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A REVIEW ON ALDEHYDE OXIDASE: AN ENZYME OF EMERGING IMPORTANCE IN NOVEL DRUG DISCOVERY

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Abstract

Aldehyde oxidase (AOX) has lately received considerable attention in drug discovery programmes due to the rising tendency in medicinal chemistry approach to decrease cytochrome P450-dependent metabolism, and the number of drug candidates that are metabolised by AOX is gradually increasing. Regions covered despite the growing significance of AOX in the drug development process, there are several significant issues with drug metabolism mediated by AOX. The issues covered in this article include failure to predict in vivo metabolic activity of AOX using conventional in vitro methods, intra- and inter-species variations in AOX activity, and the lack of trustworthy and predictive animal models using the common experimental animals. A thorough analysis of works linked to computational human AOX (hAOX) is also presented. The authors suggest using organoids technology as an efficient tool to address the basic issues connected with the assessment of AOX in drug discovery in light of current advancements in the field of stem cells. Using computer-aided drug discovery techniques, the recent breakthrough in resolving the hAOX crystal structure can also be a helpful data source for the investigation of AOX-catalyzed metabolism of novel drug candidates.

Keywords: Aldehyde Oxidase, Drug Discovery, *In-vitro*.

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Introduction

One of the crucial processes in early phase drug development, which is carried out utilising in vivo trials, in vitro screening, and computer-aided drug discovery (CADD) methodologies, is identifying an acceptable lead chemical. According to PhRMA estimates (2015), the projected average R&D expenses have increased by a factor of two over the previous 15 years, from $26.0 billion in 2000 to $51.2 billion in 2014. The U.S. Food and Drug Administration (FDA) authorised 20.2 novel molecular entities (NMEs) between 2004 and 2009, compared to 30.0 NMEs over the preceding five years. To boost the hit finding rate, many contemporary drug discovery techniques have therefore been applied, including CADD, integrated methodologies, system biology, organoids technology, high-throughput screening (HTS), and virtual screening (VS). To achieve the required effectiveness and safety, drug metabolism and pharmacokinetics (PK) assessments are crucial. The top 200 medications marketed in the USA in 2002 were metabolised in part by the enzymes cytochrome P450, UDP-glucuronosyltransferase, esterase, and flavin monooxygenase. Despite the fact that CYPs are involved in the metabolism of a large number of medications, non-CYP drug metabolism may have a substantial impact.

The goal of medicinal chemistry strategies nowadays is to decrease cytochrome P450-dependent metabolism. Adding N-heterocycle moieties to the potential medicines is one of these tactics. The

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substance is more resistant to cytochrome P450-mediated oxidation because it contains N-heterocycle moieties. This approach can also be used to reduce lipophilicity and boost solubility. As a result, during the past several years, the usage of N-heterocyclic molecules in drug screening and discovery programmes has quickly grown. N-Heterocycles have also consistently been a favourite synthetic building block in medicinal chemistry. As a result of these initiatives, the significance of enzymes that can metabolise N-heterocycles in the discovery and development of new drugs has increased.

One of these enzymes with a broad capability for metabolising N-heterocycles is aldehyde oxidase (AOX, EC 1.2.3.1). As a result, more fresh medication candidates have emerged lately for which AOX is more important. The increasing frequency of substances being broken down by AOX has drawn the attention of scientists studying drug metabolism to this enzyme.

In addition to AOX's developing importance in drug metabolism and discovery, its physiological activities have attracted increased attention. The following are some AOX-related topics that have drawn interest from a variety of angles: its use in recently developed biosensors; bioreactors for the production of drug metabolites that have undergone oxidation; and the creation of active metabolites that can bind the metabolising enzyme and inhibit its activity. Additionally, recent research has shown that cytochrome P450 and AOX work together to create and introduce mixed substrates. Toxicological aspects of AOX-mediated drug metabolism should also be considered in drug discovery studies due to their possible renal toxicity in humans because of crystallization of the AOX-derived metabolite in the kidney according to their extremely poor solubility.

The potential of the enzyme is thought to be bigger than it is now, notwithstanding the recent rise in the number of novel medication candidates for which AOX plays a more significant part.

This gap is due, in part, to inconsistencies between in vitro and in vivo research. In turn, this is due to the absence of an adequate animal model and in vivo techniques for AOX activity research. None of the experimental animals has yet been chosen as the best all-around animal model for hAOX since AOX exhibits noticeable inter- and intraspecies differences. The absence of a suitable technique for determining the in vivo enzyme activity hampers AOX investigations as well.

Interestingly, despite AOX's ability to metabolise a broad variety of substances, there isn't a straightforward, accurate, or reliable way to measure AOX activity in vivo using one of these substances. The variance in AOX activity across and between species in humans and certain common experimental animals is a major topic of this article. Another major focus of this paper is the variance in AOX activity that is substrate-based. Furthermore, the AOX crystalized form has not been solved until the year 2015 due to issues with the stability of the soluble version of the enzyme. There aren't many in silico techniques for predicting AOX activity as a result of the weak in vivo/in vitro association, lack of trustworthy experimental data, and other factors. The developed QSAR and molecular modelling approaches for AOX will also be examined in this study to solve these issues.

