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Identification of HYIpro-3-1 Metabolites, a Novel Anti-Inflammatory Compound, in Human Liver Microsomes by Quadrupole-Orbitrap High-Resolution Mass Spectrometry

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Abstract : HYIpro-3-1 is an adjuvant for preventing or treating inflammatory growth diseases. In this study, we identified the metabolic pathway of HYIpro-3-1 in human liver microsomes (HLMs) by quadrupole-orbitrap high-resolution mass spectrometry (HR-MS) and characterized the major human cytochrome P450 (CYP). Ten metabolites were identified, including one *O*-demethylation (M1), two *O*-demethylation and monohydroxylation (M2 and M3), and seven monohydroxylation metabolites (M4–M10). Based on the HR-MS^2 spectra, the metabolites are divided into two groups of monohydroxylated metabolites according to the hydroxylation position. We verified that HYIpro-3-1 is metabolized by CYP in HLMs, CYP2B6 is mainly involved in *O*-demethylation, and various CYPs are involved in the monohydroxylation of HYIpro-3-1.

Keywords : HYIpro-3-1; LC-HR/MS; cytochrome P450; human liver microsomes

Introduction

In recent decades, with technological advancement in high-resolution mass spectrometry (HR-MS), it has been employed as an essential technique in many research fields.[1] In drug metabolism, HR-MS has a great advantage in identifying the structure of metabolites by identifying the exact elemental composition of detected product ions based on its unique high resolution and high accuracy.[2] For example, an orbitrap (OT) mass analyzer provides a resolving power of up to 100,000 with mass accuracy below 1 ppm; thus, it can accelerate metabolism-guided lead identification and optimization of drug candidates in the pharmaceutical industry. Recently, HR-MS has been employed in the identification of drug metabolites.[3]

HYIpro-3-1 [(Z)-2-(2-methoxybenzylidene) benzofuran-3(2H)-one], a benzophenone derivative, is a pharmaceutical composition for preventing or treating inflammatory bowel disease comprising as an active ingredient a benzylidene benzofuranone derivative compound.[4] The benzophenone derivative compound maintains the normal state of small intestinal thickness and large intestinal length, has an excellent anti-inflammatory effect on the large intestine, and prevents inflammatory growth diseases.[4] In addition, we identified cytochrome P450 (CYP) isoforms involved in the biotransformation of HYIpro-3-1 in pooled human liver microsomes (HLMs). Analysis of the metabolic pathway may provide important information during initial drug development processes, and the data are crucial to the early development of new drug candidates. In this study, we characterized the metabolic pathway of HYIpro-3-1 in vitro by HR-MS in HLMs.

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Experimental

Materials

HYIpro-3-1 (purity > 99%) was chemically synthesized.[4] Pooled HLMs (mixed gender) were purchased from Sekisui XenoTech, LLC (Kansas City, KS, USA). Human recombinant cDNA-expressed CYP isoforms were purchased from Corning Gentest (Woburn, MA, USA). A reduced nicotinamide adenine dinucleotide phosphate (β-NADPH)-generating system (NGS) was obtained from Promega (Madison, WI, USA). All chemicals were analytical grade.

Biotransformation of HYIpro-3-1 in pooled human liver microsomes

HYIpro-3-1 (20 µM) was incubated with mixed HLMs (1 mg/mL) and 0.1 M phosphate buffer (pH 7.4) with a reaction volume of 200 µL.[5,6] The reaction was initiated by adding NGS. After 60 min incubation at 37°C, 400 µL of 100% acetonitrile (ACN) was added to terminate the reaction, and the sample was centrifuged at 4°C and 13,000 rpm for 10 min. The 550 µL supernatant was transferred to a new tube and dried by SpeedVac concentrator. The sample was stored at -80°C until subsequent analyses. The sample was redissolved in 100 µL of 20% methanol with 0.1% formic acid (FA) and centrifuged at 4°C and 13,000 rpm for 10 min. The supernatant was transferred to automatic sampler vials, and 5 µL of a mixed sample was injected into a C18 column for LC-HR/MS analysis.

Metabolism of HYIpro-3-1 in human recombinant cDNA-expressed cytochrome P450 isoforms

The reaction mixture consisted of 10 µL of human recombinant cDNA-expressed CYP isoforms (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, 10 pmole) and 20 µM HYIpro-3-1 with NGS system in a 200 µL reaction volume with a 0.1 M potassium phosphate buffer (pH 7.4).[5,6] The reactions were initiated by adding NGS and incubating at 37°C for 60 min.

