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ORIGINAL PAPER

Identification of metabolites produced from *N*-phenylpiperazine by *Mycobacterium* spp

M. D. Adjei · J. Deck · T. M. Heinze · J. P. Freeman · A. J. Williams · J. B. Sutherland

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Abstract *Mycobacterium* sp. 7E1B1W and seven other mycobacterial strains known to degrade hydrocarbons were investigated to determine their ability to metabolize the piperazine ring, a substructure found in many drugs. Cultures were grown at 30°C in tryptic soy broth and dosed with 3.1 mM *N*-phenylpiperazine hydrochloride; samples were removed at intervals and extracted with ethyl acetate. Two metabolites were purified from each of the extracts by high-performance liquid chromatography; they were identified by mass spectrometry and ^{1}H nuclear magnetic resonance spectroscopy as *N*-(2-anilinoethyl)acetamide and *N*-acetyl-*N*′-phenylpiperazine. The results show that mycobacteria have the ability to acetylate piperazine rings and cleave carbon-nitrogen bonds.

Keywords Biotransformation · Fluoroquinolones · *Mycobacterium* · *N*-phenylpiperazine · Piperazine

M. D. Adjei · J. Deck · A. J. Williams · J. B. Sutherland (✉)
Division of Microbiology, National Center for Toxicological Research, US Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA
e-mail: john.sutherland@fda.hhs.gov

T. M. Heinze · J. P. Freeman
Division of Biochemical Toxicology, National Center for Toxicological Research, US Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA

Present Address:
M. D. Adjei
Norfolk Department of Public Health, 830 Southampton Avenue, Norfolk, VA 23510, USA

Introduction

Many synthetic drugs, including antibacterial and antifungal agents, anthelmintics, anxiolytics, and antidepressants, contain a piperazine ring that plays a significant role in potency [14, 16, 18, 29]. For example, the antibacterial activity of some fluoroquinolone drugs is reduced or lost upon the substitution or partial removal of the piperazinyl moiety [33]. Also, because piperazinyl compounds are used in textile dyeing and analytical chemistry [19], they have been found in industrial wastewater [26].

Piperazine is biodegraded by several bacteria in the genera *Arthrobacter* and *Mycobacterium* [13, 15]. *Mycobacterium smegmatis, Nocardia* sp., *Pseudomonas aeruginosa, P. fluorescens*, and *Streptomyces griseus* demethylate the piperazine ring of the fluoroquinolone drug danofloxacin to produce *N*-desmethyldanofloxacin; and *M. smegmatis* and *P. fluorescens* degrade the piperazine ring of danofloxacin to produce 1-cyclopropyl-6-fluoro-7-amino-4-oxo-1,4-dihydroquinoline-3-carboxylic acid [8]. The ability of mycobacteria to biotransform piperazine rings [1, 2] may be especially important because piperazinyl fluoroquinolones are used in combination therapy to treat drug-resistant tuberculosis [7].

Several fungi metabolize piperazine rings in fluoroquinolones. For instance, *Penicillium* spp. strains cleave the piperazine ring of danofloxacin [8] and *Gloeophyllum striatum* mineralizes carbon from the piperazine rings of enrofloxacin and ciprofloxacin to CO_2 [31, 32]. *Umbelopsis ramanniana* (*Mucor ramannianus*) produces *N*-acetylated metabolites and piperazine ring-cleavage products from enrofloxacin and sarafloxacin [20, 21] and *Pestalotiopsis guepini* similarly metabolizes the piperazine rings of ciprofloxacin and norfloxacin [22].

N-Phenylpiperazine, a model compound structurally related to piperazinyl fluoroquinolones [14], is also known to have adrenergic blocking properties [9]. *Mycobacterium* sp. 7E1B1W (ATCC 29676), a non-pathogenic, fast-growing soil bacterium that degrades propane and *n*-tetradecane but not phenanthrene or pyrene [3–5], and seven other mycobacteria were investigated for the potential to metabolize *N*-phenylpiperazine.

Materials and methods

Cultural conditions

Cultures of *Mycobacterium* spp. were grown for 3 days in 125-ml flasks containing 30 ml tryptic soy broth (TSB) (Remel Inc., Lenexa, KS). They were then dosed with 3.1 mM (final concentration) *N*-phenylpiperazine hydrochloride (99%, Aldrich Chemical Co., Milwaukee, WI) that had been dissolved in water and filter-sterilized. Controls consisted of cultures that were not dosed and non-inoculated flasks of TSB dosed with *N*-phenylpiperazine. Cultures and controls were incubated aerobically at 30°C with shaking at 200 rpm. After 4 days, they were extracted with ethyl acetate for analysis by HPLC.