Aldehyde oxidase: General features and mechanism of action

Two cytosolic molybdenum-containing enzymes, aldehyde oxidase (AOX, EC 1.2.3.1) and xanthine oxidase (XO, EC 1.2.3.2), share a number of physicochemical characteristics, including cofactor composition, molecular weight, absorption spectrum, and subunit structure. They also exhibit a high degree of amino acid sequence homology. Both enzymes are homodimers made up of two 150 kDa identical subunits, each of which is separated into three different domains (Figure 1).

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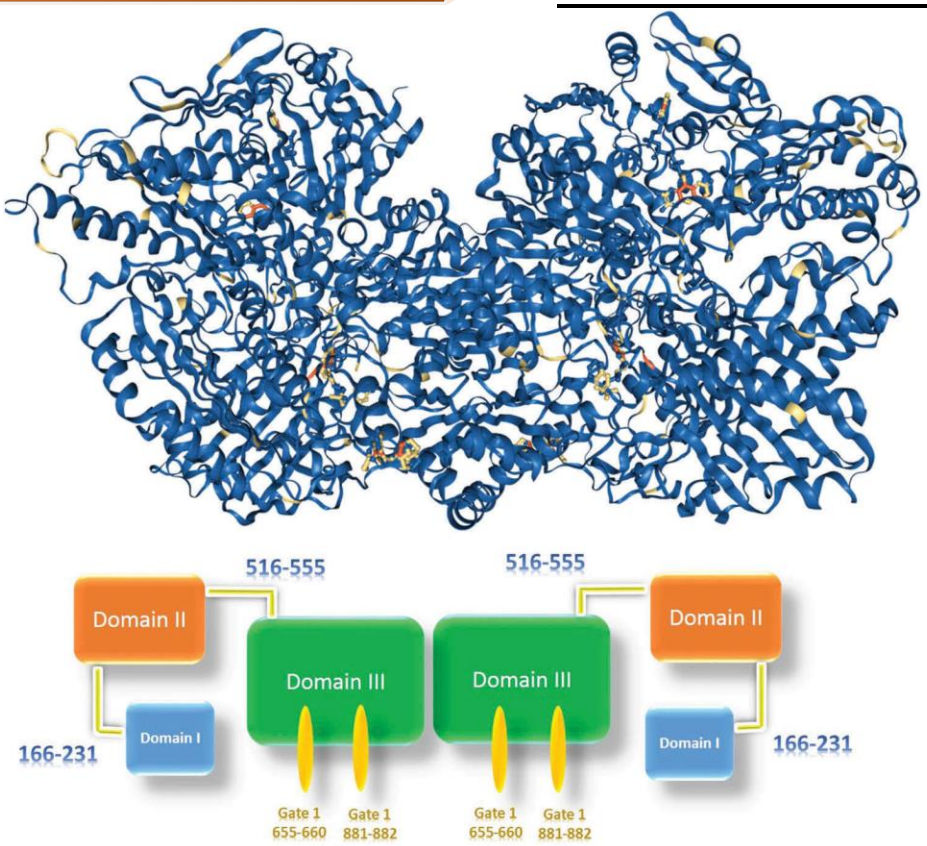

Figure 1. Schematic structural features of hAOX and its representation.

The fact that AOX is extensively spread across the animal kingdom may mean that animals value this enzyme's principal function. It is present in a variety of organisms, including man and sea anemones. For XO, only one transcriptionally active gene has been shown, but many AOX genes, each encoding a different isoenzyme, have been anticipated based on the sequencing of vertebrate genomes. It is quite likely that these two enzymes have a very close evolutionary relationship based on their structural and functional similarities, as well as their amino acid sequences with XO. Both enzymes may have descended from a single primitive enzyme through gene duplication and subsequent differential development. It is believed that the AOX gene has undergone two distinct and evolutionarily distinct duplication events from an ancestral XO gene. The AOX precursor of worm, insect, and plant enzymes was created by the first occurrence. The vertebrate homologue of AOX was created starting from fishes during the second duplication event. Thought to be a more primitive enzyme, XO was primarily responsible for oxidising endogenous purines and related substances. In general, the highest levels of AOX are found in the vertebrates with herbivores and carnivores containing the highest and lowest levels of the enzyme, respectively.

AOX is known for the ability of accommodating various compounds in its substrate pocket. Aldehyde oxidation to a carboxylic acid along with aromatic N-heterocycles oxidation is the most important role of AOX in drug metabolism. AOX as both oxidative and reductive enzyme can identify a wide range of chemical structures (e.g. aldehydes, nitroso compounds, and iminium ions to a wide range of heterocycles). Figure 2 shows a simplified scheme of AOX-catalyzed oxidation of compounds. AOX generally catalyzes the oxidation reactions which usually involve

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nucleophilic attack to an electrondeficient carbon atom of N-heterocycles that are para or adjacent to the nitrogen atom (Figure 2).

