

## Compound optimization in early- and late-phase drug discovery: Acceptable pharmacokinetic properties utilizing combined physicochemical, *in vitro* and *in vivo* screens

Gary W Caldwell

### Address

The RW Johnson Pharmaceutical Research Institute  
Drug Discovery Department  
Welsh and McKean Roads  
Spring House  
PA 19477  
USA  
Email: gcaldwel@prius.jnj.com

Current Opinion in Drug Discovery & Development 2000 3(1):30-41  
© PharmaPress Ltd ISSN 1367-6733

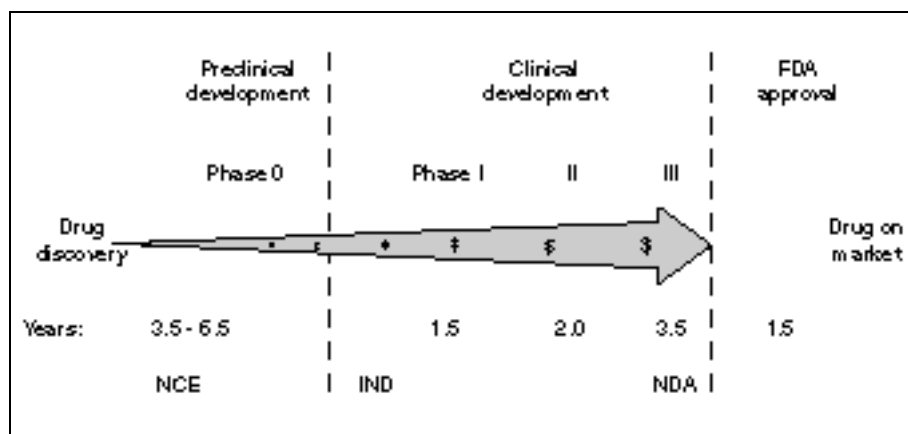
New chemical entities (NCEs) are abandoned in development primarily because of insufficient efficacy, safety issues and for economic reasons. Since efficacy and safety deficiencies are related in part to pharmacokinetics (PK), uncovering PK defects as early in drug discovery as possible would be highly valuable in reducing NCE failures in preclinical and clinical development. In this review, a strategy is put forth to integrate drug metabolism/pharmacokinetics and toxicology functions into drug discovery. Compound optimization in early- and late-phase drug discovery is covered, emphasizing physicochemical properties, *in vitro* absorption, metabolism and *in vivo* animal PK methodologies, primarily from the period 1998 to 1999. The present study also illustrates the idea of sorting oral bioavailability data into high/intermediate/low categories based on combining high/low rank-ordered information from physicochemical properties and *in vitro* absorption, metabolism and serum binding assays. It is shown that by combining the results from solubility, stability, absorption and metabolism assays, the high/intermediate/low human oral bioavailability for a series of *b*-blockers can be approximately predicted. This method has a high sample throughput and should be useful in rank-ordering the predicted oral bioavailability of large collections of compounds at the lead optimization step of drug discovery. These results are useful for selecting compounds for future *in vitro/in vivo* correlation modeling or *in vivo* animal testing. This type of approach will improve the decision making process of compound selection in drug discovery.

**Keywords**  $\beta$ -adrenoceptor blockers, assays, bioavailability, pharmacokinetics

### Introduction

The drug discovery and development process is scientifically complex and full of risk, and is therefore, expensive and time-consuming (Figure 1). Typically, a new chemical entity (NCE) is promoted from discovery into preclinical development and if it succeeds in passing all hurdles, it is submitted for an investigational new drug (IND) application and eventually enters phase I, II and III clinical development. If the compound passes all clinical trials, it is submitted for a new drug application (NDA) and eventually enters the market-place. The average cost to discover and develop a NCE into a marketable drug in the USA, is typically hundreds of millions of dollars and requires a decade or longer to reach the market-place [1]. It is clear that there is a critical need for pharmaceutical companies to become more cost- and time-efficient in light of spiraling world healthcare expenditures. A significant factor that governs the cost required for NCEs to become marketed drugs is their high attrition rates in preclinical or clinical development [2]. The proportion of IND applications that fail has been estimated to be approximately 87% in phase I, 60% in phase II and 20% in phase III clinical studies [3•]. Coupling these attrition rates with the large expenditures necessary for phase II and III clinical studies, produces the major financial problems associated with pharmaceutical research. According to data compiled by DiMasi [2], 1943 INDs were filed in the USA between 1964 and 1989; the total number of IND applications that were dropped before reaching NDA status was 1613 or 83%. In other words, during this 25-year period, approximately 1 in 6 NCEs nominated to IND status became a marketed drug. To make matters worse, only approximately 1 in 3 marketed drugs typically generates sufficient income to recover the costs

Figure 1. The pharmaceutical discovery and development process.



associated with its discovery and development [3]. Therefore, it is generally true that the majority of pharmaceutical discovery and development budgets and time are spent on drug failures, and for NCEs that do go to market, only a few are genuinely profitable. Faced with this low probability of success, the current trend of pharmaceutical companies merging is understandable.

The abandonment of IND candidates is primarily attributed to efficacy, safety and economic reasons. It has been reported for the period 1964 to 1989, that 46% of 1099 IND applications were discontinued due to unacceptable efficacy (eg, weak or lack of efficacy), 27% due to safety issues (eg, toxicity), 23% due to economics (eg, limited market) and 5% for miscellaneous reasons (eg, unclassified reasons) [2]. This trend is consistent with a smaller, but more recent study by Kennedy [3], where 46% of 121 NCEs in clinical development were discontinued due to unacceptable efficacy, 40% due to safety issues, 7% due to economics and 7% for miscellaneous reasons. Therefore, insufficient pharmacological efficacy, human adverse reactions and toxicity are estimated to account for 50% to 86% of the NCEs dropped from development. The challenges of pharmacoeconomic predictions have recently been addressed and will not be discussed here [3]. Human *in vivo* efficacy and safety predictions of NCEs are extremely difficult to forecast utilizing the present pharmaceutical process, and the vast majority of pharmaceutical companies are focused on reducing clinical development attrition rates by attempting to accurately evaluate efficacy, safety and pharmacoeconomics at a much earlier stage than before.

To address efficacy and safety attrition rates, some companies are reorganizing their traditional discovery and development departments from two independent functions, to organizations that have a drug selection interface group between these departments. There are several jargon phraseologies given to this type of interface group, namely, 'proof-of-concept' or 'proof-of-principle'. The primary mission of a drug selection interface group is to increase the quality and quantity of human data prior to full phase I clinical development. Typically, a drug candidate is tested in a limited group of human subjects (eg, 1 to 15) together with formal pharmacokinetic and toxicology analysis. It is expected that this type of information will indicate potential development problems, and thereby, significantly reduce the overall attrition rate of NCEs in future clinical studies. Another strategy is to integrate drug metabolism/pharmacokinetics and toxicology functions into a compound optimization group either partially or entirely within drug discovery. It has been argued that uncovering human pharmacokinetic defects (eg, oral bioavailability, half-life, metabolites, drug-plasma protein binding, etc) in drug discovery would be highly valuable in reducing NCE failures in preclinical and clinical development, since efficacy and safety deficiencies are related in part to pharmacokinetic problems [4]. The jargon phraseology applied to this type of compound optimization group is 'fast pharmacokinetics' or 'just-in-time pharmacokinetics'. The goal of this compound optimization group is to eliminate compounds with pharmacokinetic defects utilizing human tissues, human-derived cell lines and/or *in vivo* animal

