Identification of the Major Circulating Metabolite of Mymd-1 And Elucidation of the Mechanism of Clearance of MYMD-1 in Humans. Role of Aldehyde Oxidase and CYP2A6

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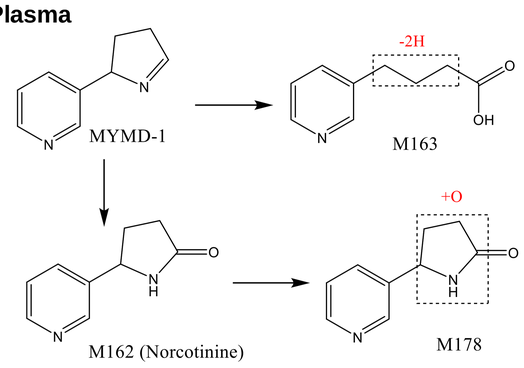
INTRODUCTION

MYMD-1 (**Figure 1**) is an immunomodulator that is currently entering Phase II clinical development for the treatment of autoimmune diseases. In preclinical and clinical studies, MYMD-1 has been shown to inhibit the inflammatory cytokines TNF-α, IL-6 and IL-17A[1,2]. The objectives of the current study were to identify the human circulating metabolites in samples obtained following multiple doses of MYMD-1 and to determine the enzymes involved in their formation.

FIGURE 1. Proposed Metabolic Pathways of MYMD-1 Based on Human Plasma



METHODS

Identification of Human Plasma Metabolites and Determination of Coverage in Safety Species

- **Samples** 1) Human plasma was collected on Day 1 and 5 (0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 24 hr) following daily oral administration of 600 mg MYMD-1 to six subjects, 2) Rat plasma was collected on Day 182 (0.083, 0.25, 0.5, 1, 2, 4, 8, 24 hr) from six animals following daily oral administration of 250 mg/kg MYMD-1, and 3) Dog plasma was collected on Day 273 (0.083, 0.25, 0.5, 1, 2, 4, 8, 24 hr) from four animals following daily oral administration of 25 mg/kg MYMD-1.
- **Plasma Pooling and Extraction.** Plasma samples were pooled by taking 100 µL from each subject/animal (N=4-6) at each time point and combined across time points to generate a time-proportional AUC pooled sample from 0 to 24 hr. Aliquots (100 µL) of pooled samples were extracted with two volumes of 1:4 MeOH:ACN, vortexed, and centrifuged (5 min at 13,000 rpm). The supernatant (250 µL) was transferred to a 96 well plate and partially dried under nitrogen to approximately 100 µL for LC-MS/MS analysis.
- **LC-MS/MS Analysis.** Aliquots of plasma extracts were analyzed by Vanquish UPLC system interfaced to Exactive Hybrid Quadrupole-Orbitrap mass spectrometer using full scan (m/z 50-700) and data-dependent MS^n (n = 4) analysis in the positive ion mode. The mass spec response factors for MYMD-1 and M162 were determined by comparing the mass spec peak area ratio and the UV peak area ratio. The linearity response in plasma was evaluated by spiking human plasma with MYMD-1 stock to final concentration of 2 µg/mL, followed by 2-fold serial dilution (2X, 4X, 8X, 16X) with control rat, dog, and human plasma.

Isolation and Identification of M162

- Rats (n=4) were administered a daily oral dose of 400 mg/kg for three days and 24 hr urine was collected after each dose. The urine samples were combined and evaporated to dryness and the residue was reconstituted in methanol and then sonicated and filtered. The filtrate was then concentrated to 10 mL solution and mixed with 1 g of silica gel and the solvent in the silica mixture was evaporated under vacuum. The dried silica gel/urine concentrate mixture was loaded onto a flash column filled with silica gel (4 g) and eluted with dichloromethane and methanol gradient. The fractions related to M162 were combined and following evaporation of the solvent, 11 mg of white solid was obtained. Identification of M162 was performed using LC-MS/MS (LTQ-Orbitrap mass spectrometer couped to Agilent 1200 HPLC) and NMR (using a Varian Mercury-300 NMR spectrometer).

