MICROBIAL MODELS OF MAMMALIAN METABOLISM

Biotransformations of HP 749 (Besipirdine) Using Cunninghamella elegans

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ABSTRACT:

HP 749 (besipirdine; Hoechst-Roussel Pharmaceuticals, Inc., Somerville, NJ) and related analogs belonging to the N-(4-pyridinyl)-1H-indol-1-amine class of compounds have shown a potential to mitigate multiple biochemical deficits associated with Alzheimer’s disease. HP 749 has demonstrated cholinergic and noradrenergic activities both in vitro and in vivo, and has potential for the symptomatic treatment of Alzheimer’s disease. The three primary metabolites of HP 749 in dogs, rats, and humans result from hydroxylation of the indole ring, N-dealkylation of the parent compound, and sequential hydroxylation and dealkylation. The fungus Cunninghamella elegans (ATCC 36112) converts 25% of HP 749 in a dextrose broth to yield four metabolites, three of which have been reported in mammalian systems. Preparative scale fermentation allowed for the isolation of the major fungal metabolite resulting from hydroxylation of the indole nucleus at position 5 (16%), which was characterized by cochromatographic (TLC and HPLC), 1H-NMR, mass spectral (chemical ionization/MS), and UV comparisons to a synthetic standard. Additional minor fungal metabolites were formed as a result of N-dealkylation (2%), and sequential N-dealkylation and aromatic hydroxylation (2.5%). C. elegans is being used as a model to help predict and generate the logical mammalian metabolites of related structural analogs of HP 749.

Alzheimer’s disease is a debilitating neurological disorder characterized by symptoms of central serotonergic, cholinergic, and adrenergic deficits (1). HP 749 [N-(n-propyl)-N-(4-pyridinyl)-1H-indol-1-amine hydrochloride] (fig. 1) enhances both cholinergic and adrenergic neurotransmission in the central nervous system (2, 3) and shows potential for the symptomatic treatment of Alzheimer’s disease. In vitro and in vivo studies in dogs, rats, and humans indicate that HP 749 is primarily metabolized by hydroxylation of the indole ring, by N-dealkylation of the parent compound, and by sequential hydroxylation and depropylation (4, 5). Additional minor mammalian metabolites are formed as a result of oxidation at position 2 of the indole ring and reductive cleavage of the hydrazine bond (4, 5). Minor amounts of a 4-hydroxy-1H-indol metabolite have been found in mammalian systems (5) (fig. 1), whereas the 6-hydroxy-1H-indol metabolite has not been seen in these mammalian systems (5). At high doses, HP 749 exerts an effect on the central nervous system leading to bradycardia in conscious dogs, rats, and monkeys (3, 6). The N-dealkylated metabolite of HP 749 exerts both α1- and α2-adrenoceptor activities by acting directly on vascular smooth muscle resulting in a vasopressor affect in conscious dogs, rats, and monkeys (3, 5). HP 749 and its two mammalian metabolites (N-dealkylated and aromatic hydroxylated; fig. 1) have shown adrenergic activity in vitro assays (5).

Microorganisms possess cytochrome P450 monooxygenase systems (7–10), and have been used as models for studying the mammalian (hepatic) metabolism of drugs and other xenobiotics. The main focus of this field has been to demonstrate parallels between the phase I metabolism of xenobiotics in mammalian and microbial systems (9–14). An advantage of using microbial systems lies in the ease with which milligram quantities of putative metabolites can be produced by large-scale fermentation and under milder conditions than those required by chemical systems.

HP 749 was chosen as a representative substrate to facilitate the development of microbial models of mammalian metabolism (12). Such a microbial model could be used to generate sufficient quantities of the logical mammalian metabolites of structural analogs of HP 749 that have not yet been tested for metabolite formation (15). Sufficient quantities of metabolites could then be isolated and used for pharmacological, toxicological, and analytical testing in conventional animal models of human metabolism, as part of the drug development process. To achieve this, attention was focused on microbes with broad metabolic capabilities.

