Metabolic Soft Spot Identification and Compound Optimization in Early Discovery Phases Using MetaSite and LC-MS/MS Validation

Markus Trunzer, Bernard Faller, and Alfred Zimmerlin*

Novartis Institutes for BioMedical Research, Metabolism and Pharmacokinetics, Postfach, CH-4002, Basel, Switzerland

Received July 14, 2008

Metabolic stability is a key property to enable drugs to reach therapeutic concentrations. Microsomal clearance assays are used to dial out labile compounds in early discovery phases. However, because they do not provide any information on soft spots, the rational design of more stable compounds remains challenging. A robust soft spot identification procedure combining in silico prediction ranking using MetaSite and mass-spectrometric confirmation is described. MetaSite's first rank order predictions were experimentally confirmed for only about 55% of the compounds. For another 29% of the compounds, the second (20%) or the third (9%) rank order predictions were detected. This automatic and high-throughput reprioritization of a likely soft-spot increases the likelihood of working on the right soft spot from about 50% to more than 80%. With this information, the structure−metabolism relationships are likely to be understood faster and earlier in drug discovery.

Introduction

Cytochromes P450 (CYP[a]) enzymes are major contributors to drug metabolism.[1] In humans, mainly five hepatic CYP isoforms, CYP1A2, 2C9, 2C19, 2D6, and 3A4 catalyze (alone or in combinations) most of the metabolic transformations that lead to drug elimination.[2] Almost three-quarters of marketed drugs have CYP pathways in their elimination process,[3] and for many of them, this pathway has a major impact on their blood levels and dosing schedule.[4] Because these enzymes are also associated with high interindividual variability and risk for drug−drug interactions, their involvement is not always welcome when developing new drugs.[5] Of particular concern is a high CYP mediated clearance, as it would not only necessitate high and frequent dosage but also carry the risk of massive variation in exposure. When drug candidates exhibit a high CYP dependent clearance, efforts should be made to stabilize them. Relatively high throughput hepatic microsomal assays measuring the in vitro CYP dependent intrinsic clearances of drug candidates have recently been established throughout the pharmaceutical industry,[6] but understanding structure−metabolism relationships (SMR) and reducing clearance is a particularly difficult task because of the relatively wide and overlapping substrate specificity of CYP enzymes.[7] The accurate identification of the soft spots of the molecules would indeed very much help identifying and modifying the culprit substructure. Currently, the elucidation of metabolic pathways involves advanced separation and structural elucidation techniques and is a highly labor-intensive task that cannot be envisaged for the many compounds investigated during lead optimization.[8] Yet it is in this early phase that the knowledge of structure metabolism relationships would be the most useful. Because chemistry efforts are near culmination, it would be much easier to invest in the synthesis of hopefully more stable (not necessarily the more potent) analogues when a reasonable hypothesis on the soft spot can be made.

In this paper, we describe how, for cytochrome P450 dependent metabolism, the combination of in silico site of metabolism (SoM) prediction combined with experimental evidence from high throughput microsomal assays can help to synthesize more stable compounds and establish SMR for chemical series much faster and much earlier in the drug discovery process.

Methods

Test Sets. A test set of 18 marketed drugs for which a main CYP dependent pathway had been described in human liver microsomes has been extracted from MDL Metabolite Database version 2.3.3 (Symyx Technologies, Inc., Santa Clara, CA). A set of 95 Novartis Research compounds was constructed by random selection within the 2007 Novartis list of compounds for which a high NADPH dependent in vitro intrinsic clearance (>150 μL/min·mg) had been measured.

Human Microsomal Incubations. The experiments were performed in 96-well glass plates at 37 °C on an automated Tecan EVO platform. Test articles at a concentration of 10 mM in pure DMSO were diluted 1:1000 in water to 10 μM. This solution (30 μL) was added to 120 μL of human liver microsomal protein (1.25 mg/mL) suspended in phosphate buffer (pH 7.4). Reactions were initiated by the addition of 150 μL of a cofactor solution containing 2 mM NADPH. At specific reaction time points (0, 5, 20, and 30 min), aliquots (50 μL) were removed and reactions were terminated by the addition to acetonitrile (100 μL) containing the analytical internal standards (1 μM alprenolol and 1.6 μM chlorzoxazone) and stored at −20 °C for at least 1 h to allow complete precipitation of proteins. The samples were then centrifuged at 5000*g* at 4 °C for 35 min, and 20 μL of the supernatants were analyzed by LC-MS/MS for quantitation of the remaining test article. The percentage of test article remaining, relative to time zero minute incubation, is used to estimate the in vitro elimination-rate constant (k_{mic}), which is used to calculate the in vitro metabolic clearance rates.[9]

LC-MS/MS Analysis. Analysis of samples was performed on a high performance liquid chromatography−tandem mass spectrometry (LC-MS/MS) system consisting of a TSQ Quantum Discovery Max mass spectrometer controlled by QuickQuan 2.0, an electrospray ion source (Ion Max electrospray interface) from Thermo Fisher Scientific Inc. (Waltham, MA), a CTC-HTS Pal autosampler

* To whom correspondence should be addressed. Phone: +41(0)613243326. Fax: +41(0)613247671. E-mail: alfred.zimmerlin@novartis.com.

