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Aldehyde Oxidase: An Enzyme of Emerging Importance in Drug Discovery

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Introduction

Aldehyde oxidase (AO,[a] EC 1.2.3.1) has for many years been recognized as a metabolizing enzyme contained within the cytosolic compartment of many tissues and in many species. The first literature reference to AO dates from the 1930s, but literature citations to this enzyme have been steadily growing ever since, especially in the past decade. It has been increasingly recognized in this past decade that AO, through its unique structure, distribution, and substrate recognition, has an important role to play in the metabolism of drugs. In this timely Perspective, we present the current knowledge of the enzyme's expression, its structure, and its distribution across species. We offer an analysis of substrates recognized by AO and highlight clinical examples where metabolism by this enzyme has had significant clinical impact or led to the termination of a drug development program. Finally, through analysis of the structure of known substrates, we present data to suggest the significant impact AO could play on future drug discovery programs along with strategies that could be employed to mitigate this.

Enzyme Structure, Function, and Expression

AO is a complex molybdoflavoprotein that belongs to a family of structurally related molybdenum-containing enzymes.[1] AO is homologous with xanthine oxidase (XO), another mammalian molybdoflavoprotein, and both AO and XO show a remarkable degree of similarity in their amino acid sequence. Like XO, AO is active as a homodimer composed of two identical subunits of about 150 kDa. Each subunit is subdivided into three distinct domains: a 20 kDa N-terminal domain that binds two iron-containing clusters, a 40 kDa domain harboring a flavin adenine dinucleotide (FAD) binding site, and a C-terminal domain that accommodates the molybdenum cofactor.[1,2] Molybdenum (Mo) is an essential component of the enzyme and is required for enzyme catalysis along with FAD.[2] It is biologically inactive until it becomes complexed by a special pterin to form the tetracyclic pteridine complex[3] termed "MoCo" (Figure 1b). A depiction of a subunit of human AO is shown in Figure 1a which highlights the three domains that make up the functional enzyme monomer.

The primary function of the pterin moiety is to position the catalytic Mo correctly within the active site of the enzyme, to control its redox behavior, and to participate in electron transfer to and from the Mo atom.[4]

Given the similarity in structures of AO and XO, the properties of the two enzymes are closely related. Both enzymes are present in the cytosol and commonly exist in all vertebrates.[5] The primary difference in the two enzymes, however, is that XO can exist in two interconvertible forms, xanthine oxidase and xanthine dehydrogenase, while AO exists only in the oxidase form. AO utilizes only molecular oxygen as an electron acceptor in contrast to XO, which can transfer electrons to both oxygen and nicotinamide adenine dinucleotide (NAD^+). Although the physiological function of XO has long been recognized as the key enzyme in the catabolism of purines, oxidizing xanthine into the terminal catabolite uric acid, the biochemical and physiological function of AO is still largely obscure.[6] Both enzymes catalyze oxidation and reduction reactions of a large array of substrates, although oxidation reactions are far more common. Notably, the substrate and inhibitor specificities of XO and AO differ.[7] In general, AO has the ability to oxidize a broader range of substrates than XO.[6,8] Typical substrates of AO are compounds containing an aldehyde function, nitro/nitroso compounds, or *N*-heterocycles,[6,9] as described below. As we will show, this last class of heterocyclic substrates are especially pertinent to drug discovery programs. AO is also known to catalyze the oxidation of intermediate products of cytochrome P450 (CYP450) or monoamine oxidase (MAO).

Tissue Distribution

AO is distributed across several tissues.[8] Liver has by far the highest expression of AO in all species including human, and AO-mediated drug clearance is dominated by liver-based AO activity. AO distribution in other tissues is highly species dependent. For example, in mouse AO activity in lung tissue is more significant than in the rat.[10−12] The distribution of AO at the cellular level has also been investigated in a variety of human tissues by immunohistochemistry and found to be widespread.[5,13] In humans, aside from the liver, AO activity

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[a] Abbreviations: AO, aldehyde oxidase; AUC, area under the curve; CYP450, cytochrome P450; FAD, flavin adenine dinucleotide; FMO, flavin-containing monooxygenase; GPCR, G-protein-coupled receptor; HLM, human liver microsomes; KDIE, kinetic deuterium isotope effects; MAO, monoamine oxidase; MoCo, molybdenum pyranopterin cofactor; NADPH, nicotinamide adenine dinucleotide phosphate; PSA, polar surface area; S9, the supernatant fraction obtained from liver homogenate; UGT, uridine 5'-diphosphoglucuronosyltransferase; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

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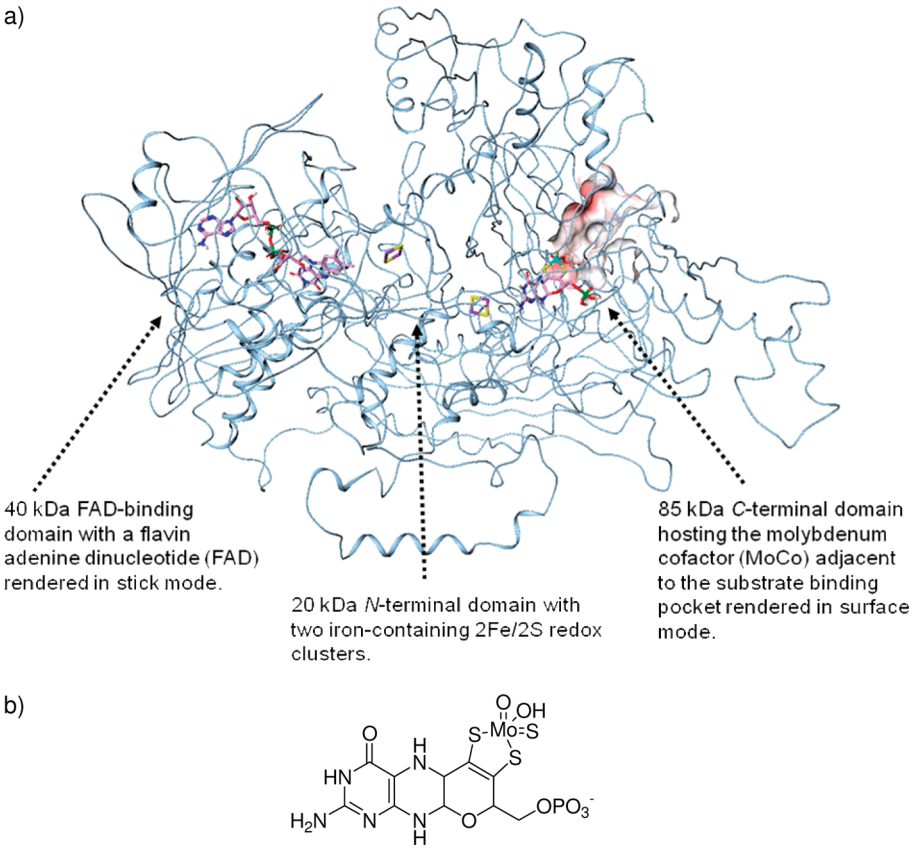

Figure 1. (a) Overview of the human aldehyde oxidase monomer structure, generated using the method described in ref 110, and (b) structure of the pyranopterin moiety MoCo.

has also been detected in excretory organs such as lung, the gastrointestinal tract, and kidney. In the gastrointestinal tract, AO activity resides primarily in the small and large intestine while in the respiratory system it is abundant in epithelial cells from the trachea and bronchium, as well as in alveolar cells. Furthermore, the proximal, distal, and collecting tubules of the kidney all show high levels of AO activity. Other organs where AO activity has been detected include endocrine tissue and brain.[14]

Species Differences

AO is found in most species including fish and insects.[2] Until a few years ago it was believed that the subfamily of molybdenum hydroxylases in humans and other mammals consisted of only two members, AO and XO. However, in the past few years, novel members of the family have been identified. In particular, at least two additional rodent molybdenum hydroxylase genes have been identified.[5] Despite the similarities between mammalian cDNA and amino acid sequence, there are marked species differences in protein expression and catalytic activity of AO.[8,15] The difference in the metabolism of substrates among species implies that several isoforms of the enzyme may exist. From a drug discovery point of view, understanding enzyme variations between species is of great importance in making accurate predictions of human pharmacokinetic parameters, particularly half-life and dose. Although the order of AO activity among animal species may vary depending on the substrate, it generally seems to be high in monkeys and humans and low in rats, whereas dogs are to a large extent deficient in activity. Such differences have been ascribed to the size of the active site in different species.[10,14] Large activity differences have also been observed among various strains in the rat and mouse.[16,17] For instance, a marked strain difference was observed when 12 different strains of rats were assessed for AO activity, with the highest activity observed in Sea:SD rats and lowest in WKA/Sea rats.[18] Further striking variations in AO activity have been noted in Sprague−Dawley rats and Wistar rats and in C129/C57 and CB57BI/6J mice.[19,20] Significant in vitro variability in AO activity has also been shown in humans[21,22] which may to some extent be caused by quantitative differences in enzyme levels. Other factors such as gender, age, cigarette smoking, drug usage, and disease states, such as cancer, also may alter AO activity. Gender has been reported to influence mouse and rat AO activity.[9,17] Male mice exhibit 2- to 4-fold higher AO activity than female mice. Reports suggesting the expression of kinetically distinct AO forms in the liver of male and female mice and rats have been published. In both species, significantly lower K_m values were observed for substrate oxidation using the male enzyme compared to female enzyme. This difference has been attributed to hormonal regulation.[8] Gender related differences in AO activity have not thus far been reported in humans. Limited clinical data on the pharmacokinetics of a known AO substrate, penciclovir, suggested no significant difference in drug exposure between male and female volunteers.[23] Disease states such as cancer, inflammatory mediators (cytokines) such as interferon, and interferon inducing agents such as lipopolysaccharides that are known to affect drug metabolizing enzymes can also affect AO activity and result in interindividual variability.[24,25] However, no reports of the impact of these factors on AO activity have been reported thus far.

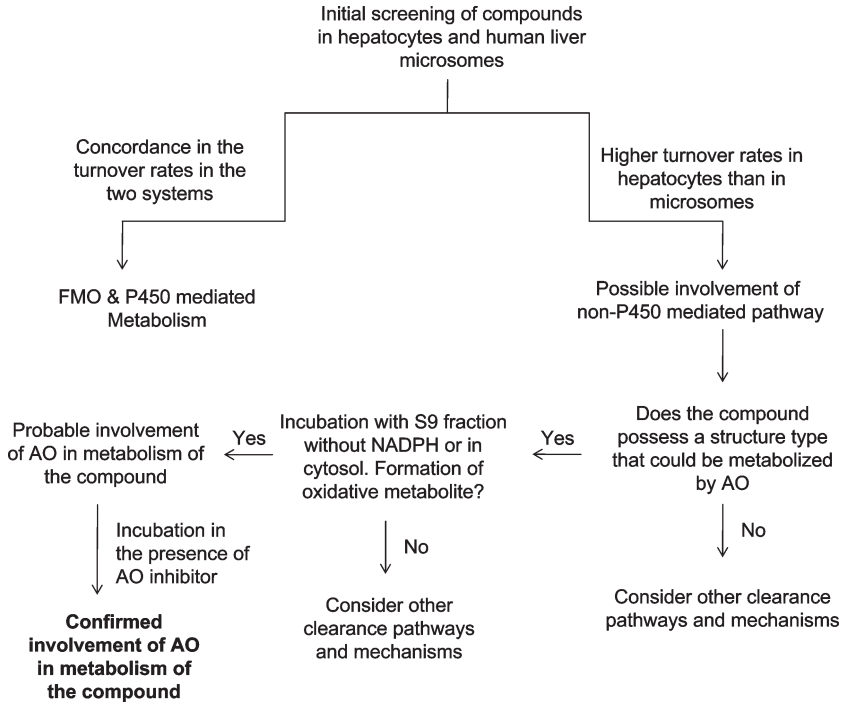

Figure 2. Decision tree to guide decision-making during the screening of potential AO substrates.

