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Aldehyde oxidase and its role as a drug metabolizing enzyme

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ABSTRACT

Aldehyde oxidase (AO) is a cytosolic enzyme that belongs to the family of structurally related molybdoflavoproteins like xanthine oxidase (XO). The enzyme is characterized by broad substrate specificity and marked species differences. It catalyzes the oxidation of aromatic and aliphatic aldehydes and various heteroaromatic rings as well as reduction of several functional groups. The references to AO and its role in metabolism date back to the 1950s, but the importance of this enzyme in the metabolism of drugs has emerged in the past fifteen years. Several reviews on the role of AO in drug metabolism have been published in the past decade indicative of the growing interest in the enzyme and its influence in drug metabolism. Here, we present a comprehensive monograph of AO as a drug metabolizing enzyme with emphasis on marketed drugs as well as other xenobiotics, as substrates and inhibitors. Although the number of drugs that are primarily metabolized by AO are few, the impact of AO on drug development has been extensive. We also discuss the effect of AO on the systemic exposure and clearance these clinical candidates. The review provides a comprehensive analysis of drug discovery compounds involving AO with the focus on developmental candidates that were reported in the past five years with regards to pharmacokinetics and toxicity. While there is only one known report of AO-mediated clinically relevant drug-drug interaction (DDI), a detailed description of inhibitors and inducers of AO known to date has been presented here and the potential risks associated with DDI. The increasing recognition of the importance of AO has led to significant progress in predicting the site of AO-mediated metabolism using computational methods. Additionally, marked species difference in expression of AO makes it is difficult to predict human clearance with high confidence. The progress made towards developing in vivo, in vitro and in silico approaches for predicting AO metabolism and estimating human clearance of compounds that are metabolized by AO have also been discussed.

Contents

Abbreviations: P450, cytochrome P450; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; MAO, monoamine oxidase; SAR, structure activity relationship; XO, xanthine oxidase; AO, aldehyde oxidase; PK, pharmacokinetics; DDI, drug-drug interactions; FAD, flavin adenine dinucleotide; Moco, molybdenum center; Mo, molybdenum; DFT, density functional theory; NAD^+, nicotinamide adenine dinucleotide; 2-PY, N^1-methyl-2-pyridone-5-carboxamide; 4-PY, N^1-methyl-4-pyridone-3-carboxamide; $t_{1/2}$, half-life; 5-IPdR, 5-iodo-2-pyrimidinone-2'-deoxyribose; 5-EP, ethynylpyrimidinone; 5-FP, 5-fluoropyrimidinone; 5-EU, 5-ethynyluracil, 5-FU, 5-fluorouracil; TDI, time-dependent inhibitor; DACA, *N*-[2-(dimethylamino)ethyl]acridone-4-carboxamide; h-chimeric mice, human-chimeric mice; NHE-1, sodium/hydrogen exchanger; AUC, area under the curve; C_{max}, maximum concentrations.

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1. Introduction

Biotransformation remains a major route of elimination for most drugs with approximately 75% of the top most prescribed drugs in the United States being cleared by metabolism (Cerny, 2016; Kola & Landis, 2004; Rendic & Guengerich, 2015; van De Waterbeemd, Smith, Beaumont, & Walker, 2001; Wienkers & Heath, 2005; Williams et al., 2004; Zhong, 2013; Zhong, Mashinson, Woolman, & Zha, 2013). Thus, identification of the metabolic pathways of a new drug candidate and characterization of the enzymes responsible for catalyzing them are major facets that are generally explored during drug discovery and development (Di et al., 2013; Lin & Lu, 1997).

Metabolism of drugs is generally categorized into phase I (i.e., functionalization reactions involving oxidation, reduction, and hydrolysis) and phase II (i.e., conjugation) reactions (R. T. Williams, 1969). Functionalization reactions are commonly catalyzed by cytochrome P450s (P450s). These are a superfamily of membrane bound heme-containing versatile enzymes that catalyze the oxidation or reduction of over 60% of drugs and xenobiotics (Guengerich, 2017; Rendic & Guengerich, 2015). Although about fifty-seven isoforms make up the P450 family in humans (Zanger & Schwab, 2013), approximately 90% of drug metabolism is attributed to CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 (Rendic & Guengerich, 2015; Walsky & Obach, 2004; Wienkers & Heath, 2005). Given their role in drug metabolism, high P450-mediated turnover of a drug can result in suboptimal pharmacokinetics due to extensive intestinal and liver first-pass metabolism (Riley, 2001).

Metabolism by P450s is partly attributed to the lipophilic nature of drug molecules. The predominant interaction of these enzymes with substrates arises from hydrophobic forces as demonstrated from structure activity relationship (SAR) studies with the major constitutively expressed human P450 isoform, CYP3A4 (Stepan, Vincent, Beaumont, & Kalgutkar, 2013). Moreover, the presence of electron rich aromatic rings (phenyl and substituted phenyl rings) makes these hydrophobic compounds even more prone to P450-mediated oxidation. Hence, the basic strategy in fixing P450 liability involves modulation of physicochemical properties like molecular weight and LogP (Yang, Engkvist, Llinas, & Chen, 2012). One strategy involves introducing azaheterocyclic rings into new molecules. Simply replacing a carbon in aromatic and non-aromatic carbocycles with heteroatoms has been increasingly successful in lowering LogD and electron density of molecules and in decreasing their lability towards P450-mediated aromatic oxidation (Dowers, Rock, Rock, Perkins, & Jones, 2004; Stepan et al., 2013). However, optimizing P450-mediated metabolism of these molecules renders them more susceptible to metabolism by several less commonly clearing non-P450 enzymes (Argikar, Potter, Hutzler, & Marathe, 2016).

Table 1 presents the non-P450 enzymes that are involved in the metabolism of drugs. Some non-P450 enzymes contributing to oxidation and reduction of xenobiotics include, flavin-containing monooxygenase (FMOs) (Cashman, 2008), monoamine oxidase (MAO) (Strolin Benedetti & Tipton, 1998), alcohol dehydrogenase (ADH) (Thompson, Sonawane, & Grafstrom, 2009), aldehyde dehydrogenase (ALDH) (Bhatt, Gaedigk, Pearce, Leeder, & Prasad, 2017; Wang, Nakajima, Kawamoto, & Honma, 2002), aldoketoreductase (AKR) (Barski, Tipparaju, & Bhatnagar, 2008), carboxylesterase (CES) (Laizure, Herring, Hu, Witbrodt, & Parker, 2013), epoxide hydrolase (EH) (Kodani & Hammock, 2015) and xanthine oxidase/xanthine dehydrogenase (XO/XDH) (Battelli, Polito, Bortolotti, & Bolognesi, 2016) and aldehyde oxidase (AO). Xenobiotics can be also substrates for phase II (or conjugative) enzymes such as glucuronosyltransferase (UGT) (Rowland, Miners, & Mackenzie, 2013), sulfotransferase (ST) (Gamage et al., 2006; Hempel, Gamage, Martin, & McManus, 2007), N-acetyltransferase (NAT) (Sim, Abuhammad, & Ryan, 2014) and glutathione transferase (GT) (Chasseaud, 1979; Hayes, Flanagan, & Jowsey, 2005). One redox enzyme that has emerged as a common drug metabolizing enzyme in the past decade is AO. Several reviews on the role of AO in drug metabolism have been published in the past decade. This is indicative of the growing interest in this enzyme and its influence in drug metabolism (Garattini & Terao, 2011, 2013; Hutzler, Obach, Dalvie, & Zientek, 2013; Mota et al., 2018; Pryde et al., 2010; Rashidi & Soltani, 2017; Sanoh, Tayama, Sugihara, Kitamura, & Ohta, 2015). Here we present a comprehensive treatise describing drugs that are AO substrates and inhibitors with the focus on the clinical relevance with respect to clearance, pharmacokinetic (PK) variability, drug-drug interactions (DDI) and toxicity. An attempt has been made to capture the latest developments in understanding AO and its impact in drug discovery and development.

2. Structure and function of AO

AO (EC1.2.3.1) is a cytosolic molybdoflavoprotein that belongs to a family of structurally related molybdenum containing enzymes like XO (EC 1.17.3.2), and utilizes molybdenum (Mo) and flavin adenine dinucleotide (FAD) for its catalytic activity in oxidizing and/or reducing substrates (Romao et al., 2017). Structurally, AO and XO are very similar in their overall topology and share up to 50% of their amino acid sequence (Terao et al., 2016). Like XO, the AO family exists as a homodimer in its catalytically active form with two identical subunits of about 145 to 150 kDa and approximately 1330 to 1340 amino acid residues. Each monomeric subunit consists of three conserved domains that are connected by two linking regions. The small 25-kDa N-terminal domain contains two non-equivalent and spectroscopically distinct iron-sulfur (2Fe-2S) centers, a 45-kDa central domain accommodates a FAD binding site and a 85-kDa carboxyl-terminal domain harbors the molybdenum cofactor (Moco) (Garattini, Fratelli, & Terao, 2008; Garattini, Mendel, Romao, Wright, & Terao, 2003; Garattini & Terao, 2012, 2013). The Moco complex is made up of Mo that is

Table 1
Non-P450 enzymes involved in the metabolism of drugs

Phase I (Functionalizing enzymes)	References
Flavin Mono-oxygenase (FMO)	(Cashman, 2008)
Monoamine Oxidase (MAO)	(Strolin Benedetti & Tipton, 1998)
Alcohol Dehydrogenase (ADH)	(Thompson et al., 2009)
Aldehyde Dehydrogenase (ALDH)	(Bhatt et al., 2017; Wang et al., 2002)
aldoketoreductase (AKR)	(Barski et al., 2008)
Carboxyesterase (CES)	(Laizure et al., 2013)
Epoxide Hydrolase (EH)	(Kodani & Hammock, 2015)
Aldehyde Oxidase	References in the review
Xanthine Oxidase/Xanthine Dehydrogenase (XO/XDH)	(Battelli et al., 2016)
Phase II (Conjugative enzymes)	
Glucuronyltransferase (UGT)	(Rowland et al., 2013)
Sulfotransferase (ST)	(Gamage et al., 2006; Hempel et al., 2007)
N-Acetyltransferase	(Sim et al., 2014)
Glutathione Transferase (GT)	(Chasseaud, 1979; Hayes et al., 2005)

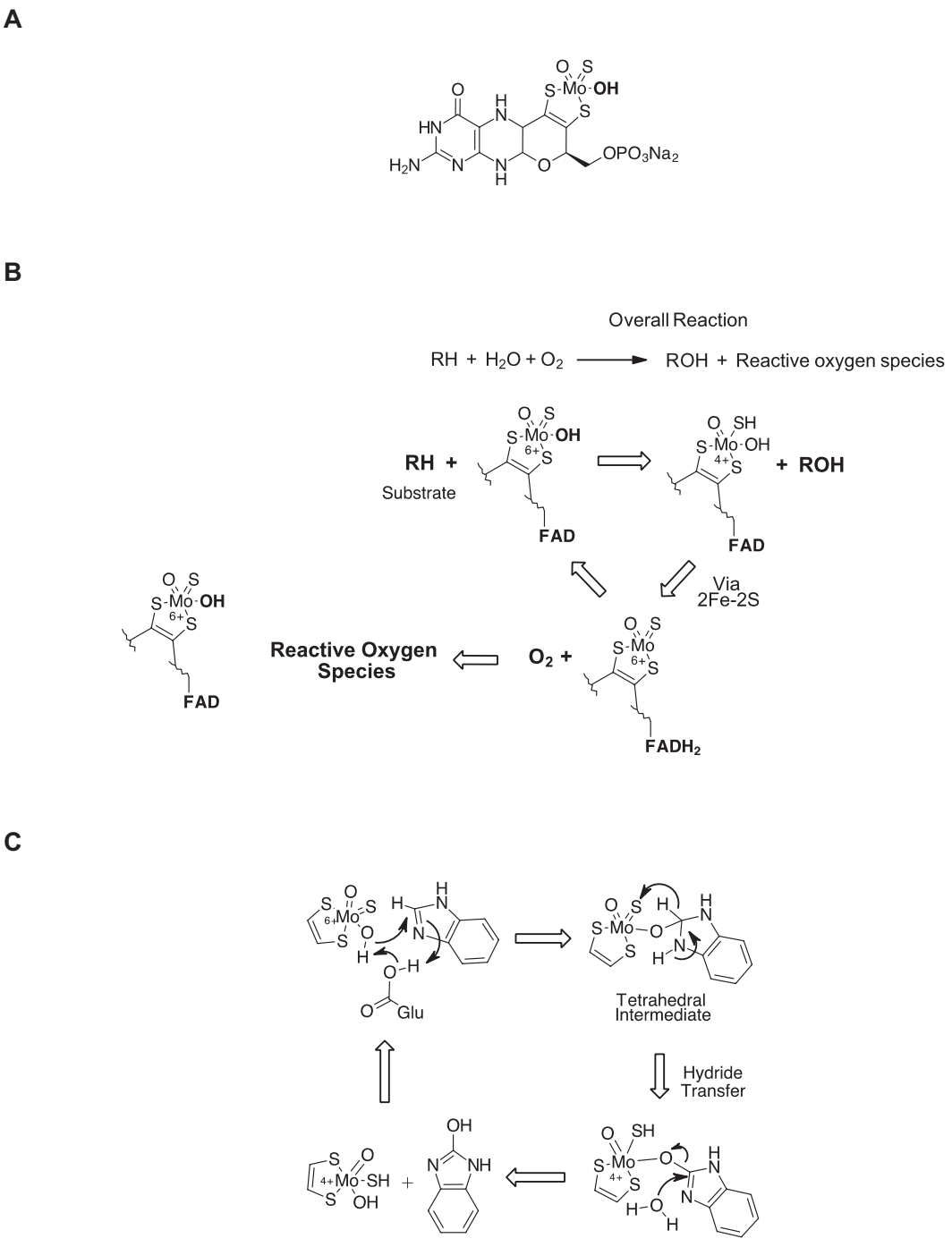


Fig. 1. A) Structure of pyranopterin complex with molybdenum in AO (Moco center). B) Simplified scheme showing the electron flow from Mo to oxygen of electron flow in AO. Oxidation of a reducing substrate converts Mo^{+6} to Mo^{+4}. The electrons are then transferred to the flavin site (FAD) via the FeS cluster and subsequently transferred to oxygen resulting reactive oxygen species. C) Mechanism detailing oxidation of heteroaromatic ring. Oxidation of the substrate involves a nucleophilic attack of hydroxyl group in the Moco center which is deprotonated by Glu residue in the active site. Subsequent hydride transfer to the sulfur atom on Moco from the resulting tetrahedral intermedate and hydrolysis of the complex yields the corresponding oxidized product. The oxidation of a heteroaromatic ring is presented for illustrative purposes. The mechanism for conversion of aldehyde to a carboxylic acid and an iminium ion to the corresponding lactam is similar.

coordinated to a pyranopterin moiety via a dithiolene group (Fig. 1A). The primary function of pyranopterin moiety is to position Mo correctly within the active site of the enzyme. It also controls the redox behavior of the enzyme and participates in the electron transfer from Mo to other prosthetic groups (Mota et al., 2018).

The role of Moco and the flow of electrons in the oxidation of substrates is depicted in Fig. 1B. The overall protein cofactor disposition is consistent with the expected electron transfer pathway. The electrons generated from the hydroxylation reaction at the Moco center are passed on via the two FeS centers to FAD. The electrons are ultimately released to the terminal electron acceptor which is molecular oxygen (O_2), resulting in the production of reactive oxygen species such as hydrogen peroxide or superoxide anion (Kundu, Hille, Velayutham, & Zweier, 2007). Even though the ultimate product of AO oxidation is the hydroxylated substrate like the P450s, the two enzymes are very diverse. Both enzymes utilize molecular oxygen as the ultimate electron acceptor but the oxygen atom incorporated into the substrate in P450-catalyzed oxidations comes from molecular oxygen (Guengerich,

2007; Meunier, de Visser, & Shaik, 2004). On the other hand, the oxygen atom in AO-catalyzed oxidation is derived from water (Hille, 2005; Hille, Hall, & Basu, 2014). AO catalyzed oxidation involves a nucleophilic attack of the hydroxyl group, whereas the oxidizing moiety of the P450s is an iron (IV)-oxo heme-porphyrin radical cation species (also called as "compound I) and more electrophilic in nature. As a consequence, only electron rich (and lipophilic) substrates are susceptible to P450s while AOs can oxidize electron deficient carbons in the heteroaromatic rings or aldehydes. A detailed mechanism of AO-catalyzed oxidation of heteroaromatic rings is described in Fig. 1C. The first step in the oxidation process is a nucleophilic addition of the deprotonated hydroxyl group of the Moco complex on to the electron deficient carbon that is adjacent to a heteroatom. A subsequent hydride transfer from the resulting transition state intermediate to the sulfur atom in Moco converts Mo from the oxidation state of +6 to +4. Following this, the product is released from the reduced Moco complex and the water molecule replenishes the vacant coordination position in Moco. The reaction cycle is closed once Mo is re-oxidized and the two reducing equivalents are transferred to molecular oxygen. This mechanism of AO oxidation was largely inferred from studies using XO (Hille, 2005; Hille et al., 2014; Skibo, Gilchrist, & Lee, 1987) but has now been confirmed by using partially purified human AO expressed in *E.coli* (Alfaro et al., 2009). Overall, these studies have suggested that nucleophilic addition and the hydride displacement are concerted and that the cleavage of the C-H bond in the transition state lags behind the nucleophilic attack and is a rate determining step. The concerted nature of the mechanism was further confirmed by computational modeling and density functional theory (DFT) calculations (Alfaro & Jones, 2008).

Despite the similarities in global structure and the oxidative mechanism, some differences exist between XO and AO. XO is the key enzyme in the catabolism of purines and primarily catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid (Fig. 2). On the other hand, the biochemical/physiological function of AO remains unclear. Some reports support the idea that AO plays an important role in adipocytic differentiation (Weigert et al., 2008). Other evidence indicates that this enzyme is involved in the conversion of endogenous substrates such as *all trans*-retinaldehyde (Ambroziak, Izaguirre, Abriola, Chern, & Pietruszko, 1999) and pyridoxal (Schwartz & Kjeldgaard, 1951) to their corresponding carboxylic acids, *all trans*-retinoic acid and pyridoxic acid (Fig. 3A and 3B).

The two enzymes also differentiate from each other in their substrate specificity. Whereas XO is highly substrate specific, AO can recognize a wide array of substrates and metabolize a broad range of natural and synthetic compounds (Garattini & Terao, 2012; Kitamura, Sugihara, & Ohta, 2006; Pryde et al., 2010). The crystal structures of mouse AO homolog 3 enzyme (mAO3) purified directly from mouse liver and human AO (hAO) expressed in *E. Coli* in its free form, and in complex with the substrate (phthalazine) and inhibitor (thioridazine) are now available

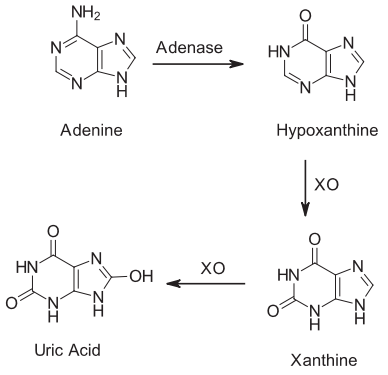


Fig. 2. Conversion of hypoxanthine to uric acid by xanthine oxidase (XO). Adenine is converted to hypoxanthine which is then oxidized by XO to the uric acid. The mechanism of oxidation is similar to that presented in Fig. 1C.

(Coelho et al., 2012; Coelho et al., 2015). These structures provide invaluable information regarding the structural determinants responsible for the differences in substrate and/or inhibitor specificity between AO and XO (Mota et al., 2018). Analysis of these structures suggest that the Moco complex sits in the active site cavity at the bottom of a deep and wide substrate access funnel. In mAO3, the funnel is ~15 Å wide at the surface and becomes narrower and tighter near the proximity of the Moco complex (Coelho et al., 2012). In the active site, the complex is oriented in such a way that it allows the catalytically important sulfur atom and the hydroxyl group (a source of oxygen that is transferred to the substrate) to be in an equatorial position and pointing towards the entrance of the funnel while the other oxygen atom occupies the apical position (Coelho et al., 2012; Mota et al., 2018; Romao, 2009).

The key amino residues detected in the active site cavity of the AO enzymes that are involved in the catalytic mechanism are glutamine (Gln), glutamate (Glu), Serine (Ser) and phenylalanine (Phe) (Table 2). These residues are conserved in XO enzymes which explains the similarity in the mechanism of oxidation of substrates by the two enzymes. The strictly conserved glutamate residue (Glu1266 and Glu1270 in mAO3 and hAO, respectively) is an important residue in the active site and plays a direct and critical role in the reaction mechanism (Coelho et al., 2012; Coelho et al., 2015; Mota et al., 2018). It is involved in the deprotonation of the hydroxyl moiety, which ultimately is transferred to the substrate in the oxidation process (see Fig. 1C). Replacement of this residue with Gln results in complete loss of enzyme activity in the mutagenesis experiments confirming its importance in catalysis (Coelho et al., 2012). The presence of each residue in the active site is important for the substrate affinity. The conserved Phe and Leu residues in mAO3 and hAO (Table 2) create a hydrophobic environment in the active site and are responsible for the orientation of the substrate molecule through stacking and hydrophobic interactions, while Gln makes a hydrogen bond with the apical oxygen ligand. The crystal structure of hAO complexed with the substrate (phthalazine) and the inhibitor (thioridazine) also provides additional insights into structural determinants of AO's active site. Analysis of the crystal structure of the complex shows that the planar phthalazine molecule is stacked with Phe and other hydrophophic residues and is placed in a way that allows the nitrogen atom to position ~3.8 Å from the hydroxyl ligand (Coelho et al., 2015).

The active site cavity and the surrounding area in AO enzymes also encompass several other important amino residues that are involved in substrate binding but which are not conserved in the XO enzymes (Table 2). For instance, charged residues like Glu 802 or arginine (Arg) 880 in the bovine XO (bXO) active site that are responsible for the correct positioning and activation of the substrate are replaced by neutral and smaller residues in the catalytic core of AOs (Coelho et al., 2012; Coelho et al., 2015). Some replacements include, valine (Val) or alanine (Ala) for Glu802 and a methionine (Met) or tyrosine (Tyr) for Arg880 in the hAO and mAO3 active site, respectively (Table 2). This change creates a more spacious active site pocket and allows the accommodation of larger substrates in the active site of AO. The importance of these amino acid residues in the catalytic activity was demonstrated by conversion of Ala807 to Val and Tyr885 to a methionine (Met) in mAO3. These changes affected the kinetic constants of bulkier and charged substrates like phenanthridine and N^1-methylnicotinamide. The effects were more pronounced with double variants (A807V/Y885M). Residues close to the catalytic core which play a role in substrate orientation also seem to vary between the two proteins. For instance, histidine (His) 884 close to the active site of XO is replaced by lysine (Lys) in AO enzymes. The Lys residue along with Glu, stabilizes the transition state intermediate via hydrogen bonding interactions (Mota et al., 2018). Molecular dynamic simulations have suggested that substrate docking into the active site causes Lys889 in mAO3 to establish new interactions with Glu1266 and/or the substrate itself (Coelho et al., 2012). Coehlo et.al. have also demonstrated the importance of Lys889 in mAO3 by exchanging this residue with His which led to a 2-3-fold decrease in catalytic efficiency

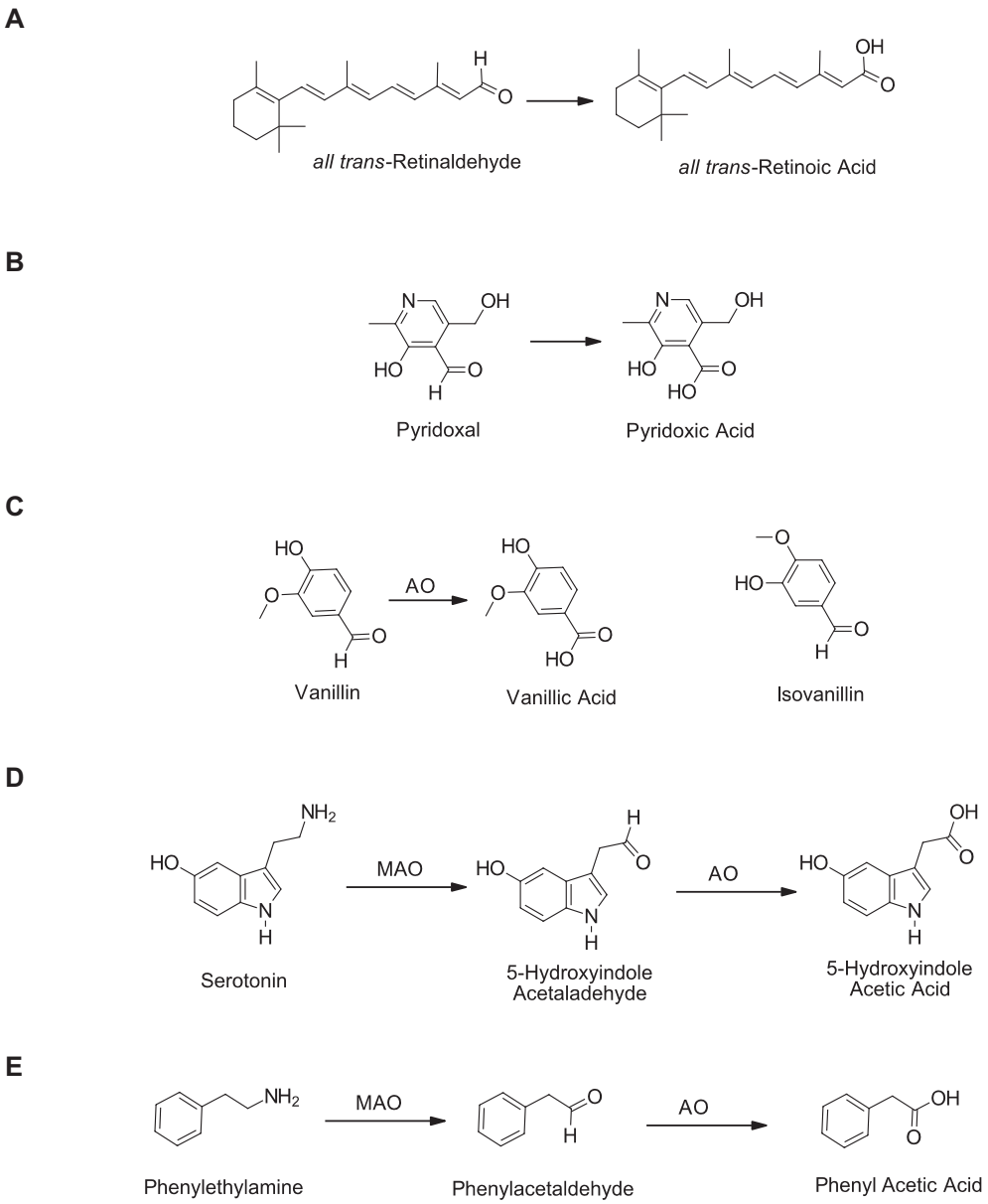


Fig. 3. Oxidation of aldehydes to carboxylic acids by AO. A) Conversion of *all trans*-retinaldehyde to *all trans*-retinoic acid. B) Conversion of pyridoxal to pyridoxic acid; C) Metabolism of vanillin to vanillic acid and structure of isovanillin, an inhibitor of AO. D) MAO and subsequent AO-catalyzed oxidation of serotonin to 5-hydroxyindole acetic acid and E) Conversion of 2-phenylethylamine to 2-phenylacetic acid.

Table 2
Comparison of important amino acid residues in mAO3, hAO and bXO enzymes (Coelho et al., 2015; Coelho et al., 2012; Mota et al., 2018).

Amino acid residues	mAO3	hAO	bXO
Conserved residues	Phe919	Phe923	Phe914
	Phe1014	Leu1018*	Phe1009
	Ser1085	Ser1089	Ser1080
	Glu1266	Glu1270	Glu1261
	Gln772	Gln767	Gln767
	Lys889	Lys893	His884
	Arg717	Arg721	Leu712
Non-conserved amino acid residues	Ala807	Val811	Glu802
	Tyr885	Met889	Arg880
	Pro1015	Gly1019	Thr1010
	Tyr1019	Ala1023	Leu1014
	Asp880	Glu872	Leu873
	Glu880	Leu884	His875
	Leu881	Phe885	Ser876
	Thr1081	Ile1085	Pro1076

when benzaldehyde and phthalazine were used as substrates (Coelho et al., 2012). Differences in the structural features of the putative gates (gate 1 and 2) at the entrance of the substrate access funnel of the two enzymes also help in explaining the broad substrate specificity of AO. The crystal structure of AOs suggests an extended and flexible gate 1 with variable length whereas the XO structures display a very ordered conformation. On the other hand, loop 2 (or gate 2) is similar in both AO and XO although it differs in amino acid residues (Coelho et al., 2012; Coelho et al., 2015). For instance, the hAO and mAO3 harbor two acidic residues in contrast to XO (Table 2). The side chains of these two acidic residues in AO allow the polar substrates orient and bind to the catalytic center of the enzyme in an appropriate manner. The flexible nature of both gates in the AO enzymes explains their promiscuous catalytic activity, allowing the entry of bulky and chemically diverse substrates into the active site cavity.

Another difference between the two enzymes is the ability of XO to readily convert to XDH form and use nicotinamide adenine dinucleotide (NAD^+) as an electron acceptor, in addition to molecular oxygen, and

reduce it to NADH (Nishino et al., 2005). On the other hand, AO can only exist as an oxidase and is unable to reduce NAD^+ (Garattini et al., 2003; Garattini et al., 2008; Olson, Ballow, Palmer, & Massey, 1974). Conversion of XO to XDH involves oxidation of cysteine residues (Cys535 and Cys992 in bXO), and formation of a new disulfide bond (Coelho et al., 2012; Enroth et al., 2000; Kuwabara et al., 2003; Nishino et al., 2005). These residues are not conserved in AO. In mAO3, these residues are substituted by tyrosine (Tyr542) and Phe997 (Tyr542 and Tyr1001 in hAO) (Coelho et al., 2012). The other difference is the absence of crucial amino acid residues in the FAD site of AO. For instance, the eleven amino acid residues that make the so called "FAD flexible loop A" close to the flavin binding site in bXO are not conserved in AOs (Coelho et al., 2012). In bXO, this loop causes changes in the hydrogen bonding network surrounding the FAD and consequently the FAD redox potential. The AO enzymes also lack residues that anchor the NADH molecule to XDH or orient the cofactor in the FAD pocket (Ishikita, Eger, Okamoto, Nishino, & Pai, 2012; Mota et al., 2018). Despite the differences, one commonality between the two enzymes is that the FAD cofactor not only participates in the protein electron transfer but can be involved in the reduction reactions that both these enzymes catalyze. For instance, reduction of organic nitrates by bXO or nitro groups by AO occurs at the FAD site of the enzymes (Doel, Godber, Eisenthal, & Harrison, 2001; Godber, Doel, Goult, Eisenthal, & Harrison, 2001; Paragas, Humphreys, Min, Joswig-Jones, & Jones, 2017).