Materials and Methods

Chemicals and Reagents. All solvents were of analytical grade or better. HP 749 [N-(n-propyl)-N-(4-pyridinyl)-1H-indol-1-amine hydrochloride], P7480 [N-(4-pyridinyl)-1H-indol-1-amine hydrochloride], P9400 [N-(n-propyl)-N-(4-pyridinyl)-N-(5-hydroxy-1H-indol)-1-amine], P9679 (N-(4-pyridinyl)-N-(5-hydroxy-1H-indol)-1-amine), and internal standards [3-ethyl-N-methyl-N-(4-pyridinyl)-1H-indol-1-amine hydrochloride] were obtained from Hoechst-Roussel Pharmaceuticals, Inc. (Somerville, NJ). 1-Bromopropane and sodium hydride (80% dispersion in mineral oil), and CD3OD for NMR studies were obtained from Aldrich Chemical Co. (Milwaukee, WI). DMF (molecular biology grade) was obtained from Fisher Scientific (Fairlawn, NJ). Triethylamine was obtained from Sigma Chemical Co. (St. Louis, MO).

TLC. Analytical TLC was performed on polyester-backed 0.25 mm silica gel GF254 plates (Whatman Ltd., Maidstone, Kent, UK), and metabolites were eluted with chloroform:methanol (90:10, v/v). Plates were visualized under UV light, and the RF values obtained in this system were as follows: HP 749 = 0.52, P7480 = 0.32, P9400 = 0.23, and P9679 = 0.08.

HPLC. HPLC was conducted using a Beckman model 110A single piston pump, a Tracor 970A variable wavelength detector set to monitor a wavelength
of 270 nm, and a Hewlett-Packard 3390A integrator for recording chromatograms. The conditions were as follows: mobile phase, 85% v/v acetonitrile and 15% v/v triethylammonium formate buffer (5.8 mM, adjusted to pH 2.5 with 90% formic acid) at a flow rate of 1 ml/min for analytical scale studies using a Hypersil-Phenyl 2 mm column (150 × 4.6 mm; Keystone Scientific, Inc., Bellefonte, PA), and 4 ml/min for semipreparative scale studies using a Hypersil-Phenyl 5 mm column (250 mm × 10 mm; Phenomenex, Torrance, CA).

**General Fermentation Procedures.** Microbial cultures (ATCC, Rockville, MD) were maintained on refrigerated slants of Sabouraud-maltose agar (Difco, Detroit, MI) at 5°C and transferred every 6 months to maintain viability. The dextrose broth (growth medium) used in these experiments consisted of: dextrose, 20 g; soybean meal (20 mesh, Capital Feeds, Austin, TX), 5 g; NaCl, 5 g; yeast extract, 5 g; and K2HPO4, 5 g. The broth was in 1000 ml of double-distilled deionized water, and the pH was adjusted to 7.0 with 6 N HCl. The broth was autoclaved in individual Bellco Delong baffled flasks at 120°C for 15 min and cooled before incubation. Fermentations were conducted using a NBS model G-25R environmental shaker (New Brunswick Scientific, Edison, NJ) operating at 250 rpm and 27°C.

The time course study (table 1) involved an analytical scale fermentation in 125 ml Bellco Delong baffled first-stage flasks containing 25 ml of dextrose broth inoculated with 1 ml of spore suspension under aseptic conditions. After a 72-hr incubation time, a 1-ml portion from the first-stage flask was used to inoculate a second-stage 125 ml flask containing 25 ml of the growth medium (13). After a 24-hr incubation time, HP 749 was added as a solution in sterile water to each flask to yield a final concentration of 250 μg/ml of growth culture (Cunninghamella elegans culture along with the growth medium). One-milliliter aliquots of the growth culture were aseptically pipetted out of the flasks at 0, 1, 2, 3, 4, 5, 6, 7, and 8 days after addition of substrate, and the

![FIG. 1. Microbial vs. mammalian metabolism of HP 749.](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>Average μg/ml Growth Culture</th>
<th>HP 749</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak A</td>
<td>Peak B</td>
<td>Peak C</td>
</tr>
<tr>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>0.76</td>
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</tr>
<tr>
<td>1.65</td>
<td>3.35</td>
<td>40.00</td>
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</tbody>
</table>

Peaks: **A**, N-(4-pyridinyl)-N-(5-hydroxy-1H-indol)-1-amine; **B**, N-(4-pyridinyl)-N-(4- or 6-hydroxy-1H-indol)-1-amine; **C**, N-(n-propyl)-N-(4-pyridinyl)-N-(5-hydroxy-1H-indol)-1-amine; **D**, N-(4-pyridinyl)-1H-indol-1-amine; and HP 749, N-(4-pyridinyl)-1H-indol-1-amine.

