A Novel Ring Oxidation of 4- or 5-Substituted 2H-Oxazole to Corresponding 2-Oxazolone Catalyzed by Cytosolic Aldehyde Oxidase

Vinod K. Arora, Thomas Philip, Stella Huang and Yue-Zhong Shu

Department of Biotransformation (V.K.A., T.P., Y.Z.S.) and Department of Discovery Analytical Sciences (S.H.), Bristol-Myers Squibb Research & Development, 5 Research Parkway, Wallingford, CT 06492, USA.
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Correspondence author: Vinod K. Arora, Tel: 203-677-7490, Fax: 203-677-6193

E. mail: vinod.arora@bms.com

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ABBREVIATIONS: HPLC, high-performance liquid chromatography; MS, mass spectrometry; AO, aldehyde oxidase; LC-UV/MS/MS: Liquid chromatography-product ion mass spectrum with UV detection; MS/MS: product ion spectrum; UV, ultra violet; \(^1\)H-NMR, proton nuclear magnetic resonance spectrum; NADPH, reduced nicotinamide adenine dinucleotide phosphate; CID, collision induced dissociation; NOE, Nuclear Overhauser Effect; 5BPO, 5-(3-bromophenyl)oxazole.)
Abstract:

The ring oxidation of 2H-oxazole, or C2-unsubstituted oxazole, to 2-oxazolone, a cyclic carbamate, was observed on various 4- or 5-substituted oxazoles. Using 5-(3-bromophenyl)oxazole as a model compound, its 2-oxazolone metabolite M1 was fully characterized by LC/MS/MS and NMR. The reaction mainly occurred in the liver cytosolic fraction without the requirement of cytochrome P450 enzymes and co-factor NADPH. Investigations into the mechanism of formation of 2-oxazolone using various chemical inhibitors indicated that the reaction was primarily catalyzed by aldehyde oxidase and not by xanthine oxidase. In addition, cytosol incubation of 5-(3-bromophenyl)oxazole in the medium containing H$_2^{18}$O led to the $^{18}$O incorporation into M1, substantiating the reaction mechanism of a typical molybdenum hydroxylase. The rank order of liver cytosols for the 2-oxazolone formation was mouse>monkey>>rat and human liver cytosol, whereas M1 was not formed in dog liver cytosol. Since the reaction was observed with a number of 4- or 5-substituted 2H-oxazoles in mouse liver cytosols, 2H-oxazoles represent a new substrate chemotype for ring oxidation catalyzed by aldehyde oxidase.
Introduction

Aldehyde oxidase (AO) and xanthine oxidase (XO) are closely related molybdoflavo proteins in cytosolic fractions and show a high degree of homology in their amino acid sequence (Beedham, 1985). However, the substrate specificities of AO and XO do differ. Physiological function of XO has long been recognized in the catabolism of purines, starting with the oxidation of hypoxanthine to xanthine, and then to uric acid (Kitamura et al., 2006). On the other hand, the biochemical and physiological role of AO is still nebulous (Kitamura et al., 2006; Garattini and Terao, 2011). AO catalyzes the oxidation of a number of aldehydes, and nitrogen containing heterocyclic xenobiotics and drug molecules such as methotrexate, cyclophosphamide, acyclovir, famciclovir, and zaleplon (Kitamura et al., 2006; Pryde et al., 2010). However, in the presence of an electron donor, both AO and XO can catalyze reduction as well, using a variety of substrates like sulfoxides, N-oxides, nitrosamines, hydroxamic acids, azo-dyes, and oximes derivatives (Tatsumi et al., 1983; Sugihara et al., 1997; Kitamura et al., 2006; Pryde et al., 2010), albeit oxidation reactions are far more common. In general, AO has the ability to oxidize a broader range of substrates than XO. Oxidations by AO generally involve nucleophilic catalysis and often occur in electron-deficient ring systems, resulting in metabolites different from those via electrophilic oxidations by cytochrome P450 system.

A number of reviews have been written on the subject of oxidation by AO (Kitamura et al., 2006; Pryde et al., 2010). More recently, Pryde et al. (Pryde et al., 2010) reviewed common aromatic heterocyclic compounds containing structural motifs which are known to be oxidized by AO. Notable structures included electron-deficient 6
membered-heterocycles such as pyridines, pyrimidines, pyrazines, and their fused ring analogues.

Five membered azoles are frequently used in medicinal chemistry as common pharmacophores and also as bioisosteres for esters and amides to prevent hydrolytic instability and improve metabolic stability and other drug-like properties (Dalvie et al., 2002; Chen and Wang, 2003; Dua et al., 2011). Compared to \( \pi \)-rich pyrroles, furans and thiophenes, azole and isoazole ring systems contain an additional nitrogen atom as a new site of basicity and have reduced \( \pi \)-electron density due to decrease in the energy levels of the \( \pi \)-orbitals. Both of these properties lead to lower susceptibility to oxidative metabolism catalyzed by cytochrome P-450. Despite AO and XO are known to catalyze reductive N-O bond cleavage of isoxazoles and isothiazoles (Kitamura et al., 2006; Pryde et al., 2010), typical azoles such as oxazoles have not been reported as a substrate for AO or XO.

