Microbial and Mammalian Metabolism Studies of the Semisynthetic Antimalarial, Anhydrodihydroartemisinin

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Microbial metabolism studies of the semisynthetic antimalarial anhydrodihydroartemisinin (1), have shown that it is metabolized by a number of microorganisms. Large scale fermentation with Streptomyces lavendulae L-105 and Rhizopogon species (ATCC 36060) have resulted in the isolation of four microbial metabolites. These metabolites have been identified as a 14-carbon rearranged product (2), 9β-hydroxynyhydrodihydroartemisinin (3), 11-epi-deoxydihydroartemisinin (4), and 3α-hydroxydeoxyxanthodihydroartemisinin (5). Microbial metabolites were completely characterized by spectral methods, including 1H-NMR and 13C-NMR spectroscopy. The structure and stereochemistry of metabolite 2 were unequivocally established by X-ray crystallographic analysis. Thermospray mass spectroscopy/high-performance liquid chromatographic analyses of plasma from rats used in mammalian metabolism studies of 1 have shown microbial metabolite 3 to be the major mammalian metabolite. In vitro antimalarial testing has shown metabolite 3 to possess antimalarial activity.

KEY WORDS: microbial and mammalian metabolism; antimalarial; anhydrodihydroartemisinin; microbial and mammalian metabolites; two-dimensional nuclear magnetic resonance (2D-NMR) techniques; thermospray liquid chromatography/mass spectroscopy (LC/MS).

INTRODUCTION

Anhydrodihydroartemisinin (1), is a semisynthetic derivative of artemisinin, the active antimalarial principle of the Chinese medicinal plant Artemisia annua L. (1). The synthesis and antimalarial activity of 1 have been reported by Lin and coworkers (2) while El-Feraly and coworkers have reported its synthesis by a different route (3). The 1H- and 13C-NMR assignments of 1 have also been reported (3,4). Metabolism studies have traditionally utilized model systems to predict metabolic pathways in humans. Microorganisms, particularly fungi, have been successfully used as in vitro models for the prediction of mammalian drug metabolism (5–12). Since there have been no previous reports on the metabolism of 1, a study of its metabolism was undertaken. Also, since some microorganisms are known to affect hydroxylation of their substrates, an active metabolite may be produced that could later be transformed into a water soluble salt. This could very well offer a solution for the water insolubility of highly potent antimalarial compounds such as 1.

In the present study, four microbial metabolites of 1, were isolated. Based on the spectroscopic data, especially two-dimensional (2D)-NMR techniques, these metabolites have been identified as the 14-carbon rearranged product 2, 9β-hydroxyxanthodihydroartemisinin (3), 11-epi-deoxydihydroartemisinin (4), and 3α-hydroxyxanthodihydroartemisinin (5). The isolation and structure elucidation of these metabolites are discussed herein.

MATERIALS AND METHODS

General Procedures

Melting points were determined in open capillary tubes using a Thomas-Hoover capillary melting point apparatus and are uncorrected. The IR spectra were recorded in KBr using a Perkin-Elmer 281 B infrared spectrophotometer. The 1H- and 13C-NMR were obtained in CDCl3 on a Varian VXR-300 FT spectrometer operating at 300 and 75 MHz, respectively. The chemical shift values are reported as ppm units, and the coupling constants are in Hz. Abbreviations for NMR signals are as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; m, multiplet. Standard Varian pulse sequences were used for COSY, HETCOR, DEPTGL, and APT experiments. Low resolution MS were obtained using LC/MS. High resolution FAB and EI MS were carried out at the University of Kansas.

Anhydrodihydroartemisinin (1), used in this study, was prepared from artemisinin by a literature procedure (3).

Chromatographic Conditions

The TLC chromatographic analysis was carried out on precoated Silica G-25 UV254 plates (Macherey-Nagel Duren). The adsorbent used for column chromatography was silica gel 60 /230-400 mesh (EM Science). The visualization of the TLC plates was performed using anisaldehyde-H2SO4 spray reagent (13).

Microorganisms

The cultures were obtained from The University of Mississippi, Department of Pharmacognosy Culture Collection, and were originally obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, or from Northern Regional Research Laboratories (NRRL), Peoria, Illinois. University of Iowa (UI) cultures were obtained from Dr. John P. Rosazza, Mucor ramannianus 1839 was obtained from Dr. Charles Sih at the University of Wisconsin. The cultures used for preliminary screening of anhydrodihydroartemisinin (1) that showed one or more metabolites by TLC are as follows: Aspergillus flavipes NRRL 6633, Aspergillus alliaceus NRRL 6633, Aspergillus flavipes ATCC 11013, Bacillus subtilis ATCC 6633 Cunninghamella echinulata NRRL 3655, Cunninghamella

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Metabolism of the Antimalarial Anhydrodihydroartemisinin

elegans ATCC 9245, Cunninghamella bainieri U1-3605, Dactylaria haptotyla ATCC 28924, Macor mucro U1-4605, Nocardia restricta ATCC 14887, Penicilium patulum ATCC 24550, Rhizopogon species ATCC 36060, Rhizopus arrhizus ATCC 11145, Streptomyces griseus L-103, Streptomyces lavendulae L-105, and Streptomyces rimosus ATCC 23955.

