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In Vitro Approaches to Human Drug Metabolism

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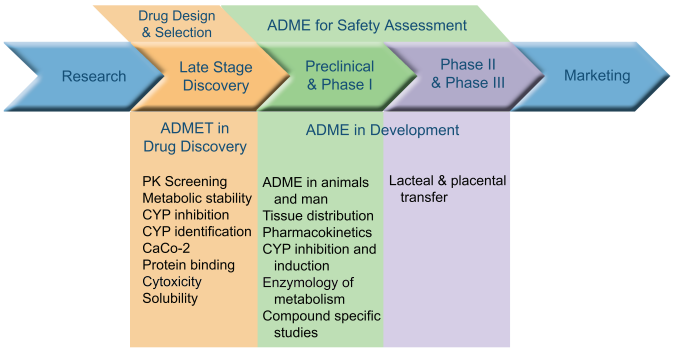
Introduction

There are a number of advantages of using *in vitro* methods in drug metabolism studies. Some of the major approaches involve cellular systems, microsomes, and recombinant products, all of which will be discussed. An important point to make here at the outset is that what we are trying to do is obtain information that will be predictive for the *in vivo* human situation. This cannot be approached directly, unless one is willing to wait until the clinical portion of the development process. A trend in recent years is to move drug metabolism to points earlier in the discovery/development process. For instance, resistance to rapid metabolism can be a criterion in screening, presumably predicting low bioavailability. During the past 10-15 years, *in vitro* methods have been developed most extensively with the cytochrome P450 (P450, or CYP) enzymes. In this period there has also been a shift of emphasis away from *in vivo* experiments with animal models to *in* vitro studies with human enzymes (1). Nevertheless, we should always remember that the human *in vivo* situation is the endpoint. All *in vitro* systems are imperfect as models-but some are useful! Having said this, the point can also be made that good *in vitro* studies can be very useful in making decisions regarding which *in vivo* studies will be most useful.



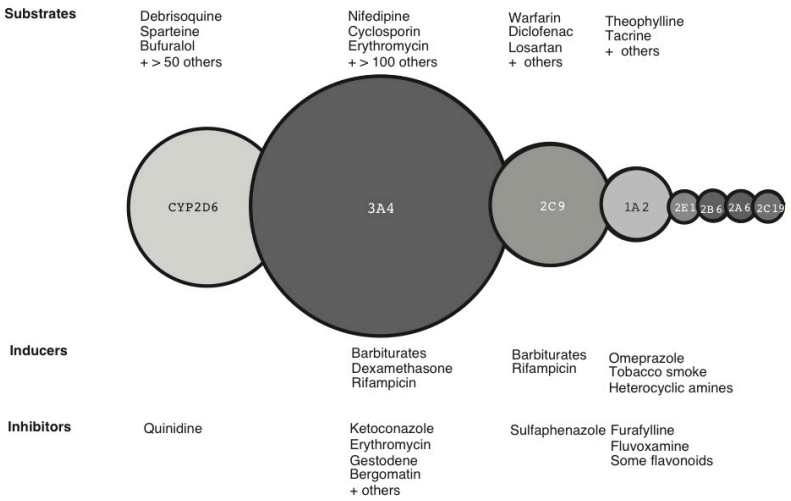
Cellular Systems

Two major approaches are discussed here, the uses of human liver slices and human hepatocytes. Both of these systems have some advantages over others. All of the enzymes are present, not only the full complement of the P450s but also other microsomal and cytosolic enzymes that contribute to metabolism. The setting is better than simpler systems in that the cellular integrity is maintained. For instance, cofactor pools generally reflect those seen in different subcellular locations in intact tissues. Another advantage is that these systems permit the use of two useful endpoints-enzyme induction and hepatocellular toxicity. However, these cellular systems also have some inherent disadvantages that limit their use. Cell cultures must be primary and cannot be passaged. Cells and slices cannot be easily frozen and subsequently thawed for use in assays, although the technology is being pursued. These cellular systems cannot be prepared from liver tissue that has previously been frozen. Also, these preparations are not easily shipped so assays must generally be done on site. Therefore, despite their advantages, cellular systems are relatively expensive. Also, because the number of liver samples used for these preparations is usually limited, a restricted sampling of the variability in biotransformation is obtained.

