10 Flavin-Containing Monooxygenase: The Role of Flavin-Containing Monooxgenase in Lead Design and Selection

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10.1 SUMMARY

The flavin-containing monooxygenase (FMO) is an important human drug-metabolizing enzyme because it oxygenates sulfides and amines, which are two functional groups very prevalent in drugs and drug candidates. FMO also oxygenates many other nucleophile-containing compounds to more polar, readily excreted metabolites. Generally, FMO-mediated metabolism constitutes a detoxication process, but sometimes it produces electrophilic metabolites that inhibit other enzyme systems because FMO is quite recalcitrant to inhibition. FMO is also not readily induced and the properties of lack of inhibition and induction contribwute to FMO being a detoxication catalyst. Because FMO is not readily inhibited or induced, most of its functional variability is not due to environmental factors but due to genetic polymorphisms. Compared with cytochrome P450 (P450), drugs and drug candidates that are metabolized by FMO possess less potential for toxicity and adverse drug–drug interactions.

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Highly nucleophilic drugs and drug candidates are oxygenated by FMO, and some of these oxygenated metabolites can be readily retroreduced to the parent compound quite efficiently. Retroreduction of drug candidate metabolites formed by FMO confounds the evaluation of the role of FMO in drug candidates and probably underestimates the amount of metabolism that FMO contributes to new chemical entities. One class of highly nucleophilic agents that are not oxygenated by FMO is the endogenous physiological nucleophiles such as glutathione or cysteine. This likely cytoprotective process probably spares the cell from unnecessary consumption of reductive equivalents of NADPH used in FMO-mediated oxygenation as well as from the loss of thiaphile cytoprotective agents such as glutathione.

Introducing a functional group into drug candidates that are metabolized by FMO may be beneficial in drug development. This might distribute the metabolism of the drug candidate over a wider set of enzymes as well as potentially provide FMO metabolites that are generally polar, nontoxic, and readily excreted. By avoiding metabolism (and bioactivation) by P450, the prospects for increasing druglike properties of a drug candidate are likely increased. Because so many drugs are metabolized by P450 3A4, decreasing the amount of metabolism through this enzyme system is likely to decrease adverse drug–drug interactions. Distributing the metabolism of drugs over a wide range of metabolic processes including FMO might decrease reliance on one metabolic pathway and decrease toxicity and adverse drug–drug interactions.

10.2 INTRODUCTION

The FMO (E.C.1.14.12.8) is a microsomal, NADPH-dependent, chemical- and drug-metabolizing enzyme that possesses broad substrate specificity [1,2]. Compared to biosynthetic enzymes with great substrate specificity, monooxygenases such as FMO appear to trade robust rates of metabolism for the ability to accept a wide variety of nucleophilic heteroatom-containing chemicals and endogenous materials [3,4]. In this regard, FMO resembles P450, although its specific rates of metabolism appear to be greater than those of P450. Generally, FMO converts lipophilic nucleophilic compounds to more polar metabolites that are more readily excreted; for example, tertiary amines are converted to their *N*-oxides, and often, the *N*-oxides possess significantly less pharmacological activity. FMO can bioactivate compounds to more reactive metabolites; however, these reactive products generally leave the product domain of FMO but can inactivate other proteins (including hemoproteins such as P450). This aspect of FMO function underscores an underlying mechanism: somehow the product-binding domain of the enzyme precludes some very reactive metabolites from inactivation of the enzyme. Amino acid residues surrounding the product-binding domain presumably are non-nucleophilic and prevent covalent binding by electrophilic metabolites. Accordingly, FMO is not easily inhibited. Lack of ready inhibition of FMO is in contrast to the relatively easily inhibited P450 class of enzymes and could suggest that FMO would have less adverse drug–drug interactions via FMO-dependent metabolism [5].

There are five forms of FMO (i.e., FMO1–5) that show 50–58% amino acid identity across species lines [1]. Additional pseudogenes (i.e., FMO6–11) have been reported in human tissue [6,7]. Because FMO pseudogene products (i.e., FMO6–11) lack functional activity, they are not discussed further in this summary. FMO utilizes a

peroxyflavin cofactor as the source of molecular oxygen, and for human FMOs, much of the FMO metabolism literature published to date suggests FMO3 as having a prominent role in FMO-dependent drug and chemical metabolism [8]. It should be pointed out, however, that FMO5 is the most prevalent form of FMO in adult human liver but its functional role is not clear, possibly because of the limited number of substrates reported [3,9]. In contrast to many small animal models of hepatic FMO where FMO1 is often a prominent hepatic form in adult human liver, very little FMO1, FMO2, and FMO4 are present [3,10]. Relatively few reports in the literature related to human FMO-mediated metabolism exist, and this is surprising in view of the fact that FMO3 is present in adult human liver to a surprisingly great extent. For a normal liver, FMO3 is present in ~65% of the amount of protein that is typically observed for P450 3A4 [10]. Adult human liver FMO3 is a prominent enzyme that likely plays a larger role in the metabolism of chemicals and drugs than has previously been reported. There are several possible reasons for this. First, FMO is thermally labile and the order of addition of cofactor (i.e., NADPH) is very important to preserve maximum enzyme activity. As much as 80% of FMO activity can be lost by treating microsomal or other sources of FMO3 with heat (even preincubation at 37°C) for as little as one minute preincubation in the absence of NADPH [11]. Accordingly, incubations initiated by the addition of NADPH to prewarmed preparations of microsomes or FMO considerably decrease the functional activity. Traditionally, initiation of microsomal incubations by the addition of NADPH has been done to decrease autooxidation of N- and S-containing substrates that are particularly prone to autooxidation. However, autooxidation of susceptible substrates can be precluded by addition of an antioxidant such as diethylaminepentacetic acid (DETAPAC). Addition of substrate to a chilled incubation containing a source of FMO, NADPH, and an antioxidant and initiation of the incubation at 37°C is an optimal means of preserving FMO functional activity. Second, the nature of the metabolite formed by FMO (i.e., *N*-oxides, *S*-oxides, and hydroxylamines) is sometimes difficult to quantify from an analytical standpoint. In addition (and this point is discussed in greater detail below), metabolites of FMO can undergo retroreduction and reform the parent drug or chemical. Thus, no apparent FMO-dependent metabolism is present when in fact, considerable metabolites are formed, but they are rapidly retroreduced. Another aspect is that sometimes, initially formed FMO-dependent metabolites (e.g., hydroxylamines) are more nucleophilic than the starting amine and are more extensively metabolized than the parent amine. Moreover, the ultimate metabolite may not be readily recognized as arising from the starting material. In rare cases, the metabolite (e.g., a tertiary amine *N*-oxide or an *S*-oxide) may be chemically unstable and may undergo a Cope-type elimination to provide a product that is also not readily recognizable as derived from a typical FMO-mediated transformation. In fact, sometimes, FMO-dependent metabolites resemble those that might arise from P450. Finally, the efficiency of FMO-mediated oxygenation is also highly dependent on the quality of the microsomes or other source of FMO. Compared to P450, for example, there are some significant differences to the approach to preparing microsomes highly enriched with FMO functional activity, but most approaches involve assuring that the microsome sample is kept at 4°C or less during preparation [11]. To further illustrate ways to characterize FMO and distinguish functional enzyme activity from P450, a few simple methodological approaches are listed below that can be used to distinguish FMO-dependent from P450 dependent metabolic processes [12].

While the dogma that FMO oxygenates highly nucleophilic compounds has been described above, there are a number of important exceptions to the rule. For example, some unusual examples of FMO-mediated carbon-centered oxygenation (e.g., 6-methylhydroxylation of the anticancer agent 5,6-dimethylxanthenone-4-acetic acid [13] and carbon oxygenation and defluorination of 4-fluoro-*N*-methylaniline [14]) have appeared in the literature. The latter example is particularly intriguing because FMO1-mediated carbon oxygenation of 4-fluoro-*N*-methylaniline is coupled with defluorination to afford 4-*N*-methylaminophenol via an electrophilic quinoneimine intermediate. Another important class of nucleophiles not oxygenated by FMO includes biological nucleophiles (e.g., cysteine or glutathione). Examined from the standpoint of biology, FMO presumably does not accept cysteine or glutathione because S-oxygenation of such nucleophiles would rapidly deplete cysteine or glutathione, putting defense mechanisms of the cell at risk. S-Oxygenation of biological thiols would also deplete NADPH, and this would be biologically wasteful and also put the cell at risk from biological electrophiles because of the inability of NADPH to otherwise participate in endogenous cellular defense mechanisms. Apparently, key amino acids in the mouth of the FMO substrate-binding channel are specifically in place to ward off biological nucleophiles. The fact that FMO is not prone to inhibition by electrophilic oxygenated metabolites (e.g., even highly reactive sulfenic acids) suggests that FMO may have evolved to detoxicate lipophilic nucleophilic N- and S-containing compounds in the primordial biota. Inhibition of FMO would have brought this vital function to a halt and predisposed any developing biology to risk. Likewise, FMO apparently evolved critical amino acids in the substrate-binding channel to disavow the metabolism of cellular protective agents (e.g., glutathione oxidized to glutathione disulfide). By using both these mechanisms, FMO presumably provides a strong detoxication presence to the cell. It is not clear that this is the physiological role of FMO, although, to date, the physiological role of FMO has eluded identification [2,15,16]. Certain physiologically important N-containing compounds are also efficiently N-oxygenated. For example, the neurotransmitters tyramine and phenethylamine are sequentially N-oxygenated by FMO3 to the oxime [17,18]. N-Oxygenation of these and other biologically relevant amines may serve as a detoxication pathway to decrease the accumulation of excess and deleterious amines by converting them into metabolites that possess considerably less pharmacological activity than the parent amine [19].

