Improving the decision-making process in the structural modification of drug candidates: enhancing metabolic stability

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The activity–exposure–toxicity relationship, which can be described as 'the rule of three', presents the single most difficult challenge in the design of drug candidates and their subsequent advancement to the development stage. ADME studies are widely used in drug discovery to optimize the balance of properties necessary to convert lead candidates into drugs that are safe and effective for humans. Metabolite characterization has become one of the key drivers of the drug discovery process, helping to optimize ADME properties and increase the success rate for drugs. Various strategies can influence drug design in the decision-making process in the structural modification of drug candidates to reduce metabolic instability.

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▼ In the past decade, advances in molecular biology, high-throughput pharmacological screens and combinatorial synthesis have resulted in a dramatic change in the drug discovery process in the pharmaceutical industry. The high cost, long development time and high failure rate in bringing drugs to market have also been important influences in this transformation. The key reasons for the low success rate of drug candidates include poor pharmacological activity, low bioavailability and high toxicity [1–11]. Thus, given the opposing requirements for absorption and metabolism, the design of drugs that not only have optimal potency and pharmacokinetic (PK) properties but also reduced toxicity is a considerable challenge: optimization of the metabolic rate of a drug and reducing toxicity are two of the key considerations during the drug discovery process [12–19]. High clearance rates, high metabolic liability (usually leads to poor bioavailability) and the formation of active or toxic metabolites (has an impact on the pharmacological and toxicological outcomes) must be avoided. Until recently, efforts in the optimization of drug candidates were directed towards the potency of the lead compounds against the target of interest and selectivity among subtypes, whereas the equally important aspects of specificity and bioavailability were left to be addressed at the end of a potency optimization cycle, which occasionally disqualified the candidates. The ability to produce this information early in the discovery phase has become increasingly important as a basis for judging whether or not a drug candidate merits further development. One approach to this dilemma would be to explore thoroughly the structural features that are required for *in vitro* pharmacological activity through combinatorial synthesis and to identify the key functional groups that are essential for activity for each chemical series. Next, modifications to the regions of the molecule that have little or no impact on the desired activity could be made in attempts to improve the metabolic properties of the candidate. Thus, an optimal level of potency can be maintained while simultaneously introducing structural features that will improve the metabolism characteristics of a compound. The rate and sites of metabolism of new chemical entities by drug-metabolizing enzymes are amenable to modulation by appropriate structural changes.

Here, strategies for the decision-making process in the structure modification of drug candidates to reduce metabolic instability are discussed and an overview of the tools used to accomplish these goals is presented. Several examples from the literature are used to demonstrate how metabolic stability has

1359-6446/04/$ – see front matter PII: S1359-6446(04)03280-5

influenced the strategies involved in drug design and has facilitated improvements in this process. In a second article in the next issue of *Drug Discovery Today*, strategies for reducing the toxicity of compounds through structural modification will be discussed.

Enhancing metabolic stability

One of the most important components in successful drug design and development is the process of identifying the optimum combination of multiple properties. Initially, it is important to determine, and then optimize, the exposure–activity–toxicity relationship, which is also referred to as 'the rule of three', for drug candidates, and thus their suitability for advancement to development. Hence, the responsibility of the drug metabolism scientist is to optimize the half-life ($t_{1/2}$) of the candidate in plasma (rate of clearance of compound), the metabolic stability of the drug and the ratio of metabolic to renal clearance. Another concern is to minimize or to eliminate gut and/or hepatic-first-pass metabolism, inhibition and/or induction of drug-metabolizing enzymes by metabolites, formation of biologically active metabolites, metabolism by polymorphically expressed drug-metabolizing enzymes and formation of reactive metabolites. There are two approaches that assess the metabolism of a compound – *in vitro* and *in vivo* investigations. The technique used depends on a variety of factors, including the nature of the program, the mindset of the company involved and the resources available. Some companies might favor high-throughput *in vitro* studies to develop SAR around metabolic stability, or even enzyme specificity, for a series of compounds. By contrast, other companies might place value on *in vivo* dosing of promising leads at the early stages, which, although of lower throughput, provides considerably more information on the fate of a particular compound than *in vitro* methods.

Various *in vitro* methods are available to evaluate metabolic stability. Among the most popular and widely used systems are liver microsomes. Microsomes retain activity of key enzymes involved in drug metabolism that reside in the smooth endoplasmic reticulum, such as cytochrome P450s (CYPs), flavin monooxygenases and glucuronosyltransferases. Isolated hepatocytes offer a valuable whole-cell *in vitro* model and retain a broader spectrum of enzymatic activities, including reticular systems, as well as cytosolic and mitochondrial enzymes. Because of a rapid loss of hepatocyte-specific functions, useful data have only been generated with short-term hepatocyte incubations or cultures. However, significant progress has been made towards enhancing the viability of cryopreserved and cultured hepatocytes. Liver slices also retain a wide array of enzyme activities and are used to assess metabolic stability. Furthermore, hepatocytes and liver slices are capable of assessing enzyme induction *in vitro*. Numerous factors, such as information about a particular chemical series and availability of tissue, influence the choice of system used in a drug discovery-screening program. For example, if a pharmacological probe has been shown to induce CYP activity, it might be preferable to use liver slices rather than microsomes for metabolic screening in this program. Cross-comparison of metabolic turnover rates in various tissues from different species can be beneficial. In addition to providing information on potential rates and routes of metabolism, interspecies comparisons could aid in the decision concerning the choice of species to be used in efficacy and toxicology models. One advantage of using *in vivo* methods to assess metabolism is that data are generated in a living species, which enables the assessment of a full early PK profile for the compound. However, *in vivo* methods are generally of low-throughput, are time-consuming and can suffer from marked species differences. In this respect, *in vitro* studies can be used in conjunction with *in vivo* experiments to select the animal model with a metabolism that is most similar to human.

