Review Article

Addressing the Challenges of Low Clearance in Drug Research

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Abstract. As a result of high-throughput ADME screening, early metabolite identification, and exploration of novel chemical entities, low-intrinsic-clearance compounds continue to increase in drug discovery portfolios. Currently available *in vitro* tools have limited resolution below a certain intrinsic clearance value, which can lead to overestimation of clearance and dose and underestimation of half-life. Significant advances have been made in recent years and novel approaches have been developed to address the challenges of low clearance in drug discovery, such as the hepatocyte relay method, use of qNMR-based standards of biosynthesized drug metabolites to permit monitoring metabolite formation, coculture hepatocyte systems, and the time depending modeling approach. Future development in the field will enable faster, more precise, and lower cost profiling of the properties of low-clearance compounds for intrinsic clearance, metabolite identification, and reaction phenotyping.

KEY WORDS: hepatocyte coculture; hepatocyte relay; IVIVC; low clearance; metabolite formation; qNMR.

INTRODUCTION

Achieving low clearance is frequently the goal of drug discovery projects in order to reduce dose, enhance exposure, and prolong half-life. However, low clearance (defined in this paper as no significant turnover of parent drug in typical *in vitro* liver microsomal or hepatocyte assays) presents a great challenge to scientists in the field of drug metabolism and pharmacokinetics (DMPK). Clinical half-lives of low-clearance compounds are frequently underpredicted due to overprediction of clearance based on the lower limit of standard human liver microsomal or hepatocyte assays or single species/allometric scaling from preclinical species. In a few cases, new drug candidates progressed to clinical research assuming the human half-life would be a few days, but turned out to be weeks or months. Many low-clearance compounds ultimately failed in the clinic due to extremely long half-lives that were incompatible with the intended usage (e.g., need short duration of action for on demand drugs or short duration on targets to minimize side effect), complex dosing regimen, safety concerns of accumulation or difficult to “wash out” when needed. The projected doses tend to be too high for low-clearance compounds because of over-prediction of clearance, which can hinder the advancement of promising drug candidates.

Additionally, gaining an understanding of potential/predicted clearance mechanisms of a low-clearance compound is challenging because of low turnover of the parent molecule. *In vitro* methods using human-derived reagents are

routinely used to identify the enzymes and transporters that could be involved in the clearance of a new drug candidate, and these data are used to help design the clinical study strategy (e.g., design of drug-drug interaction studies, development of study subject exclusion criteria, determination of the need for pharmacogenetics studies and subject genotyping, among others). If the CL_{int} for a compound is so slow as to be unreliably measured, then the various experiments that leverage measurements of parent drug consumption to predict and assign clearance mechanisms are compromised, and in some examples, impossible. Additionally, *in vitro* methods using human-derived reagents are used in early drug research to identify potential human metabolites. It is more technically challenging to identify metabolites of slowly metabolized compounds; amounts of metabolites generated will be lower which can preclude gathering enough spectral data to permit structural identification. Finally, when the human radiolabel metabolism and excretion study is eventually carried out for a low-clearance drug candidate later in the development process, the execution of this study can be more technically challenging. If a compound has a long half-life in human, the permitted dose of radioactivity in an excretion study may need to be much lower due to projected dosimetry of tissues to ionizing radiation. This can make it technically more challenging to measure excretion or require the use of high-technology accelerator mass spectrometry to measure carbon-14 instead of the much simpler liquid scintillation counting. Quantitative radiometric profiling of metabolites in biological fluids from these studies can be difficult because the drug-related material becomes too dilute. Also, it has been shown that recovery of radioactivity in an excretion study is lower when the half-life of the compound is long (1).