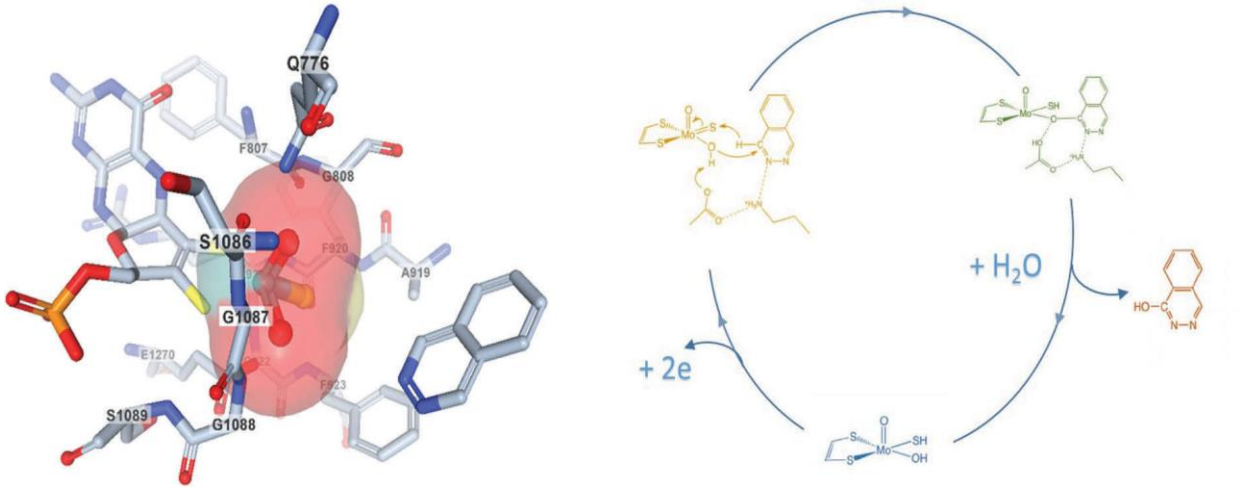

Figure 2. Simplified scheme of AOX-catalyzed oxidation of phthalazine and the interaction mode of phthalazine with the catalytic site.

The essential element for compounds to qualify as an AOX substrate is the heterocycles' receptivity to this nucleophilic assault. Recently, it was shown that AOX plays a crucial role in the hydrolysis of amid bonds.

Aldehyde oxidase catalytical binding site

The crystal structures of highly similar enzymes like XO and murine AOX were used to characterise the structure of hAOX, which had not yet been crystallised, in 2015.

With the use of a recombinant enzyme, Coelho and colleagues were able to determine the crystal structure of the human AOX in both its free form and in association with phthalazine (as the substrate) and thioridazine (as the inhibitor). Their findings supported the distinct substrate, inhibitor, and catalytic activity locations in AOX that had been anticipated from homologous enzyme structures. Each monomer, in accordance with their findings, consists of 1336 residues, which can be further subdivided into three distinct domains based on the cofactor localization: (a) two iron-thiol cluster domains (domain I), (b) a flavin adenine dinucleotide (FAD)-binding domain (domain II), and (c) a molybdenum pyranopterin cofactor (MoCo) (Figure 1).

The small N-terminal domain I contains two spectroscopically distinct iron-sulfur centers and can be further subdivided into two smaller domains, each with one [2Fe-2S] center. The linkage between domain I and domain II (residues 166–231) could be regarded as a surface-exposed region, which is similar to XO and xanthine dehydrogenase (XDH) structures (Figure 1). Two leucine residues (Leu344 and Leu438) stacked FAD's isoalloxazine within domain II (Figure 3). The loop at the entrance of the FAD pocket is flipped almost 180° in comparison with other enzyme species, which provided a unique conformation for hAOX. This loop is blocking access of the electron acceptor to the isoalloxazine ring. Domain III (catalytic domain), is linked to domain II utilizing a linker composed of residues 516–555. The O = Mo = S (-OH) catalytic center (Moco) is coordinated by the dithiolene group of a pyranopterin molecule (Figure 3).