3. Substrates of AO

AO can catalyze a wide variety of reactions. Although the oxidation and reduction of different types of substrates are the most common reactions, other reactions such as the amide hydrolysis is also catalyzed by AO.

3.1. Oxidative substrates

3.1.1. Aldehyde intermediates as substrates

As the name implies, AO is known to primarily catalyze the oxidation of aldehydes to carboxylic acid. In addition to the endogenous substrates (noted earlier), xenobiotic-derived aldehydes are also oxidized by AO to their corresponding carboxylic acids (Ambroziak, Izaguirre, & Pietruszko, 1999; Panoutsopoulos & Beedham, 2004b; Panoutsopoulos, Kouretas, & Beedham, 2004). Vanillin, an aromatic aldehyde, is a classic substrate for AO (Fig. 3C). Several groups have shown that vanillin is rapidly converted to vanillic acid by guinea pig liver AO (Panoutsopoulos, Kouretas, & Beedham, 2004; Sahi, Khan, & Black, 2008). Interestingly, a close structural analog of vanillin, isovanillin (Fig. 3C), is not oxidized by AO but on the contrary, is an inhibitor of the enzyme (Panoutsopoulos & Beedham, 2004a). This in a way points towards selectivity of AO catalyzed reactions given that a small positional change in a molecule prevents a compound from being oxidized.

Aldehydes are frequently generated as intermediates by P450s or MAO during the catabolism of endogenous (or exogenous) amines (Kalgutkar, Dalvie, Castagnoli Jr., & Taylor, 2001; Rose & Castagnoli Jr., 1983) or by ADH catalyzed oxidation of alcohols (Dalziel & Dickinson, 1966). These intermediates are oxidized to the corresponding carboxylic acids by AO. For instance, AO oxidizes the aldehyde intermediate formed by MAO mediated *N*-dealkylation of biogenic amine like serotonin, to the corresponding 5-hydroxyindole acetic acid (Fig. 3D) (Garattini et al., 2008; Weissbach, Redfield, & Udenfriend, 1957). It is important to note that aldehydes can also be oxidized by ALDH. Studies by Panoutsopoulos et al. have demonstrated that ALDH is primarily responsible for the conversion of these aldehyde intermediates to the corresponding carboxylic acid derivatives, even though AO catalyzes the oxidation of these intermediates (Panoutsopoulos, Kouretas, Gounaris, & Beedham, 2004). For example, phenylacetaldehyde intermediate, which is formed from 2-phenylethylamine by MAO catalyzed deamination (Fig. 3E), is converted to phenylacetic acid primarily by ALDH and to a lesser extent by AO. The mechanism of oxidation is similar to that shown in Fig. 1C and involves a nucleophilic attack of the hydroxyl group in the Moco complex on the electrophilic carbon next to the oxygen atom.

Citalopram and tamoxifen are two marketed drugs that exemplify the involvement of AO in the oxidation of aldehyde intermediates that are formed during their metabolism. Citalopram is an antidepressant that belongs to a class of selective serotonin reuptake inhibitors (SSRIs) and is primarily metabolized by P450 to *N*-desmethyl and *N*,*N*-didesmethyl metabolites (Fig. 4A) (Rochat et al., 1998). However, the citalopram propionic acid metabolite, which is pharmacologically inactive, has also been detected in humans and preclinical species. The primary mechanism for its formation involves MAO-A/B catalyzed deamination to an aldehyde intermediate which is subsequently oxidized to the citalopram propionic acid metabolite (Fig. 4A) (Rochat et al., 1998). Rochat and co-workers have demonstrated that the acid formation is NADP-independent and is formed in the human cytosolic fraction by AO (Rochat et al., 1998). That AO played a role in the formation of the propionic acid metabolite was confirmed using an AO inhibitor menadione, in the *in vitro* studies. Similarly, tamoxifen, a nonsteroidal antiestrogen, used in the treatment of advanced breast cancer, is extensively metabolized in humans and laboratory animals. Although the primary metabolites include hydroxylation and *N*-dealkylation of tamoxifen to the corresponding hydroxytamoxifen and *N*-desmethylamine metabolite, tamoxifen is also deaminated by MAO to the corresponding aldehyde intermediate, which is further oxidized to the oxyacetic acid metabolite in rats (Fig. 4B) (Ruenitz & Bai, 1995; Soininen, Kleimola, Elomaa, Salmo, & Rissanen, 1986).

Aldehyde products generated via P450-mediated metabolism of drugs are also subject to oxidation by AO. For instance, tolbutamide a potassium channel blocker used to treat type II diabetes, is oxidized by CYP2C9 to the benzaldehyde intermediate (Fig. 5A). Reports by McDaniel and coworkers suggests that the corresponding aldehyde intermediate is further oxidized to the benzoic acid derivative by AO (McDaniel, Podgainy, & Bressler, 1969). Cyclophosphamide, ifosfamide and trofosfamide (Fig. 5B) are prodrugs that belong to the oxaphosphorines and are non-specific alkylating agents that undergo P450-catalyzed bioactivation (Boddy & Yule, 2000). Cyclophosphamide is the most commonly used alkylating agent with broad application in cancer chemotherapy and undergoes CYP2B6, 2C and 3A4 mediated oxidation at the carbon adjacent to the nitrogen of the saturated ring as the first step, and ultimately yields the cytotoxic phosphoramide mustard via the aldehyde intermediate, aldophosphamide (Fig. 5C) (Bagley Jr., Bostick, & DeVita Jr., 1973; Hill, Laster Jr., & Struck, 1972; Kwon, 1999). The aldophosphamide is also a substrate for AO which converts it to an inactive carboxylic acid metabolite, carboxyphosphamide (Struck, Kirk, Mellett, el Dareer, & Hill, 1971). The involvement of AO in aldehyde oxidation to the carboxylic acid was confirmed by incubation with AO, which was isolated and purified from rabbit liver (Hill et al., 1972). Subsequent enzymatic studies by Domeyer and Saldek demonstrated that conversion was also catalyzed by ALDH and that this family of enzymes may contribute more to carboxyphosphamide formation than AO (Domeyer & Sladek, 1980a, 1980b).

3.1.2. Iminium ion intermediates as substrates

AO also catalyzes the oxidation of iminium ion intermediates formed during the metabolism of alicyclic amines to their corresponding lactam metabolites (Bolleddula et al., 2014; Gorrod & Aislaitner, 1994; Masic, 2011; Ulgen & Sevinc, 2017; Vickers & Polsky, 2000). Some alicyclic amine motifs that are known to undergo this conversion are, pyrrolidine, piperidine, piperazine or morpholine. Lactams have been detected as metabolites for several well-known drugs like tremorine (Hammer, Holmstedt, Karlen, Sjoqvist, & Vessman, 1968; Hammer, Karlen, Rane, & Sjoqvist, 1968), prolintane (Hucker, Stauffer, & Rhodes, 1972) and phencyclidine (Baker & Little, 1985) (Fig. 6A). Conversion

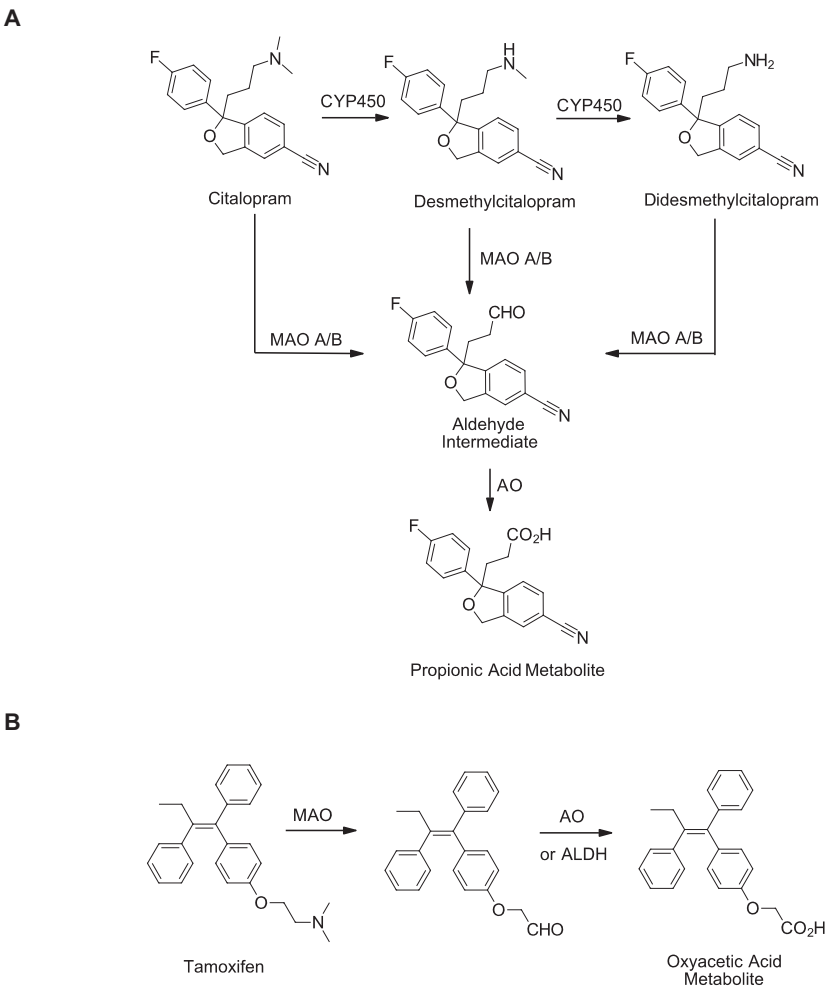


Fig. 4. A) Metabolism of citalopram to citalopram propionic acid metabolite. Conversion involves deamination of citalopram by MAO A or B followed by oxidation of the aldehyde to the corresponding propionic acid derivative. B) Conversion of tamoxifen to the oxyacetic acid derivative. Tamoxifen is deaminated by MAO and then oxidized by AO to the aldehyde. Some reports suggest involvement of ALDH in the oxidation of the aldehyde to the carboxylic acid.

of nicotine, an alkaloid and a cholinomimetic stimulant, to cotinine represents a typical example of AO involved conversion of alicyclic amines to a lactam via an iminium ion intermediate (Fig. 6B) (Benowitz & Jacob 3rd., 1994; Brandange & Lindblom, 1979; Gorrod & Hibberd, 1982; Hucker, Gillette, & Brodie, 1960; Hukkanen, Jacob 3rd, & Benowitz, 2005). The first step in the conversion to lactam involves a P450 or MAO-catalyzed two electron oxidation of the amine to an iminium ion intermediate, which can undergo hydration to the corresponding carbinolamine intermediate. Both these intermediates are in equilibrium and they can interconvert (Fig. 6B) (Brandange & Lindblom, 1979; Murphy, 1973; Peterson, Trevor, & Castagnoli Jr., 1987). Lactams metabolites are generated from iminium ions as well as carbinolamines but both have distinct mechanisms. Conversion of the iminium ions involves an AO-catalyzed nucleophilic attack of the hydroxyl group on the electrophilic carbon atom of the iminium ion (pathway a) as in the oxidation *N*-heterocycles and aldehydes (Fig. 1C). Carbinolamines on the other hand, are oxidized to lactams by P450s via an additional two electron oxidation process (pathway b). The contribution of P450 or AO in the oxidation is in part dependent on the stability of the carbinolamine intermediate. Formation of the iminium ion in the formation of the lactam has been confirmed by trapping these intermediates with sodium cyanide, which yields a stable cyano adduct (Hoag, Schmidt-Peetz, Lampen, Trevor, & Castagnoli Jr., 1988; Murphy, 1973; Peterson et al., 1987).

The Janus kinase (JAK) 1/2 inhibitor momelotinib, currently undergoing clinical trials, represents a very recent example of oxidation of alicyclic amines to lactams. Momelotinib is converted to the corresponding morpholino lactam in humans. This metabolite has been detected in systemic circulation and is pharmacologically active (Zheng et al., 2018). The authors have demonstrated that the lactam formation involves initial P450 oxidation of morpholine ring to the iminium ion followed by metabolism via AO (Fig. 6C). The identification and contribution of the enzymes in the formation of this metabolite was confirmed by incubating momelotinib with human cryopreserved hepatocytes in the presence of aminobenzotriazole (ABT, a time dependent inhibitor of P450), hydralazine (a mechanism-based inactivator of AO, discussed later) or a combination of both inhibitors (Zheng et al., 2018). The results suggested that the lactam formation is dependent on sequential metabolism by P450 and AO and that P450 enzymes are responsible for the primary metabolic event while AO catalyzes the subsequent step.

3.1.3. Drugs containing heteroaromatic rings as substrates

Metabolism of heteroaromatic rings is perhaps one of the most important metabolic reaction that AO catalyzes. N^1-Methylnicotinamide is a well-known physiological substrate that is primarily metabolized by AO (Stanulovic & Chaykin, 1971) and is converted to N^1-methyl-2-pyridone-5-carboxamide (2-PY) and N^1-methyl-4-pyridone-3-carboxamide (4-PY) by AO (Fig. 7A). A diverse set of heteroaromatic rings have been identified as AO-labile substrates (Pryde et al., 2010) that undergo oxidation at the carbon adjacent to the nitrogen atom in the ring (Fig. 1C) to the corresponding hydroxylated metabolites.

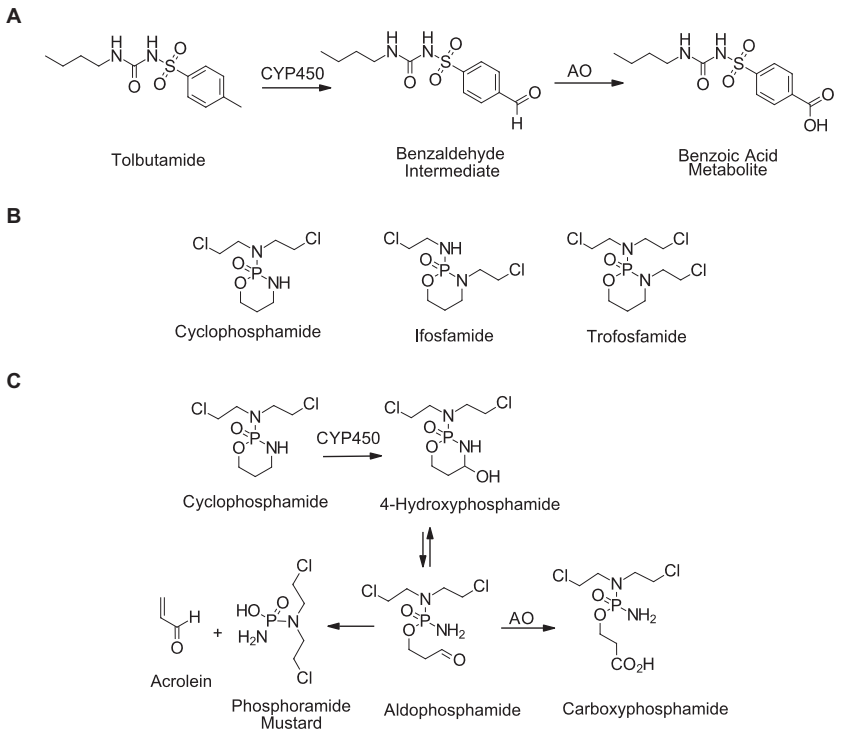


Fig. 5. A) Metabolism of tolbutamide to the benzoic acid metabolite via the benzaldehyde intermediate. B) Structures of various oxaphosphorines. C) Bioactivation of cyclophosphamide via P450. Cyclophosphamide is bioactivated to the corresponding phosphoramide mustard via hydroxylation of P450 and subsequent cleavage of the hydroxyphosphamide via aldophosphamide. However, the aldophosphamide is also a substrate of AO (and ALDH) and is converted to the inactive carboxyphosphamide.

These scaffolds are generally highly represented in a collection of drugs like the kinase inhibitors and are generally stable towards P450 metabolism (Dick, 2018; Mota et al., 2018; Sanoh et al., 2015).

Idelalisib and lenvatinib represent two recently approved drugs that are substrates of AO. Idelalisib is an inhibitor of phosphatidiylinositol 3-kinase-∂, and a first in class agent to be approved for the treatment of relapsed chronic lymphocytic leukemia, follicular B cell non-Hodgkin's lymphoma and small lymphocytic lymphoma. It is primarily metabolized by AO to GS-563117 and to a lesser extent via P450 (CYP3A4) in humans (Fig. 7B) (Jin et al., 2016; Jin, Robeson, Zhou, Hisoire, & Ramanathan, 2015; Ramanathan, Jin, Sharma, & Kearney, 2016). Following oral administration of 150 mg of [^{14}C]idelalisib, 88% of the administered dose was recovered in the feces, predominantly as GS-563117. Profiling of plasma and urine also revealed GS-563117 as the main metabolite in these two matrices.

Lenvatinib is another orally available multi-tyrosine kinase inhibitor with anti-angiogenic, and antitumor activity that is in part metabolized by AO. The drug was approved by the FDA for treatment of patients with advanced renal cell carcinoma in combination with everolimus. It exhibits a reasonable pharmacokinetic profile in humans. After oral administration of 24 mg dose, the drug is rapidly and well absorbed (Hong et al., 2015) and has a half-life ($t_{1/2}$) of 29 hr. The primary route of elimination of lenvatinib is metabolism with predominant excretion in feces and to a smaller extent in urine (Dubbelman et al., 2015). *In vitro* and *in vivo* metabolism studies have indicated that lenvatinib is mainly metabolized to glutathione conjugate and its derivatives (Fig. 7C) (Inoue et al., 2014). However, ADME studies in monkeys and humans demonstrate the role of AO in the metabolism of lenvatinib to the corresponding quinolinone metabolite (M4) and quinolinone metabolite of a P450-catalyzed oxidation product, desmethyllenvatinib (M5) (Fig. 7C). To confirm AO involvement in the elimination of lenvatinib in humans, Inoue and co-workers performed experiments with lenvatinib and desmethyllenvatinib using human S9 fraction in the presence and absence of AO inhibitors raloxifene and menadione (Inoue et al., 2014). Furthermore, ^{18}O-water and recombinant hAO were also used in these studies to demonstrate the involvement of AO.

Other well-known drugs that are metabolized by AO include zaleplon, and methotrexate. Zaleplon is a novel non-benzodiazepine sedative-hypnotic agent used in the treatment of insomnia. Pharmacokinetics of zaleplon is characterized by moderate to high plasma clearance (16 mL/min/kg) and moderate volume of distribution (1.3 L/kg), which results in a short half-life ($t_{1/2}$ in man = 1.0 hr) (Ebbens & Verster, 2010; Rosen, Fournie, Darwish, Danjou, & Troy, 1999). Metabolism studies demonstrate that zaleplon is extensively metabolized with 88% of the dose appearing in the urine (71%) and feces (17%) as metabolites. The conversion is catalyzed by CYP3A4 to *N*-desethylzaleplon and by AO to the corresponding 5-oxozaleplon (Fig. 7D). Marked species differences have been observed in the formation of the two metabolites. Whereas desethylzaleplon is common among all preclinical species (mouse, dog and rat), 5-oxozaleplon is only observed only in the monkey and humans (Kawashima et al., 1999). The role of AO in the formation of 5-oxozaleplon was confirmed by incubation of the drug with human liver cytosolic preparations and was markedly inhibited by the AO inhibitors (Kawashima et al., 1999; Lake et al., 2002).

Methotrexate is widely used in the treatment of rheumatic diseases as well as several cancers including leukemia in children as well a range of solid tumors (Bookbinder, Espinoza, Fenske, Germain, & Vasey, 1984; Furst, 1997; Hillson & Furst, 1997; Leyland-Jones, O'Dwyer, Hoth, & Wittes, 1987; Niemeyer et al., 1991; Visser & van der Heijde, 2009). Although 80 to 90% of the dose of methotrexate is excreted unchanged in the urine (Henderson, Adamson, Denham, & Oliverio, 1965; Henderson, Adamson, & Oliverio, 1965), less than 10% is also subjected to metabolism. The primary metabolites of methotrexate are methotrexate polyglutamate (Chabner et al., 1985; Koizumi, Curt, Fine, Griffin, & Chabner, 1985), 7-hydroxymethotrexate (Breithaupt & Kuenzlen, 1982; Breithaupt, Kuenzlen, & Goebel, 1982; Lankelma, van der Klein, & Ramaekers, 1980), and 2, 4-diamino-*N*-10-methylpteroic acid (Donehower, Hande, Drake, & Chabner, 1979) (Fig. 7E). Several groups have shown that formation of the 7-hydroxymethotrexate is primarily

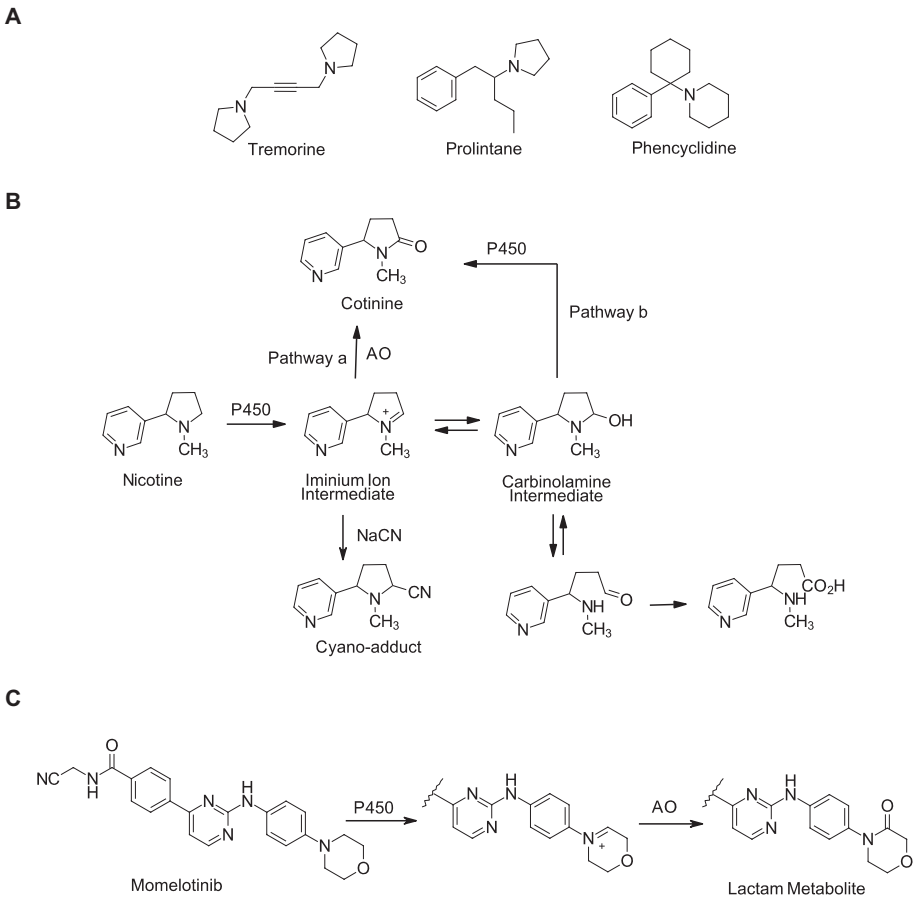


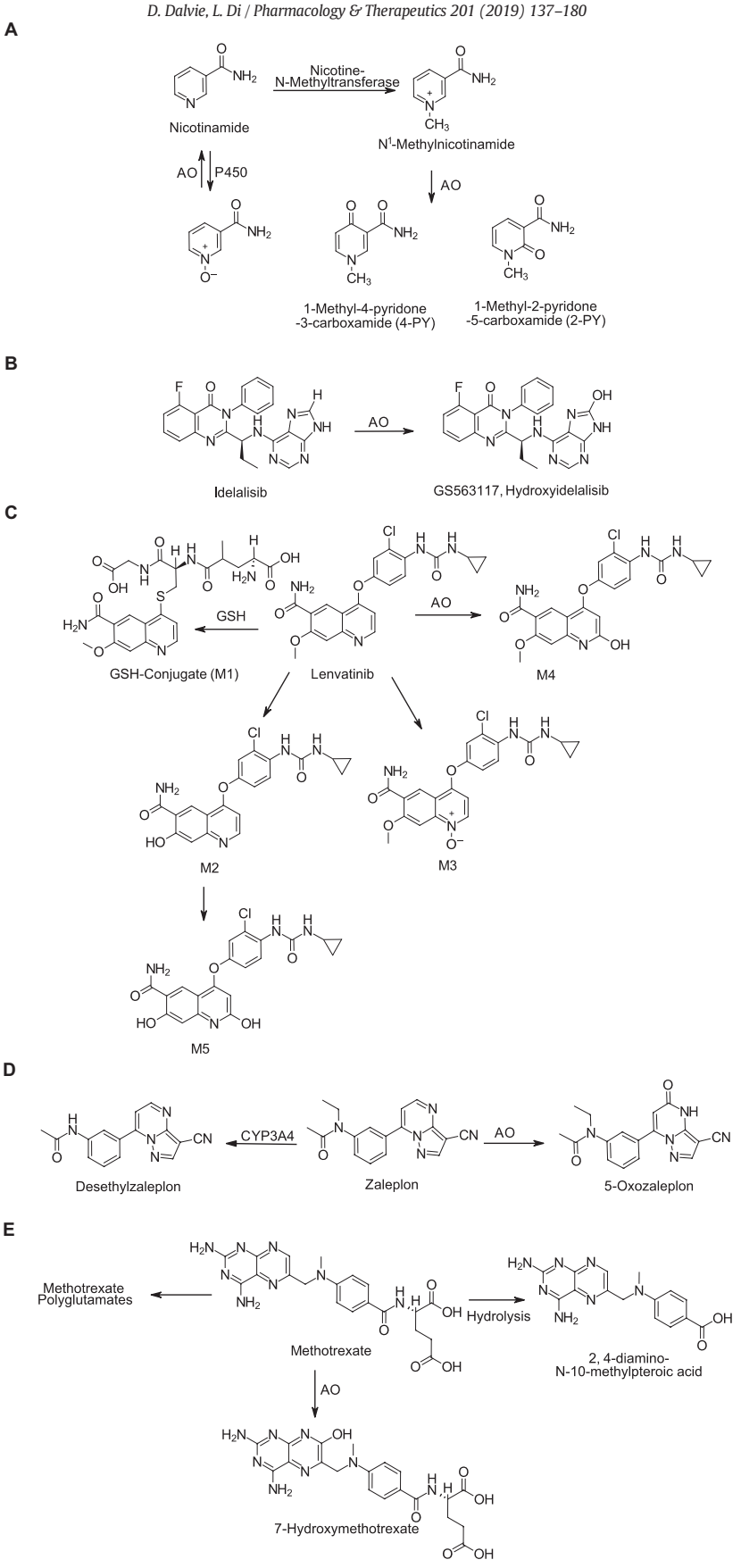
Fig. 6. Conversion of imines and iminium ions to the corresponding lactams. A) Structures of tremorine, prolintane and phencyclidine. All three alicyclic amines are converted to lactams via an iminium ion intermediate. B) Conversion of nicotine to cotinine. The conversion involves oxidation of the *N*-methylpyrrolidine moiety to an iminium ion intermediate (which can be trapped as a cyano adduct). The iminium ion is converted to the corresponding lactam (cotinine) by AO (pathway a). The carbinolamine (which is the hydrated form of the iminium ion) can also be oxidized to cotinine via P450 (pathway b). C) Conversion of momelotinib to the corresponding lactam metabolite (M1). The conversion is similar to that described for nicotine (B). Momelotinib is first oxidized to the corresponding iminium ion intermediate followd by AO-catalyzed oxidation to M1.

catalyzed by AO in almost all species including, humans (Johns, Iannotti, Sartorelli, & Bertino, 1966; Johns, Iannotti, Sartorelli, Booth, & Bertino, 1965; Kitamura, Nakatani, Sugihara, & Ohta, 1999). Human subjects and rhesus monkeys receiving methotrexate at high dose levels form significant amounts of this oxidative metabolite, which is ultimately excreted in the urine of both species. (Jacobs, Stoller, Chabner, & Johns, 1976, 1977).

Conversion of the heteroaromatic ring system to its corresponding hydroxylated metabolite, a lactam, can alter the physicochemical properties of the ring system. This change is attributed to the change in pKa of the relatively basic nitrogen atom in the ring to a neutral species, which in turn affects its ionization and solubility at physiological pH (7.4). AO-generated lactams are therefore inherently less soluble than their precursors. This can be a significant problem and can play a role in drug induced toxicity (discussed later). It is believed that the renal dysfunction caused following treatment of methotrexate is a result of poor solubility of 7-hydroxymethotrexate metabolite. The aqueous solubility of this metabolite is 1.55 mg/mL at pH 7, which is 20% of that of the parent which is 8.9 mg/mL (Jacobs et al., 1976).

