Investigation into MAO B-mediated formation of CC112273, a major circulating metabolite of ozanimod, in humans and preclinical species: Stereospecific oxidative deamination of (S)-enantiomer of indaneamine (RP101075) by MAO B

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Abbreviations: AME, Absorption, Metabolism and Excretion; CYP450, cytochrome P450; PK/PD, Pharmacokinetics/Pharmacodynamics; MPTP, 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; MS, Multiple Sclerosis, MIST, Metabolites in Safety Testing.
Abstract

Ozanimod, recently approved for treating relapsing MS, produced a disproportionate, active, MAO B-catalyzed metabolite (CC112273) that showed remarkable interspecies differences and led to challenges in safety testing. This study explored the kinetics of CC112273 formation from its precursor RP101075. Incubations with human liver mitochondrial fractions revealed $K_{Mapp}$, $V_{max}$ and $Cl_{int}$ for CC112273 formation to be 4.8 $\mu$M, 50.3 pmol/min/mg protein and 12 $\mu$l/min/mg, respectively, while $K_M$ with human recombinant MAO B was 1.1 $\mu$M. Studies with liver mitochondrial fractions from preclinical species led to $K_{Mapp}$, $V_{max}$ and $Cl_{int}$ estimates of 3.0, 35 and 33 $\mu$M, 80.6, 114, 37.3 pmol/min/mg and 27.2, 3.25 and 1.14 $\mu$l/min/mg in monkey, rat and mouse, respectively, and revealed marked differences between rodents and primates, primarily attributable to differences in the $K_M$. Comparison of $Cl_{int}$ estimates revealed monkey to be ~two-fold more efficient and the mouse and rat to be 11 and 4-fold less efficient than humans in CC112273 formation. The influence of stereochemistry on MAO B-mediated oxidation was also investigated using the $R$-isomer of RP101075 (RP101074). This showed marked selectivity towards catalysis of the $S$-isomer (RP101075) only. Docking into MAO B crystal structure suggested that even though both the isomers occupied its active site, only the orientation of RP101075 presented the C-H on the $\alpha$-carbon that was ideal for the C-H bond cleavage, which is a requisite for oxidative deamination. These studies explain the basis for the observed interspecies differences in the metabolism of ozanimod as well as the substrate stereospecificity for formation of CC112273.
Significance

This study evaluates the enzymology and the species differences of the major circulating metabolite of ozanimod, CC112273. Additionally, the study also explores the influence of stereochemistry on MAO B catalyzed reactions. The study is of significance to the DMD readers given that this oxidation is catalyzed by a non-CYP enzyme and that marked species difference and notable stereospecificity was observed in MAO B catalyzed biotransformation when the indaneamine enantiomers were used as substrates.
Introduction:

Ozanimod (Zeposia), a sphingosine-1-phosphate (S1P) receptor modulator that binds with high affinity selectively to S1P receptor subtypes 1 (S1P₁) and 5 (S1P₅), is approved for the treatment of relapsing forms of multiple sclerosis (MS) and ulcerative colitis (Cohen et al., 2016; Sandborn and Feagan, 2016; Sandborn et al., 2016; Scott et al., 2016; Sorensen, 2016; Taylor Meadows et al., 2018; Feagan et al., 2020; Harris et al., 2020; Lassiter et al., 2020). It exhibits desirable PK properties in humans and is eliminated with an elimination half-life of ~17-20 hr (Tran et al., 2017; Tran et al., 2018; Tran et al., 2020a; Tran et al., 2020b). An AME study with radiolabeled ozanimod (1 mg dose) in healthy male volunteers has revealed that metabolism is a primary route of clearance since very little unchanged ozanimod is detected in the excreta and plasma of human subjects (Surapaneni et al., 2021). While all metabolites detected in the excreta are formed via gut-microfloral reductive ring scission of the oxadiazole ring and are inactive, the majority of circulating radioactivity is attributed to oxidative metabolites, including the two main metabolites CC112273 and CC1084037 (Figure 1A) (Surapaneni et al., 2021). In vitro pharmacology studies revealed that all circulating metabolites with intact oxadiazole ring were pharmacologically active against human S1P₁ and S1P₅ receptors (Surapaneni et al., 2021).

Among the circulating metabolites, CC112273 is of great interest. Not only is it more potent than ozanimod towards S1P₁ and S1P₅ receptor subtypes (Surapaneni et al., 2021), but also accounts for 33% of circulating radioactivity after [¹⁴C]-ozanimod administration to healthy male volunteers. In clinical studies with ozanimod, the levels of CC112273 were high and its mean terminal elimination half-life was ~10 days and therefore amenable to accumulation (Tran et al., 2020a; Tran et al., 2020b). Following multiple oral doses, CC112273 exposure constituted ~73% of the total active drug exposure (parent and metabolites) (Tran et al., 2020b). Comparison of these exposures with exposures in pharmacology and toxicology species used in...
ozanimod development, showed significant interspecies differences (unpublished results). Its disproportionate nature led to challenges in evaluating this metabolite as per the MIST guidance (Surapaneni et al., 2021). The first step in CC112273 formation involves CYP3A4/1A1-catalyzed dealkylation of ozanimod to the amine RP101075, which is subsequently oxidized to CC112273 by MAO B (Figure 1A) (Surapaneni et al., 2021).