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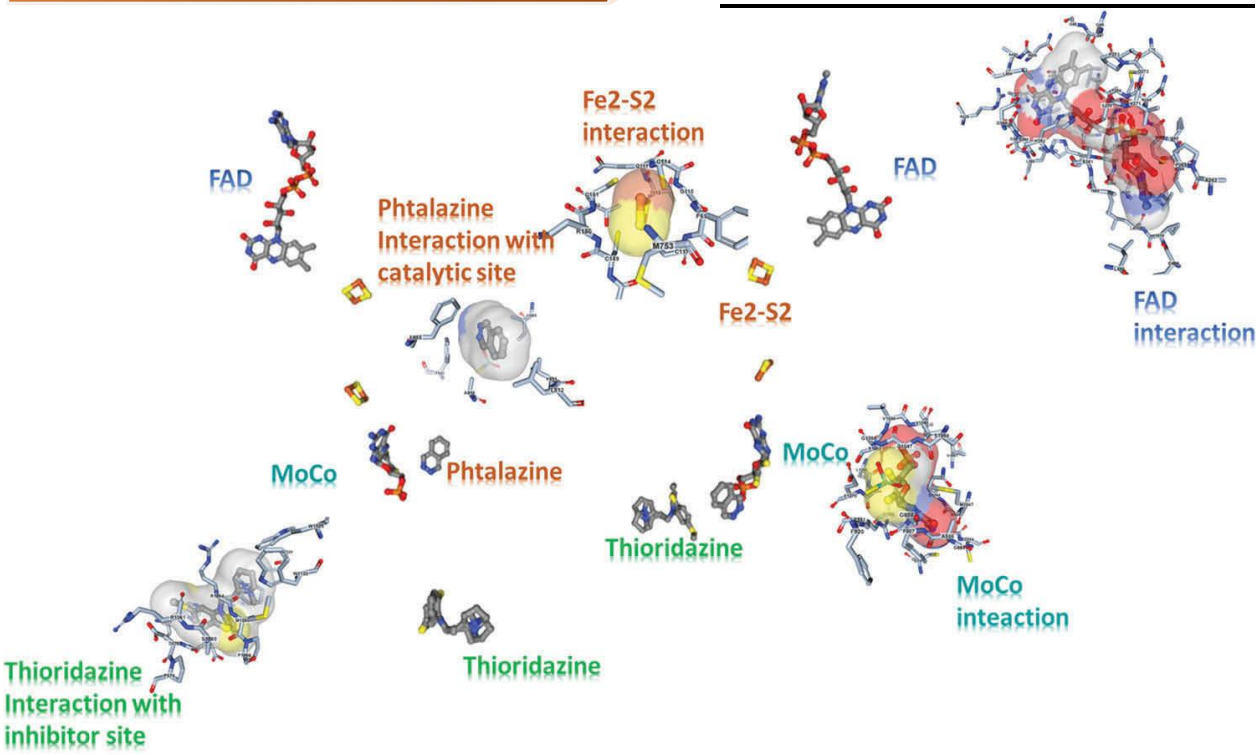

Figure 3. The arrangement of the inhibitor site, catalytic site, FAD, MoCo, Fe2-S2 moieties within the hAOX with the surrounding amino acids.

The structural characteristics of a large, deep funnel that enters the active site cavity in hAOX control the substrate access. Given that they are situated in two loop sections from domain III, some of the residues that border the funnel's entry are movable. These two putative gates (loops) are located at the protein surface. Through these loops, substrates and inhibitors may be able to reach the Mo catalytic site. The flexibility of these two gates (gates 1 and 2) is thought to play a significant role in the specificity-determining characteristics of mammalian AOX. Gate 1 features a lengthy, flexible loop that can vary in length in humans.

In hAOX, Gate 2 is home to two acidic residues. Those two residues' side chains are oriented towards the substrate channel. Polar substrate molecules can attach to the enzyme's catalytic centre in the proper way thanks to this kind of orientation. These findings imply that they could work, most likely via an induced-fit process. As a result, the AOX's promiscuous catalytic activity can be explained by the ability of the bulky and chemically varied substrates to access the active site cavity of the enzyme.

There are some differences and similarities between hAOX with other species AOX and XO in the explained gates whichhelp to explain the catalytic and specificity differences among species for the individual enzymes. The detailed differences have been discussed in some comprehensive review articles. The presence of each amino acid in the active site is important for the substrate affinity and it has been shown that single-nucleotide polymorphisms (SNPs) can result in the loss of enzyme activity. In the wild-type hAOX, the Ser1271 residue is located in the Moco active site region immediately after the catalytic Glu1270. A hydrophobic environment is created by the Phe655, Phe885, Leu1018, and Ala1023 in the active site of hAOX, which interact, with the aliphatic regions of ligands.

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The crystal structure of the hAOX-phthalazine-thioridazine ternary complex showed that the side chain of Phe-885 is mobile (establishing a π–π stacking interaction with the aromatic moiety of the ligands), and one of the two gates becomes better ordered following ligand interaction. The results confirmed the availability of distinct binding sites for the substrate and the inhibitor in hAOX. Phe923 also interacted with aromatic ring of the substrate.

A novel inhibitor-binding site in hAOX has been suggested by Coelho and coworkers. In this model, thioridazine interacts with the enzyme in a noncompetitive manner which could be confirmed with the kinetic data. Thioridazine is located near the dimer interface in a groove at the enzyme surface and is stacked between two loops. This could be regarded as the binding pocket for tricyclic phenothiazine moiety while, the methyl piperidine chain extends outside of the cleft. The tricyclic aromatic moiety interacts mostly with the main chain atoms. There are, however, local differences that may account for differences in inhibition among the various species AOX. Human and mouse crystal structures show marked differences of amino acids around the molybdenum cofactor/substrate binding site. Replacing Ala1023 and Leu1018 in hAOX by tyrosine and phenylalanine in murine AOX resulted in a wider entrance of the substrate funnel in hAOX. The findings about the possibility of metabolizing of large and bulky drugs with hAOX could be discussed using these results.

Following the ligand interaction, repositioning of Phe885 and Phe923 residues, could be crucial for the substrate orientation toward the Mo catalytic site. The structural similarity of the catalytic site between XO family members suggests that there have to be either a common inhibitor-binding site or a general mechanism of enzyme inhibition for other drugs and drug-like compounds. The existence of this inhibitor-binding site far from the hAOX active site is a useful finding for a structure-guided drug design approach and in the development of target-specific drugs.