The prediction is that FMO will oxygenate any nucleophilic heteroatom-containing compound that can be oxidized by hydrogen peroxide or peracids [20]. This is one distinction for this class of monooxygenase made on the basis of products derived from FMO: compared to P450, the structures of products are relatively easy to predict with a great deal of certainty. However, FMO does not appear to oxygenate biological nucleophiles (e.g., glutathione and cysteine), although there are some apparent exceptions (e.g., biotin [2]).

This chapter summarizes some of the general considerations of the role of FMO in preclinical drug lead selection and development. The idea that concepts of FMO drug metabolism can be used effectively to guide preclinical drug lead development is not new. Previously, some of these ideas were summarized [5]. Now, a few examples are emerging that underscore the importance of FMO3 in human drug development. The idea is that building drug lead design strategies for a wider range of metabolic pathways of a molecule to decrease reliance (or dominance) on any one metabolic pathway (e.g., P450 3A4) could have significant advantages. Engineering soft nucleophilic centers

into molecules could afford a greater amount of metabolism to go through the FMO3 detoxication route. This may improve detrimental metabolism, toxicity, and bioavailability issues that sometimes beset drug candidates and result in failure of candidates to advance to clinical drug status. Because FMO3 is not readily inhibited or induced, drug candidates that are metabolized by FMO3 may offer advantages by way of decreasing adverse drug–drug interactions that have been observed for drugs prominently metabolized by metabolic enzymes that are readily induced or inhibited. This is not to say that rapid metabolism should be built into a drug candidate. On the contrary, as we shall see with a number of examples, it is likely that FMO3-related metabolism also enables futile metabolic cycles of oxygenation and retroreduction (e.g., for *N*-oxides and *S*-oxides), thus enabling potentially greater cellular accumulation of the parent drug, greater therapeutic efficacy, and more favorable bioavailability. It is possible that the overall process of FMO3-mediated oxygenation and consequent retroreduction are a much greater dynamic than previously recognized. Of course, in addition to FMO-mediated processes, there are many other metabolic processes that can contribute to the overall biotransformation and distribution of a drug candidate. In this chapter, a summary of some of the differences between FMO and other metabolic processes is provided. To stimulate further discussion, the author takes the position that judicious use of soft nucleophiles incorporated into lead candidates and incorporation of FMO-dependent metabolism into drug design paradigms may be useful to improve the overall drugability of candidates and may decrease potential adverse drug–drug interactions.

10.3 OVERVIEW OF THE DRUG DISCOVERY PROCESS

The drug discovery process has changed dramatically over the past 20 years [21]. The drug discovery pipeline has expanded with the use of combinatorial chemistry, rapid synthetic methods, and high throughput screening. However, the success rate of moving a new chemical entity (NCE) forward to an approved drug status is low and the expense is considerable [22]. In the 1960s and 1970s, the observation that many drug candidate failures in clinical development was due to inappropriate pharmacokinetics ushered in a new approach that incorporated a greater reliance on ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties earlier in the preclinical setting [23]. Drug metabolism and pharmacokinetic studies in small animals planned and carried out earlier in the preclinical drug development paradigm and used to make predictions about the way drug candidates could be metabolized and distributed in humans have become a more accepted strategy. While predictions about the way small molecules are handled in small animals compared to humans is an inexact science, utilization of ADMET studies early in the drug development pipeline has decreased the failure rate due to inappropriate pharmacokinetic properties [22,24]. The reported success rate for drug candidates that entered clinical trials was reported as improved for the period between 1981 and 1992 because of improved pharmacokinetic properties and a greater understanding of chemical toxicology [25,26]. This may have had something to do with the improved practice of high throughput synthesis and screening coupled with *in vitro* ADMET studies that improved the metabolic properties and bioavailability for preclinical drug development during this period. However, the success rate reported for this period (i.e., 20.9%) is still unacceptably low and more recent data do not point to

an improvement [27]. The high attrition rate for drug development that still persists is likely due to unfavorable toxicologic properties of new drug candidates. Less progress has been made in understanding the fundamental chemical and biological basis underlying the reason that certain compounds cause toxicity. Compounding the problem is the uncertain nature that small animal studies extrapolate to the human situation [28]. While there is general agreement that production of metabolic reactive metabolites and intermediates is linked to impairment of cell function and cell death, it is not completely clear how small animal toxicology studies relate to human toxicology other than to follow the general idea that it is prudent to divert metabolism pathways from those that produce reactive electrophilic metabolites [29]. Metabolic bioactivation of small molecules to toxic materials as an underlying principle responsible for toxicity is not a new concept, and the idea of linking protein and DNA covalent modification with drug toxicity in *in vitro* and *in vivo* systems is becoming more and more widely accepted. New molecular and genetic approaches have emerged to identify the potential for metabolic bioactivation. Today, many departments responsible for drug safety and ADMET in the pharmaceutical industry have established protocols to identify potentially problematic compounds by a process including (i) reaction phenotyping (to identify the prominent enzymes involved in the metabolism of the drug candidate, (ii) induction or inhibition of P450 (the enzyme system that accounts for about 65% of human drug metabolism), (iii) covalent binding studies (that indicate if reactive electrophiles are being created *in vitro* or *in vivo*), and (iv) pharmacogenetic studies that point to a metabolic potential for adverse drug–drug interactions. With this information in hand, a more complete understanding of the potential for drug toxicity and adverse drug–drug interactions of a drug candidate can be assessed. However, it is likely the situation is much more complex because the biology and molecular genetics are more complex than we currently understand. To underscore this point, paradoxically, drugs of significant clinical utility that possess undesirable properties (e.g., covalently modify proteins or induce or inhibit P450) are still on the market. It is possible that the delicate and complex balance between drug candidate metabolic bioactivation and detoxication is at play under this situation in ways that we still do not fully appreciate.

While the use of combinatorial chemistry and large library screening for identifying "hits" of a pharmaceutical target in the early stages of a drug screening campaign has led to an increase in the number of compounds evaluated, many times the "hits" are of low quality from the standpoint of pharmaceutical properties (i.e., solubility, permeability, bioavailability, and chemical and metabolic stability). A key aspect of drug development is to identify compounds with ADMET and physiochemical property deficiencies as early as possible in the drug development pipeline to advance the candidates possessing the properties leading to the greatest likelihood of success. Two key developments that have spurred advances in the area of ADMET and physiochemical or pharmacokinetic properties have been in the area of genetics (e.g., genotyping to decrease idiosyncrasies) and advances in bioanalytical chemistry (e.g., LC-MS/MS to detect and quantify metabolites), respectively. However, even extensive knowledge of the enzymes involved and the types and quantities of metabolites formed do not completely address deficiencies in understanding their role in toxicity. In addition to the parent drug, the issue of the role of metabolites in the toxicity of drug candidates has emerged in the discussion to explain possible adverse drug-induced toxicities [30]. Generally, the type of metabolite under consideration is a stable drug metabolite that

can be isolated or synthesized as opposed to chemically reactive metabolites or short-lived metabolites that cannot be readily isolated or synthesized. Part of the challenge to characterize the relevance of a metabolite stems from the question as to the level of metabolite formed (i.e., should the metabolite studied be formed at a level of 5%, 10%, or 20% or more of the parent?) and how much effort should be invested in evaluating the toxicity of the metabolite and which metabolites should be studied (i.e., should just human drug metabolites or both small animal and human drug metabolites be studied?) [31]. Nevertheless, for drug approval in the United States, it is likely that increased evaluation of human drug metabolites that are also particularly prevalent in small animals administered the parent drug will be required by the US Food and Drug Administration [32]. Thus, synthesis, characterization, and quantification of drug metabolites will take on an even larger role in drug development and safety evaluation in the future. Because often metabolite synthesis is a nontrivial undertaking, in the future, it may be that use of enzyme-based or semisynthetic approaches will play a larger role in producing difficult-to-obtain metabolites.

Improving the ADMET properties earlier in the drug development paradigm will continue to play a prominent role in preclinical drug discovery. It has been suggested that medicinal chemists would prefer to work in a lead compound series that possesses tractable pharmaceutical and physiochemical properties with lower potency than the other way around [33]. Said in another way, it is more difficult to reengineer or rescue a compound with inherently poor pharmaceutical, chemical, or ADMET properties than to start with a series that possesses good druglike properties and work to improve potency [34]. Another way to express this idea is by the concept of identifying candidates with undesirable properties early in the discovery process and discarding those candidates for the ones with more favorable ADMET and physiochemical properties [35]. Thus, greater resources can be expended on drug candidates with favorable properties and considerably less time and money on candidates with poor prognosis for success. Today, considerable progress has been made to attempt to explain the mechanistic basis for metabolism in the toxicity of drugs and carcinogens. Most of the effort has been directed at P450, and this is understandable in view of the large percentage of chemicals that are prominently metabolized by P450. However, the information is not complete and is lacking in non-P450 drug-metabolizing enzymes such as FMO. A more thorough knowledge of FMO- and other non-CYP-mediated metabolism could provide additional understanding concerning predictions about metabolism, toxicity, and human drug development [36]. Accordingly, this chapter summarizes recent advances related to FMO and drug development with the hope that some of the information will be useful to solve problems and develop new approaches in the use of drug metabolism in drug development.