MAO A and MAO B are mitochondrial flavoenzymes that play an important role in the oxidative deamination of several structurally diverse amines, including biogenic amines and xenobiotics (Singer, 1987; Squires, 1997; Strolin Benedetti and Tipton, 1998; Shih et al., 1999; Kalgutkar et al., 2001; Edmondson et al., 2004; Edmondson et al., 2009; Gaweska and Fitzpatrick, 2011). Both the enzymes catalyze the deamination reaction by cleavage of the C-H bond from the $\alpha$-carbon and reducing the flavin moiety (FAD to FADH$_2$) by transfer of a hydride equivalent from the amine (Supplementary Figure S1) (Kalgutkar et al., 2001; Edmondson et al., 2004; Edmondson et al., 2009). In the process, the amine is converted to an unstable imine intermediate, which is hydrolyzed non-enzymatically to aldehyde or ketone. Despite the commonalities, these enzymes are biochemically differentiated by their substrate and inhibitor specificities (Johnston, 1968; Murphy, 1978; Cawthon and Breakefield, 1979; Trevor et al., 1987; Waldmeier, 1987; Janssens de Varebeke et al., 1990; Nair et al., 1993; Dixon et al., 1994; Kopin, 1994; Tipton, 1994; Hauptmann et al., 1996; Youdim et al., 2001; Caccia et al., 2006; Kamel et al., 2010; Finberg and Rabey, 2016). Representative substrates and inhibitors of MAO A and B are shown in Supplementary Figure S2.

Although earlier phenotyping studies suggested MAO B as the enzyme that catalyzes formation of CC112273 from RP101075 in humans (Surapaneni et al., 2021), kinetic studies are required to assess the efficiency of this reaction. Additionally, given the species differences in CC112273 exposure an understanding of the role of MAO in the metabolism of RP101075 in
animals requires further investigation. Collectively, this led to the examination of kinetics of CC112273 formation by MAO B, in humans, mouse, rat, and monkey. Additionally, since RP101075 is an S-isomer like ozanimod, we decided to explore the stereoselectivity of MAO B in catalyzing oxidative deamination by studying the R-isomer. RP101074 (Figure 1B) is an R-epimer of RP101075 that also exhibits selective modulatory activity towards S1P₁ and S1P₅ receptors and is equipotent to RP101075 and ozanimod (unpublished results). It is commonly used as a surrogate of ozanimod in pharmacology studies however, its conversion to the CC112273 has never been investigated. To this end, we also explored the metabolism of RP101074 by MAO in this study.
Material and Methods

All chemicals and solvents were reagent grade and commercially available. RP101075, CC112273 and RP101074 were synthesized at Celgene and the synthesis of these compounds has been reported previously (Surapaneni et al., 2021). RP101075 and RP101074 were dissolved in DMSO to make a stock solution of 5 mM, which was further diluted to 1 mM with acetonitrile to reduce the final concentration of DMSO in the incubation mixture and this stock solution was used for all reactions described below. The concentration of DMSO in the final incubation mixture was kept below 0.1% except when high substrate concentrations were used for kinetic studies. In these incubations, the concentration of DMSO was 0.5%. Pooled mixed gender human (n = 5) liver mitochondrial fractions (protein concentration, 20 mg/mL), pooled liver mitochondria from male IGS-Sprague Dawley rat (n = 30), male CD-1 mouse (n = 30) and male Cynomolgus monkey (n = 3) with protein concentration of 20 mg/mL were purchased from Sekisui XenoTech (Kansas City, KS) and stored at -70 °C until use. Human recombinant MAO A (Cat# M7316, enzyme activity >10 units/mg protein, protein concentration 5.0 mg/mL) and MAO B enzyme (Cat# M7441, enzyme activity >1 unit/mg protein, protein concentration 5.0 mg/mL) expressed in baculovirus infected BTI insect cells was purchased from Sigma Aldrich (St. Louis, MO). All incubations were done in triplicate.

Determination of formation of CC112273 from RP101075 or RP101074 in mitochondrial fractions from humans and preclinical species

To a pre-warmed (3 min, 37 °C) mixture containing phosphate buffer (100 mM; pH 7.4) and liver mitochondrial fraction (1 mg/mL) from human, monkey, rat and mouse, was added a solution of RP101075 or RP101074 (only human liver mitochondrial incubations) to achieve a final concentration of 2 μM and a total volume of 500 μL. The reaction mixture was allowed to shake in a water bath for 30 min at 37 °C. A 100 μL aliquot of the mixture was removed at 0
and 30 min and added to a solution of 200 µL acetonitrile containing 0.2 µM deuterated (d5) 7-ethoxycoumarin (internal standard, IS). The quenched aliquots were vortexed for 5 min and centrifuged (approximately 4000g, room temperature for 10 min) to obtain the supernatant, which was analyzed for CC112273 by LC-MS/MS as described below.