Media

All the preliminary screening and large-scale experiments were carried out in a medium consisting of the following composition: dextrose, 20g; yeast extract, 5g; peptone, 5g; NaCl, 5g; K2HPO4, 5g; distilled water, 1000 ml. Stock cultures of fungi and bacteria were stored on slants of Mycophil, (BBL, Cockeysville, MD) and Eugon agar (Difco, Detroit, MI), respectively, at 4°C.

Fermentation Procedures

Microbial metabolism studies were carried out by incubating the cultures with shaking on the model G-10 Gyrotory shaker (New Brunswick Scientific Co., NJ), operating at 250 rpm, at 25°C. Preliminary screening experiments were carried out in 125-ml stainless steel-capped Delong culture flasks containing 25 ml of medium. Fermentations were carried out according to a standard two-stage protocol (14). In general, the substrate was prepared as a 10% solution in dimethylformamide and added to the 24-hr-old stage II culture medium of the microorganism at a concentration of 0.2 mg/ml of medium. Substrate controls were composed of sterile medium to which the substrate was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions but without the substrate addition.

Animal Studies

Anhydrodihydroartemisinin (I) was given by intravenous administration of an oil/water emulsion that was prepared within 24 h of the animal dosing. The procedure used for the preparation is similar to a general procedure used for the extemporaneous preparation of the oil soluble cancer chemotherapeutic agents that are given by intravenous administration (15). Under aseptic conditions, a 100.0 mg/ml solution of the test compound, in ethanol was slowly added dropwise (10 μl/min) to a vigorously stirred commercially fat emulsion (Liposyn II®, 20%) to give a final concentration of 6.0 mg/ml. Male Wistar rats were anesthetized with sodium phenobarbital (50 mg/kg), then each animal was administered (11.6 mg/kg) of 1 by an intravenous bolus injection in the jugular vein. A blood sample (approximately 9 ml) was collected using a 20 ml syringe (containing 0.2 ml heparin solution) at 15 min after the injection. After centrifugation of the blood at 500 × g for 10 min, the plasma was collected and stored at −85°C for later analysis.

Plasma Extraction

A solid phase extraction procedure was adopted. The C-18 reverse phase extraction cartridges (BOND ELUT C-18®, Analytichem International, Harbor City, California) were first activated with 1.0 ml methanol, followed by 1.0 ml water; then 1.0 ml of the plasma sample was slowly drawn through using 5–10 mm Hg of vacuum in a VAC-ELUT® chamber. The cartridges were washed with 1.0 ml of water (discard), then the sample was eluted with 1.0 ml methanol directly into 2.0 ml conical evaporating tubes. After centrifugation at 500 × g for 5 min, the clear supernatant was transferred to a fresh conical tube, and evaporated at room temperature with a stream of nitrogen to near-dryness. The residue was taken up in 100 μl of 10% methanol in water, centrifuged at 500 × g for 5 min, then 100 ml of the sample was injected into the HPLC/MS system.

High Performance Liquid Chromatography and Mass Spectroscopy

The HPLC pump, pump controller software, injector, and column bypass switching system were a commercially available unit that had been specifically designed (Waters Associates Model 600-MS system) for interfacing with the Vestec Model 201 thermospray mass spectroscopy system. A 4.6-mm × 12.5 cm cartridge-type HPLC column packed with a 5-μm particle size, octadecyl reversed-phase material (Whatman Partisil ODS-3) was utilized, with a mobile phase (1.0 ml/min) comprised of 0.1M ammonium acetate in a methanol: water mixture. The methanol content of the mobile phase was gradient programmed from 51% (v/v) to 78% (v/v) over a 10 min period.

The Vestec Model 201 mass spectrometer with a Technivent data system was operated in the filament-on mode of operation, which yields mass spectra that are more similar to chemical ionization spectra rather than electron impact spectra of more conventional mass spectrometers. Before recording any spectra, the takeoff temperature of the thermospray vaporizer was accurately determined and the tip temperature (209°C) of the vaporizer was set 5°C below the takeoff temperature. The block temperature of the ion source was set to 195°C, which was 75–100°C lower than is commonly used for model 201 thermospray unit.

Microbial Metabolism of Anhydrodihydroartemisinin (I) by Streptomyces lavendulae L-105

Streptomyces lavendulae L-105 was grown in 11 1-liter culture flasks each containing 200 ml of medium. A total of 440 mg of anhydrodihydroartemisinin (I) (4.4 ml of DMF) was evenly distributed among the 24-hr-old stage II cultures. After 14 days, the incubation mixtures were combined and filtered to remove the cells, and the filtrate (2.2 liters) was extracted three times with EtOAc. The combined extracts were dried over anhydrous Na2SO4 and evaporated to dryness under reduced pressure to afford a dark brown residue (493 mg).

Isolation and Characterization of 2

The residue (493 mg) was purified by column chromatography over a silica gel column (50g, 3.75 × 15 cm), using hexane-ether (6:4) mixture as an eluting system and 5-ml fractions were collected. Fractions 24–48 yielding a single spot with Rf = 0.37 (TLC system, hexane-ether 1:1), were combined and evaporated to dryness to give 24 mg of 2 (5.45% yield). Crystallization from hexane gave colorless