Low-clearance compounds continue to increase through the years because of the effectiveness of high-throughput

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ADME (absorption, distribution, metabolism, and excretion) screening (2,3) and early metabolite identification (4), which enable rapid structure-activity relationship (SAR) to enhance metabolic stability. Thus, medicinal chemists have become more proficient in designing metabolically stable compounds (5,6). Plus, new targets, structure classes, and dosing paradigms are being explored, such as liver targeting (7–9) and once weekly dosing (10). All these strategies need to drive the clearance very low. A survey of our internal projects showed that about 30% of the compounds were low clearance in the drug discovery portfolios, and, for certain projects, the entire chemical series was low clearance driven by the chemotype. There is a major gap in our tools and techniques to address low-clearance DMPK science in drug discovery. In this article, new approaches that are being employed to address this challenge are discussed.

LOW-CLEARANCE METHODOLOGIES AND THEIR APPLICATIONS

In a typical human liver microsomal liability assay, test compounds are incubated with cofactors and conditions to support drug-metabolizing enzyme activity (routinely NADPH for cytochrome P450-mediated metabolism). Microsomal protein concentrations are typically kept below 2 mg/mL to prevent excessive nonspecific binding in the assay. Incubation times are usually less than an hour because enzyme activity is slowly declining and if measurements were carried out for too long, there would be underestimation of intrinsic clearance. In suspended hepatocyte assays, incubations can only be done for about 4 h (maybe as long as 6 h) because activity decreases due to decreases in cell viability. When a new compound being studied does not show measurable consumption in typical liver microsomal or hepatocyte metabolism liability assays, the simple suggestion of adding more enzyme (*i.e.*, more microsomes or more cells) or incubating for longer is not an acceptable solution. Low limits of CL_{int} (intrinsic clearance) measurements in liver microsomes is about 12 $\mu\text{L}/\text{min}/\text{mg}$ protein [assuming a limit of 120 min for a measureable *in vitro* $t_{1/2}$ (half-life)] which scales to 10 mL/min/kg body weight in human when using 0.5 mg/mL microsomal protein and 1-h incubation time. For a 4-h human hepatocyte suspension incubation containing 0.5 million cells/mL, a lower limit of CL_{int} that can be measured is about 2.5 $\mu\text{L}/\text{min}/\text{million cells}$, which scales to 6.3 mL/min/kg body weight. This is about one third of the hepatic blood flow. For a volume of distribution of 0.2, 0.7, and 2.5 L/Kg, the half-life is about 4, 13, and 47 h assuming plasma protein binding of 0.1 and blood to plasma ratio of 1. This has driven innovation in this area, and a number of approaches have been developed to address low-clearance challenges in drug discovery. Some of these are highlighted here.

Modeling Approach

One approach to be able to measure turnover of low-clearance compound is to increase the amount of enzyme in the incubation and prolong incubation time. However, nonlinear biphasic kinetics might occur due to loss of enzyme activity. Jones *et al.* reported biphasic kinetic profiles for

triazolam, diazepam, and clonazepam in rat liver microsomes (11). The parent depletion data was fitted to a biexponential model to account for loss of enzyme activity. The kinetic parameters obtained from the biexponential model were shown to be equivalent to monoexponential fit using low microsomal protein concentration and short incubation time points.

Hepatocyte Relay Method

The hepatocyte relay method has been recently developed to address the challenges of low clearance in drug discovery (Fig. 1) (12,13). The method is conceptually straightforward. In order to achieve turnover of low-clearance compounds, the incubations would need to be carried out with hepatocytes for a long time. However, as stated above, hepatocyte suspension incubations are kinetically competent for about 4 h after which significant loss of enzyme activity and cell viability occurs. The idea of the hepatocyte relay assay was to transfer the supernatant of the test compound incubations after the 4-h incubation to freshly thawed hepatocytes and incubate for another 4 h. If it is repeated for five times, a cumulative incubation time of 20 h can be achieved with hepatocyte suspensions. The low limit of the hepatocyte relay assay depends on the number of relays conducted. Increasing hepatocyte density in conjunction with the relay assay can further drive the limit lower for intrinsic clearance measurement. Increase hepatocyte density can also be applied to the conventional approach. For example, increasing cell density by twofold decreases the CL_{int} limit by twofold assuming binding to hepatocytes has minimal effect. The hepatocyte relay methodology is an extension of the standard hepatocyte metabolic stability assay with the same assay format and using the same lot of hepatocytes. Therefore, it is easy for project teams to adapt the assay to support their drug discovery programs, and it offers an advantage that it can be used with cryopreserved pooled hepatocytes to ensure consistent drug-metabolizing enzyme activities over the course of the duration of individual drug discovery projects (months to years).