**Inhibition of CC112273 formation from RP101075 and RP101074 in liver mitochondrial fraction from humans and preclinical species by MAO A inhibitor clorgyline and MAO B inhibitor, R-deprenyl.**

MAO A inhibitor, clorgyline and MAO B inhibitor, R-deprenyl is generally used to probe the contribution of these two enzymes in oxidizing MAO substrates. These inhibitors inactivate MAO A and B, respectively, when mitochondrial fractions are preincubated for 5 to 15 min with these two inhibitors at concentrations of 0.250 to 1.0 µM (Yu et al., 1986; Thull and Testa, 1994). To assess the contribution of MAO A and B in the metabolism of RP101075 and RP101074 to CC112273, a DMSO solution of R-deprenyl or clorgyline (0.5 µM each) (Kamel et al., 2012) was added to the mixture containing phosphate buffer (100 mM; pH 7.4) and liver mitochondrial fraction (1 mg/mL) from human, monkey, rat and mouse and preincubated for 5 min (Thull and Testa, 1994). After 5 min, the reaction was initiated by addition of a solution of RP101075 or RP101074 (only assessed in human liver mitochondria) to achieve a final concentration of 2 µM. The total volume of the incubation mixture was 500 µL and total incubation time following addition of the substrate was 30 min. Following this, the reaction mixture was treated in the similar manner as described earlier to assess the formation of CC112273.

**Enzyme kinetics studies for formation of CC112273 from RP101075 in liver mitochondrial fractions from humans and preclinical species**
Incubation conditions for the formation of CC112273 from RP101075 were first optimized for linearity with respect to time and protein and substrate concentration for each species (mouse, rat, monkey and human) to establish initial velocity conditions prior to the kinetic studies. The conditions selected for the final experiment were such that the turnover of the substrate was less than 10% in each incubation. Table 1 depicts the optimized times of incubation, protein concentrations, times at which aliquots were withdrawn and the range of substrate concentrations used for each species. All incubations were commenced by addition of DMSO:acetonitrile (1:1 v/v) solution of RP101075 to the pre-warmed (3 min, 37 °C) mixture containing phosphate buffer (100 mM; pH 7.4) and liver mitochondrial fraction from human, mouse, rat and monkey (total volume of the incubation mixture was 500 µL). Aliquots (50 µL) were taken at various times (Table 1) and each aliquot was added to 200 µL solution of IS in acetonitrile to stop the reaction. Following quenching of the reaction mixture, each sample was treated as described earlier to assess the formation of CC112273.

**Kinetic studies for CC112273 formation from RP101075 using recombinant human MAO B**

As discussed above, the concentration of the recombinant protein (0.02 mg protein/mL in the final incubation mixture) was first optimized to achieve linear reaction velocity conditions. The reaction was started by addition of a DMSO:acetonitrile (1:1 v/v) solution of RP101075 (0.5 - 50 µM final concentration) in a pre-warmed (3 min, 37 °C) mixture containing 100 mM phosphate buffer (pH 7.4) and recombinant protein (total volume of the incubation mixture, 500 µL). Aliquots (50 µL were withdrawn at 0, 5, 10, 15, 20 and 30 min following which each sample was treated as described in the method for kinetic studies with mitochondrial fractions.

**LC-MS/MS analysis**
Detection and quantification of CC112273 and IS was performed using API 4000 Sciex QTrap mass spectrometer (AB Sciex Framingham, MA) equipped with a Turbo Ion Spray interface. The mass spectrometer was controlled by Analyst v1.6.2. The source was operated in a positive ion mode and the experimental parameters were set as follows: ion spray voltage, 2000; source temperature, 550 °C; curtain gas, 20; collision gas, medium ion source gas 1, 55; ion source gas 2, 50. The analytes were introduced into the mass spectrometer using Waters Acquity UPLC system. The liquid chromatography was performed using a kinetex (2.6 µm particle size, 3 x 100 mm RP-C8, Phenomenex, Torrance, CA). The gradient system was used to separate the analytes with a flow rate of 0.5 mL/min using mobile phase A, water containing 0.1% formic acid and mobile phase B, acetonitrile containing 0.1% formic acid. The gradient started with 95% A and 5% B and was held for 0.5 min. It was then progressed to 5% A and 95% B in 2.5 min. This was held for 1.7 min and reverted to initial composition of 95% A and 5% B in 0.3 min. The column was equilibrated for 1 min. The total run time was 6 min. The retention times of CC112273 and IS were 3.7 and 3.3 min, respectively. The analytes were monitored using multiple reaction monitoring and the ion transitions (Q1/Q3) were 360.3/146.1 Da for CC112273 and 196/164 for the IS. The other parameters collision energy (CE) and the de-clustering potential (DP) were 40 and 61, for CC112273 and 27 and 45 for the IS and are presented in arbitrary units. All data were analyzed using AB Sciex Analyst v1.6.2 software.