* Peak areas below the quantifiable limits of the HPLC system.
* Concentrations calculated from the standard curve for P9679.
BIOTRANSFORMATIONS OF BESIPIRDINE USING C. elegans

C. elegans metabolite extract: (A) 1-(4-pyridinyl)l-N-(5-hydroxy-1H-indol)-1-amine, (B) 1-(4-pyridinyl)l-N-(4-or 6-hydroxy-1H-indol)-1-amine, (C) 1-(n-propyl)-N-(4-pyridinyl)-N-(5-hydroxy-1H-indol)-1-amine, (D) 1-(4-pyridinyl)-1H-indol-1-amine, and (HP 749) 1-(n-propyl)-1-(4-pyridinyl)-1H-indol-1-amine. (2) Standards: P9679 [1-(4-pyridinyl)-N-(5-hydroxy-1H-indol)-1-amine], P9400 [1-(n-propyl)-N-(4-pyridinyl)-N-(5-hydroxy-1H-indol)-1-amine], and P7480 [1-(4-pyridinyl)-1H-indol-1-amine hydrochloride].

remaining contents of the flasks were frozen until analysis. HP 749 (250 µg/ml broth) was also added to a second-stage flask that had been autoclaved to kill the fungi and then cooled to ambient temperature before the addition. This flask was incubated along with the rest of the flasks at 27°C for 8 days. On the 8th day, a 1-ml aliquot from this flask was obtained to serve as an inactivated (dead) culture control, whereas the remaining contents of this flask were frozen. Approximately 25% of HP 749 was metabolized over the 8-day incubation period (table 1) to form the major metabolite C (16% of the total metabolites), and the minor metabolites A, B, and D (fig. 2).

A preparative scale fermentation of C. elegans allowed for the isolation of sufficient quantities of the four fungal metabolites for structure elucidation. Five 125-ml first-stage flasks were generated as described previously, and five second-stage flasks of 1-liter capacity containing 200 ml of broth were each inoculated with the entire contents of a single first-stage flask. HP 749 was added as a solution in sterile water to each flask to yield a final concentration containing all four synthetic standards; and (c) the cochromatography solution prepared by mixing 50 µl of the 2 µg/ml standard stock with 200 µl of the 8-day bulk sample to give 250 µl.

For the analytical time course analysis (table 1), 50 µl of a 1 mg/ml methanolic solution of internal standard (16) was added to 1 ml of the culture sample harvested over the 8-day period, and to 1 ml of the inactivated culture control, and the tubes were extracted as described. The dried extracts were reconstituted with 2 ml of mobile phase for analytical HPLC analysis, and 20-µl injections were made.

Standard curves were generated separately for each synthetic standard, which were then used to quantify the metabolites formed in the time course study. Methanolic stock solutions of all synthetic standards were dried in
silanized glass test tubes (125 × 16 mm) with polytetrafluoroethylene-lined caps to obtain the following concentration ranges per milliliter of microbial growth culture: P9679 (0.625–12.5 mg/ml), P9400 (2.5–200 mg/ml), P7480 (0.625–25 mg/ml), and HP 749 (3.75–300 mg/ml). One milliliter of microbial growth culture from a first-stage flask (no added substrate) and 50 μl of internal standard solution were added. The tubes were extracted as described. The residues were reconstituted in 2 ml of mobile phase, and 20 μl injections were made onto the analytical HPLC column.

Extracts from the preparative scale fermentation were injected repeatedly onto the semipreparative HPLC column (fig. 2), and fractions containing metabolite peaks A–C (fraction 1), peak C (fraction 2), and peaks C and D (fraction 3) were collected. The retention times for standards under these conditions were: P9679 = 21.6 min, P9400 = 25.4 min, P7480 = 27.4 min, and HP 749 = 34.4 min. Metabolite peaks were observed at these retention times for the microbial extracts. Each of these fractions were then extracted from the eluent as described previously. The resulting residues were used for structure elucidation and derivatization as described.

MS and NMR. One-dimensional (fig. 4) and two-dimensional NMR (fig. 5) spectra of metabolite C and P9400 were obtained in CD3OD, using trimethylsilane as internal standard on General Electric model GN 500 MHz and Varian NT 360 MHz spectrometers. Low resolution CI (fig. 6) and FAB mass spectra were obtained on a Finnigan Mat Tsq-70 mass spectrometer using methane and glycerol as the desorption matrix, respectively, for the two types of spectrometry.