For kinetic studies, cultures and controls were incubated for 3 days in 500 ml flasks containing 100 ml TSB and then were dosed with 3.1 mM *N*-phenylpiperazine. At various intervals from 0 to 8 days, samples were withdrawn aseptically and extracted with ethyl acetate for analysis.

To determine the effect of *N*-phenylpiperazine concentration on metabolite production, cultures were grown for 3 days and then were dosed with *N*-phenylpiperazine (0, 0.4, 0.8, 1.5, or 3.1 mM). They were incubated as described above, harvested 4 days after dosing, and extracted with ethyl acetate for analysis.

Extraction and HPLC analysis

Cultures were centrifuged at 15,000 *g* for 10 min at 4°C and the supernatants were extracted three times with equal volumes of ethyl acetate. The extracts were dried in vacuo and dissolved in methanol. Metabolites were detected and purified by high-performance liquid chromatography (HPLC) using an Agilent Technologies (Palo Alto, CA) 1100 Series chromatograph; the diode array detector was monitored at 280 nm. A Phenomenex (Torrance, CA) Prodigy 5-μm ODS-3 column (250 × 4.6 mm) was used with a solvent system consisting of A (methanol: water: acetic acid, 10:90:0.2) and B (methanol: water: acetic acid, 90:10:0.2) at a flow rate of 2.0 ml/min. Solvent B was increased from 10 to 95% in a 20-min linear gradient and held at 95% for 10 min.

Mass spectrometry

Direct exposure probe/electron ionization mass spectrometry (DEP/EI-MS) was performed on a ThermoFinnigan (San Jose, CA) TSQ700 mass spectrometer in the electron-ionization (EI) single quadrupole mode. The ion source temperature was 150°C and the electron energy was 70 eV (uncorrected). The first quadrupole analyzer was scanned from *m/z* 50 to 550 in 0.7 s. The rhenium wire of the DEP was heated from 0 to 800 mA with a linear ramp of 5 mA/s.

Liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) analyses were performed on a ThermoFinnigan Quantum Ultra mass spectrometer equipped with an Agilent Technologies 1100 Series HPLC. The mass spectrometer was operated in the positive-ion ESI mode with an in-source collision-induced dissociation (CID) offset of −15 or −20 V. Other ESI conditions were: spray voltage 3.0 kV, capillary temperature 350°C, sheath gas pressure 40 psi, ion sweep gas 10, and auxiliary gas 15. MS/MS conditions were: argon collision pressure 1.5 mTorr, collision energy 15 eV, parent set masses (*m/z*) 163, 179, and 205, and Q3 scanned *m/z* 30–250 per 0.5 s. HPLC was performed with a Phenomenex Prodigy 5-μm ODS-3 column (2.0 × 250 mm). The mobile phase was a linear gradient from 5% acetonitrile to 95% acetonitrile in 40 min, with constant 0.1% formic acid, at a flow rate of 0.2 ml/min.

NMR spectroscopy

Samples were dissolved in deuterated methanol and ^{1}H nuclear magnetic resonance (NMR) spectroscopy was performed at 500 MHz using a Bruker Instruments (Billerica, MA) AM500 NMR spectrometer [21].

Statistical analysis

A one-way analysis of variance (ANOVA) was performed to determine the effect of substrate concentration on metabolite production, using the statistical software JMP v 5.1 (SAS Institute). Differences among concentration means were compared using Tukey's test.

Results

N-Phenylpiperazine was analyzed by LC/ESI-MS/MS for comparison with the metabolites. Fragmentation of the protonated molecule at *m/z* 163 gave the product-ion