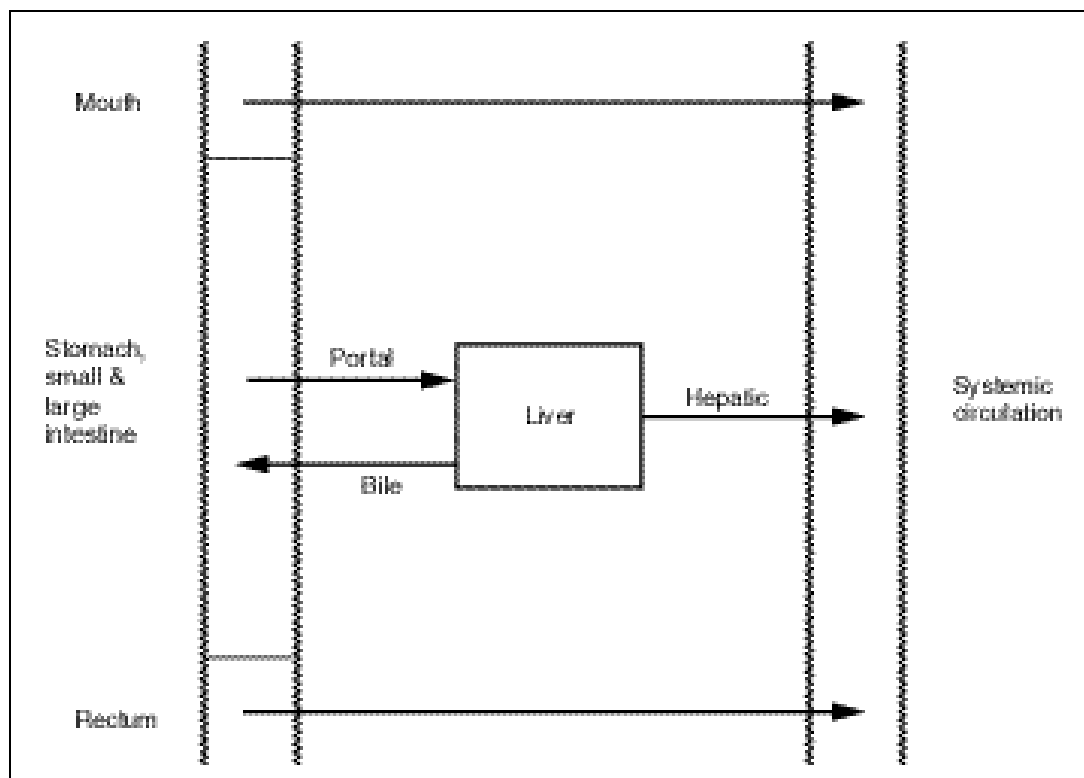
studies [5]. If defects in pharmacokinetic properties could be recognized and corrected at the drug discovery stage before they entered development, more time and resources could be allocated to projects with real potential. A compound optimization group would certainly enhance any early clinical studies such as the drug selection interface group discussed above.

In this review, the importance of a compound optimization group that is broadly integrated within a drug discovery department is discussed. Methods for compound optimization in early- and late-phase drug discovery are covered, emphasizing: (i) physicochemical properties (log P,  $pK_a$ , solubility, etc); (ii) *in vitro* and *in vivo* biophysical characteristics, such as absorption, distribution, metabolism and excretion (ADME); (iii) pharmacokinetics; and (iv) toxicology. While the importance of human oral bioavailability data for compound optimization in drug discovery is unquestioned, it is rarely available due to cost- and time-consuming experimental challenges. The present study also illustrates the idea of predicting high/intermediate/low human oral bioavailability based on rank-ordered physicochemical properties and *in vitro* absorption, metabolism and serum binding methodologies.

### Terminology and definitions

Successful drug candidates typically have good biological properties such as potency, selectivity, efficacy and oral bioavailability. It is important to have a reasonable understanding of these properties since some terms are used interchangeably in the literature, eg, potency for efficacy and absorption for bioavailability. This section will briefly review their definitions. Potency refers to the amount of compound needed to produce a given biological effect and the terms activity and potency are used interchangeably in this review. Selectivity infers that undesirable side effects are minimized or eliminated, while efficacy refers to the maximum level of biological effect a compound can produce. The oral bioavailability of compounds is primarily dictated by the following serial rates: liberation, absorption, metabolism and elimination [6]. The simple diagram shown in Figure 2 can be used to define these terms. Oral ingestion is generally the safest, most convenient and most economical method for compound administration, and when a compound is given orally, the liberation rate is defined as the net transfer of compound from the mouth and stomach to the small intestine. Typically, compounds are released from formulations that may depend upon disintegration, dissolution, solubility, surface area and chemical or enzymatic stability. In other words, the liberation rate represents the total amount of intact compound available at the small intestine after oral dosing. The absorption rate involves the net transfer of compounds from the gastrointestinal fluid across primarily the small intestine into the portal blood system. There are several mechanisms of compound uptake; the main processes available are passive diffusion and carrier transport. Intestinal P-glycoprotein (P-gp) is another transporter system that needs to be considered. P-gp is expressed at the luminal surface of the intestinal epithelium, and therefore,

Figure 2. Routes from the gastrointestinal tract into the systemic circulation.



acts to oppose the uptake of compounds into the portal blood system [7]. The amount of compound that passes through the intestinal tissues must pass through the liver and may be subjected to first-pass metabolism effects. At times, first-pass effects can prevent effective concentrations of compounds from reaching the hepatic blood system and eventually the general systemic circulation. The cytochrome P450 (CYP) enzyme system, which is primarily located in the smooth endoplasmic reticulum of liver cells, and in smaller quantities in the kidney, lung and gastrointestinal epithelium, is responsible for the monooxygenase metabolism of compounds [8]. Sometimes drug metabolites formed in the liver are excreted back into the intestinal tract via the bile. These metabolites are either excreted in the feces or reabsorbed into the portal blood system and ultimately excreted in the urine. Compounds that reach the systemic circulation either unchanged or as metabolites are excreted by the urinary system. Sublingual and rectal administration of compounds have the advantage that the compound is somewhat protected from rapid first-pass metabolism by the liver, however, these routes are not as broadly accepted for general use.

The amount of orally-administered compound reaching the systemic circulation is measured from the ratio of the area under the plasma-concentration versus time curve after oral administration  $(AUC)_{oral}$  to that after intravenous  $(AUC)_{iv}$  administration. Thus, the oral bioavailability ( $F$ ) is defined by equation 1.

$$F = [(AUC)_{oral} / (AUC)_{iv}] * [D_{iv} / D_{oral}] \quad (1)$$

This fraction or a percentage is normalized for the different compound doses ( $D$ ) given by the two routes. Alternately,  $F$  can be defined by equation 2:

$$F = f_L * f_A * f_H \quad (2)$$

Where  $f_L$  represents the net fraction of an oral dose liberated from the formulation that reaches the small intestine, the net fraction absorbed across the apical membrane of the epithelial cell is denoted by  $f_A$ , and  $f_H$  represents the net fraction escaping the first-pass hepatic effect.

After introduction into the portal circulation system, compounds can bind to various constituents such as tissue proteins, cell proteins and blood proteins. Compound binding to various blood proteins and tissue proteins is important because it can influence the therapeutic, pharmacodynamic and toxicological action of certain drugs. Competition for binding to tissues and blood proteins is likely to occur between different compounds if present at the same time. Human blood consists of three major systems: (i) formed elements (ie, erythrocytes, leukocytes and platelets); (ii) a fluid portion; and (iii) large amounts of various salts [9]. The major cell body in the blood is the erythrocyte (ie, red blood cell), which comprises approximately 95% of the total cellular fraction in the blood. There are three major components in the erythrocyte capable of binding compounds - hemoglobin, carbonic anhydrase and the cell membrane. If blood is allowed to naturally coagulate, a clear straw-colored fluid (ie, serum) can be separated from the cellular fraction by centrifugation. In contrast, plasma is obtained by

centrifugation of blood to which an anticoagulant was added immediately after removal from the body. Thus, serum contains water (90 to 92%), all blood proteins (6 to 8%) and various salts (eg, 0.1 M NaCl), while plasma contains water, proteins minus the clotting factors and salts. The concentration of various serum proteins can vary individually and on a daily basis by as much as 10% of their average value. It is also interesting to note that serum protein concentration levels can be highly affected by certain physiological and pathological conditions. Human serum albumin (HSA),  $\alpha_2$ -acid glycoprotein (AGP), the high-density lipoproteins (HDL) and the low-density lipoproteins (LDL), are the most important serum proteins responsible for the binding of compounds in serum. Typically, HSA is largely responsible for serum binding of acidic and neutral compounds, whereas AGP and lipoproteins bind mainly basic compounds. HSA and AGP serve as depots and transport proteins for numerous endogenous and exogenous compounds. Among the endogenous substances bound with high affinity to blood proteins are long chain fatty acids, L-tryptophan and bilirubin. The blood protein-exogenous complex (ie, blood protein-drug complex) acts as a transport mechanism to carry compounds to the sites of action; this transport is extremely important for compounds that exhibit low solubility in the water portion of the serum. In some cases, the circulating protein-compound complex also serves to replenish the free compound that is removed by various distribution and elimination processes. Thus, it maintains free compound concentration at its therapeutic level and provides a mechanism that prolongs the duration of compound action. Therefore, determination of the concentration of unbound or bound compound with blood proteins is an important parameter to measure to establish the importance of oral bioavailability values.

