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HIGH-THROUGHPUT SCREENING IN DRUG METABOLISM AND PHARMACOKINETIC SUPPORT OF DRUG DISCOVERY

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■ **Abstract** The application of rapid methods currently used for screening discovery drug candidates for metabolism and pharmacokinetic characteristics is discussed. General considerations are given for screening in this context, including the criteria for good screens, the use of counterscreens, the proper sequencing of screens, ambiguity in the interpretation of results, strategies for false positives and negatives, and the special difficulties encountered in drug metabolism and pharmacokinetic screening. Detailed descriptions of the present status of screening are provided for absorption potential, blood-brain barrier penetration, inhibition and induction of cytochrome P450, pharmacokinetics, biotransformation, and computer modeling. Although none of the systems currently employed for drug metabolism and pharmacokinetic screening can be considered truly high-throughput, several of them are rapid enough to be a practical part of the screening paradigm for modern, fast-moving discovery programs.

INTRODUCTION

Since the early 1990s, several new forces in drug discovery have changed the pursuit of this endeavor. We may group these forces into three main areas: chemistry, molecular biology, and robotics. Chemists have invented many new methodologies for production of large, diverse sets of novel organic compounds. We refer to these methodologies under the umbrella term combinatorial chemistry (1). The result has been a many-fold increase in the number of compounds available to sample so-called chemistry-space, that is, the multidimensional relation between molecular structure and biological activity (2). In addition, structural chemists have developed powerful new tools such as molecular docking algorithms (3), mapping of protein binding sites by nuclear magnetic resonance (SAR-by-NMR) (4), and homology modeling of proteins (5) that allow an unprecedented level of rational design to guide the synthesis of prospective drugs.

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From another direction, molecular biology and genomics have allowed identification of many important new biological targets (6) and have provided the means to express these target proteins in in vitro systems that enable them to be used in high-throughput screening (7, 8). For both combinatorial chemistry and high-throughput screening, the enabling development has been the commercial availability of reliable, highly programmable, adaptable robots (9–11) that can carry out complex microscale laboratory operations to synthesize and test hundreds of thousands of new organic compounds. Recent advances in miniaturization and assay speed and sensitivity have allowed the use of 1536-well microtiter plates instead of the conventional 96-well plates and have prompted the use of the term ultrahigh-throughput screening. These forces have synergistically increased our ability to create pharmacologically interesting compounds, at least at the in vitro level.

As a practical matter, this increase has produced an enormous pressure to determine which of these thousands of biochemically active compounds have drug-like properties (i.e. which are biologically active). We can define a compound to be drug-like when it has the following properties.

1. Efficacy: the intrinsic ability of the compound to produce a desired pharmacological effect. Efficacy comprises the absolute amount of the compound necessary to achieve the effect (potency) and the magnitude of the maximum effect that can be achieved.
2. Availability: the ability of the compound to pass through multiple biological barriers to reach the target receptor. Normally we consider availability to comprise both oral bioavailability and adequate distribution to the target organ.
3. Persistence: sufficient residence time at the target receptor so that pharmacological effects have a clinically meaningful duration. Persistence is usually expressed as the plasma elimination half-life.
4. Safety: sufficient selectivity for the target receptor so that an adequate dose range exists in which the intended pharmacological action is essentially the only physiological effect of the compound.
5. Practicality: generally thought of as the pharmaceutical properties of the compound, including solubility and rate of dissolution, chemical stability, crystallinity, and so on, which allow the drug substance to be synthesized and the drug product to be formulated, distributed, handled, and dosed in a practical manner. For this discussion, we can ignore consideration of commercial success criteria such as size of patient population, marketing, production economics, and so on.

Each of the drug-like properties must be present in a successful clinical drug, although there is considerable latitude in each property. A major deficit in any of these properties will preclude an active compound from being used as a drug. All five properties must be in the acceptable range, even if it is not possible to fully optimize each property. Of these drug properties, only efficacy and safety can be considered to be optimized in any sense during the combinatorial synthesis/in

vitro screening phase, inasmuch as the compound's intrinsic affinity for the receptor, the potential magnitude of the response (if the compound has an agonist action), and the selectivity of the compound for the particular receptor subtype are standard components of the screening phase.

Optimizing the other properties is much less straightforward for two reasons. First, to derive the full benefit from the great advances made in high-throughput screening, subsequent optimization steps must occur at a substantial fraction of the rate of production of compounds with acceptable biochemical activity, meaning that in vitro or accelerated in vivo methods must be used. Second, an in vitro method must have physiological relevance. That is, the method must be validated, or shown to have a good concordance with the desired in vivo parameter. Validation is surprisingly difficult with metabolic pathways and parameters such as intestinal absorption, half-life, or brain penetration. Often only in vivo measurements of these parameters are reliable enough to allow strategic decisions about the viability of a candidate compound. Unfortunately, in vivo measurements are generally so slow that they represent a considerable bottleneck for compound selection, largely negating the speed of creation and identification of potent compounds.

In this review we consider the problems of optimizing drug-like properties, with a focus on drug metabolism and pharmacokinetics (DMPK). We discuss the advances that have been made in recent years to increase the speed of assessment methods enough to achieve the rapidity of advancement through a drug discovery campaign that is theoretically possible. Truly high-throughput screening methods, such as those used to screen for early leads in compound libraries, may achieve rates of 50,000–100,000 compounds per week. In contrast, the fastest methods presently used in DMPK screening typically do not exceed 100 compounds per week. For in vivo methods, a throughput of 10 compounds per week might be considered high if the conventional methodology could handle only one compound in a week. Thus we use the term higher-throughput to denote the acceleration of the rate even though the absolute rate is very slow.