**Estimation of Michaelis Menten parameters of CC112273 formation from RP101075**

The formation rate of CC112273, determined as pmol/min/mg of mitochondrial or recombinant protein at each substrate concentration was used to estimate the $K_M$ (Michaelis Menten constant, a substrate concentration at half-maximum velocity) in µM and $V_{max}$ (maximum reaction velocity) in pmol/mg/min protein. The Prism 8.0 program for windows
(Graphpad software, Inc., San Diego, CA, USA) was used to fit all the kinetic data to the single binding site model described by equation 1.

\[ v = \frac{V_{\text{max}} \cdot [S]}{K_M + [S]} \]  

(1)

[S] = substrate concentration. The intrinsic clearance (Cl_{int} expressed as \( \mu \text{L/min/mg protein} \)) was then determined from the estimated \( K_M \) and \( V_{\text{max}} \) using equation 2:

\[ \text{Cl}_{\text{int}} = \frac{V_{\text{max}}}{K_M} \]  

(2)

**Structural Modeling**

A proposed structural model of compounds RP101075 and RP101074 were built in the MAO B, trans, trans farnesol structure (2BK3) (Hubalek et al., 2005) and MAO-A harmine structure (2Z5X) (Son et al., 2008). The crystal structures were imported into Maestro (Schrödinger Release 2018-3: Maestro, Glide, LigPrep, Epik, Schrödinger, LLC, New York, NY, 2018.). The structures were then prepared using the Protein Preparation workflow as implemented in the Schrödinger Suite (Sastry et al., 2013). Glide docking grids were then generated by focusing the grid box on the center of the trans, trans farnesol ligand. The size of the box enclosing the grid was set to 10 Å. As per the trans, trans farnesol structure, two positional constraints at the farnesol oxygen and C(1) of farnesol for ligand atom occupancy with a 1.0 Å radius were placed. No other constraints, rotatable groups or excluded volumes were defined. The two compounds were then prepared for docking using LigPrep and the OPLS3e force field (Harder et al., 2016) was used for minimizations; possible ionization states at pH 7.0 ± 2.0 were generated using Epik and tautomers were generated; specified chirality was retained.
Results

Kinetic studies for the formation of CC112273 from RP101075 in human liver mitochondrial fraction and recombinant human MAO B

Kinetics studies for the conversion of RP101075 to CC112273 were performed using pooled human liver mitochondria and recombinant MAO B. The formation rate of indanone was determined over a range of substrate concentrations and the relationship between initial reaction velocity and substrate concentration was used to estimate the kinetic parameters. The data obtained was fitted well to the simple Michaelis Menten model (Figure 2A) and resulted in $K_{M_{app}} = 4.8 \pm 2.2 \ \mu$M and $V_{max} = 50.3 \pm 12.0 \ \text{pmol/min/mg}$ of mitochondrial protein (Table 2) resulting in $Cl_{int}$ of $12 \pm 4.9 \ \mu$L/min/mg mitochondrial protein. Similarly, $K_M$, $V_{max}$ and $Cl_{int}$ estimates were also determined using recombinant MAO B (Figure 2B and Table 2). Like in case of the mitochondrial fraction, the rate versus concentration data could be fitted to a simple Michaelis Menten model, resulting in $K_M$ of $1.1 \pm 0.1 \ \mu$M, $V_{max}$ of $194 \pm 14.1 \ \text{pmol/min/mg}$ mitochondrial protein and $Cl_{int}$ of $176 \pm 1.38 \ \mu$L/min/mg mitochondrial protein. A 4-fold difference was observed in the $K_{M_{app}}$ and $K_M$ value following incubation with mitochondrial protein and recombinant protein.

Kinetic studies for the formation of CC112273 from RP101075 in liver mitochondrial fraction from preclinical species

As a first step, experiments were performed to evaluate if MAO B also catalyzed the formation of CC112273 from RP101075 in the preclinical species that were used in the pharmacology and toxicology studies during development of ozanimod. Thus, RP101075 (2 \ \mu$M) was incubated with pooled liver mitochondrial fractions from monkey, rat and mouse in the absence and presence of MAO A (clorgyline) and MAO B ($R$-deprenyl) inhibitors and the
formation of CC112273 was monitored in these incubation mixtures (Figure 3A). As shown, CC112273 was detected only in incubation mixtures that lacked inhibitors and the incubation mixtures that were pretreated with clorgyline. However, mitochondrial fractions when pretreated with R-deprenyl prior to addition of RP101075 to the incubation mixture led to the disappearance of the indanone peak. This confirmed that MAO B was the only ortholog involved in CC112273 formation in the preclinical species as well.