Instruments

HR-MS experiments were conducted to clarify the metabolite structures using a Q-Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific Inc., MA, USA) operated in a positive-ion electrospray mode with nitrogen as the desolvation gas, nebulizing gas, capillary voltage, and vaporizer temperature, with optimum values set at 10 L/h, 45 psi, 4,000 V and 350°C respectively. Data were acquired and analyzed using Xcalibur (Version 3.0). Full-scan MS spectra were acquired for accurate measurement of the mass of HYIpro-3-1 and its metabolites. High-performance liquid chromatography was performed in a mobile phase consisting of water (mobile phase A) and ACN (mobile phase B), both of which contained 0.1% FA, at a flow rate of 0.20 mL/min and temperature of 40°C. For metabolic profiling, the gradient conditions were as follows: 15% of B for 0-0.5 min, 15%-95% of B for 0.5-20.5 min, 95% of B for 20.5-24 min, 95%-15% of B for 24-24.1 min, and 15% of B for 24.1–30 min. The analytes were separated using 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, and temperature of 320°C. The mass spectrometer was operated in the positive-ion mode and a mass range of *m/z* 66.7–750.0.

Results and Discussion

Identification of HYIpro-3-1 metabolites in human liver microsomes

After incubating for 60 min in HLMs in the presence of NGS, the generated HYIpro-3-1 metabolites were confirmed by the representative extracted ion chromatogram (EIC). The EIC of HYIpro-3-1 and its 10 metabolites (M1-M10) are shown in Figure 1. Protonated HYIpro-3-1 ions were

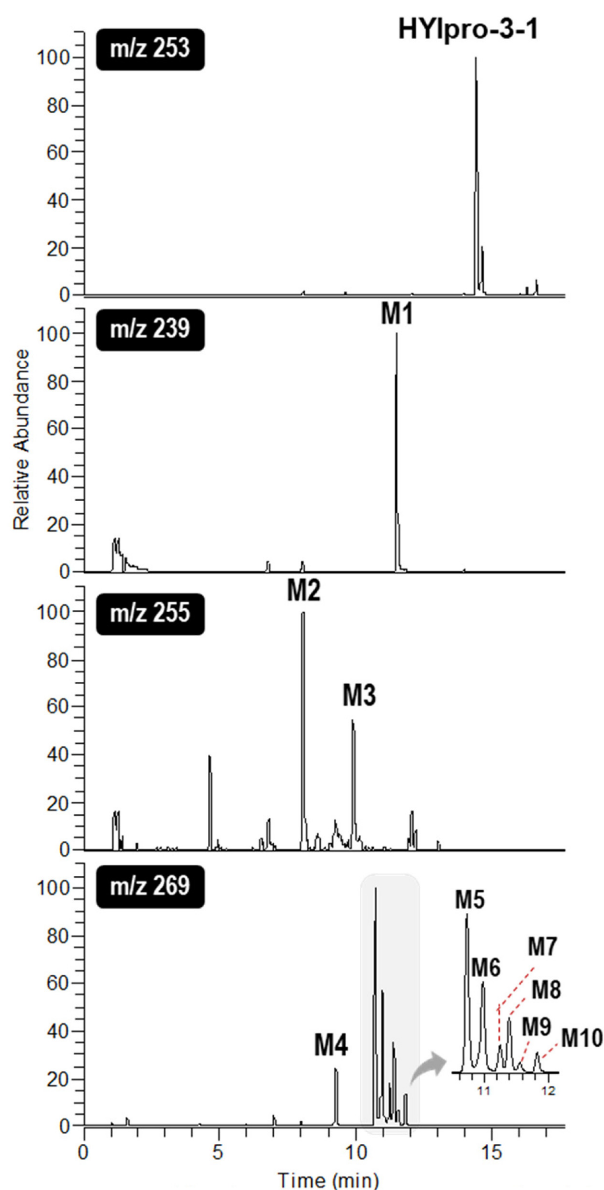

Figure 1. Extracted ion chromatograms for HYIpro-3-1, demethylated metabolite (M1), demethylated and monohydroxylated metabolites (M2 and M3), and monohydroxylated metabolites (M4-M10) after 60 min incubation with 1 mg/mL of pooled human liver microsomes.