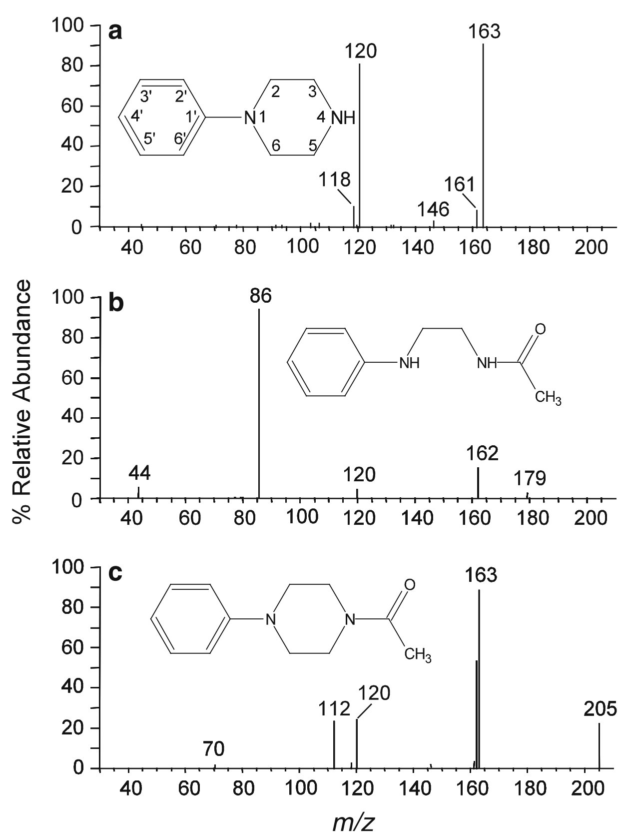

Fig. 1 Product-ion mass spectra obtained by LC/ESI-MS/MS: (**a**) *N*-phenylpiperazine (MH^+ = 163), (**b**) metabolite **I**, *N*-(2-anilinoethyl)acetamide (MH^+ = 179), and (**c**) metabolite **II**, *N*-acetyl-*N*′-phenylpiperazine (MH^+ = 205). The metabolites were produced from *N*-phenylpiperazine by *Mycobacterium* sp. 7E1B1W

mass spectrum shown (Fig. 1 a). Ions included the residual protonated molecule at *m/z* 163, a minor *N*, *N*-divinylbenzenaminium ion at *m/z* 146, and a major *N*-vinylbenzenaminium ion at *m/z* 120.

During the HPLC analysis of extracts from dosed *Mycobacterium* sp. 7E1B1W cultures, *N*-phenylpiperazine eluted at 9.8 min and two small peaks (I and II) not found in control cultures eluted at 16.8 and 22.5 min, respectively. In extracts from cultures harvested 4 days after dosing, peaks I and II formed about 1.7 and 0.9%, respectively, of the total integrated peak area at 280 nm.

The DEP/EI mass spectrum of metabolite **I** had ions at *m/z* 178 (22) [$M^{+\cdot}$], 119 (39) [C_6H_5-NH-CH = CH_2]$^+$, 118 (12), 107 (7), 106 (100) [C_6H_5-NH-CH_2]$^+$, 79 (5), and 77 [C_6H_5]$^+$. Metabolite **I** was analyzed by LC/ESI-MS/MS. Fragmentation of the protonated molecule at *m/z* 179 gave the product-ion mass spectrum shown (Fig. 1b). Ions included an extremely minor protonated molecule at *m/z* 179, a minor *N*-vinylbenzenaminium ion at *m/z* 120, a major *N*-acetylethylenaminium ion at *m/z* 86, and a minor ethylenaminium ion at *m/z* 44. The NMR resonances for metabolite **I** (Table 1) were nearly identical to those of *N*-phenylpiperazine except that the H3 resonances had been shifted slightly downfield. The H2 and H3 resonances integrated as two each, indicating that four proton resonances of the original piperazine moiety were missing. A sharp singlet at 1.92 ppm integrated as three. From the NMR spectrum, the singlet was determined to be an acetyl group attached to the terminal nitrogen.

A standard for *N*-(2-anilinoethyl)acetamide was synthesized by dissolving 50 mg *N*-phenylethylenediamine (Fisher Scientific, Pittsburgh, PA) in 4.5 ml water and adding 0.3 ml acetic anhydride (Eastman Chemical, Kingsport, TN). Of the two products formed, one was identified by LC/ESI-MS/MS as *N*-(2-anilinoethyl)acetamide (MH^+ = *m/z* 179) by the prominent product ion at *m/z* 86. Because the HPLC retention times, DEP/EI and product-ion mass spectra, and NMR spectra were identical for metabolite **I** and the synthetic *N*-(2-anilinoethyl)acetamide standard, metabolite **I** was identified as *N*-(2-anilinoethyl)acetamide (Fig. 1b).