Like XO, AO also catalyzes the oxidation of compounds with purine and pyrimidine scaffolds. Several well-known purines such as, allopurinol (Moriwaki et al., 1993), mercaptopurine (Choughule, Barnaba, Joswig-Jones, & Jones, 2014; Rashidi, Beedham, Smith, & Davaran, 2007; Van Scoik, Johnson, & Porter, 1985), azathioprine (Rashidi et al., 2007; Van Scoik et al., 1985), and the anti-viral drug famciclovir (Clarke, Harrell, & Chenery, 1995; Rashidi, Smith, Clarke, & Beedham, 1997) are metabolized by AO (Fig. 8A). Famciclovir is a di-acetyl-6-deoxy prodrug of the anti-herpesviral nucleoside analogue, penciclovir. This 9-substituted guanine derivative is rapidly hydrolyzed and oxidized in man to yield penciclovir. Following oral administration famciclovir undergoes extensive first pass metabolism to penciclovir and no parent compound is recovered from plasma or urine (Perry & Wagstaff, 1995; Vere Hodge, Sutton, Boyd, Harnden, & Jarvest, 1989). Metabolism involves sequential hydrolysis of the two acetyl groups followed by AO-mediated oxidation of the resulting 6-deoxypenciclovir to penciclovir (Fig. 8B) (Clarke et al., 1995; Vere Hodge et al., 1989). Interestingly, *in vitro* studies by Rashidi and coworkers have demonstrated that famciclovir is also a substrate for AO, and in fact the catalytic activity for oxidation of famciclovir is greater than that of 6-deoxypenciclovir (Rashidi et al., 1997).

Reports on AO catalyzed metabolism of other monocyclic heteroaromatic rings have also been published. As noted earlier, N^1-methylnicotinamide is a classic substrate of AO. Similarly, using purified rat AO, Moriwaki et. al. and Yamamoto et. al. demonstrated that pyrazinamide is also a substrate of AO and is converted to 5-hydroxypyrazinamide (Fig. 9A) (Moriwaki et al., 1993; Yamamoto et al., 1993). Interestingly, hydrolysis of pyrazinamide to the corresponding pyrazinoic acid resulted in loss of AO activity. Zebularine (Fig. 9B), a synthetic aza-analog of cytidine, is another pyrimidinone nucleoside that undergoes oxidation primarily by AO. This drug is a potent inhibitor of cytidine deaminase as well as a general inhibitor of DNA methylation (Byun et al., 2008; Laird, 2005; Yoo et al., 2008). Metabolism studies of zebularine in liver cytosol from humans and other animals suggests that the compound is primarily converted to azauridine


A
Nicotine-
N-Methyltransferase
Nicotinamide
N1-Methylnicotinamide
AO
P450
AO
1-Methyl-4-pyridone
-3-carboxamide (4-PY)
1-Methyl-2-pyridone
-5-carboxamide (2-PY)
B
AO
Idelalisib
GS563117, Hydroxyidelalisib
C
GSH
AO
GSH-Conjugate (M1)
Lenvatinib
M4
M2
M3
M5
D
CYP3A4
AO
Desethylzaleplon
Zaleplon
5-Oxozaleplon
E
Methotrexate
Polyglutamates
Hydrolysis
Methotrexate
2, 4-diamino-
N-10-methylpteroic acid
AO
7-Hydroxymethotrexate

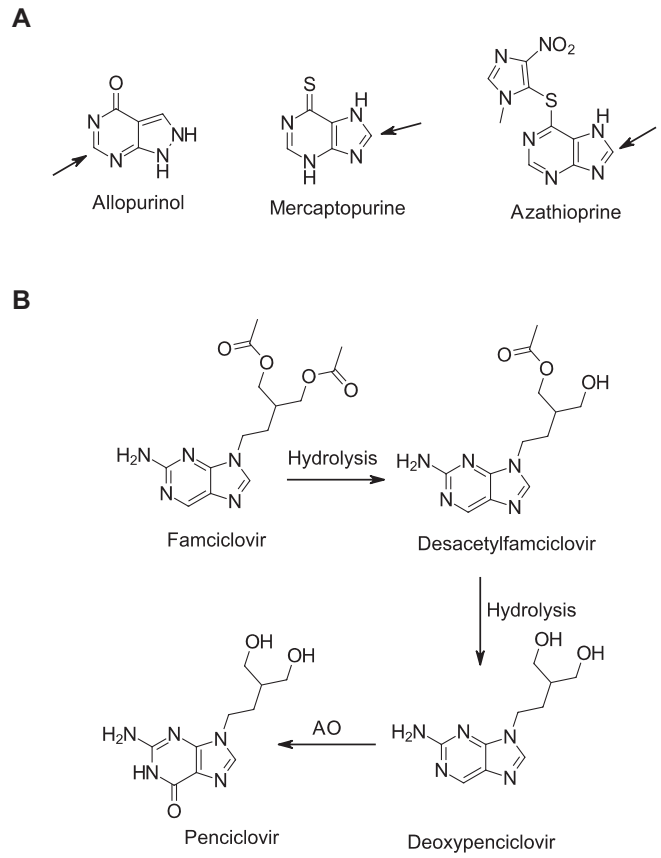


Fig. 8. A) Structures of allopurinol, mercaptopurine and azathioprine. These are purine analogues and undergo oxidation by AO (arrow indicates the site of metabolism). B) Bioactivation of the prodrug famciclovir to the pharmacologically active penciclovir, guanosine analogue antiviral drug. Famciclovir is hydrolyzed to the deoxypenciclovir intermediate, which is a substrate of AO.

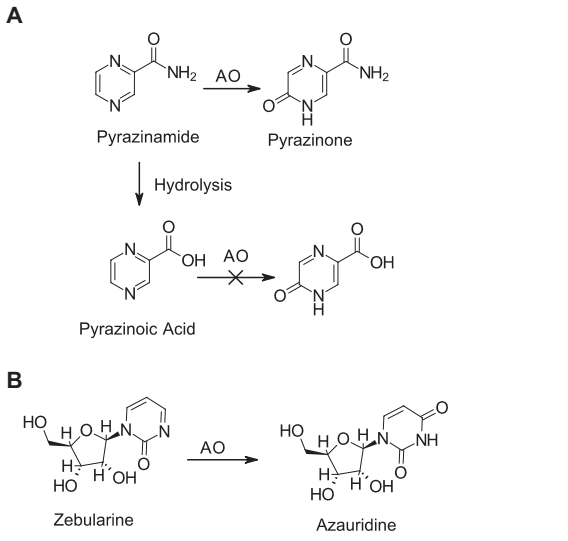


Fig. 9. Metabolism of monocyclic nitrogen heterocycles by AO. A) Conversion of pyrazinamide to pyrazinone. Pyrazinamide is also hydrolyzed to pyrazinoic acid. However, pyrazinoic acid is not oxidized by AO. B) Conversion of zebularine, a transition state analog inhibitor of cytidine deaminase, to azauridine by AO.

by AO. Contribution of AO in the catalysis was confirmed using AO inhibitor, which inhibited its conversion to the oxo-derivative (Klecker, Cysyk, & Collins, 2006).

Attempts have been made to exploit AO-mediated metabolism in activating prodrugs to their respective nucleoside analogues (Pryde et al., 2010; Rooseboom, Commandeur, & Vermeulen, 2004). Nucleoside analogues are structurally similar to natural nucleosides and function as inhibitors of cellular and viral DNA and RNA polymerases or as chain terminators (Zhang, Gao, Wen, & Ma, 2014). However, the physicochemical properties of these molecules are not optimal for passive transcellular intestinal absorption and this results in their poor oral bioavailability. The objective behind designing prodrugs of these molecules is to increase their absorption through the intestine and therefore their systemic exposure. For instance, 5-iodo-2-pyrimidinone-2′-deoxyribose (5-IPdR) was designed as a prodrug of 5-iodo-2′-deoxyuridine (IUdR) with an objective of overcoming poor bioavailability that was observed after dosing IUdR by itself (Fig. 10A). *In vitro* metabolism studies with 5-IPdR using mouse, rat, and human fractions revealed that it was rapidly and selectively converted to the corresponding active moiety by AO (Rooseboom et al., 2004). A prodrug strategy to mitigate poor absorption of acyclovir, and therefore its poor bioavailability, has also been reported by Krenitsky and coworkers (Krenitsky et al., 1984). Acyclovir is an acyclic guanine nucleoside analogue that is widely used in the clinic as an anti-herpes agent. Although the IV, PO, and topical administration provides effective therapy, only 15 to 20% of the dose is typically absorbed in humans after oral administration (de Miranda & Blum, 1983). A 6-deoxy congener of acyclovir was therefore designed as a prodrug to mitigate poor absorption characteristics and was found to be 18 times more water soluble than acyclovir (Krenitsky et al., 1984). Metabolic studies with deoxyacyclovir indicated that the compound was metabolized by both XO and AO to acyclovir and the inactive 8-hydroxy-6-deoxyacyclovir (Fig. 10B). Interestingly, the desired metabolite, acyclovir, was formed by XO whereas AO converted deoxyacyclovir to the 8-hydroxyacyclovir. The studies also showed that acyclovir as well as the 8-hydroxy metabolite were also substrates for AO (Krenitsky et al., 1984; Krenitsky, Neil, Elion, & Hitchings, 1972) and were converted to inactive 8-oxoacyclovir (Fig. 10B). Additionally, acyclovir was found to be a weak noncompetitive inhibitor of XO when xanthine was used as a variable substrate (Krenitsky et al., 1984). 5-Ethynylpyrimidinone (5-EP) and 5-fluoropyrimidinone (5-FP) have also been synthesized as liver specific prodrugs of 5-ethynyluracil (5-EU) and 5-fluorouracil (5-FU), respectively, as inhibitors of dihydropyrimidine dehydrogenase (Fig. 10C) (Guo et al., 1995; Porter et al., 1994). *In vitro* studies indicated that both 5-EP and 5-FP were efficiently converted to their respective uracil analogs by AO. The catalytic efficiency for oxidation of 5-EP to 5-EU was 60-fold higher than N^1-methylnicotinamide. Similarly, 5-FP was activated to 5-FU very efficiently by AO (K_M value of 220 μM and V_{max} of 8 nmol/min/mg). However, the prodrug approach failed due to lack of selectivity and or similar cytostatic activity as 5-FU (dosed by itself) (Guo et al., 1995; Porter et al., 1994). Despite these efforts, exploitation of AO for prodrug activation has not gained traction. As will be discussed later, the ubiquitous nature of AO as well as the marked species differences in AO expression makes it a less attractive enzyme in generating active entities from prodrugs (Rooseboom et al., 2004).

As in P450-catalyzed oxidations (Niwa, Murayama, & Yamazaki, 2011), metabolism by AO can also be stereoselective with one enantiomer being a more favorite substrate. High stereoselectivity in AO

Fig. 7. Oxidation of heteroaromatic rings. A) Oxidation *N*-methylnicotinamide to 1-methyl-4-pyridone-3-carboxamide (4-PY) and 1-methyl-2-pyridone-3-carboxamide (2-PY). B) Conversion idelalisib to hydroxyidelalisib (GS-563117) by AO. GS-563117 is the primary metabolite of idelalisib. C) Metabolism of lenvatinib and its desmethyl metabolite to the corresponding lactams by AO. Lenvatinib is also metabolized via P450 to the corresponding *N*-oxide and is also eliminated as a glutathione conjugate. D) Metabolism of zaleplon. Zaleplon is metabolized by two pathways. It undergoes CYP3A4 catalyzed *N*-dealkylation to desethylzalplon and is oxidized to 5-oxozaleplon by AO. E) Metabolism of methotrexate. Methotrexate is converted to 7-hydroxymethotrexate by AO.

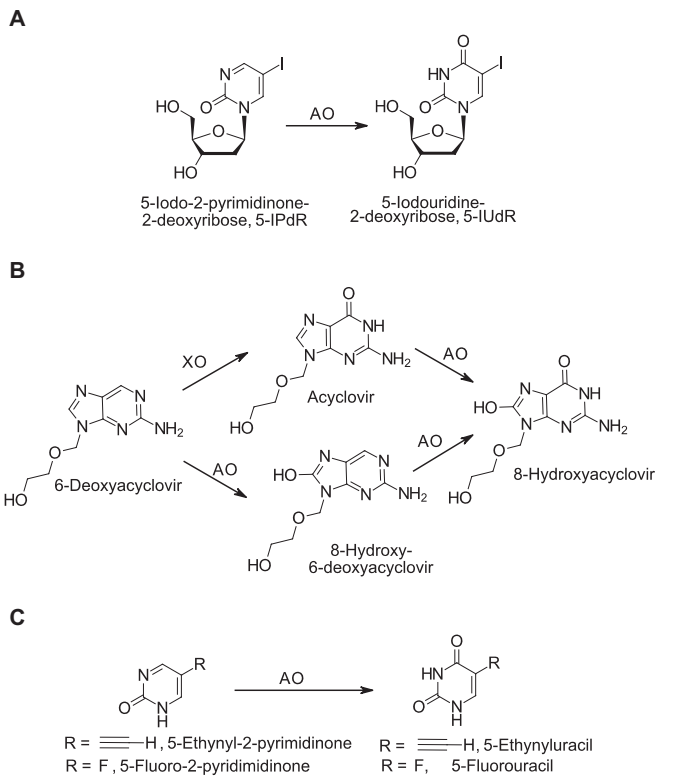


Fig. 10. Bioactivation of purine and pyrimidine nucleoside prodrugs by AO. A) 5-Iodo-2-pyrimidinone-2-deoxyribose (5-IPdR) is oxidized to 5-iodouridine-2-deoxyribose (5-IUdR). B) Oxidation of 6-deoxyacyclovir to the pharmacologically active acyclovir. AO converts 6-deoxyacyclovir to the corresponding 8-hydroxy-6-deoxyacyclovir and is subsequently oxidized to the inactive 8-hydroxyacyclovir. Acyclovir is also a substrate of AO and is converted to 8-hydroxyacyclovir. C) Conversion of pyrimidinones, 5-ethinylpyrimidinone and 5-fluoropyrimidinone to the corresponding uracil derivatives.

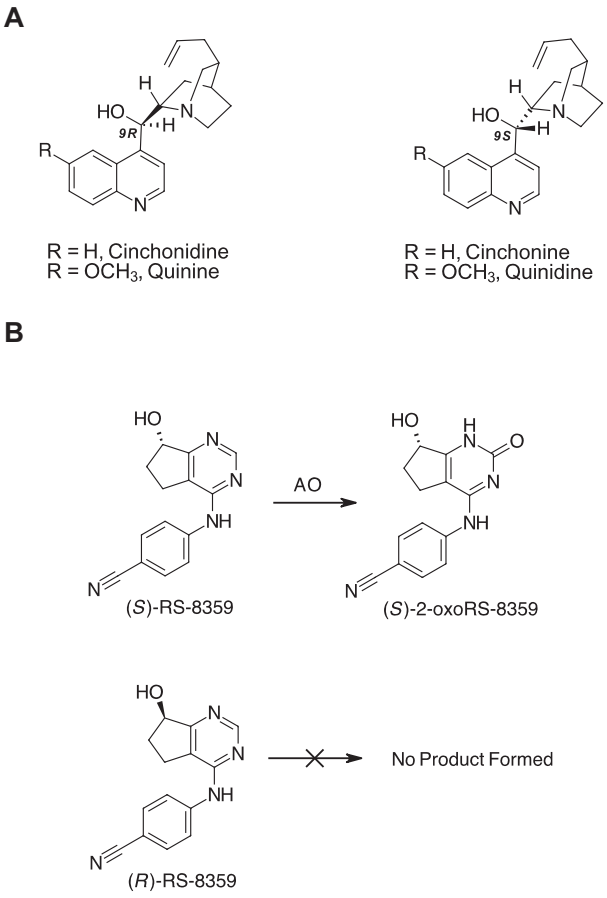


Fig. 11. A) Structures of cinchona alkaloids. The *9R*-isomers of cinchona alkaloid are predominantly metabolized compared to *9S*-isomers. B). Stereoselective oxidation of RS-8359. *S*-RS-8359 is stereoselectively oxidized by AO to the corresponding 2-oxo-*S*-RS-8359 while *R*-RS-8359 is not a substrate for AO.

catalyzed oxidation of cinchona alkaloids (Fig. 11A) (quinine, quinidine, cinchonidine and cinchonine) has been observed by Beedham and et al., and Itoh et.al. (Beedham, al-Tayib, & Smith, 1992; Itoh et al., 2006). Although all four alkaloids were oxidized at the 2′-position of the quinoline ring, the oxidation of quinine and cinchonidine was predominant. These studies also suggested that (*R*)-configuration at carbon-9 of these alkaloids was required for selectivity, which led the authors to propose that the stereoselectivity in this case is regulated by high degree of steric hindrance. Oxidation of RS-8359 (MAO-A inhibitor) by AO represents another example of stereoselective oxidation of molecules by this enzyme. Pharmacokinetic and metabolic studies with RS-8359 revealed a much lower plasma concentration of the (*S*)-enantiomer relative to that of (*R*)-enantiomer after oral administration of each enantiomer to preclinical species (Takasaki et al., 1999; Takasaki et al., 2005). Metabolite identification and phenotyping studies with the two enantiomers suggested that only the *S*-enantiomer of RS-8539 was a substrate for AO and was oxidized to the AO catalyzed 2-oxoRS-8539 metabolite in the monkeys and humans (Fig. 11B). In contrast, the *R*-enantiomer was not metabolized by AO (Itoh, Nishiya, Takasaki, Adachi, & Tanaka, 2006). It was inferred that like in the case of cinchona alkaloids, steric factors are probably responsible for selective oxidation of *S*-enantiomer of RS-8359 (Itoh, Yamamura, et al., 2006).

BILR-355, an inhibitor of HIV-1, (Fig. 12) represents an interesting case where inhibition of its major CYP3A4-mediated metabolic pathway results in metabolic switching to AO-mediated metabolite being a major metabolite (Li et al., 2012; Li, Xu, Lai, Whitcher-Johnstone, & Tweedie, 2012). Clinical studies have suggested that BILR-355 is rapidly cleared after a single oral dose to healthy volunteers resulting in low parent exposure. *In vitro* metabolism studies with this molecule demonstrated that CYP3A4 was a major contributor to the limiting systemic exposure of BILR-355 (Fig. 12) (Li, Lai, et al., 2012; Li, Xu, et al., 2012). After concomitant administration of BILR-355 with a CYP3A4 inhibitor ritonavir

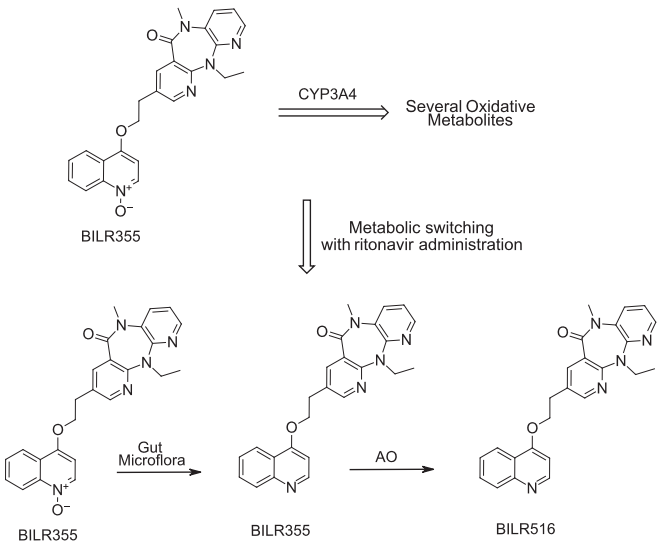


Fig. 12. Metabolism of BILR355. BILR355 is primarily metabolized by CYP3A4. However, upon co-administration with ritonavir, BILR undergoes metabolism by gut microflora to the quinoline metabolite BILR402, which is subsequently metabolized by AO to the corresponding oxoBILR (BILR516). This metabolite was the primary circulating metabolite in humans.

(RTV), BILR-355 exposure improved dramatically with AUC increasing 16- to 30-fold. However, an unexpected consequence of this boosting strategy was a disproportionate metabolite BILR-516 which was not detected previously in humans given BILR 355 alone. Studies around formation of BILR516 suggested that the formation of the oxidative metabolite was attributed to the reduction of *N*-oxide by gut microflora to quinoline (BILR-402), and subsequent oxidation to the quinolinone metabolite by AO.

Recently, Lepri et. al. have analyzed approximately 200 compounds with various hetereoaromatic scaffolds containing electron-withdrawing and electron-donating groups with regards to their susceptibility towards AO oxidation and established the structure metabolism relationship for AO (Lepri et al., 2017). Analysis of their results suggests that all heteroaromatic rings tested were substrates of AO to some degree except for pyridine rings and that the monocyclic pyrimidine and pyrazine rings were moderate substrates to AO metabolism unless they were substituted with electron-withdrawing groups. However, most bicyclic heteroaromatic rings were good substrates and were readily metabolized by AO unless they were substituted by bulky, hydrophobic groups. The authors concluded that in most cases, the carbon at the 2-position was more susceptible to oxidation and adding substituents that lower the atomic charge on the most electropositive carbon atom modulated AO activity. This also explained why N^1-methylnicotinamide, which has a quaternary nitrogen and therefore higher atomic charge, is an excellent AO substrate.

3.1.4. Impact of AO-mediated metabolism on development of drug candidates

Even though a few drugs that are metabolized by AO have been launched, the impact of AO on drug candidates leading to poor exposure and therefore their discontinuation, has been broad. Kaye and coworkers were among the first group to report the effect of AO metabolism on human exposure. Carbazeran, a phosphodiesterase inhibitor (Fig. 13), was discontinued from the clinic due to its poor exposure and lack of pharmacological effect in humans (Kaye, Offerman, Reid, Elliott, & Hillis, 1984; Kaye, Rance, & Waring, 1985). Preliminary studies in dogs indicated excellent PK properties with a moderate clearance in this species and good bioavailability (~68%). However, clinical data suggested high blood clearance in humans (~53.7 mL/min/kg) exceeding human liver blood flow and poor bioavailability. Identification of metabolites in humans indicated that carbazeran was almost completely cleared via presystemic hydroxylation of the phthalazine moiety to the corresponding hydroxycarbazeran (Fig. 13). In contrast, the primary route of metabolism in dogs was *O*-demethylation resulting in the formation of desmethylcarbazeran (Fig. 13). *In vitro* investigation into carbazeran metabolic pathways demonstrated that the compound was rapidly converted to hydroxycarbazaran in human liver cytosol by AO. Additional *in vitro* studies with carbazeran using liver cytosols of different preclinical species also showed that carbazeran was a selective AO substrate (Beedham, Bruce, Critchley, al-Tayib, & Rance, 1987; Critchley, Rance, & Beedham, 1994; Wilkinson et al., 2017). As a result of its AO selectivity, carbazeran is now commonly used as a tool compound to evaluate AO activity in cytosolic fractions (Xie et al., 2019).

Since the discontinuation of carbazeran, several examples of drug candidates that lack clinical exposure owing to rapid AO-mediated metabolic clearance, have emerged. GDC-0834, a Bruton's tyrosine kinase inhibitor (Fig. 14) and Lu AF09535, a high affinity metabotropic glutamate receptor 5 negative allosteric modulator (Fig. 15), represent two most recent cases where AO-based metabolism led to poor exposure in humans and therefore their discontinuation from the clinic. Additionally, GDC-0834 also represents a unique case of AO-catalyzed hydrolytic cleavage of the amide bond.

GDC-0834 was designed as a selective BTK inhibitor for treatment of rheumatoid arthritis. Despite its excellent preclinical PK properties, no measurable plasma levels (<1 ng/mL) were detected in human circulation after oral administration (Young et al., 2015). Preliminary metabolite identification studies in humans revealed non-P450 mediated hydrolysis of the amide bond that links the tetrahydrobenzothiophene moiety to the central aniline ring, as the primary pathway, and substantial levels of the cleaved product (metabolite M1, Fig. 14A) were observed in the urine and plasma (Liu et al., 2011). In *in vitro* studies, M1 was more prevalent in subcellular fractions from human liver than from preclinical species (mouse, rat, dog and monkey), which suggested significant species differences in this major route of metabolism (Sodhi et al., 2015). Although AO and CES were putatively identified as the

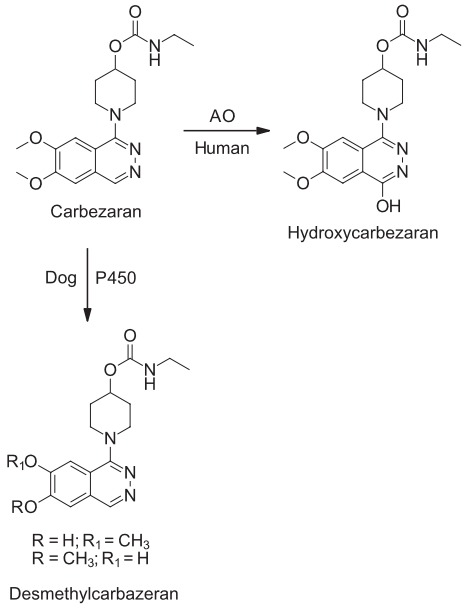


Fig. 13. Metabolism of carbazeran. Carbazeran is predominantly metabolized by AO in humans but undergoes *O*-dealkylation in the dog to the corresponding desmethylcarbazeran.

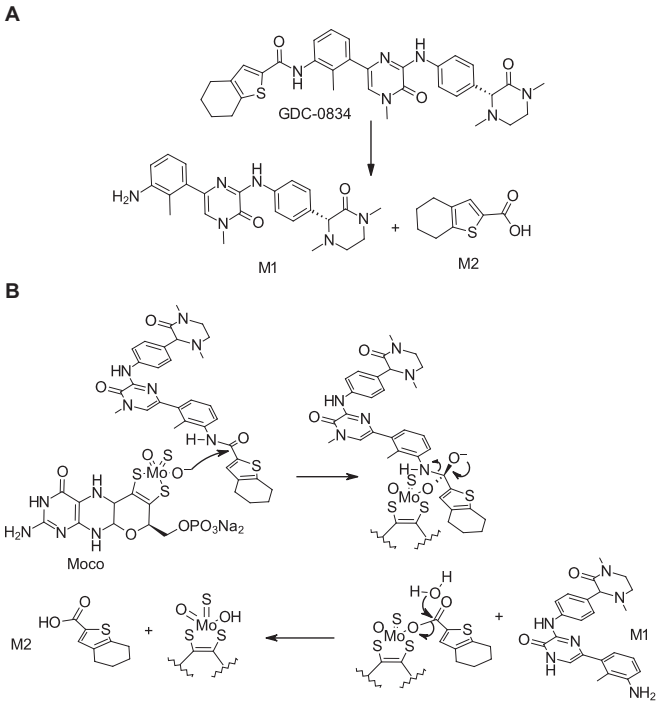


Fig. 14. A) AO-catalyzed hydrolysis of GDC-0834. GDC-0834 is hydrolyzed by AO to the corresponding aniline metabolite (M1) and the carboxylic acid metabolite, M2. B) Putative mechanism of hydrolysis of GDC-0834 by AO.

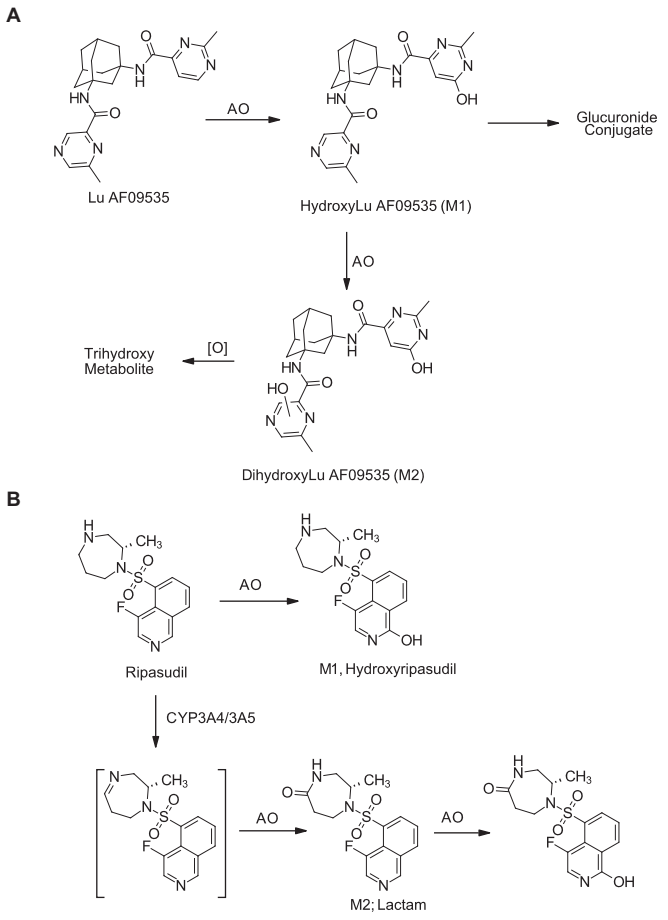


Fig. 15. A). Oxidation of Lu AF09535 by AO to its hydroxy and dihydroxy metabolites by AO. B) Metabolism of ripasudil, a rho kinase inhibitor. Ripasudil is metabolized by AO to the corresponding hydroxyripasudil metabolite (M1). It is also oxidized by CYP3A4/3A5 to an imine intermediate, which in turn converted to the lactam by AO.

enzymes responsible for the hydrolysis, further assessment with AO, CES and XO inhibitors implicated AO as the primary enzyme catalyzing GDC-0834 cleavage. Subsequent *in silico* modeling studies have suggested that GDC-0834 is capable of binding in the AO active site with the amide bond of GDC-0834 near Moco center. This orientation enables a potential nucleophilic reaction between the hydroxyl group of Moco center and the carbonyl group of the amide moiety leading to hydrolysis and liberation of M1 (Fig. 14B). Although the authors have proposed that the hydrolytic reaction by AO is probably catalyzed by the reduced Moco (+4) due to its high electron density, a direct nucleophilic attack of the hydroxyl group in its (+6 state) cannot be ruled out. Lepri and coworkers have recently investigated this hydrolytic reaction by using ~75 amide analogues (Lepri et al., 2017). Of the number of compounds screened, about 25 compounds were hydrolyzed by AO suggesting that this may actually be a general AO reaction.

The plasma exposure of Lu AF09535 was also very low after its oral administration to humans. The first measurable plasma concentrations were only detectable at higher doses (Jensen et al., 2017). The role of AO in the disposition of Lu AF09535 was identified early in the discovery process *in vitro,* in human and animal hepatocytes and cytosolic fraction, and *in vivo*, in preclinical species. Early metabolite identification studies revealed the formation hydroxyLu AF09535 metabolite (M1) (Fig. 15A) in liver cytosolic fractions from humans and preclinical species. Extrapolation of the results from these studies (*in vivo-in vitro* correlation and allometric scaling) predicted low clearance of the candidate in humans. This information coupled with a desirable pharmacological profile led to the decision of advancing Lu AF09535 to the clinic. In spite of the excellent PK and pharmacological properties in a preclinical setting, early clinical studies with radioabeled Lu AF09535 (263 nCi) coadministered with 75 mg of Lu AF09535 to healthy male volunteers and accelerator mass spectrometric analysis of the plasma samples revealed low exposure of the candidate. The poor exposure was almost exclusively attributed to AO-mediated extensive metabolism of the compound since substantial levels of the AO-catalyzed hydroxylated and the dehydroxylated metabolites (M1 and M2) were observed in circulation (Fig. 15A) (Jensen et al., 2017). This resulted in discontinuation of the candidate from development.