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observed at *m/z* 253.086 with a retention time (RT) of 14.4 min. Protonated ions of M1 corresponding to *O*-demethylation, were observed at *m/z* 239.070 in 11.5 min, and *O*-demethylated and monohydroxylated metabolites, M2 (*m/z*

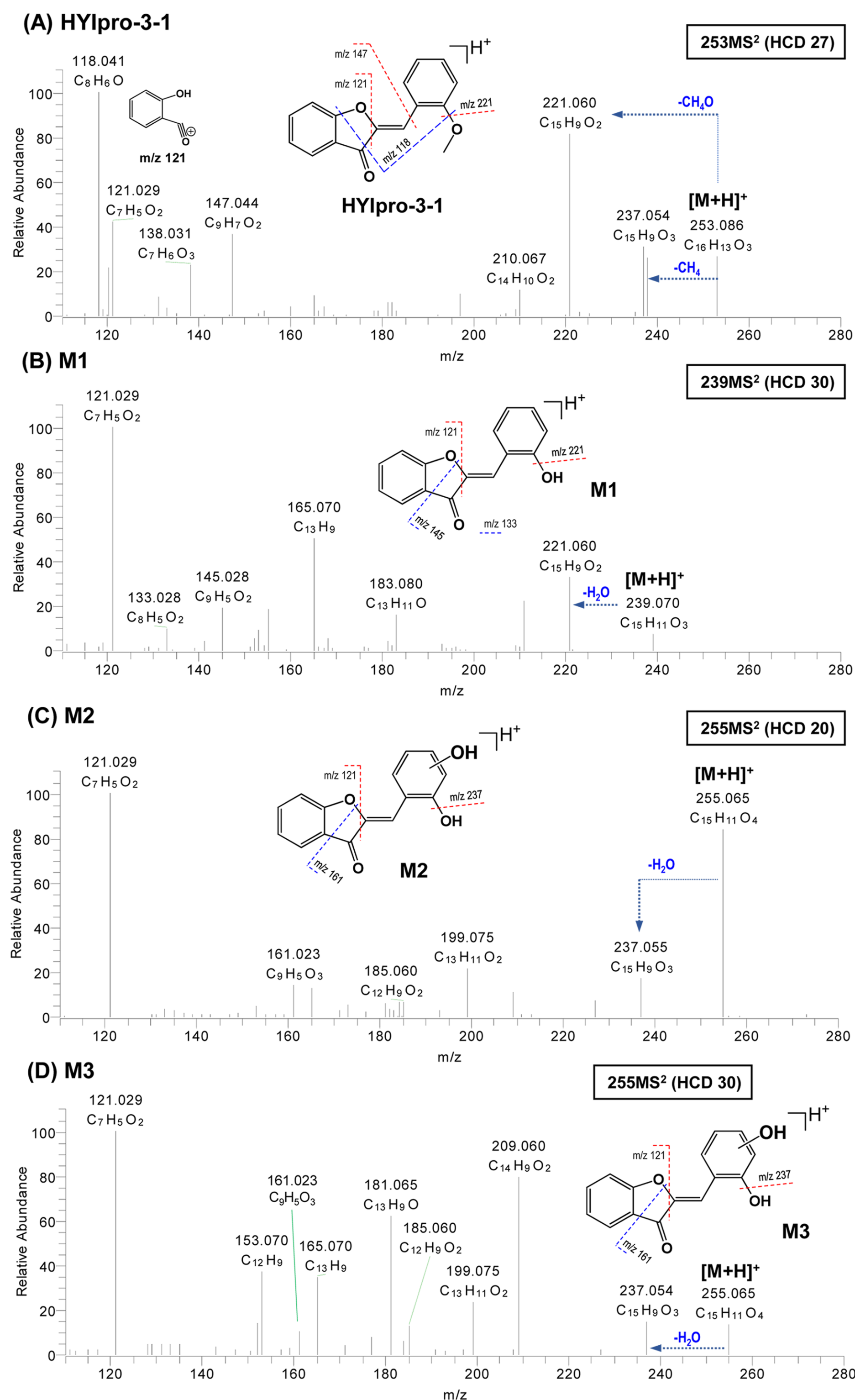

Figure 2. MS/MS spectra of protonated HYIpro-3-1 (A), M1 (B), M2 (C) and M3 (D) in quadrupole-orbitrap high-resolution mass spectrometry (Q-OT-HR-MS).