The hepatocyte relay assay has been shown to have good correlation with *in vivo* intrinsic clearance both in human and preclinical species (Fig. 2). Multiple donors of hepatocytes can be used or single donor if one wishes. The supernatant plates can be frozen down for the next relay making it flexible to fit the work schedule of scientists. The assay has a straightforward format and can be utilized by typical drug metabolism laboratories with a minimal cost. The hepatocyte relay method is being used for intrinsic clearance determination (12,13) and metabolite identification (14) for humans and laboratory animal species. The method has been developed to conduct reaction phenotyping experiments with

Fig. 1. Hepatocyte relay method (12)

clearance pathway, since there was no turnover of the compound in the standard human liver microsome or hepatocyte suspension assays. The major clearance pathway of the compound was assumed to be mainly hepatic uptake, significant biliary clearance, and moderate renal clearance based on data from preclinical species and sandwich-cultured human hepatocytes. However, in the human ^{14}C ADME study, about 50% of the dose was metabolized. The underestimation of hepatic metabolic clearance of the compound was due to a lack of low-clearance tools at the time. The compound was extensively metabolized, albeit very slowly. The hepatocyte relay assay predicted the human unbound intrinsic clearance within twofold of the clinical value.

Case 4—Reaction Phenotyping of Low-Clearance Compounds A drug candidate in development had low clearance, and reaction phenotyping was very challenging using standard approaches. Metabolites and radiolabeled material were not available at the time. Using the hepatocyte relay assay, intrinsic clearance value of the compound was determined. CYP-specific chemical inhibitors were added to the hepatocyte relay assay to determine the fraction metabolized (f_m) of the compound by various enzymes. The results were used to guide clinical protocol designs, and the f_m values determined were later confirmed by human ^{14}C ADME studies.

Metabolite Formation Method

When measuring the consumption of parent drug in an *in vitro* system, there needs to be at least 10–15% turnover to make a reliable measurement, since most bioanalytical methods will yield assay accuracy and precision values in that range. If data are tight or enough replicate assays are conducted, then statistical analyses can show whether a decline in substrate concentration is significant. This can be overcome if instead of trying to measure parent decline, one elects to measure the formation of metabolites. (For example, while it is difficult to measure the difference between 1.0 μM at time zero and 0.98 μM substrate at the end of an incubation, it is easy to reliably measure 20 nM of metabolite formed in an incubation as compared to a time-zero control where the metabolite will be less than the lower limit of detection.) Thus, measurement of metabolite(s) formation rate offers a viable approach to measuring the intrinsic clearance of low-clearance compounds.

One serious limitation to using metabolite formation measurements for low-clearance compounds is the availability of authentic standards of metabolites from which to construct calibration curves for reliable quantitation. In the absence of an authentic standard to calibrate instrument response, quantitation cannot be done. For mass spectrometry, the response factor is highly dependent on the structure and physical properties of the analyte, and the response factor for