Screening Methods

It is well-known that CYP450 is the primary enzyme responsible for metabolizing drugs and xenobiotics in the body. Thus, medicinal chemistry attempts to reduce CYP450-mediated metabolic clearance have become a norm in drug discovery and are well-documented.[26] Metabolic screens that use in vitro systems, such as liver microsomes and hepatocytes, are commonly employed early in drug discovery programs to inform iterative compound design. As strategies to reduce CYP450-mediated liabilities become increasingly successful, it is reasonable to assume that new drug molecules will increasingly become substrates for less common clearing enzymes such as AO. Although liver microsomes provide an affordable way to give a good indication of whether a compound is a CYP450 or uridine 5′-diphosphoglucuronosyltransferase (UGT) substrate, they do not provide information on the contribution of cytosolic enzymes in its metabolism.[27] Since AO is a cytosolic enzyme, normal liver microsomal assays are not useful and may underestimate the clearance of an AO substrate in vivo. Hepatocytes on the other hand contain all human phase I and phase II enzymes.[27] Thus, the parent depletion profile following incubation of a compound with hepatocytes isolated from humans or preclinical species can help to assess its metabolism by enzymes other than CYP450 when the data are used in conjunction with that generated from microsomal incubation. Two possible outcomes are feasible when the results from the microsomal and hepatocyte screen assay are compared. A concordance in the turnover rates from NADPH-supplemented human liver microsomal incubations and incubations with human hepatocytes indicates CYP450-dominated metabolism of a compound. Alternatively, a disconnect in the turnover rates of a compound by the two in vitro incubation systems, with faster turnover in the hepatocyte incubations, is a good indicator that a compound is a substrate for a non-CYP450 enzyme and possibly a cytosolic enzyme such as AO. We recommend researchers follow the line of reasoning detailed in Figure 2 to elucidate a possible involvement of AO-catalyzed oxidation in compound metabolism.

To fully enable optimization of a lead compound series and to aid in the development of structure−metabolism relationships and thereby guide optimization of compound properties, identification of metabolites and the enzymes responsible for their formation is essential. The above process should therefore be followed up with experiments in which the lead compound is incubated with a cytosolic or an S9 fraction (fraction obtained from a liver homogenate by centrifuging at 9000*g* for 20 min in a suitable medium; this fraction contains both cytosol and microsomes) in the absence of a cofactor (such as NADPH) which is generally included in incubations with liver microsomes. Formation of an oxidative metabolite in such an incubation will reveal contribution of a non-CYP450 enzyme in the oxidation of a compound. Consequently, the involvement of AO can be confirmed by carrying out the same incubation in the presence of an AO inhibitor and observing a reduction in metabolite production.[28] It should also be highlighted that compound structure should be used as a prospective indicator of possible AO metabolism, as we will show below.

In Vitro−in Vivo Correlations

Ultimately, a high confidence method of predicting clearance parameters in humans, regardless of the route of clearance, is highly desirable to make accurate dose predictions. While methods of scaling in vitro intrinsic clearance data to predict the in vivo clearance of drugs have been well-established for CYP450-dominated clearance, similar methods have not been established for drugs metabolized by AO. This may partly be due to emerging knowledge of species differences described above and to the fact that while AO is involved in the intermediary metabolism of many compounds, not many drugs have AO as the major initial clearing enzyme. Furthermore, it is highly desirable to be able to determine the fraction of total clearance that is mediated by AO (akin to CYP450

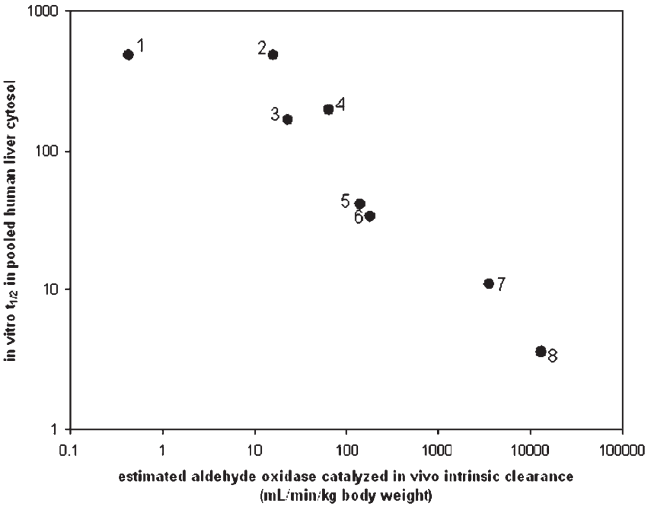

Figure 3. Correlation between in vivo intrinsic clearance mediated by aldehyde oxidase and half-life in pooled human liver cytosol, expressed in minutes: 1, methotrexate **31**; 2, **42** (XK-469); 3, **53** (*RS*-8359); 4, zaleplon **79**; 5, 6-deoxypenciclovir **82**; 6, compound **73**; 7, DACA **41**; 8, carbazeran **71**.

reaction phenotyping studies). The sedative agent zaleplon offers a well characterized example. Human liver slices demonstrated that AO was responsible for the generation of an oxidative metabolite of zaleplon which comprised approximately two-thirds of total clearance of parent drug.[29,30] However, the data were not used to predict the in vivo clearance of zaleplon. Scaling the reported enzyme kinetic parameters for conversion of zaleplon to this oxidized metabolite in human liver cytosol (K_M = 124 μM; V_{max} = 564 (pmol/min)/mg cytosol) yields a calculation of hepatic intrinsic clearance of ~11 mL/min/kg assuming 100 mg cytosolic protein per g of liver and 20 g of liver per kg of body weight. The actual human in vivo intrinsic clearance value for zaleplon is around 90 mL/min/kg, and considering that two-thirds of this is catalyzed by AO, the in vitro data underestimate the in vivo clearance by about a factor of 6.

In a recent report,[31] Zientek et al. described a cross-drug correlation for predicting AO mediated in vivo intrinsic clearance in human. In this study, a test set of compounds for which human in vivo pharmacokinetic data existed and for which AO was known to catalyze all or a portion of the total clearance were studied in pooled human liver cytosol and human liver S9 fractions. It was shown that while the in vitro intrinsic clearance data fell short of scaling to in vivo intrinsic clearance, the rank order of the compounds was preserved (Figure 3) and permitted cutoff values for the in vitro data wherein a compound could be classified as a high, medium, or low clearance compound. We will return to zaleplon and the other compounds described in Figure 3 later.

Mechanism of Oxidation

A much simplified depiction of the AO catalyzed oxidation of compounds is shown in Figure 4.[5] In a typical catalytic cycle, the substrate is oxidized to product at the Mo center. The reducing equivalents are then passed to FAD, which is reoxidized by molecular oxygen. The iron-containing centers function as mediators of electron transfer between MoCo and the flavin cofactor and serve as electron sinks, storing reducing equivalents during catalysis.

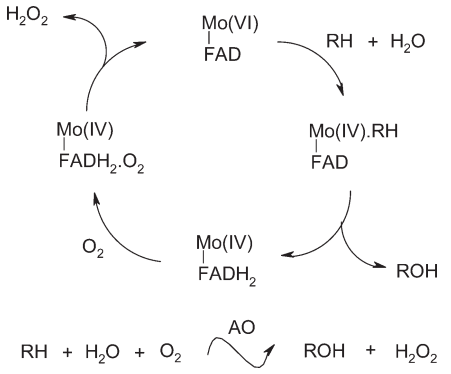

Figure 4. Proposed mechanism of AO-catalyzed oxidation of a substrate RH and the overall transformation catalyzed by AO.

The oxidative hydroxylation of substrates by AO is complementary to that mediated by CYP450, which plays a paramount role in metabolism of xenobiotics and drugs. Even though both enzymes utilize molecular oxygen as the ultimate electron acceptor,[32−35] the oxygen atom that is incorporated into the product during AO-mediated oxidative hydroxylation comes from water and not oxygen. The AO catalyzed oxidation of N-heterocycles (Figure 5) involves an initial nucleophilic attack at the carbon atom adjacent to the heteroatom. The susceptibility of a heterocycle to this nucleophilic attack therefore defines whether or not that heterocycle is a substrate for AO.

While CYP450s can also yield *N*-oxides of heteroaromatic rings, for example, pyridine *N*-oxide, no *N*-oxides are observed through the AO catalyzed oxidation of heteroaromatic compounds. Also, AO is not able to carry out other oxidation reactions such as N- or O-dealkylation that are commonly catalyzed by CYP450s. This results in many examples of orthogonal metabolite production resulting from CYP450 and AO-mediated oxidations. In contrast to oxidations, there is a significant overlap in the reduction reactions catalyzed by AO and other reducing enzyme systems such as CYP450 reductase or NAD(P)H−quinone oxidoreductase. Reductive processes include reduction of sulfoxides and nitro groups and reductive ring cleavage. The exact mechanism of reduction via AO is unknown.

Precedented AO Inhibitors

For several years, inhibitors of AO have been described in varying levels of detail. The literature on this topic can be challenging to navigate, as inhibitors have been used in several in vitro systems (i.e., partially purified enzyme, recombinant expressed enzyme, liver cytosol, liver slices) and across several species (most notably rabbit, guinea pig and human). Since AO contains multiple domains and prosthetic groups, inhibitors have been proposed to affect these different domains. However, detailed mechanistic investigations of AO inhibitors are infrequent and limit our understanding of how many compounds interact with the enzyme. Finally, an important point to make is that despite the number of inhibitors identified, there are no drug−drug interactions that have been found as arising via inhibition of AO. A list of inhibitors of AO is presented in Table 1.

This is by no means a comprehensive list but rather a representative list of compounds precedented in the literature, and a selection of inhibitor structures are shown in Figure 6. Menadione **1** is a quinone that has been used for many years as

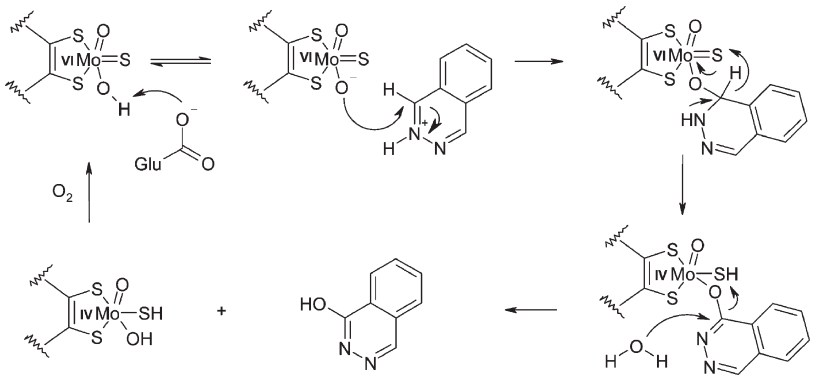

Figure 5. AO catalyzed oxidation of heteroaromatic rings (mechanism and figure adapted from the work of Alfaro and Jones[33]).