Metabolic stability and intrinsic metabolic clearance

Using the concept of intrinsic metabolic clearance (CL_{int}), a good correlation between *in vitro* metabolic rates for a selected set of model substrates and hepatic extraction ratios determined from isolated perfused rat livers has been demonstrated [20]. Thus, enzyme kinetic parameters, specifically, the Michaelis–Menton constant (K_m) and maximum velocity (V_{max}), when measured under appropriate conditions, can be used to predict the hepatic CL_{int} of human and preclinical species. CL_{int} can then be calculated using Equation 1 [21].

$$CL_{int} = \frac{V_{max}}{K_m} \qquad \text{Eqn 1}$$

However, measurement of K_m and V_{max} are time consuming. In drug discovery, a single substrate concentration ($<<K_m$) can be used for the determination of the parent drug disappearance $t_{1/2}$. The hepatic CL_{int} can be calculated from the scaling of factors from microsomes as well as hepatocytes [22]. Next, the hepatic metabolic clearance (CL_h) and hepatic extraction ratio (E_h) can be calculated [23]. Integration of metabolic stability studies with other high-throughput pharmacology screens and ADME screens, such as inhibition screening, is essential. Solving a metabolic stability problem might not necessarily lead to a compound with an overall improvement in activity or even enhanced PK properties. Compounds with improved

metabolic stability could be developed only to discover problems with absorption. Reduction in metabolic clearance could be accompanied by an increase in renal or biliary clearance of the parent drug. There is also the possibility that improvements in *in vitro* CL_{int} could result from self-inhibition of one or more drug-metabolizing enzymes. This apparent improvement is only realized during the *in vitro* screening process, whereas *in vivo* the compound might exhibit saturable metabolism, nonlinear PK or drug–drug interactions. Thus, the integration of *in vitro* metabolic stability data with inhibition screening is advised.

Advantages of enhancing metabolic stability

Several advantages are associated with the enhancement of metabolic stability [24]:

- Increased bioavailability and longer $t_{1/2}$, which in turn should enable lower and less frequent dosing, thus promoting improved patient compliance.
- Improved congruence between dose and plasma concentration, thus reducing or even eliminating the need for expensive therapeutic monitoring.
- Reduction in metabolic turnover rates from different species, which could enable a better extrapolation of animal data to humans.
- Lower patient-to-patient and intrapatient variability in drug levels, because this is largely based on differences in drug metabolic capacity.
- Diminishing the number and significance of active metabolites and thus reducing the need for further studies on drug metabolites in animals and humans.

Strategies to enhance metabolic stability

To enhance microsomal stability, *in vitro* metabolism studies can be performed to confirm formation of metabolites, as well as to provide quantitative analysis of major metabolites. After identifying moieties that contribute to activity (the pharmacophore) and other functional groups necessary for activity, several modifications can be used to enhance metabolic stability. In general, metabolism can be reduced by incorporation of stable functional groups (i.e. blocking groups) at metabolically vulnerable sites or by decreasing the lipophilicity of the compound. Substrate SARs of metabolizing enzymes have to be accommodated within the SARs of the actual pharmacological target. Several strategies have been used to modify the molecular structure of a compound (where appropriate) to enhance its metabolic stability [25,26]:

- Deactivation of aromatic rings to facilitate oxidation through substitution with strongly electron-withdrawing groups (e.g. CF_3, SO_2NH_2, SO_3^-).
- Introduction of an *N*-t-butyl group to prevent *N*-dealkylation.
- Replacement of a labile ester linkage with an amide group.
- Constraining the molecule in a conformation that is unfavorable to the metabolic pathway, more typically, protecting the labile moiety by steric shielding.
- The phenolic function has consistently been shown to be rapidly glucuronidated. Thus, avoidance of this moiety in a sterically unhindered position is advised in any compound that is intended for oral use.
- Avoidance of other conjugation reactions as primary clearance pathways would also be advised in the design stage in any drug that is to be administered orally.
- Occasionally, the optimum strategy is to anticipate the probable route of metabolism and prepare the expected metabolite if it has adequate intrinsic activity. For example, *N*-oxides are frequently as active as the parent amine but do not undergo further *N*-oxidation.

There are numerous examples from the literature of methods used to enhance metabolic stability in the molecular design (Table 1) [27–36]. In the following sections, various strategies for reducing compound lipophilicity and blocking sites of oxidative metabolism are outlined.

