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# Identification of ML106 Phase 1 Metabolites in Human Liver Microsomes Using High-Resolution Quadrupole-Orbitrap Mass Spectrometry

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**Abstract :** High-resolution quadrupole-Orbitrap mass spectrometry (HRMS), with high-resolution (> 10,000 at full-width at half-maximum) and accurate mass (< 5 ppm deviation) capabilities, plays an important role in the structural elucidation of drug metabolites in the pharmaceutical industry. ML106, a derivative of imidazobenzimidazole, decreased melanin content and tyrosinase activity in a dose-dependent manner. Here, we investigated the phase 1 metabolic pathway of ML106 using HRMS in human liver microsomes (HLMs) and recombinant *cDNA-expressed* cytochrome P450 (CYP). After the incubation of ML106 with pooled HLMs and recombinant cDNA-expressed CYP in the presence of NADPH, five phase 1 metabolites, including three mono-hydroxylated metabolites (M1-3) and two di-hydroxylated metabolites (M4 and M5), were investigated. The metabolite structures were postulated by the elucidation of protonated mass spectra using HRMS. The CYP isoforms related to the hydroxylation of ML106 were studied after incubation with recombinant cDNA-expressed CYP. Here, we identified the phase 1 metabolic pathway of ML106 induced by CYP in HLMs.

**Keywords :** ML106, high-resolution quadrupole-Orbitrap mass spectrometry, cytochrome P450, phase 1 metabolism.

## Introduction

Drug metabolism study is essential for predicting and understanding of active metabolites, toxic mechanisms, excretion pathway and therapeutic effect in drug development. Specially, the aim of drug metabolism studies is ultimately to understand cause compounds to be ineffective therapeutically or toxicity in patients.[1] Because reactive metabolites generated in the liver might contribute to the induction of drug-induced hepatotoxicity, drug metabolism, were evaluated in vitro and in vivo in this preclinical study.[2]

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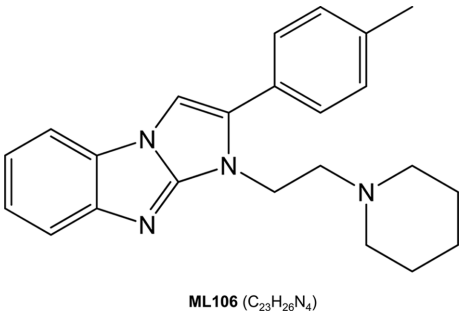
Recently, there has been continued development of high-resolution MS instruments (HRMS), which have been widely used for qualitative and quantitative analysis in many areas of research.[3] Especially, HRMS plays an important role in the structural elucidation of metabolites following the intergradation of exact elemental compositions of product ions for accelerating metabolism-guided lead finding and optimization of drug candidates in the pharmaceutical industry.[4,5] HRMS instruments providing ion measurements with high-resolution (>10,000 at full-width at half-maximum) and accurate mass (<5 ppm deviation) capabilities can distinguish drug metabolites from most isobaric endogenous components, and can determine elemental compositions of metabolite ions and their fragments.[6] Many drug metabolites have been identified using HRMS systems, therefore increasing the PubMed entries on "high-resolution mass spectrometry drug metabolism".[4]

ML106 (Fig. 1), a derivative of imidazobenzimidazole, decreased melanin content and tyrosinase activity in a dose-dependent manner.[7] ML106 has hypopigmentary activity through tyrosinase degradation via p38 MAPK phosphorylation. In the present study, we characterized the in vitro metabolic pathway of ML106 using HRMS in pooled human liver microsomes. The related CYP




**ML106** ($C_{23}H_{26}N_4$)

**Figure 1.** Chemical structure of ML106.

isoforms in ML106 metabolism were determined by incubation with human recombinant cDNA-expressed CYP isoforms. The data will be crucial to the early development of new drug candidates.