However, during our recent biotransformation studies of drug discovery compounds, we observed a novel ring oxidation on the oxazole substructure catalyzed by AO. The oxidation occurs on various 4- or 5-substituted but C2-unsubstituted oxazoles (2H-oxazoles) and forms corresponding 2-oxazolone, a cyclic carbamate (Fig. 7), as products. In this article, we demonstrate evidence of the AO-mediated oxidation using a model compound, 5-(3-bromophenyl)oxazole (5BPO), along with a number of related oxazoles.
Materials and Methods

**Chemicals and liver preparations.** All chemicals and reagents including allopurinol, oxipurinol, methotrexate, menadione, raloxifene, $\text{H}_2^{18}\text{O}$ (97 atom % $^{18}\text{O}$), were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile and water were purchased from J. T. Baker. Pooled male human and mouse liver microsomes were purchased from B.D. Gentest (Woburn, MA), and pooled male, mouse, rat, human, monkey, and dog liver cytosols were procured from B.D. Gentest and from Xenotech LLC (Lenexa, KS).

**Liver microsomal incubations.** Incubations in liver microsomes were performed in duplicates using Tecan (freedom evo model, Tecan Groups, Durham, NC) liquid handler in a 96 well plate (Costar, 96-Well Assay Blocks, #3958, Corning, NY) fitted with a te-shaker. Liver microsomes (445 µL) diluted to a concentration of 0.5 mg/mL with phosphate buffer (100 mM, pH 7.4) were pre-incubated with 5BPO (5 µL x 0.5 mM in DMSO) for 3 mins. Reaction was started by addition of NADPH (50 µL x 10 mM) and the incubation was further allowed to proceed for 30 min. The final concentration of substrate was 5 µM. Aliquots of samples were taken at 0 (immediately following addition of NADPH) and 30 min. Reactions were stopped by addition of equal amounts of acetonitrile. After precipitation of the proteins, the reaction mixture were filtered through 96 well Phenomenex Strata impact protein filtration plates (Phenomenex, CA) by centrifugation at 2000 x g for 2 min.. The resulting filtrate was analyzed using LC-UV/MS/MS for metabolite profiling.
Liver cytosol incubations and half life determinations. Incubations in liver cytosols were performed in duplicate using Tecan liquid handler in a 96 well plate as above. First a mixture of 490 µL of liver cytosol (diluted to concentration of 0.5 mg/mL in potassium phosphate buffer, 100 mM, pH 7.4) and 5 µL of 50% aq. DMSO was pre-incubated at 37 °C for 3 mins. Following which an appropriate compound/substrate (5 µL x 0.5 mM in 50% aq. DMSO) was added to the incubation mixture and subsequently, the mixture was allowed to incubate for additional 0.5 hr. The final concentration of substrate was 5 µM and the total incubation volume was 0.5 mL. Aliquots of samples were taken at 0 (immediately following addition of substrate), 15 and 30 min. Reactions were quenched by adding equal volumes of acetonitrile and were processed as in liver microsomes.

Half life of 5BPO and other 4- or 5-substituted or ring-fused 2H oxazole compounds in liver cytosols was determined using the procedures discussed above, except that incubations were carried out up to 240 min. An aliquot of samples were taken at 0, 1, 3, 5, 10, 30, 60, 90, 120, 180, and 240 mins. Percentage of parent remaining was calculated based on LC-UV (λmax) peak area of parent oxazoles at various times.

In a separate experiment effect of various molybdenum hydroxylase inhibitors on formation of M1 was studied by carrying out incubations in a similar fashion as above. Initially, a mixture of 490 µL of diluted liver cytosol (0.5 mg/mL) and 5 µL of chemical inhibitor in 50% aq. DMSO was pre-incubated at 37°C for 3 min followed by addition of substrate (5 µL, 0.5 mM in 50% aq. DMSO). The chemical inhibitors were added at final incubation concentrations of 100 µM, except for raloxifene, which was used at a final
concentration of 50 µM. For all the experiments, the final concentration of DMSO was kept at 1% (v/v).

**Incorporation of $^{18}$O into metabolite M1.** Incorporation of 18 labeled oxygen from the medium into metabolite M1 was assessed by incubation of 5BPO (5 µL x 0.5 mM in DMSO) at 37°C in mixture of 250 µL of mouse liver cytosol fraction (1.0 mg/mL, brought up in potassium phosphate buffer, 100 mM, pH 7.4) and 250 µL of H$_2^{18}$O (97% purity). Reactions were terminated at 0 and 60 min by the addition of equal volume of acetonitrile and mixed. After centrifugation at 16000 x g for 2 min., resulting supernatants were analyzed by LC-UV/MS/MS using the method detailed below for metabolite profiling, except orbitrap mass spectrometer was operated at a mass resolution of 100,000 in order to differentiate $^{81}$Br from $^{18}$O isotope. Fraction of $^{18}$O incorporated into oxygenated M1 was calculated using the metabolite isotopic profile.