Reducing lipophilicity

One strategy for improving the metabolic stability of a compound is to reduce the overall lipophilicity (e.g. logP and logD) of the structure. This is because the binding site of metabolizing enzymes is generally lipophilic in nature and hence these enzymes more readily accept lipophilic molecules. As exemplified in the study of the human rhinovirus 3C protease inhibitor by Dragovich *et al*. [27], the lead compound, which exhibited poor oral bioavailability *in vivo* in monkey, was subjected to PK optimization with the benzyl group being identified as a position for reducing lipophilicity without compromising activity. Introduction of substituents with reduced lipophilicity led to the propargyl analog **2** (Table 1) and the ethyl analog **3** (Table 1), both of which had reduced calculated logP (clogP) values when compared with the parent molecule. These compounds demonstrated improved oral exposure in monkey, highlighting a simple yet effective way to improve the metabolic profile of a compound. However, lipophilic groups are usually involved in binding to the biological target and therefore this method is not always successful.

An alternative approach towards lowering the lipophilicity of a lead compound is to introduce isosteric atoms or functional groups into the molecule that impart increased polarity. The incorporation of a heteroatom (e.g. a nitrogen atom introduced into a benzene ring to form the more polar pyridine) or instigation of transformations to produce a more polar group (e.g. conversion of a ketone to the

Table 1. Enhancement of metabolic stability through structural modification

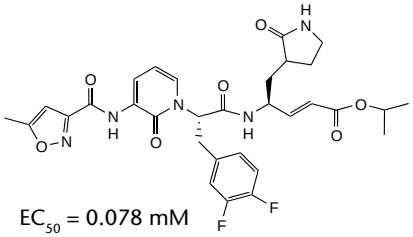
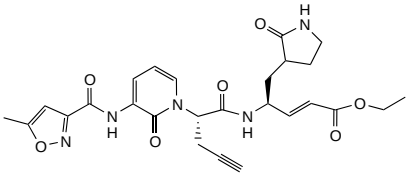
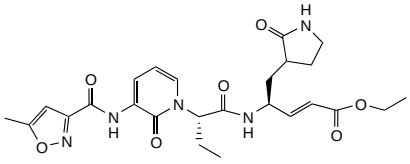
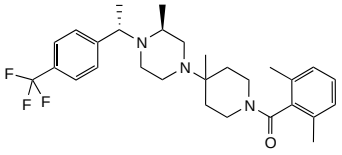
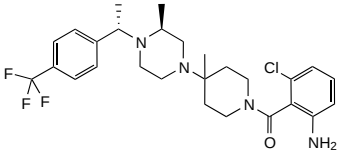
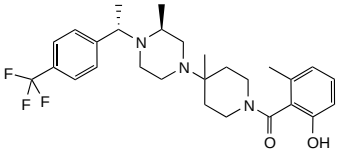
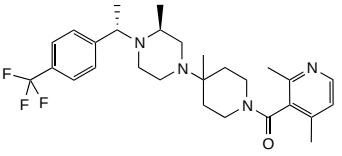
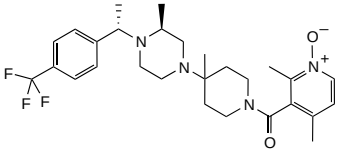
Approach and/or strategy	Enzyme or pathway	Lead compound	Optimized compound	Experimental model	Therapeutic class	Refs
Modification to improve lipophilicity and metabolic stability						
Reduce logP and logD						
	NA	EC_{50} = 0.078 mM clogP = 2.070 C 7 h = 0.012 μM	EC_{50} = 0.058 μM clogP = 0.180 C 7 h = 0.057 μM	Orally dosed monkey	3C protease inhibitor	[27]
						
	NA		EC_{50} = 0.047 μM clogP = 0.66 C 7 h = 0.896 μM	Orally dosed monkey	3C protease inhibitor	[27]
Introduce isosteric atoms or polar functional group						
	NA	K_i = 1.000 nM AUC_{0-6h} = 0.922 μg ml^{-1} h^{-1}	K_i = 5.000 nM AUC_{0-6h} = 1.872 μg ml^{-1} h^{-1}	Orally dosed rat	CCR5 antagonists	[28]
						
	NA		K_i = 5.000 nM AUC_{0-6h} = 2.543 μg ml^{-1} h^{-1}	Orally dosed rat	CCR5 antagonists	[28]
						
	NA		K_i = 2.300 nM AUC_{0-6h} = 3.905 μg ml^{-1} h^{-1}	Orally dosed rat	CCR5 antagonists	[28]
						
	NA		K_i = 7.000 nM AUC_{0-6h} = 9.243 μg ml^{-1} h^{-1}	Orally dosed rat	CCR5 antagonists	[28]

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Table 1. (continued)