GENERAL CONSIDERATIONS FOR SCREENING

Criteria for Good Screens

The following five criteria should be met when designing and implementing a discovery screen.

1. Relevance: The result of the screen should have good concordance with the corresponding in vivo property of the drug (e.g. clearance, absorption, or brain penetration). This requires validation of the screen with standard compounds of known animal or human performance.

2. Effectiveness: The cutoff criterion should eliminate a substantial fraction of compounds. If almost all compounds survive the screen, it merely adds a useless extra step that delays the discovery process (but see ''Counterscreens'' below).
3. Speed: The experimental procedure must be fast enough to keep pace with the input rate of new compounds from chemistry. Rapidity is often achieved at the expense of absolute accuracy, but for screening purposes we can afford to relax our usual criteria for accuracy. An effective means to achieve speed is to use exactly the same assay, regardless of the compound being tested, but this is seldom possible with DMPK screens.
4. Robustness: The experimental procedure must be applicable to a wide variety of chemical structures. Ideally it should also work with different biological components (e.g. animal or human microsomes, S9 fractions, hepatocytes).
5. Accuracy and reproducibility: Obviously we want the screen to give the right result the first time (see ''False Positives and False Negatives'' below), and extensive retesting degrades the method's productivity. Because of relaxed accuracy to achieve speed, it may also make sense to widen the acceptance criterion window to account for greater-than-normal uncertainty in the measured value.

Counterscreens

In many cases, we are not trying to find the compounds that have a certain property; rather, we are trying to find the ones that lack a certain property. For instance, counterscreens are used for confirming high selectivity for the receptor of interest by showing that the compound does not bind well to other receptors. Likewise, we often use a counterscreen to assure that potent inhibitors of CYP 3A4 are eliminated from further consideration. Counterscreens have the same criteria as screens, except that for counterscreens the effectiveness criterion is reversed, because we want as many compounds as possible to survive the counterscreen.

Sequencing of Screens

Often the lead optimization phase of drug discovery has a battery of successive screens that must be passed. The general rule is that the screen with the best overall combination of speed and ''veto power'' should be highest in the succession of screens. All work done on a compound that has no potential to be a clinical drug is wasted effort, except when the compound is to be used only as an investigational agent in pharmacological proof-of-principle studies. Most frequently, then, the first screen is a high-throughput in vitro assay for antagonism or inhibition of the receptor or enzyme that is the biochemical target. This is usually readily adaptable from the high-throughput lead discovery phase, and the data from this screen are clearly interpretable in an absolute sense (i.e. a compound with a K_i of 1000 nM is clearly not active enough). Thus having this screen in the first position eliminates many more compounds than other screens would.

The next screen after the in vitro activity screen might be a counterscreen against related receptor types, or a cell-culture model. However, industry is increasingly using a screen for a desirable biopharmaceutical property (e.g. Caco-2 permeability as an indicator of intestinal absorption) or a counterscreen against an undesirable biopharmaceutical property (e.g. CYP 3A4 inhibition). This second-level screen can even be an in vivo pharmacokinetic screen based on cassette dosing or pooling of plasma samples, methods that can offer enough speed to be practical even at a relatively high position in the screening sequence.

Interpretation of Screens

The biopharmaceutical properties of drugs fall into two groups: those determined entirely by the molecular structure of the compound and those that depend on interaction with a biological component. Examples of the first type are pK_a, solubility, dissolution rate, crystallinity, and chemical instability. These properties are intrinsic to the compound and are, therefore, measurable by completely abiotic methods. The interpretation of values for intrinsic properties is straightforward, because the significance of slow dissolution rate or chemical instability is clear when considering the suitability of a compound to be a drug. Even for some of the properties of compounds that depend on a biological component, for example in vitro binding affinity to the target receptor, interpretation of in vitro data is straightforward (i.e. tighter binding is better). In stark contrast, most of the DMPK properties of a compound are the result of a complex interaction of the compound with membranes, binding proteins, transporters, and metabolizing enzymes as well as higher-level phenomena such as organ blood flows, glomerular filtration rate, and tissue uptake. These biological interactions make it difficult to assess the meaning and importance of a value for a particular property that is measured in vitro, especially when a cross-species comparison is required. Consequently, it is particularly important to establish the relevancy of the in vitro–measured property to the corresponding physiological property prior to application of the screen to a new series of compounds, and to check the continuing relevance as that property is improved in successive analogs.

False Positives and False Negatives

Any screening procedure has a characteristic error rate. This is almost inevitable, because it is usually necessary to sacrifice some accuracy or precision to achieve the requisite speed. Thus when a large number of compounds is carried through a particular screen, some of the compounds will be classified incorrectly. A screen may be used in an absolute sense, so that compounds that pass a certain criterion (e.g. Caco-2 permeability greater than that of a benchmark compound) are termed positives, whereas those that fail to meet the criterion are termed negatives. Compounds that pass but should have failed are false positives. In general, false positives are tolerable, if they are not too numerous, because they will be rectified later. Compounds that fail but should have passed are false negatives. False neg-

atives may be lost forever if the failure eliminates them from further testing. However, as long as a reasonable number of true positives are being found, false negatives are also tolerable. Because the pass-fail criterion for a screen is arbitrary to a degree, the relative fractions of false positives to false negatives are adjustable by moving the acceptance criterion up or down, and the researcher may opt for more false positives and fewer false negatives for a direct screen and the converse for a counterscreen. A screen may also be used in a relative sense, to rank-order compounds for priority for subsequent testing. However, even for rank-ordering, false positives and false negatives can still cause the compound to be ranked incorrectly.