Ripasudil, a Rho Kinase (ROCK) inhibitor (Fig. 15B), represents another interesting case where the clinical development of the drug was halted due to AO-mediated poor exposure in humans. However, in this case the drug was advanced as an ophthalmic agent for the treatment of glaucoma (Isobe, Ohta, Kaneko, & Kawai, 2015). Major species differences in the intrinsic clearance estimates were observed when ripasudil was initially evaluated *in vitro* in liver S9 fractions and hepatocytes of typical preclinical species and humans. Metabolite identification revealed the AO-catalyzed hydroxylated metabolite (M1) as the primary metabolite in the monkeys and humans and the lactam metabolite (M2), which was formed by AO-catalyzed oxidation of the iminium ion intermediate (formed by P450) (Fig. 15B). Additionally, marked variation (15-fold) was also observed when three different lots of human hepatocytes, the major cause being individual differences between the expression of AO. Oral administration of ripasudil to humans resulted in high systemic levels of M1 with low levels of the parent and reflected the *in vitro* results. Poor systemic exposure and low predicted bioavailability was considered a risk for development ripasudil as an oral agent and its development was abandoned. Despite this setback, Phase I and Phase II studies showed ripasudil to be an effective and safe topical agent that lowered the intra-ocular pressure in healthy volunteers and patients suffering with primary open-angle glaucoma (Isobe et al., 2015). Given these characteristics it is being developed as an ophthalmic agent for the treatment of glaucoma.

Attempts have been made to apply pharmacogenetics analysis to understand the AO-mediated metabolism. Zhang and co-workers applied computational pharmacogenetics analysis method to facilitate rapid identification and murine characterization of the less commonly used drug metabolism pathways such as AO based oxidation (Zhang et al., 2011). They used this method in the case of a p38 kinase inhibitor, RO1, which was being developed for treatment of rheumatoid arthritis and was terminated because of its AO-based rapid clearance in human subjects. Despite the good predicted PK profile, a short $t_{1/2}$ (0.7 hr) was observed when a 50 mg dose of RO1 was administered to healthy male volunteers. This was vastly different from the $t_{1/2}$ observed in mice, rats, dogs and monkeys ($t_{1/2}$ in rats was 6.2 hr and $t_{1/2}$ in dogs and monkeys was 4 hr) (Zhang et al., 2011). The primary metabolite detected in human serum was hydroxyRO1 (Fig. 16A), which was about 220-fold greater in exposure (area under the curve, AUC) relative to the parent. In contrast, a negligible amount of the hydroxylated metabolite was observed in the sera of rat, dog and monkey. Mouse on the other hand, generated significant amounts of hydroxyRO1 that was found in humans. To assess the enzyme system that converts RO1 to hydroxyRO1, the authors determined the rate of hydroxyRO1 formation in liver cytosolic fractions of fourteen inbred strains of mice and analyzed these results by computational haplotype-based genetic analysis (Zhang et al., 2011). This led to the identification of AO as the enzyme responsible for the rapid metabolism of this drug and showed genetic variation within murine *Aox1* gene among the inbred strains. Likewise, Ramirez and coworkers performed a candidate gene study to investigate if the human *AOX* genetic variation contributes to inter-individual variability in XK-469 clearance (Ramirez et al., 2014). XK-469, a synthetic derivative of quinoxaline phenoxypropionic acid and a selective topoisomerase II beta inhibitor (Fig. 16A) was evaluated in phase I clinical trials as a single agent for treatment of refractory tumors and hematological malignancies. Despite the linear pharmacokinetics and dose

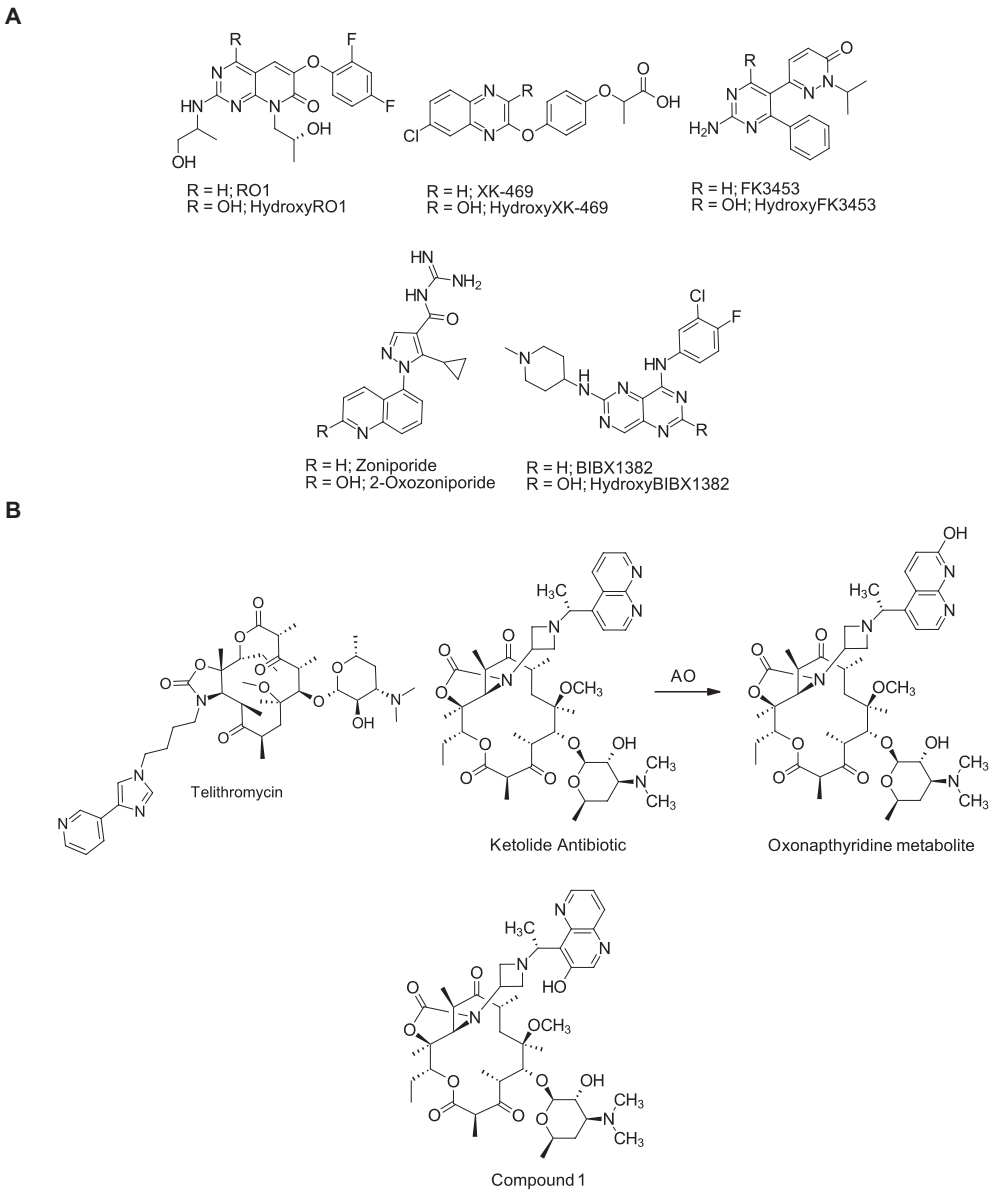


Fig. 16. Structures of drug candidates that were discontinued from development due to AO mediated metabolism. A). RO1, a p38 kinase inhibitor; XK-469, a selective topoisomerase II beta inhibitor, FK3453, an adenosine A1/2 dual inhibitor; zoniporide, inhibitor of Na+/H+ exchanger isoform 1 (NHE-1) and BIBX1382, inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase and their respective hydroxylated metabolites. B) Structures of telithromycin, ketolide antibiotic and its oxonapthyridine metabolite, and compound 1, the AO resistant ketolide antibiotic analogue.

independent drug clearance shown by XK-469 in patients, the candidate showed high inter-individual variability among patients and its development was discontinued due to lack of adequate anti-cancer activity in patients. Investigations into the metabolic pathways and enzymes responsible for metabolism suggested that XK-469 was extensively and primarily metabolized by AO to hydroxyXK-469 (Fig. 16A). The authors evaluated the functional impact of human *AOX* polymorphisms on variability by genotyping *AOX* single nucleotide polymorphism (SNP) in patients using XK469 clearance as a probe for AO activity. The results led to the conclusion that the variability observed in patients was not attributed to SNP in the *AOX* gene and that the factors other than the genetic variation in *AOX* were responsible for the variability in XK-469 clearance.

Several other examples of candidate withdrawal from development have appeared in the past decade and are briefly discussed below. Magee et. al. have published the impact of AO metabolism on exposure of a macrolide antibiotic which was designed to overcome safety liabilities that were observed with another member of azithromycin class of antibiotics, telithromycin (Fig. 16B) (Magee et al., 2009). Magee and coworkers discovered a new series of N11 ketolides (Fig. 16B) that had minimal potential for eliciting hepatotoxicity and time dependent inhibition of P450 observed with telithromycin (Magee et al., 2009). The selected compound was predicted to have low human clearance, which when coupled with its excellent efficacy and safety profile was considered as a good clinical candidate and was advanced to first-in human studies. Despite a good estimation of human PK properties, the plasma exposures were ~20% of the predicted AUC values in the single dose escalating studies. Subsequent investigation of circulating metabolites revealed the presence of AO catalyzed oxonapthyridine metabolite (Fig. 16B) that had 20-fold higher exposure than that of the parent compound. Based on these results, the authors modified the napthyridine ring and synthesized compound 1, which was devoid of AO liability (Fig. 16B). This compound was subsequently advanced to development.

The development of a novel adenosine A1/2 dual inhibitor FK3453 was also suspended due to extremely low exposure in a clinical study (Akabane, Tanaka, Irie, Terashita, & Teramura, 2011). As in the above

cases, despite promising preclinical findings, the plasma concentrations of FK3453 in humans were extremely low and the AO-mediated hydroxyFK3453 metabolite (Fig. 16A) was identified as a major circulating metabolite. Sanoh and co-workers could reproduce these results using human-chimeric (h-chimeric) mice (Sanoh et al., 2012). They also examined the species differences in oxidative metabolism of FK3453 in mice transplanted with rat hepatocytes (r-chimeric mice). Higher concentrations of human-specific AO-generated metabolite were detected in urine and feces after administration of FK3453 to h-chimeric mice compared to r-chimeric mice. The observed clearance in h-chimeric mice was also higher than that of r-chimeric mice and corroborated with the species differences observed in humans and rats in *in vitro* and *in vivo* studies (Akabane et al., 2011).

Zoniporide, a highly selective inhibitor of sodium/hydrogen exchanger (NHE-1), was another drug candidate that was discontinued from development. The candidate was developed for the treatment of perioperative myocardial ischemic injury in high risk surgery patients and was a very selective AO substrate. While the exact reason for withdrawal from clinic is unknown, the candidate exhibited high clearance (21 mL/min/kg) and short $t_{1/2}$ (2 hr) in humans (Dalvie et al., 2010). Metabolism and excretion studies with radiolabeled zoniporide in humans suggested that the drug was primarily cleared by metabolism and the primary metabolite was the AO-catalyzed 2-oxozoniporide (Fig. 16A). The exposure of the hydroxylated metabolite was ~2-fold greater than that of zoniporide in plasma and it was detected in large quantities (~52% of the dose) in the excreta. 2-Oxozoniporide was also pharmacologically active against NHE-1 receptor but its potency was 3-fold less potent than zoniporide. Comparison of the unbound exposure of 2-oxozoniporide with that of zoniporide, suggested that the metabolite contributed in the overall pharmacological activity of the drug (Dalvie et al., 2010).

Poor PK properties as a result of AO mediated metabolism were also observed when BIBX1382 (Fig. 16A), an epidermal growth factor receptor inhibitor, was advanced to clinic (Dittrich et al., 2002). As with other failed candidates, preclinical profile of BIBX1382 indicated ideal drug like properties such as great solubility and permeability. Disposition studies in rat, mice and dog suggested absolutely bioavailability to be high, ranging from 50 to 90%. BIBX1382 was also stable in human liver microsomes with a low estimated clearance. However, the clearance of BIBX1382 after intravenous infusion to humans was 25–55 mL/min/kg, a rate in excess of liver blood flow (~21 mL/min/kg). In addition, after oral administration, plasma levels of BIBX1382 were well below target concentrations expected for efficacy (5% mean absolute oral bioavailability) and highly variable (15 to 25-fold range of maximum concentration, C_{max} and AUC) resulting in the rapid attrition of this drug candidate. Investigation into the reasons and mechanisms for low exposure uncovered an oxidative metabolite (hydroxyBIBX1382) in human plasma. The role of AO in the formation of M1 was confirmed when cytosolic incubations with BIBX1382 were performed in the presence of AO-selective inhibitor hydralazine. In an effort to evaluate the appropriate preclinical surrogate for humans, Hutzler and co-workers conducted metabolism studies using liver cytosol and cryopreserved hepatocytes from multiple species (Hutzler et al., 2014). These studies revealed that the estimated clearance in the liver cytosol fraction of cynomolgus and rhesus monkeys was 42 and 43 mL/min/kg and comparable to that in humans (≥93% of the liver blood flow). In addition, an IV and PO PK study of BIBX1382 in cynomolgus monkey exhibited high clearance (118 mL/min/kg) and low oral exposure (C_{max} of 12.7 nM and 6% bioavailability), with exposure of hydroxyBIBX1382 exceeding the parent after oral dosing. These results agreed favorably with the PK parameters in humans suggesting that the monkey is a suitable surrogate for humans.

3.2. Reductive substrates

AO can also catalyze reduction of selective functional groups (Kitamura et al., 2006). However, in comparison to oxidative transformation, AO-mediated reductive reactions and mechanisms have not been studied extensively. Several functional groups are susceptible to reduction by AO. Groups like nitrite, aromatic and aliphatic nitro groups, as well as *N*-oxide, sulfoxide and hydroxamate can also act as alternative electron acceptors. These groups can act as oxidizing substrates and compete favorably with oxygen to get reduced by AO. Likewise, a few five membered heteroaromatic rings such as isoxazoles and isothiazoles can also act as electron acceptors and undergo reductive ring cleavage yielding ring opened products (Beedham, 1985; Kitamura et al., 2006; Pryde et al., 2010). In many cases, these reactions occur under anaerobic conditions and are more effective in the presence of saturating concentrations of electron donor substrates (reducing substrates) such as 2-hydroxypyrimidine or N^1-methylnicotinamide.

3.2.1. Reduction of nitrite to nitric oxide (NO)

Nitric oxide radical (NO) is a signaling molecule involved in several physiological and pathological processes (Bender & Schwarz, 2018; Maia & Moura, 2018; Zweier, Li, Samouilov, & Liu, 2010). NO is commonly generated by specific nitric oxide synthetases that convert L-arginine to L-citrulline (Bredt et al., 1991). However, studies in the past two decades suggest that a nitrite ion (NO_2^-) can also be reduced to NO by AO and XO under hypoxic conditions (Li, Kundu, & Zweier, 2009; Maia, Pereira, Mira, & Moura, 2015; Weidert et al., 2014). The XO and AO-catalyzed NO generation is dependent on the simultaneous presence of enzyme, nitrite and one reducing substrate (Maia & Moura, 2018). The kinetics of AO-catalyzed nitrite reduction with the molybdenum site electron donor cinnamaldehyde as well as flavin site electron donor NADH has been studied by Li et al. under aerobic and anaerobic conditions (Li et al., 2009). The rate of NO formation is a function of the concentration of protein, nitrite and reducing substrate. The NO_2^- reduction to NO can be triggered in the presence of several reducing substrates of a variety of chemical nature such as heterocyclic compounds or aldehydes which act in the catalytic domain at the Moco center or by NADH that reacts at the FAD center.

The molecular mechanism for reduction of NO_2^- has been reviewed by Maia and Moura (Maia & Moura, 2018) and shown in Fig. 17. The nitrite reduction to NO is presumed to occur at the molybdenum center of AO or XO with a sequential transfer of one electron at a time and by conversion of $Mo^{+6} \rightarrow Mo^{+5} \rightarrow Mo^{+4}$. This was unequivocally demonstrated by several studies combining EPR and NO electrode assays, using molybdenum-specific inhibitors. Additional assays with diphenyleneiodonium chloride, a FAD specific inhibitor, and also with deflavo-XO and deflavo-AO (Maia et al., 2015) provided the "negative confirmation" that nitrite reduction is not dependent on the FAD center.

In mammals, NO controls a number of functions, including vasodilation, neurotransmission, platelet aggregation, apoptosis, gene expression, immune response, and mediates a wide range of both anti-tumor and anti-microbial activities. Given the importance of NO, any processes that affect the generation of NO can be deleterious. Since it is clear that

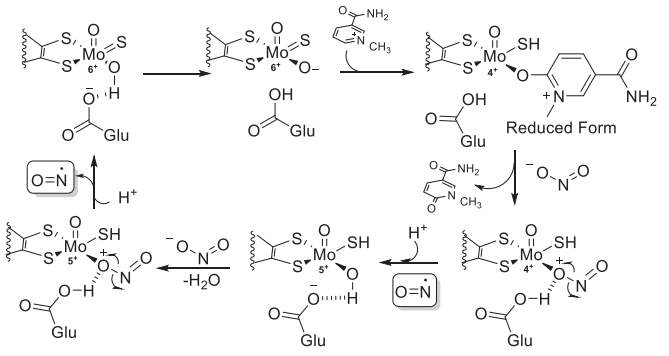


Fig. 17. Mechanism of reduction of nitrite to nitric oxide (NO) by AO in the presence of N^1-methylnicotinamide. The nitrite gets reduced at the Moco center in the active site of AO (Maia & Moura, 2018).

NO formation takes place at the Moco center, inhibitors that bind to this site and inhibit AO can affect the concentrations of NO formation. Additionally, NO itself can cause modifications of enzymes that can alter its concentration. For instance, modification of cysteine residues and metal centers (molybdenum and iron/sulfur) and can affect enzyme activity of AO. Since NO formation is a one-electron reduction process, and AO proceeds via a two-electron reduction to regenerate the catalytically competent enzyme form ($Mo^{4+} \rightarrow Mo^{6+}$), the chances of molybdenum center being trapped in an intermediate oxidation state (Mo^{5+}) exist. This can potentially stop the catalysis process. As discussed by Maia and Moura, the "classic" oxidizing substrates such as dioxygen can act as strong competitive inhibitors of NO formation. Therefore, factors such as hypoxia that change in the levels of dioxygen can potentially affect the amount of reduced enzyme formed and may affect AO reactions in general (Maia & Moura, 2018). Since nitrite reduction occurs at the Moco active site, nitrite itself can compete with the oxidative substrates to access the enzyme and can possibly decrease the NO production. Similarly, inhibitors that bind to the Moco active site can alter the NO levels in tissues leading to unintended pharmacodynamic effects (Paragas et al., 2017).

3.2.2. Reduction of N-oxides to amines

N-Oxide groups are reduced back to their parent amines by AO (Fig. 18A) (Bickel, 1969; Kitamura & Tatsumi, 1984a, 1984b; Takekawa, Sugihara, Kitamura, & Ohta, 2001). In the early eighties, Kitamura and Tatsumi reported the reduction of tertiary amine *N*-oxides as well as nicotinamide *N*-oxide to nicotinamide using mammalian liver AO (Kitamura & Tatsumi, 1984a, 1984b). Rabbit liver AO-supplemented with an electron donor exhibited significant nicotinamide N-oxide reductase activity under anaerobic conditions (Fig. 7A) (Kitamura & Tatsumi, 1984a). While this AO-reductase activity was exhibited by several other species in the presence of an electron donor of AO, XO did not reduce nicotinamide *N*-oxide. Reduction of *N*-oxides of drugs such as imipramine *N*-oxide or cyclobenzaprine *N*-oxide by AO in the liver cytosol of a variety of species such as rabbits, guinea pigs, rat and mice has also been reported (Fig. 18B) (Kitamura & Tatsumi, 1984b). Sugihara and co-workers have shown that AO can function as a nicotine *N*-oxide reductase in the presence of 2-hydroxypyrimidine (Sugihara, Kitamura, & Tatsumi, 1996b). Recently, *N*-oxide reduction of quinoxaline-1,4-dioxides, catalyzed by porcine AO has been demonstrated by Mu et.al. (Fig. 18C) (Mu et al., 2014). Quinoxaline-1,4-dioxides are a class of quinoxaline derivatives that possess two N-O bonds at positions N1 and N4, respectively. This class of derivatives have been used in various indications and possess a wide range of biological properties such as antibacterial, antiviral, antifungal, antiprotozoal and anticancer activities. *N*-oxide reduction is one of the main metabolic pathways for several drugs including Cyadox, quinocetone and mequindox (Fig. 18D) (Liu et al., 2010; Mu et al., 2014).

3.2.3. Reduction of nitro groups

Tatsumi and co-workers were the first to report AO-catalyzed reduction of carcinogenic compounds like 2-nitrofluorene, 4-nitrobiphenyl and 1-nitronapthalene to their respective hydroxylamines and arylamines in the presence of 2-hydroxypyrimidine under anaerobic conditions (Fig. 19A) (Tatsumi, Kitamura, & Narai, 1986; Ueda, Sugihara, Ohta, & Kitamura, 2005). Reduction of nitro groups by AO is perhaps one of the most important reduction reactions given that this conversion can result in metabolites with toxic implications. The hydroxylamine intermediate formed during the reduction process is known to cause adverse effects via binding to DNA or proteins or reactive oxygen species. In fact several nitroaromatic drugs have been shown to elicit hepatotoxicity following their administration and several of them have been withdrawn from the market due to adverse drug reactions (Boelsterli, Ho, Zhou, & Leow, 2006). Recent reports have shown that AO-mediated reduction of the nitro group can occur under normal oxygen conditions (Konishi, Fukami, Gotoh, & Nakajima, 2017). Nitrazepam is a hypnotic agent that is reduced to potentially toxic 7-*N*-hydroxylaminonitrazepam, 7-aminonitrazepam and converted to 7-acetylaminonitrazepam via acetylation of 7-aminonitrazepam in humans and rats (Fig. 19B). Studies by Konishi et. al. demonstrated that nitrazepam reductase activity resides in human liver cytosol and can be inhibited by AO inhibitors (Konishi et al., 2017). The role of AO was confirmed when the reductase activity increased upon addition of electron donor like N^1-methylnicotinamide.

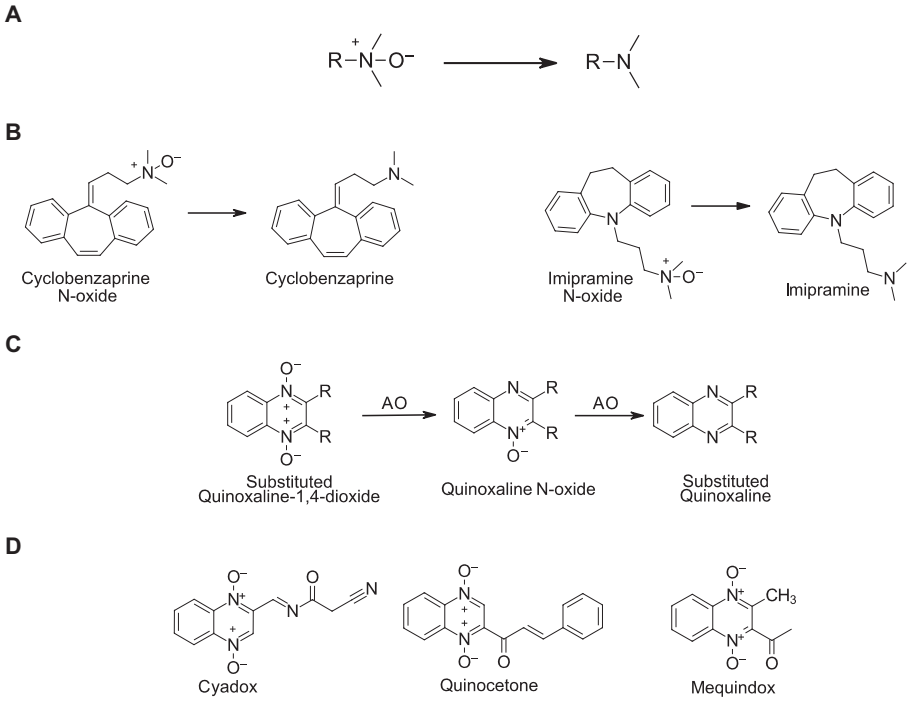


Fig. 18. A) Reduction of *N*-oxides. B) Reduction of cyclobenzaprine *N*-oxide and imipramine *N*-oxide. C) Sequential reduction of substituted quinoxaline-1,4-dioxide to the quinoxaline. D) Structures of quinoxaline-1,4-di-*N*-oxides containing antimicrobial agents that undergo reduction to their respective quinoxaline derivatives.

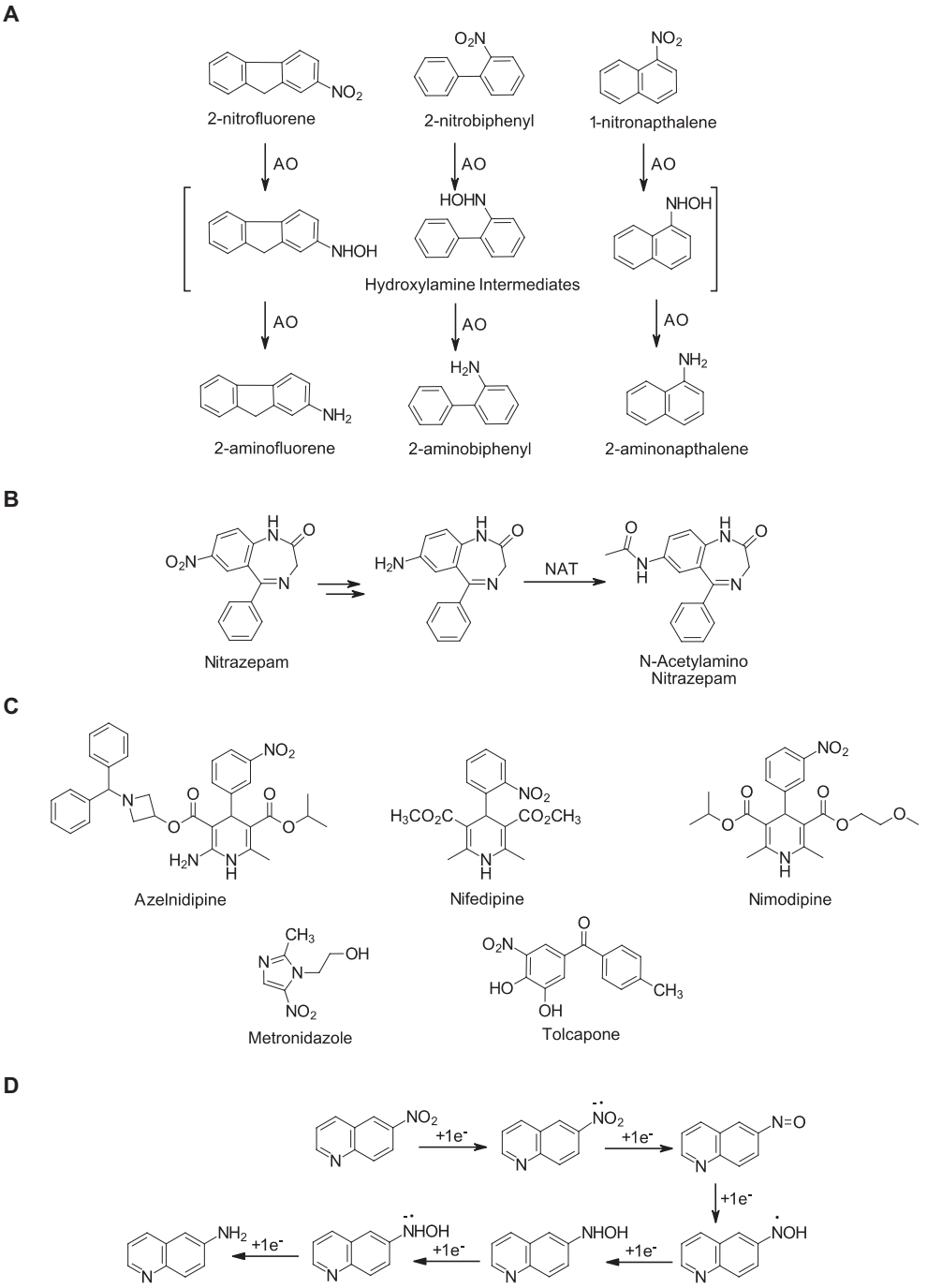


Fig. 19. Reduction of compounds aromatic and aliphatic nitro functionality by AO. A) Conversion of 2-nitrofluorene, 2-nitrobiphenyl and 1-nitronapthalene to their corresponding aromatic amines via hydroxylamine intermediates. B) Reduction of nitrazepam to amino nitrazepam by AO, which is subsequently converted to *N*-acetylaminonitrazepam. The AO-catalyzed reduction of nitrazepam to aminonitrazepam takes place under normal oxygen conditions. C) Structures of nitroaromatics that are not reduced by AO. It is speculated that nitroaromatics with electron donating groups do not undergo reduction by AO. D) Mechanism of reduction of 6-nitroquinoline by XO. The nitro groups undergo six electron reduction to the corresponding amino derivative. E) Conversion of 5-nitroquinoline to the 5-aminoquinoline by AO under aerobic conditions. 2-Oxo-5-nitroquinoline and 2-oxo-5-aminoquinoline metabolites are also detected in the reaction. 2-Oxo-5-aminoquinoline is a product of AO-mediated oxidation of aminoquinoline, which is formed via reduction of the nitro group in the molecule. F) Reduction and oxidation of imidacloprid by AO and P450, respectively. AO-catalyzed reduction of imidacloprid results in aminoimidacloprid via the nitrosoimidacloprid intermediate.