Terao and coworkers proposed two different regions for substrate binding sites in mice AOX; (a) one region characterizing the inner active site.

(b) an isoform-specific region located in the distal half of the catalytic tunnel which shows remarkable differences in the four mice Aox isoforms (they determine the nature and chemical shape of as yet undetermined ligands recognized selectively by AOX isoforms).

Animal models for AOX

Animal models have been used in medical research for a long time, and they are frequently used in drug discovery and preclinical investigations of medication safety and efficacy. Finding a suitable model for people is a difficult undertaking, nevertheless, given the significant variances and discrepancies between human models and animal models. Since enzymes are crucial to drug metabolism, there are considerable species differences in the activity of the enzymes that account for a large portion of the disagreement between animal models and humans.

Significant inter- and intraspecies heterogeneity in AOX activity has been thoroughly reported, as it was previously indicated. Because of this, it has been challenging to extrapolate human PK characteristics of substances that may behave as AOX substrates from preclinical animal model data. To determine the best animal model for hAOX, it is therefore interesting and crucial to explore the enzyme activity of diverse species towards various AOX substrates. Although several animals have been proposed as animal substitutes for hAOX by various researchers throughout the years (Table 1), there is no universal agreement on this matter. We will concentrate our attention in this review on those species that have been commonly exploited as AOX sources in investigations of drug metabolism.

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Table 1. Metabolism of some compounds by aldehyde oxidase from different species.

	Human	Guinea pig	Rat	Monkey	Mouse	Rabbit	Dog	The best model for human enzyme
Famciclovir	√	√	√ (SD)	–	–	√		Guinea pig
Zaleplon	–	–	√ (SD)	√ (C)	–	–		Monkey
Methotrexate	√	√	–	–	–	√		None
SB-277011	√	–	√ (SD)	√ (C)	–	–	√	Monkey
RS-8359	√	√	√ (W)	√ (C)	√	√		None
Zebularine	√	–	√ (SD)	√ (C)	√		√	None
GABA agonist	√	–	√ (SD)	√ (R)	–	–	√	Monkey
SGX523	√	–	√ (SD)	√ (C)	–	–	√	Monkey
p38 kinase inhibitor	√	–	√ (W)	√ (C)	√	–	√	Mouse
Zoniporide	√	√	√ (SD & W)	√ (C & R)	√	√	√	Rat
Phthalazine & DACA	√	√		√	–	–		None
Lenvatinib	√	–	√ (SD)	√ (c)	–	–	√	None
BIBX1382	√		√ (W)	√ (C & R)			√	Monkey
Ripasudil	√	–	√ (SD)	√ (C)	–	√	√	Monkey
Methotrexate	√	√	√	√	–	√		None

Monkey

The expression of AOX in monkeys has been demonstrated by several studies. Cynomolgus and rhesus are two monkey species that have been used as an AOX source in different studies. Sugihara et al. have shown high cynomolgus monkey liver AOX activity toward several known AOX substrates, including benzaldehyde, phthalazine, and N1-methylnicotinamide. These authors proposed cynomolgus monkey liver AOX as a model for human hepatic

AOX in drug metabolism studies. Later, this was further supported by Hutzler et al by in vitro study of BIBX1382 (an epidermal growth factor receptor inhibitor) metabolism in liver cytosol and cryopreserved hepatocytes from multiple species and comparison of the BIBX1382 PK in cynomolgus monkey with the reported human clinical data of this compound. Based on these studies, they suggested cynomolgus monkeys as a suitable surrogate for the observed hAOX metabolism of BIBX1382. However, according to some other studies and the results they yielded, cynomolgus monkey could not be served as an appropriate general animal model for hAOX. For example, in the conversion of a candidate p38 kinase inhibitor to its 4-hydroxylated metabolite as the major metabolite, cynomolgus monkey liver AOX was not found to be quite a suitable surrogate for hAOX; rather, it was the mouse AOX that produced better results.

Another example that is significantly metabolized via AOXmediated pathways in a species-specific manner is SGX523, a mesenchymal-epithelial transition factor inhibitor, which is oxidized to 2-quinolinone SGX523. It has been shown that cynomolgus monkeys make a better animal model than rats or dogs for hAOX in the metabolism of SGX523. However, the inhibitory effects of certain AOX inhibitors on SGX523 oxidation by monkey AOX were not similar to those obtained with the human enzyme. Similar results have been reported for the inhibition of AOX-catalyzed oxidation of lenvatinib by AOX inhibitors, menadione, and raloxifene.

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Rhesus is another monkey species that has been introduced as the best animal model for hAOX for both in vitro and in vivo studies. However, the results have been somehow controversial. In addition, the data and evidence used for this proposition are not very strong.
Human liver expresses only AOX1, cynomolgus monkey liver expresses AOX1 as the major isoform with a small amount of AOX3. Rhesus monkeys have two functional (AOX1 and AOX3L1) as well as one or two inactive (AOX3 and AOX4) pseudogenes.
Taking into account the quality of data and the practical aspects of drug metabolism studies, cynomolgus monkey may be considered as a more suitable animal model than rhesus for hAOX. However, it might yet be difficult to consider cynomolgus as the best all-round model for hAOX.