the parent drug cannot be assumed to be the same for the metabolites. This can be less of a concern for UV/VIS as the detection technique; nevertheless, if the metabolism results in a change in the chemical structure of the chromophore, then UV/VIS response also cannot be used without calibration using an authentic standard of the metabolite. If a radiolabelled analog of the test compound is available (typically ^{14}C or ^3H) and the isotope is not present in a metabolically labile position, then quantitation of metabolite formation can be done using chromatographic separation of radioactive parent from radioactive metabolites and measurement of the metabolite peaks by liquid scintillation counting. However, it is not routine to have radiolabelled material available for compounds early in drug discovery due to cost and resources needed for radiosynthesis. In the absence of a radiolabelled material, in order to follow metabolite formation to measure CL_{int} for a low- CL_{int} compound, authentic standards of metabolites must be available. But like radiolabelled material, chemical synthesis of authentic standards of drug metabolites can be challenging, costly, and long. It is not typical to possess authentic standards of metabolites in drug discovery unless the metabolite happens to be a synthetic precursor to the parent drug (e.g., carboxylic acid metabolite for an ester parent compound; 2° amine metabolite of a 3° amine parent, *etc.*).

To meet this challenge, we have devised methods to obtain stock solutions of known concentrations of metabolites utilizing biosynthesis and quantitative NMR (15). Biosynthetic incubations of 10–50 mL containing 10–100 μM parent compound are incubated with an appropriate source of drug-metabolizing enzyme(s), such as liver microsomes, S9 fraction, or recombinant enzymes and cofactors (NADPH, UDPGA, *etc.*), extracted, and metabolites are isolated by HPLC. The individual fractions containing metabolites are evaporated, reconstituted in deuterated solvent, and the solution strength is determined using NMR spectroscopy. This procedure has been enabled by the advances in cryomicroprobe technology and magnet strength for NMR such that solutions of less than 50- μL total volume and concentrations of 50 μM can be reliably analyzed (16,17). The resulting solution of metabolite in deuterated solvent can then be used as a mother stock from which dilutions can be made to construct a standard curve for HPLC-MS analysis of rate of metabolite formation in metabolic incubations.

For an example of this approach, a discovery project had a lead candidate that showed a low CL_{int} value in liver microsomes; yet, it was important to gain an accurate prediction of clearance in humans as well as to determine which CYP enzymes were involved in metabolism. Small signals for five metabolites were observed in metabolite identification experiments *via* HPLC-MS analysis of human liver microsomal extracts. Stock solutions of these metabolites were prepared using the biosynthesis and qNMR approach described above, and these stock solutions were used to develop a quantitative HPLC-MS/MS method whereby the enzyme kinetics for formation of each metabolite in pooled human liver microsomes were measured. The V_{max} and K_M values for each reaction were used to calculate CL_{int} values which were summed to yield the total CL_{int} for the compound. Furthermore, these same standards were used to conduct reaction phenotype experiments that yielded an

assignment that CYP3A4, CYP1A2, and CYP2C19 were involved in metabolism at an approximate ratio of 0.85:0.10:0.05, respectively. While such an approach is not suitable for hundreds of compounds, it is very applicable for dealing with low-clearance drug candidates later in the drug discovery process (proximate to nomination into preclinical drug development) or for specific individual lead molecules earlier in drug discovery.

Hepatocyte Culture Systems

It is known that drug-metabolizing enzyme activities in hepatocyte suspensions or standard culture methods are not long-lived. Thus, efforts have been made toward specializing culture systems to sustain the endogenous drug-metabolizing capabilities of intact hepatocytes. The innovations have included leveraging engineered apparatus to better mimic the multicellular structure and flow in intact liver (e.g., LiverChip™, HμREL®) and/or cell coculture structures that better mimic the cell population of intact liver (HepatoPac®).