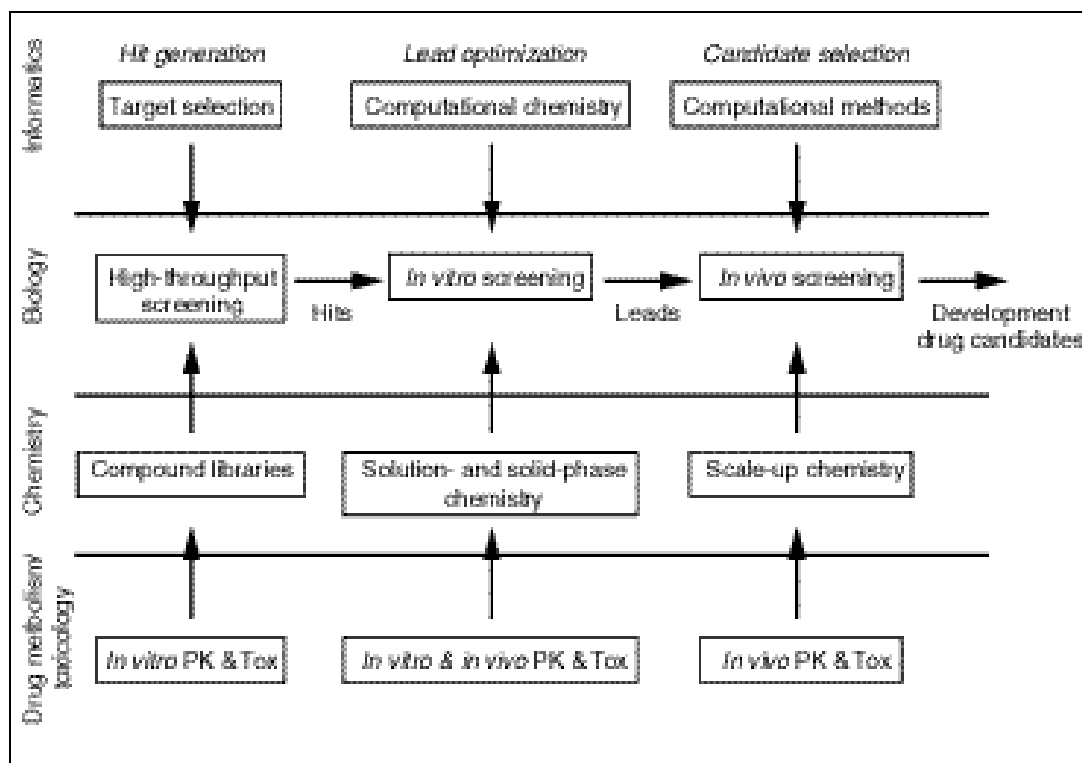
### The drug discovery process

Traditionally, drug discovery groups have focused primarily on compound synthesis and target screening. In this type of environment, medicinal chemists synthesize compounds in order to maximize *in vitro* or *in vivo* potency, selectivity and efficacy for a relevant biological target. Optimized structure-activity relationships (SARs) are generated based on *in vitro* potency versus structural modifications; however, in many cases, all other physicochemical properties (log P,  $pK_a$ , solubility, etc), ADME, pharmacokinetic and toxicological properties have been ignored until a later time. As mentioned earlier, uncovering pharmacokinetic defects is highly valuable in reducing NCE failures since efficacy and safety deficiencies are related in part to pharmacokinetics [4]. The consequence of the exclusion of these properties in drug discovery has been to waste time and resources in development groups by putting them in a 'patch and mend' strategy. It is clear that the way to avoid this situation is to perform many of the traditional development physicochemical, ADME, pharmacokinetics and toxicological studies in drug discovery [10]. Unfortunately, many of these traditional *in vitro* and *in vivo* assays are not well adapted for the higher-throughput screening that is necessary in drug discovery. Using a hierarchical organizational approach and with recent methodological advances, it is feasible to add some

relevant physicochemical, ADME, pharmacokinetics and toxicology criteria in the decision making process of drug discovery. In Figure 3, the organizational format of a broadly-based drug discovery department that is used to illustrate this idea is shown. Compound hit generation, lead optimization and candidate selection steps are the three main decision points used to drive the overall process to produce drug development candidates. The points have input from informatics (ie, bio- and chemoinformatics, molecular modeling, and computer and automation science), biology (ie, biochemistry, molecular biology, cell biology and pharmacology), chemistry (ie, combinatorial, parallel and scale-up synthesis) and drug metabolism/toxicology (ie, pharmacy, pharmacokinetics). This type of format naturally allows the composition of the department to change as the discovery process advances, ie, early drug discovery emphasizes more basic research in biology and chemistry, while late stage discovery shifts to more applied research. It allows for the elimination of compounds utilizing a combination of activity and biophysical data at each decision point. This type of organization also provides a maximum feedback loop between medicinal chemists and drug metabolism/toxicology scientists. In other words, structural modifications suggested by metabolic and toxicity data are incorporated into the synthetic plan. We will describe each decision point emphasizing the input from physicochemical, ADME and *in vivo* pharmacokinetic assays for the selection of drug development candidates. It is not the intention of this report to exhaustively review the array of important drug metabolism/toxicology and absorption assays that abound in the literature [11,12,13-16], rather, it is to highlight some important assays in the drug discovery setting and make general comments concerning their use.

The goal of the 'hit generation' step is to screen large compound libraries in a relatively short amount of time in an attempt to find compounds that cause a specific biological response, ie, 'hits'. This step includes target identification, selection, validation and high-throughput screening (HTS) of large structurally-diverse compound libraries. The productivity in target identification and selection has improved with the development of automated DNA sequencing, genomics databases and bioinformatic tools [17]. However, target validation remains a time-consuming process where assays are typically performed without automation. HTS operations are highly automated to handle sample preparation, assay procedures and large volume data processing. After each step is optimized to operate efficiently, it is common to screen 100,000 compounds in a 1- to 6-month period [18,19]. As new 'mix and read' detection assays are developed, HTS is moving toward ultra-HTS which will screen over 100,000 compounds per day [20,21]. Compound libraries are typically created by combinatorial and parallel synthesis paradigms [22], and HTS can identify thousands of hits or only a few from these libraries. The hit rate for HTS bioassays is set somewhat arbitrarily, that is, the activity cut-off is lowered until an adequate number of hits is obtained. Cluster analyses can also be performed on thousands of hits in order to reduce

Figure 3. The organizational format of a broadly-based drug discovery department.



the number of hits to an adequate number [23]. It is interesting to note that libraries of either virtually assembled compounds or actual compound libraries have been computationally screened utilizing three- (3-D) or four-dimensional (4-D) quantitative structure-activity relationship (3-D/4-D-QSAR) techniques to generate hits [24,25]. Virtual screening is a promising new technique for the discovery of high-affinity hits [26]. Hits can also be produced from 'me-too' compounds (close structural derivatives of a known active drug). The *de novo* design of new ligands bound in 3-D receptor protein structures using molecular modeling methods represents another source of hits [27]. The exact number of hits chosen is largely dictated by the *in vitro* potency, the compound patentability, the complexity of the analog chemistry, chemistry head count and the duration of the drug discovery program. In addition to these criteria, it could be of interest for certain projects to incorporate physicochemical, ADME, pharmacokinetics and toxicology selection criteria at this decision point. Unfortunately today, there are no *in vitro* or *in vivo* HTS methods capable of assaying 100K compound libraries in a timely manner for physicochemical, ADME, pharmacokinetics and toxicology properties. DNA microarray technology or DNA chips is a promising method used to monitor changes in expression at the mRNA level [28]. While unproven at this time, it is conceivable that the DNA chip will discover pertinent toxicodynamic markers [29] that can be used in reporter gene, branched-DNA or other HTS assays for early toxicology assessment [30]. There has been considerable effort made in computer-aided physicochemical property [31], solubility [32,33], intestinal uptake [33], permeability [34], metabolism [35] and toxicity [36] predictions. These

approaches typically use QSAR techniques, knowledge-based systems or neural network modeling to predict physicochemical properties and relevant biological parameters. While these calculations are well established, the accurate prediction of reliable data is still debatable. There is a serious disadvantage in selecting or eliminating hits based on calculated or screening data of physicochemical, ADME, pharmacokinetics or toxicology data at this point. It should be remembered that it is rare for a hit compound to have all of the desirable properties necessary for it to proceed directly to phase I clinical studies without structural modifications, furthermore, it is generally true that any single property of a compound can be optimized; however, this optimized property is often achieved at the expense of other properties. If substantial structural manipulation to the prototype hit is required to obtain the desired potency, selectivity and efficacy, then all favorable physicochemical, ADME, pharmacokinetics and toxicology properties obtained in the original hit may be reduced or forfeited in the process. Therefore, it is conceivable that nothing will be gained by screening for biophysical characteristics at this point in drug discovery. It is more advantageous to screen the analogs for favorable physicochemical, ADME, pharmacokinetics and toxicology properties during the lead optimization step.