Based on this information, kinetics studies for CC112273 formation by MAO B were performed using liver mitochondrial fractions obtained from the rat, mouse and monkey. Like humans, all species displayed Michaelis Menten kinetics. Figures 3B-D show the relationship of reaction velocity and RP101075 concentrations in liver mitochondria from the three preclinical species. Table 2 lists the mean $K_{Mapp}$, $V_{max}$ and $Cl_{int}$ estimates for the formation of CC112273 in the mouse, rat and monkey. A ~12-fold variation was observed in the $K_{Mapp}$ value estimated using liver mitochondrial fractions from all species (Table 2). The $K_{Mapp}$ of RP101075 in monkeys was the lowest among the three species (3.0 ± 0.1 μM), whereas rat and mouse had a higher $K_M$ of 35 ± 4.8 and 33 ± 8.3 μM, respectively. The $V_{max}$ values for CC112273 formation showed a ~3-fold variation among preclinical species. The rat and mouse had highest and lowest $V_{max}$ with values of 114 ± 9.7 pmol/min/mg mitochondrial protein and 37.3 ± 4.1 pmol/min/mg mitochondrial protein, respectively. When the $Cl_{int}$ values were compared among preclinical species, the monkey was the most efficient in the conversion of the amine to the ketone with an estimate of 27 ± 0.8 μL/min/mg protein, whereas the mouse was the least efficient (1.1 ± 0.15 μL/min/mg protein). When compared to humans, the apparent $K_{Mapp}$ in monkey was almost similar to humans (less than 2-fold difference between the two species), while the apparent $K_{Mapp}$ values of rat and mouse were 7-fold higher than in humans. Comparison of $Cl_{int}$ estimates indicated that the monkey was ~2-fold more efficient compared to human, while the mouse and rat were 11 and 4-fold less efficient than human.
**In vitro studies with the R-enantiomer, RP101074 in human liver mitochondria.**

While enzymology of the S-enantiomer was the primary focus of our work, we also wanted to investigate the stereoselectivity of MAO in catalyzing the oxidative deamination of the indaneamine. The goal was to see if inverting the stereochemistry would also influence the selectivity of oxidation of the indaneamine by MAO B. Therefore, RP101074 was incubated with human liver mitochondria to assess its conversion to CC112273. Incubations were also performed by treating the mitochondrial fraction with clorgyline and R-deprenyl.

As shown in Figure 4, RP101074 showed negligible formation of the indanone when incubated with human liver mitochondria without or with MAO inhibitors. RP101075 was used as a positive control in this experiment to compare the formation of CC112273 by RP101074 relative to CC101075 under similar conditions. The lack of formation of CC112273 in incubations with RP101074 suggested stereospecificity in deamination of with preference for the S-enantiomer.

**Structural modeling of RP101075 and RP101074 in MAO B**

To understand the differences in oxidative deamination between RP101075 and RP101074, both enantiomers RP101074 (R-enantiomer) and RP101075 (S-enantiomer) were docked into the MAO B crystal structure. Although high resolution crystal structures with several MAO B inhibitors are available, the structure complexed with *trans, trans* farnesol (2BK3) was used in this study (Hubalek et al., 2005). *trans, trans* Farnesol is a natural isoprenoid alcohol that is a component of tobacco smoke and is an inhibitor of MAO B (Khalil et al., 2006). Given the conjugated nature of RP101075 (due to the three aromatic rings), we surmised that this molecule resembled *trans, trans* farnesol and would occupy the active site of MAO B in the same way as *trans, trans*-farnesol. Figure 5A depicts an overlay of RP101075 and *trans, trans*
farnesol suggesting a similar orientation of the amino group of RP101075 to that of the hydroxy group of trans, trans farnesol in the active site near the covalently bound FAD. We hypothesized that the key interaction(s) for hydride abstraction by FAD is both degree of fit in the binding site (as computed by Glide docking scores, Supplementary Table S1), the orientation of the hydrogen appropriately with correct distance and geometry to the cofactor FAD for hydride abstraction (specifically N5 atom), the \( \pi - \pi \) stacking interaction of the indane ring with Tyr 495 and Tyr 398 and the edge-\( \pi \) interaction with Phe393 which orients the hydrogen near FAD. Modeling studies showed that both epimers bound in the active site but only RP101075 orients the hydrogen near FAD in an orientation ideal for hydride transfer (Figure 5B) in contrast, RP101074 does not (Figure 5C).
Discussion:

This study explored the kinetic properties of CC112273 formation by MAO B in humans and preclinical species. Saturation kinetic studies revealed that RP101075 was a good substrate of MAO B with a \( K_M \) and \( K_{Mapp} \) value of 1.1 and 4.8 \( \mu \text{M} \) in incubations containing recombinant human MAO B or human liver mitochondria, respectively (Table 2). A 4-fold higher \( K_{Mapp} \) was possibly attributed to non-specific binding of RP101075 to the mitochondrial proteins. Lipophilic, basic drugs can bind non-specifically to membranes due to their enhanced affinity for phospholipid membranes. This can lead to overestimation of the measured \( K_M \) and consequently underprediction of the \textit{in vitro} \( \text{Cl}_{\text{int}} \) (Austin et al., 1995; Obach, 1997; Obach, 1999; McLure et al., 2000; Venkatakrishnan et al., 2000; Austin et al., 2002; Burns et al., 2015). Since RP101075 is a lipophilic base (cLog \( P = 4.5 \) and pKa = 8.7 ACD/Percepta, ACD labs, Toronto, Ontario, Canada), it could bind to mitochondrial protein resulting in higher \( K_M \). Using the equation described by Halifax and Houston (equation 3) we estimated the free fraction (\( f_{\text{inc}} \)) of RP101075 in the incubation containing the mitochondrial fraction to be 0.23 (Hallifax and Houston, 2006; Wood et al., 2017).

\[
\begin{align*}
f_{\text{inc}} &= \frac{1}{C \cdot 10^{0.072 \cdot \text{LogP/D}^2 + 0.067 \cdot \text{LogP/D} + 1.126}} \quad (3)
\end{align*}
\]

where \( C \) is the protein concentration in the incubation in mg/mL. We assumed that a cLog \( P \) value would provide a better estimation of \( f_{\text{inc}} \), as is the case in estimation of \( f_{\text{inc}} \) in microsomes for basic drugs (Austin et al., 2002; Wood et al., 2017). With this estimated \( f_{\text{inc}} \), the unbound \( K_M \) of RP101075 in human liver mitochondrial incubations was projected to be \( \sim 1.1 \) \( \mu \text{M} \) and was consistent with the experimental value obtained using recombinant human MAO B. On the other hand, the \( f_{\text{inc}} \) in incubations with recombinant MAO B was estimated to be 0.92. Hence, even if one assumed the composition between the mitochondrial fraction and...
recombinant protein prep to be similar, the high $f_{u_{inc}}$ had very little influence on $K_M$ in this incubation.

Notable species variation was observed in the kinetic parameters of RP101075 oxidation by MAO B, especially when the $K_{M_{app}}$ and $Cl_{int}$ values in rodents were compared to primates (monkey and human). While the difference in the $Cl_{int}$ of CC112273 formation was about two-fold between monkey and human, the rodents were less efficient (~ four-fold) in the deamination of the amine. This disparity was primarily attributed to $K_{M_{app}}$ of the substrate in these species. Assuming similar composition in the mitochondrial preparation among species and lack of species differences in $f_{u_{inc}}$ as in microsomal binding (Zhang et al., 2010), the $K_{M_{app}}$ of RP101075 in rodents was ~7-fold higher than that in humans suggesting weak interactions with mouse and rat MAO B. On the other hand, a less than two-fold difference in the $K_{M_{app}}$ value between monkey and human suggested possible similarities in the MAO B binding pocket in these two species. When an attempt to compare the formation of CC112273 in this study with CC112273 levels from the *in vivo* studies in preclinical and clinical studies after ozanimod administration was made, we found good concordance between the *in vitro* and *in vivo* results. Although such a comparison can be challenging, the CC112273 exposures in the rodents and primates trended with the $Cl_{int}$ values of CC112273 formation. As reported in the summary basis of approval of Zeposia, the systemic exposures of CC112273 in mouse, rat, monkey and human were 2.4%, 6%, 48%, and 73% of the total active drug related material at steady state, respectively, and this was consistent with higher and lower efficiency of CC112273 formation observed in the primates and rodents, respectively (www.accessdata.fda.gov/drugsatfda_docs/nda/2020/209899Orig1s000PharmR.pdf).

It is known that a difference in the substrate binding pocket of an enzyme can lead to changes in interactions of the substrate with the active site. Even though the overall primary amino acid sequences of MAO B from different species are similar (Hsu et al., 1989), evidence
of species differences in substrate and inhibitor specificity has been reported (Garrick and Murphy, 1980; Reid et al., 1988; Krueger et al., 1995; O’Brien et al., 1995; Hubalek et al., 2005). A comprehensive study published by Inoue et al., which investigated the MAO-catalyzed species dependent oxidation of MPTP and its analogs in liver and brain mitochondrial preparations (Inoue et al., 1999), also reflects upon the differences in their MAO B specificities in various species. However, our results contradicted those from Inoue and co-workers. As noted above, our studies showed a clear difference in kinetic parameters for oxidative deamination of RP101075 between humans and rat, and a similarity between monkey and humans. In contrast, studies by Inoue et al. reported that the MAO B activity of humans and rat was similar but difference in the catalytic properties was observed between human and monkey when MPTP was used as a substrate (Inoue et al., 1999). Although the reason for this is unknown, the disconnect could be ascribed to the physicochemical and structural properties of a substrate being metabolized.