Rat

Rat is often employed in metabolism studies during the development of potential drugs and in vivo studies. However, this animal usually does not serve as an appropriate animalmodel for hAOX. This has been reported by different authors using different substrates such as 6-methylpurine, zaleplon, p38 kinase inhibitor, SGX523, and BIBX1382.
Sprague-Dawley rat liver AO activity toward 6-methylpurine is 10- and 5-fold lower than that of guinea pig and rabbit, whereas it is 8-fold greater than that of human. On the other hand, Kawashima et al. have found low Sprague-Dawley rat AOX activity toward zaleplon, whereas this compound was oxidized to 5-oxo-Zaleplon as the major metabolite by cynomolgus monkeys and human AOX. Besides, rat shows significant variations in AOX activity, which makes it difficult to use this animal as a suitable experimental model for predicting PK of drugs in human. The existence of a marked variation or a lack of AOX activity in Sprague-Dawley rats has been demonstrated using N1-methylnicotinamide, pyridoxal, 6-methylpurine, phenazine methosulfate, phthalazine, carbazeran, and famcyclovir as substrates. Marked intraspecies variation in AO activity has also been reported in Wistar rat liver by Gluecksohn-Waelsch et al. They found appreciable N1-methylnicotinamide oxidase activity in only 36 out of 76 animals examined.
In addition, significant variations in AOX activity in different strains of rat have been reported for the conversion of methotrexate to 7-hydroxymethotrexate, N1-methylnicotinamide to its 2- and 4-pyridone metabolites, RS-8359 to its 2-oxo metabolite and zaleplon to 5-oxo-zaleplon. A possible genetic basis for the variation in AOX activity in both Sprague-Dawley and Wistar rats has been postulated. Rat AOX also differs from that of human in terms of its isoforms. As with rabbit, two isoforms have been reported for rat as Aox1 and Aox3 with the later enzyme as the predominant AOX form, whereas humans possess a single enzyme (Aox1).

Guinea pig

As with rat and rabbit, the highest level of AOX in guinea pig is found in the liver using 6-methylpurine, phthalazine, or phenanthridine as substrates with kidney and small intestine containing descending levels of enzyme activity. Guinea pig AOX activities in the liver and intestine are higher than those in rat. Unlike rat liver, guinea pig hepatic AOX activity measured with either 6-methylpurine, phthalazine, or phenanthridine showed less intraspecies variation. According to some studies, guinea pig liver AOX has a closer resemblance to human liver. A close resemblance of guinea pig liver AOX to that of man in terms of kinetic parameters and drug interactions has been reported in the famciclovir metabolism by Rashidi coworkers (1997). They showed a similar in vitro metabolic pathway for famciclovir with guinea pig and human liver AOX and the interaction of 13 drugs with 6-deoxypenciclovir oxidation catalyzed by guinea pig liver AOX was found to be very similar ($r = 0.96$) to that catalyzed by human liver enzyme.

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Guinea pig liver synthesizes a sole active Aox1, which is an ortholog of humans; however, this animal is characterized by three active Aox1, Aox4, and Aox2 genes, which makes guinea pigs different from humans in terms of AOX isoenzymes.

In general, a combination of factors including the high level of AOX activity, less intraspecies variation in the enzyme activity, closer resemblance to human liver AOX, and other practical considerations, such as availability, usability, and costs may make guinea pig hepatic AOX as the best animal model for AOX study.

Rabbit

As it was mentioned, rabbit liver was commonly used as a source of AOX as it was thought to have a high oxidation capacity with a broad substrate specificity. High levels of AOX are found in the rabbit liver, small intestine, lungs, and kidneys, the relative activities of which depend on the substrate used. With 6-mercaptopurine, the liver appears to contain the highest AOX levels, whereas with methotrexate similar amounts are found in the lungs, kidneys, and liver. It has been employed as an AOX source in some comparative studies of AOX specificity to assay contribution of AOX in drug metabolism and to predict metabolism of heterocycles in man. However, there is a large body of evidence that the rabbit enzyme is not always representative of the human liver AOX activity. Thus, carbazeran is a good substrate for baboon, guinea pig, and hAOX, but it is not oxidized by rabbit enzyme. On the other hand, methotrexate is rapidly converted to 7-hydroxymethotrexate by the rabbit liver AOX, whereas oxidation occurs very slowly with rat, guinea pig, mouse, and human liver. The results obtained from isoelectric focusing analysis of liver enzyme fractions from various species have indicated that rabbit liver AOX has marked differences from the human liver enzyme in terms of the pI values. Isovanillin, which has been frequently used as AOX inhibitor in various studies, is a substrate of rabbit liver AOX undergoing oxidation to isovanillic acid with a Km = 45 μM and Vmax = 0.211 μmol/min/mg protein. The metabolic pathway of famciclovir with rabbit liver AOX is different from that of human, guinea pig, and rat liver AOX. Rabbit liver AOX also differs from that of human's in that both Aox1 and Aox3 have been identified as the major isoforms of AOX expressed in the rabbit liver, whereas human liver synthesizes Aox1 as the only AOX isoform. Therefore, it appears that rabbit liver AOX is not a good representative of AOX activity from other mammalian species and may fail to serve as a good animal model for the human enzyme in spite of its high activity toward some heterocyclic and aldehyde substrates.