[a] Abbreviations: CYP, cytochrome P450; SMR, structure−metabolism relationship; SoM, site of metabolism; NCE, new chemical entity; SRM, single reaction monitoring; PM, predicted metabolite; LC-MS/MS, liquid chromatography−tandem mass spectrometry.

10.1021/jm8008663 CCC: $40.75
Published on Web 12/24/2008

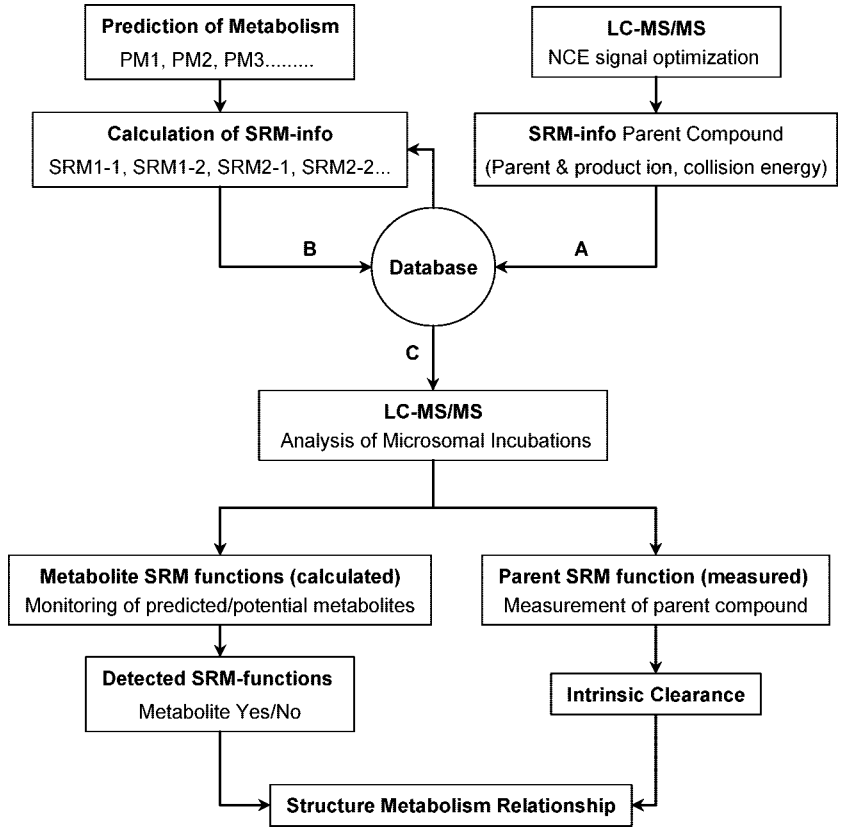

Figure 1. Experimental confirmation of site of CYP dependent metabolism and construction of structure metabolism relationship. Tuning parameters for the test articles are gained by automatic optimization of the compound signal and stored in a database (A). The metabolite monoisotopic masses predicted by MetaSite (PM) are combined with the SRM-info of parent compound to generate metabolite specific SRMs (B). For acquisition, the tuning parameter are taken from the database and used to measure the test article and monitor the potential/predicted metabolites (C). Information on soft spots is compiled within research programs or for specific chemical scaffolds to derive structure metabolism relationships and initiate problem solving.

(CTC Analytics, Zwingen, Switzerland) with a sample cooling unit (10 °C), and a Rheos pump model 2000 (Thermo Fisher Scientific Inc., Waltham, MA). Compound specific parameters (tube lens and precursor ion, collision energy, and product ion for single reaction monitoring (SRM)) were obtained by automatic tuning using the QuickQuan software. These parameters were stored in the QuickQuan database to be used for selective quantitation of each test article.

Samples were separated on a Zorbax SB-C18 Rapid Resolution HT, 2.1 mm × 30 mm, 3.5 μm (Agilent Technologies, Basel, Switzerland). The components were eluted with a gradient of 0.1% formic acid (mobile phase A) versus 0.1% formic acid in acetonitrile (mobile phase B) at a flow of 250 μL/min using the following gradient: 0 min 5% B; 1 min 5% B; 3 min 100% B; 4.8 min 100% B; 4.81 min 5% B; 5.8 min 5% B. The injection volume was 20 μL. The first 1.5 min of eluent were diverted to waste to protect in ion source from salts and polar impurities from the incubation.

Predictions of Metabolites. Predictions of metabolites were done with a beta version of MetaSite 3.0 (Molecular Discovery Ltd., Middlesex, UK, www.moldiscovery.com) using the P450 liver model, reactivity correction, and a minimal mass threshold of 100 Da for predicted metabolites. The P450 liver model displays a consensus of the SoM predictions of the three major liver isoforms CYP3A4, CYP2D6, and CYP2C9. Stereochemistry was ignored and the smaller part of complementary metabolites (cleavage) was rejected. The predicted metabolites were exported as text-file, which was used to automatically calculate, together with parent fragment-information, metabolite specific selected reaction monitoring functions (SRM) for mass spectrometry analysis. The most likely structure of the product ions was determined with the help of using Mass Frontier when needed (Thermo Fisher Scientific Inc., Waltham, MA).