Table 1. Selected Aldehyde Oxidase Inhibitors and Their Inhibitory Potency[a]

inhibitor	human	mouse	rat	guinea pig	rabbit	monkey
amsacrine[45−48]	3.2		6.4−7.7, 3.0[b]	7.7	0.06−2.5[b]	
6,6′-azopurine[49]					3.3[b]	
chlorpromazine[37,38,43,50]	0.57−5, 2.3[b]			0.86[b]	20	
cimetidine[30]	155[b]					
cyanide[51,52]		> 190	1.5		30	
diethylstilbestrol[37,38]	0.46−0.5					
estradiol[37−39,53]	0.08−0.33		3[b]		40	12
ethinyl estradiol[38]	0.57					
genestein[38]	0.34					
7-hydroxy-DACA[47]	0.04		0.32	0.03		
isovanillin[54]	15					
menadione[38,39,47,51,54]	0.20−7.0	0.15	0.19−0.73	3.1	2.0	0.11
methadone[55]			0.03−0.57[b]			
myricetin[41]				0.3−2.5[b]		
perphenazine[38,39]	0.033−0.79	6.1	0.74			3.6
proadifen[46,47]	>200		1.1, 0.3−4.9[b]	> 500		
quinacrine[38,51]	3.3				10	
raloxifene[38,39,28,56]	0.003−0.008, 0.0009−0.0014[b]	0.5−0.61	1.1−2.8			0.5−1.0

[a] All values are in μM, and all values are IC_{50} unless otherwise noted. [b] Denotes K_i.

an in vitro tool AO inhibitor. While it has been cited as a clinically used compound in the past as vitamin K3,[36] this use is obscure and its ability to elicit a possible drug interaction via inhibition of AO is limited by its very short half-life. It inhibits AO from all species tested with potency values of about 1 μM but not XO (Table 1). Its use as an investigative tool has focused mostly on distinguishing whether the in vitro oxidation of an investigative compound is mediated by AO vs XO, although it should be noted that menadione is also a potent inhibitor of NAD(P)H−quinone oxidoreductase.

Several sterols and other phenolic compounds have been shown to be inhibitors of AO (Figure 6). These have included estradiol **2** and ethinyl estradiol **3** as well as natural and synthetic estrogenic compounds, such as genistein **4**, raloxifene **5**, diethylstilbestrol **6**, and others.[37,38] Raloxifene offers an interesting example because of its extremely high inhibitory potency of human AO and because more mechanistic work has been done using this agent than others of its class. The potency of inhibition of human AO by raloxifene has been reported in the single digit nanomolar range,[28] while for other species this potency is 3 orders of magnitude greater.[39] At least one of the two phenolic moieties was essential for raloxifene to exhibit its potent inhibition, as the dimethoxy derivative suffered from a 1000-fold loss in potency. The hydrophobic 2-piperidinylphenoxyethyl portion was also needed for inhibition; however, the basicity of the piperidine nitrogen was not.[28] Several phenothiazine-containing drugs have been shown to potently inhibit AO, with potency values of about 1 μM and lower, for example, chlorpromazine **7** and perphenazine **8**. These drugs are known to inhibit AO in several species.[37] Other structural motifs that have been shown to cause inhibition of AO include acridine and quinoline-containing compounds, for example, amsacrine **9** and quinacrine **10**.[38,39] Recently, there has been attention paid to the inhibition of AO caused by flavonoids, which are present ubiquitously in the diet.[40] It is hypothesized that flavonoids in the diet and herbal supplements could inhibit AO and thereby reduce the generation of reactive oxygen species (e.g., H_2O_2) and have a modulatory impact on disease processes. In particular, myricetin **11** has a potency at guinea pig AO of less than 1 μM.[41] An interesting inhibitor example is provided by hydralazine **12**, which has been shown to inhibit AO from rabbit, guinea pig, baboon, and human.[42,43] In this case, the inhibition has been shown to be time-dependent. Hydralazine is a hydrazine-containing analogue of the AO substrate phthalazine. A mechanism for the inactivation has not been delineated, although the loss of hydrazine does not appear to be responsible.[42,44]

Several further AO inhibitors **13**−**18** are shown in Figure 6, encompassing a wide range of structural motifs including cyanoguanidine, aldehyde, diphenylmethyl, and hydroxyacridine.

Precedented AO Substrates

AO is capable of both oxidation and reduction transformations and recognizes an extensive range of chemical structures

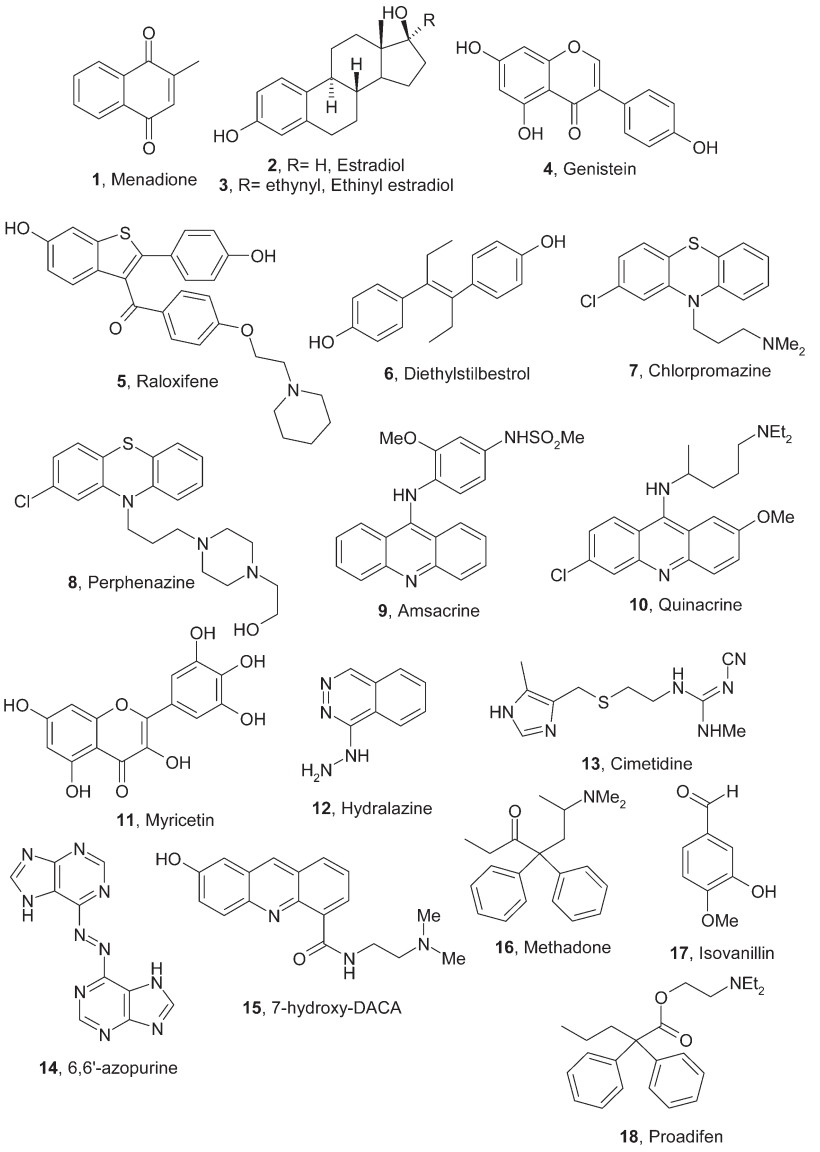

Figure 6. Inhibitors of aldehyde oxidase. For inhibitory potency values, see Table 1.

from aldehydes, nitroso compounds, and iminium ions to a wide range of heterocycles.[6,9]

Oxidative Transformations

AO will catalyze the oxidation of aldehydes to carboxylic acids, alicyclic iminium ions to lactams, and aromatic azaheterocyclic compounds to oxoheterocycles. The first two types of reactions can be important in drug metabolism; however, since drugs themselves rarely possess aldehyde or iminium moieties, the role of AO in the metabolism of such compounds is really only important in the secondary metabolism of intermediate metabolites. Aldehydes can be intermediate metabolites of alcohols and amines, and imines can be intermediate metabolites of amines. Furthermore, aldehydes can also be oxidized to carboxylic acids by aldehyde dehydrogenase, an enzyme completely unrelated to AO in structure and mechanism.

Aldehyde Oxidation. AO is known to metabolize a number of aliphatic and aromatic aldehydes to the corresponding carboxylic acids (Figure 7). Early reported examples include the oxidation of retinal **19** into retinoic acid[57] and pyridoxal **20** into 4-pyridoxic acid.[58] Some acid metabolites that have been observed are proposed to result from the combined action of other enzymes and AO. For example, acetaldehyde **21** is produced from ethanol by alcohol dehydrogenase or CYP2E1 and its subsequent oxidation to acetic acid can then be catalyzed by AO.

Tolbutamide **22**, a potassium channel blocker used to treat type II diabetes, is biotransformed into an aldehyde intermediate by the combined action of CYP450 and alcohol dehydrogenase and is then further oxidized to carboxytolbutamide by AO.[59] The nonsteroidal antiestrogen tamoxifen **23** is oxidized first by monoamine oxidase (MAO) to an aldehyde intermediate and subsequently oxidized to the corresponding carboxylic acid by AO.[60] Similarly, the antidepressant citalopram **24** is oxidized by CYP450 to an aldehyde before being oxidized by AO.[61] Vanillin **25** has been shown by several groups to be rapidly converted to vanillic acid by guinea pig liver AO.[39] Interestingly, close structural analogues of vanillin, for example, isovanillin **17** shown above in Figure 6, have been shown to be inhibitors of AO[62−64] while other close analogues such as phenylacetaldehyde **26** are substrates.[65]

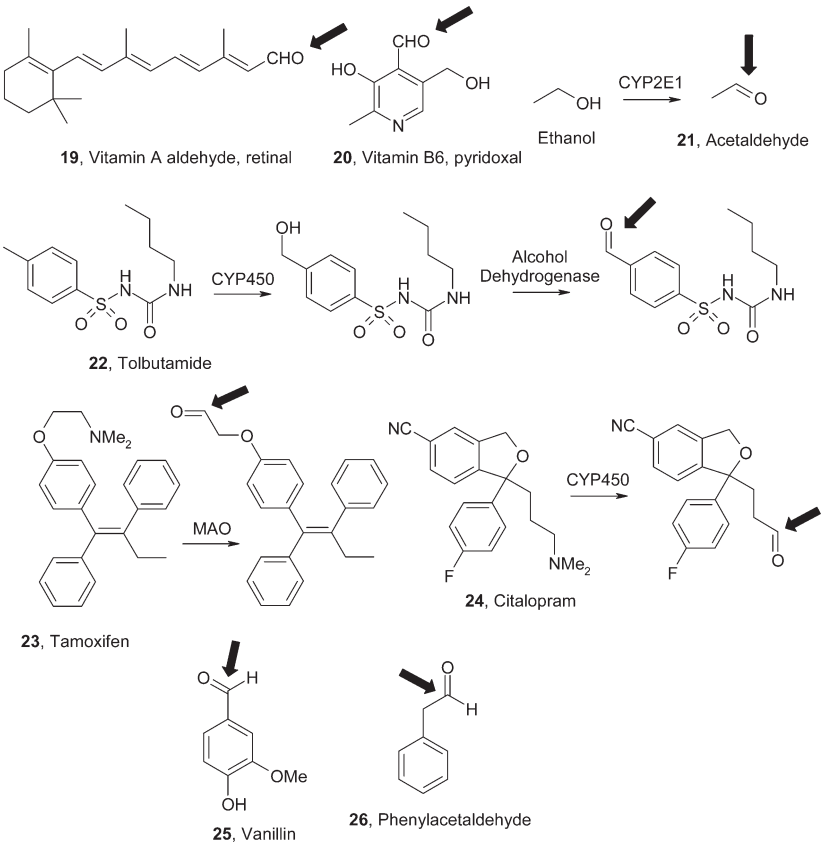

Figure 7. Aldehyde containing substrates of AO.

