appropriate *in vitro* or *in vivo* tools to optimize the ADME properties. During the HTL stage of the drug discovery process optimizing potency still should be considered as the main priority. Without low nM or even pM potency and high specificity, a compound with the best PK properties still can be terminated because of narrow safety window. The current technologies available to scientists and the learnings from successes and failures of drugs in the market, have greatly improved the ADME optimization and selection process in the past few years. This has resulted in significant decline in the attrition rate in drug development due to ADME [18, 19, 20]. The toxicity and efficacy have now become the prime candidates for attrition.

ABBREVIATIONS

DABS = Discovery assay by stage

LO = Lead optimization

DC = Development candidate

HTS = High throughput screening

ADME = Absorption, distribution, metabolism and elimination

DDI = Drug-drug interaction

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