## Experimental Methods

### Materials

ML106 (purity > 99%) was chemically synthesized.[7] Pooled HLMs (mixed gender) were purchased from Sekisui XenoTech, LLC (Kansas City, KS, USA). Glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reduced β-nicotinamide adenine dinucleotide phosphate (β-NADPH) was obtained from the Oriental Yeast Co., Ltd. (Tokyo, Japan). All chemicals used in the experiment were of analytical grade.

### Biotransformation of ML106 in pooled human liver microsomes

ML106 (1-(2-(piperidin-1-yl)ethyl)-2-(p-tolyl)-1H-benzo [d]imidazo[1,2-a]imidazole, 50 μM) was incubated with pooled HLMs (1 mg/mL) in 200 μL reaction volume in the presence of 0.1 M potassium phosphate buffer (pH 7.4). The reactions were initiated by adding an NADPH-generating system (NGS) containing 0.8 mM *B*-NADPH, 10 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase. After 60 min incubation at 37°C, 400 μL of acetonitrile (ACN) was added to stop reactions, the samples were centrifuged at 13,000 rpm for 10 min at 20°C. An amount of 500 μL supernatant was transferred and dried via vacuum concentration. After evaporation, samples were stored immediately at -80°C until further analysis. The samples were reconstituted with 100 μL 50% methanol in 0.1% formic acid and centrifuged at 13,000 rpm for 10 min at 20°C. Supernatants were transferred to auto-sampler vials, and 5 μL aliquots were injected into the LC system.

### Reaction phenotyping in ML106 metabolism

The reaction mixture consisted of 10 μL of human recombinant *cDNA-expressed* CYP isoforms (CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5, 10 pmol) and 50 μM ML106 with NGS system in 200 μL reaction volume in the presence of 0.1 M potassium phosphate buffer (pH 7.4) and incubated at 37°C for 60 min. The procedure for the sample preparation was described in the previous section.

### Instruments

High-resolution MS (HR/MS) experiments were conducted to elucidate metabolite structures on a Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific Inc., MA, USA) operated in a positive-ion electrospray mode. Acquisition and analysis of the data were performed using Xcalibur (Version 3.0). Full scan MS spectra were acquired for the measurement of accurate masses of ML-106 and its metabolites. HPLC methods in the mobile phase consisted of water (mobile phase A) and ACN (mobile phase B), both of which contained 0.1% formic acid, at a flow rate of 0.22 mL/min at 40°C. For metabolic profiling, the gradient conditions were as follows: 10% of B at 0-3 min, 10-90% of B at 3-26 min, 90% of B at 26-28 min, 90-10% of B at 28-29 min, and 10% of B at 29-30 min. The analytes were separated with a Kinetex® 2.6 μm C18 100 Å (150 × 2.1 mm, Phenomenex Inc., CA, USA). The ion spray voltage was adjusted to 3,500 V. Nitrogen was used as an aux gas and sheath at 10 and 40 (arbitrary unit), respectively, at 320°C. The mass spectrometer was operated in the positive ion mode in a mass range of 66.7-1,000 m/z.

## Results and Discussion

Representative extracted ion chromatograms following the incubation of pooled human liver microsomes and human recombinant *cDNA expressed*-CYP isoforms with ML106 are shown in Fig. 2 and Fig. 3. After 60 min incubation in the presence of an NGS, five metabolites (M1-5) were generated, and the protonated ions were observed at m/z 375.21664 (M1) and 375.21716 (M2) corresponding to mono-hydroxylation, at m/z 391.21204 (M4) and 391.21213 (M5) corresponding to di-hydroxylation in pooled human liver microsomes (Table 1 and Fig. 2). M3 was strongly generated in *cDNA-expressed* recombinant CYP 3A4 and 3A5, in which protonated ions were observed at m/z 375.21674 corresponding to mono-hydroxylation (Table 2 and Fig. 3).