Human

According to previous studies, human liver is considered as a low AOX level species; however, recent in vitro and in vivo studies have provided a large body of evidence indicative of high human liver AOX capacity in drug metabolism.

Compared to XO, there are not many studies on the activity and substrate specificity of human liver AOX, but the number of these types of studies is increasing rapidly. One of the reasons for these observations arises from the enzyme instability, which has been more dominant in the past studies. Duley et al. observed high activity of human liver AOX in surgical biopsy samples toward benzaldehyde on cellulose acetate electropherograms, which rapidly disappeared with storage, or in postmortem tissues. The unstable nature of human liver AOX may lead to an underestimation of the enzyme activity and a marked inter-sample variability. The problems arising from instability of human liver AOX could become more significant in drug screening programs. Purified enzymes are usually most common source for in vitro studies in drug discovery, but as it was mentioned

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before, hAOX is a relatively unstable enzyme and may lose a significant part of its activity during the purification process and thus become inactivated very rapidly upon surgical excision, death, freezing-thawing of the samples. Furthermore, AOX activity in human donors is highly variable and this variability becomes even more pronounced when different substrates are used in the enzyme activity measurements.

Therefore, it is not clear whether the reported variation in hAOX activity arises from its stability or intrinsic activity and developing appropriate enzyme assay methods can help clarify the issue and provide more valid and reliable data concerning AOX activity in drug screening and discovery studies. Furthermore, the selection of an appropriate animal model for hAOX is a problem because of the different substrate specificity of enzyme from each species while this enzyme shows extreme substrate-dependent inter- and intraspecies variation. Genetic considerations will not be enough to determine the best animal model for hAOX. For example, in humans, there is only a single active gene, Aox1, which is the ortholog of rodent Aox1. Although this protein has over 90% similarityto the rhesus Aox1 counterpart, this species has shown the least activity in converting zoniporide to M1 out of 10 species used in the study. In addition, Fu et al. (2013) have shown very low human liver AOX activities in several donors despite having normal Aox1 protein expression, which indicates that Aox1 protein expression levels alone cannot be a precise indicator of AOX activity. According to Choughule et al. (2015), human, monkey, and rabbit livers have almost similar expression levels of Aox1; however, their AOX shows significantly different activity toward methotrexate.

Overall, the in vitro and in vivo evidence indicates that human liver AOX activity is higher than it was previously assumed to be and that the enzyme is capable of handling those xenobiotics, which have a reasonable affinity to the enzyme. That is why the number of reviews indicating the importance of AOX in drug metabolism and development is growing quite fast.

Substrate-based variation in AOX activity

Some part of the species variation in the AOX originates from the selection of its substrate for the enzyme activity assays. Different substrates have been used to assay AOX activity whereas XO activity has been usually monitored with a single compound, xanthine. However, hepatic AOX shows a marked variation in substrate specificity which may lead to an exaggeration of interspecies variation of AOX activity. Furthermore, it is difficult to extrapolate the results obtained for the assay of enzyme activity from one animal species to another and ultimately to man. For instance, carbazeran is a good substrate for guinea pig, baboon, and human liver AOX, whereas it does not serve as a substrate for rabbit enzyme. Conversely, methotrexate is a more efficient substrate for rabbit liver AOX than that of other species. Furthermore, the site of substrate oxidation catalyzed by AOX may be species dependent. Taylor et al. have indicated that the N-phenylquinolinum perchlorate is oxidized at two alternative positions by both rabbit and guinea pig liver AOX but the ratio of the two-oxidation products is species dependent. Similarly, N1-methylnicotinamide is oxidized to two EXPERT OPINION ON DRUG DISCOVERY 311 metabolites, a 2-pyridone (N1-methyl-2-pyridone-5-carboxamide) and a 4-pyridone (N1-methyl-4-pyridone-3-carboxamide) by liver AOX from various species; however, the ratio of the 4-pyridone to the 2-pyridone metabolite differs from one species to another.

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Prediction of aldehyde oxidase activity

The ability to predict the extent of biotransformation via a particular pathway is very important from drug development point of view. AOX activity prediction is more difficult comparing with other metabolizing enzymes e.g. cytochrome P450. Lack of suitable animal models is one of the main reasons for difficult predictions. Thanks to two decades of in vitro study on aldehyde oxidase, which has made available a large body of data, there is presently a growing interest in developing in silico methods for aldehyde oxidase substrate prediction. Resolving of its crystal structure would lead to the more computational study. There are currently very few methods described in the literature to predict the metabolism (site of AOX oxidation, or AOX clearance) of xenobiotics by AOX and according to the literature review, there is no comprehensive QSAR study or molecular modeling method for the activity prediction of hAOX. A qualitative comparison between amination sites of 1- alkyl-3-carbamoylpyridinium chlorides in liquid ammonia with the sites of oxidation of them by rabbit liver AOX were reported by Angelino et al. Although the developed method correlated well with amination and oxidation patterns, its substrate dependency limited its application. Dastmalchi et al. developed a homology model of hAOX (using the crystal structure of bovine XDH as a template) to study the mode of interaction between the enzyme and its substrates. They applied the developed model for a 3D-QSAR model construction to predict the sites of metabolism phthalazine and quinazoline compounds. Their method showed about 65% accuracy for the test set compounds.