Once useful structural prototypes have been obtained from the hit generation step, analog construction around these templates utilizing traditional solution- and solid-phase chemistries are initiated so as to improve, primarily, *in vitro* potency [22]. This process is referred to as the 'hit-to-lead optimization step' or 'lead optimization step'. The types of structural modifications incorporated into the hit are usually governed by *in vitro* potency data and by the wisdom of the

medicinal chemist. If the hit has a 3-D X-ray crystallographic compound-receptor protein structure, computational chemistry (ie, molecular modeling) techniques are an important method for lead optimization [27]. Typically, hundreds to thousands of analogs are required in the lead optimization step to select compounds worthy of advancement to the candidate selection step and receive more extensive *in vivo* animal testing. The exact number of analogs synthesized per biological target is largely dictated by the potency, efficacy and selectivity deemed necessary for the target, the personnel available, the complexity of the analog chemistry and the duration of the drug discovery program. Since the total number of analogs for any particular target is synthesized over some time period, an iterative strategy can be used to organize the workload. For example, if 1000 analogs per target are synthesized over a 1-year period, then for 30 targets, roughly 2500 samples per month need to be tested. There are only a few pertinent physicochemical properties, ADME and toxicology assays that have medium sample throughput capable of handling 2500 samples per month. Turbidimetric methods for measuring kinetic solubilities of compound have been estimated to handle 6000 samples per month [33]. Permeation properties are related to transcellular compound absorption. The use of artificial membranes in a 96-well plate-based format to predict permeation properties of compounds has been estimated to handle 10,000 to 20,000 samples/month [37]. Hepatotoxicity studies, which give information on the maximum compound concentration compatible with cell survival, can be performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method [38]. The assay is based on the ability of mitochondrial enzymes in live cells to reduce a tetrazolium salt into a colored formazan dye. A 96-well plate-based assay using a multi-well scanning spectrophotometer has been described [39]. Recently, an HTS technique utilizing rat liver microsomes and a pulsed ultrafiltration mass spectrometer has been developed [40,41]. Pulsed ultrafiltration-electrospray mass spectrometry is an attractive method for *in vitro* formation and mass spectrometric characterization of metabolites in an automated fashion. It was stated that the method had the potential for automated HTS of up to 60 samples (ie, profiles) per hour. Fluorometric assays for assessing cytochrome P450 drug-drug inhibition for the five principal drug-metabolizing enzymes, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 have been developed in a 96-well plate-based assay [42,43,44]. The judicious application of these techniques in rank-ordering compounds for more extensive *in vitro/in vivo* correlation modeling or *in vivo* animal testing may be useful; however, one should be cautious since the data will contain many false-positive and false-negative results. Experience indicates that physicochemical, ADME, pharmacokinetic and toxicology information is only essential for the subset of analogs that demonstrate the correct *in vitro* potency range deemed necessary for the project to be viable. Using the example above, for 2500 samples per month, we have found it necessary to assay approximately 10% of these compounds or 250 compounds per month. If we only assay those compounds that are considered to have the correct activity, then there are many low-throughput *in vitro* assays at our disposal. Approaches for measuring

$pK_a$  [45], octanol-water partition coefficients [46], thermodynamic solubility-pH profiles [47], and liposomal membrane-water partition coefficients [48] utilizing potentiometric techniques have been developed. Immobilized artificial membrane (IAM) chromatography can be used to model membrane partitioning of compounds [49]. IAMs are solid-phase membrane mimetics that are prepared by covalently immobilizing monolayers of cell membrane phospholipids to silica particles at high molecular surface densities, and are used as a chromatographic stationary-phase to mimic the lipid environment of a fluid cell membrane [50]. The analyte retention (capacity) factors on IAM chromatographic columns have been shown to predict drug permeability across the blood-brain barrier [51]. An alternative to IAM has been suggested recently utilizing lysophospholipid micellar electrokinetic chromatography [52]. The Caco-2 (immortalized human colon adenocarcinoma) and the MDCK (Madin-Darby canine kidney) cell lines have been used as an *in vitro* model for assessing membrane permeability [53,54]. These cell lines differentiate on microporous filter membranes into columnar epithelium and form tight cellular junctions. The utility of liquid chromatography/mass spectrometric (LC/MS) methods to measure the apparent permeability ( $P_{app}$ ) coefficients of compounds from a Caco-2 cell culture intestinal model has been demonstrated [55]. Capillary electrophoresis/frontal analysis has been used to screen drugs interacting with human serum and human serum proteins [56]. The rate of metabolism utilizing rat, monkey and human primary hepatocytes has been demonstrated using a sample handling system for 96-well plates directly coupled to a LC/MS, ranking compounds based on their resistance towards metabolism [39]. The rationale behind these experiments is that compounds that are resistant to metabolism are more likely to exhibit low *in vivo* first-pass effects. Reverse transcription-polymerase chain reaction (RT-PCR) assays have been developed to study the induction of human cytochromes P450 (1A1, 2A6, 2E1 and 3A3/4) mRNA in cell culture systems [57]. The various assays outlined above can be used to simply rank-order compounds or to select compounds based on a validated *in vitro/in vivo* correlation. The ranking of compounds based on these assays should be considered as a first step to reduce the number compounds for future *in vitro/in vivo* correlation modeling or *in vivo* testing. To be successful, compounds with the desired activity and the fewest *in vitro* biophysical defects or the best *in vitro/in vivo* correlations must be selected. Based on our experience, the combination of a thermodynamic solubility assay [47], a Caco-2 permeability assay [55], a hepatocyte or microsome assay to estimate hepatic extraction ratios [39], a drug-blood protein binding assay [56] and a P450 drug-drug inhibition assay [42], gives the most pertinent information. Valuable information is also gained from studying the profile of metabolites produced in rat and human hepatocytes or microsomes. It should be noted that interpretation of results from all of the assays listed above could be hotly debated, since it will be necessary to eliminate compounds with activity at this point. In a later section, it is shown how a combination of rank-order screening assays can provide a high/intermediate/low prediction of human oral bioavailability.

Once the selection criteria have been defined in the lead optimization step, compounds are promoted to the 'candidate selection' step where the goal is to assay compounds utilizing primarily *in vivo* animal models. Using *in vivo* data obtained from animal species and allometric scaling, human pharmacokinetic parameters can be predicted, such as volume of distribution, clearance, half-life and oral bioavailability [58]. Note that at this stage, the function of the chemistry group has shifted to scale-up chemistry (Figure 3) that is necessary, since larger quantities of compounds are required for *in vivo* studies. The number of candidates at this point is totally arbitrary. For example, assume we have rank-ordered 250 compounds from one analog series, our experience indicates that we only need to assay approximately 10% of these compounds or 25 compounds per target. There are again several low-throughput *in vivo* assays at our disposal. An effective approach for screening a large number of samples is to simultaneously measure multiple analytes in a single analysis. *In vivo* cassette-dosing pharmacokinetic studies utilizing LC/MS have been successful via the oral [59] and intravenous routes [60]. Alternatively, by using automated procedures to handle plasma preparation, and rapid LC/MS techniques to perform quantitation, we measure the oral bioavailability of singly-dosed compounds in a rapid manner. With this technique, many hundreds of compounds per year can be measured. Utilizing these fast quantitation techniques, it is possible to perform short-term *in vivo* toxicity experiments in order, for example, to study exposure levels. Microdialysis sampling is a flexible technique for the study of *in vivo* pharmacokinetics of the extracellular fluid in the brain [61]. In recent years, microdialysis with LC/MS detection has emerged as an important tool in biochemical research [62]. The various *in vivo* assays outlined above can be used to identify potential pharmacokinetic defects, such as low oral bioavailability, short half-life, active metabolites, etc. Based on *in vivo* and *in vitro* data, attempts can be made to correct these problems, and if successful, these compounds are promoted to drug development candidacy and enter preclinical development.