LiverChip™ refers to a microfluidically perfused 3D hepatocyte culture system and has shown some promise in making low clearance measurements (18). Culture medium recirculates through the system to better mimic the natural environment of hepatocytes in the liver. The system is “dosed,” and serial samples of medium can be taken and analyzed for drug concentrations. Early studies in our laboratory showed the feasibility of measuring the CL_{int} of low-clearance drugs including theophylline, tolbutamide, and disopyramide using a substrate depletion approach over a 72-h continuous incubation (19). [Note that instead of determining the *in vitro* $t_{1/2}$ value and calculating CL_{int} from that which is routinely done for short-term microsomal and hepatocyte suspension liability experiments, in the LiverChip™ experiment, the data were treated like an *in vivo* intravenous pharmacokinetic experiment in that the C (concentration) vs t (time) data were measured, area under the curve (AUC) was calculated, and clearance was determined from that. AUC can be a preferred method of calculating CL_{int} for these systems to take into account declines in substrate concentration that do not follow a simple monoexponential function.] Clearance prediction was actually more accurate for the low- CL_{int} drugs, and the high- CL_{int} drugs tested (e.g., midazolam, diclofenac, verapamil) were underpredicted (Table I). Recently, a report by Vivares *et al.* (20) described the characterization drug-metabolizing enzymes in LiverChip™ and its use in CL_{int} measurements, as compared to a static 2D 24-h hepatocyte culture incubation. Drug disappearance was

maintained over 48 h, and CL_{int} was determined from *in vitro* $t_{1/2}$. Five drugs were tested, but most were high CL_{int} . Tolbutamide CL_{int} was measured in cells from three donors in the LiverChip™, and the overall value was 0.056 mL/h/million cells, which scales to about 2 mL/min/kg body weight. Work by Novik *et al.* (21), using the HμREL system, looked at the consumption of drugs, including low- CL_{int} drugs indomethacin, antipyrine, and carbamazepine. While only the data are shown for indomethacin, the authors show a plot of CL scaled from the *in vitro* data to *in vivo* CL across the drugs tested and demonstrate a good *in vitro*–*in vivo* correlation (IVIVC) that was improved in the coculture-flow system relative to static and monoculture systems.

HepatoPac® refers the commercially available hepatocyte-fibroblast coculture system including a novel micropatterned lattice array wherein the cells form hepatocyte “islands.” When cultured in this way, it was shown that drug-metabolizing enzyme activities were sustained for several days without a change in medium, thereby yielding the potential to carry out long drug metabolism incubations potentially ideal for measuring reliable consumption rates of low- CL_{int} compounds. This was demonstrated by Chan *et al.* (22), for several drugs, including low- CL_{int} drugs theophylline, diazepam, tolbutamide, meloxicam, among others using hepatocytes from three individual donors. As with the Liverchip method, it was low- CL_{int} drugs that were best predicted, with high- CL_{int} drugs underpredicted.

Thus, hepatocyte culture systems appear to offer an approach to measure CL_{int} values for slowly metabolized compounds. Modifications over standard 2D culture systems, such as microfluidic flow, stationary support, and coculture, appear necessary for maintaining drug-metabolizing enzyme activities over periods of days. Adaptation of these approaches to more routine use in a drug discovery setting is yet to be realized but should be achievable. Routine use would require somehow ensuring a stable supply of hepatocytes over the long term wherein the panel of drug-metabolizing enzyme activities are not different from year to year.

CONCLUSIONS

Significant advances have been made in the field of low clearance in past few years. A number of novel approaches have been developed to address the challenges of low clearance in drug discovery, including the hepatocyte relay method, metabolite monitoring using qNMR to quantify metabolite standard concentration, coculture hepatocyte

Table I. IVIVE of Intrinsic Clearance for LiverChip™ System (19)

Compounds	Ionization class	CL_{int}	<i>In vivo</i> $CL_{int,u}$ (mL/min/kg)	LiverChip™ $CL_{int,u}$ (mL/min/kg)
Tolbutamide	Acid	Low	4.9	0.95
Meloxicam	Acid	Moderate	40	0.61
Diclofenac	Acid	High	700	17
Disopyramide	Base	Low	5.9	2.6
Metoprolol	Base	Moderate	15	1.5
Verapamil	Base	High	190	12
Theophylline	Neutral	Low	2.6	0.76
Methylprednisolone	Neutral	Moderate	27	5.1
Midazolam	Neutral	High	310	23

systems, and the modeling approach. Refinement of these tools and innovation in the field will continue to meet the needs of the increasingly difficult chemical entities of new therapeutic targets.

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