This activation of reduction, shown by an increased in V_{max} in the presence of AO oxidative substrates, is concerning specifically on determining drug-drug interactions.

It is important to note that not all nitroaromatics are substrates of AO. A recent study by Ogiso et.al. demonstrates substrate selectivity in the AO-catalyzed reduction of nitroaromatic drugs (Ogiso et al., 2018). The authors selected eleven drugs that had a nitroaromatic moiety and incubated these compounds with recombinant human AO and human liver cytosol in the presence of N^1-methylnicotinamide. While six drugs, clonazepam, flunitrazepam, flutamide, nilutamide, nimesulide and nimetazepam, were substantially reduced (Table 3), azelnidipine, nifedipine, nimodipine, metronidazole and tolcapone (Fig. 19C) were not reduced at all or slightly reduced. Assessment of the structural features showed that drugs that underwent significant turnover by AO possessed a relatively electron-deficient nitroaromatic ring. For instance, flutamide or nilutamide have a strongly electron-

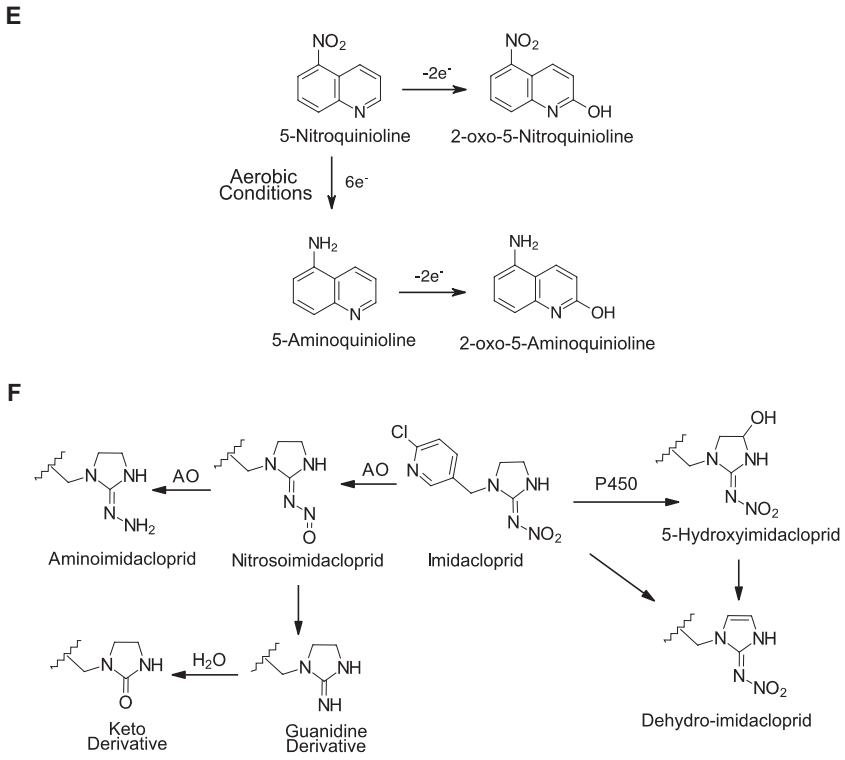
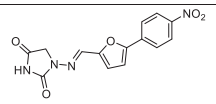
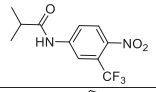
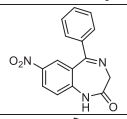
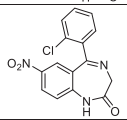
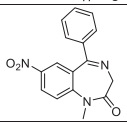
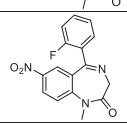
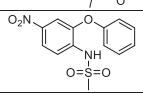


Fig. 19 (*continued*).

Table 3
Drugs containing a nitro group that are reduced by AO in the presence of N^1-methylnicotinamide (Ogiso et al., 2018).

Drugs	Structures	CL_{int} (μL/min/mg protein)
Dantrolene		3.8
Flutamide		2.4
Nitrazepam		2.3
Clonazepam		1.5
Nimetazepam		1.3
Flunitrazepam		0.5
Nilutamide		0.2

withdrawing trifluoromethyl group at the *ortho* position of the nitro group. Whereas the compounds like metronidazole or tolcapone, which possessed a relatively electron rich nitroaromatic ring were not reduced (or weakly reduced) by AO.

The mechanism of reduction of nitro groups by AO is currently unknown. Rajapakse et.al. have used xanthine/XO system to study the conversion of 6-nitroquinoline to the corresponding 6-aminoquinoline (Fig. 19D) (Rajapakse et al., 2013). Their studies suggest that XO-catalyzed reduction of 6-nitroquinoline to 6-aminoquinoline possibly proceeds via a series of 6-one-electron reduction steps with nitroso and hydroxylaminoquinoline as intermediates (Fig. 19D) and the nitro functionality acting as an electron acceptor in place of oxygen. However, this conversion only occurred under strictly hypoxic conditions (Rajapakse et al., 2013) as opposed to AO mediated reduction of nitrazepam which was shown to occur normal aerobic conditions (Konishi et al., 2017). In a recent study, Paragas et. al. used 5-nitroquinoline (Fig. 19E) as a substrate to understand aerobic reduction of AO-based reductive transformations using human liver cytosol and purified expressed human AO (Paragas et al., 2017). This study demonstrated that the nitro group can compete favorably with reduction of oxygen when AO catalyzed reductions are carried out under normal atmospheric oxygen concentrations. Consistent with the results of the Konishi study, addition of saturating concentrations of DACA or phthalazine to the incubation mixture increased the yield of the reduction product. *In vitro* incubation with AO under aerobic conditions also generated oxidative 2-quinolinone metabolites of the parent and 5-aminoquinoline as was expected (Fig. 19E). The experiments conducted by these authors produced results that revealed some interesting facts about AO in cytosolic preparations. Studies conducted with higher concentrations of human liver cytosol yielded higher concentrations of reduced product, aminoquinoline, compared to the corresponding 5-nitroquinolinone metabolite. The authors attributed this result to the presence significant concentrations of allopurinol in cytosolic

preparation. They proposed that since allopurinol is a reducing substrate of AO, the enzyme exists in its reduced form in the resting state. The hypothesis was confirmed by incubating nitroquinoline with purified AO that lacks allopurinol, which showed the formation of oxidative product in excess of the reducing product as expected. The results by Paragas et.al. also supported the presence of two kinetically distinct AO sites and suggested that the reductive transformation occurs at a second site that is in the vicinity of the flavin moiety or one of the two iron-sulfur clusters and not in the Moco active site (Paragas et al., 2017).

AO-based reduction of a series of nitroguanidine and nitromethylene class of insecticides to their corresponding amine metabolites has also been demonstrated by Dick et al. (Dick, Kanne, & Casida, 2005, 2006). These studies have suggested that AO is also capable of reducing compounds containing an aliphatic nitro group in addition to the aromatic nitro groups. The metabolism of imidacloprid to aminoimidacloprid exemplifies the role of AO in the reducing aliphatic nitro groups (Swenson & Casida, 2013). Neonicotinoids, imidacloprid and thiamethoxan, account for about 25% of the total worldwide insecticide market. The nitro substituent in the neonicotinoids is believed to be an important motif in their activity, potency and selectivity for the insect nicotinic acetylcholine receptor. Imidacloprid is oxidized to 5-hydroxy-imidacloprid, unsaturated imidacloprid by P450 and is reduced to nitrosoguanidine, aminoguanidine, iminoguanidine and imidacloprid-urea via AO (Fig. 19F). The role of AO in the reduction was established by reducing the liver AO activity by adding tungsten or hydralazine in the drinking water or by using AO-deficient DBA/2 mouse strain. These approaches did not reduce the P450 activity but reduced AO activity by 45% with tungsten and by 61% by hydralazine and 81% in AO deficient mice relative to controls.

3.2.4. Reduction of sulfoxides to sulfides

The role of AO in the reduction of sulfoxides in the presence of electron donors was demonstrated by Tatsumi and coworkers and Yoshihara and coworkers (Tatsumi, Kitamura, & Yamada, 1982; Yoshihara & Tatsumi, 1986, 1990, 1997). Sulindac (Fig. 20A) represents a good example of a drug that undergoes reductive bioactivation to the sulfide metabolite (Duggan, Hooke, Risley, Shen, & Arman, 1977). Although involvement of several enzymes in this metabolic activation has been reported, Tatsumi showed that sulindac is reduced by AO in the presence of certain small molecule substrates (like NADPH, NADH or even acetaldehyde) in liver subcellular fractions from several mammalian species under anaerobic conditions (Tatsumi et al., 1982). The ability of AO to reduce sulfoxides was exploited by Sun and coworkers in design of nitrogen mustards as hypoxia directed bioreductive cytotoxins (Sun et al., 2000). This group synthesized and evaluated a series of diaryl and alkylaryl sufoxide-containing nitrogen mustards (Fig. 20B) for their hypoxia-selective cytotoxicity against V-79 cells in vitro. Structure-activity relationship suggested that the diarylsulfoxide mustards were better substrates for AO than alkylaryl sulfoxides. The reduction was inhibited in the presence of menadione and enhanced in the presence of benzaldehyde, acetaldehyde or 2-hydroxypyrimidine suggesting the involvement of AO in the reduction of sulfoxides. Fig. 20C depicts reductive bioactivation of sulfoxide-tethered nitrogen mustards via reduction to sulfides and its subsequent covalent binding to DNA.

3.2.5. Reduction of heteroaromatic rings

Five-membered heteroaromatic rings such as isoxazoles and isothiazoles are also subject to reduction by AO. This transformation of reductive cleavage is of great importance since five-membered heteroaromatic scaffolds are considered to be excellent replacements of phenyl rings in several drugs.

Ziprasidone and zonisamide are two drugs undergoing metabolism via the reductive pathway in humans. Ziprasidone (Geodon) (Fig. 21A) is an atypical antipsychotic agent that undergoes extensive metabolism in humans. Although ziprasidone is mainly metabolized

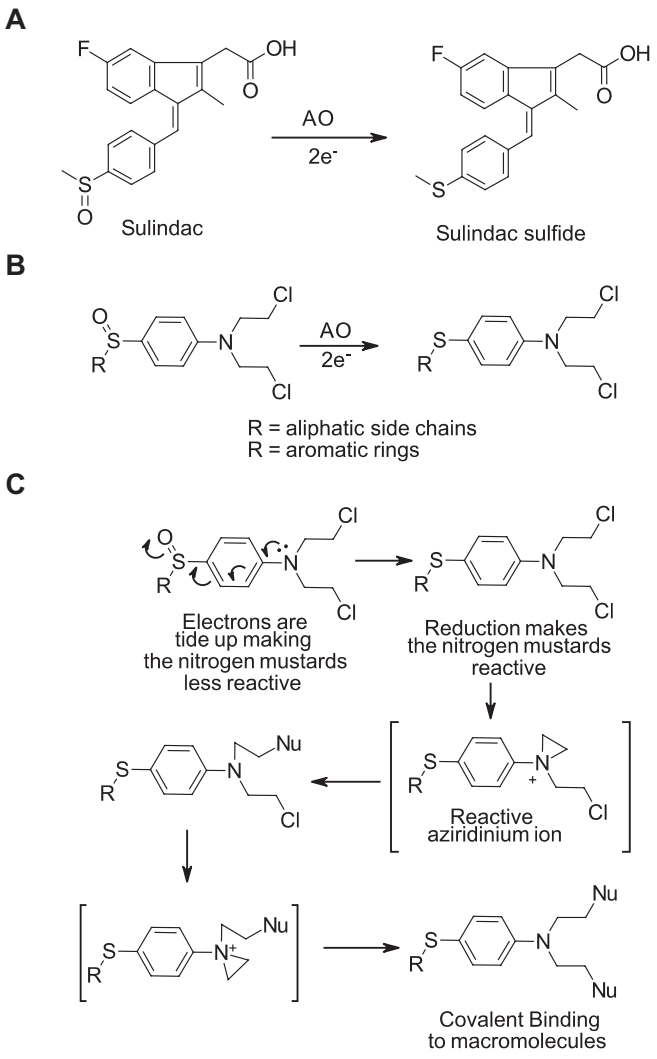


Fig. 20. Reduction of sulfoxides to sulfides. A) AO-mediated reduction of sulindac, an anti-inflammatory agent, to sulindac sulfide. B) Exploitation of sulfoxides in the design of bioreductive nitrogen mustards. C) Illustration of reductive bioactivation of sulfoxide-tethered nitrogen mustards and its covalent binding to nucleophiles.

by P450s (primarily CYP3A4), it is also an AO substrate and is converted to *S*-methyldihydroziprasidone via reductive cleavage of the benzisothiazole ring. The *S*-methyldihydroziprasidone metabolite has been identified as the major circulating metabolite in humans and is formed via 2-sequential reactions (Prakash, Kamel, & Cui, 1997; Prakash, Kamel, Gummerus, & Wilner, 1997). First step involves the AO-catalyzed ring opening of benzisothiazole to dihydroziprasidone. This is followed by methyltransferase catalyzed conversion of the thiophenol derivative to the corresponding *S*-methyldihydroziprasidone metabolite (Fig. 21A) (Beedham, Miceli, & Obach, 2003). Similarly, zonisamide, an anticonvulsant used to treat the symptoms of epilepsy and Parkinson's disease, also undergoes AO-catalyzed reductive ring opening of the 1,2-benzisoxazole ring to yield the corresponding phenolic metabolite (Fig. 21B) (Sills & Brodie, 2007; Stiff, Robicheau, & Zemaitis, 1992; Sugihara, Kitamura, & Tatsumi, 1996a). Although the initial reports suggested involvement of CYP3A4/5 in this reduction (Nakasa, Komiya, Ohmori, Rikihisa, & Kitada, 1993; Nakasa et al., 1993), in depth investigation into the reduction of the isoxazole ring by Sugihara and co-workers demonstrated that rabbit liver cytosol supplemented with an electron donor exhibited significant menadione sensitive zonisamide reductase activity (Sugihara et al., 1996a).

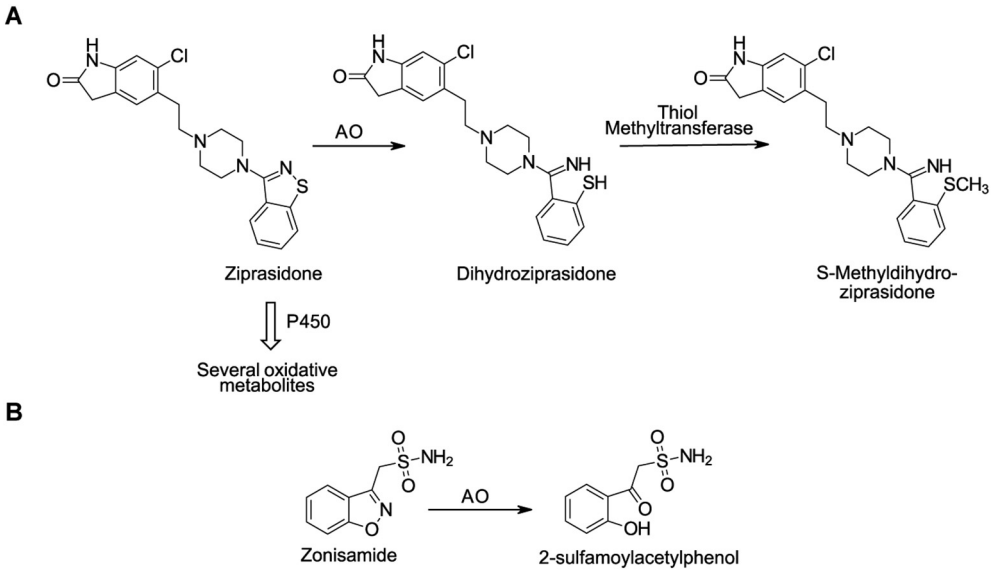


Fig. 21. Ring cleavage of 5-membered heterocyclic rings by AO. A) Reduction of ziprasidone by AO to the corresponding dihydroziprasidone metabolite, which is then methylated by thiol methyltransferase to *S*-methyldihydroziprasidone. B) Reduction of benzisoxazole ring of zonisamide by AO.

3.2.6. Reduction of hydroxamic acid to amide

AO-mediated reductive metabolism of hydroxamic acids to amides (Fig. 22A) has also been reported (Sugihara & Tatsumi, 1986). A matrix metalloproteinase 13 (MMP13) inhibitor CP-544439 is an experimental drug, which represents a good example of a drug containing hydroxamate motif that undergoes AO-mediated reduction to the amide (Dalvie et al., 2008). Metabolic profiling in matrices of rat, dog revealed that CP-544439 was mainly metabolized via glucuronidation, reduction and hydrolysis of the hydroxamate moiety in the molecule (Fig. 22B). While glucuronidation of CP-544439 was the primary pathway of metabolism in dogs, reduction to the corresponding amide was the major metabolite in rats (Dalvie et al., 2008). When plasma and urine obtained from healthy human volunteers was analyzed, the metabolic pathways were similar to those observed in the preclinical species. *In vitro* studies using pooled human liver cytosol supplemented with N^1-methylnicotinamide as an electron donor yielded the corresponding amide metabolite of CP-544439 (Obach, 2004). The reaction was inhibited by raloxifene further confirming that AO was involved in the reduction. As in the case of nitro groups (Paragas et al., 2017), the hydroxamate reduction was also observed under normal oxygen conditions suggesting the hydroxamate reduction can compete favorably with oxygen reduction even in aerobic conditions.

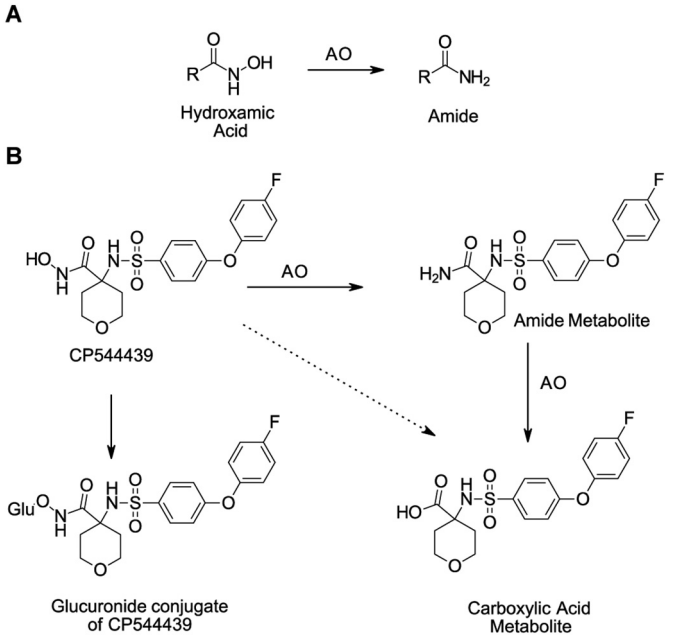


Fig. 22. A) Reduction of hydroxamic acid group by AO to the corresponding amide. B) Conversion CP-544439, a MMP13 inhibitor, to the amide metabolite by AO. CP-544439 also undergoes glucuronidation and hydrolysis forming the glucuronide conjugate and the carboxylic acid metabolite.

4. AO metabolism and toxicity

Drug metabolites have been implicated as causative agents that can manifest serious adverse effects (Baillie & Rettie, 2011). One perceived notion is that drug induced toxicities can result from bioactivation of drugs to reactive metabolites that are electrophilic in nature. These electrophilic metabolites can covalently bind to critical cellular macromolecules resulting in adducts that can either trigger cellular damage, oxidative stress or an immune response through haptenization of protein adducts. Drug-mediated organ damage (for instance kidney damage) can also occur indirectly by accumulation of metabolites and physical deposition in the tissues (Besenhofer et al., 2011; Drayer, 1977; Osborne, Joel, & Slevin, 1986; Reidenberg & Drayer, 1977). The P450 and peroxidase enzyme systems are generally considered to be the most important group of enzymes involved in the bioactivation of drugs and xenobiotics, however non-P450 enzymes can also yield metabolites that could result in severe toxicities (Gan, Ma, & Zhang, 2016; Ioannides & Lewis, 2004; Walsh & Miwa, 2011). Species differences in drug metabolism can add another layer of complexity especially when differences in toxicity are observed.

Two recent examples of AO-mediated metabolism and renal toxicity in humans exemplify the role of AO in drug related adverse effects. SGX-523 and the structurally similar JNJ-38877605 (Fig. 23A) were two orally available, nanomolar active and highly selective c-Met tyrosine kinase inhibitors that were advanced into clinical development for the treatment of solid tumors. However, acute renal failure as evidenced by increased serum creatinine was observed in all patients when SGX523 or JNJ-38877605 administered (Diamond et al., 2010; Lolkema et al., 2015). This compromised renal function was attributed to the precipitation of crystals of the oxidative metabolite(s) in the renal tubule. Investigation into the metabolism of SGX-523 and JNJ-38877605 revealed hydroxylation of the quinoline ring and formation

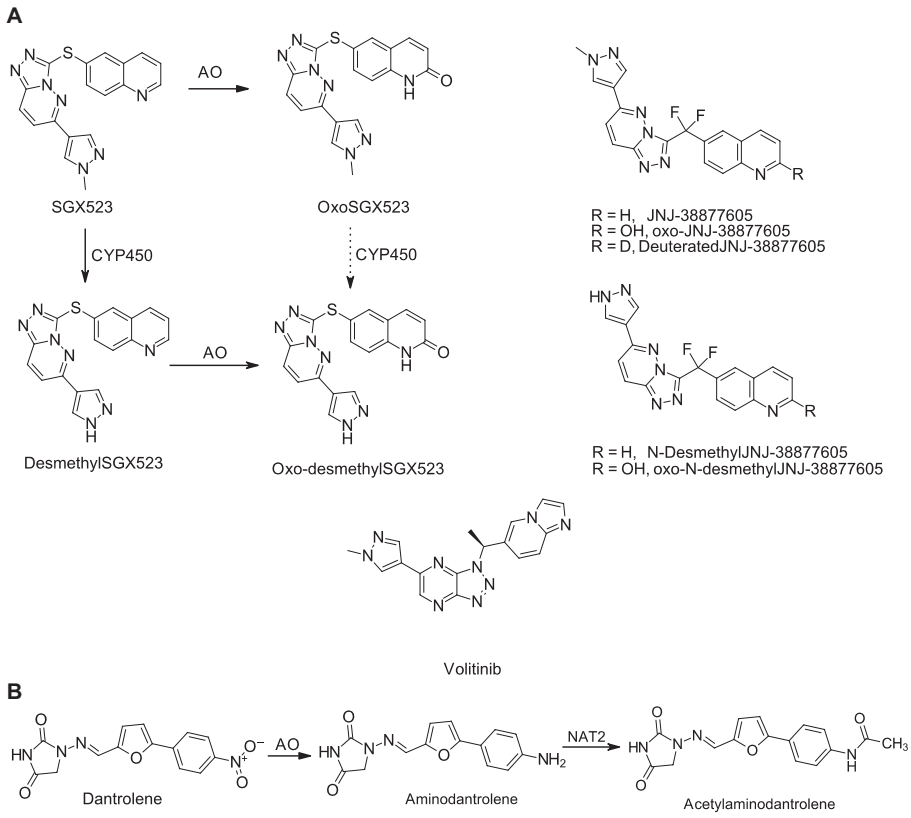


Fig. 23. Toxicity of AO metabolites. A) Conversion of SGX523, a cMET inhibitor, and its P450-catalyzed N-desmethyl metabolite to the corresponding oxo-derivatives. JNJ38877605, a structural analogue of SGX523 also undergoes a similar reaction to give the lactam metabolite. The lactam metabolites of both these compounds are insoluble and precipitate in the kidney, resulting in nephrotoxicity. Substitution of a deuterium decreases the production of hydroxyJNJ38877605. Volitinib is an analogue of SGX523 and JNJ38877605 in which the quinoline ring is substituted by an imidazopyridine ring. This prevents the oxidation of the compound by AO and avoids toxicity. This candidate is currently in clinical trials. B) Reduction of dantrolene to aminodantrolene by AO. This metabolite further converted to *N*-acetylaminodantrolene. The hepatotoxicity observed with dantrolene is believed to be a result of the hydroxylamine intermediate formed during the reduction of dantrolene to aminodantrolene.

of 2-quinolinone metabolite. Subsequent phenotyping studies suggested that the 2-quinolinone formation was catalyzed by AO. In both cases, the metabolism was species dependent. *In vitro* studies with SGX-523 showed that the late eluting 2-quinolinone metabolite was formed in major amounts in the monkey and human S9 fraction and to a lesser extent in rats S9 fraction, whereas this oxidative metabolite was totally absent in dog S9 incubations. This *in vitro* data corroborated well with the results from *in vivo* studies. Metabolic profiling of urine and plasma following an oral dose of SGX-523 to monkeys showed the presence of 2-quinolinone metabolite of SGX-523 and its *N*-desmethyl metabolite in significant amounts (~70-fold greater than the parent) and showed obstructive neuropathy with intratubular crystal formation which was consistent with the human data. Similar interspecies differences were also observed when metabolism of JNJ-38877605 was assessed in rat, dog and rabbits. Comparative mass balance and metabolism studies in these species showed that like the humans, the 2-quinolinone metabolite of JNJ-38877605 and its *N*-desmethyl-JNJ-38877605 were the major constituents in the plasma and urine of rabbits. Additional toxicology studies in rabbits and detection of quinolinone metabolites as the two main components in the renal crystals led the authors to infer that these two metabolites play a critical role in the explanation of the renal findings. Crystallization of the metabolites was attributed to low solubility of quinolinones. Like hydroxymethotrexate, in both cases, the quinolinone metabolites of SGX523 and JNJ38877605 displayed poor solubility than the parent compound under acid, neutral and basic conditions with solubility values under 2 μg/mL. As discussed earlier, oxidation of the quinoline moiety in SGX523 and JNJ38877605 altered the pKa of this heteroaromatic ring and therefore its potential to ionize. In the case of SGX523 and JNJ38877605, the pKa of the nitrogen atom in the quinoline ring was changed from 3.6 to 11.0 when it was converted to a corresponding quinolinone thus making it neutral at physiological pH (7.4).

Several approaches that block AO metabolism of cMet inhibitors and reduce the formation of quinolinone metabolites have been reported recently. For example, replacement of the quinoline scaffold with an imidazo[1,2-a]pyridine maintained pharmacological activity but avoided AO metabolism leading to the discovery of volitinib (Fig. 23A), which is in phase III clinical trials (Gavine et al., 2015; Jia et al., 2014). Also, metabolism studies with deuterated-JNJ38877605 (Fig. 23A) have been reported in which the hydrogen atom at AO metabolic site of JNJ38877605 is replaced with a deuterium atom. This substitution has shown to significantly reduce AO oxidation and improve oral exposure as well as *in vivo* antitumor activity (Zhan, Peng, Sun, Ai, & Duan, 2018).

Dantrolene constitutes another example where a metabolite generated by AO-catalyzed reduction of the parent drug has been implicated as a causative agent in the liver toxicity of this drug (Amano et al., 2018). Dantrolene is a postsynaptic muscle relaxant used in the treatment and prevention of malignant hyperthermia, a rare and life-threatening disorder triggered by general anesthesia. The primary metabolite of this drug is acetylaminodantrolene, which is formed via reduction of the parent drug to aminodantrolene that subsequently undergoes *N*-acetyltransferase (NAT2) catalyzed acetylation to the corresponding acetylaminodantrolene (Fig. 23B). Phenotyping studies revealed that dantrolene reductase activity mainly resided in the liver cytosolic fraction and that formation of aminodantrolene was increased in the presence of an electron donor, N^1-methylnicotinamide. Inhibitors of AO inhibited the reduction of the drug suggesting that this reaction is primarily mediated by AO. Potent inhibitors of AO and a correlation study with phthalazine oxidase activity in a panel of 28 human liver

Table 4
Estrogenic compounds as inhibitors of AO (Obach, 2004; Obach et al., 2004).

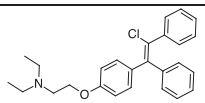
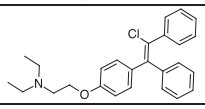
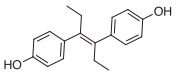
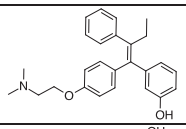
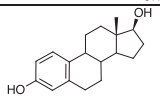
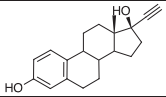
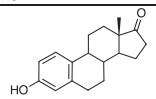
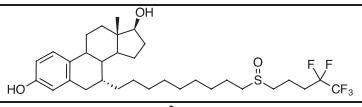
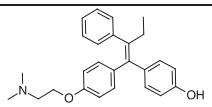
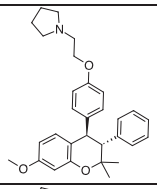
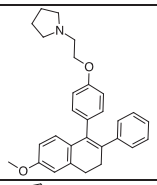
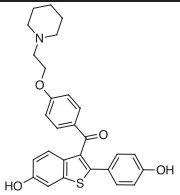
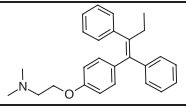
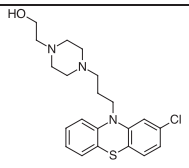
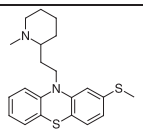
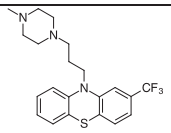
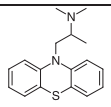
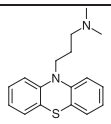
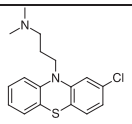
Drugs	Structure	IC_{50} (μM)
Clomifene		2.5
Coumestrol		0.48
Diethylstilbestrol		0.46
Droloxifene		0.79
ß-Estradiol		0.29
Ethinyl estradiol		0.43
Estrone		0.57
Fulvestrant		0.66
4-Hydroxytamoxifen		0.45
Levormeloxifene		1.0
Nafoxidine		0.5
Raloxifene		0.0029 - 0.0057
Tamoxifen		2.2

Table 5
Phenothiazine related class of drugs that are inhibitors of AO (Obach et al., 2004).

Drugs	Structure	IC_{50} (μM)
Perphenazine		0.033
Thioridazine		0.16
Trifluperazine		0.24
Promethazine		0.51
Promazine		1.6
Chlorpromazine		0.57

cytosol samples supported the role of AO in dantrolene reduction. A glutathione adduct formed in the glutathione trapping assay revealed that the hydroxylamine intermediate formed via AO-dependent reduction of dantrolene may be associated with liver injury.