10.4 ACADEMIC DRUG DEVELOPMENT USING ADMET

With the advent of the NIH Roadmap and funding of university and research institute high throughput molecular probe discovery (http://pubchem.ncbi.nlm.nih.gov/), considerably greater attention has been focused on probe and drug discovery in the nonprofit setting. However, in academia, application of ADMET concepts early in the probe or medications development approach has not been as widely utilized. The author suspects that as more and more academic laboratories begin to work on probe and

drug discovery and drug repurposing (i.e., drug rescue to improve a known drug that possesses a weakness or the improvement of a clinical candidate that needs removal of a deficiency to become a drug), application of ADMET concepts early in the drug discovery paradigm may come into vogue and result in more druglike materials from the academic setting. Out of economic necessity and because of the expense of large chemical libraries, the Human BioMolecular Research Institute (HBRI) has embraced using concepts of dynamic medicinal chemistry in its molecular probe and medications development programs [37]. This is because typical academic laboratories including HBRI do not have the financial or other resources for extremely large-scale library screening and pharmacological compound evaluation throughput that a typical for-profit entity has at its disposal. Dynamic medicinal chemistry is an iterative process of recursive chemical synthesis, testing, and optimization by utilizing ADMET principles early in the discovery phase to focus on relatively modest-sized "smart libraries" of druglike compounds that have a much greater chance of surviving and possessing suitable properties for *in vivo* efficacy testing. At HBRI, progress has been made turning high throughput screening "hits" into drug leads with a very modest budget [38,39]. Synthesizing "smart libraries" of druglike materials early in the preclinical drug development paradigm could in principle lead to more favorable candidates with fewer liabilities. The goal of this approach is not to predict the toxicity of a series of compounds but rather to avoid compounds that could afford toxicity and develop more promising candidates in the first place. Such candidates would be more likely to be useful in preclinical animal model efficacy studies because they are designed on the basis of ADMET properties to possess appropriate pharmacophores that are associated with greater bioavailability and other favorable pharmaceutical properties. To illustrate this point, an example from one of our in-house NIH Roadmap Projects is considered. Figure 10.1 shows the structure of a potent nuclear factor-kappa B (NF-κB) inhibitor.

NF-κB is a member of the family of transcription factors that play a critical role in many pathways including host defense, immune responses, inflammation, and cancer. The NF-κB pathway activated by antigen receptors is critical for acquired (as opposed to innate) immunity and contributes to T- and B-lymphocyte activation, proliferation, survival, and effector functions. Dysregulated NF-κB activation in lymphocytes can contribute to the development of autoimmunity, chronic inflammation, and lymphoid malignancy [40,41]. The NF-κB activation pathway linked to antigen receptors involves a cascade of adapter and signal-transducing proteins, which minimally includes Carma1, Bcl-10, Paracaspase (MALT1), TRAF6, and Ubc13. In T and B cells, the NF-κB pathway is initiated by protein kinase C (PKC)-θ and PKC-β, respectively, that induces phosphorylation of components of this signaling pathway,

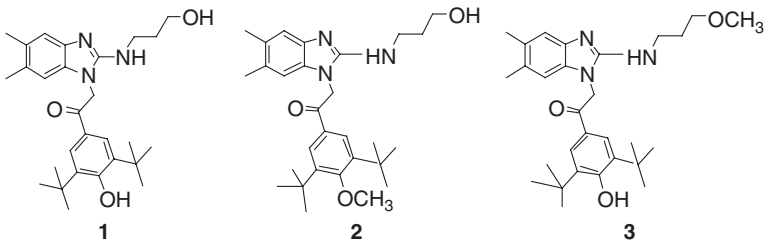

Figure 10.1 Structure of NF-κB inhibitor **1** and analogs **2** and **3**.

leading ultimately to inhibitor of κβ Kinases (IKKs) activation through a mechanism involving IKK-γ [42]. Dysregulated antigen-receptor-mediated NF-κB activation can contribute to the development of autoimmunity, chronic inflammation, and malignancy. A chemical biologic screening strategy for identification of chemical compounds that selectively inhibit antigen-receptor-mediated NF-κB activation was devised and implemented [39]. Chemical libraries comprising a total of 110,000 compounds (http://mlsmr.glpg.com/MLSMR_HomePage/) were screened for inhibitors of antigen-receptor-mediated NF-κB activation. Compounds with an $IC_{50} < 3\,\mu M$ were further characterized by 12 additional counter-screens that helped determine inhibition selectivity. From the initial screens, three hits with IC_{50} values $<2\,\mu M$ were identified and confirmed as selective for the antigen receptor activation pathway by the counter-screen assays. Of the three hits, benzimidazole (**1**) (Fig. 10.1) was found to be a highly potent and selective inhibitor of antigen-receptor-mediated NF-κB activation.

Because the next goal was to test the NF-κB inhibitor *in vivo*, and because a few sites of **1** could be prone to metabolism and decrease *in vivo* potency, we examined **1** and a few analogs for metabolic stability. As is standard in our dynamic medicinal chemistry approach, *in vitro* metabolic stability studies in the presence of both human liver microsomes (HLMs) and S9 and a reduced NADPH generating system was done with compounds **1, 2**, and **3**.

In vitro metabolic stability studies showed compounds **1**–**3** were quite stable to hepatic metabolism. Generally, if a compound has a half-life of >60 min in these types of *in vitro* metabolism studies, it is judged sufficiently stable to move the compound into *in vivo* studies. Of course, if Phase II conjugation metabolites could be formed, addition of the appropriate cofactor is necessary to observe optimal phase II metabolism. Another possible explanation for metabolic stability of **1**–**3** was that **1, 2**, or **3** inhibited its own metabolism. This was a distinct possibility because imidazole-containing compounds are known to inhibit P450. However, significant inhibition of P450 3A4-mediated microsomal hydroxylation of testosterone by **1, 2**, or **3** was not observed [38], suggesting that inhibition of its own metabolism was not occurring. However, it is possible that some of the metabolic stability of compounds **1**–**3** was due at least in part to inhibition of other P450s that are more prominent in the metabolism of **1**–**3**.

That metabolic deficiencies do not appear to be present in **1**–**3** have thus encouraged more extensive biological studies of these compounds [39]. Metabolic liabilities have significantly contributed to the failure rate of NCEs as clinical candidates and because P450-mediated bioactivation has been associated with P450 inhibition and induction (two features that can confound clinical candidate advancement), improving the metabolism by distributing the metabolism of a drug candidate over a wider range of metabolic enzymes may have significant advantages. As described above for the NF-κB inhibitor **1**, during lead evaluation and optimization, several steps were taken to improve the druglike properties of **1**, including testing for metabolic stability and inhibition of P450. Appropriate ADMET properties for **1** have afforded a compound that can be tested for efficacy *in vivo* and can possibly lead to a candidate with a favorable bioavailability profile. Even in an academic setting, employing concepts in drug metabolism and applying informed approaches for acceptable physiochemical drug properties can lead to elaborating more druglike materials with decreased potential for adverse drug–drug interactions. This can ultimately lead to biological probes or drug candidates with more favorable properties and to increased success rate for clinical candidates.

10.5 ENHANCING DRUG CANDIDATE SELECTION

Because many examples of drugs with adverse drug–drug interactions have been reported for drugs that are prominently metabolized by one enzyme system (e.g., P450 3A4), one potential strategy to avoid this situation is to distribute the metabolism of a drug over a wide variety of monooxygenases including FMO. In principle, this could also lead to a decrease in potential toxicity, if toxicity was related to one major metabolite. One of the purposes of this chapter is to point out examples where optimization of the role of FMO in drug metabolism and drug design could lead to advantages in drug development owing to decreased toxicity and decreased adverse drug–drug interactions. One recent example comes from the development of a multitargeted Src kinase inhibitor called TG100435 by TarGen, Inc., (San Diego, CA). Src is dysregulated in several types of cancers and is involved in tumor progression and metastases [43]. Anticancer activity was observed in the parent drug, TG100435, **4**, and its tertiary amine *N*-oxide metabolite, **5** (Fig. 10.2).

The tertiary amine *N*-oxide metabolite was formed by FMO in preclinical species including human, dog, rat, and mouse liver. FMO-mediated formation of tertiary amine *N*-oxides is not that unusual, but it is unusual that the *N*-oxide possessed potent anticancer potency. Generally, polar, readily excreted *N*- or *S*-oxide metabolites arising from the action of FMO possess less biological activity than the parent compound. This is because *N*- or *S*-oxides, being polar metabolites, tend to part away from lipophilic centers that are often associated with molecular drug targets. However, *N*- or *S*-oxides can be retroreduced to their parent compounds, and this complicates matters somewhat insofar as determining which compound is the active species. In the

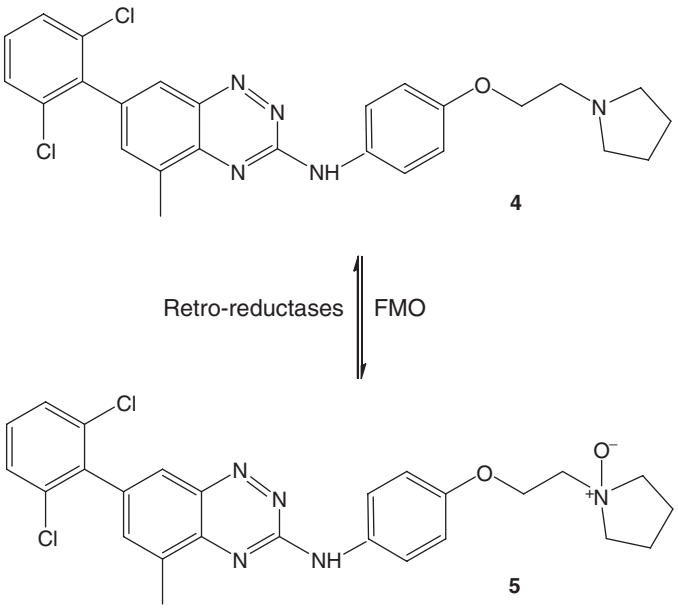

Figure 10.2 Chemical structure of the Src kinase inhibitor, TG100435 (compound **4**), and its tertiary amine *N*-oxide, TG100855 (compound **5**).