Single Reaction Monitoring Information of Metabolites. The calculation of specific SRM information of predicted metabolites was done with Microsoft Excel using the measured optimal SRM-information of parent compound and the predicted mass changes for the metabolites. For every metabolite, two specific SRM functions (SRM1−1 and SRM1−2 for example) were generally calculated based on the precursor and product ion of parent compound. For the first SRM function of predicted metabolite 1 (PM1), the original mass of the product ion was used (SRM1−1), and for the second SRM-function, the difference corresponding to the metabolic transformation was incorporated (SRM1−2). The calculated SRM information for the metabolites were exported to the QuickQuan database to monitor all predicted metabolites. When samples were analyzed, QuickQuan automatically loaded the parameters needed to quantify parent compound and to monitor predicted metabolites. LC peaks of internal standards, parent compound, and potential metabolites were automatically integrated by Xcalibur 2.0 (Thermo Fisher Scientific Inc., Waltham, MA) and peak areas ratios were used to calculate parent disappearance and metabolite appearance rates.

Results and Discussion

Metabolite identification studies are performed relatively late in the compound optimization process because they are work intensive and generally aimed to understand the metabolic pathway (generally in vivo) of an already potent and optimized drug candidate.[8,10] This work is also needed to identify species differences that may hamper extrapolation to humans. At this late stage, the investment in the development of sophisticated bioanalytics and structure elucidation techniques is fully justified. On the other hand, information on potentially deleterious

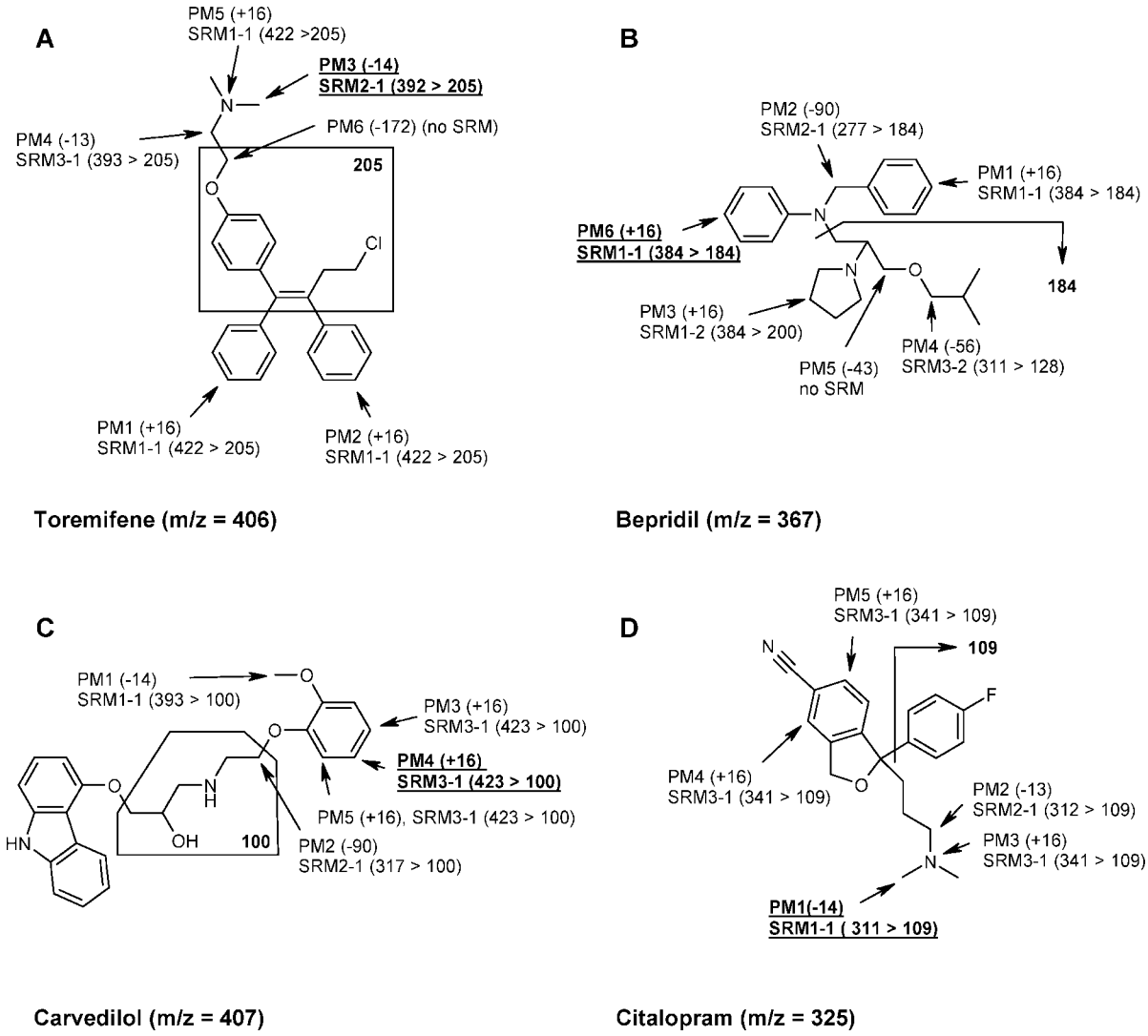

Figure 2. Structure, MS fragmentation and SoM prediction of Toremifene, Bepridil, Carvedilol and Citalopram. The predictions of the site of CYP dependent metabolism by MetaSite is given with rank (PM) order and the corresponding expected SRM-functions with precursor and product ions. The published main metabolite is underlined and the postulated fragments are given in bold.