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255.065) and M3 (*m/z* 255.065), were observed at 8.1 and 9.9 min, respectively. Protonated ions of monohydroxylated metabolites (M4-M10) were observed at *m/z* 269.080-269.081 with RT of 9.3 (M4), 10.7 (M5), 11.0 (M6), 11.3 (M7), 11.4 (M8), 11.6 (M9), and 11.8 (M10) min.

Chemical structure of metabolites

Although at EIC of *m/z* 255, new peaks with potential metabolites, including M2 and M3, were obeserved, LC-HR-MS analysis did not provide interpretable MS^2 spectra. Therefore, other peaks were not identified as metabolites except M2 and M3; M2 and M3 are metabolites that have undergone both *O*-demethylation and monohydroxylation, and the various peaks shown in EIC of *m/z* 255 are attributed to various metabolites obtained by mixing hydroxylation and *O*-demethylation, like M2 and M3. Therefore, since it is a

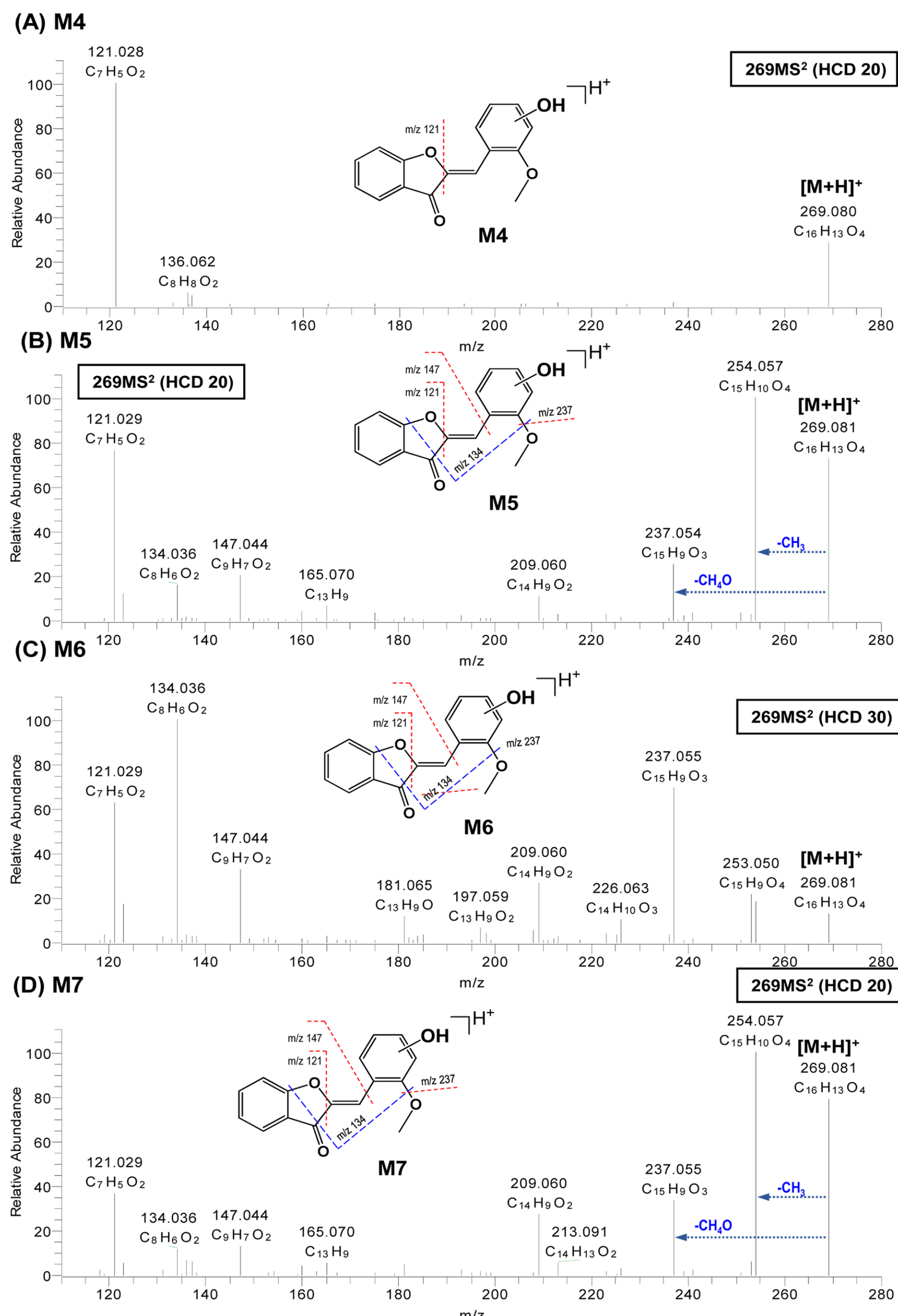

Figure 3. MS/MS spectra of protonated M8 (A), M9(B), and M10 (C) by Q-OT-RHMS.

two-step metabolism, the amount of the final product is lower than that of *O*-methylated metabolites (M1) and monohydroxylated metabolites (M4-M10), and the MS^2 spectra could not be obtained.

The structures of **HYIpro-3-1** and its metabolites were evaluated and characterized by quadrupole-orbitrap high-resolution mass spectrometry (Q-OT-HR-MS). Based on the high resolution analysis, the product ions and fragments indicated that their elemental composition was less than 5 ppm. The precursor ion spectra of