DRUG METABOLISM AND PHARMACOKINETICS SCREENING

General Considerations

The main subject of this review is the extensive effort presently occurring in industrial drug discovery organizations to increase the probability of ultimate success for a compound entering clinical trials (12). Obviously when a compound fails in clinical trials, failure was not expected. In fact, the three main reasons for clinical failure (lack of efficacy, toxicity, and unfavorable DMPK properties) are poorly understood and inherently difficult to predict. To illustrate, let us look at these reasons for failure. In contemporary drug discovery and development, most companies are investigating new pharmacology—that is, a novel approach, a novel molecular target, or a previously untreatable disease. Of course, the reason for this is that to be ''first-in-market'' with a new therapy is the sine qua non of the pharmaceutical business. The difficulty with being first is that clinical trials carry the additional burden of ''proof-of-principle'' with the new therapeutic concept, and sometimes the new pharmacology simply does not work in humans. This is something that can be determined only by clinical experience; thus, it is unpredictable preclinically. Similarly, a particular clinical toxicity is not normally expected, except in the special case of exaggerated pharmacology. For example, if the compound had shown hepatotoxicity in the preclinical animal toxicology studies, it would not have been taken to Phase I clinical trials, except for poorly treated, life-threatening indications. So, in most cases, an observed human toxicity was not predicted by animal toxicity.

The prediction of DMPK properties is similarly difficult, partly because of the vast number of possible metabolic pathways (13) and partly because of interspecies differences (14). At the same time, good DMPK properties are fundamental to the success of a drug candidate, and it is important to have some sort of procedure to assure their presence in the clinical candidate. For instance, although efforts continue to quantitatively predict pharmacokinetics, the current industry trend is to settle for categorization of candidates as clearly bad, marginal, or probably good (15). Candidates that are clearly bad are eliminated, and the good

candidates are rank-order prioritized for more in-depth study. If enough good candidates are found, it may be unnecessary to make hard decisions about the marginal ones. As we discuss the screening for various DMPK properties in the next sections, we will see how the categorization concept is applied in each case.

Absorption Potential

Although in vivo studies are clearly the most reliable way to determine intestinal absorption, current in vivo methodology is far too slow to allow its application in discovery screening. Although a high-throughput in situ rat intraduodenal dosing model was used to guide synthesis of absorbable renin inhibitors (16), this method has not been applied widely, and researchers have mainly sought in vitro methods. The two chief determinants of in vivo intestinal absorption are solubility and intestinal permeability. Solubility is readily estimated on small quantities of a compound following synthesis, but permeability is much more difficult to assess. The most popular approach is to measure rates of compound diffusion down a concentration gradient across cultured Caco-2 cell monolayers (17).

Caco-2 permeability screening is used widely to screen drugs (18), prodrugs (19), and combinatorial libraries (20, 21). It is generally accepted that good permeability through Caco-2 monolayers is a reliable indicator of good absorption in vivo (22, 23), unless dissolution is a problem. The interpretation of low and intermediate Caco-2 permeabilities is not clear. Low molecular weight hydrophilic compounds, sugars, nucleosides, or small peptides may show very low permeabilities in vitro yet be well absorbed in vivo because alternative mechanisms of absorption are not well modeled by the Caco-2 monolayer, including paracellular passage or active transport (24). Even for drugs that have good Caco-2 permeability, in vivo absorption can occasionally be limited by the P-glycoprotein (PGP) efflux pump (see below). This ambiguity in interpretation makes it difficult to predict with confidence the net in vivo absorption of a candidate. Most groups eschew quantitative predictions entirely, preferring either to categorize compounds as having low, medium, or high permeability or to compare them to a structurally related benchmark compound with known in vivo absorption. A genetically engineered Caco-2 variant has been developed that stably expresses high levels of CYP 3A4, the intestinal P450 enzyme responsible for some first-pass metabolism, to allow a concurrent assessment of permeability and gut wall metabolism (25). It is difficult to quantitatively relate the degree of metabolism in such a system to the in vivo first-pass effect.

Reports of increasing throughput of the Caco-2 permeability assessment via automation are just beginning to appear in the peer-reviewed literature (26), but this technology is already becoming widely applied, with several automation companies offering specialized robots for the purpose. The usual approach is to separate the overall process into two regimens: (*a*) culture of the cells until confluency and tight junctions are achieved, and (*b*) the actual permeation experiment. Although neither of these steps can be considered truly high-throughput, the cell culture process is by far the slower of the two. This stage is accelerated by the

simple expedient of maintaining many cultures in parallel. After an initial 3-week delay, monolayers can be staggered to be ready for a permeability experiment each day. If the automation is set up properly, every step from initial seeding of cells, through daily maintenance and medium changes, through running the permeability experiment, to the LC/MS/MS (liquid chromatography/tandem mass spectrometry) assay can be carried out with only a little human intervention, such as occasionally replenishing media reservoirs or manually moving microtiter plates from the robotic liquid handler to the LC sample-injector robot. Special 24-well plates are now available for Caco-2 cultures, allowing rates of a few hundred compounds per month to be realized. Although such a rate is far below that of true high-throughput screening, it is far better than the rate typically achievable with purely manual cell-culture methods, and it is usually sufficient to keep pace with the delivery of potent compounds through the primary in vitro activity screen. Another advantage is that automation allows cells to be maintained during holiday and vacation periods, which otherwise create blackout periods for Caco-2 determinations.