case of TG100435, N-oxygenation and *N*-oxide retroreduction plays a large part in its metabolism. FMO, and not P450, is apparently reported to be solely responsible for formation of the *N*-oxide, TG100855, although P450 3A4 contributed to other parent compound metabolism [44]. This conclusion was based on studies using some of the standard methods currently used to distinguish FMO from P450-mediated metabolism, including (i) use of microsome preparations enriched in P450 and/or FMO, (ii) incubation with selective inhibitors of P450 or alternate competitive substrate inhibitors of FMO, (iii) thermal inactivation studies in the presence or absence of NADPH under conditions that largely abrogate FMO activity and retain P450 functional activity, and (iv) use of highly purified recombinant P450 and FMOs [12]. That the *N*-oxide TG100855 was observed as a prominent metabolite followed from investigations of the kinetics of N-oxygenation versus *N*-oxide retroreduction back to the parent amine. In microsomes, *N*-oxide retroreduction was 15-fold slower than FMO-mediated N-oxygenation.

To examine the metabolic retroreduction of TG100855, compound **5**, to its parent, (i.e., TG100435, compound **4**), several approaches were tried. The experimental approaches included (i) synthesizing the authentic *N*-oxide metabolite and incubating it with liver microsomes in the presence or absence of NADPH, (ii) incubating the *N*-oxide in heat-inactivated liver microsomes to show that FMO was not responsible for *N*-oxide retroreduction, (iii) incubating the *N*-oxide in the presence of recombinant P450 with or without P450 inhibitors to show that P450 was not responsible for *N*-oxide retroreduction (in fact, the P450 inhibitor 1-aminobenzotriazole (ABT) actually increased retroreduction from three- to fourfold), and (iv) varying the amount of P450 reductase present in the microsomal and P450 incubations, showing that retroreduction was tracked with the P450 reductase amount (although recombinant P450 reductase in phosphate buffer alone did not cause retroreduction of **5** to **4**). Because P450 reductase alone did not retroreduce **5** to **4**, additional factors, presumably involving other cofactors or microsomal or lipidoidal factors, are involved in retroreduction of **5**. In addition, the results did not indicate whether other enzymes associated with retroreduction (i.e., aldehyde oxidase, quinone reductase, or other hemoproteins) contribute to *N*-oxide retroreduction of **5** [44]. Regardless, this is the example of a futile metabolic cycle involving retroreduction of an FMO metabolite that may be very useful from a clinical standpoint.

10.6 FMO IN DRUG CANDIDATE SELECTION

To date and to the author's knowledge, there have been no published reports specifically utilizing FMO in a drug candidate selection process. However, there have been several drugs that have been recognized as having FMO as a prominent contributor to their metabolic profile and more often than not, this has resulted in a useful outcome or at least an advantage from the standpoint of more favorable metabolism or decreased adverse drug–drug interactions. Utilizing functional groups in drug development candidates that are metabolized by FMO may have some advantages compared to a molecule predominantly metabolized by P450 (and, in particular, P450 3A4). This is because in contrast to P450, FMO is not readily inhibited or induced. Accordingly, drug candidates that avoid prominent P450 metabolism may possess more attractive pharmacokinetics and have a better safety profile. Generally, FMO is not readily inactivated but can undergo alternate substrate competitive inhibition by chemicals that

contain functional groups with low K_m values and are readily oxygenated by FMO (i.e., thiones, thioureas, thioamides, and thiols), but these functional groups are not commonly present in drugs. True competitive inhibition of FMO is rare. In contrast to P450, no reports of a time-dependent irreversible inactivator of FMO have appeared in the literature. In rare cases, apparent FMO inhibition is actually the result of interference with the NADPH cofactor-binding site. One example of this is inhibition of FMO by indomethacin [45]. For nitrogen-containing functional groups that are widely present in drugs, frequent alternate substrate competitive inhibition is not observed probably because the commonly present nitrogen-containing functional groups in drugs (i.e., tertiary and secondary amines) generally do not possess K_m values that are low enough for FMO, which would afford apparent inhibition. In contrast to P450, the lack of apparent inhibition of FMO may contribute to decreased adverse drug–drug interactions for drugs prominently metabolized by FMO because a detoxication role of FMO is unaffected.

A few reports of FMO induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment have been noted in mice [46,47]. In addition, in experimental small animal models of diabetes, significant increases in FMO have been observed [8], and in one study, elevated levels of FMO were restored to normal after treatment with insulin in the diabetic rat [48]. In another study, significant downregulation of FMO was observed in different mouse models of inflammation [49]. However, the general lack of extensive examples of induction of FMO also suggests that compared with inducible forms of P450, adverse drug–drug interactions will also be less for drugs mainly metabolized by FMO. Because human FMO is not readily induced or inhibited, it is likely that most of the variation in metabolism due to human FMO is not dependent on environmental factors but on genetic polymorphisms. Because the pharmacogenetic basis for FMO variability has been described in recent reviews [8,50], it is not discussed further in this summary, except to highlight some of the functional consequences of drug metabolism.

There are several examples of currently used drugs that are extensively metabolized by FMO and show significant differences in efficacy or bioavailability apparently due to FMO genetic polymorphisms. For example, the prodrug sulfoxide (sulindac) is retroreduced to sulindac sulfide, which is an anti-inflammatory agent and sulfide substrate of FMO [51]. Retro-S-oxygenation of sulindac sulfide decreases the efficacy of the anti-inflammatory agent in familial adenomatosis polyposis (FAP) [52]. Individuals with a genetic polymorphism of FMO3 have a defective ability to convert the active sulfide to the inactive *S*-oxide prodrug, resulting in greater concentrations of the active agent. Those individuals with FMO3 genetic polymorphisms show greater efficacy in the treatment of FAP [53].

Two examples of an FMO3 genetic polymorphism that decreases metabolism and increases the efficacy of drug administration stem from the N-oxygenation of ranitidine [54] and the antiulcer agent and benzydamine [55]. Tamoxifen is an example of a tertiary amine substrate of FMO3 that shows an interesting relationship between tissue-selective metabolism and toxicity [56]. In the adult human liver, where P450 is primarily responsible for tamoxifen hydroxylation, a sulfate metabolite that is produced covalently labels hepatic DNA and produces cellular toxicity. Because FMO1 preferentially N-oxygenates tamoxifen and is present in the kidney but not in the adult liver, detoxication by FMO1 protects the renal tissue from covalent binding of tamoxifen metabolites. The lack of FMO1 in the adult human liver predisposes the adult liver to P450-mediated bioactivation and covalent binding.

Itopride is another example of a second-generation drug that was reengineered to decrease a deficiency and optimize its drug efficacy [57]. Itopride is a gastrokinetic agent that is related to mosapride. In contrast to mosapride that is predominantly metabolized by P450 3A4, itopride is primarily converted to the tertiary amine *N*-oxide by FMO3. Because itopride is not metabolized by P450 3A4, it does not engage in adverse drug–drug interactions that have been described for mosapride [58].

Finally, a considerable number of reports have shown a relationship between FMO3 genetic polymorphisms and defective trimethylamine N-oxygenation [59–61]. Conversion of trimethylamine (TMA) to its *N*-oxide is a detoxication and deoderation process resulting in the formation of a polar, readily excreted metabolite. For certain individuals with causative FMO3 mutations, trimethylamine is not N-oxygenated and accumulates and causes the individual to possess an offensive odor. It is notable that no apparent retroreduction of trimethylamine *N*-oxide occurs. This may have been an important evolutionary advantage to increase the clearance of trimethylamine from the body. What is more likely is that the various reductases that retroreduce tertiary amine *N*-oxides to the tertiary amine do not accept a polar material such as trimethylamine *N*-oxide (TMA *N*-oxide). Regardless, defective TMA metabolism has provided a keen insight into the functional aspects of FMO because the naturally occurring genetic mutations have shed light on amino acids in FMO3, which are critical for function.