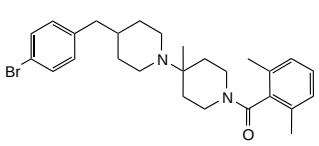
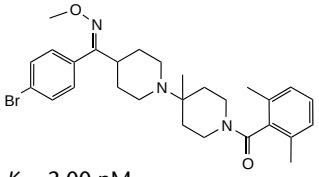
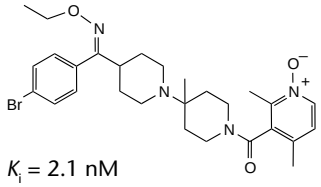
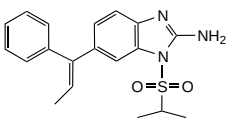
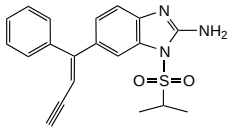
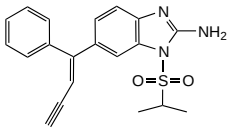
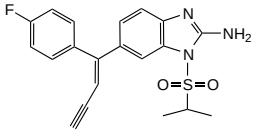
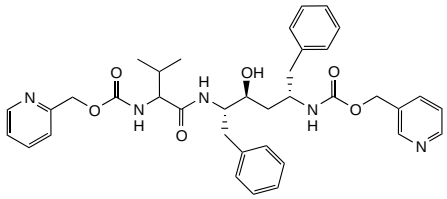
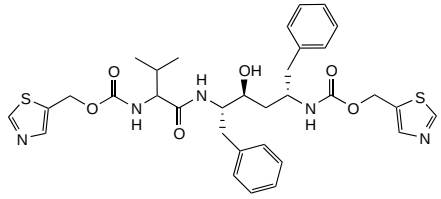
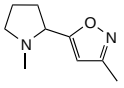
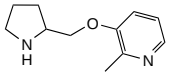
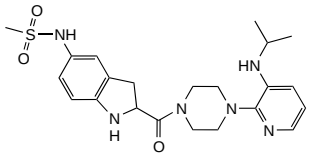
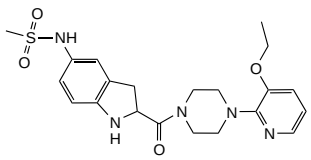
Modification of metabolically labile groups					
Remove or block the vulnerable site of metabolism					
Benzylic oxidation	K_i = 66.00 nM AUC $_{0-6h}$ = 0.04 μg ml^{-1} h^{-1}	K_i = 2.00 nM AUC $_{0-6h}$ = 1.40 μg ml^{-1} h^{-1}	Orally dosed rat	CCR5 antagonists	[29]
					
	K_i = 2.1 nM AUC $_{0-6h}$ = 6.5 μg ml^{-1} h^{-1}		Orally dosed rat	CCR5 antagonists	[29]
					
Allylic oxidation	IC_{50} = 0.06 μg ml^{-1} C_{max} = 14–140 ng ml^{-1}	IC_{50} = 0.02 μg ml^{-1} C_{max} = 70–300 ng ml^{-1}	Orally dosed monkey	Vinylacetylene antivirals	[30]
					
Phenyl oxidation	IC_{50} = 0.02 μg ml^{-1} %F = 9.00	IC_{50} = 0.04 μg ml^{-1} %F = 23.00	Orally dosed monkey	Vinylacetylene antivirals	[30]
					
N-oxidation	AUC = 1.98 μg ml^{-1} h^{-1} %F = 26.00	AUC = 4.24 μg ml^{-1} h^{-1} %F = 47.00	Orally dosed rat	HIV protease inhibitors	[31]
					
N-demethylation	$t_{1/2}$ = 3.0 h %F = 1.2	$t_{1/2}$ = 24.0 h %F = 61.5	Dog liver slices	nAChR ligands	[32]
					
N-dealkylation	$t_{1/2}$ = 10.8 min	$t_{1/2}$ = 46.8 min	Rat liver microsomes	BHAP reverse transcriptase inhibitors	[33]

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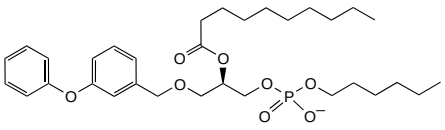
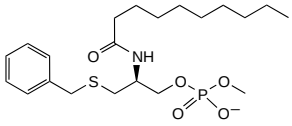
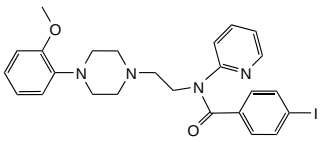
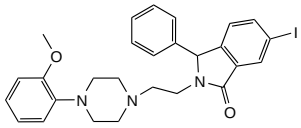
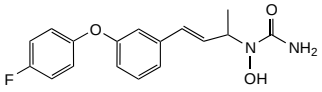
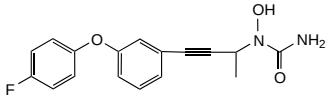
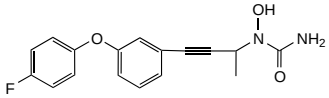
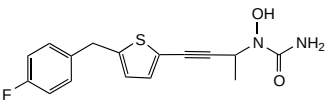
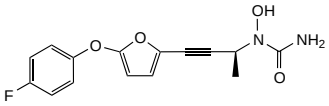
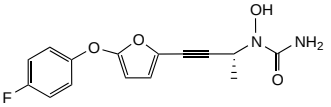
Table 1. (continued)