protonated HYIpro-3-1 were observed at *m/z* 253.086 and those of the major product ions were observed at *m/z* 237.054 ($C_{15}H_9O_3$), 221.060 ($C_{15}H_9O_2$), 147.044 ($C_9H_7O_2$), 121.029 ($C_7H_5O_2$), and 118.041 (C_8H_6O) (Figure 2A). HR-MS analysis revealed that the dominant fragment ions of MS^2 were produced at *m/z* 221.060 and 118.041, indicating the loss of the CH_4O group from anisole moiety (32 Da) and the loss of benzaldehyde in *m/z* 221.060 (103 Da), respectively, which were confirmed by the MS^3 spectra of the *m/z* 221 of product ion of HYIpro-3-1 (data not shown). Other key ions were at *m/z* 147.044 and 121.029, indicating the loss of anisole and 1-methoxy-2-vinylbenzene moiety, respectively, which shows the metabolism in the benzofuran part of HYIpro-3-1.

M1 produced protonated ions at *m/z* 239.070, which are 14.016 Da smaller than the protonated ions of HYIpro-3-1, indicating *O*-demethylation. M1 yielded product ions at *m/z* 221.060 ($C_{15}H_9O_2$, loss of H_2O), 121.029 ($C_7H_5O_2$, loss of 1-methoxy-2-vinylbenzene moiety), and 145.028 ($C_9H_5O_2$) (Figure 2B). The product ion at *m/z* 145.028 (loss of phenol in the benzofuran part) was the key ion to estimate the metabolism in phenol group of *O*-demethylated HYIpro-3-1.

M2 and **M3** were observed at *m/z* 255.065, corresponding to *O*-demethylation and monohydroxylation. The product ions at *m/z* 237.055 ($C_{15}H_9O_3$, loss of H_2O), 121.029 ($C_7H_5O_2$), and 161.023 ($C_9H_5O_3$) determined the structure

