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Report

Fungal Metabolism of 4-Methylprimaquine

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4-Methylprimaquine (4-MPQ), an 8-aminoquinoline antimalarial drug, was subjected to microbial metabolism studies in an effort to isolate, identify, and predict the probable mammalian metabolite(s). Preliminary screening with a number of strains of *Aspergillus* indicated that several species were capable of metabolizing 4-MPQ to one major metabolite based on thin-layer chromatographic analysis. Preparative scale conversion of 4-MPQ using whole-cell suspensions of *Aspergillus ochraceus* afforded one major metabolite (4-MPQ-I). Based on spectroscopic and chemical evidence, the structure of the metabolite is proposed as 6-methoxy-8-(3-carboxyl-1-methylpropylamino)-lepidine.

KEY WORDS: 4-methylprimaquine, fungal metabolism; 8-aminoquinoline, metabolism; *Aspergillus ochraceus*, 4-methylprimaquine metabolism.

INTRODUCTION

4-Methylprimaquine (4-MPQ; I) (Fig. 1) is an 8-aminoquinoline antimalarial drug which was first synthesized by Elderfield *et al.* in 1955 (1). More recently, renewed interest in 4-MPQ has been sparked by reports that it possesses radical curative activity superior to that of the parent antimalarial, primaquine (II), and is less toxic as well (2). This has also resulted in a number of studies directed toward the synthesis and biological evaluation of derivatives of 4-MPQ (2–6). Since the metabolism of 4-MPQ was unknown, it was of interest to determine the metabolic fate of 4-MPQ in biological systems.

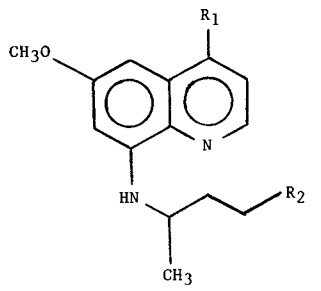
While many authors have documented that the microbial metabolism of certain xenobiotics closely parallels the previously known mammalian metabolism, recent studies with the antimalarial drug primaquine have also shown that microbial systems may be utilized to predict reliably the metabolic fate of drugs whose mammalian metabolism is not known (7). Although it was known that primaquine (II) was extensively metabolized, the identity of the metabolites was not established. The successful use of fungi as a model for the mammalian metabolism of primaquine led to the isolation and identification of 8-(3-carboxyl-1-methylpropylamino)-6-methoxyquinoline as the major fungal metabolite (III) (8). Subsequent studies also identified the carboxyprimaquine (III) as the major metabolite in the plasma of rat (9), monkey (10), and human (11).

Utilizing the fungal models for the preliminary metabolism study of 4-MPQ (I), one major, more polar metabolite has now been isolated and tentatively identified as the deaminated carboxylic acid metabolite (IV).

MATERIALS AND METHODS

General

Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Infrared (IR) spectra were determined on a Perkin–Elmer 281B spectrophotometer. ^1H nuclear magnetic resonance (NMR) spectra were obtained on either a Varian EM 390 or a JEOL-FX60 FT NMR spectrometer, using tetramethylsilane (TMS) as the internal standard. ^{13}C NMR spectra were obtained at 15.03 MHz on a JEOL-FX60 FT NMR spectrometer, using TMS as the internal standard and $CDCl_3$ as the solvent. Mass spectra were obtained on a Finnigan Model 3200 spectrometer with the INCOS data system. 4-Methylprimaquine was provided by Walter Reed Army Institute of Research.



I	$R_1 = CH_3$;	$R_2 = CH_2NH_2$
II	$R_1 = H$;	$R_2 = CH_2NH_2$
III	$R_1 = H$;	$R_2 = CO_2H$
IV	$R_1 = CH_3$;	$R_2 = CO_2H$
V	$R_1 = CH_3$;	$R_2 = CO_2CH_3$

Fig. 1. Structure of (I) 4-methylprimaquine, (II) primaquine, (III) 8-(3-carboxyl-1-methylpropylamino)-6-methoxyquinoline, (IV) the deaminated carboxylic acid metabolite, and (V) the metabolite methyl ester.

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Cultures and Fermentation Screening Procedures

Fungal stock cultures were maintained on slants of Mycophil (BBL Microbiology Systems) agar and potato dextrose agar containing 2% yeast extract. Stock slants are stored at 4°C.

Initial screening was carried out as previously described (8). After 72 hr of incubation in a dextrose peptone medium, a portion of the stage I cultures was used as the inoculum for fresh stage II cultures. After 24 hr of incubation of stage II cultures, 4-methylprimaquine diphosphate was added (0.5 mg/ml medium) as a suspension in dimethylformamide (250 mg/ml DMF).