					
Ester hydrolysis	$t_{1/2}$ = 33 min C_{max} = 465 ng ml^{-1} %F = 4	$t_{1/2}$ = 39 min C_{max} = 3261 ng ml^{-1} %F = 90	Rat blood and plasma, liver microsomes and homogenate	Hospholipase A inhibitors	[34]
					
Amide hydrolysis	K_i = 0.200 nM 40% and >60% degradation in human liver cytosol and microsomes, respectively	K_i = 0.069 nM 10% and <5% degradation in human liver cytosol and microsomes, respectively	Human liver cytosol and microsomes	5-HT$_{1A}$ receptor ligand	[35]
					
Glucuronidation (effect of linker)	UDPGA rate = 0.19 nM, min^{-1} mg^{-1} of protein $t_{1/2}$ = 4.70 h	UDPGA rate = 0.05 nM, min^{-1} mg^{-1} of protein $t_{1/2}$ = 5.50 h	Monkey liver microsomes and plasma	5-LO inhibitors	[36]
					
Glucuronidation (effect of template)	UDPGA rate = 0.050 nM, min^{-1} mg^{-1} of protein $t_{1/2}$ = 5.500 h	UDPGA rate = 0.012 nM min^{-1} mg^{-1} of protein $t_{1/2}$ = 14.500 h	Monkey liver microsomes and plasma	5-LO inhibitors	[36]
					
Glucuronidation (effect of stereochemistry)	UDPGA rate = 0.02 nM, min^{-1} mg^{-1} of protein $t_{1/2}$ = 7.70 h	UDPGA rate = 0.01 nM, min^{-1} mg^{-1} of protein $t_{1/2}$ = 8.70 h	Monkey liver microsomes and plasma	5-LO inhibitors	[36]

Abbreviations: AUC, area under curve; BHAP, bis(heteroaryl)piperazine; C 7 h, concentration at 7 h; CCR5, chemokine (cc motif) receptor 5; clogP, calculated logP; C_{max}, maximum plasma concentration; %F, % bioavailability; 5-HT$_{1A}$, 5-hydroxytryptamine 1A receptor; 5-LO, 5-lipoxygenase; NA, not available; nAChR, nicotinic acetylcholine receptor; $t_{1/2}$, half-life of compound; UDPGA, uridine 5'-diphosphoglucuronic acid

corresponding carboxamide) could lead to increased polarity. Tagat and co-workers [28] used this approach to enhance the pharmacokinetics of a chemokine (cc motif) receptor (CCR)5 antagonist lead in their HIV-1 inhibitor investigations [28]. The benzamide of the lead compound was identified as a modification site for reducing lipophilicity and changes were made to alter the substitution pattern of the ring. The more polar anthranilamide and salicylamide afforded significant improvements in rat oral blood levels, with a resultant substantial increase in the area-under-curve. However, the optimum results, in terms of improving metabolic stability, were achieved by introducing a heteroatom into the phenyl ring, with the more polar nicotinamide showing satisfactory oral blood levels in rat. In addition, the rapid formation of a single metabolite of nicotinamide in the plasma samples was reported, which was thought to be a product of *N*-oxidation. Thus, the pyridine *N*-oxide was prepared and found to be an excellent compound in terms of potency and oral bioavailability in rat, dog and monkey.

Blocking metabolically labile groups

A much more elegant approach towards improving metabolic stability is to remove or block the vulnerable site of metabolism. For example, sites that have been identified as being potentially labile towards oxidation (e.g. benzylic or allylic positions) can be blocked through the introduction of a halogen atom onto the carbon atom of the site, or by replacing the benzylic CH_2 with an isostere such as an oxygen atom. Using this approach, Palani and colleagues [29] conducted a metabolism-driven optimization of a second-generation lead from CCR5 antagonist investigations [28]. The lead compound exhibited good potency against the receptor, but showed poor oral bioavailability in rat. The benzylic position was identified as a metabolically labile site and a variety of isosteres were introduced to this position, including O, CHOH and C=O. Eventually, the methoxime was identified as a potent, metabolically stable derivative and formed the next generation lead compound.

The work of Victor *et al.* [30] provides another example of the strategy of blocking sites of potential oxidative metabolism to improve metabolic stability. Replacing a methyl group vulnerable to allylic oxidation with acetylene resulted in up to a fivefold improvement in blood levels of the compound in monkey. Replacement of a methyl group with an acetylenyl moiety and *p*-fluoro substitution on the phenyl ring led to an improvement of approximately twofold in oral bioavailability in monkey. It is possible that the electron-withdrawing character of fluorine deactivates the aromatic ring towards metabolic oxidation. Alternatively, the somewhat lipophilic character of fluorine, coupled with its ability to form hydrogen bonds, could potentially provide a unique mixture of physical properties that assist in the absorption process.

Kempf and co-workers [31] used the principle of bioisosterism in their efforts to synthesize the potent HIV protease inhibitor ritonavir. Pyridyl *N*-oxidation was a limiting route of metabolism in one of their early lead compounds. Thus, a systematic search for other heterocyclic groups that would simultaneously decrease the rate of metabolism and maintain adequate solubility to ensure absorption as well as inhibitory activity was performed. Ultimately, pyridyl groups were replaced with metabolism-resistant thiazole groups, resulting in an analog (ritonovir) that exhibited a nearly twofold improvement in bioavailability while sustaining potent inhibition of HIV protease.