**HPLC/MS analysis.** Chromatographic separations were carried out using an Agilent 1100 Series Separation Module (Palo Alto, CA) and an YMC Pro C8 column (2.0 x 150 mm, 5µ) maintained at room temperature at a mobile phase flow rate of 0.3 mL/min. The mobile phase consisted of 95/5 v/v mixture of 0.1% aq. formic acid and acetonitrile (Solvent A) and 70/30 v/v mixture of acetonitrile and methanol (Solvent B). LC separation was performed using the following gradient conditions: initially solvents were held isocratically at 0% solvent B for 0.5 min, followed by a linear gradient to 100% solvent B over 23 min. Solvent B was then held isocratic at 100% for 3 min, followed by immediate return to 0% solvent B in 0.1 min, and re-equilibrated for 4.9 min. Total LC
Purification of metabolite M1 of 5-(3-bromophenyl)oxazole. 5-(3-bromophenyl)oxazole (5BPO, 10 µM) was incubated in 30 mL (6 x 5mL) of mouse liver cytosol at higher protein concentrations, 1 mg/mL for 60 min. Under the scale-up incubation conditions, the LC-UV profile showed that 5BPO was completely consumed and converted to M1. Reaction was terminated by the addition of equal volume of acetonitrile as above. After centrifugation at 16000 x g for 2 min., resulting supernatant was dried under stream of dry nitrogen and reconstituted in 800 µL of 50/50 mixture of methanol:water, mixed, and centrifuged. Resulting supernatant was injected onto HPLC system by multiple injections for isolation and purification of M1 using a published methodology (Johnson et al., 2010). Fractions (0.4 min/well) were collected in a 96 well plate. The fractions containing M1 as determined by LC-UV were combined and evaporated to dryness under nitrogen.

NMR analysis of M1 of 5-(3-bromophenyl)oxazole. The isolated M1 was analyzed using a Bruker AV500 NMR spectrometer. The sample was dissolved in 150
μL of DMSO-d$_6$ and transferred to a 3 mm NMR tube. The data collected included a $^1$H (at 500 MHz) and $^{13}$C (at 125 MHz) spectra in 1D and 2D formats. All chemical shifts were referenced to TMS at 0.00 ppm. Nuclear Overhauser Effect (NOE) experiments were performed in order to obtain through space connectivity. Structure of M1 was elucidated by comparing its $^1$H and $^{13}$C chemical shifts with that of 5BPO under similar conditions.

**Results**

**Biotransformation of 5BPO in liver preparations.** After incubation in mouse liver microsomes (0.5 mg/mL) supplemented with NADPH, 5BPO (5 μM) was metabolized to a major mono-oxidation metabolite M2 (39%, Rt 15.4 min) and a minor oxidation metabolite M1 (1%, Rt 16.9 min). A representative LC-UV/MS chromatogram of 5BPO incubation is shown in Fig.1 (panel C). Both M1 and M2 eluted earlier than 5BPO. Percentages of metabolites are calculated based on the LC-UV peak area at $\lambda_{max}$ 261 nm of 5BPO.

Interestingly, the microsomal incubation of 5BPO without addition of NADPH only yielded M1 (6%) but not M2 (Fig. 1, panel A), suggesting that while M2 appeared to be a cytochrome P450 mediated metabolite, the formation of M1 was likely due to the catalysis by residual cytosolic components present in the mouse liver microsomes.

A subsequent incubation of 5BPO in mouse liver cytosol was carried out and LC-UV/MS/MS analysis revealed M1 as a single major metabolite (Rt 16.9 min) in the
HPLC chromatogram, accounted for 89% of 5BPO-related components. (Fig. 2, panel C).

**Identification of metabolites.** Structure of M1 and M2 were established on the basis of their LC/MS/MS product ion mass spectra compared to that of 5BPO. The mass spectrum of 5BPO (Fig. 3) furnished a molecular ion containing bromine isotope pair at m/z 223.9700/225.9680 (calculated accurate mass: 223.9706/225.9685, error: -3 ppm). The product ion mass spectra (MS/MS) of m/z 223.9 gave a major daughter ion at m/z 168.9644 (calculated accurate mass: 168.9647, error: -2 ppm) suggested it to be bromophenyl moiety and an adjacent carbon that is a part of the oxazole ring in the molecule. The molecular ion of metabolite M1 and M2 both appeared at 16 mass units higher than that of 5BPO (Fig. 4A and 4C) and showed a bromine isotope pair; high resolution mass spectrum proved that both metabolites contained one more oxygen atom than 5BPO. M1 isotopic pair appeared at m/z 239.9649/241.9628 (calculated accurate mass: 239.9655/241.9634, error: -2 ppm). Product ion mass spectra of M1 (m/z 239.9) upon CID gave diagnostic fragment ions at m/z 193.9595 and 168.9644. The composition formula of m/z 168.9 ion was same as that appeared in 5BPO, representing the intact bromo-benzylic substructure. The ion at m/z 193.9 (Fig. 4B) of M1 represented further extension of the bromo-benzylic substructure to include oxazole C5-C4-N3 atoms (calculated accurate mass: 193.9600, error: -3 ppm) of oxazole. Collectively, these ions suggested that the additional oxygen atom was likely introduced to the oxazole at C2 position in M1. It is known that C2-hydroxyl on oxazole exists in its thermodynamically stable 2-oxazolone form rather than the 2-hydroxyl tautomeric
form (Joule et al., 1995). The conversion from oxazole to 2-oxazolone is also implicated by the marked bathochromic shift of UV maxima to 279 nm (a relatively smaller shift was observed for M2 to 272 nm) of M1 from 261 nm of 5BPO. The proposed structure of M1 is shown in Fig. 4B.