**Derivatization of Metabolites A, B, and D for Structural Analysis.** The minor metabolites of HP 749 (A, B, and D) were derivatized to propoxy-HP 749 (fig. 7) and HP 749, and then chromatographically compared with corresponding standards synthesized from P9400 and P7480 using HPLC, UV, and MS. For the derivatization of the metabolites, 1-ml aliquots from each of HPLC fractions 1 and 3 were reconstituted in 2 ml of DMF and subjected to alkylation using excess amounts of sodium hydride (dissolved in 1 ml DMF) and 1-bromopropane (diluted with 1 ml DMF), and stirred for 3 hr at 20°C in round bottom flasks (17). The reaction flasks were then quenched after 3 hr by adding distilled water dropwise with stirring. One-milliliter aliquots of the derivatized product mixtures were extracted as described (see section on extraction of metabolite cultures), after which the residues were reconstituted in 1 ml of mobile phase, and 20 μl injections were made on the analytical HPLC column.

For preparation of derivatized standards, P9400 and P7480 (5 mg reconstituted in 2 ml of DMF) were subjected separately to nucleophilic substitution as described to prepare the Propoxy-HP 749 standard and HP 749, respectively. These products were then used to characterize the derivatives of HPLC fractions 1 and 3 by chromatography.

**Results**

An initial screening of microbial cultures was conducted on the analytical scale (HP 749, 250 μg/ml). Fungi including *Streptomyxa affinis* (ATCC 6737), *Aspergillus ochraceous* (ATCC 18500), *Curvularia lunata* (ATCC 38850), *Rhizopus oryzae* (ATCC 11145), *Cunninghamella echinulata* (ATCC 8688A), and *C. elegans* (ATCC 36112) were screened for their ability to metabolize HP 749. Gram-positive bacteria—including *Arthrobacter simplex* (ATCC 6946), *Nocardiya corallina* (ATCC 19070), and *Streptomyces roseochromogenus* (ATCC 13400)—were also screened for their HP 749-metabolizing ability. A TLC analysis of the metabolite extract from each of the above indicated that the filamentous marine fungus, *C. elegans* was the only microorganism that produced all three metabolites of HP 749 corresponding to the available standards and was chosen for this study. An 8-day incubation was required to convert 25% of HP 749 in
the C. elegans dextrose broth to four fungal metabolites (table 1): C, the 5-hydroxyindole product (16% of total metabolites); A, the 5-hydroxy and N-dealkylated product (1% of total metabolites); B, a hydroxylated and N-dealkylated metabolite (1.5% of total metabolites); and D, the N-dealkylated product (2% of total metabolites). Three of these fungal metabolites (A, C and D) have also been reported in mammalian systems (1, 4, 5).

A cochromatographic analysis of 8-day bulk metabolite extracts of C. elegans (fig. 3) and the available standards was conducted to confirm the results of the TLC screening, as well as to serve as an initial step in the structure elucidation of the phase I metabolites produced. A preparative scale fermentation of C. elegans then allowed for the production of sufficient quantities of the fungal metabolites for structure elucidation. The metabolites were collected as HPLC fraction 1 (A, B, and C), 2 (C) and fraction 3 (C and D) using a semipreparative HPLC column (fig. 2).

HPLC fraction 2 consisted entirely of metabolite C, which shared the following characteristics with synthetic standard P9400: $R_F = 0.23$ (TLC), $R_T = 25.4$ min (HPLC, fig. 2), one-dimensional NMR (18, 19) (fig. 4), and two-dimensional NMR spectra (fig. 5), CI/MS $[(M+H)^+ = 268]$ (fig. 6), and UV $\lambda_{max} = 260–270$ nm. The peak at $[(M+29)^+ = 296]$ arises due to the addition of an ethyl fragment (originating from the methane gas used for CI). To confirm this, a FAB mass spectrum of standard P9400 was obtained, which showed a peak corresponding to only the $[(M+H)^+ = 268]$ as expected. The aromatic region of metabolite C in the two-dimensional COSY (fig. 7) shows the expected upfield shift of the signals from protons 4 and 6 (compared with HP 749) consistent with the hydroxylation of carbon at position 5 of the indole ring, and shows the expected doublets at 7.0 $\delta$ and 6.7 $\delta$ for protons 4 and 6, respectively, with a coupling constant $(J_{4,6})$ of 2.34 Hz. Proton 7 shows a doublet at 6.92 $\delta$ as expected $(J_{6,7} = 8.7$ Hz). Thus, the spectroscopy data are consistent with the hydroxylation at position 5 on the indole ring in metabolite C.