Aromatic Heterocycle Oxidation. The oxidation of aromatic azaheterocyclic groups to oxoheterocycles is important in the metabolism of drugs, since such substituents are present in many drug molecules. Pyridines, pyrimidines, pyrazines, and their fused-ring analogues are all typical targets for oxidation by AO. Examples are discussed in this section.

Ghafourian et al. have reported on structure−activity relationship (SAR) studies of phthalazine **27** and quinazoline **28** derivatives[66] and the influence of substituents on AO mediated oxidation (Figure 8). The findings of this study are consistent with a nucleophilic attack on an electron-deficient sp^2-hybridized carbon atom by AO with electron deficient groups on either the R2 or the R4 position favoring the oxidation reaction. A similar conclusion was reached in the case of the related phthalazine core. Similarly, Hodnett et al. have described a set of experiments that examined the rabbit liver AO turnover of various pteridine analogues, for example, 2,4-diaminopteridine **29** to its 7-hydroxylated derivative.[67] This study is also useful in that it provides SAR details of pteridine oxidation catalyzed by XO in addition to AO and indicates that the pattern of oxidation supported by both enzymes is largely orthogonal.[9]

The selective dopamine D3 receptor antagonist **30** (SB-277011)[68] was incubated in liver microsomes and liver homogenates from several species. The compound was found to be quite stable to liver microsomes from rat, dog, cynomolgus monkey, and human, while in total liver homogenate, **30** was metabolized much more rapidly in cynomolgus monkey and human than it was in rat and dog homogenates. The human in vitro data were especially striking, with clearance values some 35-fold higher in homogenates than in microsomes. These data were confirmed in vivo, whereby **30** was shown to have a high clearance and low (2%) bioavailability in cynomolgus monkey compared with moderate to good bioavailability (35−43%) in rat and dog. The metabolic route for the compound was shown to be NADPH-independent and inhibited by isovanillin, confirming AO to be the responsible enzyme. This careful and comprehensive preclinical evaluation of **30** (very similar to the recommended process outlined in Figure 2) allowed for a high confidence prediction that the bioavailability of the compound would be low in human, and its progression as a drug candidate was terminated.

Methotrexate **31** is a potent inhibitor of dihydrofolate reductase and used extensively for the treatment of leukemia and a range of other malignancies.[69] A major metabolite of methotrexate is its 7-hydroxylated derivative, formed by the action of hepatic AO on the parent drug. This metabolite has been observed in human and monkeys but much less so in rats.[70] Interestingly, different strains of rats were shown to produce vastly differing levels of this metabolite, approaching some 2 orders of magnitude difference, and then results were closely correlated with strain differences in cytosolic AO activity.

AO is known to oxidize a number of substrates related to purine and pyrimidine bases and, as such, is involved in catalyzing the oxidation of various nucleobases, nucleobase analogues, and nucleosides. 6-Mercaptopurine **32**, an agent used to treat childhood acute lymphoblastic leukemia, is oxidized to 6-thiouric acid via 6-thioxanthine **33**. The first and rate limiting step in this biotransformation is catalyzed solely by XO, whereas both XO and AO are involved in the oxidation of 6-thioxanthine to 6-thiouric acid.[71] It is

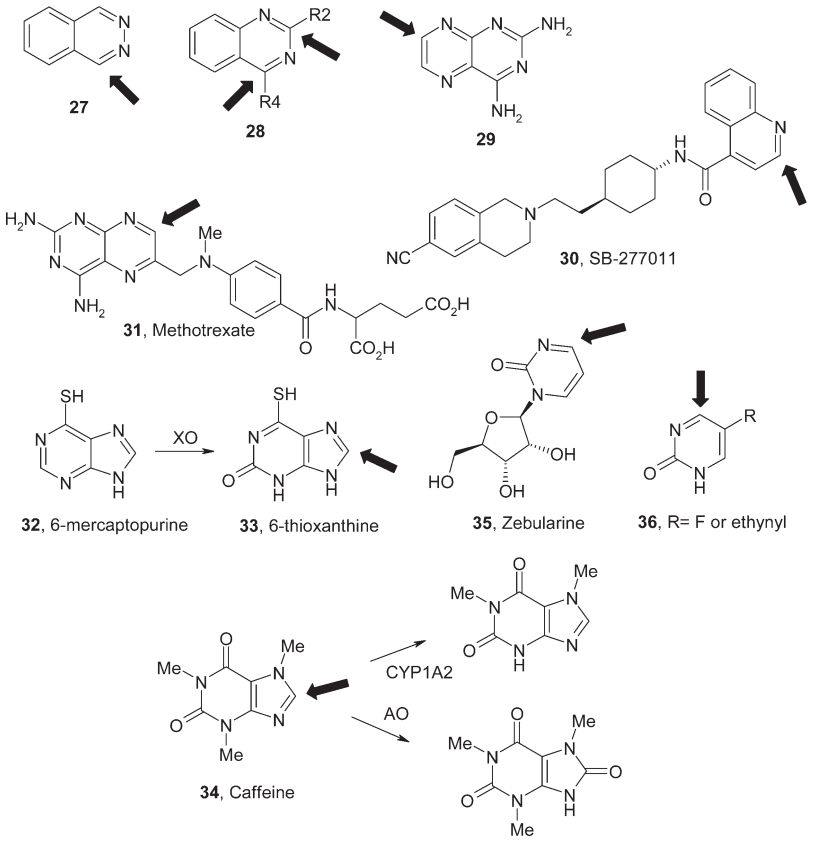

Figure 8. Heterocycle containing substrates of AO.

worthwhile to note that 6-mercaptopurine is subject to a large and clinically meaningful drug−drug interaction when coadministered with allopurinol. This is mediated through inhibition of XO and interestingly only was evident when 6-mercaptopurine was dosed orally and not intravenously. This suggests that the impact is on first-pass extraction and could also be occurring in the intestine.[72]

Caffeine **34** is N-demethylated by CYP1A2 and oxidized at the 8-position by a combination of XO and AO.[73] Zebularine **35**, a pyrimidinone nucleoside that targets epigenetic modulation of DNA methylation, was found to be an excellent substrate for hepatic AO.[56] Preclinical pharmacokinetic evaluation confirmed its poor oral bioavailability in rhesus monkey, rat, and mouse. Comprehensive investigation of species and sex differences in the AO-mediated metabolism of zebularine showed that metabolic activity in the male mouse was particularly high, with no AO turnover observed in Beagle dogs. AO oxidizes several 5-substituted pyrimidinones to the corresponding pyrimidine nucleoside bases and has been used as a means to overcome low and/or inconsistent oral bioavailability of this compound class. For example, both 5-fluoro- and 5-ethynylpyrimidinone **36** are efficiently converted to the uracil analogues by AO.[74,75] Krenitsky et al. have described a prodrug strategy to mitigate the low absorption of the antiherpetic agent acyclovir when adiministered orally in human. Using 6-deoxyacyclovir **37** (Figure 9), a much more water-soluble congener, the authors confirmed that two major metabolites were produced in vitro following oxidations at either the 6 or the 8 position. Interestingly, the desired 6-oxidation product was formed predominantly by XO, while the alternative 8-oxidation of **37** which deactivates the acyclovir template was favored by AO. The resulting 8-oxo derivative **38** is also a substrate for AO oxidation at the 6-position. This approach has been validated in a small human volunteer study in which oral administration of 6-deoxyacyclovir showed a much improved plasma exposure of acyclovir.[76]

Purine analogues such as allopurinol **39** and pyrimidine analogues such as pyrazinamide **40** have also been shown to be substrates of AO in rat liver cytosol, although in vivo these conversions were found to be more significantly influenced by XO activity than by AO.[77,78] The acridine carboxamide DACA **41**, an experimental antitumor agent, is metabolized efficiently to 9-acridone carboxamide in vitro in rat and mouse hepatic cytosolic fractions.[79] Using menadione and amsacrine to inhibit the formation of the 9-acridone metabolite, the authors demonstrated that this reaction was catalyzed by AO. Interestingly, the authors also showed that introducing a simple hydroxy substituent into the ring at the 7-position of **41** (see compound **15**) was enough to block AO metabolism and to turn the parent compound from a substrate into an inhibitor of AO.

A potential new anticancer agent **42** (XK-469)[80] underwent extensive metabolism in vitro in human hepatocytes and in vivo in a phase 1 patient study, with an AO-catalyzed oxidation at C-3 of the pyrazine ring of **42**, the major metabolite observed both in vitro and in vivo. Dibenz[*b*,*f*]-1,4-oxazepine **43** is a potent sensory irritant being developed for possible use in riot control. The compound is oxidized to its lactam analogue when incubated in rat cytosol fraction but not in rat microsome fraction. In addition, this metabolism is inhibited by the presence of menadione, allowing the authors to identify AO as the enzyme responsible for this transformation.[81]

Hepatic oxidation of brimonidine **44** to a mixture of 2-oxobrimonidine, 3-oxobrimonidine, and 2,3-dioxobrimonidine

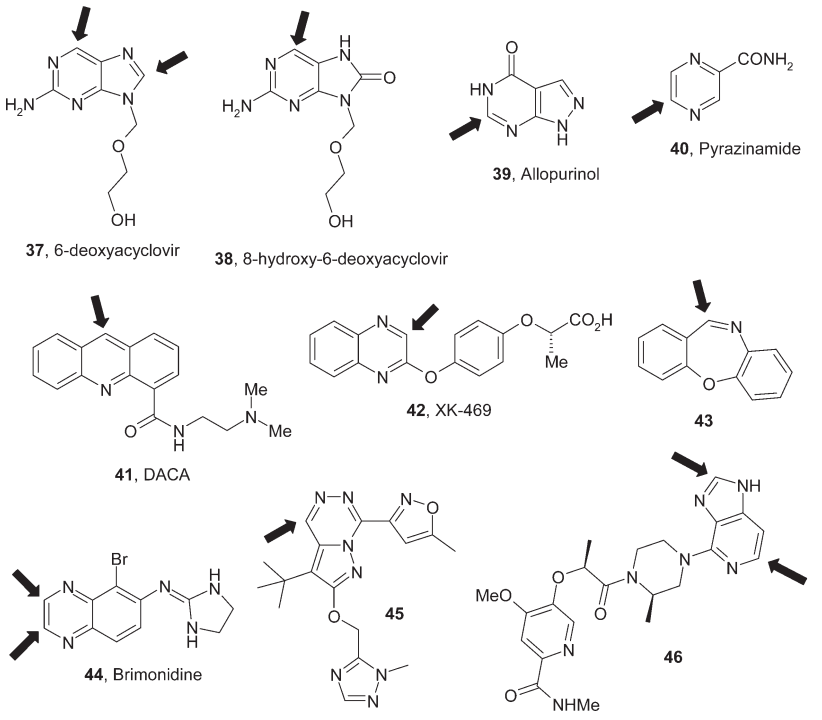

Figure 9. Additional heterocycle containing substrates of AO.

has been observed in vitro in rat, rabbit, monkey, and human liver fractions, catalyzed by AO.[82]

A group at Merck has disclosed a series of $GABA_A$ α5 inverse agonists as substrates for AO production of a pyrazolotriazinone core.[83,84] For example, compound **45** was shown in several species to produce a range of oxidized metabolites, including a triazinone derivative. Subsequent work in various in vitro metabolic systems showed that this metabolite was only produced in vivo and in hepatocyte preparations, but not in microsomes, and pointed toward AO as the responsible enzyme for its production. Similarly, the azabenzimidazole **46**, synthesized and studied as part of an effort to identify new therapeutic agents for the treatment of HIV infection, was oxidized when incubated in rat cytosol but not in rat microsomes. Raloxifene inhibited the metabolism of parent compound, suggesting it was a substrate for AO.[85] Although the authors did not identify the site of metabolism, there are two vulnerable electron deficient sp^2 carbons on the azabenzimidazole moiety, as indicated, and possibly a third on the pyridine ring.