The DEP/EI mass spectrum of metabolite **II** had ions at *m/z* 204 (48) [$M^{+\cdot}$], 189 (4), 161 (14), 132 (100) [C_6H_5-N(CH_2)CH = CH_2]$^+$, 120 (21), 119 (26) [C_6H_5-NH-CH = CH_2]$^+$, 106 (12) [C_6H_5-NH-CH_2]$^+$, 105 (21), 104 (21), 77 (15) [C_6H_5]$^+$, and 56 (18). The mass

Table 1 ^{1}H NMR data for *N*-phenylpiperazine and metabolites **I** and **II** produced by *Mycobacterium* sp. 7E1B1W

Compound[a]	Chemical shifts (ppm)[b]						Coupling constants (Hz)[b]
	H2′, H6′	H3′, H5′	H4′	H2, H6	H3, H5	Acetyl	
N-Phenylpiperazine	6.99	7.26	6.89	3.22	3.29		$J_{2,3}$ = 5.2, $J_{2',3'}$ = 8.6, $J_{2',4'}$ = 1.1, $J_{3',4'}$ = 7.3
N-(2-Anilinoethyl)-acetamide **(I)**	6.63	7.07	6.60	3.21	3.36	1.92	$J_{2,3}$ = 6.5, $J_{2',3'}$ = 8.6, $J_{2',4'}$ = 1.1, $J_{3',4'}$ = 7.3
N-Acetyl-*N*′-phenyl-piperazine **(II)**	6.98	7.24	6.85	3.12, 3.18	3.68, 3.72	2.13	$J_{2,3}$ = 5.2, $J_{2',3'}$ = 8.6, $J_{2',4'}$ = 1.1, $J_{3',4'}$ = 7.3

[a] Compounds were dissolved in deuterated methanol

[b] For NMR assignments, piperazine-ring positions are numbered from 1 to 6 and phenyl-ring positions from 1′ to 6′. (The piperazine ring in metabolite **I** is incomplete and lacks positions 5 and 6.)

spectrum matched that of 1-(4-phenyl-1-piperazinyl)ethanone (CAS# 2155713-1, National Institute of Standards and Technology reference library) with a reverse fit of 94%. Metabolite **II** was analyzed by LC/ESI-MS/MS. Fragmentation of the protonated molecule at *m/z* 205 gave the product-ion mass spectrum shown (Fig. 1c). Ions included the residual protonated molecule at *m/z* 205, a major *N*-phenylpiperazinium ion at *m/z* 163, an *N*-vinylbenzenaminium ion at *m/z* 120, and an *N*-acetyl-*N*-vinylethylenaminium ion at *m/z* 112. The NMR data for metabolite **II** (Table 1) showed a downfield chemical shift for the H3 resonance when compared to that of *N*-phenylpiperazine. A resonance representing the three protons of an acetyl group had been added. The H2, H6 and H3, H5 resonances of the piperazine ring were split into two triplet resonances each due to the asymmetry of the acetyl group.

A standard for *N*-acetyl-*N*′-phenylpiperazine was synthesized by dissolving 50 mg *N*-phenylpiperazine hydrochloride in 4.5 ml water and adding 0.3 ml acetic anhydride. Because the HPLC retention times, DEP/EI and product-ion mass spectra, and NMR spectra were identical for metabolite **II** and the synthetic standard and matched the data from the reference library, metabolite **II** was identified as *N*-acetyl-*N*′-phenylpiperazine (Fig. 1c).

In kinetics experiments with *Mycobacterium* sp. 7E1B1W, metabolites **I** and **II** were detected simultaneously 6 h after the addition of *N*-phenylpiperazine to duplicate cultures (Fig. 2) and were at approximately equal concentrations at 12 h. After 18 h, about twice as much of metabolite **I** as of metabolite **II** was found. Between 1 and 4 days, the rates of formation of the metabolites were approximately linear; both compounds continued to increase in concentration until the experiment was terminated at 8 days.

The rates of formation of metabolites **I** and **II** appeared to be correlated with substrate concentration (Fig. 3). Increasing the concentration of *N*-phenylpiperazine from 0.4 to 3.1 mM in triplicate cultures led to a significant increase at 4 days in metabolite **I** ($P < 0.0001$ using Tukey's test) but not in metabolite **II**. Starting with 1.5–3.1 mM *N*-phenylpiperazine, the amount of metabolite **I** detected was about twice that of metabolite **II**.

To find whether other mycobacteria would produce metabolites from *N*-phenylpiperazine, seven other strains were tested, including *M. frederiksbergense* FAn9 (DSM 44346), *M. gilvum* ATCC 43909, *M. gilvum* BB1 (DSM 9487), *M. gilvum* PYR-GCK (ATCC 700033) [17], *M. smegmatis* mc^{2}155 (ATCC 700084), *Mycobacterium* sp. PYR100 [17], and *Mycobacterium* sp. RJGII-135 [28]. The same two metabolites (**I** and **II**) were detected in extracts from dosed cultures of all strains; the identities were confirmed by HPLC retention times and product-ion spectra (data not shown).