Two other methods for rapid assessment of absorption potential should be mentioned, both of which are based on completely artificial membrane-mimic systems. Immobilized artificial membrane (IAM) chromatography offers the advantages of experimental simplicity and familiarity to most chemists, compared with Caco-2 permeabilities. Good correlations have been shown between IAM chromatographic k′ values and both Caco-2 permeabilities and in vivo intestinal absorption (27). Because retention times increase with increasing absorption potential, the chromatography sets limits on throughput that would have to be addressed in a high-throughput screening application. Recently, the parallel artificial membrane permeation assay (PAMPA) was introduced for in vitro assessment of passive absorption (28). The method utilizes permeation through an artificial phospholipid bilayer formed on a filter within the wells of a 96-well microtiter plate. The assay, based on simple ultraviolet absorbance of the effluent, allows high rates of compound assessment, in the range of hundreds per day. Like the Caco-2 permeability method, IAM and PAMPA may underestimate the absorption of compounds subject to active or paracellular transport in vivo. However, both methods, especially PAMPA, seem well suited to early drug discovery absorption counterscreening, in which the goal is to eliminate poorly absorbable compounds.

As mentioned above, a potential limitation of net absorption of compounds with good transport properties is the PGP efflux pump (29). For instance, PGP has been shown to limit the absorption of several HIV protease inhibitors by as much as fivefold (30). Thus there is interest in screening compounds as PGP substrates during discovery. A higher-throughput assay based on competition against ^{3}H-verapamil binding in PGP-overexpressing Caco-2 cells has been described (31). Two other methods are based on inhibition of efflux of a fluorescent PGP substrate (32–34), and these methods have been applied in an industrial

setting. A limitation of all three methods is that they do not distinguish between PGP substrates and inhibitors.

The case of PGP screening illustrates the difficulty of screening for DMPK properties. Because PGP both limits intestinal absorption and excludes xenobiotics from the brain (30), a compound in which PGP recognition has been eliminated may be better absorbed but also show greater net brain penetration. Although brain penetration will be desirable for central nervous system (CNS) drugs, it is generally undesirable for other indications because of the potential for CNS side effects or toxicity. Also, a PGP substrate may competitively inhibit the brain efflux of a second co-dosed drug, increasing its potential for CNS toxicity (35). Therefore, one must carefully consider the therapeutic indication and likely co-dosed drugs before applying a PGP screen in a drug discovery program.

Blood-Brain Barrier

The ability of compounds to penetrate the blood-brain barrier is almost always of interest, either as a property to screen for in a CNS indication or to counterscreen against in non-CNS indications. Although this property is not usually considered an object of primary screening, requiring higher-throughput methodology, it is often set as the object of secondary or tertiary screening in CNS discovery programs, because many pharmacological models of CNS diseases are long and tedious. It is prudent to determine whether a compound can get past the blood-brain barrier before investing time in the in vivo assay. A good in vitro model is available for this purpose, bovine brain microvessel endothelial cell (BMEC) monolayers, which are used in a manner analogous to that of Caco-2 permeability screening (36, 37). BMEC monolayers have been applied to higher-throughput drug discovery screening for good brain penetration (38). Although BMEC permeability is determined quantitatively, it is usually sufficient to express the results qualitatively (i.e. good or poor brain penetration). A major disadvantage is that BMECs are not an immortal cell line; therefore, they must be primary-cultured from cow brain. Although the cells may be frozen after harvesting, they are an exhaustible resource that must be replenished regularly. Recent studies show that BMECs express a functional PGP system (39, 40) that may allow them to be used as an in vitro model of PGP-limited brain penetration in a screening mode. The BMEC system has not yet been reported to have been automated.

Enzyme Inhibition

Inhibition of a shared metabolic enzyme is a common source of adverse clinical interaction between co-dosed drugs (41–43). At the same time, enzyme inhibition is one of the easiest phenomena to measure in a higher-throughput mode, because of experimental simplicity and because the same assay can be used regardless of the test compound. As a result, most companies conduct enzyme inhibition counterscreening, and the main difference among companies is in the stage in the discovery process at which screening occurs. The only enzymes that have been

subjected to higher-throughput inhibition screening are the cytochrome P450 enzymes, although others are certainly amenable if there were interest. Because the majority of drugs metabolized by P450 are substrates of either CYP 3A4 or CYP 2D6, these two enzymes have been subject to the most screening efforts, but CYP 2C9 has recently become recognized as an important enzyme for inhibition screening (44). It is also possible to determine which P450 enzyme is mainly responsible for the metabolism of a compound (45), but this is more difficult experimentally and is of less interest for counterscreening purposes. Flow diagrams to guide the application of hepatocytes in profiling of discovery compounds as both inhibitors and substrates of P450 have been published (46), and similar flow diagrams might be useful to those investigators contemplating higher-throughput screening.

The commercial availability of single P450 enzymes has made possible rapid inhibition assays in a 96-well format that are readily adaptable to automation (47, 48). Measurement of the enzymatic rate can be accomplished by a fluorimetric method (49, 50), but industrial laboratories are increasingly using LC/MS/MS because of advances in the speed of this technique and because of its ready availability in the industrial environment (51, 52).