The product ion mass spectra of M2 (m/z 239.9) upon CID gave diagnostic fragment ions at m/z 184.9597 and 196.9597 (Fig. 4D). The daughter ion at m/z 184.9 indicated loss of the oxazole C4-N3-C2 portion, leaving charge on the bromophenyl-oxazole C5-O1 moiety (calculated accurate mass: 184.95965, error: -0.4 ppm). Further evidence of C4 hydroxylation came from loss of oxazole O1-C2-N3 moiety leading to daughter ion at m/z 196.9 (calculated accurate mass: 196.95965, error: -0.2 ppm), leading to a proposed structure of M2 in Fig. 4D.

**Confirmation of M1 structure by NMR.** In order to confirm the proposed structure of M1 (Fig. 4B), it was isolated and purified from a larger incubation of 5BPO (10 µM, 6 x 5mL) in mouse liver cytosol using a higher protein concentration (1 mg/mL). The purified M1 was then subjected to 1D and 2D NMR analysis. Proton NMR spectrum of M1 was very diagnostic and the modification on the oxazole ring was indicated by the absence of the singlet proton, at either position C2-H at δ8.28 or C4-H at δ7.58 present in the 1H NMR spectrum of 5BPO (Fig. 6). All proton resonances and coupling pattern belonging to the bromophenyl group in M1 observed were same as in 5BPO (Table 1), confirming that the bromophenyl group was intact. NOE experiments showed correlations between C4-H and C7-H and between C4-H and C11-H, demonstrating the oxidative modification to be present at the C2- position of oxazole as
2-oxazolone. The $^{13}$C chemical shift changes of C4, C5, and C6 of M1 were significant compared to those of 5BPO. These changes were in agreement with chemical shifts predicted by ACD software (Pagenkopf, 2005) for 2-oxazolone (Table 1).

**M1 Formation in mouse liver cytosol in the presence of AO and XO inhibitors.** 5BPO was incubated in mouse liver cytosol in presence of chemical inhibitors of XO and AO (Table 2). Formation of M1 from 5BPO was compared to that of control incubation without an inhibitor. Raloxifene and menadione, two selective inhibitors of AO, caused significant inhibition of M1 formation as compared to that of the control. Amount of M1 formed was reduced to 7% by raloxifene and to 0% by menadione. Allopurinol, oxipurinol, and methotrexate, all selective inhibitors of XO, exhibited minimal to weak inhibition of M1 formation. Amount of M1 formed was reduced to 74% by allopurinol, and was little changed by oxipurinol and methotrexate.

**M1 Formation in mouse liver cytosol in $^{18}$O-Labeled water and LC/MS analysis.** To further assess the involvement of molybdenum hydroxylases, the incorporation of $^{18}$O labeled oxygen into M1 from the incubation medium was studied. 5BPO was incubated in mouse liver cytosol containing a 50/50 mixture of H$_2^{16}$O/H$_2^{18}$O and samples were analyzed by LC-UV/MS/MS. Orbitrap mass spectrometer utilized for MS and MS/MS analysis was operated using a mass resolution of 100,000 to distinguish the mass difference between $^{18}$O and $^{81}$Br isotopes. Analysis of the cytosol incubation mixture showed a MS spectrum with an isotopic pattern of M1 characteristic
of $^{18}$O incorporation (Fig. 5). Major isotopes present under the M1 peak were m/z 239.96546 ($C_9H_6^{79}$Br$^{16}$O$_2$, error: -0.03 ppm), m/z 241.96337 ($C_9H_6^{81}$Br$^{16}$O$_2$, error: -0.2 ppm), m/z 241.96956 ($C_9H_6^{79}$Br$^{16}$O$_{18}$O, error: -0.6 ppm), and m/z 243.96762 ($C_9H_6^{81}$Br$^{16}$O$_{18}$O, error: -0.2 ppm). The total amount of $^{18}$O incorporation was 37% ($=48.5\% \times 0.76$) based on abundance of m/z 241.96956 (76%) relative to m/z 239.96546. 

**5BPO Incubation in liver cytosol from different animal species.** 5BPO (5 µM) was incubated in liver cytosols (0.5 mg/mL) from various animal species including the rat, mouse, dog, monkey, and human and samples were analyzed by LC-UV/MS/MS at multiple time points up to 240 min. Table 3 shows T1/2 obtained from measuring 5BPO disappearance in cytosols across species. Maximum turnover of 5BPO was observed in mouse liver cytosol ($T1/2 = 7$ min) followed by monkey liver cytosol (217 min). M1 was the only metabolite detected in these incubations. Relative percentage of M1, based on LC-UV area was 89% in mouse and 10% in monkey liver cytosol at 30 min of incubation. Whereas only trace amount of M1 was detected in rat and human liver cytosols. Dog liver cytosol, however, did not show any formation of M1.