The HPLC retention times for metabolites A and D corresponded to that of the synthetic standards P9679 and P7480, respectively (fig. 2). However, because semipreparative HPLC did not give sufficient baseline resolution that was needed to isolate metabolites A, B, and D as single peaks, they were collected as peak mixtures in HPLC fractions 1 and 3, and identified by derivatization and comparison to derivatized standards.

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The HPLC retention times for metabolites A and D corresponded to that of the synthetic standards P9679 and P7480, respectively (fig. 2). However, because semipreparative HPLC did not give sufficient baseline resolution that was needed to isolate metabolites A, B, and D as single peaks, they were collected as peak mixtures in HPLC fractions 1 and 3, and identified by derivatization and comparison to derivatized standards. Specifically, it was concluded that a N-depropylated metabolite would be converted to HP 749 on chemical N-propylation. Furthermore, a metabolite that was monohydroxylated and N-depropylated would undergo bis-alkylation, resulting in formation of propoxy-HP 749. Keeping this in mind, HPLC fractions 1 and 3 were derivatized separately with 1-bromopropane and sodium hydride (fig. 7).

Derivatization of HPLC fraction 1 with 1-bromopropane resulted in a product that gave a single peak on HPLC analysis (fig. 8). The product was then isolated from the reaction mixture by HPLC and
characterized by comparison with standard propoxy-HP 749 using HPLC (fig. 8, R_T = 37.5 min), MS [(M+H)^+] = 310], and UV spectral analysis (λ_{max} = 220–230). These results confirmed that metabolites A and B were the monohydroxylated and N-dealkylated products of HP 749. Because metabolite A is a 5-hydroxyindole product, metabolite B could be either the 4 or the 6-hydroxyindole product of HP 749 (5).

HPLC fraction 3 (mixture or metabolites C and D) gave two products upon reaction with 1-bromopropane (fig. 9). These two derivatives were then isolated by semipreparative HPLC. The product peak with an HPLC R_T = 33.5 min showed the following characteristics: UV λ_{max} = 260–270 nm, and CI-mass chromatogram [(M+H)^+] = 252], all of which were consistent with the data obtained from HP 749 (also prepared by derivatizing P7480). This confirmed the presence of a N-dealkylated metabolite (D) in HPLC fraction 3. Metabolite C, which was present in a minute amount in HPLC fraction 3, was converted to propoxy-HP 749 (fig. 9, R_T = 37.5 min) and was characterized in the same manner as the 5-hydroxyindole metabolite.

Discussion

Fungi belonging to the genus Cunninghamella have demonstrated broad metabolic capabilities in catalyzing a range of phase I biotransformations characteristic of the mammalian liver (13). Cytochrome P450 monooxygenases have been implicated in the metabolism of selected xenobiotics in C. elegans (7–11), and direct parallels have been observed between the fungal and mammalian metabolism with a wide range of drugs and other chemicals (10, 11, 13). Therefore, these microbial systems show promise for application in the early phases of drug development, and in xenobiotic metabolism studies for predicting potential routes of mammalian biotransformation of drug candidates. The goals of the present study were to identify a culture with sufficient ability to metabolize HP 749, and to explore similarities in HP 749 metabolism between reported mammalian and microbial systems. Such a microbial system could then be used as a model to predict and generate mammalian metabolites of other analogs of HP 749. The fungus C. elegans (ATCC 36112) metabolized HP 749 to give four fungal metabolites, three of which have been reported in mammalian systems (1, 4, 5). An inactivated control showed no spontaneous formation of these metabolites under the same conditions. Preparative scale fermentation allowed for the isolation and unequivocal identification of the 5-hydroxyindole product as the major fungal metabolite (C) based on mass spectral, chromatographic, UV, and NMR comparisons to a synthetic standard (P9400). The minor fungal metabolites resulting form the N-dealkylation (D), and sequential phase I reactions (A) were characterized based on the comparison of mass spectra, HPLC, UV λ_{max} (P7480 and P9679), and derivatization to yield HP 749 (for metabolite D) and propoxy-HP 749.
(for metabolite A). Furthermore, it can be speculated that metabolite B in HPLC fraction 1 is a monohydroxylated (at the 4- or 6-position of the indole ring) and N-dealkylated metabolite, based on its HPLC retention time (compared with P9679), and its derivatization to form propoxy-HP 749.

In conclusion, the results from this study show that C. elegans (ATCC 36112) produces the known mammalian metabolites A, C, and D, and has potential as a microbial model for generating mammalian metabolites of the related analogs of HP 749 (15) for the structural and toxicological analysis in this series of compounds.

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References