Another aspect of P450 screening is whether the observed inhibition is direct or metabolism/mechanism-based, the distinction being that direct inhibition is the reversible, noncovalent binding of the test compound to a site on the enzyme that alters its Michaelis-Menten kinetic parameters, whereas metabolism/mechanism-based inhibition is the binding of a metabolic product of the test compound. To further elaborate this distinction, metabolism-based inhibition results from a metabolite that is a tighter binding reversible inhibitor, whereas mechanism-based inhibition results from covalent binding to the enzyme by a chemically reactive intermediate, which inactivates the enzyme (42). Metabolism/mechanism-based and direct inhibition are readily distinguished experimentally because the former increases with time whereas the latter is constant or may decrease with time.

If necessary, metabolism-based and mechanism-based inhibition may also be distinguished by removal of the metabolites through dilution or dialysis and rechallenge of the enzyme with fresh substrate. The enzyme would be affected reversibly by metabolism-based inhibition but irreversibly by the mechanism-based type. Because of the propensity of the P450 oxidative reaction mechanism to produce reactive intermediates, it is not unusual for a compound to be a weak direct inhibitor but a strong metabolism/mechanism-based inhibitor. Therefore, a complete assessment of the inhibition potential of a test compound should look for both types of inhibition. Commonly this is accomplished in the higher-throughput mode by determining the apparent IC_{50} of the test compound toward a standard substrate with and without a preincubation period of the enzyme with the test compound (53). Generally, compounds are classified as potent ($IC_{50} < 1$ μM), marginal (1 μM $< IC_{50} <$ 10 μM), or weak inhibitors($IC_{50} > 10$ μM). Most companies are reluctant to advance a potent inhibitor. A large decrease (greater than 10-fold) in the apparent IC_{50} after preincubation with the test compound is

evidence of metabolism/mechanism-based inhibition and usually terminates interest in the compound.

Enzyme Induction

Although a variety of drug-metabolizing enzymes are inducible by xenobiotic compounds, the main industrial interest is in the cytochromes P450 (54). There are two main, though disparate, reasons for this interest in P450 induction. The first reason is the obvious one, namely the involvement of P450 induction in some clinical drug interactions (42, 43). The second reason is that P450 induction appears to be related to the production of liver tumors in rodent oncogenicity studies during drug development (55), which provides a compelling reason to screen out this property in the discovery phase. Of course, rodent induction can be determined directly in discovery, but compound requirements and the necessity for days of dosing eliminate this approach for screening purposes, and it can be used only in the final testing phase of a potential clinical candidate just prior to selection for development. Therefore, as we discuss below, much faster methods requiring only milligram amounts of compound are required for practical screening. Unfortunately, rat enzyme induction does not predict human induction. For instance, phenobarbital induces mainly 2B enzymes in rats but 3A enzymes in humans (42). If human induction is also to be assessed preclinically, a separate, human-based in vitro system must be used.

Screening methods for P450 induction must necessarily use in vitro systems, of which two basic types exist: hepatocytes and cell-based gene-reporter constructs. Hepatocytes are used widely and can be applied to both rodent and human induction problems, now that human hepatocytes are commercially available (56, 57). Hepatocytes cannot be considered to be high-throughput or even higher-throughput methods. Even if we ignore the preincubation culture period of several days, the basic experiment requires at least 2 days of incubation of the cells with the test compound. As with Caco-2 culture, the throughput can be increased by parallel incubation of many cultures and test compounds, but the subsequent analysis of enzyme activities and Western blotting also offers a considerable barrier to rapid operation.

The utility of hepatocytes lies not in their speed but in their comparison to alternative methods for assessment of P450 induction. First, the amount of compound required is small in comparison to in vivo rodent studies. For instance, to conduct a 5-day induction study in three rats at doses of 30 and 100 mg/kg, a total of 600 mg of test compound is needed, compared to the $\sim$2 mg needed for hepatocytes. Although this difference may seem unimportant when investigating the induction patterns of established drugs, for discovery purposes the total compound supply may be a paramount consideration, because the combinatorial synthesis may have produced only 10 mg of each of 500 compounds. In general, a compound must pass many in vitro and in vivo tests before it is of sufficient interest to warrant the synthesis of gram quantities, meaning that in vivo rat

induction testing is limited to late-stage discovery compounds and is not suitable for early-stage screening. Second, there is no in vivo alternative to hepatocyte testing for human induction. Thus it is not surprising that discovery research has embraced the use of both rat and human hepatocytes for induction screening (58).

To achieve a higher-throughput induction assay, companies are now turning to promoter-gene-reporter constructs. Such cell-based systems may incorporate a fluorescent or luminescent reporter product (59, 60) to allow an automated spectrophotometric endpoint, and the entire induction experiment may require only a few hours. Each specific enzyme to be investigated would have a separate gene-construct-transfected cell line. We currently understand enough of the induction mechanisms for the 1A (61) and 3A families (62) for these systems to be practical realities. A method to investigate the 4A family has been reported, because CYP 4A is closely related to peroxisome proliferation (63). Although the induction of the 2B family has been poorly understood (64), recent advances in our understanding of the phenobarbital-response mechanism may also allow a construct that is useful for the assessment of the CYP 2B induction potential (65, 66). Based on the results of the induction screen, whether from hepatocytes or a reporter-gene system, the test compound would be categorized as a strong, weak, or non-inducer of P450 and as phenobarbital-like or methylcholanthrene-like.