Genotoxic (clastogenic) effect of imidacloprid has also been attributed to AO-mediated reduction of the insecticide in New Zealand rabbits (Vardavas et al., 2018). As described previously, imidacloprid undergoes reduction the compound to the corresponding amine metabolite by AO (Fig. 19F). Evaluation of DNA damage in the micronucleus and single cell electrophoresis assays following administration of imidacloprid to New Zealand rabbits co-exposed with an AO inhibitor, sodium tungstate dihydrate, showed reduction in the frequency of binucleated cells compared to the control animals that were not exposed to sodium tungstate. Based on these results, the authors inferred that AO-mediated reductive metabolic pathway plays a more important role in the systemic toxicity of imidacloprid.

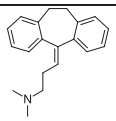
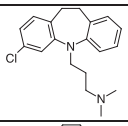
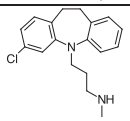
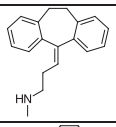
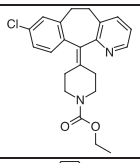
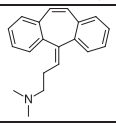
5. Inhibition of AO by xenobiotics

5.1. Reversible inhibitors

Like the P450s, AO is also subject to inhibition by xenobiotics in a reversible and irreversible manner. Reversible inhibitors of AO span a wide range of chemical space with various sizes and functional groups covering many classes of drugs. Earlier work has led to the identification of some agents like menadione, and the endogenous hormone like β-estradiol as inhibitors of AO (Johns, 1967; Johnson, Stubley-Beedham, & Stell, 1985; Rajagopalan, Fridovich, & Handler, 1962). In 2004, Obach et.al. investigated more than 200 drugs and other xenobiotics for their potential to inhibit phthalazine oxidation by hAO in cytosolic fractions (Obach, Huynh, Allen, & Beedham, 2004). The results of these studies revealed that thirty six compounds of certain drug classes were potent AO inhibitors. These included the estrogenic compounds (Table 4), phenothiazines (Table 5) tricyclic antidepressants (Table 6), tricyclic atypical antipsychotic agents, and dihydropyridine calcium channel blockers (Table 7) (Obach et al., 2004). Some compounds (Table 7) that could not be classified into a particular class, also inhibited AO.

Raloxifene of the estrogen compound class is by far the most potent inhibitor and currently remains the most potent inhibitor known with an IC_{50} of 2.9-5.7 nM (Obach, 2004). As a result, it is commonly used as an *in vitro* tool to selectively inhibit AO activity in cytosol or S9 fractions. Reports suggests that AO inhibition by raloxifene is atypical and that the process often occurs via either a competitive, mixed (inhibitor binds to both the free enzyme and the enzyme-substrate complex) or uncompetitive mode (Barr & Jones, 2013; Barr, Jones, Oberlies, & Paine, 2015; Wang, Abdul-Hadi, Cohen, & Xia, 2013). Mechanistic evaluation of inhibition of phthalazine, vanillin, and nicotine-Δ1′(5′)-iminium ion oxidase activities by raloxifene indicated that it is an uncompetitive inhibitor (Obach, 2004). In contrast, studies with *N*-[2-(dimethylamino)ethyl]acridone-4-carboxamide (DACA, Fig. 24A) as a substrate found raloxifene to be a competitive inhibitor of AO (Barr &

Table 6
Tricyclic antidepressant class of drugs that are inhibitors of AO (Obach et al., 2004).

Drugs	Structure	IC_{50}
Amitriptyline		0.26
Clomipramine		0.48
Norclomipramine		0.6
Nortriptyline		0.85
Loratidine		0.49
Cyclobenzapine		3.1

Jones, 2013). These results indicate that raloxifene inhibition of AO is substrate dependent. Studies by Obach have also demonstrated that raloxifene is a potent inhibitor of AO reductase activity. However, inhibition of the reductive process is 50-fold less potent than inhibition of the oxidation reaction (Obach, 2004). Mechanistic studies also suggest that raloxifene inhibits reduction of the hydroxamate CP-544439 (Fig. 22B) in a non-competitive manner. Since the reduction process requires saturating concentrations of the reducing substrate (N^1-methylnicotinamide), the authors have speculated that the inhibition results from binding of raloxifene to the reduced form of the enzyme.

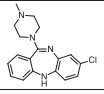
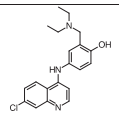
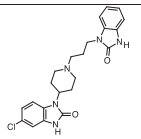
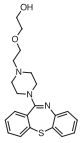
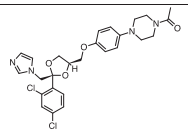
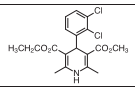
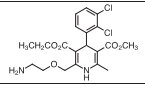
Obach has investigated the inhibitory potency of several drugs belonging to selective estrogen receptor modulators category (Table 4) (Obach, 2004). Although all these drugs inhibited AO, none of these compounds were as potent as raloxifene. A closer look into the structure-inhibitor relationship suggests that the phenolic groups in raloxifene are important for inhibitory activity. Likewise, the 3-aroyl-*p*-ethoxypiperidinyl substituent is also important for potency. Molecular docking studies using mAO3 by Coehlo et. al. with raloxifene as an inhibitor suggests two different binding modes for this molecule. In one binding mode, one of the phenol moieties enters the pocket and interacts via the hydrogen bond with Glu1266 and aromatic stacking interaction with Phe919. In the other mode, the piperidinyl group replaces the phenol and the molecule is held together by van der Waals interactions between the piperidinyl group of the inhibitor and Phe919 in the pocket (Coelho et al., 2012).

Phenothiazine related class of antipsychotic drugs also inhibit AO (Table 5). Obach et.al. have shown that several phenothiazine drugs inhibit AO with an IC_{50} ranging from 33 nM to 1.6 μM. Perphenazine was identified as the most potent inhibitor among all the phenothiazine containing compounds tested, and displayed an IC_{50} of 33 nM (Obach et al., 2004). The crystal structure of hAO complexed with phthalazine and thioridazine has revealed the molecular details of the binding site for phenothiazine class of compounds (Coelho et al., 2015). The complex has shown that thioridazine binds near the interface in a groove at the enzyme surface which is 34 Å away from the active site. Steady state kinetic studies with hAO and mAO3 suggested that thioridazine is a noncompetitive inhibitor of both enzymes when phthalazine is used as a substrate. Comparison of K_i values suggests that thioridazine is a 16-fold more potent inhibitor of hAO than mAO3 and this is perhaps attributed to structural differences in the two proteins (Coelho et al., 2015). The binding site in hAO contains a proline residue (Pro576) that confers extra flexibility to the loop in the binding pocket and allows inhibitor access. On the other hand, the Leu residue observed in that location, in mAO3, probably alters the binding affinity. The authors have proposed that this is a phenothiazine binding site and that possibly all inhibitors belonging to the phenothiazine class will bind to this site and inhibit the enzyme in a non-competitive manner.

Using eight AO inhibitors belonging to different classes and phthalazine and DACA as a probe substrate, Barr and coworkers gained further insight into the substrate dependent inhibition by AO. In their studies seven of the eight inhibitors showed a mixed-type inhibition profile and inhibited AO in a competitive and uncompetitive manner. When the substrate was changed from phthalazine to DACA, the inhibitors became less uncompetitive in nature, especially for domperidone, clozapine and chlorpromazine (Barr & Jones, 2013). While the binding of these molecules has not been studied, the authors proposed that these inhibitors can bind to one or more of the domains and prevent or slow down enzyme turnover. More importantly, these results led the authors to conclude that the inhibition profile is affected by the selection of the substrate used to probe the kinetics (Barr & Jones, 2013).

A wide range of natural components including polyphenols and flavonoids are known to interfere with AO-catalyzed reactions. The effects

Table 7
AO inhibitors belonging to class of atypical antipsychotic agents and calcium channel blockers as well as other drugs that inhibit AO (Obach et al., 2004).

Drugs	Structure	IC_{50} (μM)	Drugs	Structure	IC_{50} (μM)
Atypical Antipsychotic Agents			Other Drugs Inhibiting AO		
Clozapine		4.4	Menadione		0.2
Loxapine		2.3	Amodiaquine		0.74
Olanzapine		6.0	Domperidone		3.0
Quetiapine		1.4	Ketoconazole		3.5
Calcium Channel Blockers					
Felodipine		0.30			
Amlodipine		5.5			

of 12 flavonoids from three subclasses of flavon-3-ol, flavan-3-ol and flavanone on the oxidation of vanillin and phenanthridine by AO were studied by Pirouzpanah et. al. and Hamzeh-Mivehroud et. al. Among the flavonoids studied by the two groups, quercetin, myricetin and genistein (Fig. 24B) were the most potent inhibitors of AO, whereas naringin displayed negligible inhibition. In both studies, the inhibition of vanillin oxidation by flavonoids was stronger than that of phenanthridine oxidation suggesting substrate-dependent inhibitory effects (Hamzeh-Mivehroud, Rahmani, Feizi, Dastmalchi, & Rashidi, 2014; Hamzeh-Mivehroud, Rahmani, Rashidi, Hosseinpour Feizi, & Dastmalchi, 2013). Analysis of the structural features indicate that planar flavonoids were more potent inhibitors and any substitution by sugar moieties reduced the inhibitory effects (Pirouzpanah, Hanaee, Razavieh, & Rashidi, 2009). Also, the size of the flavonoids was an important factor and inversely affected their potencies. Barr and coworkers have also investigated the inhibitory properties of a panel of chemically diverse diet-derived constituents towards AO using O^6-benzylguanine (Barr et al., 2015). These studies have demonstrated that polyphenols like epicatechin gallate and epigallocatechin gallate (Fig. 24B) inhibited AO with sub-micromolar potency. Similarly, Siah and co-workers have investigated the inhibitory effects of selected phenolic compounds from three subclasses of aurone, flavanone and phenolic lactone compounds on the activity of AO (Siah et al., 2016). Among the phenolic compounds tested, ellagic acid (Fig. 24B) was the most potent agent with higher inhibitory action than menadione.

5.2. Irreversible inhibitors

A few time-dependent inhibitors (TDI) and/or mechanism-based inactivators (MBI) of AO have been reported. These are hydralazine (Fig. 24C) (Critchley et al., 1994; Johnson et al., 1985), quinazoline (Fig. 24C) (McCormack, Allen, & Hodnett, 1978), nitroso-imidacloprid (Fig. 19F) (Dick, Kanne, & Casida, 2007) and cyanide (Wahl & Rajagopalan, 1982). Among the different time-dependent inhibitors noted above, hydralazine is the most studied *in vitro* and *in vivo*. Johnson et al were the first ones to investigate the selective influence of hydralazine on AO but not XO. Potent inhibition of rabbit liver AO by hydralazine was observed *in vitro* but no effect was seen with bXO suggesting that hydralazine is extremely selective towards AO. The authors reproduced this result in *in vivo* studies when levels of AO were significantly decreased following administration of hydralazine in rabbits at a dose of 10 mg/kg/day for seven days. However, the hepatic XO activity was unaltered by hydralazine treatment (Johnson et al., 1985). Recent *in vivo* studies by Takaoka et. al. in h-chimeric mice have also shown that pretreatment with hydralazine increases the exposure of O^6-benzylguanine after multiple administration compared to single administration suggesting that hydralazine inhibits via mechanism based inactivation of AO (Takaoka et al., 2018). Investigations by Strelevitz et al and Hutzler et al have demonstrated that hydralazine possesses suitable properties as a selective inhibitor of AO in human hepatocytes (Hutzler et al., 2012; Strelevitz, Orozco, & Obach, 2012). Using zaleplon oxidase and deethylase activities as simultaneous probes for AO and P450 activities, respectively, Strelevitz and coworkers showed that hydralazine demonstrated the necessary selectivity for AO at 25 μM. However, recent studies by Yang et.al. have demonstrated that hydralazine is found to inhibit CYP1A2, 2B6, 2D6, and 3A4 in human suspension hepatocytes under reaction phenotyping assay conditions, at concentrations that chemically knocked out most of the AO activities (≥50 μM) (X. Yang, Johnson, & Di, 2018).

Irreversible inhibition of AO by iminium ion intermediate generated as a result of metabolism of KW-2449 has also been reported by Hosogi et. al (Hosogi et al., 2018). KW-2449 (Fig. 24D), was being developed as

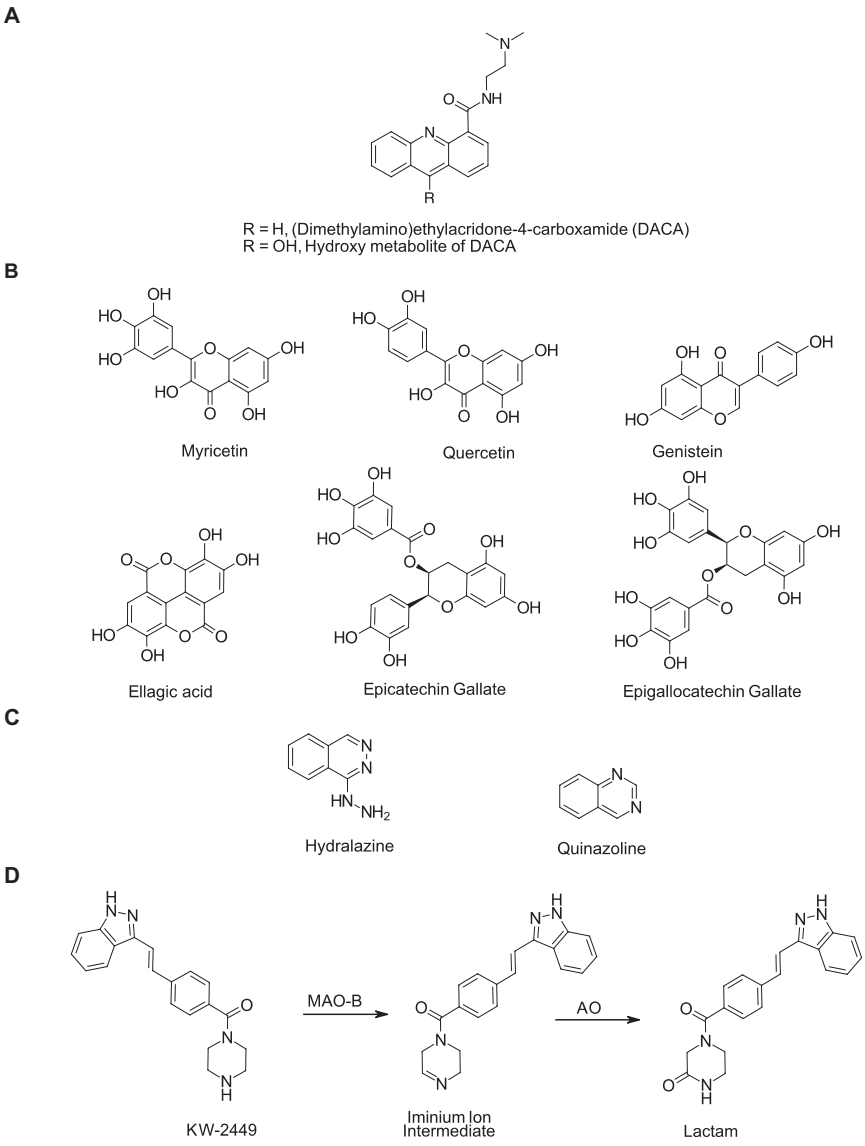


Fig. 24. A) Structure of (dimethylamino)ethylacridone-4-carboxamide (DACA) and its hydroxylated metabolite. HydroxyDACA is generated by AO mediated oxidation of DACA. B) Structures of flavonoids and polyphenols that inhibit AO. C) Irreversible inhibitors of AO. Structure of hydralazine and quinazoline. Hydralazine is an irreversible inhibitor of AO. Mechanism of irreversible inhibition by hydralazine is unknown. D) Conversion of KW-2449, a novel multikinase inhibitor, to the lactam metabolite by MAO-B and AO. The iminium ion intermediate formed by MAO-B catalyzed oxidation is an irreversible inhibitor of AO.

a novel multi-kinase inhibitor for treatment of leukemia patients (Hosogi et al., 2017). *In vivo* and *in vivo* metabolism studies indicated that the compound was primarily metabolized to the pharmacologically active lactam metabolite (Fig. 24D), first via oxidation of the piperazine ring by MAO B followed by oxidation of the corresponding iminium ion intermediate by AO. Interestingly, repeated administration of KW-2449 significantly decreased the lactam metabolite levels and therefore hampered the effectiveness of KW-2449 in primates. Subsequent investigation into the underlying mechanism of this observation led to the findings that the AO activity was inhibited in a time-dependent manner when KW-2449 was co-incubated with MAO B and led to the conclusion that the iminium ion intermediate was an irreversible AO inhibitor. Studies with ^{14}C-KW-2449 also showed that the reactive iminium ion also formed covalent adducts with proteins *in vivo* and *in vitro* suggesting that the intermediate was not only an irreversible AO inhibitor but also had the potential to cause idiosyncratic adverse drug reactions (Hosogi et al., 2018).

In vitro methods have been developed to screen AO inhibitors (Obach et al., 2004). In a sensitive, moderate throughput AO inhibition assay, phthalazine is primarily used as a substrate and the AO-catalyzed oxidation product (1-phthalazinone) formation is monitored in pooled human liver cytosol by LC-MS with and without inhibitors. Another reported method uses O^6-benzylguanine as an AO probe substrate and monitors the formation of 8-oxo-benzylguanine in human liver cytosol with LC-MS in the presence and absence of inhibitors (Barr et al., 2015).

5.3. AO inhibitors and clinical drug-drug interactions

Even though highly potent AO inhibitors have been identified *in vitro*, their relevance in terms of clinical DDI has yet to be established. To date, only one clinically relevant pharmacokinetic interaction due to inhibition of AO has been reported (Lake et al., 2002; Renwick et al., 2002). Concomitant administration of zaleplon (10 mg) and cimetidine (800 mg) produces an 85% increase in the mean C_{max} and AUC of zaleplon. As noted earlier, even though zaleplon is metabolized by CYP3A4 to *N*-desethylzaleplon, AO plays a major role in its metabolism. Strelevitz et. al. have shown that the contribution of AO in the

metabolism of zaleplon is ~60% or greater (Strelevitz et al., 2012). *In vitro* studies by Renwick et al demonstrated that even though cimetidine inhibited both CYP3A4 and AO, it was more potent in inhibiting AO (Renwick et al., 2002). These results led the authors to conclude that cimetidine is likely to have a marked effect on AO-catalyzed 5-oxozaleplon formation and warranted inclusion of a moderate cimetidine related drug-drug interaction in the zaleplon label (https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/020859s011lbl.pdf).

Combination of *in vitro* inhibitory potency, dose and long term treatment would suggest that drugs like raloxifene or thioridazine can act as potential precipitants of DDI in the clinic. However, given the uncompetitive mode of inhibition by these drugs, the risk of interaction with AO substrates can be low (Barr & Jones, 2013; Obach, 2004). The extent of drug interactions observed by an uncompetitive inhibitor are more magnified when the concentration of the substrate is greater than its K_M value. Since the concentrations of a drug at efficacious doses are typically low (lower than the K_M), the possibility of a DDI for uncompetitive inhibitors is automatically mitigated. However, substrate dependent inhibition profiles can have a large impact on *in vitro* DDI predictions. As mentioned earlier, the mode of raloxifene inhibition is substrate dependent. Although raloxifene is not considered a high risk for DDI because of its uncompetitive nature of inhibition, it inhibits the metabolism competitively when probed with DACA (Barr & Jones, 2013). DDI prediction of competitive inhibitors can be made by the ratio of concentration of the inhibitor [I], and inhibitor constant K_i ($[I]/K_i$) (Barr & Jones, 2013). An $[I]/K_i$ greater than or equal to one is generally indicative of a high risk of DDI. Raloxifene concentrations in the liver during first-pass, after a standard dose of 60 mg are likely to exceed its K_i (Obach, 2004). Also, literature sources indicate an *in vivo* C_{max} of 3 to 6 nM for raloxifene following a chronic dosing regimen (Hochner-Celnikier, 1999). Using these estimates, Barr and coworkers have shown that raloxifene falls within a high-risk range for DDI when probed DACA, whereas it has no risk when probed with phthalazine. These examples shows that the selection of substrate can have a substantial impact on the prediction of DDI (Barr & Jones, 2013).

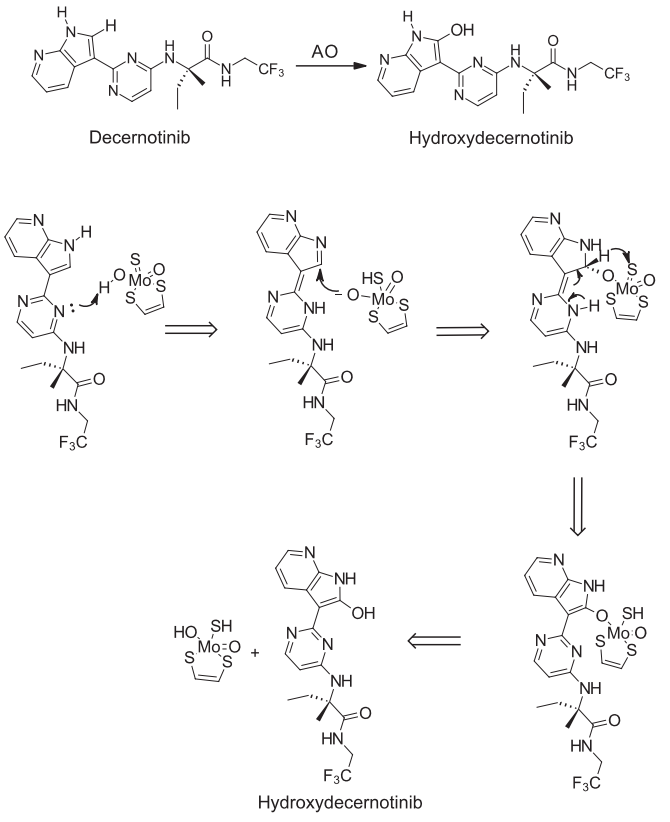


Fig. 25. A) Conversion of decernotinib, JAK inhibitor, to hydroxydecernotinib by AO. Hydroxydecernotinib is a mechanism based inactivator of CYP3A4. B) Putative mechanism for conversion of decernotinib to hydroxydecernotinib by AO.

5.4. Inhibition of P450 enzymes by AO metabolites

Metabolites formed by non-P450 enzymes have been shown to inhibit P450s and subsequently cause DDIs. There are a couple of instances where an AO-mediated metabolite has inhibited P450s and has resulted in an increase in exposure of the victim drug. For instance, GS-563117 (Fig. 7C), an AO-catalyzed metabolite of phosphoinositide 3-kinase ∂ inhibitor idelalisib, is a time dependent inhibitor of CYP3A4 ($K_I = 0.2$ μM; k_{inact} 0.033 min^{-1}) (Jin et al., 2015; Ramanathan et al., 2016). Consequently, administration of 150 mg idelalisib twice daily increased the midazolam exposure (138% and 437% for C_{max}, and $AUC_{(0\text{-}inf)}$) (Jin, Robeson, Zhou, Hisoire, & Ramanathan, 2015). A significant drug interaction of idelalisib with diazepam has also been reported recently. Co-administration of idelalisib and diazepam, a 3A4 substrate, to patients has resulted in altered mental status and type II respiratory failure resulting in hospitalization (Bossaer & Chakraborty, 2017).

Inhibition of CYP3A4 by decernotinib (an oral Janus kinase 3 inhibitor) (Fig. 25) is the second example that highlights the role of AO in the formation of hydroxylated metabolite of decernotinib, which is involved in clinically significant DDI (Zetterberg et al., 2016). Initial study that evaluated competitive inhibition of decernotinib using human liver microsomes (HLM) indicated a low risk for CYP3A4-mediated DDI with this candidate. Decernotinib was not only found to be a weak competitive inhibitor of CYP3A4 ($IC_{50} = 10$–28 μM) but there was no discernible inactivation of CYP3A4 with exposure to the compound overtime. However, coadministration of decernotinib increased the AUC of midazolam, atorvastatin and methylprednisolone by approximately 12.0-, 2.7-, and 4.3-fold, respectively, in the clinic. In contrast to the parent molecule, the hydroxylated metabolite (hydroxydecernotinib), which was formed by AO-catalyzed oxidation, showed a strong TDI response in HLM with a $k_{obs} = 0.047$ min^{-1}. Hydroxydecernotinib also inhibited recombinant CYP3A4 with IC_{50}s of 0.5 and 2.4 μM when testosterone and midazolam were used as substrates.

Decernotinib also represents an interesting example where the oxidative addition by AO occurs on the sp^2 carbon adjacent to nitrogen of the azaindole instead of the carbon atom in the pyrimidine ring in the molecule. One possible mechanism for this unique AO oxidation may involve an initial protonation of the pyrimidine nitrogen followed by a nucleophilic attack by hydroxyl group in the Moco center on the resulting imine intermediate (Fig. 25).

6. Regulation and induction of AO

Studies on regulation of AO are rather limited however, some reports have suggested the role of endogenous factors and steroid hormones in regulating *AOX* gene (Huff & Chaykin, 1967). Androgens have been shown to increase mouse AO mRNA and protein expression, while estrogens have an opposite effect (Garattini & Terao, 2012; Gluecksohn-Waelsch, Greengard, Quinn, & Teicher, 1967). Reports by Kurosaki et. al. and Yoshihara and Tatsumi have suggested that AO activity is primarily regulated by testosterone and growth hormone. Treatment of female mice with testosterone increases the amounts of AO mRNA and the relative translation product to levels similar to those in male animals (Kurosaki, Demontis, Barzago, Garattini, & Terao, 1999; Yoshihara & Tatsumi, 1997). Some studies have shown that the human *AOX* gene is regulated by the NrF2 pathway (Maeda et al., 2012). Molecular analyses using reporter transfection analysis, electrophoretic mobility shift assay for detection of proteins, and chromatin immunoprecipitation analysis show that Nrf2 binds to and activates *AOX* gene in the rat.

Xenobiotics have also been known to induce AO via modulation of genetic factors that control their expression in humans and preclinical

species. Johnson et al have demonstrated a concomitant rise in the concentration of Mo and activity of AO following administration of phthalazine to rabbits for seven days (Johnson, Stubley-Beedham, & Stell, 1984). However, the authors found that the induced form of AO differed from the control enzyme. This form had higher affinity towards phthalazine than the control enzyme suggesting that the increased AO activity was a consequence of combined effects on both K_M and V_{max} values in contrast to typical chemical induction where the rise in specific activity resulted from elevated V_{max} values due to higher enzyme levels. Since phthalazine is extensively metabolized in rabbits the authors also speculated that the observed increased in AO activity was attributed to the oxidative metabolite of phthalazine rather than the parent itself (Johnson et al., 1984). Rivera et. al. have shown that the AO mRNA is induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in the mouse hepatoma cell line (Hepa-1) which has functional aryl hydrocarbon (AhR) receptor and aryl hydrocarbon receptor translocator (ARNT) proteins. Induction of *Aox* gene was also observed in mouse liver after administration of dioxin to mice (Rivera et al., 2005). The increase in AO activity was assessed by employing an AO substrate and was confirmed using tungstate, known inhibitor of AO. Sugihara et al have also shown AhR-mediated induction of XO/xanthine dehydrogenase activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin (Sugihara et al., 2001). Increased exposure to alkylating agents like *N*-methyl-*N*-nitrosoguanidine to rats has also been shown to cause AO induction and increase N^1-methylnicotinamide oxidase activity (Ohkubo, Sakiyama, & Fujimura, 1983).

7. Tissue distribution

Garattini and others have reported on the tissue and cell-specific distribution and function of mammalian AO (Beedham, 1985; Beedham, Bruce, & Rance, 1987; Garattini et al., 2008; Garattini, Fratelli, & Terao, 2009; Garattini & Terao, 2011; Krenitsky, Tuttle, Cattau Jr., & Wang, 1974). Briefly, AO is widely distributed in the body. Like the P450s, highest levels of AO have been observed in the liver of humans and most preclinical species (Beedham, Bruce, & Rance, 1987). Gene expression and immunohistochemistry studies also show a relatively large extrahepatic presence of AO in the preclinical species and humans (Moriwaki, Yamamoto, Takahashi, Tsutsumi, & Hada, 2001; Nishimura & Naito, 2006). For instance, Moriwaki et. al. showed the presence of AO in respiratory, digestive, urogenital, and endocrine tissues (Moriwaki et al., 2001). An immunoreactive AO band was also detectable in the eye, thymus, testis, duodenum, heart and esophagus in their studies. However, some tissues like the striated muscle and pancreas lacked AO protein. Using an anti-AO polyclonal antibody, Calzi et.al. also showed a significant amount of AO in the lung and spleen (Calzi et al., 1995). In humans, AO was detected in the brain, adrenal glands, the prostrate and the epididymis with very low expression in the peripheral leukocytes. Recent studies have also shown the AO expression (mRNA and protein expression) in human skin (T. Hu et al., 2010; Manevski et al., 2014; van Eijl et al., 2012). Despite the high levels, the AO activity in the liver of humans is much lower compared to that in rat, rabbit or guinea pig livers (Moriwaki et al., 2001). AO distribution in other tissues is also highly species dependent. For example, in mouse, AO activity is highest in lung tissue while in rats it is greatest in the liver (Beedham, Bruce, Critchley, et al., 1987; Kitamura, Nakatani, et al., 1999; Shimada, Mishima, Nikaido, Kitamura, & Tatsumi, 1989).