Oxidation of Iminium Ion Intermediates. In many AO substrates, iminium ions are generated as intermediates during the metabolism of cyclic amines such as pyrrolidines or piperidines via CYP450 or MAO. These iminium ions can be further oxidized to cyclic lactams by AO (Figure 10). For example, nicotine **47** is metabolized to a nicotine iminium ion by CYP450, then to cotinine **48** by AO.[86] A similar biotransformation has been noted with the related compound **49** (ABT-418).[87] Prolintane **50**, an agent used to stimulate appetite, is also oxidized by CYP450 to an iminium ion intermediate and then to oxoprolintane by AO.[88]

Similarly, azapetine **51**, an antihypertensive agent, is transformed into the iminium ion by CYP450 and subsequently converted to a lactam metabolite, oxazapetine, by AO.[6] In some heterocyclic cases, for example, *N*-methylnicotinamide **52**, which is formed by the N-methylation of nicotinamide by methyltransferase, oxidation of the quaternary ammonium

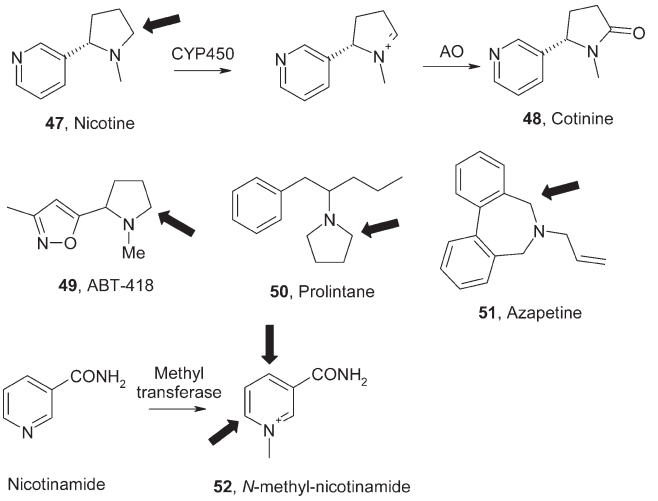

Figure 10. Iminium ion substrates of AO.

ion intermediate by AO can occur. Depending on the substitution pattern on the original heterocyclic system, isomeric metabolite production is possible.[89]

Stereospecific Oxidation. For the oxidation of both enantiomers of **53** (*RS*-8359),[90] a selective and reversible MAO inhibitor was studied by Itoh et al. using rat liver cytosolic fraction. The authors showed in this study that AO exhibited a remarkable stereoselectivity for the *S*-enantiomer of **53** (Figure 11). When **53** was then dosed in vivo in rats, mice, dogs, monkey, and then humans, plasma concentrations of the *R*-enantiomer **55** were substantially higher than those of the *S*-enantiomer in all species studied, reflecting a significantly higher clearance and shorter half-life of the *S*-enantiomer in vivo driven by metabolism by several enzymes, including AO, to the oxidized analogue **54**.[91]

The same group also extended their study to a range of cinchona alkaloids and showed that AO oxidized stereoselectively

the 9*R*-configuration of the cinchona alkaloid **56**. A detailed explanation for the observed stereoselectivity of the oxidation was not offered.

Reductive Tranformations

Reduction reactions catalyzed by AO have not been as extensively examined as oxidation reactions. In vitro, reduction reactions of N−O and N−S bonds have been described as catalyzed by AO for drugs containing nitro groups, hydroxamic acids, isoxazoles, and isothiazoles, among others. While such reactions have been described in vitro, whether these are actually catalyzed by AO in vivo remains uncertain. In vitro, the incubations must be done anaerobically, since the drug substrate is now taking the place of oxygen. Furthermore, the presence of a reducing substrate must be present (e.g., *N*-methylnicotinamide) for these reactions to occur, and the identities of such substrates that could participate in this reaction in vivo are unknown. Finally, there are other capabilities within the body that can carry out reduction reactions of N−O bonds, such as CYP450 and enzymes in gut microflora. As an example, there are reports of the benzisoxazole-containing antiepileptic agent zonisamide being reduced not only by AO but also by CYP450 enzymes in vitro and bacteria, and it is not clear which enzyme(s) is responsible for this biotransformation in vivo.[92]

***N*-Oxide Reduction.** Kitamura et al. have described the reduction of nicotinamide *N*-oxide **57** to nicotinamide when incubated in liver cytosols from rabbits, hogs, guinea pigs, hamsters, rats, and mice. Using menadione to inhibit the cytosolic *N*-oxide reductase activity, the authors identified AO as the enzyme responsible for this transformation (Figure 12).[93]

In a separate paper and using the same experimental protocols, Kitamura et al. also identified AO as the cytosolic enzyme responsible for reducing imipramine *N*-oxide **58** to imipramine and cyclobenzaprine *N*-oxide **59** to cyclobenzaprine.[94]

Sulfoxide Reduction. An extensive study was carried out on the guinea pig liver AO mediated reduction of the nonsteroidal anti-inflammatory drug, sulindac **60**, using a range of small molecules as electron donors (Figure 12).[95] Fenthion **61** is widely used throughout the world as a broad-spectrum crop insecticide. Using liver preparations of sea bream, goldfish, and rats, Kitamura et al. have shown that fenthion was oxidized to its sulfoxide by CYP450 and flavin-containing monooxygenase (FMO), that fenthion sulfoxide was easily reduced to fenthion by AO, and therefore that these metabolites are interconverted in fish and mammalian species.[96]

Nitro Reduction. A range of nitroguanidine and nitromethylene insecticides have been shown to undergo nitro group reduction by rabbit liver AO (Figure 13).[97] Interestingly, nitroguanidines such as **62** are capable of undergoing both two and six electron reductions to the nitroso **63** and the amino derivatives **64**, respectively. By comparison, nitromethylenes such as **65** only undergoes a two electron reduction to the corresponding nitroso derivative **66**, although it has been noted that in some cases, further metabolites are observed through subsequent loss of water, for example, in the case of the nitenpyram nitrile analogue **67**. In a related investigation the reduction by AO of the nitroguanidine, imidacloprid **68**, to its nitrosoguanidine and aminoguanidine was described.[98]

The study also reported large species differences in the AO reductase activity in liver cytosol. While rabbit and monkey gave the highest levels of total metabolite formation, human, mouse, cow, and rat are all capable of metabolizing imidacloprid rapidly. In contrast dog, cat, and chicken liver cytosols did not metabolize imidacloprid at significant rates.

Heterocycle Reduction. The antipsychotic agent ziprasidone **69** undergoes extensive metabolism in human (Figure 14). While CYP3A4 catalyzes both N-dealkylation of the piperazine ring and S-oxidation of the benzisothiazole ring, AO was found to reduce the benzisothiazole ring. Subsequent S-methylation of the produced thiol moiety has also been observed to give a circulating metabolite in vivo.[99,100] The authors point out that a combination of metabolic pathways is one means to reduce the potential for pharmacokinetic interactions with other drugs. This is especially pertinent because, as was pointed out earlier, AO activity appears to be unaffected by comedications.

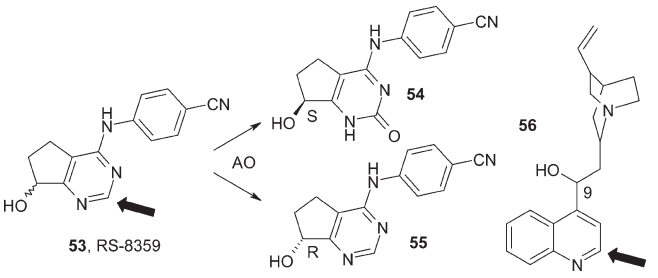

Figure 11. Stereospecific substrates for AO-mediated oxidation.

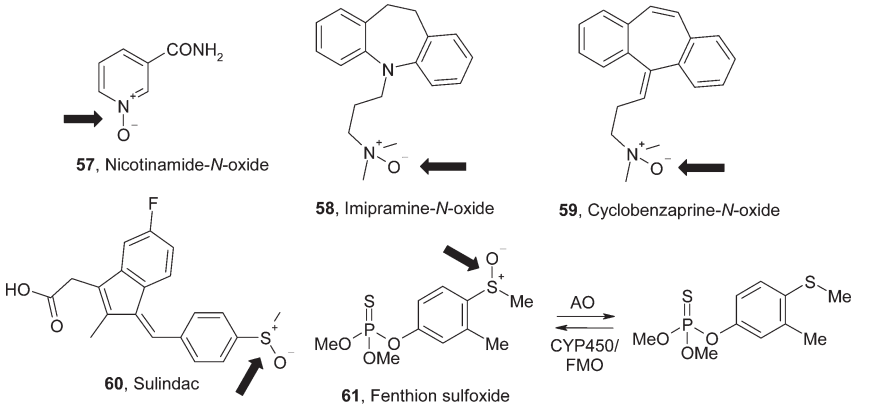

Figure 12. *N*-Oxide and *S*-oxide substrates of AO.

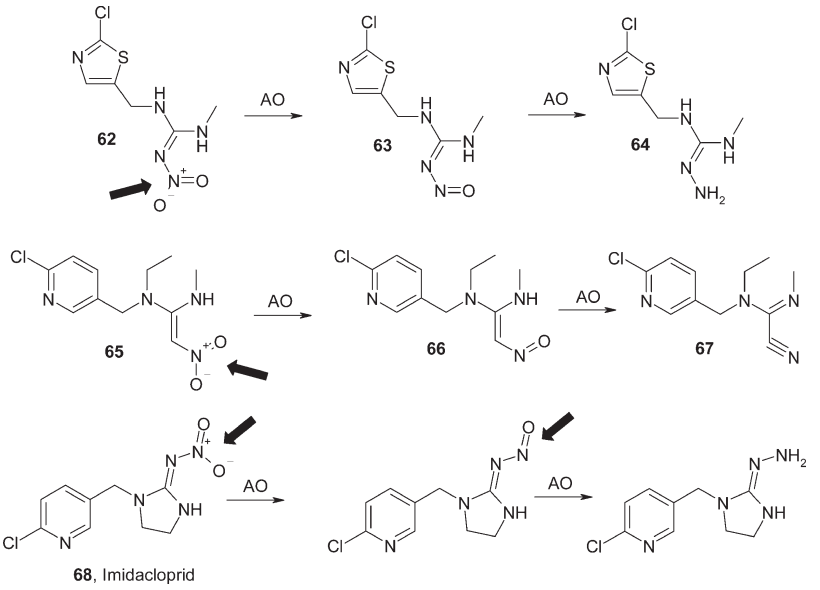

Figure 13. Nitro-containing substrates of AO.