Pharmacokinetics

In Vitro Screening

Metabolic Stability The kinetic susceptibility of a compound to biotransformation is one determinant of oral bioavailability and systemic clearance. Although rates of in vitro biotransformation can be used in a more rigorous fashion to extrapolate in vivo clearances (see below), it is often adequate and useful merely to set up an appropriate in vitro metabolism model system and rank-order compounds in terms of their relative metabolic stability. The in vitro model system may be microsomes, cDNA-expressed enzymes, liver slices, or hepatocytes, and the availability of these biological components from common animal species and humans makes this a powerful technique for assessing metabolic stability through a long series of compounds as well as across species. When adapted to a 96-well plate format and to LC-MS assay of disappearance of parent compound, it is possible to implement a metabolic stability screen with throughput appropriate to apply in a fast-moving drug discovery program (67). In fact, throughput can be high enough that informatics software may be needed to capture, manipulate, and distribute the data. A helpful variation is to index the rate of metabolism of the test compounds to that of a benchmark compound whose in vivo persistence is to be matched or exceeded. The system can be adjusted to provide an intermediate rate of metabolism of the benchmark compound (e.g. 30% loss of parent). Compounds that are substantially more or less stable than the benchmark are discerned readily.

Two caveats are important when interpreting data of this type. First, it is easily possible to have no correlation between the in vitro stability data and the in vivo clearance of the drug. Thus it is important to carry out in vivo checking of the in vitro stability order before incorporating this method into the screening process. Second, the measured rate of disappearance of parent drug should ideally be the initial rate but likely is not because of substrate and cofactor depletion, accumulation of inhibitory products, and decay of enzyme activity. Therefore, the observed relative rates of decline of two compounds may be quite different from their true relative intrinsic clearances. Consequently, relative stability data through a compound series should not be interpreted too quantitatively. Nonetheless, microsomal stability screening is experimentally simple, and the data lend themselves to intuitive interpretation, making this method widely utilized.

In Vitro/In Vivo Scaling A great deal of effort has been expended on the theory and practice of quantitative prediction of human hepatic clearance of drugs from rates of metabolic conversion observed in various in vitro systems such as microsomes, hepatocytes, and liver slices (15, 68–70). From the point of view of discovery screening, we can make two comments. First, scientific understanding of the in vitro and in vivo components of the predictions is presently insufficient for them to be more accurate than within a factor of two of the true value, on average. However, this situation may change as our understanding improves, and, indeed, hepatocytes may be used to rank compounds by their predicted hepatic extraction ratios (71). The problem of quantitative uncertainty is alleviated by lowering the expectation of the results and merely classifying a compound as low, medium, or high extraction. Second, although these methods may be useful for selecting among a few final clinical candidates at the end of a discovery program (72), they are not fast enough to serve as a practical means of screening compounds in the early phase. Thus these methods are not more useful in discovery of a suitable clinical candidate than is simple metabolic stability ranking, and they are considerably slower.

In Vivo Screening

Multicompound Dosing This term refers to simultaneous administration of several compounds to a single animal and is also called cassette or *N*-in-one dosing. Multicompound dosing has become widely applied in DMPK screening because it offers enhanced rates of examination of compounds in vivo, avoiding the problem of in vitro/in vivo correlation (73–76). The enabling technology is LC/MS/MS, which allows the investigator to cleanly assay a particular analyte in the presence of many structurally similar compounds (77, 78). The rate acceleration comes from two efficiencies: (*a*) far fewer animals must be prepared and dosed, and (*b*) far fewer samples are generated for assay. The degree of acceleration depends on how many compounds are co-dosed (*N*), but there are practical limitations on *N* so that most applications use *N* of 10 or less. Both rats and dogs

have been used. Because medicinal chemists have been very receptive to the introduction of multicompound dosing into discovery DMPK screening, it is worthwhile to discuss several caveats that accompany its use.

1. Care must be taken in selecting the mixture of compounds to be dosed because of the homogeneity of the typical compound set and the possible presence of metabolites in the plasma samples. Isomers may be co-dosed as long as they exhibit distinct daughter ions because the MS/MS technique is normally used. However, it is easily possible for a metabolite to have a structure identical to one of the original test compounds. For instance, if the test mixture contains both a tertiary methyl amine and the corresponding secondary amine, the *N*-desmethyl metabolite of the first compound is identical to the second compound. Similarly, if the test mixture contains both a phenyl compound and the corresponding phenol, the phenolic metabolite of the former will confound the assay of the latter.
2. Drug interactions can cause a distortion of the pharmacokinetics of one or more components of the co-dosed test mixture. Although this phenomenon is generally recognized, the following misconceptions exist: (*a*) One may guard against competitive inhibition of a shared metabolic enzyme (the main reason for drug interactions) by keeping doses small; (*b*) even if the absolute values are wrong, the correct rank order will be observed; (*c*) one can detect drug interactions by always including a control compound; and (*d*) drug interactions can lead only to false positives, which will be discovered later in the process. Consideration of pharmacokinetics shows that none of these assumptions is true.
3. The total pharmacological and toxicological load delivered to the animal must be considered. Most investigators take the position that it is better to keep the total amount of all compounds low so that pharmacological and toxicological effects do not become large enough to physiologically compromise the animal's ability to clear the drugs. The consequence is a limit to the total number of compounds that can be co-dosed, because the sensitivity of the assay will become limiting when the dose of each compound is very low.