Torres has reported the application of density functional theory methods to geometry optimization of tetrahedral intermediates resulting from the nucleophilic attack of a hydroxyl nucleophile to predict the regioselectivity of oxidation of drugs and drug-like compounds by AOX. This predictive method showed >90% accuracy for heterocyclic compounds. Barr and coworkers developed the first molecular modelling study, using hAOX on diet-derived chemicals. Molecular modeling was conducted using homology-modelled hAOX. The results showed interaction between studied compounds both with AOX active site and between inhibitor sites. The docking score correlated well with the measured activity (r^2 = 0.5). According to their results, ECG interacted by its phenyl rings using a π- stacking interaction with the Phe923 and Phe885. In addition, hydrogen bonding occurred between two phenol moities and the carboxylate group of Glu882. Similar interactions were reported for AOX substrates zoniporide, DACA, and substituted phthalazines. The results confirmed previous reports about the competitive inhibiting mechanism for the AOX inhibitors, while other results showed evidences of noncompetitive inhibition too. They also developed a QSAR model for inhibitory potency prediction by the application of 44-structural descriptors. The best-fitted model (r^2 = 0.85) contained three molecular descriptors: dipole, the hydrophilic solvent-accessible surface area (FISA), and hydrogen bonding-accepting capacity (accpt HB).

They predicted the AOX-mediated dietary substance (epicatechin gallate and epigallocatechin gallate as prominent constituents in green tea) – drug interactions. According to their results, there is a moderate-to-high-risk interaction between green tea and AOX-mediated drug metabolism.

A homology model for hAOX was produced by Sodhi and coworkers. Induced-fit docking was used to place N-[(2- dimethylamino) ethylacridine-4-carboxamide into the active site of AOX and the results suggested a new role (amide hydrolysis) for AOX-catalyzed metabolism of N-heterocyclic compounds.

Molecular docking of methotrexate into the active site of the human and rabbit AOX enzymes studied recently showed that the hAOX has only three residues in the active site that are different from rabbit AOX, while the experimental studies showed that the metabolism of methotrexate in

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rabbit liver cytosol was several orders of magnitude higher than that in other species. The extent of variations has been studied by SNPs and the results have confirmed the importance of the active site amino acids. The methods described here only allow a prediction of suitable position of AOX catalyzed oxidation, while quantitative models to estimate AOX oxidation mechanism have not been reported.

Jones and Korzekwa have developed an in silico model to predict both in vitro and in vivo human intrinsic clearance for 8 drugs with combined steric and electronic properties. Their models could explain the differences between applied data well. They used computational estimates of the electronic and steric features of the reaction. They concluded that AOX is able to metabolize large molecules. They proposed out of plain steric bulk group's conformation in accordance with MoCo reaction centre, which can slowdown the metabolism. None of the developed models can predict whether a compound will be an AOX substrate.

Conclusion

Recently, AOX has drawn more attention from drug development programmes, and more novel drug candidates have emerged for which AOX has a more significant role. The incongruence between in vitro and in vivo investigations is a problem for AOX studies. This is a result of the insufficient in vivo techniques and suitable animal model for AOX activity research. Accordingly, it appears that the assessment of AOX's significance in drug discovery based on these in vitro approaches is unreliable and that its true relevance may be far more than what was previously believed to be the case. More recently, extremely encouraging developments in the realm of stem cells have made it feasible to develop complex multicellular, almost physiological, and self-renewing tissues known as "organoids." With the use of this new technique, it would be possible to measure human extrahepatic AOX activity, figure out how to anticipate this enzyme's metabolic clearance, and prevent underestimating human clearance of AOX substrates. While the present models do not allow for the categorization of chemicals into AOX substrate and non-substrate, the established CADD approaches can predict the AOX activity. Using hAOX crystal structure in 3D models might aid in the development of models with substrate selection capabilities. The real levels of AOX in human liver are now clearly higher than those previously reported, and the enzyme activity may be shown using the right animal model or in vitro and in vivo techniques. Numerous factors contribute to underestimating AOX activity. Since AOX is an unstable enzyme, the enzyme activity is lost significantly throughout the purifying processes. As a result, judging the significance of AOX in drug discovery based only on these in vitro approaches is unreliable, and its true significance may be substantially more than previously thought. Providing concrete evidence that this enzyme is involved in the metabolism of novel medicines is another challenge with hAOX. Finding an adequate animal model for these investigations has proven challenging due to the wide species diversity in AOX activity. This challenge may be regarded as one of the major bottlenecks in the development of AOX-oriented drugs. Unfortunately, solutions to the issues with in vitro models for hAOX haven't proven very effective. Therefore, the availability and selection of a suitable and accurate in vitro test is a requirement to elucidating the function of AOX in drug metabolism.

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