Results in the study by Surapaneni et al. (Surapaneni et al., 2021) and the phenotyping results presented in this study showed that neither RP101075 nor RP101074 were metabolized by MAO A (RP101074 was not a substrate for MAO B either, discussed later). This is consistent with the subtle differences that are observed in the residues present in the active site A and B. Reports by Geha et al. and Hubalek et al. suggest the importance of Ile-199 in the active site of human MAO B, which acts as a gate residue between the entrance and substrate cavities of the enzyme (Geha et al., 2000; Geha et al., 2001; Ma et al., 2004; Hubalek et al., 2005). The ability of Ile-199 to exist in open (both entrance and substrate cavities are fused) and closed (where the two cavities are separate) determines the specificity towards this isozyme. MAO A active site on the other hand, has phenylalanine (Phe 208) in the analogous position, the size of which prevents alternate conformations (Binda et al., 2003; Hubalek et al., 2005). It is suggested that compounds that occupy both cavities with Ile-199 in an open
conformation, exhibit specificity for MAO B (Edmondson et al., 2004; Hubalek et al., 2005). Superimposition of \textit{trans}, \textit{trans} farnesol with RP101075 suggests that RP101075 has the potential to occupy both sites in the MAO B active site. Hence, as in the case of \textit{trans}, \textit{trans} farnesol, the presence of Phe 208 in MAO A might perhaps cause steric clashes in the orientation of RP101075 (and RP101074) to MAO A and therefore making them weak substrates (Supplementary Figure S3). These steric interactions could prevent the compound to bind in an orientation that can result in hydrogen transfer mechanism.

Another interesting facet of this study was the marked stereoselectivity observed in MAO B catalyzed in deamination of only the S-enantiomer (RP101075). Even though docking studies suggested that both, RP101075 and the R-enantiomer (RP101074) occupied the MAO B active site similarly (Figure 5B and 5C) (Supplementary Table S1), RP101074 was not converted to CC112273. These results were consistent with reports from earlier studies that showed stereospecific conversion of only the S-(\(\alpha\)-\(2^H\))-deuteroamines (Belleau et al., 1960; Belleau and Moran, 1963; Battersby et al., 1979; Yu and Davis, 1988). Mechanistic studies on MAO catalyzed deamination suggest C-H bond cleavage to be a requisite and rate determining step in the oxidation process (Belleau et al., 1960; Yu et al., 1986; Yu and Davis, 1988; Ottoboni et al., 1989; Silverman et al., 1993; Edmondson et al., 2004; Edmondson et al., 2007; Orru et al., 2013). While three mechanisms for C-H bond cleavage have been proposed (Edmondson et al., 2007; Edmondson et al., 2009; Vianello et al., 2012), the exact mechanism for oxidation of RP101075 is unknown. Recent studies with selegiline and various benzylamine derivatives have suggested that MAO B exhibits a C-H bond cleavage via the hydride transfer (Vianello et al., 2012; Orru et al., 2013; Tandaric et al., 2020). Thus, one proposed mechanism of CC112273 oxidation could be abstraction of the hydride anion from the \(\alpha\)-carbon of RP101075 and hydrolysis of the corresponding imine to CC112273 (Figure 6). To this end, the substrate should position itself such that the C-H moiety should be in the proximity of the N(5) nitrogen of
the flavin for the hydride transfer to occur. Thus, even though both epimers can occupy the binding site, docking studies suggested that only the S-isomer (RP101075) is able to position the \( \alpha \)C-H close to the N(5) nitrogen with correct distance and geometry (Supplementary Table 1S) for hydride abstraction and facilitation of the oxidation process, which is not the case for RP101074 (Figure 5C).

Overall, these studies revealed marked species differences in MAO-B-catalyzed oxidation of RP101075 to the major human circulating metabolite CC112273. Since this difference reflected in the exposure of CC112273 in preclinical studies, one could speculate that early \textit{in vitro} metabolism studies could help in selecting appropriate species, especially when MAO B is involved in the metabolism. The results of these studies also reiterate that not only is it important to assess the metabolic profile of drug candidates in animal species \textit{in vitro} prior to selection of an animal model for a pharmacology or toxicology study, but also necessary to identify the enzymes involved in generating key metabolites, especially if the metabolite is pharmacologically active.
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**Contributed to writing of manuscript:** Deepak Dalvie, April Bai, Sekhar Surapaneni, Veerabahu Shanmugasundaram, Julie V. Selkirk.
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Multiple Sclerosis: A Comprehensive Review of Disease, Drug Efficacy and Side Effects.
Neurol Int 12:89-108.