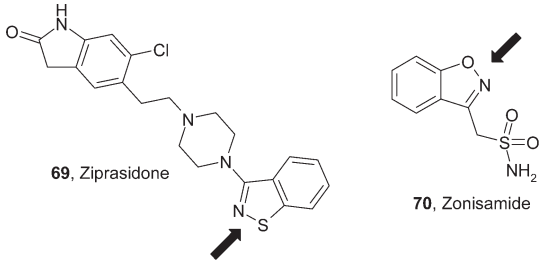

Figure 14. Heterocycle reduction catalyzed by AO.

The anticonvulsant agent zonisamide **70** is primarily metabolized to sulfamoyl acetyl phenol via an initial reduction of the benzisoxazole ring.[101] AO has been proposed as accounting for this metabolite in rat and rabbit cytosol, while in vivo, the reductase activity was found to be highly strain dependent, being especially high in Sea:SD rats and especially low in WKA:Sea rats. As pointed out previously, it is unclear if AO is entirely responsible for this biotransformation of zonisamide in vivo.

Heterocycle Oxidation by Aldehyde Oxidase Is of Greatest Relevance to Drug Discovery Programs

In the reviewing of all of the published substrates for metabolism by AO and the filtering for structures that were directly derived from human drug discovery programs, it quickly became apparent that heterocycle oxidation was the single most important modality affecting compound progression through clinical evaluation. Several well-characterized examples of AO-mediated heterocycle oxidation impacting clinically precedented drugs are presented in this section (Figures 15−17). In some cases, impact is in the form of preclinical species chronically underpredicting for human clearance, and in some cases this has led to termination of a compound. An orthogonal example details an activation approach in which AO is used to produce the active drug component in vivo. In all cases, clinical impact relates to an N-heterocycle oxidation, which is the most relevant substrate type for human drug discovery programs.

Termination of Compound Progression. Carbazeran. Carbazeran **71**, a potent inotropic agent, is primarily metabolized by AO in humans to the phthalazinone **72**. Functional efficacy was initially demonstrated in the dog at 5 mg/kg, which was not reproduced in man at similar doses (350 mg, i.e., approximately 5 mg/kg). It was subsequently found that while oral bioavailability in the dog was ∼68%, the oral bioavailability in man was too low to be measurable, sealing the fate of the compound. Comparison of the metabolic rates of this compound in baboons, humans, and dog cytosolic fractions showed that it was rapidly inactivated in baboons and humans but not in the dog, suggesting minimal AO activity in the latter species.[102] In addition, the major metabolic pathway in dog was found to be O-demethylation, not the oxidation that was the prevalent pathway in man.

Ketolide Antibiotics. In a recent account, Magee et al. have described a series of heterocycle-substituted azetidinyl ketolide analogues based on the structure of clarithromycin[103] as potent antibacterial agents with minimal hepatic turnover and time dependent inhibition of CYP3A4. An extensive investigation into heterocycle analogues identified a number of systems that appeared to combine favorable efficacy and metabolism properties. Compound **73** was predicted to have low human clearance, which combined with its favorable in vitro profile led to its being taken into a healthy volunteer study. Upon oral dosing of 300−1000 mg of **73**, measured plasma exposures were ∼20% of the predicted AUC values, leading to the termination of further clinical investigation with the compound. Human liver cytosolic assessment of turnover with and without raloxifene confirmed it as a substrate of human AO. The 1,8-naphthyridine ring system in **73** was subsequently shown to be vulnerable to AO, although the authors do not describe which position(s) on the naphthyridine system undergoes oxidation. This metabolic vulnerability was not mitigated by additional polar functionality as in **74** but was through modification of the heteroatom arrangement. The 1,5-naphthyridine system **75** was stable in human liver cytosol, and **75** has been advanced into clinical trials.

p38 Inhibitor. Recently, clinical development of a p38 kinase inhibitor for the treatment of rheumatoid arthritis was terminated because of unexpectedly rapid clearance and short half-life in man. The observed half-life in healthy male

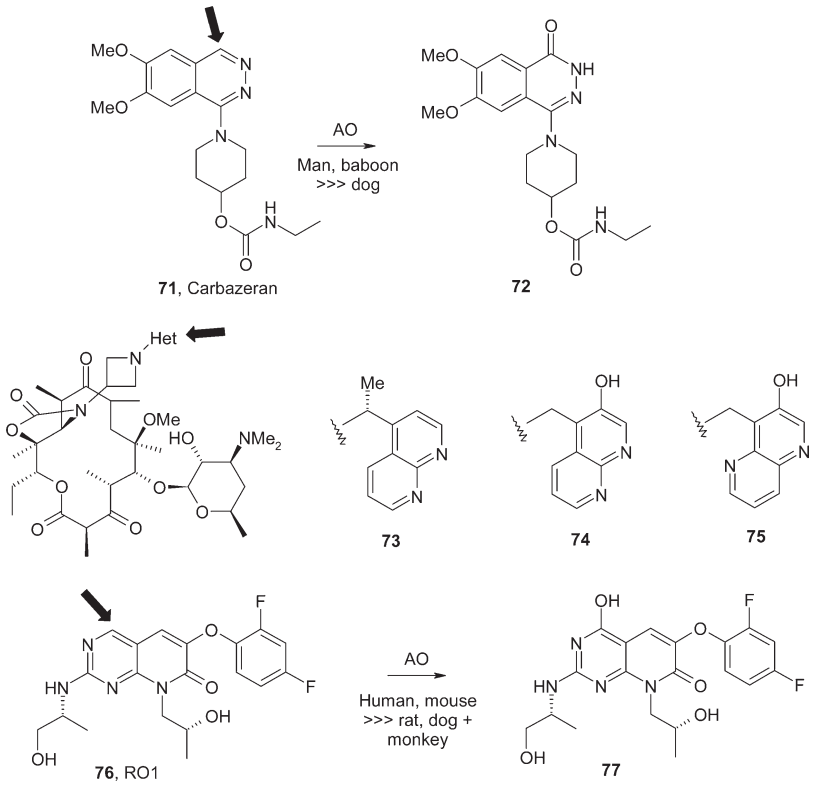

Figure 15. Selected examples where AO metabolism has led to compound termination.

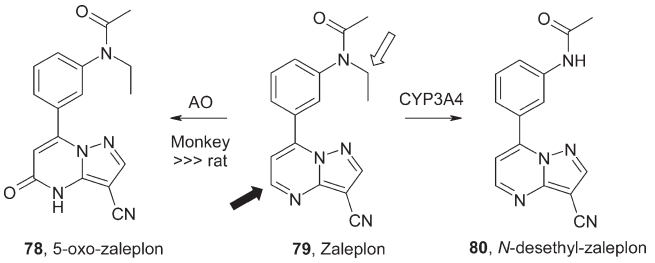

Figure 16. Example where AO metabolism issues have greatly underpredicted human clearance.

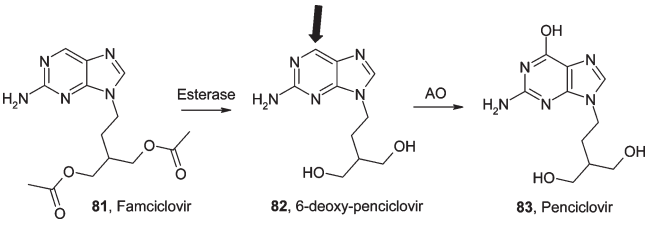

Figure 17. Example of AO-mediated activation.

volunteers of **76** (RO1)[104] was just 0.7 h compared to the predicted value of some 6 h obtained from allometric scaling of preclinical species. The clearance rate of the compound in man was subsequently shown to be significantly different from that in rat, dog, and monkey because of differing AO activity across these species. Notably, mice generated the same hydroxylated metabolite **77**, which was found to be the major metabolite in human but not observed in any other species. This unexpected oxidative clearance pathway was compounded by the observation in man that **77** underwent rapid glucuronidation and excretion in the urine. AO was initially implicated in the metabolism of **76** via a murine in vitro biotransformation assay and haplotype-based computational genetic analysis, and its involvement confirmed when the compound was co-dosed with isovanillin.

Underpredicting Human Clearance. Zaleplon. Zaleplon **79** is a nonbenzodiazepine hypnotic mainly used for insomnia. A marked species difference was found in the metabolism of zaleplon administered orally, i.e., the major metabolite in rat, mouse, and dog plasma is *N*-desethylzaleplon **80**, and in that of the monkey and human is 5-oxozaleplon **78**.[105] Using monkey and rat livers, Kawashima et al. demonstrated that zaleplon is transformed into 5-oxozaleplon selectively by cytosolic AO and to *N*-desethylzaleplon by microsomal CYP450s.[106] The authors provided evidence that the marked difference in zaleplon metabolism in the monkey and rat in vivo is due to the marked difference in the hepatic AO activity between these two species.

As mentioned above, the human in vivo intrinsic clearance of zaleplon was underpredicted some 6-fold,[29,30] resulting in a much shorter human half-life (approximately 1 h) than predicted. However, in the case of zaleplon, a short half-life does offer some advantages for a sedative agent, in avoiding residual "hang-over" sedation, and zaleplon continues to be a clinically useful agent.

AO-Mediated Activation. Famciclovir. While the above examples show the impact of AO in clearing parent drug, the example in this section is of using AO to *generate* the active drug component. This example also serves to illustrate that while it is generally reported that AO-mediated metabolites produced in many species are seldom reproduced in the dog,[10,14] there is at least one exception. Famciclovir **81** is a viral polymerase inhibitor prodrug that is converted to penciclovir **83** in vivo by hydrolysis of two acetyl groups

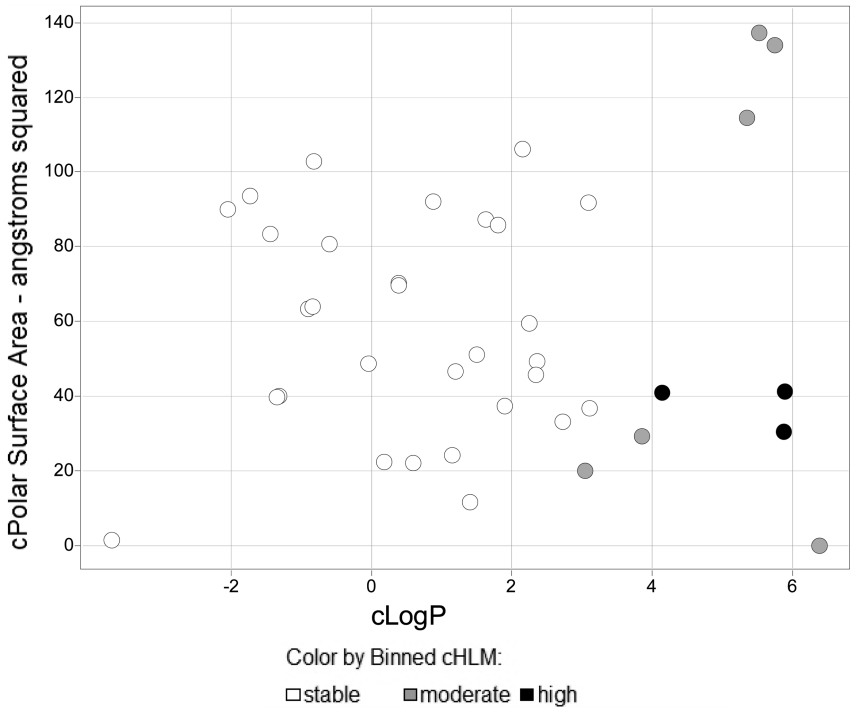

Figure 18. Calculated molecular properties of 40 known AO substrates.

and an oxidation of the heterocyclic moiety to produce the corresponding hydroxylated derivative. This type of activation approach was introduced in Figure 9 above. Rashidi et al. provided evidence that in human and guinea pig liver extracts, oxidation of both famciclovir **81** and deoxypenciclovir **82** is catalyzed by AO.[107] When famciclovir was dosed orally to both rat and dog, in each case plasma analysis indicated efficient metabolism to penciclovir **83**.[108] In contrast to the majority of reported AO substrates, not only is the AO-mediated oxidation of famciclovir and deoxypenciclovir efficient in the dog, it also mirrors the metabolic profile observed in rat.