**Biotransformation of other 4- or 5-substituted or ring-fused 2H oxazoles in mouse liver cytosol.** The compounds investigated were C5-substituted oxazole, 5-(3-chlorophenyl)oxazole and merimepodib, and C4-substituted oxazole: 4-(2-bromophenyl)oxazole, and 4, 5-di-substituted 2H-oxazoles (Table 4). These compounds were incubated in mouse liver cytosol at a substrate concentration of 5 µM
using the conditions as described above for 5BPO. Both C4- and C5-substituted oxazoles formed corresponding 2-oxazolone as the only metabolite in the incubation mixture. T1/2 measuring disappearance of parent oxazoles are listed in Table 4. Maximum turnover of parent was observed in 5-(3-chlorophenyl)oxazole and 4-(2-bromophenyl)oxazole with T1/2 of 4 min. Relative amount of oxazolone metabolite was ≥98% with both oxazoles at 30 min of incubation. Lower turnover was seen with merimepodib, which had T1/2 of 201 min and 15% relative amount of oxazolone metabolite at 30 min of incubation, whereas the two 4, 5-di-substituted 2H-oxazoles were stable in mouse liver cytosol.

Discussion

In the present work we reported, for the first time, the oxidation of 2-oxazole to 2-oxazolone (Fig. 7) catalyzed by aldehyde oxidase, as exemplified by conversion of 5-(3-bromophenyl)oxazole (5BPO) to its corresponding 2-oxazolone (M1) in mouse liver cytosol. Structure of M1 was characterized by LC-UV/MS/MS and confirmed by NMR. The conversion of 2-oxazole to 2-oxazolone was found applicable to a number of other 4- or 5-substituted 2H oxazoles.

When incubated in mouse liver microsomes supplemented with NADPH, 5BPO gave rise to two oxidative metabolites, M1 (1%) and M2 (39%). Surprisingly, metabolite M1 (6%) was also present in the incubation devoid of NADPH (Fig. 1, panel A), suggesting that the formation of M1 was likely due to the catalysis by residual cytosolic components present in the mouse liver microsomes. The initial finding prompted us to
investigate the metabolizing enzyme and reaction mechanism for M1 formation, especially, cognizant of the fact that typical oxazoles have not been reported to undergo non-cytochrome P450 mediated oxidation. When 5BPO was subsequently, incubated in mouse liver cytosol, in absence of NADPH cofactor, LC-UV/MS/MS analysis revealed M1 as a single major metabolite (Fig. 2) and accounted for 89% of 5BPO-related components based on the total UV peak areas.

Most oxazole ring metabolic transformations are mediated by cytochrome P450s, and often led to ring opened products (Dalvie et al., 2002). In early 1950s, Bray and co-workers (Bray et al., 1952) reported conversion of benzoxazole and 2-substituted benzoxazole to an O-aminophenol as a result of oxazole ring opening in rabbits. The benzoxazole ring in L-696,229, an HIV reverse transcriptase inhibitor, was found to undergo ring oxidation and subsequent hydrolysis to an amide in rat liver slices (Balani et al., 1994). Similarly, oxazole ring opening to a diketoamide was observed in metabolism studies of muraglitazar in rat, dog, and human (Zhang et al., 2007) and its formation was mediated via cytochrome P450 in liver microsomes (Li et al., 2006). Oxazole ring opened products such as carboxamidoacetic acid from C2-phenyl substituted oxazole were also reported in rat, dog, monkey, and human (Chando et al., 1999). More recently, Kalgutkar and coworkers showed cytochrome P450 mediated ring opening of a 2H-oxazole to a diol as a primary route of biotransformation of 6-(4-(2,5-difluorophenyl)oxazol-5-yl)3-isopropyl-[1,2,4]-triazolo[4,3-a]pyridine (Kalgutkar et al., 2006; McClure et al., 2006) in liver microsomes from rat, dog, and human. Interestingly, there were only a few examples of oxazole ring oxidation at the C2 position to 2-oxazolone (Marchetti et al., 1973; Maurer and Kleff, 1988; Dalvie et al.,
Ditazole, a 2-amino substituted oxazole, was reported to undergo sequential N-dealkylations to a 2-amino-oxazole, which was further hydrolyzed to 2-oxazolone via a proposed imine tautomer (Dalvie et al., 2002). Similarly, 2-oxazolone has also been observed in the biotransformation of zoxazolamine, a 2-amino-5-chlorobenzoxazole (Van der Graaff et al., 1986) in the rat. However, its formation mechanism remained unclear. In the present study, 5BPO underwent oxazole C4 oxidation (M2) in liver microsomes, however, there was no evidence of oxazole ring opened metabolites. Interestingly, 5BPO also underwent oxidation to 2-oxazolone, M1, in mouse liver microsomes, in the presence or absence of NADPH. A significant amount of M1 formed in mouse liver cytosol incubation with 5BPO strongly suggested that the M1 formation may be catalyzed by aldehyde oxidase (AO) or xanthine oxidase (XO) but not by cytochrome P450. This preliminary finding was rather intriguing as typical oxazoles have never been known as substrates for AO.