Rapid Pharmacokinetic Screening To avoid the problems of in vitro/in vivo scaling and drug interactions, several laboratories have developed methods to accelerate the acquisition of focused data from singly dosed animals. The approach in each case is to reduce the overall time for assessment of each drug candidate by minimizing both the in vivo and bioanalytical phases of the experiments. To minimize the in vivo phase, the collection period is reduced to 6–8 h, which is much shorter than that in conventional pharmacokinetic studies. The bioanalytical phase is minimized by the reduced number of samples accruing from the shorter collection period, by pooling at time points or across all time points, and by the use of an abbreviated standard curve. All higher-throughput in vivo pharmacokinetics methods depend on an accelerated assay technology to achieve meaningful rate increases (79). Interestingly, one group has reversed the trend to

use LC/MS as the assay tool and has revived the use of bioassay of plasma concentrations for screening purposes because of the ease of automation of enzyme-based methodologies (80). Shortened, ''one-in-one'' in vivo pharmacokinetics procedures can result in a throughput that is still adequate to support many drug discovery programs, as long as the in vivo pharmacokinetic screening is somewhat downstream in the screening sequence so that no more than about 20–30 compounds are to be tested each week.

The first such procedure to be reported used pooling of plasma from several animals at each time point up to 8 h, LC/MS for sample assay, and normal pharmacokinetic analysis of the concentration-time data (81). This process was followed quickly by a variation in which samples were collected for 24 h and then pooled across all time points, yielding a single sample per animal per compound dosed (82). LC/MS/MS compound assay provided a single concentration that was multiplied by the collection period to yield an approximation of the AUC_{0-t} (t = time of last sample) for the test compound. A final variation, theoretically capable of the highest speed, was introduced later, in which the in vivo phase is reduced to 6 h, yielding a truncated area under the curve (AUC) value (83). Because of the truncated AUC value, a 6-h time point is also assayed to provide an indication of whether most of the area has been captured. The rapid AUC method is particularly useful for screening for plasma levels after oral dosing of a series of drug candidates because oral bioavailability is frequently an issue in discovery support. A simple method has been described for deriving the true AUC from these pooling methods and for approximating the fraction of the AUC_{∞} captured within the collection period (84). Another advantage of using LC/MS/MS for sample assay is that it is easy to concurrently examine the plasma for simple metabolites such as hydroxylated derivatives, which are monitored at M + 16 (i.e. 16 mass units greater than the parent molecular weight) (85). A related but experimentally more difficult method is the continuous withdrawal technique, in which blood is drawn from the animal slowly but continuously for the entire collection period. The concentration of this sample, multiplied by the collection period, is the true AUC_{0-t} for the test compound (86).

Metabolic Transformation

Although there has been no pressure to add metabolite structure elucidation to the screening process, this does not mean that metabolite identification is without value in early-phase discovery. On the contrary, it can often be a critical component of the discovery of DMPK-competent drug candidates because every discovery program has periods in which high-speed screening is not producing compounds with adequate pharmacokinetics, and synthesis must be directed toward solving the pharmacokinetics problem. Usually the problem is poor absorption or a metabolic hot spot; in the latter case, rapid identification of the major metabolites is required, normally without the benefit of radiolabeled parent compound (87). The methods described in the next two sections are intended for this situation.

Methods for Metabolite Generation The simplest system to use involves liver microsomes, which are easily obtainable from laboratory species and from humans. Because many metabolites are the result of P450-catalyzed oxidation, microsomes often give good results in generating the correct metabolites. However, in some cases liver microsomes fail to metabolize the test compound or they give a different pattern of metabolites than would be observed in vivo. Consequently it is a good idea to use hepatocytes (88, 89) or liver slices (90, 91) in conjunction with or in place of microsomes. Hepatocytes and liver slices have the additional advantage of being more integrated than microsomes, meaning that the full complement of metabolic enzymes and cofactors are present. Thus the metabolism is not biased toward oxidation, as occurs with microsomes. Incubation of the test compound with the microsomes, hepatocytes, or liver slices provides a relatively clean matrix to bring to the next step, metabolite isolation and identification. The highest level of integration is in vivo, and the use of whole animals has the advantage that all physiological processes that may affect metabolic pathways are present, including membrane transporters, nonhepatic clearance, blood flows, protein binding, and tissue distribution. Bile, urine, and plasma can be collected from animals, but these matrices are complex and make subsequent instrumental analysis of metabolites difficult. Modern methods such as LC/MS and LC/NMR (nuclear magnetic resonance) can accomplish metabolite analysis even in these fluids.

New Rapid Technology Several ingenious new techniques have been described recently that accelerate or facilitate metabolite identification. These techniques involve innovative coupling of existing technologies, one of which is always a variant of mass spectrometry. For instance, a method has been devised in which liver microsomal incubations are conducted inside an ultrafiltration apparatus that is infused continuously with buffer (92). The effusate is free of protein, avoiding the slow extraction step, and can be introduced directly into an electrospray mass spectrometer for real-time analysis of metabolites. The authors suggested that very high rates of sample throughput are theoretically possible, up to 60 incubations per hour, limited by the number of ultrafiltration chambers that can be operated in parallel. Of course, until human operators are able to interpret the results of 60 mass spectrometer runs in an hour, such rates cannot be fully realized in the overall throughput.