pharmacokinetic properties like high in vitro cytochrome P450 dependent metabolic stability is available much earlier when synthesis efforts and compound chemical optimization is at its maximum. At Novartis, more than one-quarter of the compounds tested for human CYP dependent turnover exhibit high in vitro clearances. For some programs or chemical scaffolds, this proportion can increase to more than 80%. To help chemists in designing new compounds with improved pharmacokinetics, the knowledge of the soft spot position is needed. The systematic search for potential metabolites and their relative quantification both in vitro and in vivo has been described previously.[11−13] The approach described herein is new in that it introduces the use of MetaSite, a new in silico tool, to (i) predict the most likely metabolites and (ii) allow their automatic search, detection, and experimental validation via LC-MS. While in silico tools supporting pharmacodynamic structure−activity relationships are relatively well established, there is little available for the medicinal chemist to understand pharmacokinetic SMRs in parallel. Several in silico tools have been used to predict metabolic pathways and have been reviewed recently in detail.[14,15] Some like MDL Metabolite are databases for metabolism, others are empirically based systems that rely on expert rules as the basis of their predictions (Meteor, MetaDrug). MetaSite is a unique new computational procedure to predict the site of CYP dependent metabolism starting from computed 3D compound structures and GRID-based representations of the CYP enzymes.[16] For each atom in the molecule, the probability to be the site of metabolism is derived from its likely distance to the reactive oxygen in the CYP450 binding site and its intrinsic reactivity. MetaSite is unique as a metabolite prediction software because it does not depend on a training set of compounds from which expert rules are derived. Cruciani et al.[16] described success rates of first rank SoM predictions greater than 70% for selected CYP2C9 substrates. More recently, Zhou et al.[17] found that top-ranked site predictions were correct for only about 42% of CYP3A4 substrates. Caron et al.[18] ultimately demonstrated that the success rate is indeed linked to the test set design and can be as high as 82% for small series of congeneric drugs. In early compound profiling phases, nothing is known about the CYP isoforms likely to be responsible for microsomal clearance. Predicting soft spots with each of the nine isoform available in the software would increase dramatically the number of likely metabolites. The (beta) Version 3.0 of MetaSite solves this issue with the help of a consensus "liver" model, which has been used to generate the data presented here. When chemists need to optimize the metabolic stability of their drug candidates they would indeed start with the first rank soft spot prediction. Because of the use of a consensus model with