AO and XO are capable of catalyzing oxidations of wide range of endogenous compounds such as retinaldehyde, pyridoxal, purine and N-methylnicotinamide (Dastmalchi and Hamzeh-Mivehrod, 2005) and drug molecules of pharmacological and toxicological relevance (Terao et al., 2006). Despite similarity of AO and XO molecular structures and >80% homology, AO carries out majority of oxidative reactions. Oxidation of N-heterocycles by AO generally involves nucleophilic catalysis, resulting in metabolites different from those formed via electrophilic oxidation by cytochrome P450 system. Pryde et. al. (Pryde et al., 2010) in a recent review summarized structural motifs of aromatic heterocyclic compounds which are known to be oxidized by AO, all of which contained one or more nitrogen heteroatoms in six membered ring alone or fused
with five or six membered aromatic ring structure. Unlike cytochrome P450, oxidation catalyzed by AO-like molybdenum hydroxylases generates reducing equivalents and although the enzyme uses molecular oxygen (O₂) as a co-substrate, the ultimate source of the oxygen atom inserted into the substrates comes from water and not from molecular oxygen. AO oxidoreductases mechanism involve attack by oxygen at electron deficient sp² carbon atom adjacent to a ring nitrogen atom such as pyridine, pyrazine, purine, pyrimidine, quinoline, and pteridine (Kitamura et al., 2006). However, no published literatures have demonstrated or implicated isolated or fused oxazole ring system as a substrate structure feature for AO mediated oxidation.

In order to further clarify the potential role played by AO or XO as catalyzing enzyme in the oxidation of 5BPO to 2-oxazolone (M1), two approaches were taken; the first approach was to assess the involvement of molybdenum hydroxylases through incorporation of ¹⁸O into M1 during the mouse liver cytosol incubation of 5BPO prepared with 50/50 mixture of H₂¹⁶O/H₂¹⁸O (97% purity). Samples were analyzed using an orbitrap mass spectrometer with resolution of 100,000 to distinguish the mass difference between ¹⁸O and ⁸¹Br isotopes. Theoretically, a maximal incorporation of ¹⁸O of 48.5% would be expected. Analysis of the cytosol incubation mixture by LC-UV/MS/MS showed an isotopic pattern of M1 characteristic of ¹⁸O incorporation (Fig. 5). The amount of ¹⁸O incorporation was found to be 37% (=48.5% x 0.76) and was calculated based on abundance of isomeric peak representing ¹⁸O incorporation (m/z 241.96956, C₉H₇¹⁸BrN₁⁶O₁₈O), which was 76%, compared to that representing ¹⁶O incorporation (m/z 239.96546, C₉H₆¹⁶BrN₁⁶O, considered 100%). Different levels of ¹⁸O incorporation
have been reported in the literature (Hutzler, et al., 2012) for the AO mediated oxidation of BIBX1382 (33% incorporation), carbazeran (47.5%), and zaleplon (40%).

The second approach was to assess the effect of chemical inhibition on the reaction of 5BPO to M1. Raloxifene was reported to be the most potent inhibitor (Obach, 2004; Obach et al., 2004) of AO found thus far among other known inhibitors, such as cimetidine, isovanillin, methadone, etc (Obach et al., 2004; Dastmalchi and Hamzeh-Mivehrod, 2005). Obach further demonstrated that raloxifene could be an uncompetitive or a noncompetitive inhibitor depending on the substrate (Obach, 2004; Obach et al., 2004). In the chemical inhibitor studies, the formation of M1 from 5BPO after incubation in the mouse liver cytosol in the presence of an inhibitor was compared with that of control incubation without an inhibitor (Table 2). Under these conditions, two AO inhibitors, raloxifene and menadione, caused more than 90% inhibition of M1 formation, whereas three XO inhibitors, allopurinol, oxipurinol, and methotrexate exhibited minimal to weak (3-16%) inhibition. Consequently, results from the two approaches established the role of AO, but not XO, as the major enzyme responsible for metabolizing the 2H-oxazole in 5BPO to its corresponding 2-oxazolone (M1) (Table 2).

Notable difference is known among mammalian species in protein expression and catalytic activity of AO (Moriwaki et al., 1999; Beedham, 2001). AO activity among species may vary depending on the substrate as well. However, in general AO catalytic activity seems to be high in monkeys, mice and humans and low in rats, whereas it is either minimal or absent in dogs (Beedham et al., 1987; Dalvie et al., 2010; Diamond et al., 2010; Pryde et al., 2010). For example, a marked species difference was reported
in metabolism of Zaleplon in human, monkey, and rat when administered orally. 5-Oxo-zaleplon resulting from oxidation of zaleplon by AO was observed as a major circulating metabolite in monkey and human than rat and was also a major liver S9 and cytosolic metabolite in monkey over rat (Kawashima et al., 1999). Similarly, metabolite resulting from oxidation of Zoniporide by AO in the quinoline ring was a primary metabolite in humans and rats, but was not observed in dogs (Dalvie et al., 2010). On the other hand, zebularine was found to be metabolized by AO with higher V\textsubscript{max} observed in mouse than human and monkey liver cytosols (Klecker et al., 2006), and the order of AO activity, measured by the rate of vanillic acid formation from vanillin, was monkey > mouse > human > rat liver cytosol, with no activity seen in dog cytosol (Sahi et al., 2008). When 5BPO was incubated in liver cytosols from various species including the rat, mouse, dog, monkey, and human, maximum turnover of 5BPO was observed in mouse liver cytosol (T\textsubscript{1/2} = 7 min) followed by monkey liver cytosol (217 min). M1 was the only metabolite detected in these incubations. Relative percentage of M1, based on LC-UV area was 89% in mouse and 10% in monkey liver cytosol at 30 min of incubation. Whereas, only trace amount of M1 was detected in rat and human liver cytosols. Dog liver cytosol, however, did not show any formation of M1. Similar results of M1 formation were obtained with liver cytosols acquired from different suppliers, BD Gentest and Xenotech, and with increased cytosolic protein concentration to 2 mg/mL (data not shown).