### Prediction of human oral bioavailability at the lead optimization step

Based on equation 2, if accurate human *in vitro/in vivo* correlation models to predict the  $f_l$ ,  $f_A$  and  $f_H$  were available, human oral bioavailabilities (F) could be estimated. There are several good reviews outlining methods to predict human oral bioavailability based on *in vitro* (human/animal)/*in vivo* (animal) metabolism data [63,64]. The individual variation in oral bioavailability measurements can be very large due to genetic and environmental factors, and thus, cannot be accurately or readily predicted. To take into account this natural variability, our strategy is simply to sort oral bioavailability data into high/intermediate/low categories based on combining rank-ordered information from several *in vitro* assays. This method has a higher sample throughput than *in vitro/in vivo* correlation modeling, and therefore, should be useful in sorting large collections of compounds at the lead optimization step for future *in vitro/in vivo* correlation modeling or *in vivo* animal testing.

Intuitively, one would suspect that maximum oral bioavailability (eg,  $\geq 90\%$ ) should occur when a compound has a high liberation rate (eg, the net transfer of compound from the mouth and stomach to the small intestine), a high absorption rate (eg, net transfer of compounds from the gastrointestinal fluid across primarily the small intestine into the portal blood system) and low metabolism rate (eg, first-pass effect). Of course the minimum concentration of compound in the systemic system (eg, low oral bioavailability,  $\leq 10\%$ ) should occur when the compound has a low liberation rate, a low absorption rate and a high metabolism rate. All other combinations of liberation, absorption and metabolism can be considered as having intermediate oral bioavailability values using the above arguments. Thus, if we could devise liberation, absorption and metabolism assays that ranked compounds in a high/low manner, it should be possible to predict into which oral bioavailability range a compound would fall by utilizing the scheme in Table 1. The liberation rate of a compound can be approximated by examining the dissolution rate and the compound's chemical stability. Since compounds are primarily solutions or suspensions in drug discovery, the dissolution rate can be approximately assessed by the solubility of the compound assuming that the surface area of the compound is not a factor. Thus, the dissolution rate will not be a limitation for compounds having reasonable aqueous solubility ( $> 0.1$  mg/ml) [33]. The chemical stability of compounds at low gastric pH values can be approximately assessed by stability studies at pH 2 over a time period equivalent to the compound's mean residence time in the stomach (human  $\approx 75$  min). That is, we can measure the disappearance of compounds at pH 2 for 75 min. Thus, compounds with aqueous solubility  $> 0.1$  mg/ml and chemical stability  $> 50\%$  (arbitrarily selected) can be considered to have high liberation rates, while those with solubilities  $< 0.1$  mg/ml and chemical stability  $< 50\%$  can be considered to have low liberation rates (Table 1). All other combinations of solubility and stability will be considered to have a low liberation rate. The Caco-2 cell line is used as an *in vitro* model to study drug transport in the intestinal epithelium [55]. Compounds that are completely absorbed in humans have permeability rates typically  $> 1.0 \times 10^{-6}$  cm/s, compounds that are absorbed  $> 1\%$ , but  $\leq 100\%$ , have permeability rates of  $0.1 \times 10^{-6}$  to  $1.0 \times 10^{-6}$  cm/s and compounds that are absorbed  $< 1\%$  have permeability rates  $\leq 1.0 \times 10^{-7}$  cm/s. Thus, compounds with permeability rates  $> 1.0 \times 10^{-6}$  cm/s can be considered to have high absorption rates, while those with  $< 1.0 \times 10^{-6}$  cm/s can be considered to have low absorption rates (Table 1). It should be emphasized, that these permeability cut-offs are somewhat variable depending upon the experimental condition (eg, stirred versus unstirred water layers) [55], however, the separation of the Caco-2 data into high/low categories is typically not a problem. Once the compound has passed from the small intestine to the portal system, the liver may metabolize a considerable portion of the compound prior to entering the systemic circulation. The prediction of metabolism rates can be studied using isolated human hepatocyte cells and/or various liver subcellular fractions [39,65]. The rate of first-pass metabolism can be roughly estimated into high/low

**Table 1. Oral bioavailability classified into high/intermediate/low categories based on *in vitro* rank-ordered liberation, absorption and metabolism data.**

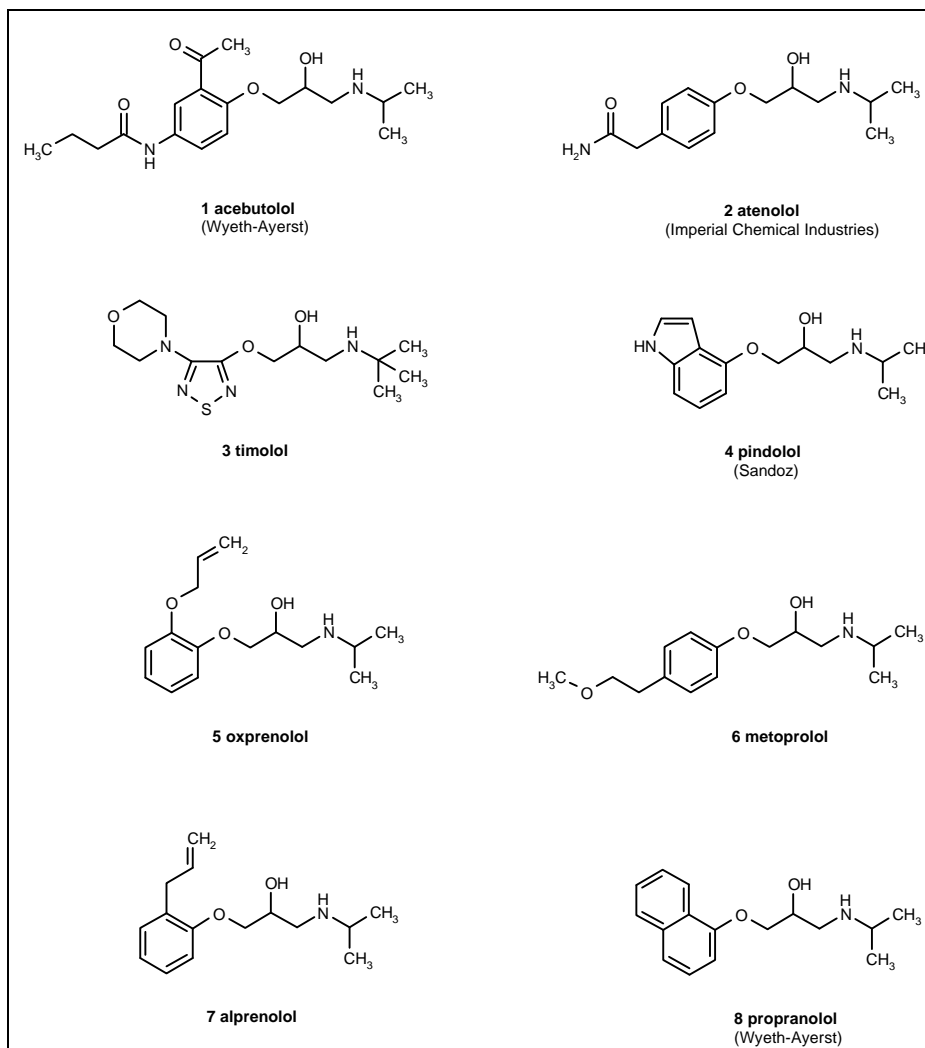
Liberation ranking	Absorption ranking	Metabolism ranking	Oral bioavailability
High	High	Low	High
High	Low	Low	Intermediate
Low	High	Low	Intermediate
Low	Low	Low	Intermediate
High	Low	High	Intermediate
High	High	High	Intermediate
Low	High	High	Intermediate
Low	Low	High	Low

categories by examining the rate of drug disappearance (or the rate of appearance of the metabolites) from human hepatocyte or microsomal suspensions. Thus, compounds with hepatocyte stability > 50% (arbitrarily selected) can be considered to have low metabolism rates, while those with hepatocyte stability < 50% can be considered to have high metabolism rates (Table 1). Thus, a simple high/low strategy could be used to roughly estimate first-pass metabolism effects. Alternately, when concentration studies and scaling techniques are performed, it has been found that drugs that had high *in vivo* extraction ratios had *in vitro* hepatocyte clearance values > 30  $\mu\text{l}/\text{min}/10^6$  cells, while drugs that had low *in vivo* extraction ratios had *in vitro* hepatocyte clearance values < 10  $\mu\text{l}/\text{min}/10^6$  cells [65].