Identification of the Enzymes Involved in the Metabolism of M162

- Incubation mixtures containing MYMD-1 or M162 (1 µM), $MgCl_2$ (3 mM), rCYP (100 pmol/mL) in 0.1 M phosphate buffer (pH 7.4) were placed in a shaking water bath kept at 37ºC and pre-incubated for 5 min before the addition of NADPH (2 mM). Aliquots (50 µL) were removed in duplicate at 0, 5, 10, 15, 30, and 60 min and treated with 200 µL ACN containing the internal standard (d4-nicotine, 100 ng/mL). The samples were then centrifuged (4,000 rpm for 10 min) and aliquots of the supernatant were analyzed by LC-MS/MS using the peak area ratio approach. Similar incubations were also carried out with human liver microsomes and cytosol.

RESULTS

Identification of Circulating Human Metabolites

- MYMD-1 was extensively metabolized in humans, with unchanged parent accounting for <1% of total of drug-related components in $AUC_{0\text{-}24hr}$ pooled plasma samples (<1% and <4% based on MS and UV response, respectively). The oxidative metabolite M162 was the dominant species accounting for >97% of total drug-related related components (**Table 1**).

TABLE 1. Identified Human Metabolites

Name	Identity	% Abundance	
		Day 1 AUC	Day 5 AUC
MYMD-1	Parent	0.3	0.2
M163	-(NH)+2O	0.5	0.9
M178	+2O	0.6	1.2
M162	+O	98.6	97.7

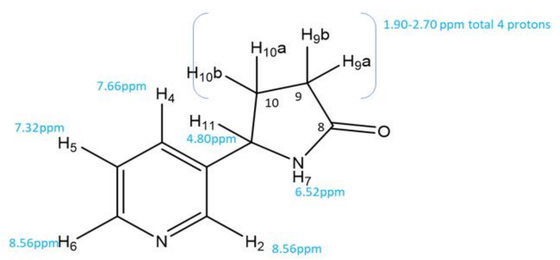
*Abundance is based on MS data

- Two other metabolites detected were M163 (proposed to be due to oxidation followed by deamination) and M178 (oxygenated derivative of M162) that together represented <2% of circulating species.
- Coverage of M162 in the safety species was evaluated and the results indicate adequate coverage in both safety species (**Table 2**).

TABLE 2. Plasma Exposure of M162 in Rat, Dog and Human

Peak Area Ratio of AUC0-24 hr Sample (±SD, n=3)			Ratio	
Rat	Dog	Human	Rat/Human	Dog/Human
10.79±0.05	5.15±0.2	10.38±0.4	1.04	0.5

FIGURE 2. ^{1}H-NMR Characterization of M162



Isolation and Identification of M162

- To determine the structure of M162, the metabolite was isolated and purified from urine of rats orally administered MYMD-1. The purified M162 was then characterized by high resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy. Based on HRMS and NMR data (**Figure 2**), and by comparison with synthetic standard, M162 was unambiguously identified as the known compound norcotinine.
- In the ^{1}H-NMR spectrum of M162, H2 and H6 were observed at 8.56 ppm, H4 was observed at 7.66 ppm and H5 was observed at 7.32 ppm. All the pyridine protons were observed, indicating no modification occurred on the pyridine ring. An NH or OH proton was found at 6.52 ppm. The methine proton, H11, was observed at 4.80 ppm with triplet pattern, indicating there are two neighboring protons on carbon 10 (H10a or H10b). If hydroxylation occurred on carbon 9, the chemical shift for H9a or H9b will be 3-4 ppm, which were not present in ^{1}H-NMR spectrum of M162, indicating carbon 9 remained unchanged in M162. With all other potential sites for hydroxylation excluded, the only possible position for hydroxylation was determined to be on carbon 8, which tautomerizes to the more stable lactam structure.