Disclosure and Conflicts of interest

All the authors are employees of BMS. This work did not receive external funding.
Figure Legends:

Figure 1. A) Circulating metabolites of ozanimod in humans (Surapaneni et al., 2021). CBR, Carbonyl reductase; AKR, Aldo-keto reductase; HSD, 11-β-hydroxysteroid dehydrogenase, NAT, N-Acetyl transferase; ADH, Alcohol dehydrogenase; ALDH, Aldehyde dehydrogenase. B) Structure of the R-epimer of RP101075 (RP101074).

Figure 2. Michaelis Menten kinetics of CC112273 formation from RP101075 in human liver mitochondria (A) and recombinant MAO B (B).

Figure 3. A) Conversion of RP101075 to CC112273 in pooled liver mitochondria from monkey, rat and mouse. B) Michaelis Menten kinetics of CC112273 formation from RP101075 using pooled liver mitochondria from monkey, rat and mouse.

Figure 4. Incubation of RP101074 and RP101075 with human liver mitochondria in the presence of clorgyline (MAO A inhibitor) and deprenyl (MAO B inhibitor).

Figure 5. A) Overlay of CC101075 and trans, trans farnesol in the MAO B active site. Both molecules resemble in their overall orientation and positioning in the active site. B) Structural models of RP101075 (B) and RP101074 (C) in the active site of MAO B.

Figure 6. Proposed mechanism of oxidation of RP101075 by MAO B.
Table 1 – Conditions used in the enzyme kinetics studies of MAO B-catalyzed conversion of RP101075 to CC112273 in the mitochondrial fractions.

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein concentration (mg/mL)</th>
<th>Final incubation time (min)</th>
<th>Aliquots taken at times (min)</th>
<th>Concentration range used in the study (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0.75</td>
<td>15</td>
<td>0, 5, 10, 15</td>
<td>0.5 to 50</td>
</tr>
<tr>
<td>CD-1 Mouse</td>
<td>0.75</td>
<td>20</td>
<td>0, 5, 10, 15, 20</td>
<td>1.0 - 80</td>
</tr>
<tr>
<td>Sprague Dawley Rat</td>
<td>0.5</td>
<td>20</td>
<td>0, 5, 10, 15, 20</td>
<td>1.0 - 80</td>
</tr>
<tr>
<td>Cynomolgus Monkey</td>
<td>0.5</td>
<td>20</td>
<td>0, 5, 10, 15, 20</td>
<td>0.5 - 50</td>
</tr>
</tbody>
</table>
Table 2. Mean kinetic parameters ($K_{\text{M app}}$, $V_{\text{max}}$ and $Cl_{\text{int}}$) for CC112273 formation from RP101075 using human, monkey, rat and mouse liver mitochondrial fractions and recombinant human MAO B.

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_{\text{M app}}$</th>
<th>SD</th>
<th>$V_{\text{max}}$</th>
<th>SD</th>
<th>$Cl_{\text{int}}$</th>
<th>SD</th>
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<tr>
<td></td>
<td>$\mu$M</td>
<td></td>
<td>pmols/min/mg protein</td>
<td></td>
<td>mL/min/mg protein</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>4.8</td>
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<td>7.42</td>
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<tr>
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<td>0.09</td>
<td>80.6</td>
<td>0.4</td>
<td>27.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Rat</td>
<td>35</td>
<td>4.8</td>
<td>114</td>
<td>9.70</td>
<td>3.25</td>
<td>0.18</td>
</tr>
<tr>
<td>Mouse</td>
<td>33</td>
<td>8.27</td>
<td>37.3</td>
<td>4.1</td>
<td>1.14</td>
<td>0.15</td>
</tr>
<tr>
<td>Recombinant MAO B</td>
<td>1.1*</td>
<td>0.1</td>
<td>194</td>
<td>14.1</td>
<td>176</td>
<td>1.38</td>
</tr>
</tbody>
</table>

* $K_M$
Human liver mitochondrial fraction

Human recombinant MAO B
Figure 3

A. Formation of CC112273 (metabolite/IS area ratio)

- No Inhibitor
- Clorgyline
- Deprenyl

B. Monkey

C. Rat

D. Mouse

RP101075 concentrations (µM)

Reaction velocity (pmol/min/mg protein)
Figure 4

Formation of CC112273 (metabolite/IS ratio)

- without Inhibitors
- With Clorgyline
- With Deprenyl
Figure 5

trans, trans Farnesol

C-H alpha to the amine or alcohol

RP101075 (S-isomer)
Figure 5

RP101074 (R-isomer)

FAD

MAO-B

Ile199

Gln206

Tyr435

Tyr326

Phe343

Tyr398

Cys397

N5

C-H in proximity to N5 nitrogen of FAD

RP101075 (S-isomer)

C-H away from N5 nitrogen of FAD

MAO-B

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Figure 6