Lin and colleagues [32] observed up to a ~50-fold improvement in potency at the nicotinic acetylcholine receptor when a *N*-desmethyl analog was used instead of a *N*-methyl analog, which was susceptible to *N*-demethylation. Genin and colleagues [33] realized an approximate fourfold improvement when they replaced a metabolically susceptible 3-isopropylamino substituent on the lead compound with an ethoxy substituent. Although it could be reasoned that this substituent would also be rapidly metabolized, in this case it proved to be stable, at least when compared with the stability of the lead compound.

Modification of metabolically labile groups

Functional groups are open to other types of metabolism in addition to oxidation: the presence of amidases and esterases in the liver is well-documented and these enzymes can hydrolyze amides and esters, respectively. Furthermore, Phase II metabolism, where polar functionality (e.g. glucuronides and sulfates) is imparted on a molecule to make it more water-soluble and hence easier to clear, can be targeted as a method for improving overall metabolic stability.

Replacing a labile ester linkage with an amide group is known to impart added stability with respect to esterase activity. A study performed by Blanchard *et al.* [34] exemplifies the applicability of this approach – a 22-fold improvement in bioavailability for an amide when compared with the corresponding ester was observed.

During a metabolism-driven optimization of a series of new arylpiperazine benzamido derivatives as potential ligands for 5-hydroxytryptamine-1A receptors, the low brain uptake observed in the human subject was attributed to rapid metabolism (amide hydrolysis) [35]: in other species (rats and monkeys), the metabolic pathway of the compounds (cleavage of the amide bond by liver microsomes) appears to be much slower. It was also reported that the cyclized amide derivatives afforded >twofold improvement in amide stability compared with the open-chain lead compound.

An extensive body of work on *N*-hydroxyurea inhibitors of 5-lipoxygenase related to zileuton provides an excellent opportunity to examine SARs with respect to conjugation with glucuronic acid [36]. It was discovered early on in this investigation that glucuronidation of the *N*-hydroxyl moiety was an activity-limiting step and it therefore became crucial to impart metabolic stability towards this conjugation. The *N*-hydroxyurea compounds were divided into three areas for structural modification; the template, the linking group and the pharmacophore. In zileuton, the *N*-hydroxyurea moiety was identified as an optimal pharmacophore for potency and selectivity; therefore, it was not modified in the search for metabolically stable compounds. The template and link components were the major focus of medicinal chemistry efforts to optimize *in vivo* duration in the cynomolgus monkey. First, the linker group joining the template with the *N*-hydroxyurea pharmacophore was systematically modified, and then the procedure was repeated with the benzthiophene template. Each compound was screened for stability to glucuronidation of *N*-hydroxyurea. Of the linker moieties,

Table 2. Tools for the identification and characterization of metabolites

Stage	Tools	Uses
Presynthesis compound for drug discovery	*In silico*	Assistance with chemical synthesis efforts to select and/or eliminate compounds
Compounds available for screening and early drug discovery	*In silico* LC–MS–MS	Assistance with synthesis efforts to block or enhance metabolism Identify simple and major metabolites (e.g. dealkylations and conjugations such as glucuronide) Prediction of metabolites likely to be formed *in vivo*
Late drug discovery and candidate selection	LC–MS–MS QTOF (high resolution and exact mass measurement) Ion trap (MS^3) H–D exchange	Determination of metabolic differences between species Identification of potential pharmacologically active or toxic metabolites
Preclinical and clinical development	LC–MS–MS QTOF Ion trap (MS^3) H–D exchange Radioactivity detector LC–MS–NMR	Determination of the percentage of metabolite formed *in vitro* or *in vivo* Most definitive presynthesis confirmation available for metabolite structure Synthesis of metabolites for toxicology testing Comparison of human pathways Identification of drug–drug interactions

Abbreviations: LC–MS, liquid chromatography–mass spectrometry; LC–MS–MS, liquid chromatography–mass spectrometry–mass spectrometry; MS^3, tandem mass spectrometry; QTOF, quadrupole time-of-flight.

compounds with acetylene linker groups were generally found to have lower uridine 5′-diphosphoglucuronic acid (UDPGA) rates than any of the links tested. This lower UDPGA rate results in the longest *in vivo* duration in monkey. Apparently, the rigid conformation required by the acetylene group disrupts binding in the active site of uridine 5′-diphosphate-glucuronosyltransferase and diminishes conjugation.

Many variations of the template group were also tested in the UDPGA assay. Compounds containing the benzthiophene template found in zileuton demonstrated more rapid rates of glucuronidation. Conversely, biaryl templates such as phenoxyphenyl, phenoxyfuran and phenoxythiophene demonstrated reduced rates of glucuronidation. These different metabolic stability results could be attributed to changes in lipophilicity and/or remote electronic effects.