The activation approach is useful in counteracting low absorption of polar compounds, which is especially pertinent to the nucleoside class of compounds. Penciclovir is poorly absorbed when administered orally, but when famciclovir is dosed orally, the plasma exposure of penciclovir is an order of magnitude greater than with an equivalent oral dose of penciclovir itself.

Molecular Properties of Substrate Recognition by Aldehyde Oxidase

Taking all of the structures of the compounds from the above sections, we calculated the common molecular properties of cLogP and polar surface area (PSA). It is apparent that substrates for AO do not correlate with any specific area of molecular space, and there is a wide spread of properties for AO substrates across structure, polarity, and lipophilicity ranges, as illustrated in Figure 18.

This lack of correlation for AO turnover is interesting given the high correlation that exists between CYP450-mediated turnover and higher cLogP, as measured by microsomal clearance; i.e., while CYP450-mediated oxidation may be correlated more highly with a lipophilic property space, AO oxidation is spread across a wide range of cLogP and PSA values. To illustrate this contrasting behavior, we calculated predicted clearance values by human liver microsomes (cHLM) for the AO substrates contained in Figure 18.[127] This shows clearly the much higher correlation that exists between cLogP and expected turnover in HLM. For AO, it is clear that specific structural features alone are a good indicator of AO turnover.

We next assessed the structures of all of the heterocyclic groups known to be oxidized by AO, which all without exception featured an aromatic carbon−hydrogen bond adjacent to an aromatic nitrogen atom. *For any structural feature of this type, there is a reasonable chance of AO oxidation across all physicochemical space*. In the context of drug discovery, molecules containing at least one aromatic nitrogen based heterocycle with an unsubstituted aromatic carbon atom adjacent to the nitrogen atom are highly prevalent, and it is anticipated that AO metabolism is likely more common than perhaps reported. The heterocyclic motifs represented in the above review sections were combined to provide the following group of heterocyclic motifs, which are correlated with a high risk of AO turnover in the molecules where they appear, regardless of overall compound physicochemistry (Figure 19).

Modeling Studies To Predict Susceptibility to Oxidation by Aldehyde Oxidase

There are currently very few methods described in the literature to predict the metabolism of xenobiotics by AO. Angelino et al. reported a qualitative comparison between the sites of amination for 1-alkyl-3-carbamoylpyridinium chlorides in liquid ammonia with the sites of oxidation of these compounds by rabbit liver AO.[109] While this method showed a good correlation between the amination patterns and the observed oxidation patterns found on these compounds, this empirical model is very substrate dependent and of limited value for general use.

Dastmalchi et al. reported the construction of a three-dimensional model of human AO to study the mode of

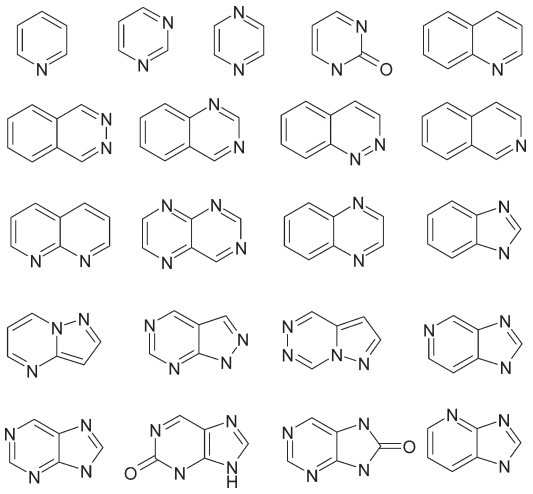

Figure 19. Common substrates for oxidation by AO.

interaction between the enzyme and its substrates.[110] Human AO was modeled using the crystal structure of bovine xanthine dehydrogenase (XDH) as a template. The construction of the homology model was guided by multiple alignments using the sequences of AO from bovine, rabbit, and rat as well as the sequence of chicken XDH and the structure then refined by molecular dynamics simulation and energy minimization. A 3D-QSAR model was then developed using 22 phthalazine and quinazoline compounds as a training set and seven compounds as a test set. The correlation coefficient (r^2) between measured and predicted binding constants (K_m) for the test set of seven compounds was reasonable at 0.65.

To illustrate the potential utility of this model in a predictive sense, penciclovir and 6-deoxypenciclovir were computationally docked into the homology model. Their 2D structures and experimentally observed sites of metabolism are shown in Figure 20, alongside the docking pose of each compound placed within the homology model of human AO. In the case of 6-deoxypenciclovir, the dock shows the predicted site of

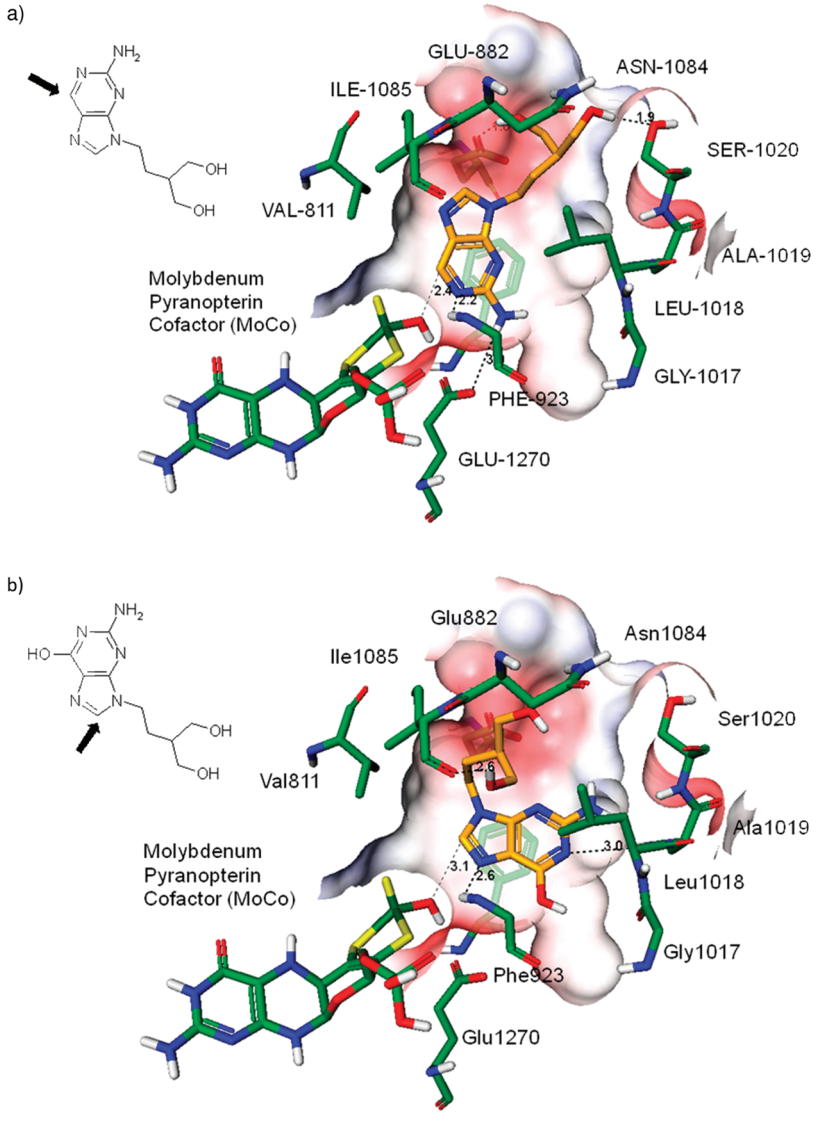

Figure 20. Docking poses for (a) 6-deoxypenciclovir and (b) penciclovir in the modeled active site of AO using the method described in ref 110. (a) The docking pose of 6-deoxypenciclovir shows the predicted site of metabolism to be 2.4 Å away from the molybdenum pyranopterin cofactor (MoCo). The bicyclic core is sandwiched in between Phe923 and Leu1018. The amino substituent is interacting with Glu1270, and the two hydroxyl groups make hydrogen bond interactions with Ser1020 and Glu882. (b) The docking pose of penciclovir shows the predicted site of metabolism to be 3.1 Å away from the MoCo. The bicyclic core is sandwiched between Phe923 and Leu1018. One of the nitrogen atoms on the core is interacting with Ala1019, and one of the hydroxy lgroups makes a hydrogen bond interaction with Ile1085.

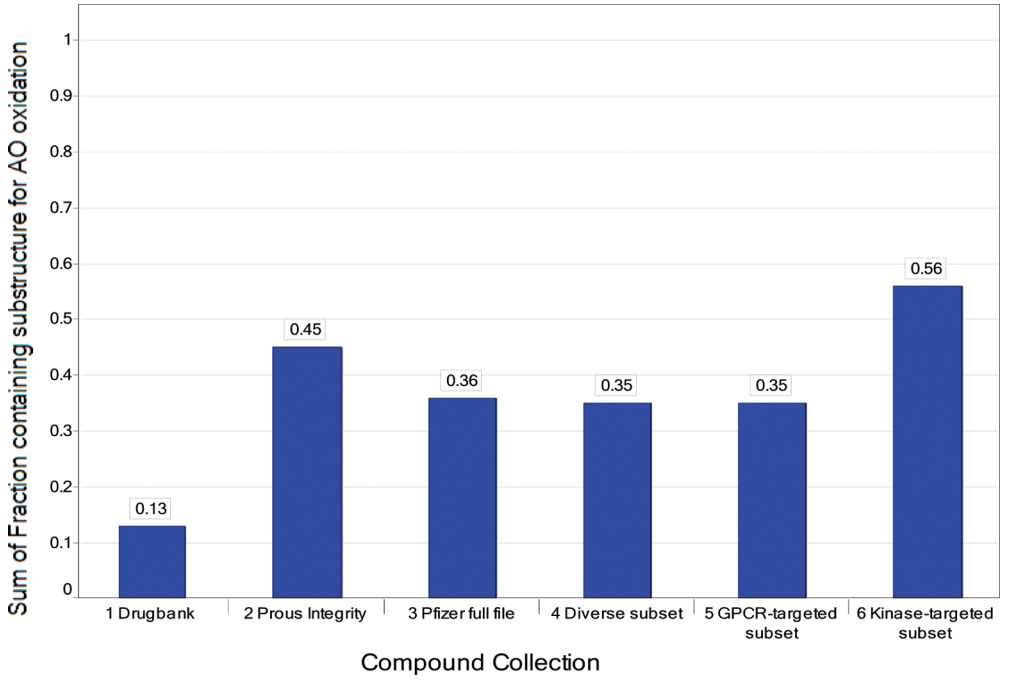

Figure 21. Fraction of compound collections that could be substrate for AO oxidation.

metabolism to be 2.4 Å distance away from MoCo and consistent with the observed in vitro metabolism pattern.[2] Similarly, the docking pose of penciclovir shows the predicted (and experimentally observed) site of metabolism to be 3.1 Å away from MoCo and in good agreement with the experimentally observed site of metabolism. With the addition of hydroxyl substitution on the core structure, the predicted site of metabolism for penciclovir switched completely compared to 6-deoxypenciclovir and the homology model offers an entirely plausible means to prospectively assess the most likely AO-mediated oxidation sites on a molecule.