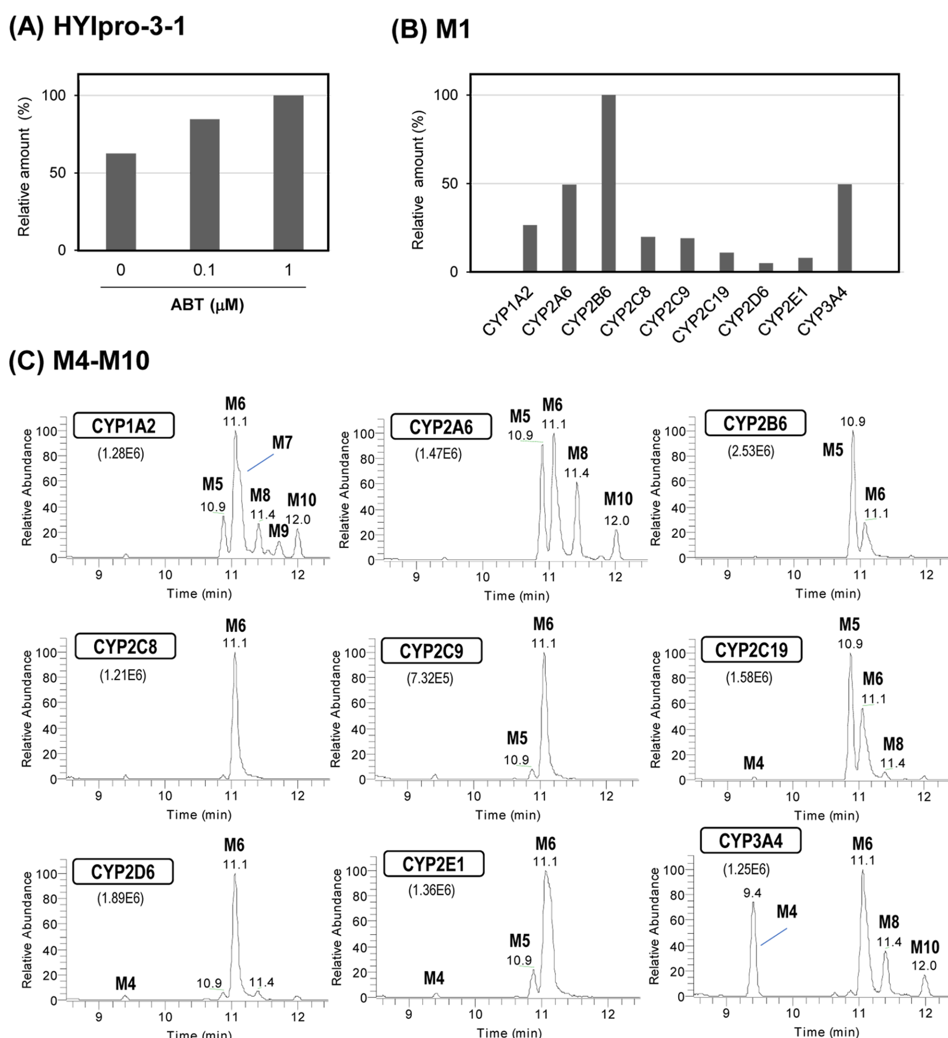

Figure 4. Inhibition of HYIpro-3-1 metabolism by 1-aminobenzotriazole (ABT) as a non-specific inhibitor of cytochrome P450 (CYP) (A). The relative formation of M1 (B) and M4-M10 (C) in human recombinant cDNA-expressed CYP isoforms.

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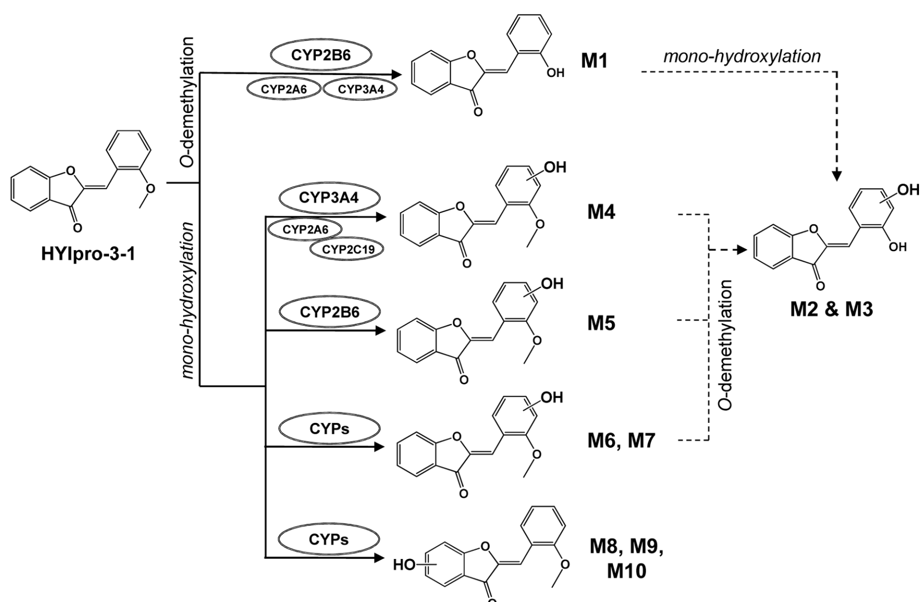

Figure 5. Proposed metabolic pathway of HYIpro-3-1 in human liver microsomes.

of M2 and M3, and they were detected in the MS^2 spectra (Figure 2C and D). The product ions at *m/z* 121.029 were the same as those of HYIpro-3-1, indicating that the benzofuran part was not metabolized. The *m/z* 161.023 indicates monohydroxylation of the phenol moiety as an ion with an increase of 16 Da compared to *m/z* 145.028 observed in M1.