An alternative approach to using a complete mixture of enzymes involves working with simple homogenates of liver or other tissues. All of the enzymes are present but their cofactors are diluted.

Microsomal Preparations

Microsomes are readily prepared from frozen liver tissue and a number of different experiments are possible. Patterns of biotransformation of a particular P450 substrate (usually from several individuals) can be quickly obtained and compared to profiles from experimental animals. This approach can be used to help select the most appropriate animal model on the basis of metabolism. If sufficient amounts of human microsomes are available and a particular product (initially identified only as an HPLC peak) is formed only in humans, microsomal incubations can be used to obtain enough material for chemical characterization.



The relative impact of various human liver CYP isoforms in human drug metabolism.

Several approaches can be used to identify which P450s make major contributions to a new reaction (formation of a particular product of a new drug), as outlined elsewhere (2). (i) In a correlation approach, a set of microsomal samples obtained from different individuals (usually >10) is compared with regard to rates of the new reaction and reactions known to be catalyzed by individual P450s (or immunochemically-determined levels of the P450s). If a

particular P450 is involved, the new reaction will correlate with the marker. (ii) Chemical inhibitors have reasonably good diagnostic value for individual P450s. Lists have been published elsewhere (1,3). (iii) Antibodies known to inhibit individual P450s can be added to the microsomes and their effects on a particular reaction can be determined. (iv) Microsomes can be used as starting material for the purification of a previously unknown P450 if other attempts at identification fail. However, many P450s have already been identified and current knowledge argues that this approach will no longer be used very often.

Human liver microsomes are relatively available, both from some academic scientists and from commercial companies that sell preparations. If properly handled, liver tissue from surgery or organ donors can be frozen (in liquid N2 and held at -80 °C) essentially indefinitely without apparent loss of P450 enzyme activity. All of the P450s are present in the material. The microsomal preparations can also be stored for long periods. The availability of human microsome preparations does allow for insight into the variability among the population. The disadvantages are that human liver microsomes are still not quite as available as one might desire, the information is not as complete as from the cellular systems, and some of the approaches to identifying the roles of the individual P450s may not always be straightforward, particularly if more than one P450 is involved or if good antibodies and diagnostic inhibitors are not available (2).

Recombinant Systems

Many early studies utilized P450 enzymes purified from human liver, but the availability of tissue and technical difficulties in purification has required a shift to heterologous expression

methods to satisfy the demands for single P450 systems for use in practical applications, i.e., drug metabolism. A number of heterologous systems have now been used for expression of human and other P450s, and all have some advantages and disadvantages.

Systems based on mammalian cells usually have relatively low levels of expression. Cell culture is also expensive. Transient systems used include transformed African green monkey kidney (COS) cells and vaccinia virus-infected HepG2 cells. These systems require cell transfection each time before expression, although microsomes can be isolated from the cells (at peak expression and stored frozen). Most permanent cell lines of human liver origin do not express P450s or, if they do, it may only be P450 1A1. Stable heterologous expression of individual human P450s has been achieved in several cell lines, including V79 Chinese hamster and human lymphoblastoid cells, which are both commercially available (4,5). In addition, some pharmaceutical companies are developing other systems of their own. These systems have the advantage that they have the normal integrity found in mammalian cell lines and have accessory proteins such as NADPH-P450 reductase and cytochrome *b*5. They can also be used to address endpoints relevant to toxicity (6). The disadvantages are that expression levels are low, so work is expensive and sensitive assays are needed. In making comparisons of transformation rates with such systems containing different P450s, it is important to estimate the level of P450 expression; this is usually done by immunoblotting except in some cases for which levels of expression are greater.

The other general area includes higher levels of expression in simpler systems. There are three major vector systems-yeast, baculovirus, and bacteria.

Heterologous expression of mammalian P450s in yeast was first introduced by Ohkawa and his colleagues in Japan (7) and this approach was also employed in this laboratory in the late 1980s. In principle, yeast have the advantage that they have NADPH-P450 reductase and cytochrome *b*5, and yeast microsomes should be capable of transformation. However, these yeast enzymes (reductase, *b*5) do not always couple well with the mammalian P450s. Levels of P450 expression are not generally as high as desirable, and yeast expression has not been very popular in this country. Nevertheless, Pompon and others have made some major improvements in the yeast system and use these systems in France (8).