In a related development, a means to find and identify active metabolites has been described in which chromatographic peaks from a biological sample are exposed to a receptor in an ultrafiltration device (93). After filtering the unbound (i.e. inactive) materials through the ultrafiltration membrane, the bound materials (i.e. parent and active metabolites) are released and introduced into the mass spectrometer for structural characterization. Although the method is easily applied in a 96-well format, the identification of active metabolites has not been a screening priority. Also, recently introduced innovations in instrument design, such as ion-trap and quadrupole-time-of-flight mass spectrometers, have facilitated

metabolite identification, allowing it to be accomplished at speeds suitable for rapid discovery work, even from complex ex vivo samples (94).

As anyone conducting LC/MS can attest, it is difficult to determine, in the absence of radiolabel, that a particular chromatographic peak is a metabolite and not a background component. To determine the chemical structure is harder yet. Toward that end, industrial mass spectrometrists have begun introducing artificial intelligence into the process, taking advantage of the ability of modern mass spectrometric systems to monitor numerous ions and fragmentation processes during a chromatographic run. The computer is programmed to watch for various relationships among masses of the parent ions of the peaks and the resulting fragment ions. For a human, this kind of numerology is tedious and prone to error, whereas computers excel at such tasks. For example, automated simultaneous monitoring of the incremental differences from the molecular weight of the test compound due to common biotransformations such as hydroxylation (M + 16), oxidation of a methyl group to a carboxyl (M + 30), and demethylation (M − 14) considerably reduced the time required by the human operator to deconvolute the metabolic profile of in vitro incubations (95). A more sophisticated elaboration of this idea was described (96) in which the mathematical technique of correlation analysis was brought to bear, allowing the metabolites from mixtures of drugs to be recognized and characterized.

Just beginning to be developed widely in industry is the technique of LC/NMR, which generates a completely different kind of information to apply to the problem of metabolite recognition and identification (97). NMR has not previously been considered as useful as mass spectrometry for metabolite identification because of the need to present purified, concentrated samples to the instrument due to the relative insensitivity of the NMR technique. However, the intrinsic power of NMR to elucidate molecular structure has kept interest in the technique alive and has spurred development of new microflow cell designs that have improved dramatically the sensitivity. An advantage of NMR is that for molecules containing fluorine, phosphorus, or deuterium atoms, or functional groups uncommon in biology, the NMR signal is as definitive of drug-relatedness and as easy to find as a radiolabel. There is no reason to discuss whether LC/MS or LC/NMR is the better technique, because LC/MS/NMR has already been demonstrated (98), and one may expect its widespread implementation in the coming years.

Virtual Screening

A trend in higher-throughput discovery DMPK screening is the increasing applicability of computer models. The earliest such approach was a completely empirical and statistical method known as the Rule of Five, or Lipinski's Rules (99). The Rule of Five is summarized as follows: A molecule is more likely to be poorly absorbed when the molecular weight is greater than 500, the sum of OH and/or NH groups is more than 5, the sum of N and O atoms is more than 10, and the log P is greater than 5. Because these criteria are easily calculable from

the molecular structure without experimental measurements, the Rule of Five is easy to incorporate into the compound registration process to provide an alert that the compound may exhibit poor absorption, and it may even be used prospectively when planning a combinatorial chemistry campaign. More theoretically derived estimates of absorption can be made with the use of physiologically based absorption models (100, 101), but these models presently require experimental input such as Caco-2 permeability and rate of dissolution.

Molecular modeling is now starting to be used to predict substrate recognition by P450 enzymes. Homology models are available for most liver microsomal forms, including 2B (102), 2C9 and 2C19 (103), 2D6 (104), and 3A4 (105). This approach has been most successful with CYP 2D6 (106, 107), but so far it has been applied only to the drug design process and not to screening. Pattern recognition has been applied to the identification of substrates for PGP (108). Two structural motifs were proposed, and simple small-molecule modeling can identify whether those structural features are present in a real or hypothesized molecule, allowing rapid prospective screening against PGP substrates and inhibitors. Two industrial groups have introduced computational methods for scoring molecules (109, 110). In this automated neural network process, numerous molecular descriptors are compared to the values for molecules in databases defined as drug-like (e.g. the World Drug Index) and non-drug-like (e.g. the Available Chemicals Directory) to create an individual score for each molecule that allows ranking of molecules in terms of drug-like properties. As with the Rule of Five, the utility of this approach is that no experimental measurements are needed, so that scoring may be done during the planning process for combinatorial synthesis to avoid wasting effort in making molecules that lack favorable DMPK properties. However, the scoring approach has not been applied widely, and it is probably too early to assess its ultimate value.

CONCLUSION

Although the fastest DMPK screening methods do not come within two orders of magnitude of the rates routinely achieved by truly high-throughput discovery screening, they are nonetheless absolutely essential in the screening paradigm. The technology for higher-throughput screening in most cases is already mature, and additional large rate enhancements will probably be achieved only at the cost of increasing the scientific gap between the measured quantity and its human in vivo correlate. The greatest need in DMPK screening for discovery support is a much better understanding of the integrated operation of in vivo absorption, first-pass metabolism, organ uptake and efflux mediated by transporters, plasma protein binding, competition among metabolic enzymes, and cellular determinants of intrinsic clearance. In the short term, we would be able to use current in vitro systems more reliably to anticipate the in vivo disposition of drug candidates. Discovery DMPK screening would then be accelerated as we would be able to

reduce our reliance on slower in vivo work. Ultimately it might be possible to create physiologically based DMPK models that would use animal in vitro/in vivo data for training and validation and then be able to support rapid screening of drug candidates with only human in vitro data for input.

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