8. Species differences

Although AO is widely expressed in all species, marked differences in the protein expression and AO activity have been observed between species (Beedham, Bruce, Critchley, et al., 1987; Garattini & Terao, 2012). The number of genes coding for distinct AO isoenzymes in different species has been discussed in great detail by Garattini et. al. (Garattini et al., 2008, 2009; Garattini et al., 2003; Kurosaki et al., 2013; Mota et al., 2018; Terao et al., 2016). In humans and non-human primates, a single active gene (*AOX*) codes for a single active form of AO, while the rats, mice and even rabbits contain a cluster of four AO genes, *Aox1, Aox3, Aox4* and *Aox3l1* that express an equal number of catalytically active forms of the enzyme (Garattini et al., 2009; Kurosaki et al., 2013; Terao et al., 2016). The products of the four mouse *Aox* genes (mAO1, mAO3, mAO4 and mAO3l1) are expressed in a tissue- and organ-specific fashion (Garattini et al., 2008). The mAO1 and mAO3 are mainly synthesized in the liver (Terao et al., 2001), whereas the richest source of mAO4 is the Harderian gland which is a major structure located in the orbital cavity (Terao et al., 2009; Terao et al., 2000). Finally, mAO3l1 protein expression is restricted to the Bowman's gland of the nasal mucosa (Kurosaki et al., 2004). In mice and rats, the main drug metabolizing enzyme is AO3 as this is the prevalent form of AO present in liver. Canines are devoid of AO activity despite the presence of two functional genes (orthologs of rodent *Aox4* and *Aox3l1*) and two pseudogenes (the orthologs of mouse *Aox1* and *Aox3*) (Garattini et al., 2009; Terao et al., 2006). Human AO protein (hAO) shows the highest amino-acid identity (85%) to that of rodent AO protein and only 60% similarity with the other three AO isoforms (mAO3, mAO3l1 and mAO4). Some reports have proposed that AO expressed in guinea pigs is an ortholog of hAO (Garattini & Terao, 2013). Recent studies have shown that marmosets are a promising primate model for preclinical drug metabolism because of the similarities of drug metabolism enzymes in this species and humans (Uehara et al., 2017). Studies of *AOX* gene in this species and deduction of the amino acid sequence of the expressed protein revealed high sequence identities (92-93%) with AO protein in other primates but not with the protein in pig, rabbit, rat and mouse (Uehara et al., 2017). Tissues expression analyses by real-time reverse-transcription polymerase chain reaction and immunoblotting also show that marmoset AO mRNA and protein were abundantly expressed in liver like the cynomolgus monkey and humans.

As noted earlier, the overall structure and the catalytic mechanism of AOs in all preclinical species and humans is similar. Despite this similarity, the activity to a single substrate has been shown to vary among the species. Reports suggests that this is perhaps attributed to the amino acid variation in the Moco active site and in substrate funnel, and the differences in the size and shape of the active site (Beedham, Critchley, & Rance, 1995; Garattini et al., 2008; Terao et al., 2016). The sequence alignment of various analogues of AO from different species has been reviewed by Mota et.al. (Mota et al., 2018). This analysis indicates that there are differences among the residues in the active site. For instance, Val residue in hAO, which is involved in substrate binding, is conserved in guinea pig, mAO1 or mAO4 but is replaced by Ala in chimpanzee, rhesus monkey and mAO3. Similarly, Phe885 in hAO, which is important in the orientation of the substrate is replaced by residues of different sizes and shapes in other animal species. The region located above the Mo active site in the entrance of the catalytic cavity also presents high variability. For example, when comparing the funnel of hAO and mAOs, gate 1 of hAO is mostly composed of bulky hydrophobic residues, with the exception of one residue (Glu660) none of the residues are conserved in mAOs and the gate is shorter. Replacing Tyr and Phe in mAO3 with Ala (1023) and Leu (1018) in hAO results in a wider entrance of the substrate funnel in hAO (Rashidi & Soltani, 2017). Comparison between the crystal structures of hAO and mAO3 has also shown variation in the position of one loop at the FAD site (the site of oxygen reduction) between the two enzymes. The loop close to the FAD pocket is flipped by almost 180° in hAO relative to mAO3 (Coelho et al., 2015). Previous reports by Beedham et al. and Liu et al. have also suggested that the difference in the size of the binding site in the AO enzyme relates to the variation in binding affinity among animals (Beedham et al., 1995; Liu, Liang, Wang, & Guo, 2009; Schumann et al., 2009; Taylor, Stubley-Beedham, & Stell, 1984). Investigation into the substrate specificity of several phthalazine and quinazoline analogs towards rabbit, guinea pig, baboon and human liver AO showed a good correlation

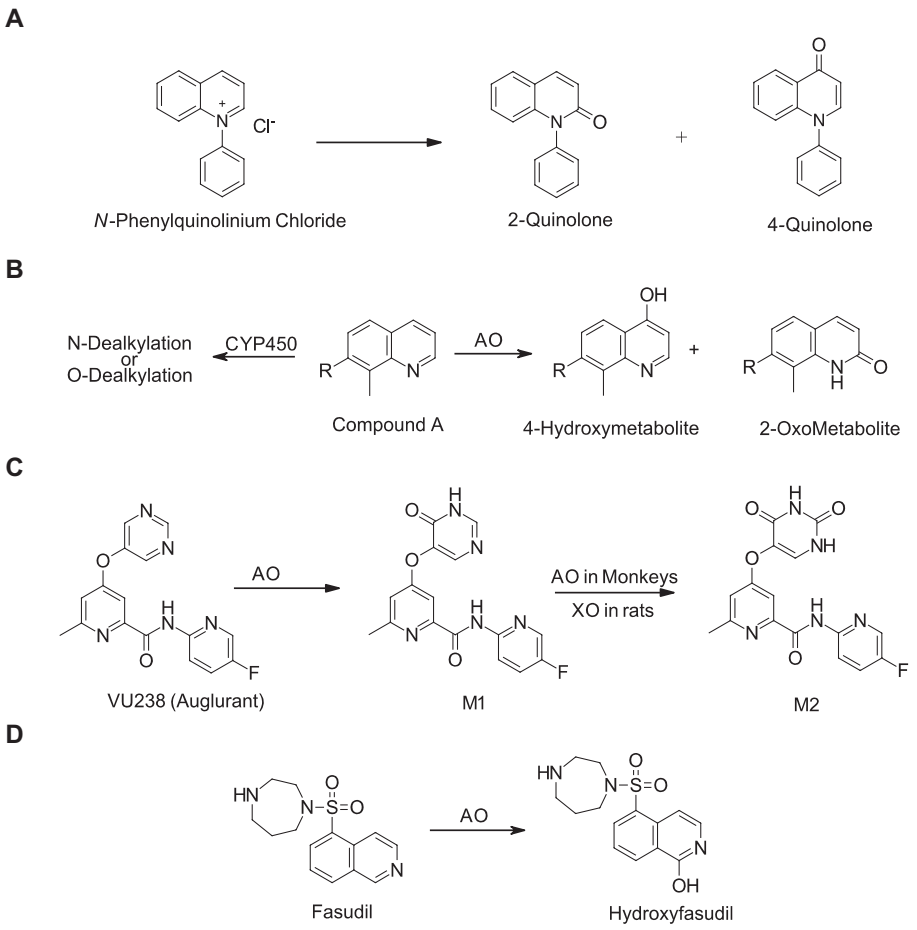


Fig. 26. A) Conversion of *N*-phenylquinolinium chloride to 2- and 4-quinolone. B) Oxidation of compound A by AO to the corresponding 4-hydroxy and 2-hydroxyquinoline metabolites. Compound A is also metabolized by P450. C) Metabolism of VU238 (auglurant), a negative allosteric modulator of metabotropic glutamate receptor subtype 5, to hydroxy (M1) and dihydroxy (M2) metabolites. VU238 is an interesting case where species differences are observed in enzymes oxidizing the compound. VU238 is first converted to M1, which is then oxidized to M2 by AO in monkeys but by XO in the rats. D) Conversion fasudil, a potent Rho-kinase inhibitor, to hydroxyfasudil by AO.

between molecular size of the substrate and size of the binding site of AO (Beedham et al., 1995). These results revealed that the rabbit liver AO shows marked differences from hAO. The rank order based on the activity is rabbit < guinea pig < baboon < man (Beedham et al., 1995). Similarly, remarkable difference in cinchonidine and methotrexate oxidation activity has been observed between rabbit and monkey AO. As in the case of quinazolines, the bulky cinchonidine and methotrexate molecules are metabolized rapidly by rabbit AO than by monkey AO and this has been ascribed to the structure of the active site (Fukiya et al., 2010; Liu et al., 2009). Also, following oxidation of *N*-phenylquinolinium chloride (Fig. 26A) by AO decreases dramatically from rabbit to guinea pig liver enzyme (Taylor et al., 1984). Interestingly, carbazeran, which is rapidly metabolized by hAO, is refractory to rabbit AO (Beedham, Bruce, Critchley, et al., 1987). All these examples suggest indicate the variation in the size of the active site among species can alter AO-mediated metabolism.

In addition to substrate dependency and size of the active site, differential expression of AO in preclinical species can also result in significant species differences in the metabolism and clearance of AO substrates (Beedham, Bruce, Critchley, et al., 1987). The general trend based on the literature is high AO activity in monkey and human, medium to low in rabbit, guinea pig, rat or mouse and lack of AO activity in dog (although some reports suggest that AO activity in guinea pigs is similar to that in monkey and humans). Kawashima and co-workers have shown a marked species difference in the AO-metabolism of the sedative-hypnotic zaleplon between monkeys and rats (Kawashima et al., 1999). Similarly, Sahi et al. have compared the AO activity in cytosol and hepatocytes of from humans, monkey, rat and mouse (Sahi et al., 2008). In these studies, the AO activity was measured using the formation of vanillic acid from vanillin. The results indicated that the AO activity (as measured by V_{max}) was highest in monkey and lowest in rat, while mouse liver cytosol exhibited the highest efficiency (highest CL_{int}) for the formation of vanillic acid. High AO metabolic activity in monkey and human is also consistent with the expression and sequence similarity of AO in non-human primates (chimpanzee, rhesus monkey and cynomolgus monkey) and humans (Garattini & Terao, 2012). A good correlation between the estimated CL_{int} values has been observed between monkey and humans for several experimental drugs that are primarily metabolized by AO. To better understand the AO-mediated species differences and their correlation to humans, Choughule et. al. have studied metabolism of phthalazine and DACA (Choughule, Barr & Jones, 2013). These studies showed high CL_{int} of both these substrates in the rhesus monkey and guinea pig than humans. Despite the general trend of higher AO activity in monkey and humans, there are cases where other preclinical species exhibit high CL_{int} for AO substrates. Recently, Choughule and co-workers have evaluated the AO-mediated metabolism in human, guinea pig, monkey rat and rabbit using methotrexate as a probe substrate (Choughule, Joswig-Jones, & Jones, 2015). Even though equal amounts of human and rabbit AO protein was used in this study, the methotrexate hydroxylase activity was several orders of magnitude higher in the rabbit liver cytosol followed by monkey, guinea pig, rat and finally human. Overall, this discussion suggests that each species does not necessarily have the same substrate specificity and that the metabolism and variation in different species is

substrate dependent. As a result of this wide specificity, it is often important to monitor enzyme activity with different substrates.

Inter-species difference metabolism of enantiomers has also been observed when RS8359 (Fig. 11B) has been tested for its oxidation by AO in liver fractions of different species (Itoh, Yamamura, et al., 2006). Investigation into the 2-oxidase activity of the enantiomers of RS-8359 indicates that the CL_{int} of *S*-RS8359 was extremely high in monkeys and humans, moderate in guinea pigs and low in rats and mice. *R*-RS8359 on the other hand was only oxidized at a low rate in guinea pigs, monkeys and humans, and not in rats, mice and rabbits.

Interspecies AO activity variation among different animal species has also been extensively probed by Dalvie and co-workers using zoniporide as a substrate (Dalvie, Xiang, Kang, & Zhou, 2013). Sixteen species (including humans) were probed for zoniporide hydroxylase activity by estimating the CL_{int} for the formation of 2-oxozoniporide in the liver S9 fraction. Approximately 22-fold variation in the estimated CL_{int} of zoniporide was observed among species. The rabbit and Gottingen minipig were more efficient in metabolizing zoniporide compared to humans, while other species rhesus monkey, cynomolgus monkey and Fischer rats were less efficient than humans in the biotransformation of the drug. The difference in the efficiency was more likely related to the variation in V_{max} estimates for 2-oxozoniporide formation among species. This low efficiency of zoniporide metabolism in monkeys contradict the reports that monkeys are generally good surrogates for predicting clearances of AO substrates. Recently, Li and coworkers have reported species differences in regioselectivity of oxidative metabolism of compound A by AO (Li et al., 2018). In their studies, a methyl quinoline analog (compound A) was oxidized by AO at the 2- and 4-position resulting in the formation of 2- and 4-quinolinone (Fig. 26B). Although all species formed the 2-quinolinone metabolite, a larger variation was observed in formation of 4-quinolinone metabolite. Species differences have also been observed in reductive cleavage of zonisamide. Studies by Kitamura and co-workers have demonstrated that zonisamide AO-reductase activity in the monkey and rabbit was relatively higher compared to other animals when benzaldehyde and 2-hydroxypyrimidine as an electron donor (Kitamura et al., 2001).

An interesting case of species differences has been presented by Crouch et.al. (Crouch, Blobaum, Felts, Conn, & Lindsley, 2017). *In vitro* metabolism studies with VU238 (Auglurant, negative allosteric modulator of metabotropic glutamate receptor subtype) in liver S9 fractions revealed that it was oxidized to hydroxyVU238 (Fig. 26C) by AO in most species, including monkeys and rats. However, an apparent species difference was uncovered when the conversion of hydroxyVU238 to the corresponding dihydroxyVU238 was phenotyped. With the use of hydralazine and allopurinol (AO and XO inhibitor), the authors observed that this conversion was catalyzed by AO in the monkeys and predominantly by XO in rats. The development of VU238 was halted due to adverse events observed in the 28-day toxicologic assessment in monkeys. Although the mechanisms responsible for the toxicity were unknown, the authors speculated that species differences in the involvement of AO and XO in VU238 metabolism could play a role in the observed monkey-specific toxicity.

Along with species differences, striking variation in AO activity has been observed among various strains of rat and mouse. Studies by Al-Salmy have shown that the AO activity in different mouse strains with DACA as a substrate, varied 15-fold variation in K_M and10-fold variation in apparent V_{max}. (Al-Salmy, 2002). Nude mice and C129/C57 had the highest intrinsic clearance compared to Swiss CD mice. Similarly, a striking variation in AO activity has been observed between C129/C57 and CB57BI/6J mice. The reason for this variation is unknown but perhaps could be because of variability in the expression of different orthologs of AO among different strains. Interestingly, differences in AO metabolism were also observed in xenograft models. For instance, the clearance of DACA in non-tumor bearing nude mice was twice as fast compared to tumor bearing nude mice. In rats, a marked strain difference has also been observed in the conversion ratio of 2-PY and 4-PY and N^1-methylnicotinamide when 12 different strains of rats examined *in vitro* and *in vivo* by Sugihara and coworkers (Sugihara, Kitamura, & Tatsumi, 1995; Sugihara et al., 2006). Also, studies with zoniporide by Dalvie et.al. also demonstrated differences in CL_{int} of formation of 2-oxozoniporide when Sprague-Dawley, Wistar and Fischer rats were compared. The CL_{int} estimates in Wistar rats were approximately two to three-fold higher in the Sprague Dawley and Wistar rats when compared to the CL_{int} in the Fischer rats (Dalvie et al., 2013). The AO activity also appears to vary with gender. In rodents, AO activity varies between the males and females with the males exhibiting higher activity. Klecker and coworkers have reported major differences in the AO-mediated metabolism of zebularine between genders of mice (Klecker et al., 2006). Their studies have shown that male CD-1 mouse is 50-fold more efficient in metabolizing zebularine (Fig. 9B) as compared to the female mouse. Similarly results by Dalvie et al. have demonstrated that the CL_{int} estimates in the male mouse were 6-fold greater than the female mouse (Dalvie et al., 2013). In Al-Salmy's study, a gender difference was also observed among male and female Swiss CD mice. the male mice had higher CL_{int} than the female Swiss CD mice when AO activity was assessed using DACA and benzaldehyde (Al-Salmy, 2002). Akabane and coworkers have also observed a difference in the metabolism of FK3453 to hydroxyFK3453 (Fig. 16D) between male and female Sprague-Dawley rats (Akabane et al., 2011). However, their study showed that the CL_{int} of FK3453 oxidation in the female rats was 11 -fold greater than that in male rats (Akabane et al., 2011). More recent study on metabolism of fasudil to hydroxyfasudil (Fig. 26D) by AO has also demonstrated a significant gender difference between male and female rats (Mao et al., 2018).

It is well-known from the P450 literature, that different isoforms of an enzyme can exhibit substantial differences in the kinetic parameters for the same substrate. Kücükgöze and Leimkühler have recently shown differences in the enzymatic properties of four mAO isozymes using different substrates (Kucukgoze & Leimkuhler, 2018; Kucukgoze, Terao, Garattini, & Leimkuhler, 2017). In this report, the authors used purified isoforms and compared the parameters of all substrates. While some compounds like retinaldehyde, vanillin or salicylaldehyde were efficient substrates for all four mouse isoforms, *N*-methylnicotinamide was not a substrate for mAO1 or mAO4 and pyridoxal was not metabolized any isoforms. The results also suggested that mAO4 displayed higher substrate selectivity among the four isoforms. When the kinetic parameters were compared, mAO1 showed highest catalytic turnover for most substrates (Kucukgoze & Leimkuhler, 2018).

Species differences in AO inhibition have also been reported. For instance, the mechanism of inhibition of raloxifene varies depending on the species. In guinea pigs, raloxifene inhibits phthalazine and DACA oxidase activity uncompetitively, whereas in the rhesus monkey, the mode of inhibition was a mixed-type, for both substrates (Choughule, Barr, & Jones, 2013). Inhibition studies with purified mAO3 using raloxifene as an inhibitor have also revealed a mixed-type inhibition (Coelho et al., 2012). Takaoka and co-workers have tested compounds for species-specific differences in the inhibitory effect of AO activity using genetically engineered HEK293 cells over-expressing hAO and mAO1 or mAO3 (Takaoka et al., 2018). Their studies also revealed that typical inhibitors like β-estradiol, menadione and raloxifene exhibited marked differences in inhibitory effects between the hAO and mAO when the phthalazine substrate was used. The IC_{50} values correlated well with the rank order of the minimum binding energy estimated from docking simulations of these compounds with these enzymes. Menadione is another well studied AO inhibitor that exhibits differences in mechanism of inhibition depending on the species. Inhibition studies with purified mAO3 have shown that menadione is an uncompetitive inhibitor with a $K_i = 1.25$ μM when phthalazine is used as a substrate (Coelho et al., 2012). In contrast, studies by Barr and Jones have suggested that menadione inhibits human AO is mixed fashion (Barr & Jones, 2013).

In addition to the inter-species differences, the literature is replete with examples of inter-individual variability in AO activity. Studies by

Kitamura et.al. demonstrated a 48-fold variation in methotrexate 7-hydroxylase activity in liver cytosol from six human donors (Kitamura et al., 1999). Similarly, the CL_{int} of DACA varied 18-fold when fifteen human cytosolic fractions were examined for DACA hydroxylase and benzaldehyde oxidase activity by Al-Salmy (Al-Salmy, 2001). This seems to correlate with the clinical data, which showed that the clearance of DACA in humans across twenty eight individuals ranged from 5.2 to 35.8 mL/min/kg (Kestell et al., 1999). In other studies, ~50-fold and 90-fold spread has been observed in the cytosolic activity when used be benzaldehyde or carbazeran has been used as a substrate (Fu et al., 2013; Sugihara, Kitamura, Tatsumi, Asahara, & Dohi, 1997). Recent studies by Hutzler and co-workers also observed significant variability in AO activity in human cryopreserved hepatocytes from 75 donors (Hutzler, Yang, Brown, Heyward, & Moeller, 2014). These studies demonstrated that the activity varied by at least 17-fold with 63% of the donors having higher activity (Hutzler, Yang, et al., 2014). Likewise, an approximately 5-fold variation in the AO activity was observed between the hepatocytes evaluated from five human subjects (Sahi et al., 2008).

9. Factors affecting AO expression and activity

As in the case of other drug metabolizing enzymes, several factors can affect the expression and activity of AO and therefore contribute to the observed inter-individual variability. Single nucleotide polymorphism (SNP) is one of the important genetic determinants of variation in expression and active protein levels. Numerous SNPs have been identified with the human *AOX* gene. Hartmann and coworkers sequenced 35 coding exons of the human *AOX* gene in a sample of 180 Italian individuals and identified relatively frequent, synonymous, missense and nonsense SNPs, which could lead to functionally inactive AO allelic variants or variants encoding AO with different catalytic activities (Hartmann et al., 2012). The SNP information of the human *AOX* gene is available in the National Center for Biotechnology Information dbSNP data base (http://www.ncbi.nlm.nih.gov/snp). Foti and coworkers selected ten novel SNPs resulting in amino acid exchanges close to the FAD site of human *AOX*. This data revealed significant alterations in the production of superoxide anion among the variants and suggested that SNP-based amino acid exchange close to the isoalloxazine ring of the FAD factor resulted in increased rate of superoxide radical production (Foti et al., 2016). Hartmann et al have also studied the impact of some commonly identified amino acid changes (Hartmann et al., 2012). Their data show that the mutation of each amino acid residue has a variable impact on the ability of AO to metabolize some selected substrates and provides evidence for the existence of frequent AO allelic variants defining fast and poor metabolizers in the human population. Itoh et. al. have conducted functional studies on *Aox* SNPs in Donryu rats using RS-8359 as a substrate (Itoh et al., 2007). They found that the activity was clearly divided into low and high groups with a frequency of approximately 1:1 suggesting a dimorphic pattern for the hydroxylation of RS-8359. Further functional characterization of the identified SNPs permitted the classification of these rats into ultrarapid, extensive and poor metabolizers according to the *Aox* mutation.

Age and developmental changes have been shown to affect AO expression and activity as well. Although *in vitro* studies in human hepatocytes by Hutzler et. al. did not show any age related changes in AO activity (Hutzler, Yang, et al., 2014), *in vivo* studies by Tayama et. al. clearly showed effect of developmental changes on variability of AO in postnatal rat liver. These studies demonstrated that AO protein expression and activity in rats increases rapidly from birth and reaches a plateau within four weeks (Tayama, Moriyasu, Sugihara, Ohta, & Kitamura, 2007). Similarly, investigation of developmental changes of AO activity in 101 Japanese children also showed significant correlation N^1-methylnicotinamide hydroxylase activity and age. The results suggested that AO activity begins to increase soon after birth (Tayama et al., 2007). The studies also recommended an adjustment of dose based on individual AO activity for children below 1 year of age. When the same group assessed AO activity in human livers ranging from 13 days to 45 years of age, no activity was observed in human livers obtained from children whose ages ranged from 13 days to 4 months (Tayama et al., 2012). However, the AO activity markedly increased after four months reaching an adult level by about two years of age. Immunoblotting analysis correlated with AO activity suggesting that the activity is regulated at the protein expression level. Recently, the effect of age on AO activity has also been investigated in male domestic pigs by Hu and coworkers (Hu, 2018). In these studies, the O^6-benzylguanine activity was assessed using liver cytosolic fractions from pigs at five different ages (1 day, 2, 5, 10 and 20 weeks). As in the studies by Tayama et.al., the porcine hepatic AO activity was minimal at the age of one day but increased gradually to a maximum in five weeks and remained constant to the age of 20 weeks (Hu, 2018).

Inter-individual variation and differences in species differences have led development of sensitive methods that can quantify the amount of AO protein in liver. LC-MS-based quantitation approaches of proteins are now becoming popular owing to their selectivity, robustness, high-throughput and capacity of multiplexing. Barr et. al. and Fu et.al. have developed a LC-MS/MS method for quantifying absolute amounts of AO (Barr, Jones, Joswig-Jones, & Rock, 2013; Fu et al., 2013). The method is highly efficient and sensitive for determination of levels of AO protein in liver cytosol. A typical LC-MS quantification work-flow involves the following steps. First, the protein is digested into proteolytic peptides by proteases (for example, trypsin). A set of unique peptides are then selected as surrogate measures of protein concentrations. Only those peptides are selected that are singly or doubly charged and have high signal intensity in MS analysis. Also, these peptides should behave optimally in human liver cytosol and other preparations with minimal signal interference and high sensitivity. Finally, multiple reaction monitoring acquisition method is employed for peptide quantification. Both groups used recombinant human AO expressed in *E. coli* as a surrogate for peptide mapping and compared this with peptide map from the crude liver cytosol digest. The peptides that were specific to human AO (^{446}VFFGEGDGHR^{456}, ^{779}YIQDIVASTLK^{789}, ^{813}TGIIAAVTAFAANK^{826}) and had favorable ionization and fragmentation characteristics were then selected as surrogate measures for AO quantification in human liver cytosol. Correlation of AO levels to those measured using the traditional ELISA assay provided confidence on the LC-MS/MS based quantitation method in complex matrices.

Several mechanisms have been put forth that can lead to disparity between the expression levels of AO protein and AO catalytic activity. Fu et.al. have shown that even though the AO protein levels among 20 human liver cytosolic samples are consistent with very little variability in the total protein, the spread of AO activity in these samples is substantial (Fu et al., 2013). Studies by Itoh and coworkers have suggested that low AO activity in some rat strains could be due to lack of ability to produce a dimer (Itoh et al., 2007a). This is perhaps attributed to the 377G to A nucleotide substitution (Itoh et al., 2007b). Another possibility for poor activity is attributed to the deficiency in the Moco complex in the final AO protein. As described before, AO has different cofactors in the full protein and relies on a number of enzymes to incorporate these and finally produce an active enzyme (Hartmann et al., 2012). Schwarz and coworkers have reported that AOs require Moco sulfurase catalyzed addition of a terminal sulfido ligand to the molybdenum center in the final step of maturation in order to gain enzymatic activity (Schwarz, Mendel, & Ribbe, 2009). Impairment of this process can lead to protein with weak activity. Also, the Moco complex needs to be inserted correctly in the active site domain in order for the enzyme to be active (Garattini et al., 2008). Fu and coworkers indicated that at least one of human liver cytosolic sample among the 20 samples that they assessed had a lower concentration of Mo which probably resulted in poor incorporation of Moco in the protein and hence led to decrease in activity (Fu et al., 2013). Heavy and chronic alcohol consumption can also lead to abnormally low AO activities. Evaluation of demographics of donors in

the study by Hutzler et.al. suggested that at least three out of the five donors with no measurable AO activities had immediate medical histories of extensive alcohol abuse (Hutzler, Yang, et al., 2014). Likewise, demographic details of liver cytosol donors used by Fu et.al. showed abnormally low AO activities in two of the donors that were alcoholic (Fu et al., 2013).

10. Prediction of AO metabolism and clearance

10.1. Prediction of AO metabolism

Recent interruptions in the clinical development of AO substrates and the lack of correlation in AO liability with the physicochemical properties (Dalvie et al., 2012; Linton et al., 2011; Pryde et al., 2010; Pryde et al., 2012) have resulted in generation of computational and chemical and *in vitro* approaches to discern the role of AO in its ability to metabolize new candidates. Several computational approaches have been developed in the past decade that can help to identify compounds that are susceptible to AO metabolism. Since AO catalyzed oxidation of a heteroaromatic ring involves a nucleophilic attack of the hydroxyl group on the electron deficient carbon, most methods that compute the site of oxidation are based on assessing the electronic deficiency at the metabolic site. Consequently, all methods have exploited parameters that determine the electrophilicity at the reaction site. The electrostatic potential (ESP) of the targeted carbon atom adjacent to the nitrogen atom in the heteroaromatic ring is one such parameter that can predict the most positively charged carbon atom adjacent to the nitrogen atom in a heteroaromatic ring. Higher positive value of ESP signifies more positive charge at the carbon and therefore higher reactivity and susceptibility to a nucleophilic attack (Chan, Poon, & O'Brien, 2008; Cronin, Manga, Seward, Sinks, & Schultz, 2001). Another parameter, energy of the lowest unoccupied molecular orbital (E_{LUMO}), also predicts the overall electron deficiency of the heteroaromatic ring. A more negative value of E_{LUMO} indicates greater reactivity (Chan et al., 2008; Cronin et al., 2001) of the heteroaromatic ring and it potential to undergo oxidative modification by AO. These parameters have been used by Ghafourian and Rashidi to assess QSAR of compounds containing a phthalazine or a quinazoline ring (Ghafourian & Rashidi, 2001). Dalvie and co-workers have also used this method to explore the relationship between structures of zoniporide analogs and their metabolism by AO (Dalvie et al., 2012). Cruciani and coworkers have tried using Parr's index to estimate the electrophilicity of the molecule and correlate with AO metabolism (Cruciani et al., 2018). However, this method did not provide good discrimination between compounds that are substrates and those that did not undergo metabolism by AO.

Torres et al. developed a simple method to predict the regioselectivity in AO metabolism (Torres, Korzekwa, McMasters, Fandozzi, & Jones, 2007). The method was based on the formation of the tetrahedral intermediate at the carbon resulting from the oxygenation reaction by AO. The authors used DFT quantum chemical methodology to optimize the geometry of the tetrahedral intermediate formed following an attack of a hydroxyl group from water at all possible sites of metabolism and calculate its relative energy. They based their approach on the ability of the molecule to accept an equivalent of a hydroxide at the site of metabolism and that the relative energies of the tetrahedral intermediate formation is proportional to the activation enthalpy of AO oxidation. The heat of reaction of the tetrahedral intermediate of a given reaction was assessed from the difference in energy between the drug and the tetrahedral intermediate after addition of water to the parent drug. The formation energies of putative tetrahedral intermediates (ΔG) resulting from AO mediated hydroxylation of the carbon atom in the heteroaromatic ring were then correlated with the site of hydroxylation determined experimentally. A lower (negative) value of ΔG suggested enhanced stability of the tetrahedral intermediate and favored oxidation of the carbon atom in the ring. Thus, estimation of formation energies can serve as an indicator of reactivity and susceptibility of the carbon atom towards nucleophilic attack by AO and hence predict the site of hydroxylation on the heteroaromatic ring. The method showed greater than 90% accuracy in predicting AO metabolite and also appears to be able to distinguish the products of AO vs. XO (Torres et al., 2007). Dalvie et al. used this method to estimate the susceptibility of the quinoline ring in various zoniporide analogs towards AO-mediated oxidation (Dalvie et al., 2012). In their studies, zoniporide analogs that were oxidized at rates equal to or faster than zoniporide formed tetrahedral intermediates with a ΔG value that was lower whereas, compounds that displayed higher ΔG values relative to the tetrahedral intermediate observed in the case of zoniporide, were resistant to oxidation. Pryde and coworkers also applied this method to predict the AO lability of new designed molecules in the Pfizer TLR7 agonist program and successfully switch off AO metabolism that was observed (Pryde et al., 2012). Montefiori et al. have extended the methodology using molybdenum cofactor as a model for the reactive part of the enzyme (Montefiori, Jorgensen, & Olsen, 2017). Using concerted mechanism as a model for the enzymatic reaction, the group determined the transition state energies for the formation of all possible metabolites for a series of known AO substrates. The lowest activation energies corresponded to all experimentally observed sites of AO metabolism (Montefiori et al., 2017).