10.7 FMO-MEDIATED METABOLISM: STRONG NUCLEOPHILES

10.7.1 Thiones

Electron-rich highly polarizable nucleophilic functional groups are generally accepted as substrates for FMO [62]. Barring steric effects that limit approach to the substrate-binding domain of FMO, highly nucleophilic thiones, thioamides, thioureas, thiocarbamides, thiocarbamates, and the like are the substrates most efficiently S-oxygenated by FMO. The same is also likely for nucleophilic phosphorus- and selenium-atom-containing counterparts, but this has not been exhaustively examined. This substrate selectivity can be most easily understood in terms of FMO-accepting lipophilic highly polarizable nucleophilic compounds as substrates. A distinction between endogenous substrates and dietary substrates should be made for both N- and S-containing compounds metabolized by FMO. As described previously [2], an endogenous substrate (e.g., tyramine) or a substrate that results from a conjugate (e.g., cysteine conjugate) is synthesized in the body. Dietary constituents (e.g., alkaloids or plant-derived sulfides) are not synthesized in the body but derived from external sources. Sometimes an FMO substrate could come from both sources (e.g., phenethylamine or trimethylamine), and the definition of endogenous and dietary substances is somewhat ambiguous. Previously, the role of the metabolism of endogenous sulfur-containing substrates by FMO has been summarized [2]. Highly nucleophilic S-containing compounds can be substrates for FMO, including *S*-cysteine conjugates (i.e., mercapturic acids). Generally, however, mercapturates of xenobiotics are sufficiently polar to be excreted per se. It may be that highly lipophilic *S*-cysteine conjugates require additional metabolism by FMO to increase metabolite polarity for excretion [63]. Because the FMO oxygenation mechanism utilizes a peroxyflavin intermediate, if a sulfur-containing compound is treated with hydrogen peroxide in an enzyme model condition and produces an *S*-oxide

product, it is likely (barring steric limitations) that the compound will be a substrate for FMO. From the standpoint of drug development, such S-oxygenated thione products also have potentially interesting pharmaceutical and metabolic properties. Once formed, an *S*-oxide often possesses sufficient nucleophilicity to be S-oxygenated a second time, thus forming a thione *S*,*S*-dioxide. Generally, thione *S*,*S*-dioxides are more electrophilic than a thione *S*-oxide and can undergo reaction with nucleophiles, although thione *S*-oxides sometimes possess sufficient electrophilicity to be attacked by nucleophiles at the thione carbon atom. This has implications for predicting the toxicity of thione-containing compounds and their S-oxygenated metabolites. Often, if toxicity is observed with thione-containing compounds, it is associated with nucleophilic attack on electrophilic S-oxygenated metabolites and not necessarily with the parent compound. Neighboring atoms can control the electrophilicity of the oxygenated sulfur atom by either electron donation or electron withdrawal. For example, primary or secondary thioamides are more likely to form *S*,*S*-dioxides than simple thioketones because the neighboring nitrogen atom increases the nucleophilicity of the sulfur atom on the basis of resonance properties [64]. The thioamide nitrogen atom also helps stabilize the incipient *S*,*S*-dioxide by hydrogen-bonding interactions. Evidence for certain thione *S*-oxides (e.g., carbon disulfide) to form cyclic structures with the extrusion of elemental sulfur has been reported via a three-membered intermediate [65].

One important consideration in the overall metabolism, disposition, and toxicity of sulfur-containing compounds is the retroreduction of the *S*-oxide metabolite. An initial *S*-oxide metabolite of a thione is also electrophilic and can be attacked on the sulfur atom by thiaphiles (Fig. 10.3).

An attack by a thiol generally results in the formation of a disulfide and liberation of water, and often the disulfide is converted to the parent compound (Fig. 10.3). Thioamide *S*-oxides can be retroreduced, and this process is also apparently dependent on the electron-donating versus electron-withdrawing nature of the neighboring groups. For example, the electron-deficient *para*-trifluoromethyl thiobenzamide *S*-oxide is metabolically retroreduced to its thioamide more rapidly than the electron-rich *para*-methoxy thiobenzamide *S*-oxide [64]. The oxygenation–reduction dynamic plays an important role in the potential for thione-mediated toxicity. For thione *S*-oxides that are efficiently retroreduced, less metabolite is available to proceed to the more electrophilic (and hence potentially more toxic) *S*,*S*-dioxide. A generality often observed is that decreased *S*,*S*-dioxide metabolite formation likely results in less potential for toxicity. Thioketone-containing compounds as well as other sulfur-containing compounds are also subject to autooxidation and from an analytical standpoint, this can be very challenging. In any case, it is important to clearly distinguish enzymatic from autooxidative processes in any metabolic incubation or biotransformation process.

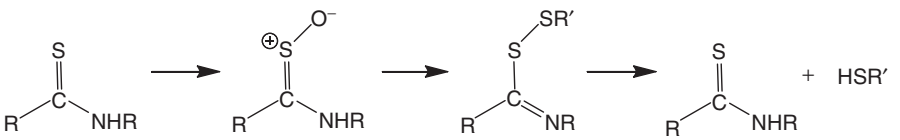

Figure 10.3 S-Oxygenation of a thioamide to the sulfoxide, and thiaphile attack by HSR′ to ultimately reform the thioamide.

10.7.2 Thioamides

Thioamides are among the most efficiently metabolized substrates of FMO because of their pronounced nucleophilicity. But thioamide functionality generally does not appear in drugs to a great extent because of the potential for toxicity. Oxidative bioactivation of thioamides represents an example of a functional group that can produce toxicity, but this is dependent on a number of factors including steric constraints and electronic properties. S-Oxygenation of thioamides involves two sequential steps that afford *S*-oxide and subsequently *S*,*S*-dioxide as major metabolites (although the latter is generally too unstable to isolate and characterize). Thioamide S-oxygenation may be mediated by both FMO and P450s. An initially formed thioamide *S*-oxide metabolite is often relatively stable and in many cases isolable. Thioamide *S*-oxides possess capacity for toxicity because they are electrophilic but more likely because they are one step closer to a subsequent metabolite (i.e., thioamide *S*,*S*-dioxide) that is extremely chemically reactive. Thioamide *S*-oxides possess some potential toxicity in their own right because they are electrophilic enough to be attacked by thiaphiles to form an imino disulfide (Fig. 10.3).

Reduction of the imino disulfide by glutathione would result in the formation of the parent thioamide but is wasteful to the cell because of the consumption of NADPH. In addition, the cell could become susceptible to toxicity under conditions where glutathione depletion was to occur. However, not all thioamide *S*-oxides are toxic likely owing to steric factors that prevent thiaphile attack or decrease formation of *S*,*S*-dioxide formation [64]. Another factor that comes into play concerns the nucleophilicity of the *S*-oxide sulfur atom determined by neighboring electronic effects. If an *S*-oxide is relatively non-nucleophilic or electron deficient, the *S*-oxide sulfur atom can be the site for reduction. As discussed earlier, thioamides are generally not present in drugs to a great extent. However, some examples of second-line antitubercular drugs are available, including ethionamide and thiacetazone (a hydrazinecarbothioamide), that are sequentially S-oxygenated by the mycobacterial enzyme EtaA [66] as well as mammalian FMOs [67]. Like other thioamide *S*-oxides, ethionamide *S*-oxide has greater biological activity than ethionamide, but the observation of hepatotoxicity has limited its use clinically.

10.7.3 Thiols

Thiols are highly nucleophilic functional groups that probably do not occur extensively in drugs because of rapid metabolism. Thiols are substrates for FMO and are initially converted to the sulfenic acid (RSOH) [68]. Once formed, sulfenic acids can be attacked by thiaphiles such as glutathione and rapidly converted to disulfides in the presence of thiols. Sometimes the disulfides are S-oxygenated one or more times to produce materials that can undergo additional transformations, both oxidative and reductive. In the case where the thiol that reacts with a sulfenic acid metabolite is glutathione, a mixed disulfide is formed, representing a detoxication process. The corresponding *S*-glutathione conjugate that is formed can be metabolized to a mercapturic acid. However, in the case where thiol S-oxygenation and glutathione attack are extensive, glutathione depletion can occur, and this may have toxicological consequences because glutathione depletion allows other electrophilic metabolites to accumulate. In the presence of depleted glutathione, additional metabolites that are sufficiently electrophilic could covalently modify important nucleophiles and lead to cell damage and

Figure 10.4 S-Oxygenation of thiol RSH to sulfenic acid, and retroreduction to the thiol via a disulfide.

eventually cellular necrosis. As discussed above, disulfide metabolites can be further oxygenated by FMO or other oxidases. In addition, disulfides can be reduced. This is another example of a futile metabolic cycle because the parent thiol is regenerated (Fig. 10.4).

In the case of FMO-mediated thiol S-oxygenation and subsequent reduction of the disulfide, the overall process consumes NADPH and potentially leads to loss of glutathione. Under such cellular conditions, glutathione needs to be biologically resynthesized, and hence, this overall process is physiologically wasteful. Disulfide S-oxygenation could also produce an electrophilic center (i.e., R-S-(O)-S-R) that is susceptible to further S-oxygenation and/or reduction. In addition, S-oxygenation of either a thiol or a disulfide affords susceptibility to displacement by another thiol (i.e., disulfide exchange). If the attacking thiol is a protein thiol, this could lead to protein disulfide formation and potentially could result in toxicological (or immunological) consequences. Disulfides arising from oxidation of thiol-containing xenobiotics that are recognized as foreign can elicit immune reactions and immunotoxicity. However, disulfides can be utilized as prodrugs in drug development. Because disulfide reduction in the body is relatively facile, thiol drugs can be liberated relatively efficiently. However, disulfide prodrugs have not been utilized that extensively, quite possibly because of the downstream metabolism outlined above. Thiols can be enzymatically S-methylated by an *S*-methyltransferase, and this tends to prolong the lifetime of a thiol-containing drug because of the types of metabolism available to the *S*-methyl functional group described below. An *S*-methyl metabolite is a thioether and, as discussed below, has attractive physiochemical and drug metabolism properties compared to the corresponding thiol drug. There are a few examples of endogenous thiol-containing substrates for FMO. Cysteamine is S-oxygenated to a sulfenic acid that is attacked by another molecule of cysteamine to produce the disulfide cystamine [69]. Currently, the physiological role of cysteamine S-oxygenation by FMO is not known but it could be involved in protein disulfide isomerization or FMO could have evolved as a general cellular protectant to ward the cell from reactive oxygen species by forming mixed disulfides [70]. It is interesting to speculate that cysteine conjugates of S-oxidative electrophiles produced by FMO (e.g., cysteamine) could serve as the necessary precursor for biosynthesizing glutathione because cysteine is an essential precursor that is transported into the cell to elevate cellular glutathione levels [2], and glutathione requires de novo synthesis after it is exported from the cell.