Despite our overall results were in agreement with species difference of AO catalytic activity reported in the literature (Kitamura et al., 2006; Sahi et al., 2008; Pryde et al., 2010), more robust AO activity oxidizing 5BPO and related oxazoles was
observed in mouse liver cytosol over monkey or human liver cytosol. This could possibly be an oxazole-chemotype specific or compound specific phenomenon. Nevertheless, there are implications to drug discovery due to these findings because wild type or genetically engineered mouse models are frequently used in pharmacological studies of drug discovery, particularly in disease areas such as cancer and CNS. Thus, robust mouse AO-mediated metabolism and formation of significant metabolite may play a potential role in pharmacodynamics, toxicology, and pharmacokinetics in the mouse. Awareness of the reaction and necessary follow-up investigations using human matrices (cytosol and hepatocytes) in the preclinical setting will be certainly helpful to avoid surprise or buy down risk in the clinic.

Intrigued by the possibility that 2H-oxazole may represent a new substrate chemotype for AO mediated oxidation to 2-oxazolone, we further extended the investigation of this unique biotransformation to a number of 2H-oxazoles using mouse liver cytosol. As shown in Table 4, among the compounds surveyed, C5-substituted oxazole: 5-(3-chlorophenyl)oxazole and C4-substituted oxazole: 4-(2-bromophenyl)oxazole showed the maximum turnover of parent compound with T1/2 of 4 min. Merimepodib showed T1/2 of 201 min. Oxazolone was the only metabolite detected in mouse liver cytosol and modification sites in various substrates were assigned by LC/MS/MS data and are indicated by arrows in Table 4. Relative percentage of oxazolone metabolite was ≥98% in case of both 5-(3-chlorophenyl)oxazole and 4-(2-bromophenyl)oxazole and 15% in merimepodib at 30 min of incubation based on LC-UV area.
The two 4, 5-di-substituted 2H-oxazoles did not yield any corresponding 2-oxazolone metabolite. Since the finding in Table 4 was obtained from only a limited set of 2H-oxazoles, it would be difficult to elaborate mechanistic aspect of substrate specificity. Suffice it to say that 2H-oxazoles, particularly, 4- or 5-substituted oxazoles, represent a new substrate chemotype for AO while each substrate requires careful experimental examination.

In summary, results from the present study revealed a novel ring oxidation of 2H oxazole to 2-oxazolone (Fig. 7) catalyzed by AO. The catalytic activity of AO varied among animal species but was overall consistent with the literature reports as shown in the rank order of mouse>monkey>>rat and human liver cytosol. Consequently, 4- or 5-substituted 2H oxazoles represent a new substrate chemotype for ring oxidation catalyzed by AO.
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Authorship Contributions

*Participated in research design:* Vinod K. Arora, Yue-Zhong Shu, and Stella Huang

*Conducted experiments:* Vinod K. Arora, Thomas Philip, and Stella Huang

*Contributed new reagents or analytic tools:* Vinod K. Arora, Thomas Philip, and Stella Huang

*Performed data analysis:* Vinod K. Arora, Thomas Philip, and Stella Huang

*Wrote or contributed to the writing of the manuscript:* Vinod K. Arora and Yue-Zhong Shu
References


Legends for Figures:

Figure 1: LC-UV/MS chromatogram of 5BPO incubation in mouse liver microsomes with NADPH (C and D) or without NADPH (A and B). Panel A and C represent LC-UV chromatograms at 261 nm; panel B and D represent XIC at m/z 223-242.

Figure 2: LC-UV/MS chromatogram of 5BPO incubation in mouse liver cytosol at 0 min (A and B) and 30 min (C and D). Panel A and C represent LC-UV chromatogram at 261 nm; panel B and D represent XIC at m/z 223-242.

Figure 3: MS (A) and MS/MS (B) spectra of 5BPO in mouse liver cytosol.

Figure 4: MS (A) and MS/MS (B) spectra of M1 in mouse liver cytosol and MS (C) and MS/MS (D) spectra of M2 in mouse liver microsomes.

Figure 5: MS spectra of M1 in mouse liver cytosol incubation containing 50/50 mixture of H$_2^{16}$O and H$_2^{18}$O.

Figure 6: $^1$H NMR spectrum of 5BPO and M1.

Figure 7. Aldehyde oxidase mediated conversion of 2H-oxazole to 2-oxazolone.
Table 1: $^1$H and $^{13}$C NMR chemical shifts of 5BPO and M1.