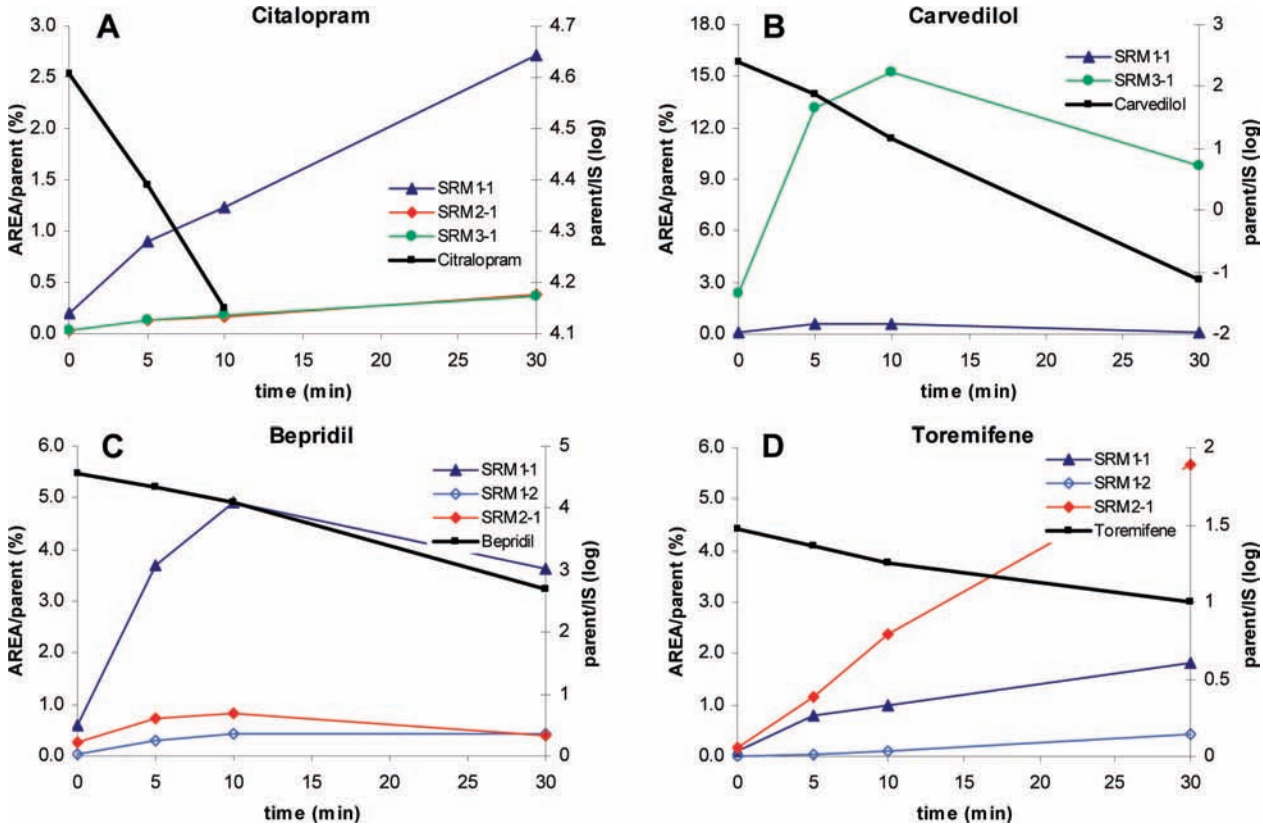

Figure 3. Kinetics of parent and detected metabolites. Relative areas ratios of metabolites for Citalopram (A), Carvedilol (B), Bepridil (C), and Toremifene (D) in human liver microsomes incubations and disappearance of parent compound. Metabolites are expressed as percent of parent area ratio at time zero incubation. Parent compound is given as area ratio to internal standard in logarithmic scale.

unbiased test sets, the success of first rank predictions is likely to be lower than what is described in the literature (lower than 50%). Improving the confidence in MetaSite's predictions would therefore be of high added value. Figure 1 describes a procedure allowing experimental validation of MetaSite's predictions to increase confidence and better prioritize rational synthesis efforts. Standard high throughput metabolic clearance assays classically measure the time dependent disappearance of NCEs in the supernatants of liver microsomal incubations using LC-MS/MS setups.[19,20] However, modern mass spectrometers allow following of much more than a single molecule with high selectivity and sensitivity without significant loss of performance. Selectivity and sensitivity is achieved through the optimization of the mass spectrometer parameters on the NCE ionization and fragmentation pattern (Figure 1A). This selected reaction monitoring (SRM) information can be combined with the MetaSite predictions to generate and store SRM data to monitor potential metabolites (Figure 1B). Sample analysis (Figure 1C) is done in parallel for the quantification of parent compound to determine intrinsic clearance and the search and detection of predicted metabolites. Ultimately, the experimental detection of the predicted metabolites is used to reprioritize the likelihood of the soft spot location and to quickly establish a structure CYP dependent metabolism relationship within a chemical series. Figure 2 shows examples of such a procedure with four marketed drugs: Toremifene, Bepridil, Carvedilol, and Citalopram. The metabolite predicted as most likely by MetaSite (PM1) is monitored by two functions: SRM1−1 is the function calculated when the predicted mass change is not on the measured fragment and SRM1−2 when it is (see Methods). In some cases, the SRMs calculated for the PMs may be equivocal and precise assignment of the soft spot can only rely on MetaSite's prediction. For SRM1−1 of Bepridil, the predicted hydroxylation may affect one or the other of the two aromatic rings present in the structure. Increasing the number of measured SRM functions (additional fragments) would allow distinguishing which of the rings is concerned primarily. Although the resulting MS signal for the metabolites may not be quantitative, their detection is a likely valid proof for the existence of the predicted metabolic pathway. Conversely, because of the high sensitivity of the LC-MS/MS technology, when predicted metabolites are not detected, the pathway is unlikely to be present. Because mass spectrometry parameters optimized for parent compound are used to monitor PMs, the measurement may not be quantitative. Especially when PMs are likely to ionize or fragment differently, the quantitative relationship is likely to be lost. The kinetics of PMs (rate of appearance) may however provide information on their relative importance, and contribution to clearance as the initial rate of PM appearance should be directly proportional to parent disappearance rate (Figure 3). For example, the rate of appearance of SRM1−1 of Citalopram (Figure 3A) is similar to the parent disappearance rate, suggesting that this is the major metabolic pathway and the principal soft spot of the molecule. For Toremifene (Figure 3D), it is the sum of SRM2−1 and SRM1−2 appearance rates, which accounted for almost all of parent disappearance rate. In this case, one not only gets information about two soft spots but also about their relative importance (SRM2−1 > SRM1−2). Finally, it is relatively likely that PMs are further metabolized, especially with high clearance compounds. This is likely exemplified with Carvedilol and Bepridil (Figure 3B,C) as the rate of metabolite appearance declines with time.

Table 1 shows the results obtained for a set of marketed drugs for which a known major CYP dependent metabolic pathway has been described. The range of intrinsic clearance in this test set spanned from very low (Pefloxacin) to very high (Mida-

Table 1. Success of Prediction and Detection of Main Microsomal Metabolites of Marketed Drugs[a]