An homologous series of  $\beta$ -adrenoceptor blocking drugs ( $\beta$ -blockers, Figure 4 and Table 2) were chosen as model compounds to illustrate the above scheme, since their pharmacokinetic parameters display a wide variability [66,67]. These compounds have molecular weights that range from 248 Da (4, pindolol) to 336 Da (1, acebutolol), and dissociation constants ( $\text{pK}_a \approx 9$ ) which minimize the influence of these parameters on solubility, stability, absorption and metabolism. The pseudo-thermodynamic solubility was measured using a simple LC/MS technique. Briefly, the compound was saturated at pH 7.4 (ionic strength = 0.15), shaken for 30 min, filtered through a nylon filter and assayed by LC/MS. The stability was measured by preparing the sample at pH 2 and measuring the disappearance over a 75 min period. Data for the  $\beta$ -blockers are listed in Table 2 and indicate that these drugs are all highly liberated. For the Caco-2 transport in the apical to basolateral direction, 50  $\mu\text{M}$  of the  $\beta$ -blocker of interest was placed in the insert (apical side) [55]. The insert was moved seven times over a 1 h period to wells containing fresh buffer. These well (basolateral) samples were spiked with an internal standard and analyzed by LC/MS. The  $P_{\text{app}}$  coefficients obtained from this experiment are listed in Table 2. The results for the  $\beta$ -blockers indicated that acebutolol and atenolol (2) had a low absorption rate, while all of the other drugs were highly absorbed. If we compare the  $P_{\text{app}}$  coefficients to the *in vivo* human absorption data of the  $\beta$ -blockers (Table 2), we observe that the  $P_{\text{app}}$  coefficients correctly distinguish the poorly absorbed  $\beta$ -blockers (acebutolol and atenolol) from the highly absorbed  $\beta$ -blockers. After known drug concentrations (5  $\mu\text{M}$ ) were incubated with hepatocytes for 6 h, LC/MS procedures were developed to determine the amount of drug which was metabolized (ie, % metabolized) [39]. These results are listed in Table 3 and indicate that propranolol (8), alprenolol (7) and oxprenolol (5) were highly metabolized. If we compare the *in vitro* human hepatocyte cell data with the *in*

*vivo* first-pass effect data, we observe that the *in vitro* human hepatocyte cell data could approximately differentiate the *in vivo* first-pass effect data in a high/low manner. That is, propranolol, alprenolol and oxprenolol were highly metabolized *in vitro* and also exhibited a high *in vivo* first-pass effect. Timolol (3), pindolol, acebutolol and atenolol were resistant towards metabolism by human hepatocytes and also exhibited a low *in vivo* first-pass effect. However, it should be noted that metoprolol (6) did not fit this pattern, suggesting *in vitro* human hepatocyte cell data obtained at a single concentration and time point do not completely correlate with *in vivo* first-pass effect data. Finally, if we combine the high/low liberation, absorption and metabolism result from Table 2 with the scheme outlined in Table 1, we can predict the high/intermediate/low oral bioavailability for the  $\beta$ -blockers. Note that timolol and pindolol were correctly chosen as having high bioavailability, however, metoprolol was predicted to have a high oral bioavailability, while the experimental data suggests its value is closer to an intermediate value. Clearly, the *in vitro* methods discussed here are able to distinguish  $\beta$ -blockers with favorable characteristics. We believe the above procedure will work reasonably well for other analog series and probably worse for structurally-diverse sets of compounds.

As mentioned earlier, the prediction of drug-serum binding is an important factor to understand along with oral bioavailability. It has been reported that capillary electrophoresis/frontal analysis (CE/FA) can be used in an automated manner to estimate the blood protein-drug binding association constants [56]. The percentage of drug bound in serum samples or artificial mixtures of serum proteins samples (eg, HSA + AGP) can also be determined. Briefly, CE/FA is used to determine the free drug concentration in a drug-protein binding equilibrium. In CE/FA, sample volumes that represent approximately 5 to 7% of the total volume of the capillary are injected onto the capillary to result in a frontal peak shape for the drug. Based on the frontal theory, the free drug concentration is directly determined from the height of the frontal peak. In the normal CE polarity mode, the basic  $\beta$ -blockers elute first and are resolved from the negatively charged serum proteins. We have shown that the binding capacity of the  $\beta$ -blockers with human protein mixtures of AGP, HSA, HDL and LDL has the same high/low ranking order as that obtained with human serum samples. The results, from this experiment indicated that propranolol had significantly higher binding to these proteins than the other  $\beta$ -blockers. Therefore, timolol and pindolol have higher oral bioavailabilities and lower serum binding profiles than propranolol [66].

Figure 4.  $\beta$ -adrenoceptor blocking agents.Table 2. *In vitro* and *in vivo* biophysical data for a series of  $\beta$ -adrenoceptor blocking drugs.

Drug	Solubility <sup>1</sup>	Stability <sup>2</sup>	Liberation ranking	Caco-2 <sup>3</sup>	<i>In vivo</i> f <sub>a</sub> <sup>4</sup>	Absorption ranking	Hepatocytes <sup>5</sup>	<i>In vivo</i> f <sub>H</sub> <sup>6</sup>	Metabolism ranking
acebutolol	200	100	High	3	78	Low	36	50	Low
atenolol	93	100	High	3	56	Low	26	17	Low
timolol	70	100	High	33	100	High	41	42	Low
pindolol	147	100	High	41	100	High	21	22	Low
oxprenolol	319	100	High	66	100	High	67	67	High
metoprolol	8	100	High	89	100	High	33	83	Low
alprenolol	46	100	High	93	103	High	133	150	High
propranolol	40	100	High	100	100	High	100	100	High

<sup>1</sup>The drugs were prepared at pH 7.4 (ionic strength = 0.15) in an aqueous solution, shaken for 30 min, filtered through a nylon filter and assayed by LC/MS. The units are mg/ml.

<sup>2</sup>The stability (% remaining) was measured by preparing the sample at pH 2 and measuring the disappearance over a 75 min period. The data are normalized to the starting concentration at time = 0.

<sup>3</sup>Caco-2 P<sub>app</sub> coefficients (see reference [55] for experimental details). The data are normalized to propranolol ( $7.5 \pm 1.2 \times 10^{-5}$  cm/s).

<sup>4</sup>*In vivo* human absorption (%) (see references [66] and [67]). The data are normalized to propranolol (90%). The net fraction absorbed across the apical membrane of the epithelial cell is denoted by f<sub>a</sub>.

<sup>5</sup>*In vitro* human hepatocyte cell data (% metabolized in 6 h at 5  $\mu$ M of drug). See reference [39] for experimental details. The data are normalized to the starting concentration at time = 0 for propranolol. *In vivo* human first-pass effect (%) (see references [66] and [67]). The data are normalized to propranolol (60%).

<sup>6</sup>The notation f<sub>H</sub> represents the net fraction escaping the first-pass hepatic metabolism effect.

Table 3. Comparison of the predicted and experimental oral bioavailability values for a series of  $\beta$ -adrenoceptor blocking drugs.

Drug	Liberation ranking <sup>1</sup>	Absorption ranking <sup>1</sup>	Metabolism ranking <sup>1</sup>	Predicted oral bioavailability <sup>2</sup>	Experimental oral bioavailability <sup>3</sup>
acebutolol	High	Low	Low	Intermediate	50
atenolol	High	Low	Low	Intermediate	50
timolol	High	High	Low	High	75
pindolol	High	High	Low	High	87
oxprenolol	High	High	High	Intermediate	50
metoprolol	High	High	Low	High	50
alprenolol	High	High	High	Intermediate	40
propranolol	High	High	High	Intermediate	30

<sup>1</sup>The ranking of the drugs was based on results from Table 2.

<sup>2</sup>The ranking of the oral bioavailability was based on the system used in Table 1.

<sup>3</sup>The *in vivo* human oral bioavailability (%) values are from references [66] and [67].