Finally, the importance of the stereochemistry of the carbon center immediately adjacent to *N*-hydroxyurea was investigated. *N*-hydroxyurea compounds containing a methyl branch on the linking group had already shown longer *in vivo* durations than their methylene analogs, which encouraged further work with this methyl branch series. The addition of the methyl group introduced a chiral center and resulted in a compound with separable *R*- and *S*-enantiomers. A ~twofold difference in the glucuronidation rate, depending on the configuration of the adjacent carbon center, was reported.

Analytical tools

In recent years, tools available for the development of new drugs have dramatically improved [12–19]. New screening methodologies have contributed considerably to the efficiency of the drug discovery process. In addition, *in silico* screening methods enable the evaluation of virtual compounds. As a consequence, the lead identification, optimization and development process has shown a marked enhancement in terms of speed and efficiency. The use of a robotic system for sample preparation in conjunction with *in silico* software to predict and identify potential metabolites, as well as to postulate hypothetical metabolite chemical structures, works well to accomplish rapid, accurate metabolite identification. This can be combined with liquid chromatography–mass spectrometry (LC–MS) to determine exact mass measurements (accurate mass) for sample analysis, and LC–MS–NMR and online H–D exchange for further metabolite structure confirmation and elucidation. A summary of the tools that can be used for metabolite characterization is presented in Table 2 [17]; it should be noted that any of the tools shown for use during a particular stage can be applied to any of the tasks listed.

Conclusions

Structural information on metabolites is a considerable aid in enhancing, as well as streamlining, the process of developing

new drug candidates, which in turn has significant value in several important aspects of drug discovery and development. By improving the identification of beneficial and harmful metabolites, suggestions for structural modifications will optimize the probability that other compounds in the series are more successful. *In silico* and *in vitro* techniques are available to screen compounds for key ADME characteristics, which, when applied within a rational strategy, can make a major contribution to the design and selection of successful drug candidates. Structural modifications to solve a metabolic stability problem might not necessarily lead to a compound with enhanced PK properties. Solving metabolic stability problems at one site could result in the increase in the rate of metabolism at another site, a phenomenon known as metabolic switching. Furthermore, reduction in hepatic clearance could lead to increased renal or biliary clearance of a parent drug or inhibition of one or more drug-metabolizing enzymes. Therefore, it is advisable that *in vitro* metabolic stability data be integrated with other ADME screening results.