The product ion spectrum of protonated ML106 (359.22209 m/z) is depicted in Fig. 4A, and shows the major ion product ion at 112.11120 m/z. The major ion product at 112.11220 m/z indicated the ethylpiperidine moiety representing the even electron cation $C_7H_{14}N^+$ with an error of 1.1 ppm, which was the key ion for the structural elucidation. The ion product spectra of three protonated mono-hydroxylated metabolites (M1-3) showed the same pattern. The dominantly generated metabolite in HLMs, M2, was observed at 375.21716 m/z, and was 16 Da heavier than protonated ML106, indicating mono-hydroxylation. The ion at 112.11220 m/

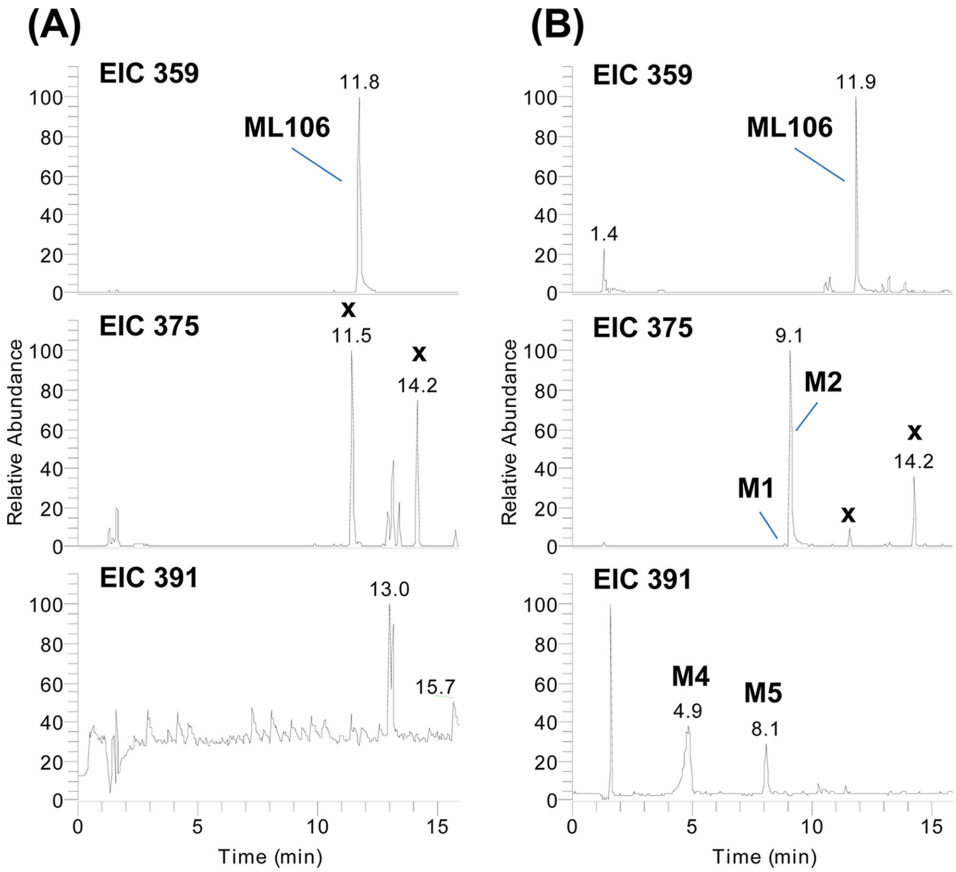



**Figure 2.** Extracted ion chromatograms for ML106 (50 μM), its mono-hydroxylated metabolite (M1 and M2), di-hydroxylated metabolites (M4 and M5) and after 60 min incubation with 1 mg/mL of pooled human liver microsomes in the absence (A) and presence of an NADPH-regenerating system (B). The symbol "x" indicates endogenous interference peaks.