## Conclusion

This review has focused on integrating drug metabolism/pharmacokinetics and toxicology functions into drug discovery in order to reduce the attrition rates in clinical development. Three main decision points are used in drug discovery to drive the overall process to produce superior drug development candidates. These include hit generation, lead optimization and candidate selection steps. The elimination of compounds occurs at each decision point utilizing a combination of activity, physicochemical properties, absorption, metabolism, *in vivo* pharmacokinetics and toxicity assays. This type of organization provides a maximum feedback loop between medicinal chemists and drug metabolism/toxicology scientists. Literature has been cited primarily between 1998 to 1999. At the lead optimization step, a combination of a thermodynamic solubility assay [47], a Caco-2 permeability assay [55], a hepatocyte or microsome assay to estimate hepatic extraction ratios [39], a drug-blood protein binding assay [56], and a cytochrome P450 drug-drug inhibition assay [42] gives the most pertinent information. The profile of metabolites produced in rat and human hepatocytes or microsomes is also suggested at this step. These higher-throughput assays can be rank-ordered to prioritize compounds for more extensive *in vitro/in vivo* correlation modeling or *in vivo* animal studies. A simple example was used to illustrate this idea. Utilizing a series of  $\beta$ -adrenoceptor blocking drugs, a strategy was developed to sort oral bioavailability data into high/intermediate/low categories based on combining rank-ordered information from several *in vitro* assays. This oral bioavailability ranking procedure worked reasonably well for the  $\beta$ -adrenoceptor blocking drugs, and therefore, should work for other analog series. The predictions will probably be worse for structurally-diverse sets of compounds. The candidate selection step is used to assay compounds utilizing primarily *in vivo* animal models to gain information on key pharmacokinetic parameters and drug exposure levels [60]. While the above procedure appears to be a reasonable start, clearly much remains to be done to improve drug selection in discovery and development.

## Acknowledgements

The author would like to acknowledge the contribution of Per A Peterson, Michael Ernest, Keith Demarest, Joseph Gunnet, William Hageman, Eric Ericson, John A Masucci, Patrick Sasso, William Jones, Amy Maden, Mary Evangelisto, Zhengyin Yan, Scott M Easlick and Patricia A McDonnell. Allen Reitz and Edward L Tolman are gratefully acknowledged for reviewing the manuscript.

## References

- of outstanding interest
  - of special interest
1. DiMasi JA: **Risks, regulation, and rewards in new drug development in the United States.** *Regul Toxicol Pharmacol* (1994) **19**:228-235.
  2. DiMasi JA: **Success rates for new drugs entering clinical testing in the United States.** *Clin Pharmacol Ther* (1995) **58**:1-14.
  3. Kennedy T: **Managing the drug discovery/development interface.** *Drug Disc Today* (1997) **2**:436-444.
  - A good review of the skill sets necessary to manage an interface between discovery and development.
  4. Caldwell J: **The importance of drug metabolism studies for efficient drug discovery and development.** *Yakubutsu Dotai* (1996) **11**(1):119-126.
  5. Caldwell GW: **Novel biophysical analytical methods: The utility of combining physicochemical and *in vitro* physiological properties of drug compounds for estimating *in vivo* oral bioavailability.** *Second International Symposium on Drug-Drug Interaction Potential.* Baltimore, MD, USA (1997).
  6. Benet LZ, Mitchell JR, Sheiner LB: *Goodman and Gilman's - The Pharmacological Basis of Therapeutics.* 8th Edition. Gilman AG, Rall TW, Nies AS, Taylor P (Eds), Pergamon Press, New York (1990):1-62.
  7. Spahn-Langguth H, Baktir G, Radschuweit A, Okyar A, Terhaag B, Ader P, Hanafy A, Langguth P: **P-glycoprotein transporters and the gastrointestinal tract: Evaluation of the potential *in vivo* relevance of *in vitro* data employing talinolol as model compound.** *Int J Clin Pharmacol Ther* (1998) **36**:16-24.
  8. Lewis DF (Ed): *Cytochromes P450: Structure, Function and Mechanism.* Taylor & Francis Ltd, London (1996):1-348.
  9. Reif OW, Lausch R, Freitag R: **High-performance capillary electrophoresis of human serum and plasma proteins.** *Adv Chromatogr* (1994) **34**:1-56.
  10. Heykants J, Meuldermans W: **Nonclinical kinetics and metabolism studies in support of the safety assessment of drugs.** *Drug Inf J* (1994) **28**:163-172.
  - A good review of the studies necessary for preclinical and clinical toxicology.
  11. Rodrigues AD: **Use of *in vitro* human metabolism studies in drug development. An industrial perspective.** *Biochem Pharmacol* (1994) **48**(12):2147-2156.

12. Rodrigues AD: **Preclinical drug metabolism in the age of high-throughput screening: An industrial perspective.** *Pharm Res* (1997) **14**(11):1504-1510.
- A good review of some of the potential problems encountered in preclinical drug metabolism and rational screening strategies.
13. Rodrigues AD: **Rational high-throughput screening in preclinical drug metabolism.** *Med Chem Res* (1998) **8**(7/8):422-433.
14. Tarbit MH, Berman J: **High-throughput approaches for evaluating absorption, distribution, metabolism and excretion properties of lead compounds.** *Curr Opin Chem Biol* (1998) **2**:411-416.
15. Smith DA, van de Waterbeemd H: **Pharmacokinetics and metabolism in early drug discovery.** *Curr Opin Chem Biol* (1999) **3**:373-378.
16. Sinko PJ: **Drug selection in early drug development: Screening for acceptable pharmacokinetic properties using combined *in vitro* and computational approaches.** *Curr Opin Drug Discovery Dev* (1999) **2**(1):42-48.
17. Benton D: **Integrated access to genomic and other bioinformation: an essential ingredient of the drug discovery process.** *SAR QSAR Environmental Research* (1998) **8**(3-4):121-155.
18. Broach JR, Thorner J: **High-throughput screening for drug discovery.** *Nature* (1996) **384**:14-16.
19. Silverman L, Campbell R, Broach JR: **New assay technologies for high-throughput screening.** *Curr Opin Chem Biol* (1998) **2**:397-403.
20. Mere L, Bennett T, Coassin P, England P, Hamman B, Rink T, Zimmerman S, Negulescu P: **Miniaturized FRET assays and microfluidics: key components for ultra-high-throughput screening.** *Drug Disc Today* (1999) **4**(8):363-369.
21. Winkler T, Ketting U, Koltermann A, Eigen M: **Confocal fluorescence coincidence analysis: an approach to ultra high-throughput screening.** *Proc Natl Acad Sci USA* (1999) **96**(4):1375-1378.
- An interesting paper outlining an approach to screen 100,000 compounds/day.
22. Fecik RA, Frank KE, Gentry EJ, Menon SR, Mitscher LA, Telikepalli H: **The search for orally active medications through combinatorial chemistry.** *Med Res Rev* (1998) **18**:149-185.
- A good review of combinatorial chemistry with ideas concerning the incorporation of ADME properties in the design of libraries.
23. Stanton DT, Morris TW, Roychoudhury S, Parker CN: **Application of nearest-neighbor and cluster analyses in pharmaceutical lead discovery.** *J Chem Inf Comput Sci* (1999) **39**(1):21-27.
24. de Julian-Ortiz JV, Galvez J, Munoz-Collado C, Garcia-Domenech R, Gimeno-Cardona C: **Virtual combinatorial syntheses and computational screening of new potential anti-herpes compounds.** *J Med Chem* (1999) **42**(17):3308-3314.
25. Ekins S, Bravi G, Binkley S, Gillespie JS, Ring BJ, Wikel JH, Wrighton SA: **Three and four dimensional-quantitative structure activity relationship (3D/4D-QSAR) analyses of CYP2D6 inhibitors.** *Pharmacogenetics* (1999) **9**(4):477-489.
26. Wang J, Ramnarayan K: **Toward designing drug-like libraries: A novel computational approach for prediction of drug feasibility of compounds.** *J Combinatorial Chem* (1999) **1**(6):524-533.
27. Bohm H-J: **Computational tools for structure-based ligand design.** *Prog Biophys Mol Biol* (1996) **66**(3):197-210.
28. Gerhold D, Rushmore T, Caskey CT: **DNA chips: promising toys have become powerful tools.** *Trends Biochem Sci* (1999) **24**(5):168-173.
29. Johnson DE, Braeckman RA, Wolfgang GH: **Practical aspects of assessing toxicokinetics and toxicodynamics.** *Curr Opin Drug Discovery Dev* (1999) **2**(1):49-57.
30. Todd MD, Ulrich RG: **Emerging technologies for accelerated evaluation of potential drug candidates.** *Curr Opin Drug Discovery Dev* (1999) **2**(1):58-68.
31. Wildman SA, Crippen GM: **Prediction of physicochemical parameters by atomic contributions.** *J Chem Inf Comput Sci* (1999) **39**(5):868-873.
32. Huuskonen J, Salo M, Taskinen J: **Aqueous solubility prediction of drugs based on molecular topology and neural network modeling.** *J Chem Inf Comput Sci* (1998) **38**(3):450-456.
33. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ: **Experimental and computational approaches to estimate solubility and permeability in drug discovery and development setting.** *Adv Drug Deliv Rev* (1997) **23**:3-25.
34. Stenberg P, Luthman K, Ellens H, Lee CP, Smith PL, Lago A, Elliott JD, Artursson P: **Prediction of the intestinal absorption of endothelin receptor antagonists using three theoretical methods of increasing complexity.** *Pharm Res* (1999) **16**(10):1520-1526.
35. Erhardt PW: **Drug metabolism data: Past and present status.** *Med Chem Res* (1998) **8**:400-421.
36. Cronin MT: **Computer-aided prediction of drug toxicity in high throughput screening.** *Pharm Pharmacol Commun* (1998) **4**:157-163.
37. Kansy M, Senner F, Gubernator K: **Physicochemical high throughput screening: Parallel artificial membrane permeation assay in the description of passive absorption processes.** *J Med Chem* (1998) **41**(7):1007-1010.
38. Plumb JA: **Cell sensitivity assays: the MTT assay.** *Methods Mol Med* (1999) **8**(Cytotoxic drug resistance mechanisms):25-30.
39. Caldwell GW, Masucci JA, Chacon E: **High throughput liquid chromatography-mass spectrometry assessment of the metabolic activity of commercially available hepatocytes from 96-well plates.** *Comb Chem High-Throughput Screen* (1999) **2**(1):39-51.
40. Van Breemen RB, Nikolic D, Bolton JL: **Metabolic screening using on-line ultrafiltration mass spectrometry.** *Drug Metab Dispos* (1998) **26**(2):85-90.
- An interesting approach for doing online drug metabolism.
41. Nikolic D, Fan PW, Bolton JL, Van Breemen RB: **Screening for xenobiotic electrophilic metabolites using pulsed ultrafiltration-mass spectrometry.** *Comb Chem High-Throughput Screen* (1999) **2**(3):165-175.
42. Crespi CL, Miller VP, Penman BW: **Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450.** *Anal Biochem* (1997) **248**(1):188-190.
43. Crespi CL: **Higher-throughput screening with human cytochromes P450.** *Curr Opin Drug Discovery Dev* (1999) **2**(1):15-19.