References

1 Kantharaj, E. *et al.* (2003) Simultaneous measurement of drug metabolic stability and identification of metabolites using ion-trap mass spectrometry. *Rapid Commun. Mass Spectrom.* 17, 2661–2668
2 Watt, A.P. *et al.* (2003) Metabolite identification in drug discovery. *Curr. Opin. Drug Discov. Devel.* 6, 57–65
3 Taylor, E.W. *et al.* (2002) Accelerating the drug optimization process: identification, structure elucidation, and quantification of *in vivo* metabolites using stable isotopes with LC/MSn and the chemiluminescent nitrogen detector. *Anal. Chem.* 74, 3232–3238
4 Lesko, L.L. *et al.* (2000) Optimizing the science of drug development: opportunities for better candidate selection and accelerated evaluation in humans. *Pharm. Res.* 17, 1335–1343
5 Kubinyi, H. *et al.* (1977) Quantitative structure–activity relationships. 7. The bilinear model, a new model for nonlinear dependence of biological activity on hydrophobic character. *J. Med. Chem.* 20, 625–629
6 Meyboom, R.H.B. *et al.* (2000) An ABC of drug-related problems. *Drug Saf.* 22, 415–423
7 Greene, N. *et al.* (2002) Computer systems for the prediction of toxicity: an update. *Adv. Drug Deliv. Rev.* 54, 417–431
8 Pirmohamed, M. *et al.* (1998) Adverse drug reactions. *Br. Med. J.* 316, 1295–1298
9 Richard, A.M. *et al.* (1998) Structure-based methods for predicting mutagenicity and carcinogenicity: are we there yet? *Mutat. Res.* 400, 493–507
10 Lasser, K.E. *et al.* (2002) Timing of new black box warnings and withdrawals for prescription medications. *J. Am. Med. Assoc.* 287, 2215–2220
11 Bakke, O.M. *et al.* (1995) Drug safety discontinuations in the United Kingdom, the United States and Spain from 1974 through 1993: a regulatory perspective. *Clin. Pharmacol. Ther.* 58, 108–117
12 Tiller, P.R. *et al.* (2002) Liquid chromatography/tandem mass spectrometric quantification with metabolite screening as a strategy to enhance the early drug discovery process. *Rapid Commun. Mass Spectrom.* 16, 1225–1231
13 Uetrecht, J.P. *et al.* (2002) Screening for the potential of a drug candidate to cause idiosyncratic drug reactions. *Drug Discov. Today* 5, 832–837
14 Nassar, A.E. and Lopez-Anaya, A. (2004) Strategies for dealing with reactive intermediates in drug discovery and development. *Curr. Opin. Drug Discov. Devel.* 7, 126–136
15 Nassar, A-E.F. *et al.* (2003) Metabolite characterization in drug discovery utilizing robotic liquid-handling, quadrupole time-of-flight mass spectrometry and *in silico* prediction. *Curr. Drug Metab.* 4, 259–271
16 Nassar, A-E.F. *et al.* (2003) Online hydrogen-deuterium exchange and a tandem quadrupole time-of-flight mass spectrometer coupled with liquid chromatography for metabolite identification in drug metabolism. *J. Chromatogr. Sci.* 41, 398–404
17 Nassar, A-E.F. *et al.* (2004) Strategies for dealing with metabolite elucidation in drug discovery and development. *Drug Discov. Today* 9, 317–327
18 Plumb, R.S. *et al.* (2003) Use of liquid chromatography/time-of-flight mass spectrometry and multivariate statistical analysis shows promise for the detection of drug metabolites in biological fluids. *Rapid Commun. Mass Spectrom.* 17, 2632–2638
19 Kostiainen, R. *et al.* (2003) Liquid chromatography/atmospheric pressure ionization-mass spectrometry in drug metabolism studies. *J. Mass Spectrom.* 38, 357–372
20 Rane, A. *et al.* (1977) Prediction of hepatic extraction ratio from *in vitro* measurement of intrinsic clearance. *J. Pharmacol. Exp. Ther.* 200, 420–424
21 Segel, I.H. *et al.* (1993) Kinetics of Unireactant Enzymes. In *Enzyme Kinetics*. pp. 18–89, John Wiley & Sons
22 Obach, R.S. *et al.* (1999) Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: an examination of *in vitro* half-life approach and nonspecific binding to microsomes. *Drug Metab. Dispos.* 27, 1350–1359
23 Obach, R.S. *et al.* (1997) Nonspecific binding to microsomes: impact on scale-up of *in vitro* intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol. *Drug Metab. Dispos.* 25, 1359–1369
24 Ariens, E.J. and Simonis, A.M. (1982) Optimization of Pharmacokinetics, an Essential Aspect of Drug Development, by 'Metabolic Stabilization'. In *Strategy in Drug Research (Pharmacochemistry Library),* pp. 165–178, Elsevier
25 Testa, B. and Meyer, J.M. (1990) Drug metabolism and pharmacokinetics: implications for drug design. *Acta Pharm. Jugosl.* 40, 315–350
26 Humphrey, M.J. *et al.* (1992) Role of metabolism and pharmacokinetic studies in the discovery of new drugs - present and future perspectives. *Xenobiotica* 22, 743–755
27 Dragovich, P. *et al.* (2003) Structure-based design, synthesis, and biological evaluation of irreversible human rhinovirus 3C protease inhibitors. 8. Pharmacological optimization of orally bioavailable 2-pyridone-containing peptidomimetics. *J. Med. Chem.* 46, 4572–4585
28 Tagat, J.R. *et al.* (2001) Piperazine-based CCR5 antagonists as HIV-1 inhibitors. II. Discovery of 1-[(2,4-dimethyl-3-pyridinyl)carbonyl]-4-methyl-4-[3(*S*)-methyl-4-[1(*S*)-[4-(trifluoromethyl)phenyl]ethyl]-1-piperazinyl]- piperidine N1-oxide (Sch-350634), an orally bioavailable, potent CCR5 antagonist. *J. Med. Chem.* 44, 3343–3346
29 Palani, A. *et al.* (2002) Synthesis, SAR, and biological evaluation of oximino-piperidino-piperidine amides. 1. Orally bioavailable CCR5 receptor antagonists with potent anti-HIV activity. *J. Med. Chem.* 45, 3143–3160
30 Victor, F. *et al.* (1997) Synthesis, antiviral activity, and biological properties of vinylacetylene analogs of enviroxime. *J. Med. Chem.* 40, 1511–1518
31 Kempf, D. *et al.* (1998) Discovery of ritonavir, a potent Inhibitor of HIV protease with high oral bioavailability and clinical efficacy. *J. Med. Chem.* 41, 602–617
32 Lin, N.H. *et al.* (1997) Structure-activity studies on 2-methyl-3-(2(*S*)-pyrrolidinylmethoxy) pyridine (ABT-089): an orally bioavailable 3-pyridyl ether nicotinic acetylcholine receptor ligand with cognition-enhancing properties. *J. Med. Chem.* 40, 385–390
33 Genin, M. *et al.* (1996) Synthesis and bioactivity of novel bis(heteroaryl)piperazine reverse transcriptase inhibitors: structure–activity relationships and increased metabolic stability of novel substituted pyridine analogs. *J. Med. Chem.* 39, 5267–5275
34 Blanchard, S.G. *et al.* (1998) Discovery of bioavailable inhibitors of secretory phospholipase A2. *Pharm. Biotechnol.* 11, 445–463
35 Zhuang, Z.P. *et al.* (1998) Isoindol-1-one analogues of 4-(2′-methoxyphenyl)-1-[2′-[N-(2″-pyridyl)-*p*-iodobenzamido]ethyl]piperazine (p-MPPI) as 5-HT1A receptor ligands. *J. Med. Chem.* 41, 157–166
36 Bouska, J.J. *et al.* (1997) Improving the *in vivo* duration of 5-lipoxygenase inhibitors: application of an *in vitro* glucuronosyltransferase assay. *Drug Metab. Dispos.* 25, 1032–1038