In an alternative approach, Torres[111] has reported the use of density functional theory methods[34] and geometry optimization of tetrahedral intermediates resulting from the nucleophilic attack of a hydroxyl nucleophile to predict the regioselectivity of oxidation of a substrate by AO. This predictive method showed 93% accuracy for a wide variety of heterocyclic compounds examined.

The methods described here only allow an assessment of likely position of AO catalyzed oxidation of a heterocycle substrate but offer little quantitative direction on whether an oxidation by AO will actually take place. Further development of methods to accurately make predictions of this type is anticipated. The stereoselective systems described in Figure 11 also seem to lend themselves very well to testing the accuracy of the docking models, studies that again are anticipated. In the meantime, the empirical list of "at-risk" substructures shown in Figure 19 could be used as a first port of call in prospectively assessing the potential liability of a compound.

Proportion of Potential Aldehyde Oxidase Substrates in Current Compound Collections

In recent decades, attrition rates due to human pharmacokinetic failure for compounds entering the clinic have been reduced dramatically.[112] In part, this is due to a decrease in the number of predominantly CYP450-cleared compounds being progressed to clinical evaluation and the availability of predictive assays and models for human clearance mediated mainly through CYP450-based metabolism.[113] Current estimates suggest that only 10% of potential drug targets are currently being addressed,[114] and in the future, new target classes for drug discovery will be actively pursued. For example, in recent years the number of compounds in clinical trials targeting kinase inhibition has risen dramatically.[115] Additionally, it is predicted that the number of compounds targeting ion-channel modulation will also increase significantly.[116] The introduction of reliable methods to predict for CYP450-based clearance, together with a move to new target space, will inevitably result in drug candidate molecules with inherently different structural and physicochemical properties compared to established drugs. Clearance of such compounds will more than likely be mediated by non-CYP450 based mechanisms, and alternative metabolic pathways, for example, mediated by AO, will become more important. Given this context and the structural features highlighted in Figure 19 as indicators of possible AO-mediated turnover, we estimated the proportion of compounds contained within several in-house and external collections that could be a substrate for AO oxidation, from a simple structural point of view. In Figure 21, the fraction of compounds within each collection containing at least one aromatic nitrogen based heterocycle incorporating an unsubstituted aromatic carbon atom adjacent to the nitrogen is depicted. This simple analysis did not take into account electron deficiency or steric environment of the adjacent carbon atom but was based purely on the structural scaffold.

Looking at external compound collections, we first used the Drugbank database (http://www.drugbank.ca/) and examined approximately 1400 approved drugs with molecular weight less than or equal to 600. The proportion of potential AO substrates is low, 0.13, suggesting that relatively few compounds have progressed to market that could be AO substrates. We next looked at the Prous Integrity source, a competitor intelligence database supplied by Prous Science. It provides a wide range of information about drugs in research and development across the industry (http://integrity.prous.com). Examining this collection of approximately 295 000

compounds showed that the proportion of potential AO substrates in current research and development was significantly higher than that of approved drugs, 0.45. This suggests that AO clearance may be more of a problem with compounds currently in development and could reflect the aforementioned discussion predicting a move to compounds that are stable to CYP450-mediated clearance as well as compounds designed for new target classes. Looking at our own internal compound collections, we first looked at the full Pfizer file of approximately 4 million compounds. The fraction of potential AO substrates, 0.36, is moderate and likely reflects the large contribution from historical compounds in the collection, as well as more current compounds designed for newer target space. This fraction is mirrored by that found in a smaller diverse compound subset of approximately 150 000 compounds[117] selected from across the complete compound collection, which gave a fraction of 0.35. We next looked at two smaller compound collections that have been designed for specific drug target classes, namely, GPCRs and kinases. The GPCR targeted subset of approximately 75 000 compounds was selected from the full Pfizer file and contains compounds with known activity against GPCR targets from in-house data and compounds with high similarity to literature compounds with published GPCR activity. The fraction of potential AO substrates in the GPCR collection, 0.35, closely correlates with that already noted for the Pfizer full file and the diverse subset collection. This likely reflects the more historical nature of this type of target and the compounds designed to interact with a typical GPCR.[118] The kinase targeted subset of approximately 140 000 compounds was selected from across the Pfizer full file and includes compounds with known activity against kinase targets from in-house data, as well as compounds with high similarity to literature compounds with published kinase activity, incorporating both classical and nonclassical kinase inhibitors.[119] Interestingly, the fraction of potential AO substrates in the kinase-targeted subset, 0.56, was significantly higher than all other collections examined. This likely reflects the types of compounds that are designed to interact with kinases, for example, through polar nitrogen based heterocyclic motifs binding to the ATP binding "hinge" region of the kinase domain.[120,121] Thus, the very nature of the structural motifs required to inhibit kinase targets through such binding modes appears to lead to compounds with a much greater potential to be metabolized by AO.

As noted earlier, it is difficult to make confident pharmacokinetic predictions for drug candidates if they are cleared predominantly through AO mediated oxidation, because of marked inter- and intraspecies differences and lack of good in vitro and in vivo assays and models. Our analysis suggests that the fraction of drug candidates metabolized by AO is likely to increase with time as we move away from compounds cleared by CYP450-mediated mechanisms and into compounds that address new target space such as kinase inhibitors. It remains to be seen whether this will be reflected in a return to higher attrition rates for clinical compounds due to human pharmacokinetic failure, an example of which was highlighted above.[105] A major challenge for the industry in the future will be to devise high confidence in vitro prediction methodologies for non-CYP450 cleared compounds such as those that are oxidized by AO. Additionally, given emerging evidence that AO oxidation could lead to liver injury,[122] renal toxicity,[123] and Lou Gehrig's disease[124] through generation of reactive oxygen species or toxic metabolites, one also needs to be vigilant in assessing likely toxicity risks of any compounds that are turned over predominantly by AO.

Mitigation Strategies

Having established that structure is an important indicator of potential for a compound to undergo AO-mediated oxidation, having identified a range of "at-risk" substructures, and having proposed that AO related metabolism issues are likely to increase in drug discovery programs as the industry increasingly targets less traditional chemical space, we now turn to how best to address AO metabolism.

Given the clear substrate preferences outlined in Figure 19, it does appear that avoiding AO metabolism should be quite straightforward. However, the literature reviewed above offers few real examples where drug discoverers have successfully moved away from AO oxidation. Some strategies that the authors think should be effective based on the information gleaned from writing this Perspective are proposed below.

Evaluate Alternative Heterocycles. The example of Magee et al.[103] wherein an alternative stable heterocycle **75** with a different nitrogen atom arrangement compared to the original labile moiety **73** was identified is a very sensible pragmatic approach to take. Alternative heterocycle motifs that either simply remove an adjacent C−H or those that make the heterocycle less electrophilic at the position adjacent to the aromatic N atom should be especially effective, provided these changes are compatible with primary target SAR.

Heterocycle Substitution. Another approach is to add substitution to the heterocyclic ring which turns off any oxidation by AO, for example, the DACA case, **41**, in which a single hydroxyl group turned off all AO metabolism, but as a cautionary note, 7-hydroxy-DACA **15** then became an inhibitor of AO. It should also be possible to place sterically encumbering groups onto the vulnerable heterocycle to disrupt recognition by AO. The modeling approach shown in Figure 20 should be useful in guiding this approach.

Blocking Groups Adjacent to the Aromatic N Atom. A simple approach would simply be to block the labile position on the nitrogen-containing heterocycle with a substituent (for example, a halogen, alkyl, or alkoxy group) to replace the adjacent C−H bond, thereby negating any AO metabolism. An interesting additional blocking group that should be carefully considered is deuterium. Vaz et al.[125] have described experiments to derive kinetic deuterium isotope effects (KDIE) for several known substrates of AO. This work showed that a full KDIE was expressed with a deuterated version of carbazeran **71** primarily through elevations in V_{max}, but that this only translated into modest KDIE for both AUC and C_{max} in the rat. Despite in vitro KDIE in cytosolic fractions in the order of 6, this was not mirrored by half-life extensions either in human hepatocytes or when dosed in vivo to rats, indicating alternative clearance mechanisms at work across the species and test systems used. This work shows the crucial importance of knowing the clearance mechanism in the species and test system when evaluating AO SARs, but in theory, for a fully AO-cleared compound that has been well-characterized for human pharmacokinetics, deuteration is an option, provided this strategy does not simply redirect metabolism to a non-AO dominated pathway.

Conclusion

AO has been known as a cytosolic metabolizing enzyme for decades, but it is relatively recently that it has been recognized

as playing an important role in the metabolism of drugs. In this Perspective, we have proposed a basis for an increasing role of AO in the metabolism of new chemical entities emerging from modern drug discovery programs, primarily through nitrogen-containing heterocycle oxidation, and identified particular substructures that are "at-risk" of oxidation mediated by AO.

Our analysis has highlighted a straightforward process that can be followed to elucidate the role of AO in the metabolism of drugs and predicts that advances in modeling and screening methods should make the prospective design of AO-stable compounds more commonplace in the future.

Materials and Methods

The molecular docking studies described in this article were carried out as follows. The ligands were preprocessed with Corina [Sadowski, J.; Schwab, C. H. *Corina*, version 2.61; Molecular Networks GmbH Computerchemie: Erlangen, Germany] to add hydrogen and to convert to a 3D conformation. The aldehyde oxidase (AO) homology model published by S. Dastmalchi et al. was used in the docking study, which was prepared with the protein preparation wizard within the Maestro program [*Maestro User Manual*, version 9.0; Schrodinger Co. Ltd.]. A special core docking routine was used to mimic the transition state. To allow these conformations in a normal docking program, the catalytic OH from the MoCo complex is removed and the van der Waals radius is scaled by 0.2 and charge is scaled to 0.5 for the whole MoCo complex. Core-based docking was performed with Glide,[126] version 55211, in standard precision mode (SP) with expanded sampling. Core was defined as [#1][c-0X3][n-0X2], which is placed according to the overlay of the transition-state structure of AO-metabolized 6-methyl-4-quinazolinone-modithiolene[33] into the AO homology model with some manual adjustment within the AO-binding site, and tolerance is set to 1.5 Å. The final poses of each compound were visually inspected to ensure that the carbon atom from substrate known to be involved in the oxidation is at a reasonable distance (< 3.5 Å) from the OH group in the molybdenum pyranopterin cofactor (MoCo).

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