Baculovirus expression in insect cells has gained in popularity. Systems expressing both NADPH-P450 reductase and a single P450 have been reported (9), and the microsomes prepared from the insect cells are capable of oxidizing P450 substrates at reasonable rates. Baculovirus systems have some inherent drawbacks-heme or heme precursors are needed and media is expensive, although there are some prospects for use of serum-free media that may lower the cost. As with many of the systems, the levels of expression of individual P450 forms are not always very predictable. Nevertheless, P450s can be purified on a reasonably large scale from these insect cell systems and used directly in various studies.

Although early attempts to express mammalian P450s in bacteria were disappointing, a number of technological modifications have been employed successfully (10). At least 35 mammalian P450s (16 from humans) have now been expressed in *Escherichia coli* (11). In most cases, some modification of the N-terminus (5') is necessary for optimal levels of expression. However, no studies to date have shown an effect of such changes on catalytic activity. Studies in this laboratory (12) and by Pernecky and Coon (13) indicate that a large

portion of the N-terminus can be removed without altering catalytic activity. One unexplained observation in heterologous bacterial expression is that expression of some of the P450s requires addition of the heme precursor d-aminolevulinic acid to the medium (11). However, this is apparently not due a requirement for heme synthesis, since some of the highest-expressing P450s do not need such supplementation.

Purification of bacterially-expressed P450s has been relatively straightforward (14). The purified proteins can be used with NADPH-P450 reductase and, when necessary, cytochrome *b*5 to reconstitute catalytic activities at expected rates. The expected patterns of catalytic specificity are seen. In particular, bacterial recombinant P450 3A4 shows the partitioning between aflatoxin B1 8,9-epoxidation and 3a-hydroxylation expected from microsomal studies and the unusual modulating effects of a-naphthoflavone, plus the sigmoidal plots of reaction velocity *vs* substrate concentration (15).

Another feature of bacterial expression is that *Salmonella typhimurium* can also be utilized. This is not a complete surprise given the similarities of these enteric bacteria, Salmonella and Escherichia. However, even without any precautions regarding levels of protease activity, the Ames' test systems have been used to express human P450 1A2 and the bacteria show mutations from aryl and heterocyclic amines in the absence of added S9 and cofactors (16). This aspect of bacterial expression remains to be further developed but has potential in terms of improved and more useful mutagenicity test systems.

Advantages of the bacterial expression systems are the low cost of growing bacteria and the use of large amounts of recombinant human P450. For example, the bufuralol oxidation products generated in a 20 min incubation with 35 nmol of recombinant P450 1A2 could be

isolated and characterized by mass and 1H-NMR spectroscopy (17). The large amounts of purified P450s have also been used as antigens for the preparation of polyclonal antibodies (18). This approach offers potential in the development of antibodies as reagents to be utilized with human liver microsomes in the discernment of roles of P450s in particular reactions.

One current disadvantage of bacterial systems is that the individual proteins are still expressed and purified, in the historic manner used with proteins purified from liver. One approach to circumventing this problem is the use of fusion proteins, i.e., a single protein containing both a P450 and NADPH-P450 reductase. Several of these have been produced in bacteria and some have been purified (19). These tend to be self-sufficient entities, although their rates of oxidation can often be further stimulated by adding NADPH-P450 reductase. To date none seem to have the unusually high rates of substrate oxidation seen with the normal bacterial P450 BM-3 (P450 102), however. The catalytic selectivity of these available fusion proteins has not been extensively investigated, but to date the patterns seem to be holding well (20). Another approach to development of systems involves co-expression of both a P450 and NADPH-P450 reductase (and cytochrome *b*5) in a single bacterial vector. This approach has been taken by Porter (21) although the expression level in this first effort was low. A polycistronic approach may be advantageous.

Conclusions

A number of *in vitro* approaches have been developed for the study of P450s and other enzymes in human drug metabolism. Different systems are available and rely on human

tissues and heterologously expressed proteins. Information can be obtained about patterns of metabolism, which P450s are capable of catalyzing a reaction, which P450s predominate in a typical liver cell, whether metabolism generates toxicity, chemical characterization of products, and possibly even induction. The choice of systems is dictated by the goals. Heterologous expression products (P450s and antibodies raised to them) offer many advantages and will continue to be developed as reagents with improvements in technology and lowered cost in high-level expression systems.

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