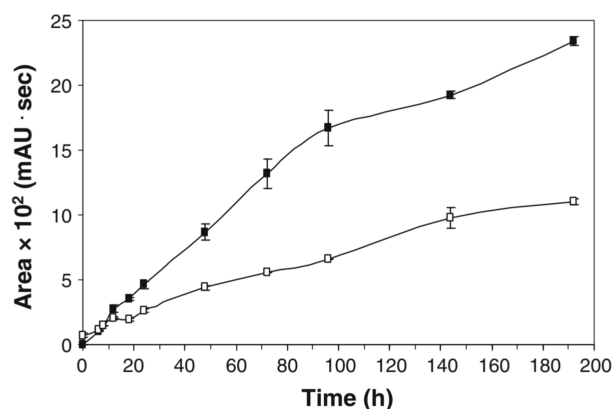

Fig. 2 Time course of *N*-phenylpiperazine metabolism by *Mycobacterium* sp. 7E1B1W incubated in TSB for 8 days after dosing. Results are means ($n = 2$), with error bars, of the peak areas at 280 nm for metabolites **I** (*filled squares*) and **II** (*open squares*)

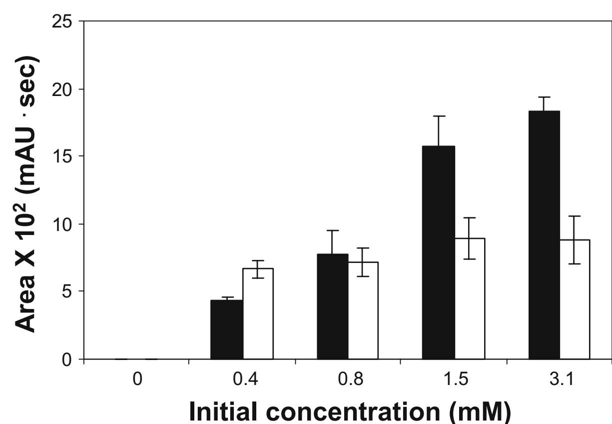

Fig. 3 Formation of metabolites **I** (*filled bars*) and **II** (*open bars*) by *Mycobacterium* sp. 7E1B1W incubated for 4 days in TSB with different concentrations of *N*-phenylpiperazine. Results are means ($n = 3$), with error bars, of the peak areas at 280 nm

Discussion

Eight different *Mycobacterium* spp. strains, when tested for the ability to metabolize *N*-phenylpiperazine, produced *N*-acetylated metabolites. The piperazine rings of fluoroquinolone drugs may be *N*-acetylated by fungi [20–22] and mycobacteria [1, 2]; similar reactions also occur in experimental animals [11] and in humans [23]. The acetylated drug metabolites found in animals and human urine could also result from microbial metabolism. *N*-Acetyl-*N*′-phenylpiperazine has been synthesized

previously for use as an activator in the radical polymerization of methyl methacrylate [30].

Although arylamine *N*-acetyltransferases from bacteria metabolize arylamines, arylhydrazines, and arylhydroxylamines [6, 24, 25], these enzymes are not known to acetylate piperazine rings. *Mycobacterium* sp. 7E1B1W also *N*-acetylates the piperazine rings of norfloxacin and ciprofloxacin [1, 2] but it did not acetylate *p*-aminobenzoic acid (data not shown).

Carbon–nitrogen bond cleavage, which occurred in the formation of *N*-(2-anilinoethyl)acetamide, is known in the metabolism of other cyclic secondary amines by mycobacteria [10, 12, 27]. It is typical of the metabolism of piperazine-containing fluoroquinolones by fungi [20–22], animals [11], and humans [23].

The metabolism of *N*-phenylpiperazine by *Mycobacterium* sp. 7E1B1W may proceed in two possible sequences, depending on whether *N*-acetylation precedes or follows C–N bond cleavage. Because of the simultaneous appearance of the two metabolites, no evidence is available at this time to show the sequence.

In summary, eight strains of *Mycobacterium* spp. modified the piperazine ring of *N*-phenylpiperazine, forming *N*-(2-anilinoethyl)acetamide and *N*-acetyl-*N*′-phenylpiperazine as products.

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