**Table 1.** Elemental composition of protonated ions of ML106 and its hydroxylated metabolites in pooled human liver microsomes using high-resolution quadrupole-Orbitrap mass spectrometry.

| Compounds | Parent ions (m/z) | Elemental composition | Error (ppm) | Product ions (m/z) | Elemental composition | Error (ppm) |
|---|---|---|---|---|---|---|
| ML106 | 359.22209 | $C_{23}H_{27}N_4$ | -2.6 | 112.11220 | $C_7H_{14}N$ | 1.1 |
| M1 | 375.21664 | $C_{23}H_{27}ON_4$ | -3.5 | 112.11208 | $C_7H_{14}N$ | 0.03 |
| M2 | 375.21716 | $C_{23}H_{27}ON_4$ | -2.1 | 112.11220 | $C_7H_{14}N$ | 1.1 |
| M3 | 375.21674 | $C_{23}H_{27}ON_4$ | -3.2 | 128.10678 | $C_7H_{14}ON$ | -3.2 |
| M4 | 391.21204 | $C_{23}H_{27}O_2N_4$ | -3.5 | 112.11223 | $C_7H_{14}N$ | -3.5 |
| M5 | 391.21213 | $C_{23}H_{27}O_2N_4$ | -3.3 | 128.10698 | $C_7H_{14}ON$ | -4.4 |

z was detected as the same as that of the parent, indicating a hydroxyl group on the 2-(p-tolyl)-1H-benzo[d]imidazo[1,2-a]imidazole moiety, but not the ethylpiperidine group (Fig. 4B).

M4 and M5 were observed at 391.21204 and 319.21213 m/z, corresponding to di-hydroxylation at retention times of 4.9 and 8.1 min, respectively. The product ions of protonated M4 at 112.11223 m/z indicated di-hydroxylation of 2-(p-tolyl)-1H-benzo [d]imidazo[1,2-a]imidazole moiety, whereas the product ion at 128.10698 m/z of M5 was a characteristic ion for the presence of a hydroxyl group at one of the positions of the ethylpiperidine group and 2-(p-tolyl)-1H-benzo[d]imidazo [1,2-a]imidazole moiety, respectively (Figs. 4C and 4D).

To characterize ML106 metabolism in *cDNA-expressed* recombinant CYPs, the metabolism of ML106 was performed using incubation with 10 specific human