		parent SRM		major metabolite[c]			detected metabolites SRMs				
compd	CL_{int}[b]	precursor	product	Δ	expected SRM	rank in MetaSite	>10%[d]	1−10%	0.1−1%	SRMs not detected	new rank
Acenocoumarol	37	352	265	16	368 > 281	2		368 > 281		368 > 265	1
Bepridil	94	367	184	16	383 > 184	6		383 > 184	383 > 200 277 > 184	277 > 94, 311 > 184/128	1
Carvedilol	235	407	100	16	423 > 100	4	423 > 100		393 > 100	393 > 86, 315 > 100, 423 > 116	1
Chlorpromazine	117	319	214	16	335 > 230	4	335 > 230	305 > 214	335 > 214	305 > 200, 306 > 214/201	1
Cibenzoline	28	263	115	16	279 > 115	1			279 > 115	279 > 131, 295 > 115/147	1
Citalopram	36	325	109	−14	311 > 109	1		311 > 109	312 > 109 341 > 109	311 > 95, 312 > 96, 341 > 125	1
Kaempferol	269	285	285	16	301 > 301	1	319 > 319	301 > 301		301 > 285, 319 > 285	2
Ketamine	136	238	125	−14	224 > 125	6	254 > 125 224 > 125	254 > 141		224 > 111	1
Maprotiline	31	278	250	−14	264 > 236	2		294 > 266		294 > 250, 264 > 250/236	nd[e]
Mequitazine	34	323	212	16	339 > 228	1	339 > 228			339 > 212, 355 > 212/244	1
Midazolam	554	326	291	16	342 > 307	1			342 > 307	342 > 291	1
Pefloxacin	5	334	316	−14	320 > 302	2		320 > 302		366 > 316/348, 320 > 316, 350 > 316/332	1
Propranolol	80	260	116	16	276 > 116	3		276 > 116	218 > 116	218 > 74, 276 > 132, 145 > 116	1
Tadalafil	49	390	135	−14	376 > 135	1			376 > 135	376 > 121, 406 > 135/151, 422 > 135/167	1
Toremifene	31	406	205	−14	392 > 205	3	392 > 205	422 > 205	422 > 221	392 > 191, 294 > 205/93	1
Trichostatin-A	107	303	148	−14	289 > 134	1	289 > 134			289 > 148, 319 > 148/164, 337 > 148/182	1
Tropisetron	22	285	124	−14	271 > 110	1			301 > 140	271 > 124/110, 301 > 124	nd[f]
Venlafaxine	59	278	260	−14	264 > 246	1			264 > 246	264 > 260, 265 > 260/247, 294 > 260/276	1
				Success rate (Rank 1):		50%					83%

[a] The six first predicted metabolites were searched via their corresponding SRMs and reprioritized according to their relative importance versus parent compound after 10 minutes incubation in standard conditions. nd: not detected. [b] μL/min·mg protein. [c] MDL metabolite. [d] The highest area ratio of the metabolite corresponding to this SRM was greater than 10% of the highest area ratio of parent compound. [e] The major metabolite, desmethyl-maprotiline does not fragment in a similar way than Maprotiline. When another parent fragment (SRM) is used or exact mass spectrometry (data not shown), the main metabolite was detected with high relative abundance (1−10%). [f] The major metabolite, desmethyl-tropisetron, was below limit of detection. This metabolite was detected in the incubation when exact mass spectrometry was used (data not shown).

zolam). In accordance with previous studies,[16] the principal authentic SoM is found within MetaSite's top three predictions in about 78% of the cases (14/18). This proportion increases to 100% when predictions are taken into account up to rank 6. However, only 50% (9/18) of the true soft spots are ranked first. Therefore, when chemistry efforts are deployed to reduce metabolic clearance, chemical modification are likely to be erroneously oriented in every second case. When using the automatic high throughput experimental confirmation of prediction described here, the incorrect first rank predictions are declassified and the authentic soft spot is promoted to first rank in 83% of the cases (15/18). In addition, the relative abundance of the detected signals for the metabolites is in agreement with the intrinsic clearance of parent compound. With the exception of midazolam, the detected metabolite signals of all high clearances compounds (>100 μL/min·mg) had a relative abundance of >10%. For midazolam, the lower relative abundance of its main metabolite is explained by the greater propensity of the metabolite versus parent to lose water upon fragmentation. For Kaempferol, the MS signal corresponding to olefinic epoxidation (M+34) and its appearance rate is slightly higher than the aromatic hydroxylation producing Quercetine, which was originally described as major pathway by Breinholt et al.[21] However, only quercetin was quantified in Breinholt's study, which does not exclude the existence of another major pathway as suggested here. It is also likely that Quercetine is further metabolized.[22] The major metabolites of Maprotiline and Tropisetron could not be measured. The LC-MS/MS search and detection fails to detect the metabolites when they fragment differently from parent compound (Maprotiline) or when parent compound clearance and/or the relative MS signal of the metabolites are too low to detect the metabolites (Tropisetron). Although the success rate of LC-MS/MS detection is high, it may be further increased by the use of more characteristic fragments or the use of high resolution and more sensitive mass spectrometry technologies. Indeed, the metabolites of Maprotiline and Tropisetron could be detected using an Orbitrap (data not shown). When another typical, although less intense, fragment of Maprotiline is used (264 > 169), the des-methyl metabolite was detected with a relatively high apparent abundance/appearance rate.