<table>
<thead>
<tr>
<th>Carbon atom number</th>
<th>$^{13}$C NMR shifts</th>
<th>$^1$H NMR shifts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parent</td>
<td>M1</td>
</tr>
<tr>
<td>C2</td>
<td>152.9</td>
<td>156.3</td>
</tr>
<tr>
<td>C4</td>
<td>122.9</td>
<td>109.4</td>
</tr>
<tr>
<td>C5</td>
<td>151.3</td>
<td>138.6</td>
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<tr>
<td>C6</td>
<td>130.6</td>
<td>122.1</td>
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<tr>
<td>C7</td>
<td>127.8</td>
<td>125.1</td>
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<tr>
<td>C8</td>
<td>123.7</td>
<td>129.9</td>
</tr>
<tr>
<td>C9</td>
<td>132.4</td>
<td>130.2</td>
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<tr>
<td>C10</td>
<td>131.6</td>
<td>130.3</td>
</tr>
<tr>
<td>C11</td>
<td>123.8</td>
<td>121.1</td>
</tr>
</tbody>
</table>

* ACD NMR software (Advanced Chemistry Development, Toronto, ON) was used to predict NMR chemical shifts.
Table 2: Effect of chemical inhibitors on M1 formation from 5BPO (5µM) in mouse liver cytosol (protein conc. 0.5 mg/mL).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Concentration (µM)</th>
<th>Target Enzyme</th>
<th>% M1 formation at 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Inhibitor (Control)</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>100</td>
<td>XO</td>
<td>74</td>
</tr>
<tr>
<td>Oxipurinol</td>
<td>100</td>
<td>XO</td>
<td>90</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>100</td>
<td>XO</td>
<td>97</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>50</td>
<td>AO</td>
<td>7</td>
</tr>
<tr>
<td>Menadione</td>
<td>100</td>
<td>AO</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3: Incubation of 5BPO (5μM) in liver cytosols (0.5 mg/mL) across species

<table>
<thead>
<tr>
<th>Species</th>
<th>T1/2 (min) in liver cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>7</td>
</tr>
<tr>
<td>Rat</td>
<td>&gt;240</td>
</tr>
<tr>
<td>Dog</td>
<td>N/A</td>
</tr>
<tr>
<td>Monkey</td>
<td>217</td>
</tr>
<tr>
<td>Human</td>
<td>&gt;240</td>
</tr>
</tbody>
</table>

N/A: not applicable, no biotransformation was observed in dog liver cytosol
Table 4: Incubation of various oxazoles (5 µM) in mouse liver cytosol (0.5 mg/mL).

<table>
<thead>
<tr>
<th>Compound Types</th>
<th>Compounds</th>
<th>Structures</th>
<th>T1/2 (min) in mouse liver cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-substituted oxazole</td>
<td>5-(3-bromophenyl)oxazole (5 BPO)</td>
<td><img src="image1" alt="Structure" /></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>5-(3-chlorophenyl)oxazole</td>
<td><img src="image2" alt="Structure" /></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>merimepodib</td>
<td><img src="image3" alt="Structure" /></td>
<td>201</td>
</tr>
<tr>
<td>4-substituted oxazole</td>
<td>4-(2-bromophenyl) oxazole</td>
<td><img src="image4" alt="Structure" /></td>
<td>4</td>
</tr>
<tr>
<td>4,5-di-substituted oxazole</td>
<td>4-phenyloxazole-5-carboxamide</td>
<td><img src="image5" alt="Structure" /></td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>7-bromo-5-methoxybenzo[d]oxazole</td>
<td><img src="image6" alt="Structure" /></td>
<td>N/A</td>
</tr>
</tbody>
</table>

Arrows indicate oxidation site based on LC/MS/MS assignment; N/A: not applicable, no biotransformation was observed.
Figure 2
Figure 4

M1

\[ m/z \ 193.9595 \]

M2

\[ m/z \ 184.9597 \]

\[ m/z \ 196.9597 \]

A

\[ m/z \ 239.9649 \]

\[ m/z \ 241.9628 \]

\[ m/z \ 238.9571 \]

\[ m/z \ 242.9660 \]

B

\[ m/z \ 104.1489 \]

\[ m/z \ 133.0518 \]

\[ m/z \ 211.9700 \]

\[ m/z \ 168.9644 \]

C

\[ m/z \ 239.9656 \]

\[ m/z \ 241.9635 \]

\[ m/z \ 237.8364 \]

\[ m/z \ 236 \]

\[ m/z \ 238 \]

\[ m/z \ 240 \]

\[ m/z \ 242 \]

\[ m/z \ 244 \]

D

\[ m/z \ 133.0523 \]

\[ m/z \ 121.6858 \]

\[ m/z \ 161.0473 \]

\[ m/z \ 196.9597 \]

\[ m/z \ 184.9597 \]

\[ m/z \ 74.7331 \]

\[ m/z \ 121.6858 \]

\[ m/z \ 161.0473 \]

\[ m/z \ 204.4025 \]

\[ m/z \ 193.9595 \]

\[ m/z \ 196.9597 \]
Figure 5
Figure 6:

5BPO

Formic Acid

M1
Figure 7

2H-Oxazole → 2-Oxazolone

R = R' = Substitutions either on C4-, or C5-positions