10.7.4 Thioethers

Thioethers possess many favorable druglike properties, and it is for this reason that the thioether functional group appears in many drugs. Replacement of a thiol with a thioether moiety in a drug candidate generally increases the lipophilicity of the parent drug, and because thioethers are typically not exceptional hydrogen-bond participants, this feature also contributes to improved cell permeability and bioavailability compared to the corresponding thiol. In addition to favorable physiochemical properties,

thioethers have beneficial metabolic properties. Barring steric constraints, lipophilic thioethers are generally good substrates for FMO, and S-oxygenation affords sulfoxides. When the two substituents adjacent to the sulfide are not the same, an enzymatically prepared *S*-oxide metabolite can possess a center of chirality [71]. Often, FMO catalyzes *S*-oxide formation with significant stereoselectivity. Of course, nonenzymatic oxidation (or autooxidation) of a sulfide with two different substituents can produce a racemic sulfoxide. In some cases, use of prochirality of a sulfide that produces an *S*-oxide with a center of chirality can provide information about the enzymatic process because often P450 and FMO work with opposite stereoselectivity [71]. Enzymatically formed *S*-oxides are zwitterionic in character and tend not to penetrate cells as efficiently as their parent sulfides. Consequently, sulfoxide metabolites can accumulate in cells and tend to have favorable cellular distribution if the target of the parent sulfide is inside the cell. This is because retroreduction of a sulfoxide inside the cell can elevate the relative concentration of a drug inside the cell. *S*-Oxides can be retroreduced to their sulfides, and this constitutes another example of a futile metabolic cycle. Aldehyde oxidase has often been implicated as the reductase for this process [72]. After retroreduction of an *S*-oxide to a sulfide, enzymatic reoxygenation completes the cycle and again tends to facilitate accumulation inside the cell, thus prolonging the action of the drug within the cell. In addition to retroreduction, *S*-oxide metabolites can be further oxidized to sulfones. Sulfone formation has been associated with FMO, P450, and other oxidases probably because the nucleophilicity is not always sufficient for FMO S-oxygenation. For FMO, the propensity for S-oxygenation of an *S*-oxide to a sulfone depends on the nucleophilicity of the *S*-oxide sulfur atom. In the case of P450, formation of sulfones from *S*-oxides involves additional considerations that are not easy to predict.

Sulfones tend to be relatively benign metabolites and are not reduced to their parent sulfides. However, there are examples of sulfones of aryl sulfides that participate in additional chemical and enzymatic transformations including displacement reactions. Unlike sulfoxides, sulfones are not charged and do not accumulate in cells to as great an extent compared with their corresponding sulfoxides. Sulfones do participate in extensive hydrogen bonding and thus possess slightly different permeability properties than sulfides.

Where a center of chirality can be formed on S-oxygenation, an investigation of the stereochemistry of the *S*-oxide itself should take place to rule out the role of autooxidation. In this regard, a chemical or biochemical method to produce sufficient amounts of the chiral *S*-oxide is desirable so that the chiral *S*-oxide can be incubated with the metabolic system to examine retroreduction and serve as a control for possible racemization. In the case of the sulfide cimetidine, the issue of enzymatic versus nonenzymatic oxidation was examined in some detail [73]. Cimetidine *S*-oxide is a prominent metabolite formed by FMO in humans. Although not explicitly studied, there is some evidence that cimetidine *S*-oxide is retroreduced to the sulfide or undergoes interhepatic recycling because pharmacokinetic studies in some humans show a biphasic profile [73]. It is notable that even HPLC grade solvents contain oxidizing agents, and solvents used in analytical work related to chiral *S*-oxide analysis and the like should be scrupulously treated to remove oxidizing agents, so that the analytical procedure does not contribute to confounding the determination of stereochemistry of the metabolite examined. As a practical matter, chiral HPLC and similar approaches

can be used to examine the contribution of enzymatic versus nonenzymatic oxidation as well as the stability of the *S*-oxide chiral center [71].

10.7.5 Sulfoxides

As discussed above, both P450 and FMO contribute to further oxidation of sulfoxides. One sulfoxide-containing chemical widely used in the drug discovery industry is dimethylsulfoxide (DMSO) and is worthwhile to discuss in some detail. With the advent of high throughput screening, DMSO has become a widely used vehicle to administer compounds for both *in vitro* and *in vivo* evaluation. This is because many compounds or closely related congeners from high throughput screening campaigns do not possess good solubility properties but are soluble enough in DMSO for *in vitro* evaluation or *in vivo* administration. The issue with *in vivo* administration of DMSO is that it is rapidly retroreduced to dimethylsulfide (DMS), which is an excellent substrate for FMO and is efficiently S-oxygenated by FMO. This sets up a metabolic futile cycle [74]. In addition, at the doses typically used as a vehicle for *in vivo* studies, DMS saturates FMO and effectively inhibits the enzyme as an alternate substrate competitive inhibitor. Thus, after administration to an animal, DMSO sets up a futile cycle, but because DMS is such a good FMO substrate, it effectively abrogates contribution to metabolism of most compounds coadministered with DMSO because (as discussed below) many of these compounds are amines. Thus, administration of DMSO (e.g., 1 mL/kg) to a small animal serves as a chemical knockdown that is similar to the more eloquent molecular knockout of FMO recently reported [75]. Generally, but not always, amines are poorer FMO substrates than DMS. From the perspective of evaluating the contribution of FMO in the metabolism of an amine drug candidate coadministered with DMSO, this represents quite a confounding situation. Likely, coadministration of an amine with DMSO will significantly decrease FMO-dependent metabolism of the amine. In addition, futile metabolic cycling of DMSO/DMS may also influence other NADPH-requiring metabolic pathways because of the consumption of NADPH in the reoxygenation of DMS. However, to my knowledge, this has not been extensively examined.

10.7.6 Amines

As a generality, nitrogen-containing functional groups are less nucleophilic than sulfur-containing ones, and while amines are well-recognized substrates for FMO, they tend to be N-oxygenated less efficiently than sulfur-containing substrates. Hence, avidity of an amine to FMO tends to be less, and, as a generality (although there are notable exceptions), K_m values tend to be greater for tertiary amines than for sulfur-containing FMO substrates. There are notable exceptions where amines are excellent substrates for FMO. In fact, the original name for FMO was *N*,*N*-dimethylaminoaniline-*N*-oxidase because *N*, *N*-dimethylaminoaniline was such a good substrate for the originally isolated FMO from pig liver [76]. Today, we recognize that FMOs N-oxygenate primary, secondary, and tertiary amines, depending on the form of the enzyme. Because many drugs employ amine functionality as an intrinsic part of their pharmacophoric composition, evaluation of the role of FMO in the metabolism of amines constitutes an important endeavor.

10.7.7 Hydrazines

Hydrazines comprise a group of highly nucleophilic nitrogen-containing compounds that are substrates for FMO. Metabolism of hydrazines by FMO illustrates a number of important points. Because of the lone pair on the adjacent nitrogen atom, the nitrogen atom that is oxygenated possesses increased nucleophilicity. This is the so-called alpha effect of the hydrazine nitrogen atom to oxygenation via a through-space lone pair–lone pair assisted mechanism. The alpha effect is also present in other molecules that have heteroatoms alpha to a nucleophilic center (i.e., hydroxylamines). Of course, the relative alpha effect for substituted hydrazines is dependent on the substituent pattern and the degree of electron-withdrawing or electron-donating properties of the neighboring substituents. For FMO-mediated N-oxygenation of substituted hydrazines, the extent of N-oxygenation may include some steric considerations. Generally, the tertiary nitrogen atom of a hydrazine is the site of N-oxygenation by FMO [77]. However, hydrazines are among the most toxic substances known, and extensive studies examining the role of human FMO in their metabolism have not been reported. Nevertheless, depending on the substitution pattern of the hydrazine, N-oxygenation could lead to several unusual azo metabolites and some of the metabolites could contribute to the toxicity of these compounds.

10.7.8 Hydroxylamines

Primary and secondary aliphatic amines are converted to hydroxylamines by FMO [17–19]. Generally, aliphatic hydroxylamines are more nucleophilic than the parent amine (because of the alpha effect), and often, they are N-oxygenated a second time to produce an *N*,*N*-dihydroxy intermediate that is not stable (Fig. 10.5).