In an attempt to use this methodology to better understand metabolic clearance of drug candidates in early discovery, 95 drug candidates from diverse research programs showing high intrinsic clearances in human liver microsomal incubations were investigated (Figure 4). For most of the NCEs (84.4%), at least one metabolite out of the top three predicted metabolites is detected. More than half (55%) of MetaSite's top predictions

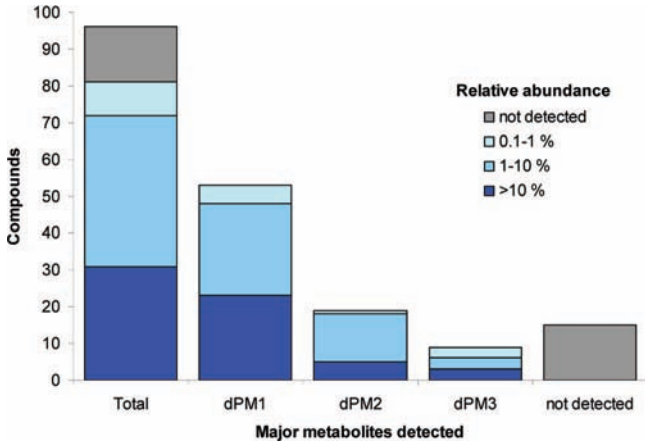

Figure 4. Relative abundance of predicted metabolites detected in incubations of high clearance new chemical entities ($n = 95$). Relative abundance of predicted metabolites with probability rank 1 to 3 detected in human liver microsomal incubations (dPM1 to dPM3). The most abundant (major) metabolite of each compound was expressed as a percentage to the time zero parent compound area ratio and distributed into four categories: >10%, 1−10%, 0.1−1%, and not detected. "Not detected" describes compounds for which no signal compatible with either of the first three most probable predicted metabolites was detected.

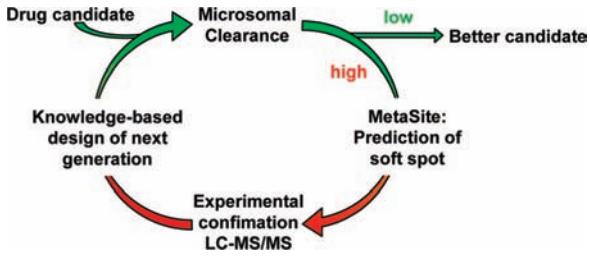

Figure 5. The proposed new loop in the drug optimization process.

were confirmed experimentally (dPM1). In 20% of the cases, the top predictions were not detected but the second most likely (dPM2) and for 9% the third most likely metabolites were detected as the most abundant metabolite (dPM3). For about 16% of the compounds, none of the top three predicted metabolites could be detected (not detected). Experimental confirmation of the top 3 predicted metabolites increases therefore the likelihood of identifying the soft spot in the first instance from 55% (PM1) to 84%. (dPM1 + dPM2 + dPM3). Without experimental validation, the poorly predicted soft spots (PM3) would have had very few chances to be identified and modified to reduce clearance. The proportion of detected metabolites representing a high relative abundance versus parent compound (>10%) is highest for dPM1 and decreases with dPM2 and dPM3, suggesting that MetaSite is performing particularly well in its rank 1 prediction. The fact that the test set used is composed of high clearance NCEs only might explain this, as these compounds are more likely to have an "obvious" weak point. The automatic search and detection of predicted metabolites allows also gathering of information on secondary soft spots. High clearance may in fact be the result of the combination of two or more pathways. For about 1/3 of the NCEs for which a major pathway was detected, there was indeed evidence that a second pathway was also contributing significantly to clearance (data not shown). When more than one soft spot can be identified, it becomes eventually more difficult to design stable, pharmacologically active drug candidates and the decision may be made to abandon the incriminated scaffold as a whole.

Experimental monitoring of the first three (or six) most likely metabolites predicted by MetaSite in the standard microsomal stability assay allows dramatic increase of the confidence in SoM prediction. The information on prominent metabolic pathways can been obtained using existing biological samples (standard microsomal incubations to measure metabolic clearance) and standard analytical tools. The data can be generated for all compounds going through high throughput clearance assays with no loss of quality. When NCEs are found to be high clearance, the chemists can immediately access the data on the most likely experimentally confirmed soft spots and establish a rational stabilizing strategy (Figure 5). Whereas detailed pathway elucidation studies are reserved to confirmed drug candidates, the current procedure applies to compounds synthesized much earlier in the search for the optimal potency. An obvious limitation of the procedure described here is that it is only addressing compound instability due to CYP dependent metabolism, although this pathway is predominant in drug metabolism. A clear indication of the relative importance of the detected pathway(s) is also lacking when authentic metabolite references are not available. Whether semiquantitative information can be derived from metabolite kinetics (rate of appearance) remains to be explored. The high throughput LC separation performance and the limit in the number of fragments modern mass spectrometer can analyze simultaneously are reducing the possibilities to discriminate between metabolites having the same mass (i.e., PM1 and PM6 of Bepridil in Figure 1). More sophisticated systems using UPLC separation, more fragmentation information (MS^n), or high mass resolution would help to further increase the success rate.

Beyond the identification of soft spots, the procedure described here may also be used to evaluate the risk for reactive metabolite generation or for mechanism-based CYP inhibition. MetaSite version 3 includes a module predicting time dependent inhibition combining the identification of substructures likely to be reactive and their SoM probability. Information on metabolite structure may be used to synthesize authentic references, which can be used to quantify metabolites both in samples from in vitro and in vivo experiments and to characterize their activity or toxicity in early research. Last but not least, SMR information gathered for thousands of compounds can be stored in databases and ultimately be used to develop in silico models that would not only identify soft spots with a high success rate but also suggest the best stabilizing substitutions.

Acknowledgment. We sincerely thank Prof. G. Cruciani from the University of Perugia, Italy, and Riccardo Vianello and Massimiliano Pippi (Molecular Discovery, UK) for providing the beta version of MetaSite's metabolite structure prediction tool and the software upgrades that made this work possible.