Identification of the Enzymes Involved in the Metabolism of M162

- Following incubation of MYMD-1 or norcotinine (1 µM) with a panel of human recombinant CYP enzymes (100 pmol/mL), MYMD-1 was metabolized by recombinant CYP2A6 with half-life and intrinsic clearance of 6.7 min and 1.03 µL/min/pmol, respectively. No turnover was observed with CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 or 3A5 (**Figure 3**).
- MYMD-1 was metabolized in human liver microsomes and cytosol with an intrinsic clearance of 36.1 and 91.2 µL/min/mg, respectively (**Table 3**). The depletion of MYMD-1 in cytosol was accompanied by robust formation of norcotinine (**Figure 4**). The formation of norcotinine in cytosol was strongly inhibited by the aldehyde oxidase inhibitor menadione. No norcotinine was formed by CYP2A6, indicating that the formation of norcotinine is dependent exclusively on the cytosolic enzyme aldehyde oxidase.
- No measurable turnover of norcotinine was observed in any of the nine recombinant CYP enzymes evaluated. However, when OH-norcotinine (M178) was monitored it was found to be formed only by CYP2A6.
- Norcotinine did not show measurable turnover in human liver microsomes or cytosol. Monitoring M178 indicated that the metabolite is formed in NADPH-fortified human liver microsomes.

FIGURE 3. Percent MYMD-1 Remaining Following Incubation with Recombinant CYP Enzymes

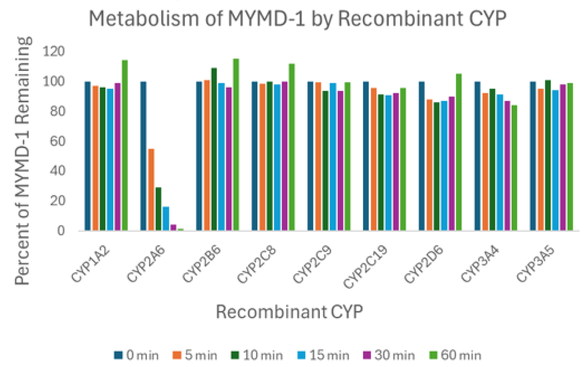
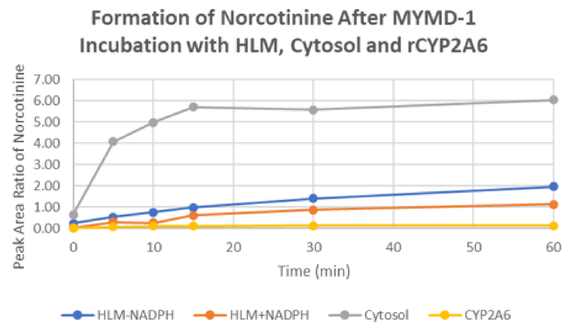


TABLE 3. Percent Remaining, Half-Life and Intrinsic Clearance of MYMD-1 Following Incubation in Human Liver Microsomes or Cytosol

Time (min)	Liver Microsomes		Liver Cytosol
	-NADPH	+NADPH	
0	100	100	100
5	97.1	82.7	33.0
10	97.6	67.7	13.1
15	92.7	52.6	6.55
30	90.8	29.9	4.34
60	84.1	11.5	3.83
$t_{1/2}$ (min)	ND	19.2	3.8
R^2	-	1.00	0.99
CL_{int} (µL/min/mg)	-	36.1	91.2

FIGURE 4. Formation of Norcotinine by rCYP2A6, Human Liver Microsomes and Cytosol



CONCLUSIONS

- The major circulating human metabolite of MYMD-1 accounting for >97% of drug-related exposure was identified as the known compound norcotinine and coverage of the metabolite in safety species was demonstrated. The formation of norcotinine was exclusively dependent on aldehyde oxidase. Studies with transporters[3] indicated that MYMD-1 is not a substrate of the common uptake and efflux transporters, and there was minimal elimination of unchanged MYMD-1 in human urine[2]. Thus, aldehyde oxidase is the main determinant of the clearance of MYMD-1 in humans, producing the major circulating and excretory metabolite M162, and CYP2A6 is predicted to play only a minor role in the disposition of MYMD-1.

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2. J. Brager, C. Chapman, L. Dunn and A. Kaplin. A double-blind, placebo-controlled, randomized, single ascending, and multiple dose Phase I study to evaluate the safety, tolerability, and pharmacokinetics of oral dose of Isomyosamine capsules in healthy adult subjects. *Drug Res* (Stuttg) 2023, 73(02):95-104.
3. In vitro interactions of MYMD-1 with drug transporters. Frontage report, July 2021