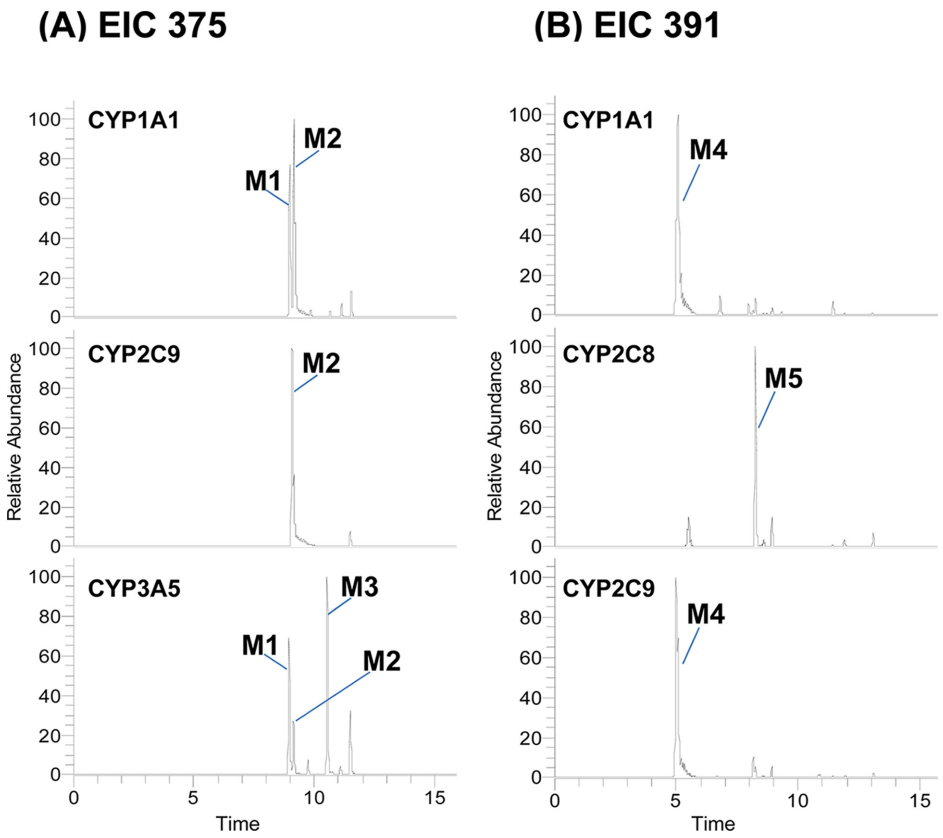



**Figure 3.** The formation of M1-M3 (A), and M4 and M5 (B) in human recombinant cDNA-expressed CYP isoforms for 60 min incubation in the presence of an NADPH-regenerating system.

**Table 2.** The formation of metabolites in human recombinant CYPs.

| Metabolite | Relative formation of metabolites (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CYP1A1 | CYP1A2 | CYP2B6 | CYP2C8 | CYP2C9 | CYP2C19 | CYP2D6 | CYP2E1 | CYP3A4 | CYP3A5 |
| M1 | 83.7 | 50.6 | 2.0 | 1.9 | 0.6 | 100 | 0.2 | 22.2 | 21.4 | 23.0 |
| M2 | 33.7 | 24.6 | 17.4 | 17.3 | 100 | 53.1 | 0.6 | 2.6 | 2.9 | 3.2 |
| M3 | 8.0 | 5.8 | 24.8 | 25.0 | 3.8 | 2.4 | 0.5 | 51.5 | 100 | 93.2 |
| M4 | 100 | 22.4 | 0.9 | 2.8 | 56.8 | 68.9 | - | 0.3 | 0.3 | 0.3 |
| M5 | 50.4 | 5.5 | 5.9 | 100 | 21.5 | 24.2 | - | 4.8 | 5.1 | 9.4 |

recombinant CYPs in the presence of NGS (Fig. 2). ML106 was incubated in 10 pmol human recombinant CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5, respectively (Table 2). Two mono-hydroxylated metabolites (M1 and M3) were newly generated in specific CYP isoforms, whereas were weakly generated metabolites in HLMs. M1 was significantly generated in CYP 1A1 and 2C19, and M3 was selectively generated in the CYP3A family. M2 was mainly generated by the CYP2C family, such as CYP2C8, 2C9, and 2C19, with a slight contribution from CYP1A1. For the di-hydroxylation of ML106, CYP1A2, 2D6, and 3A4 mainly generated the formation of M4 and M5.

## Conclusion

In the present study, ML106 was metabolized in a CYP-dependent manner in pooled human liver microsomes. ML106 was metabolized to two kinds of metabolites, i.e., mono- (M1-3) and di-hydroxylated forms (M4 and M5). The five metabolites of ML106 were proposed using high-resolution quadrupole-Orbitrap mass spectrometry. The postulated metabolic pathway was shown in Fig. 5. Knowledge of the proposed structures of the metabolic pathway of ML106 will be helpful in studies of in vivo metabolism in future research.