Fermentation Sampling and Thin-Layer Chromatographic (TLC) Analysis

The fermentations were sampled by extraction of 5 ml of culture broth (following adjustment to pH 8) with 5 ml of ethyl acetate. After evaporation of the solvent, the residue was spotted on precoated silica gel G TLC plates (0.25 mm). The plates were developed in 8% methanol in benzene–ethyl acetate–diethylamine (5:4:1) and visualized by spraying with 5% aqueous diazo blue B. The R_f value of 4-methylprimaquine is 0.56 and the *Rf* of IV is 0.07.

Preparation of 6-Methoxy-8-(3-carboxyl-1-propylamino)-lepidine (IV) Using Resting Cell Suspensions of *Aspergillus ochraceus* ATCC 22947

A total of 336 g of cell biomass was obtained following suction filtration of 10-liter stage II cultures of *A. ochraceus* ATCC 22947 grown in dextrose–peptone medium (2 × 5-liter stirred fermentor cultures; 200 rpm; 2 liter air/min). The cells were suspended in 2.73 liters of phosphate buffer (pH 7.2) held in a 5-liter fermentor jar. A total of 400 mg of 4-methylprimaquine diphosphate was added to the suspension, which was then stirred (300 rpm) at room temperature with an air flow of 6 liters/min. After incubation for 48 hr, the suspension was filtered, the cells were washed with distilled water, and the filtrate (ca. 3 liters) was extracted with 3 × 1 liters of ethyl acetate. The ethyl acetate layer was dried with Na_2SO_4 and evaporated *in vacuo* (40°C) to give 284 mg of residue. Crystallization from methanol afforded 70 mg pure IV, mp 180–182°C; IR, ν_{max}^{KBr} 3425 (N-H), 2975, 2925, 2400–3000 (br, CO_2H), 1700 (CO_2H); MS m/z (% RA) 288 (M^+, 12.4) 273 (2.4) 229 (45.3), 215 (100), 200 (17.1); ^1H NMR (deuterochloroform) δ 8.41 (1H, d, J = 4.5 Hz, H-2), 7.14 (1H, d, J = 4.5 Hz, H-3), 6.42 (1H, d, J = 3.0 Hz, H-5), 6.33 (1H, d, J = 3.0 Hz, H-7), 3.87 (3H, s, $ArOCH_3$), 3.4–3.7 (1H, m, H-1′), 2.7 (1H, br t, H_b-4′), 2.53 (3H, s, 4′-CH_3), 1.5–1.7 (3H, m, H-3′, H_a-4′), 1.9–2.1 (2H, m, H-2′), 1.28 (3H, d, J = 6.0 Hz, 1′-CH_3). Calcd. for $C_{16}H_{20}N_2O_3$: % C, 66.65; H, 6.99; N, 9.72. Found: % C, 66.28; H, 6.98; N, 9.39

6-Methoxy-8-(3-carboxyl-1-methylpropylamino)-lepidine Methyl Ester (V)

A solution of metabolite IV (17 mg) in 5 ml methanol was treated with ethereal diazomethane (3 ml) at room temperature for 2 hr. After evaporation of the solvent, the oily residue was purified by flash chromatography over alumina (neutral for dry column chromatography) using benzene as the solvent to give the methyl ester (V) as an oil, IR, $\nu_{max}^{CHCl_3}$ 3390 (N-H), 2920, 2845, 1723 (CO_2CH_3); MS m/z (% RA), 302 (M^+, 13.4), 229 (32.8), 215 (100); ^1H NMR (deuterochloroform) δ 8.53 (1H, d, J = 4.5 Hz, H-2), 7.25 (1H, d, J = 4.5 Hz, H-3), 6.40 (1H, d, J = 3.0 Hz, H-5), 6.50 (1H, d, J = 3.0 Hz, H-7), 3.97 (3H, s, $ArOCH_3$), 3.67 (3H, s, CO_2CH_3), 3.6–3.8 (1H, m, H-1′), 2.62 (3H, s, 4′-CH_3), 2.5 (2H, m, H-3′), 1.8–2.2 (2H, m, H-2′), 1.33 (3H, d, J = 6.0 Hz, 1′-CH_3); ^{13}C NMR data, see Table II.