44. Crespi CL, Miller VP, Penman BW: **High throughput screening for inhibition of cytochrome P450 metabolism.** *Med Chem Res* (1998) **8**(7/8):457-471.
- *An outstanding paper for screening the inhibition of P450 metabolism.*
45. Avdeef A, Box KJ, Comer JE, Gilges M, Hadley M, Hibbert C, Patterson W, Tam KY: **pH-Metric log P 11. pK<sub>a</sub> determination of water-insoluble drugs in organic solvent-water mixtures.** *J Pharm Biomed Anal* (1999) **20**(4):631-641.
46. Takacs-Novak K, Avdeef A: **Interlaboratory study of log P determination by shake-flask and potentiometric methods.** *J Pharm Biomed Anal* (1996) **14**(11):1405-1413.
47. Avdeef A: **pH-Metric solubility. 1. Solubility-pH profiles from Bjerrum plots. Gibbs buffer and pK<sub>a</sub> in the solid state.** *Pharm Pharmacol Comm* (1998) **4**(3):165-178.
48. Avdeef A, Box KJ, Comer JE, Hibbert C, Tam KY: **pH-Metric logP 10. Determination of liposomal membrane-water partition coefficients of ionizable drugs.** *Pharm Res* (1998) **15**(2):209-215.
49. Yang CY, Cai SJ, Liu H, Pidgeon C: **Immobilized artificial membranes - screens for drug-membrane interactions.** *Adv Drug Deliv Rev* (1997) **23**(1-3):229-256.
50. Caldwell GW, Masucci JA, Evangelisto M, White R: **Evaluation of the immobilized artificial membrane phosphatidylcholine. Drug discovery column for high-performance liquid chromatographic screening of drug-membrane interactions.** *J Chromatogr A* (1998) **800**(2):161-169.
51. Ducarne A, Neuwels M, Goldstein S, Massingham R: **IAM retention and blood brain barrier penetration.** *Eur J Med Chem* (1998) **33**:215-223.
52. Masucci JA, Caldwell GW, Foley JP: **Comparison of the retention behavior of  $\beta$ -blockers using immobilized artificial membrane chromatography and lysophospholipid micellar electrokinetic chromatography.** *J Chromatogr A* (1998) **810**(1-2):95-103.
53. Yee S: ***In vitro* permeability across Caco-2 cells (colonic) can predict *in vivo* (small intestinal) absorption in man - fact or myth.** *Pharm Res* (1997) **14**(6):763-766.
54. Irvine JD, Takahashi L, Lockhart K, Cheong J, Tolan JW, Selick HE, Grove JR: **MDCK (Madin-Darby canine kidney) cells: A tool for membrane permeability screening.** *J Pharm Sci* (1999) **88**(1):28-33.
55. Caldwell GW, Easlick SM, Gunnet J, Masucci JA, Demarest K: ***In vitro* permeability of eight  $\beta$ -blockers through Caco-2 monolayers utilizing liquid chromatography/electrospray ionization mass spectrometry.** *J Mass Spectrom* (1998) **33**(7):607-614.
56. McDonnell PA, Caldwell GW, Masucci JA: **Using capillary electrophoresis/frontal analysis to screen drugs interacting with human serum proteins.** *Electrophoresis* (1998) **19**(3):448-454.
57. Mattes WB, Li AP: **Quantitative reverse transcriptase/PCR assay for the measurement of induction in cultured hepatocytes.** *Chem Biol Interact* (1997) **107**:47-61.
58. Sanwald-Ducray P, Dow J: **Prediction of the pharmacokinetic parameters of reduced-dolasetron in man using *in vitro/in vivo* and interspecies allometric scaling.** *Xenobiotica* (1997) **27**(2):189-201.
59. Olah TV, McLoughlin DA, Gilbert JD: **The simultaneous determination of mixtures of drug candidates by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry as an *in vivo* drug screening procedure.** *Rapid Commun Mass Spectrom* (1997) **11**:17-23.
60. Bayliss MK, Frick LW: **High-throughput pharmacokinetics: Cassette dosing.** *Curr Opin Drug Discovery Dev* (1999) **2**(1):20-25.
61. Wong PS, Yoshioka K, Xie F, Kissinger PT: ***In vivo* microdialysis/liquid chromatography/tandem mass spectrometry for the on-line monitoring of melatonin in rat.** *Rapid Commun Mass Spectrom* (1999) **13**(5):407-411.
62. Masucci JA, Ortegon ME, Jones WJ, Shank RP, Caldwell GW: ***In vivo* microdialysis and liquid chromatography/thermospray mass spectrometry of the novel anticonvulsant 2,3,4,5-bis-O-(1-methylethylidene)- $\beta$ -D-fructopyranose sulfamate (topiramate) in rat brain fluid.** *J Mass Spectrom* (1998) **33**(1):85-88.
63. Obach RS, Baxter JG, Liston TE, Silber BM, Jones BC, MacIntyre F, Rance DJ, Wastall P: **The prediction of human pharmacokinetic parameters from preclinical and *in vitro* metabolism data.** *J Pharmacol Exp Ther* (1997) **283**(1):46-58.
64. Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA, Sugiyama Y: **Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data.** *Pharmacol Ther* (1997) **73**(2):147-171.
65. Houston JB, Carlile DJ: **Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices.** *Drug Metab Rev* (1997) **29**(4):891-922.
66. Meier J: **Pharmacokinetic comparison of pindolol with other beta-adrenoceptor-blocking agents.** *Am Heart J* (1982) **104**(2):364-373.
67. Hinderling PH, Schmidlin O, Seydel JK: **Quantitative relationships between structure and pharmacokinetics of beta-adrenoceptor blocking agents in man.** *J Pharmacokinet Biopharm* (1984) **12**(3):263-287.