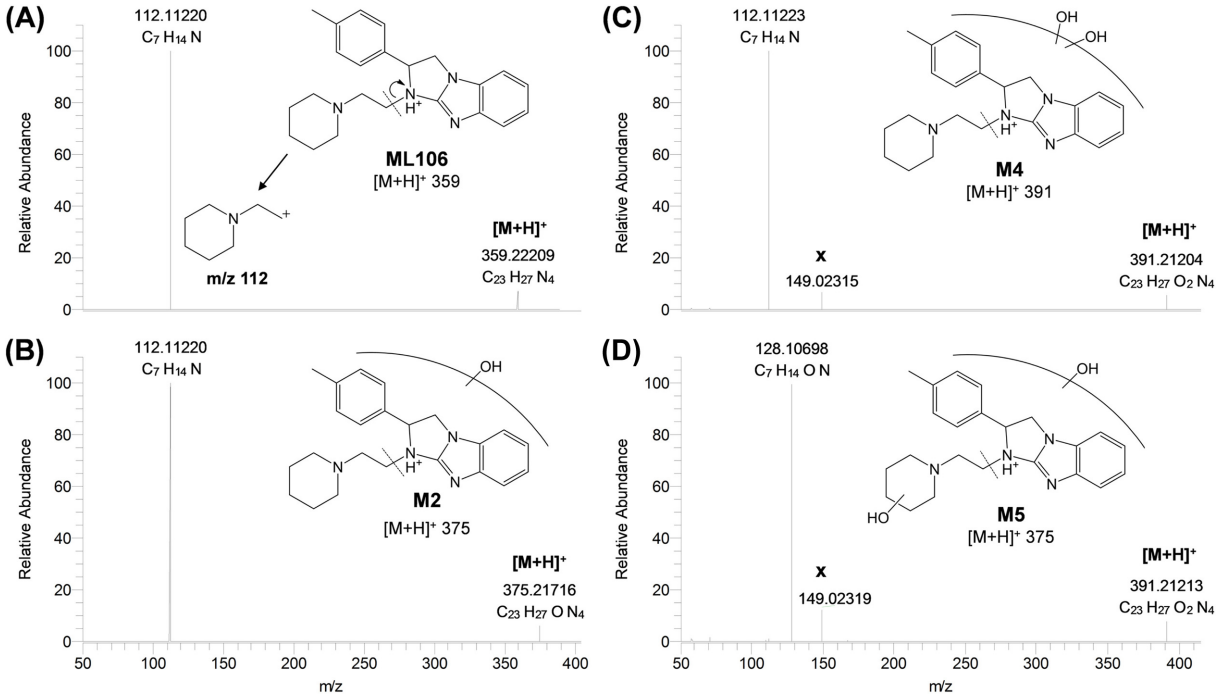



**Figure 4.** MS/MS spectrum of protonated ML106 (A), M2 (B), M4 (C), and M5 (D) in high-resolution quadrupole-Orbitrap mass spectrometry. The symbol "x" are endogenous interferences peaks.

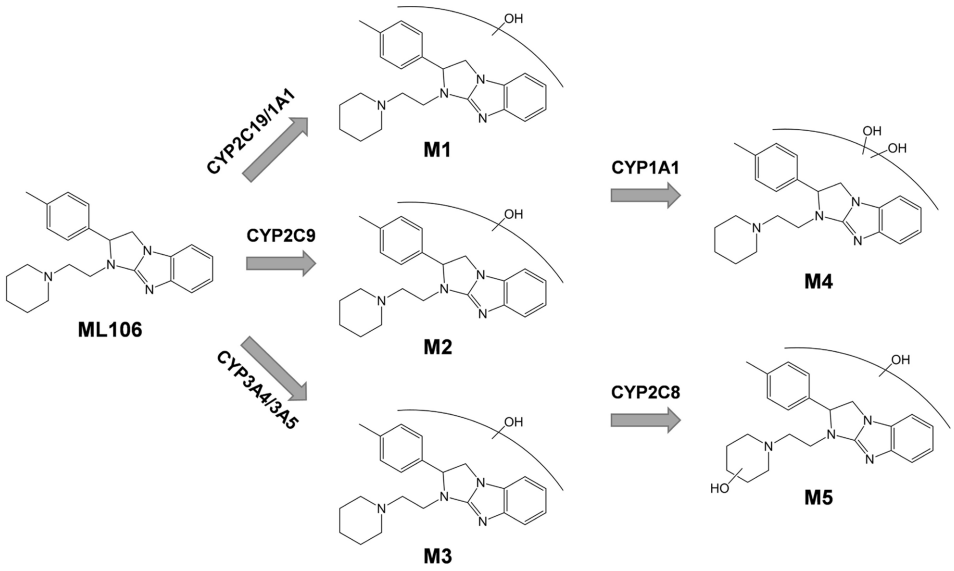



**Figure 5.** Proposed metabolic pathways of ML106 in human liver microsomes.

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