M4-M10 produced protonated ions at *m/z* 269.080-269.081, 16 Da greater than the protonated ions of HYIpro-3-1, indicating monohydroxylation of HYIpro-3-1. Based on the MS^2 spectra, the metabolites were divided into two groups according to the hydroxylation positions. M4-M7 are metabolites with monohydroxylated to the anisole part, whereas M8-M10 are monohydroxylated metabolites in the benzofuran part. **M4-M7** commonly yielded product ions at *m/z* 121.029 ($C_7H_5O_2$), which are the same as those of the parent material indicating that the benzofuran part was not hydroxylated (Figure 3). In the MS^2 spectra of M5-M7, *m/z* 147.044 ($C_9H_7O_2$) and 134.036 ($C_8H_6O_2$) were observed. The ion at *m/z* 147.044 nonhydroxylation in the benzofuran moiety, and that at *m/z* 134.036 indicates the monohydroxylation of anisole moiety as an ion with an increase in 16 Da compared to the *m/z* 118.041 observed in the parent material.

In **M8-M10,** monohydroxylation of benzofuran moiety was identified from the generated product ions at *m/z* 163.039 ($C_9H_7O_3$), 137.023 ($C_7H_5O_3$), and 118.041 (C_8H_6O) (Figure 4).

The product ions at *m/z* 163.039 and 137.023 were 16 Da higher than those at *m/z* 147.044 and 121.029 observed in parent compound, respectively. The ion observed at *m/z* 118.041 indicates the phenol moiety of HYIpro-3-1. Therefore, M8-M10 are considered monohydroxylated metabolites in benzofuran moiety.

Cytochrome P450-dependent metabolism of HYIpro-3-1

To verify the dependence of the HYIpro-3-1 metabolism on CYP, HYIpro-3-1 was incubated with 1-aminobenzotriazole (ABT) as nonspecific inhibitor of CYP in HLMs in the presence of NGS for 60 min.[7] After the reaction, the remaining amount of HYIpro-3-1 varied with the ABT concentration (Figure 5A), and the production of metabolites decreased (data not shown).

For further identification of HYIpro-3-1 metabolic enzymes in HLMs, we incubated HYIpro-3-1 with nine human *cDNA-expressed* recombinant CYP (10 pmole) isoforms in the presence of NGS. First, M1, as an *O*-demethylated metabolite, was generated by CYP2B6 (Figure 5B). Other CYPs, such as CYP3A4, CYP2A6, and CY1A2, were partially involved in the formation of M1. Various CYP isoforms were involved in the monohydroxylated metabolite (M4-M10; Figure 5C). M4 was mainly metabolized by CYP3A4. M5 was metabolized by CYP2A6, CYP2B6, and CYP2C19. M6 was produced in most isoforms, M8 was metabolized by CYP2A6, and M10 was metabolized by CYP1A2, CYP2A6, and CYP3A4. M2 and M3, which were subjected to both *O*-demethylation and monohydroxylation, were not detected in a single CYP isoform, and M7 and M9, which produced a low amount of monohydroxylation metabolites, could not identify by related CYPs.

Conclusions

In this study, HYIpro-3-1 was metabolized in pooled HLMs,

and the metabolism varied with the CYP concentration. HYIpro-3-1 was metabolized to three metabolites, including *O*-demethylation (**M1**), *O*-demethylated and monohydroxylation (**M2** and **M3**), and monohydroxylation (**M4-M10**) (Figure 6). The 10 metabolites of HYIpro-3-1 were observed by Q-OT-HR-MS. Based on the results, we propose a pathway for metabolism. Knowledge of the proposed structures of HYIpro-3-1 would be helpful to study on in vivo metabolism.

Acknowledgments

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