In the case of primary amines, elimination of the elements of water produces an oxime. Both *cis*- and *trans*-oximes are capable of being formed from FMO, but often considerable stereoselectivity is observed [19]. Because loss of water from the *N*,*N*-dihydroxy intermediate is a spontaneous reaction and not strictly speaking enzyme

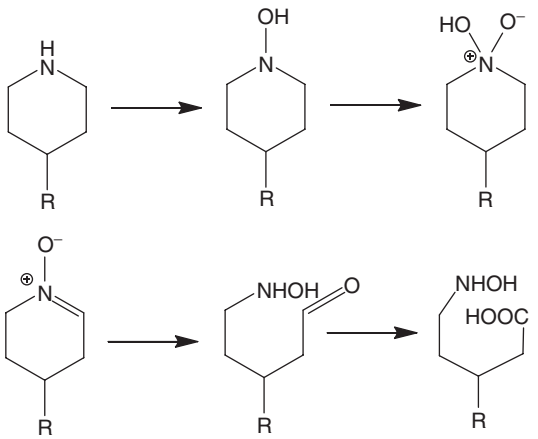

Figure 10.5 Sequential N-oxygenation of a cyclic secondary amine, with eventual ring opening and oxidation to the carboxylic acid.

catalyzed, it is somewhat surprising that very often a great degree of stereoselectivity in the FMO-derived product is observed. The large degree of stereoselectivity observed for oxime formation has been interpreted as an FMO enzyme "template effect" on product formation because the final oxime-forming reaction is not apparently FMO catalyzed [17,18]. It is also somewhat surprising that in the formation and disposition of aliphatic hydroxylamines, this transformation can go on in the presence of the strongly reducing environment of the cell. Like tertiary amine *N*-oxides, hydroxylamines can be efficiently retroreduced to the parent amine [78]. While the enzymatic basis for retroreduction of hydroxylamines is somewhat controversial (i.e., some favor a system of cytochrome b_5, NADPH-cytochrome b_5 reductase, and a P450, while others favor a hydroxylamine reductase), the fact that oxime formation can out-compete retroreduction suggests that FMO-mediated formation of oxime is quite robust. Similarly, formation of nitrones from aliphatic secondary hydroxylamines suggests that N,N-dihydroxylation of secondary hydroxylamines by FMO can effectively compete with hydroxylamine retroreduction (Fig. 10.5). An exhaustive examination of primary and secondary hydroxylamine retroreduction has not been reported. It is likely that different retroreduction systems are at work for different hydroxylamines (and for different tertiary amine *N*-oxides). But obviously, N-oxygenation may play a much larger role in primary and secondary aliphatic amine metabolism than previously recognized, if retroreduction is a facile process. It may be that considerable futile cycling occurs in many cycles of amine oxygenation and reduction. Because in some cases, the ultimate metabolite arising from FMO-mediated processes is quite distinct from the starting amine, careful evaluation of the metabolic pathway should be considered before the relative contribution of FMO in the metabolism of an amine is adjudged. For example, after formation of a nitrone from a secondary amine, unless the nitrone is highly conjugated or stabilized in some manner, it is prone to hydrolysis to the corresponding aldehyde and hydroxylamine [79]. Generally, because aldehydes are converted to carboxylic acids that are conjugated and excreted, this type of metabolism presents quite a distinct product metabolite profile compared to the starting amine. In the case of cyclic secondary amines, even more complex metabolites can be formed because of the propensity for cyclization and intramolecular reactions of the metabolites that are formed (Fig. 10.6).

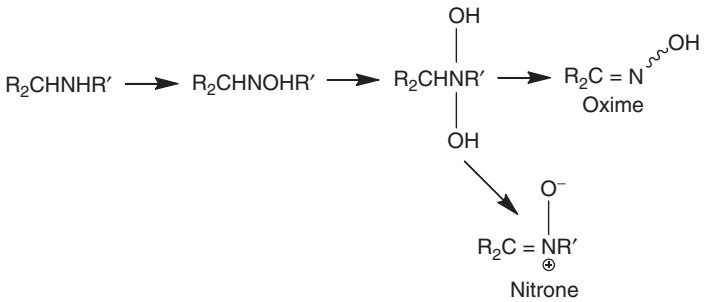

Figure 10.6 N-Oxygenation of primary (R′ = H) and secondary (R′ = substituent) amine to *cis*- or *trans*-oximes and a nitrone, respectively, via the intermediacy of a *N*, *N*-dihydroxy intermediate.

Regardless, and in agreement with tertiary amine metabolism, the role of FMO in primary and secondary aliphatic amine metabolism is potentially quite complex. Further confounding the metabolism of primary and secondary aliphatic amines is the role of retro-reductase systems in hydroxylamine (or tertiary amine *N*-oxide) retroreduction. Additional studies are needed to clarify the oxidative and retroreductive nature of primary and secondary hydroxylamine metabolism.

10.7.9 Cyclic Amines

Certain cyclic tertiary amines such as piperidines, piperazines, morpholines, and tetrahydropyridines are commonly found in biologically active drugs and drug candidates. Cyclic amines such as these are often found in drugs and drug candidates because of their favorable pharmaceutical properties and the propensity of pharmacological targets to bind these types of tertiary amines with high avidity. It is a common drug design strategy to decrease the metabolism of aliphatic secondary and tertiary amines and increase their bioavailability by incorporating the secondary and tertiary amines as a cyclic amine. In addition, other proximal substituents appended to the nitrogen atom or ortho to the nitrogen atom in the ring often further decrease metabolism and increase bioavailability. Sometimes, by decreasing metabolism via N-demethylation (in the case of aliphatic tertiary amines), N-oxygenation mediated by FMO (in the case of cyclic tertiary amines) becomes a more prominent metabolic pathway. One of the reasons for this is that cyclic tertiary amines such as piperidines are preferentially N-oxygenated by FMO because of the increased nucleophilicity of the tertiary amine. In the case of piperidines, for example, the through-space influence of the neighboring nitrogen atom lone pair increases the nucleophilicity of the tertiary amine. This is analogous to a through-space alpha effect discussed earlier for hydrazines and hydroxylamines. However, cyclic tertiary amines are not impervious to further metabolism, including N-demethylation. Cyclic tertiary amine *N*-oxides can also enter into further metabolism after retroreduction. After N-dealkylation, a des-alkyl cyclic amine metabolite can also, in principle, be a substrate for FMO (although it tends to be a less avid substrate than cyclic tertiary amines), but cyclic secondary amines can sometimes participate more in extensive metabolism than cyclic tertiary amines. This is particularly true in comparison to cyclic tertiary amine *N*-oxides (that, once formed *in vivo*, often possess sufficient water solubility to be directly excreted in the urine). For cyclic secondary amines, the initial FMO-dependent metabolism produces a hydroxylamine that often undergoes N-oxygenation a second time to afford an *N*,*N*-dihydroxy metabolite because the hydroxylamine is more nucleophilic than the parent cyclic secondary amine (Fig. 10.6).

Of course, this assumes that facile retroreduction of the cyclic secondary hydroxylamine does not occur to provide the parent amine. Nevertheless, the N,N-dioxygenated metabolite is not indefinitely stable and eliminates the elements of water to afford a cyclic nitrone. Generally, unless highly conjugated, nitrones are unstable to hydrolysis and result in ring opening to produce an aldehyde and a hydroxylamine (Fig. 10.6). Typically, the aldehyde and hydroxylamine portions of the metabolite undergo further metabolism. Sometimes, the ultimate metabolites are quite distinct from the structures of the initial metabolites and are not obviously related to classical FMO-mediated products. Considerable care needs to go into deciphering some of these more complex metabolic profiles.

10.7.10 Pyrrolidines

Cyclic tertiary amines such as pyrrolidines are also often N-oxygenated by FMO. Like some of the six-membered heteroatom-containing compounds discussed earlier, the compact structure of a pyrrolidine creates a pinned-back nitrogen atom lone pair that markedly increases the nucleophilic nature of the tertiary amine (e.g., N-oxygenation of nicotine is an example of a probe substrate of human FMO3 [80]). Similar to piperidine *N*-oxides, pyrrolidine *N*-oxides are relatively stable and in the *in vivo* setting are often excreted unchanged because of the polarity of the molecule. In principle, pyrrolidine *N*-oxides can be metabolically retroreduced in animals but this may be dependent on the route of administration; for example, a pyrrolidine *N*-oxide (e.g., nicotine-*N*-1′-oxide) administered i.v. is excreted unchanged [81] in humans but nicotine *N*-oxide administered orally to small animals is retroreduced [82]. One possible explanation to this phenomenon is that bacterial reductases of the gut reduce nicotine-*N*-1′-oxide after oral administration. The relative polarity and stability of cyclic tertiary amine *N*-oxides contribute to the lack of toxicity in these types of metabolites. While no evidence was observed for retroreduction of nicotine-*N*-1′-oxide after administration to humans [81], this is likely not the case for other cyclic tertiary amine *N*-oxides *in vivo*. However, the polarity of the *N*-oxide generally decreases pharmacological activity observed for the parent amine, and these types of metabolites are generally not potent and are excreted in the urine. Although there are some notable exceptions that tertiary amine *N*-oxides possess pharmacologic activity, it is unclear whether this is due to retroreduction to the parent amine or functional activity of the tertiary amine *N*-oxide in its own right.

10.7.11 Aliphatic Tertiary Amines

Aliphatic tertiary amines have long been associated as the prototypical FMO substrate. Conversion of tertiary amines to their *N*-oxide results in polar, readily excreted metabolites that are usually devoid of pharmacological potency. However, the zwitterionic nature of tertiary amine *N*-oxides can in some cases bestow pharmacological function. Although not extensively examined, aliphatic tertiary amine *N*-oxides can be retroreduced to the parent tertiary amine [5]. This is another example of a futile metabolic cycle. As described, the overall process consumes NADPH and leads to the regeneration of the tertiary amine. It is likely that many more examples of retroreduction of aliphatic tertiary amine *N*-oxides to tertiary amines have not been reported or observed due to analytical challenges or lack of direct evaluation. To date, a number of retro-reductases have been reported for tertiary amine *N*-oxides [83], and examples for tamoxifen *N*-oxide include aldehyde oxidase, cytochrome reductase, P450, and other various hemoproteins (e.g., hemoglobin and catalase) [84]. The importance of aldehyde oxidase as a general tertiary amine *N*-oxide retro-reductase is notable [85] because its presence in small animals is gender specific (i.e., males $\gg$females) and estimates of the relative amount of tertiary amine *N*-oxides can be confounded as a consequence of gender [86]. Thus, comparison of metabolism between both genders can provide circumstantial evidence for the formation of tertiary amine *N*-oxides as well as evidence for retroreduction. Further confounding the situation for retroreduction of tertiary amine *N*-oxides is the observation that many commonly used drugs inhibit aldehyde oxidase [87].