Recently, Cruciani et al. have developed a novel computational method to the predict metabolism lability of newly designed compounds for human AO (Cruciani et al., 2018). This model was developed using more than 270 compounds with high quality data were used to develop a model to predict AO site of metabolism using both electronic and exposure factors (Cruciani et al., 2018; Lepri et al., 2017). The method considers both, the nucleophilic attack by the hydroxyl group bound to molybdenum and therefore the electrophilicity of the sp^2 carbon, and the subsequent hydride displacement step. Additionally, this method also helps predict the possibility of amide hydrolysis that is mediated by AO. Lately, Xu et al demonstrated the use of electronic and steric parameters and proposed a decision tree model to predict the most reactive sites in a molecule that are susceptible to AO metabolism (Xu et al., 2017).

Ability to predict AO based metabolism has also been explored using a chemical approach. O'Hara et al have reported the use of bis ((difluoromethyl)sulfinyl)oxy)zinc (DFMS) as a source of CF_2H radical for a rapid and early identification of heteroaromatic drug candidates that have a high probability of metabolism by AO (O'Hara et al., 2014). The method was developed on the findings that the carbon-hydrogen bonds of heteroarenes can be readily functionalized using radicals derived from alkylsulfinate salts and that the reactivity of an azaheteroarene toward a nucleophilic radical species might closely approximate the susceptibility to AO-based metabolism. The authors used this method on five known AO substrates. These compounds were subjected to reaction with DFMS and the reaction mixtures were examined by LC-MS for a characteristic M+50 peak associated with the addition of a -CF_2H group. The product formation correlated well with the experimentally determined site of metabolism as well as AO substrate properties. This simple so called "litmus test" procedure represents a chemical reaction that rapidly decipher innate reactivity of a drug candidate and hence its susceptibility toward AO. Li et. al. utilized this method to confirm that more than one oxidative product could be formed from Compound A (Fig. 26B) as indicated by the formation of at least three such products in this reaction (A. C. Li et al., 2018).

Molecular docking approaches are commonly used to elucidate important interactions between substrates and active site residues of drug-metabolizing enzymes (H. Sun & Scott, 2010). These have also been extended to characterize the binding of substrates in the AO active site. A three-dimensional homology model of human AO that was constructed by Dastmalchi and Hamzeh-Mivehrod early on to decipher the major sites of interaction within the active site (Dastmalchi & Hamzeh-Mivehrod, 2005). Dalvie and coworkers have used this approach in rationalizing the differences in substrate properties of zoniporide analogs (Dalvie et al., 2012). A combination method of energy calculations and

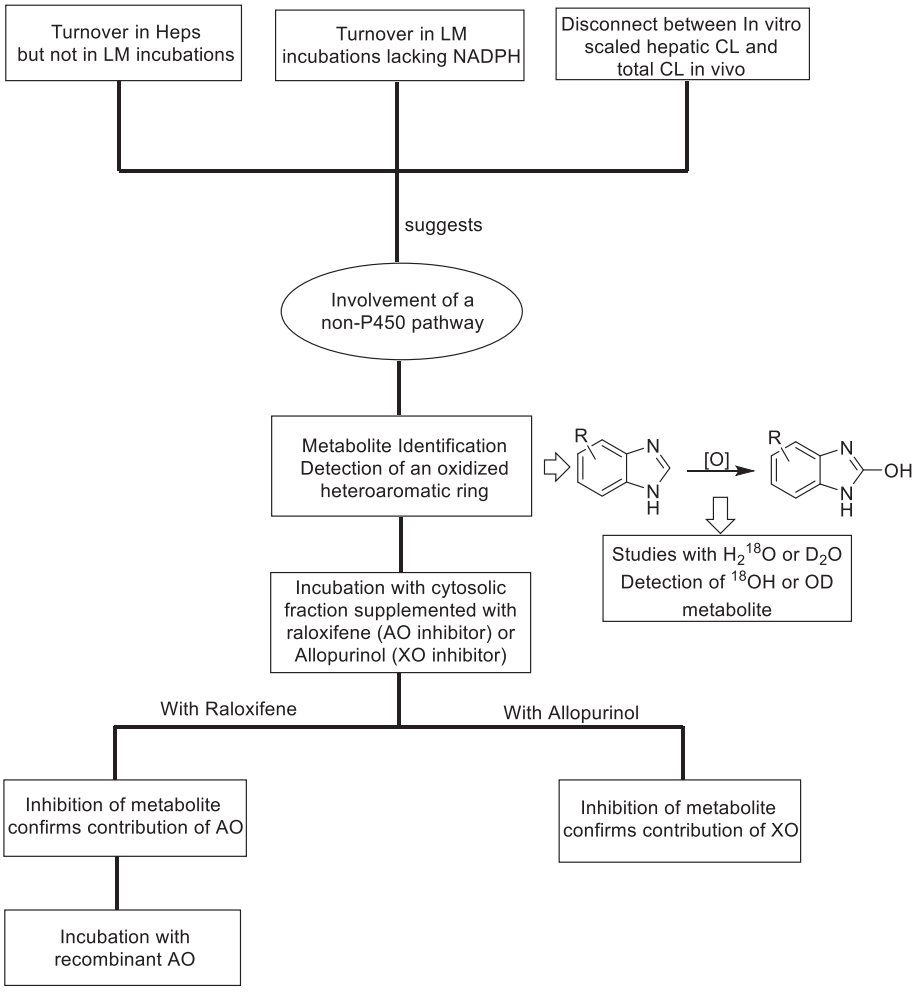


Fig. 27. Flow chart depicting systematic identification and confirmation of AO in the metabolism of a new candidate.

docking studies showed good success for to predict rate of AO metabolism for a series of zoniporide analogues. Likewise, the approach was also used by Pryde et al and Linton et al demonstrated the utility of docking as a predictive method to rationalize the site of metabolism penciclovir and 6-deoxypenciclovir (Pryde et al., 2010) and the site of metabolism of the imidazopyrimidine containing androgen receptor antagonists (Linton et al., 2011).

Quantitative structure-activity relationship (QSAR) model of human AO inhibitors has also been developed to enable prediction of AO inhibitors with *in silico* approach based on molecular properties. In studies by Barr et. al., the three key descriptors that best explained inhibitory potency using a panel of twenty-four structurally diverse diet-derived constituents are, dipole moment, hydrophilic solvent-accessible surface area, and hydrogen bonding-accepting capacity (Barr et al., 2015). QSAR model developed by Hamzeh-Mivehroud et.al using 15 flavonoids identified two key descriptors, Glu (related to size, shape, symmetry and atom distributions) and Mor17v (related to molecular volume) (Hamzeh-Mivehroud et al., 2014; Hamzeh-Mivehroud et al., 2013).

10.2. In vitro methods to identify AO substrates

AO substrates can be identified experimentally by incubating the molecule with a cytosolic or S9 fraction as well as cell systems like the hepatocytes. More recent addition to arsenal of biological systems is the availability of human recombinant AO enzymes (Foti et al., 2016). A detailed protocol to identify AO as a drug metabolizing enzyme has been described in detail by Hutzler et al. (Hutzler et al., 2012) and is depicted in Fig. 27. An early indication that a compound containing a heteroaromatic ring is susceptible to AO (or XO)-mediated oxidation originates from the increased rate of metabolism in liver microsomal incubations that are not supplemented with NADPH. Recent studies have demonstrated a substantial level (up to 40% of that in liver cytosol) of catalytically active AO protein in multiple human liver microsomal preparations by multiple commercial suppliers (Hutzler et al., 2013; Xie et al., 2019). An increased rate of metabolism in hepatocyte incubations compared to an industrial standard NADPH-fortified microsomal assay may also point toward the involvement of a non-P450 enzyme in metabolism of the compound. In addition to the above *in vitro* observations, a significant *in vitro* and *in vivo* disconnect in the total clearance of the compound in preclinical species when comparing scaled liver microsomal or hepatocyte data with total clearance also suggests a possibility of a non-P450 and potentially a non-hepatic clearance. Follow up metabolite identification studies and detection of a metabolite with modification of the heteroaromatic ring indicates the contribution of either AO or XO in the biotransformation of the compound. Additional studies, including incubation of the compound in cytosol, S9 fraction or cryopreserved hepatocytes with and without a specific AO inhibitor like raloxifene (Obach, 2004) or hydralazine (Strelevitz et al., 2012) or incubation of the compound with recombinant AO enzyme (Alfaro et al., 2009; Rodrigues et al., 2014) should be conducted to confirm the involvement of AO in the metabolism. It is important to note that raloxifene is also an inactivator of CYP3A4. Because of the lack of selectivity caution should be used in interpreting the data if the compound being tested is metabolized CYP3A4 and AO. This is especially, when

sources containing both cytosolic and microsomal fractions such as the hepatocytes are used. Also, as mentioned before, hydralazine, which is considered to be a selective inhibitor of AO, inhibits CYP1A2, 2B6, 2D6, and 3A4 in human suspension hepatocytes under reaction phenotyping assay conditions, (Yang et al., 2018). Hence, precautions need to be taken when using raloxifene or hydralazine as AO inhibitors for *in vitro* studies because fraction metabolized by AO is likely to be overestimated and the likelihood of false positives in identifying AO substrates increases (Yang et al., 2018). Since AO catalyzed oxidation of a substrate involves a nucleophilic attack of a hydroxyl moiety that is derived from a water molecule (Alfaro et al., 2009), biotransformation studies using $H_2^{18}O$ or D_2O have also been conducted to assess the involvement of AO or XO (Li et al., 2018; Morrison et al., 2012). In these studies, incorporation of either ^{18}O or a deuterium into the molecule can indicate the contribution of molybdenum hydroxylases in the reaction. As discussed above, these studies have to be followed up with incubations that are supplemented with raloxifene or allopurinol to discern the contribution of AO or XO. More recently, a sensitive *in vitro* method which is amenable to high throughput screening, has been developed to effectively identify AO substrates by using high resolution mass LC-MS. This method monitors the parent depletion and AO metabolite formation in liver cytosol simultaneously (Dick, 2018).

10.3. Prediction of human clearance of AO substrates

Predicting human pharmacokinetics of new drug candidates is of primary importance in a discovery setting to prevent candidate attrition in a phase I study. Hence, the pharmacokinetic profile of a drug candidate is comprehensively assessed during the discovery and pre-clinical development phase using *in vivo* and *in vitro* approaches and the human PK parameters as well as the dose is estimated before advancing it to the clinic (Di et al., 2013; Hosea & Jones, 2013; Kang & Lee, 2011).

The use of *in vitro* metabolism in scaling human clearance of new chemical entities has become a routine in the development of new drugs (Obach, 2011). Generally, CL_{int} data obtained from microsomal systems fortified with cofactors or hepatocytes are scaled to estimate human *in vivo* clearance and these predictions are within two-fold of the observed clearances in the clinic (Kilford, Stringer, Sohal, Houston, & Galetin, 2009; Naritomi, Nakamori, Furukawa, & Tabata, 2015). However, as mentioned earlier, dramatic variation in the AO expression and catalytic activity among humans poses a major challenge in estimating clearance of candidates that are AO substrates. All *in vitro* reagents including human liver cytosol, S9 fraction and hepatocytes tend to underestimate the *in vivo* AO clearance. For instance, an attempt to scale *in vitro* CL_{int} for eleven AO substrates by Zientek et. al. (M. Zientek, Jiang, Youdim, & Obach, 2010) using pooled human liver cytosol and human liver S9 fractions resulted in significant under-prediction of unbound *in vivo* CL_{int}. Similarly, an attempt to evaluate pooled human cryopreserved hepatocytes by Hutzler et. al. and Akabane et. al. as a means to estimate *in vivo* clearance was not successful (Akabane, Gerst, Masters, & Tamura, 2012; Akabane, Gerst, Naritomi, Masters, & Tamura, 2012; Hutzler et al., 2012). As a solution to this, a semi-quantitative approach to bin compounds into high, medium, and low clearance using marker drugs that have human clearance data has been developed (Zientek et al., 2010). In this method, Zientek and co-workers used a set of weak, moderate and strong AO substrates and determined their unbound CL_{int} in human liver cytosol and liver S9 fraction. The results were then correlated with the unbound *in vivo* CL_{int} that was estimated from *in vivo* human clearances for these compounds. The study provided a relative scale that can be used for *in vitro-in vivo* correlation of AO-mediated clearance. The approach has been effective in guiding early discovery. It alerts the medicinal chemists on the impact that the AO contribution may have on the overall clearance in humans. Jensen et. al. used this method retrospectively to address the high clearance of Lu AF6503 in humans (Jensen et al., 2017). Their results demonstrated that Lu AF09535 was metabolized almost as fast as carbazeran, which classified the candidate as an excellent substrate of AO and indicated that the *in vivo* clearance of Lu AF09535 was in the same order of magnitude as carbazeran. These results also supported the observed extensive human *in vivo* clearance of Lu AF09535.

Reasons for under-prediction of clearance of AO substrates by *in vitro* systems have been proposed. First, the under-estimation could be in part due to ubiquitous expression of AO in extrahepatic tissues. The lack of contribution of these tissues in the estimation exercise can lead to a significant disconnect in *in vivo* and estimated *in vitro* clearances. Secondly, clearance estimation is highly dependent on the system used (cytosolic or S9 fraction). AO activities between cytosol and S9 fractions are generally variable (Hutzler et al., 2012; Zientek & Youdim, 2015). Variability has also been observed between different lots and batches of cytosolic fractions or S9 fractions from different vendors and is attributed to donor characteristics associated with each pool (Garattini & Terao, 2013; Hutzler, Yang, et al., 2014; Zientek & Youdim, 2015). Third, differences in the preparation and handling of cells and subcellular fractions can also lead to varying degree of enzyme denaturation. Also, the conditions during preparation of cellular fractions such as buffer components, process of homogenization of the liver, handling of the cells and/or storage conditions as well as freeze/thaw cycles can affect the activity and stability of AO (Al-Salmy, 2001; Duley, Harris, & Holmes, 1985; Garattini & Terao, 2013; Hutzler, Yang, et al., 2014; Johns, 1967; Sahi et al., 2008; Zientek & Youdim, 2015). One study has suggested that AO activity may decline rapidly with storage or in post-mortem tissues (Barr et al., 2013). It is therefore advisable to use more than one batch of liver cytosol fractions or hepatocytes from multiple vendors for estimation of CL_{int}. One way is prescreening donors for AO activity and selecting systems with highest level of AO activity. Creating a custom high activity pooled lot can potentially minimize under-predictions of clearance. Measuring the absolute AO concentrations in liver cytosols and hepatocytes using recently developed the highly sensitive and rapid LC-MS/MS can also allow for direct comparison of AO levels between enzyme sources and provide a means to scale between different *in vitro* enzyme sources (e,g. recombinant AO to cytosol) (Barr et al., 2013). Finally, it is important to select *in vitro* reagents (e.g., human cytosol, S9 or hepatocytes) that are representative of the average of the human population.

Allometric scaling of PK parameters obtained from *in vivo* studies in preclinical species to humans is another approach that is generally employed for estimating human PK parameters. Allometric modeling assumes that anatomical, physiological and biochemical variables can be scaled across mammalian species as a power function of the body weight (Poggesi, 2004). However, given the marked differences in the AO activity among species, this approach is not ideal to project human clearances of AO substrates (Garattini & Terao, 2012; Mota et al., 2018; Rashidi & Soltani, 2017). Despite this, attempts have been made to use cynomolgus and rhesus monkey and guinea pig, which express a similar AO ortholog, to predict human clearance on a limited number of compounds. For instance, prediction of human clearance has been shown to be significantly better when using monkey or guinea pig as a model, than using mouse or rat for BIBX1382 and zoniporide (Crouch, Hutzler, & Daniels, 2018). It is important to note that precise

Table 8
Observed and predicted human total CL using h-chimeric mice (Miyamoto et al., 2017).

Candidates	Observed human clearance (mL/min/kg)	Predicted human clearance using h-chimeric mice (mL/min/kg)
BIBX1382	40	44
Carbazeran	37	23
Fasudil	73	105
O^6-Benzylguanine	13	11
XK-469	0.12	0.05
Zaleplon	16	8.0
Zoniporide	21	46

prediction of human clearance can become problematic using these species as well. Firstly, because AO metabolism is substrate dependent. Secondly, contribution of other non-P450 enzymes to clearance or a different route of elimination in humans compared to monkey or guinea pigs can lead to wrong prediction and force discontinuation of the candidate from development.

In the recent years, h-chimeric mice are being considered as an attractive animal model for human clearance prediction (Miyamoto et al., 2017; Sanoh & Ohta, 2014). Over 80% of the liver is replaced with human hepatocytes in these mice and hence these animals are endowed with major human drug-metabolizing enzymes as well as transporters in the liver (Katoh et al., 2004; Katoh et al., 2005). Studies have shown that metabolites formed in humans have been detected in urine and plasma after administration of various drugs in h-chimeric mice (Sanoh & Ohta, 2014). Kitamura and co-workers have examined the conversion of N^1-methylnicotinamide to the respective 2-PY and 4-PY metabolites in humanized mice developed by transplanting human hepatocytes into urokinase-type plasminogen activator-transgenic severe combined immunodeficient ((uPA/SCID) mice (Kitamura et al., 2008). The ratio of pyridones (2-PY/4-PY) excreted in the urine of these mice was resembled that of humans than the control mice suggesting that the AO activity in chimeric mice had human-type functional characteristics. Liu et al. retrospectively evaluated the metabolism and pharmacokinetics of GDC-0834 in h-chimeric mice with SCID mice as controls and showed significant hydrolysis of the compound in this strain (Liu et al., 2011). Likewise, Sanoh et al have used these mice for assessment of human hepatic clearance of four model AO substrates, 6- deoxypenciclovir, fasudil, sulindac and zaleplon (Sanoh et al., 2012). The CL_{int} values estimated from IV studies in these mice were in concordance with the observed human *in vivo* CL_{int}. Although the relative rank order of clearance for the four compounds was similar to those observed in the clinic, a precise prediction of human clearance could not be made from this approach. Miyamoto and co-workers also used h-chimeric mouse for prediction of total human clearance at steady state for additional AO substrates and demonstrated that there was a good correlation between these values and human clearance observed *in vivo* (Table 8) (Miyamoto et al., 2017). Jensen et. al. could also predict human clearance of Lu AF09535, using the pharmacokinetic data obtained from the h-chimeric mice. Analysis of metabolites in this study also showed a significantly higher exposure of hydroxyLu AF09535 and dihydroxyLu AF09535 (Fig. 15A) compared to the parent, which was consistent with that observed in human plasma (Jensen et al., 2017). Overall, all these studies have demonstrated that h-chimeric mouse offers a good solution for projecting human PK parameters for AO substrates. One disadvantage in using h-chimeric mice for human PK prediction is their cost and availability. However, it can help in assessing human clearance of promising candidates that are advanced to the clinic.

In silico models that combine the stability of the intermediates with a steric factor have been developed to explore the possibility to predict intrinsic clearance (Jones & Korzekwa, 2013; Xu et al., 2017). The thought is that this could fill the void of lack of a proper *in vitro* assay to predict CL_{int}. This *in silico* approach also allows to predict if a new synthesized molecule is a potential AO substrate. Jones and Krozekwa used seven compounds to assess the ability to use electronic and steric features of drugs to model the CL_{int} of these compounds. The authors correlated the rates of formation with the stability of the tetrahedral intermediates formed in the reaction. The values estimated from this model performed better than *in vitro* metabolism studies at predicting in vivo intrinsic clearance of compounds that were used in the study (Jones & Korzekwa, 2013).

11. Conclusion

The present article provides a comprehensive review of AO as a drug metabolizing enzyme. In the past two decades, AO has a played a pivotal role in metabolism of important drug candidates which has led to the failure of these candidates in the clinic. Consequently, AO has emerged as one of the most studied enzymes in the past fifteen years in the DMPK field. Significant progress has been made in terms of understanding the structure and function of this enzyme, and in identifying and predicting AO liabilities. However, accurate prediction of AO substrates, rates and sites of metabolism from structures still pose a challenge due to substrate dependency of AO metabolism. Additionally, while the oxidation mechanism and site of binding of AO substrates has been well studied, gaps in the mechanistic knowledge of reduction reactions that are catalyzed by AO still remain. Recent work on the mechanism of AO-mediated reduction nitroaromatics has emerged (Paragas et al., 2017), this information is lacking for other functional groups that undergo AO-mediated reduction. Also, findings that the nitro group of can be reduced under normal oxygen conditions suggests that the reduction process can possibly compete with dioxygen. Thus, the dioxygen availalibility and the conditions that affect the levels of oxygen needs further scrutiny.

Clinical DDI occurring via inhibition of AO is another issue that has not been well recognized despite the fact that several important marketed drugs have been shown to inhibit AO (Obach et al., 2004). This could be due to the fact that a few drugs are cleared primarily by AO and that the nature of inhibition of most potent inhibitors is uncompetitive and therefore resulting low risk of clinical DDI for AO. Also, the drugs that are known substrates for AO like zaleplon, ziprasidone or methotrexate also exhibit other routes of metabolism (P450 for zaleplon and ziprasidone, and renal excretion for methotrexate). Thus, the significance of DDI due to inhibition of AO is reduced. However, this landscape is changing and the newly discovered drugs that possess nitrogen containing heterocycles may have higher contribution of AO in their elimination and potentially make them susceptible to AO DDI (e.g., idelalisib). Given that the mode of inhibition by AO inhibitors is substrate dependent, the risk of DDI for drugs that are exclusively metabolized by AO cannot be ignored. Also, the inhibition of AO-catalyzed reduction reactions requires further investigation since most of the *in vitro* inhibition work done so far, focuses on the AO-catalyzed oxidation reactions. Understanding the risk of DDI for AO-catalyzed reduction is important given the fact AO catalyzes reduction of five membered scaffolds like isoxazole and isothiazole that are present in a number of drugs.

Another major gap relates to estimation of clearance of AO substrates. Extra-hepatic AO contribution, and marked intra and interspecies variation in AO activity has made it difficult to find an appropriate animal model for AO substrates and predict human PK. In addition, AO is an unstable enzyme and although human recombinant AO enzymes are available, the activities tend to be low. Thus, the use of *in vitro* methods is not reliable and a direct quantitative translation of *in vitro* CL_{int} of AO substrates to human *in vivo* clearance is challenging. Although recent advances in the quantitation of AO protein and measurement of absolute AO concentration in human liver cytosol has helped (Barr et al., 2013), the extension of the quantitation of AO levels in other tissues is also required. Absolute concentrations of AO protein levels in extrahepatic tissues may lead to better physiologically based pharmacokinetic (PBPK) models for AO and in turn help in predicting human clearance with higher confidence. AO studies also suffer from the lack of appropriate method for the assessment of the *in vivo* enzyme activity and research in correlating AO levels in preclinical toxicology and pharmacology species with those in humans can is essential.

Major gaps of knowledge also remain in understanding the factors that affect the activity of AO. For example, deeper understanding of AO protein misfolding, chemical/enzymatic modification, disruption of AO homodimer and Mo cofactor deficiency may help in appreciating the protein expression and activity relationship (Fu et al., 2013). This will not only increase our understanding of how individuals respond to AO catalyzed drugs, but shed some light on disconnect between *in vivo* and the estimated *in vitro* clearance. Another critical area of

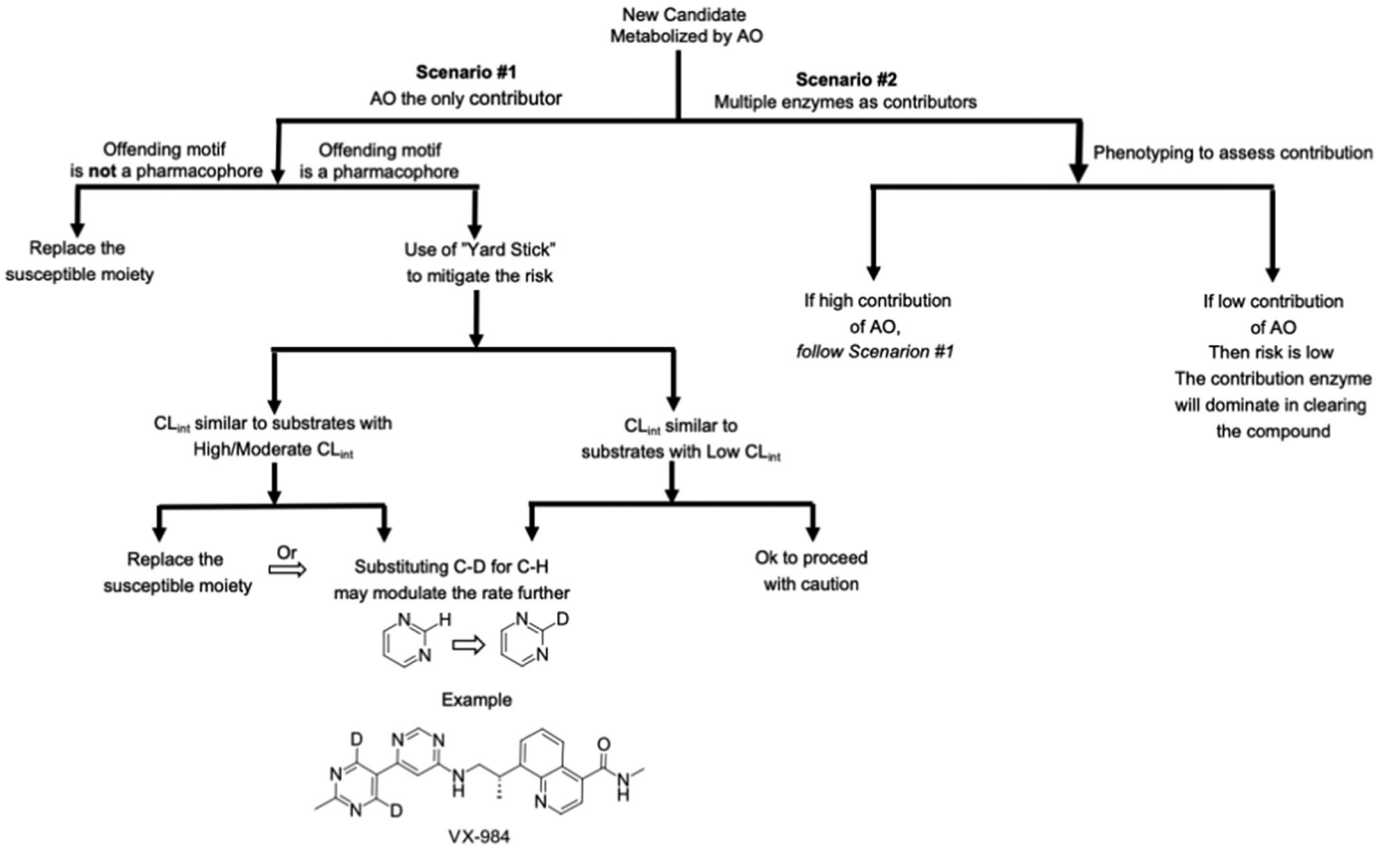


Fig. 28. Flow chart depicting the one possible strategy to mitigate the risk of AO mediated metabolism of a compound.

future research is in furthering our knowledge of the physiological function of AO. Apart from the role of AO in drug metabolism there has been renewed interest in the physiologic function of the enzyme especially, its role in production of NO. This certainly is an area of interest given the role of NO in several important pathophysiological processes and the possibilities for new therapeutic opportunities. To summarize, despite the progress made in AO research in the past decade, additional insight into the structural, mechanism (especially reductive mechanism) and genetics is required to completely understand this enzyme and its role in pathophysiology and drug metabolism.

Given the problems associated with AO based substrates, the current tactic used by the medicinal chemistry community is to minimize AO contribution to clearance through structural modification. Even though a semi-quantitative yardstick approach has been applied in drug discovery to classify compounds into high, medium and low clearance categories and guide SAR, the primary question that still remains is "when is the AO liability a show stopper for the molecule"? Fig. 28 proposes one possible flow chart that can guide in deciding the fate of an AO substrate. The decision to proceed or stop the development of a compound depends on the contribution of AO in the metabolism of the candidate. Two scenarios can be envisioned if a candidate has AO liability. In scenario one, AO is the primary and only contributor to metabolism of the compound. In this case, one can consider replacing or modifying the labile scaffold in the molecule to reduce AO liability (for instance, replacement of the quinoline ring with imidazopyridine ring in volitinib). Alternatively, if the AO-susceptible scaffold in the compound is a pharmacophore and cannot be replaced, the use of the "yard stick" approach proposed by Zientek et. al. (2010) to assess the level of AO metabolism can help in the decision making. A more recent strategy of substitution of the hydrogen atom with a deuterium can also help in modulating the rate of metabolism (Pirali, Serafini, Cargnin, & Genazzani, 2019). This is exemplified by the success of VX-984, a selective DNA-dependent protein kinase inhibitor, where deuterium incorporation mitigates AO-driven metabolism. VX-984 has now completed a phase I trial for the treatment of recurrent or metastatic endometrial cancer (Khan et al., 2018).

In scenario two, the compound can be metabolized by multiple enzymes (e.g., AO and P450s). In this case, if the contribution of AO is less and the primary metabolizing enzyme is P450, it might be okay to advance the compound since the clearance of the compound will be mostly driven by P450s. However, if AO is the major contributor among the enzymes metabolizing the compound, one could follow steps in scenario #1 to mitigate the possibility of AO liability.

In summary, unanticipated AO-mediated clearance and clinical failure of drugs has greatly increased the awareness of AO as an important drug metabolizing enzyme. Consequently, the knowledge of AO has significantly increased over the years and new tools are being continuously developed to address the challenges. Despite the progress, there is still much to be learnt about AO and much more research of this fascinating enzyme is expected in the coming years.

Declaration of conflict of interest

Dr. Deepak Dalvie and Dr. Li Di are employees of Celgene and Pfizer respectively.

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