RESULTS AND DISCUSSION

There have been no reports on the metabolism of 4-methylprimaquine (I). Since microbial models were utilized so successfully for the production and identification of the major mammalian metabolite of the parent drug primaquine (7–10), this approach was also utilized for the production, isolation, and identification of potential mammalian metabolites of 4-methylprimaquine (4-MPQ; I). Earlier studies with primaquine (7) had shown that *Aspergillus* species were capable of metabolizing primaquine to one major metabolite, which was later identified as the major mammalian plasma metabolite (8–10). Therefore, a total of 17 strains representing nine different *Aspergillus* species was screened for the ability to metabolize 4-MPQ (Table I). Of these 17 strains, all but 6 were capable of metabolizing 4-MPQ to one major metabolite (same by TLC). Based on the apparent efficiency of conversion (based on TLC analysis), *Aspergillus ochraceus* ATCC 22947 was selected for further study. Utilizing whole-cell suspensions of the fungus, a preparative scale conversion of 4-MPQ to one major metabolite (29% yield) was accomplished in 48 hr. The structure of the metabolite was formulated primarily on the basis of a compar-

Table I. Fungi Screened for 4-MPQ Metabolism

Fungus (Number)[a]		Metabolic Production[b]
Aspergillus alliaceus	NRRL 315	–
Aspergillus flavipes	ATCC 10307	+
Aspergillus flavipes	ATCC 11013	+
Aspergillus flavipes	ATCC 16795	+
Aspergillus flavus	ATCC 9170	+
Aspergillus flavus	ATCC 24741	+
Aspergillus flavus	NRRL 501	+
Aspergillus flavus	NRRL 626	+
Aspergillus foetidus	NRRL 377	+
Aspergillus niger	ATCC 11394	–
Aspergillus niger	ATCC 16888	–
Aspergillus niger	ATCC 10581	–
Aspergillus niger	ATCC 10549	–
Aspergillus ochraceus	ATCC 18500	+
Aspergillus ochraceus	ATCC 22947	+
Aspergillus parasiticus	ATCC 15517	–
Aspergillus species	NRRL 5694	+

[a] ATCC = American Type Culture Collection
NRRL = Northern Regional Research Laboratories

[b] Metabolite production denoted by + indicates one major metabolite was produced, as evidenced by TLC.

Table II. ^{13}C NMR Spectral Assignments

Carbon No.	I	V
2	143.8	143.9
3	122.6	122.7
4	142.6	142.8
4a	129.7	129.7
5	88.3	88.7
6	159.4	159.4
7	96.2	96.5
8	145.7	145.6
8a	134.9	134.9
1′	48.1	47.6
2′	34.1	31.8[a]
3′	30.1	30.9[a]
4′	42.1	174.0
5′	20.5	20.5
$ArOCH_3$	55.1	55.2
$4-CH_3$	19.0	19.2
CO_2CH_3	—	51.5

[a] Interchangeable assignments.

ison of its spectral data with those of the parent compound, 4-MPQ.[3]

The molecular formula of the metabolite as $C_{16}H_{20}N_2O_3$ was established by elemental analysis and by mass spectral analysis, which showed the parent ion at m/z 288, with the base peak at m/z 215, indicating that no change had occurred in the aromatic portion of the molecule. The IR spectrum showed the presence of a carbonyl absorption band at ν_{max} 1700 cm^{-1} and a broad hydroxyl absorption band (ν_{max} 2400–3000 cm^{-1}), which supported the presence of a carboxylic acid group. Confirmation of the presence of the carboxylic acid functionality was achieved by methylation of the metabolite with ethereal diazomethane. The methyl ester product showed the parent ion at m/z 302, with the base peak again at m/z 215. The carbonyl absorption band in the IR spectrum of the methylated product was found at ν_{max} 1723 cm^{-1}, consistent with the formation of an aliphatic methyl ester. The structure of the metabolite was proposed as IV, based primarily on a comparison of the ^{13}C NMR spectral data of the metabolite methyl ester (V) with those of the parent drug, 4-MPQ (I).

[3] The ^{13}C NMR spectral assignments of 4-MPQ were made by comparison with primaquine (8) and by long-range coupling experiments. A manuscript detailing these assignments has been accepted for publication (*Spectroscopy Letters*).

The ^{13}C NMR spectral data of the methyl ester (Table II, V) showed essentially no differences in the aromatic region of the spectrum compared to 4-MPQ (I). The major differences in the spectra were found in the aliphatic region. In the spectrum of 4-MPQ (I), there are three triplet signals at 30.1, 34.1, and 42.1 ppm for the 3′, 2′, and 4′ methylene carbons, respectively. However, in the spectrum of the metabolite methyl ester (V), the signal at 42.1 ppm is absent and only the two triplets (31.0 and 32.0 ppm) for C-2′ and C-3′ are evident. Two additional signals at 174.0 ppm ($-CO_2CH_3$) and 51.5 (CO_2-CH_3) were evident in the spectrum of the metabolite methyl ester. Based on these data, together with the mass spectral and IR data, the structure of the metabolite was proposed as 6-methoxy-8-(3-carboxyl-1-methylpropylamino)-lepidine (IV).

4-Methylprimaquine undergoes the same metabolic transformation as primaquine by *Aspergillus* species. Although mammalian metabolic studies with this drug are not complete, it may be speculated that 4-MPQ will follow a metabolic pathway similar to that of primaquine in mammalian systems as well, in which the terminal primary amine group is deaminated to yield the carboxylic acid metabolite.

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