Certain tertiary amines are prone to nonenzymatic autooxidation, and this can confound analysis of products formed and contribution of enzymes to the process. In the

case where *N*-oxide metabolites can produce a stereochemical outcome (e.g., in the case of *cis*- vs *trans*-nicotine-*N*-1′-oxide), ruling out the involvement of autooxidation is important for correct stereoselectivity determination. To prevent autooxidation in *in vitro* metabolic experiments, antioxidants such as DETAPAC can be added to the incubation [11]. DETAPAC is superior to EDTA because EDTA can also serve as a prooxidant under certain conditions and exacerbate the autooxidation situation.

10.7.12 Aliphatic Secondary Amines

Aliphatic secondary amines can be N-oxygenated by FMO to produce the hydroxylamine. The hydroxylamine metabolite, being more nucleophilic, is often N-oxygenated again by FMO to produce a nitrone (after loss of water) because the hydroxylamine is generally a better substrate for FMO than the parent amine. Generally, aliphatic nitrones are unstable to hydrolysis and afford the aldehyde that can be further metabolized to a carboxylic acid and other metabolites. Hydroxylamines are also efficiently retroreduced to the parent amine. In the case of FMO-mediated N-oxygenation, N,N-dioxygenation affords a metabolite (i.e., a nitrone after elimination of water) that is not subject to ready retroreduction. However, the initially formed hydroxylamine is efficiently retroreduced. For FMO substrates that do not desorb from the enzyme surface, substrates can be converted to nitrones without the intermediacy of retroreduction because the second N-oxygenation is kinetically faster than the initial one. In cases where steric inhibition to hydroxylamine N,N-dioxygenation is apparent, hydroxylamine retroreduction can be observed and little net N-oxygenation observed. To experimentally examine the role of retroreduction, saturating amounts of another hydroxylamine (e.g., dimethylamine hydroxylamine) can be added to the incubation to enable study of the test agent hydroxylamine formation. Other approaches to examining the role of retroreduction in secondary amine metabolism include varying the NADH/NADPH cofactor because certain retro-reductases preferentially require NADH to NADPH [88].

10.7.13 Primary Amines

Aliphatic primary amines are N-oxygenated by FMO. To date, compared with FMO1, FMO3 has shown much greater activity as a primary amine *N*-oxygenase. The initially formed hydroxylamine is more nucleophilic than the parent amine and is N-oxygenated a second time to an N,N-dioxygenated material. The *N*,*N*-dioxide is not indefinitely stable and eliminates the elements of water to produce an oxime as a stable metabolite (Fig. 10.5).

For sterically unhindered aliphatic primary amines, generally, the hydroxylamines are not observed because the second N-oxygenation is more efficient than the initial hydroxylamine formation. It is possible that an enzyme template effect is in operation for FMO-mediated oxime formation where the intermediate does not desorb from the enzyme surface until the oxime is formed. Because dehydration is fast and nonenzymatic for primary amine N,N-dioxygenated intermediates, the relative stereochemistry of the oxime formed is probably determined by a template effect and not via true enzyme catalysis [17,18]. Somewhat surprising to the nonenzymatic nature of the oxime formation process is that some primary oximes are formed with great stereoselectivity. In the case where this has been studied in detail, considerable stereoselectivity has been observed for a number of primary amine substrates [17,18].

In other cases, much lower stereoselectivity has been observed [19]. It is not clear as to the precise substrate structural features required that affect great stereoselectivity, but it underscores the utility of FMO in forming oximes from primary amines with sometimes considerable stereoselectivity.

10.8 NON-NUCLEOPHILES

In a few cases, relatively non-nucleophilic substrates have been reported to be oxygenated by FMO. Generally, non-nucleophilic substrate oxygenation by FMO is very rare, and considerable care needs to be applied to rule out nonenzymatic autooxidation. Another possible origin for FMO-dependent oxygenation of a non-nucleophilic substrate involves uncoupling of FMO (i.e., where the normal mechanism employing the peroxyflavin becomes uncoupled from the enzyme catalytic cycle and acts as a hydrogen peroxide generator to oxidize substrate). In the case where a center of chirality can be generated, a check of the stereochemistry of the product can indicate if uncoupling has occurred for FMO because if hydrogen peroxide or lipid peroxides are the oxidants, a racemic product will result. On the other hand, if a center of chirality can be formed, FMO produces products often with marked stereoselectivity [71]. If a center of chirality cannot be formed, standard incubation of FMO and comparison with the presence or absence of 3–5% hydrogen peroxide and product analysis can indicate if nonenzymatic hydrogen peroxide is responsible for product formation because racemic product will form in the case of the nonenzymatic process. Incubations in the presence of hydrogen peroxide alone will provide an indication of the nonenzymatic rate of oxidation. Of course, a true control incubation without hydrogen peroxide (and without NADPH) must also be run because certain hepatic microsome preparations (i.e., those that are prepared without antioxidants or at elevated temperatures) contain significant quantities of lipid peroxides that can readily autooxidize nucleophilic organic compounds. More complex experiments involving the use of $^{18}O_2/^{16}O_2$ and labeled water can help distinguish the role of FMO in substrate oxidation because FMO incorporates molecular oxygen and not water into enzyme-derived products. Thus, examination of a non-nucleophilic substrate in the presence of $^{18}O_2$ should show incorporation of 18-oxygen into the product if the product is formed in an NADPH- and FMO-dependent process. An oxidation product arising from aldehyde oxidase would incorporate oxygen from water and not molecular oxygen. Using commercially available recombinant FMO (e.g., BD Gentest, Woburn, MA) in these types of experiments would get around any potential confounding reaction with microsomal monooxygenases.

10.9 DRUG OPTIMIZATION

While functional groups primarily metabolized by FMO generally are not associated with apparent toxicity, there are a few such functional groups (e.g., thiones, thioureas, thioamides) that if present in a drug candidate likely should be replaced because of their propensity to cause toxicity via oxidative bioactivation. Generally, the corresponding oxygen-containing compound possesses less potential for toxicity. In principle, replacement of functional groups with pharmacophorically similar moieties that can be

metabolized to reactive metabolites by FMO should take place early in the development paradigm. Another strategy is to purposely introduce functional groups into drug candidates that are selectively metabolized by FMO. This approach would accomplish the objective of distributing the metabolism of the drug over a wider set of enzymes as well as potentially affording a variety of metabolites including FMO metabolites that are generally polar, nontoxic, and readily excreted. By avoiding metabolism (and bioactivation) by P450, the prospects for increasing the druglike properties of a drug candidate is likely increased. Because so many drugs are metabolized by P450 3A4, decreasing the amount of metabolism through this enzyme system is likely to decrease adverse drug–drug interactions. Enhancing the metabolism of a drug by FMO could take a few different forms. One approach might be to increase the propensity for FMO-mediated oxygenation, and another approach might be to decrease the retroreductive pathways that tend to decrease FMO product formation. Yet another method is to use a prodrug approach to fundamentally change the nature of the drug properties to improve some aspect of ADMET or bioavailability. A concept that has not been discussed herein could be to target drugs to different tissues by taking advantage of the distribution of FMOs. Because FMO1, for example, is prevalent in kidney and not present in adult human liver, developing drugs with FMO1 selectivity could potentially and preferentially target drugs to the kidney.

10.10 CONCLUSIONS AND FURTHER PERSPECTIVES

With the introduction of greater and greater numbers of preclinical drug candidates because of high throughput synthesis and screening, the bottleneck in drug discovery has shifted to include safety considerations. Applying metabolism and drug safety concepts earlier in the drug discovery and development process is likely to yield greater numbers of druglike materials that have a chance to become clinically useful agents.

One of the concepts presented herein is the idea that FMO can be utilized in the preclinical paradigm to facilitate the process of improved “hit” rate in drug discovery. FMO possesses some attractive features as a metabolic system: FMO is neither readily inhibited nor induced and the likelihood for adverse drug–drug interactions is likely decreased. It is likely that most of the variability of FMO is due to pharmacogenetics and genetic polymorphisms. As more clinical studies are done with drugs prominently metabolized by FMO, more information about the variability of FMO in the human setting will be revealed.

While not discussed to any great depth in this chapter, the structural biology of FMO has begun to show advances. Several crystal structures of FMO-like proteins have been reported, including the FMO from *Schizosaccharomyces pombe* [89], the FMO from *Methylophaga* sp. strain SK1 [90], and a Baeyer–Villiger oxidation catalyst called *cyclohexanone monooxygenase* from bacteria [91,92]. These FMO-like proteins are considerably smaller than mammalian FMOs; nevertheless, considerable new information has been forthcoming that can be related to the mammalian FMO structure and mechanism of action. In the future, additional progress with the structural basis of mammalian FMOs is anticipated, which will undoubtedly aid in understanding this important monooxygenase and designing new and more efficacious drug candidates.

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FURTHER READING

The Scripps Research Institute from the NIH compound collection (http://mlsmr.glpg.com/MLSMR_HomePage/).

The Sanford-Burnham Medical Research Institute's small molecule collection (http://bccg.burnham.org/Assays/ScreeningCompoundCollection.aspx).