References

(1) Williams, J. A.; Hyland, R.; Jones, B. C.; Smith, D. A.; Hurst, S.; Goosen, T. C.; Peterkin, V.; Koup, J. R.; Ball, S. E. Drug−drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUC(i)/AUC) ratios. *Drug Metab. Dispos.* **2004**, *32*, 1201–1208.

(2) Bertz, R. J.; Granneman, G. R. Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin. Pharmacokinet.* **1997**, *32*, 210–258.

(3) Kennedy, T. Managing the drug discovery/development interface. *Drug Discovery Today* **1997**, *2*, 436–444.

(4) Kumar, G. N.; Surapaneni, S. Role of drug metabolism in drug discovery and development. *Med. Res. Rev.* **2001**, *21*, 397–411.

(5) Murray, M.; Petrovic, N. Cytochromes P450: Decision-making tools for personalized therapeutics. *Curr. Opin. Mol. Ther.* **2006**, *8*, 480–486.

(6) Carlson, T. J.; Fisher, M. B. Recent advances in high throughput screening for ADME properties. *Comb. Chem. High Throughput Screening* **2008**, *11*, 258–264.
(7) Rendic, S. Summary of information on human CYP enzymes: Human P450 metabolism data. *Drug Metab. Rev.* **2002**, *34*, 83–448.
(8) Zhang, N. Y.; Fountain, S. T.; Bi, H. G.; Rossi, D. T. Quantification and rapid metabolite identification in drug discovery using API time-of-flight LC/MS. *Anal. Chem.* **2000**, *72*, 800–806.
(9) Obach, R. S. Prediction of human clearance of 29 drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metab. Dispos.* **1999**, *27*, 1350–1359.
(10) Castro-Perez, J. M. Current and future trends in the application of HPLC-MS to metabolite-identification studies. *Drug Discovery Today* **2007**, *12*, 249–256.
(11) Li, A. C.; Alton, D.; Bryant, M. S.; Shou, W. Z. Simultaneously quantifying parent drugs and screening for metabolites in plasma pharmacokinetic samples using selected reaction monitoring information-dependent acquisition on a QTrap instrument. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1943–1950.
(12) Shou, W. Z.; Magis, L.; Li, A. C.; Bryant, M. S. A novel approach to perform metabolite screening during the quantitative LC-MS/MS analyses of in vitro metabolic stability samples using a hybrid triple-quadrupole linear ion trap mass spectrometer. *J. Mass Spectrom.* **2005**, *40*, 1347–1356.
(13) Tiller, P. R.; Romanyshyn, L. A. Liquid chromatography/tandem mass spectrometric quantification with metabolite screening as a strategy to enhance the early drug discovery process. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1225–1231.
(14) Madden, J. C.; Cronin, M. T. D. Structure-based methods for the prediction of drug metabolism. *Expert Op. Drug Metab. Toxicol.* **2006**, *2*, 545–557.
(15) Anari, M. R.; Baillie, T. A. Bridging cheminformatic metabolite prediction and tandem mass spectrometry. *Drug Discovery Today* **2005**, *10*, 711–717.
(16) Cruciani, G.; Carosati, E.; De Boeck, B.; Ethirajulu, K.; Mackie, C.; Howe, T.; Vianello, R. MetaSite: Understanding metabolism in human cytochromes from the perspective of the chemist. *J. Med. Chem.* **2005**, *48*, 6970–6979.
(17) Zhou, D. S.; Afzelius, L.; Grimm, S. W.; Andersson, T. B.; Zauhar, R. J.; Zamora, I. Comparison of methods for the prediction of the metabolic sites for CYP3A4-mediated metabolic reactions. *Drug Metab. Dispos.* **2006**, *34*, 976–983.
(18) Caron, G.; Ermondi, G.; Testa, B. Predicting the oxidative metabolism of statins: An application of the MetaSite (R) algorithm. *Pharm. Res.* **2007**, *24*, 480–501.
(19) Carlson, T. J.; Fisher, M. B. Recent advances in high throughput screening for ADME properties. *Comb. Chem. High Throughput Screening* **2008**, *11*, 258–264.
(20) Drexler, D. M.; Belcastro, J. V.; Dickinson, K. E.; Edinger, K. J.; Hnatyshyn, S. Y.; Josephs, J. L.; Langish, R. A.; McNaney, C. A.; Santone, K. S.; Shipkova, P. A.; Tymiak, A. A.; Zvyaga, T. A.; Sanders, M. An automated high throughput liquid chromatography−mass spectrometry process to assess the metabolic stability of drug candidates. *Assay Drug Dev. Technol.* **2007**, *5*, 247–264.
(21) Breinholt, V. M.; Offord, E. A.; Brouwer, C.; Nielsen, S. E.; Brosen, K.; Friedberg, T. In vitro investigation of cytochrome P450-mediated metabolism of dietary flavonoids. *Food Chem. Toxicol.* **2002**, *40*, 609–616.
(22) Nielsen, S. E.; Breinholt, V.; Justesen, U.; Cornett, C.; Dragsted, L. O. In vitro biotransformation of flavonoids by rat liver microsomes. *Xenobiotica* **1998**, *28*, 389–401.

JM8008663