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Microbial models of mammalian metabolism: microbial transformation of naproxen

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Preparative-scale fermentation of *S*-naproxen, the known antiinflammatory, analgesic and antipyretic drug, with *Cunninghamella elegans* ATCC 9245 afforded *S*-demethylnaproxen, the known human active metabolite of naproxen, in a 90% yield. Demethylnaproxen was also detected as the major metabolite of naproxen using *Cunninghamella blakesleeana* ATCC 8688a. A review of the previous microbial metabolism studies using the fungi *Cunninghamella* species suggested that it could be a plausible *in vitro* predictor for mammalian metabolism.

1. Introduction

The use of microbes as possible models for mammalian metabolism is extensively studied and reviewed [1–9]. Several reports, reviewed in this paper, indicated that the fungi *Cunninghamella* species was able to mimic the mammalian metabolism of many drugs. The present study also represents the first microbial metabolism study of *S*-naproxen, the known antiinflammatory, analgesic and antipyretic drug, and provides further support for the use of *Cunninghamella* species as a plausible predictor for mammalian drug metabolism.

2. Investigations, results and discussion

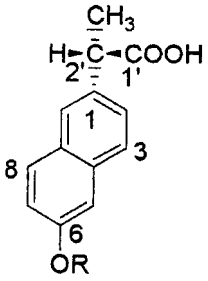
Twenty-eight growing cultures were screened for their ability to bioconvert *S*-naproxen (**1**). Few cultures were observed to transform **1** partially or completely into metabolites of greater polarity. Both *Cunninghamella elegans* ATCC 9245 and *C. blakesleeana* ATCC 8688a were able to transform **1** into the same more polar metabolite, as suggested by TLC. *C. elegans* ATCC 9245 was chosen for preparative-scale fermentation of **1** because it entirely depleted and converted **1** into the more polar metabolite **2**.

The high resolution FT-ICR MS of **2** displayed a molecular ion peak $(M + Na)^+$ at *m/z* 239.0647, suggesting the molecular formula $C_{13}H_{12}O_3$ and eight degrees of unsaturation. The FT-IR spectrum of **2** ($CHCl_3$) showed a strong absorption bands at 3596 and 3478 cm^{-1}, suggesting the presence of carboxy and hydroxy groups. The ^{13}C- and ^1H-NMR spectra of **2** (Table) suggested that **2** is the known active human metabolite, demethylnaproxen [10]. The quaternary carbon signal absorbed at δ 155.2 (Table) is assigned C-6, bearing a free phenolic group. This was based on its HMBC couplings with both proton doublets at δ 7.09 and 7.72 (H-7 and H-8, respectively) as well as with the proton singlet at δ 7.14 (H-5). Since the NMR spectra were recorded using $CDCl_3$–CD_3OD (9.5 to 0.5) as a solvent, the expected exchangeable signal due to the free phenol at C-6 is lost. However, the lack of methoxy proton and carbon signals, as compared with the NMR data of **1**, supported the C-6 *O*-demethylation. Hence compound **2** was proved to be *S*-demethylnaproxen, the fact which suggests the validity of using *Cunninghamella* species as a plausible predictor for mammalian drug metabolism. A literature survey for the use of this microbe in various drug metabolism studies further supported this assumption. In many instances, the microbial model system yielded oxidative patterns of metabolites similar to those reported with cytochrome P450 monooxygenases of hepatic microsomes or the *in vivo* mammalian system [11]. The potential of selected microorganisms, including *Cunninghamella*, to hydroxylate aromatic substrates, e.g., acetanilide, acronycine, aniline, anisole, benzene, benzoic acid, biphenyl, chlorobenzene, coumarin, naphthalene, nitrobenzene and *trans*-stilbene is reported [11]. The *O*-dealkylation of 10,11-dimethoxy-aporphine into isoapocodeine, in a fashion similar to the mammalian system, using *C. blakesleeana* and *C. bainieri* is also reported by Rosazza *et al.*, 1975 [12]. Similarly, the *O*-demethylation of papaverine using *Cunninghamella* species and other organisms in a similar manner to that of mammalian metabolism is reported [13]. The *O*-demethylation of the natur-

Table: ^{13}C- and ^1H NMR spectral data of *S*-naproxen (1) and *S*-demethylnaproxen (2)[a]

Position	1		2	
	δ_C	δ_H	δ_C	δ_H
1	134.9, s	–	136.1, s	–
2	127.3, d	7.70, d (8.8)	126.9, d	7.65, d (8.4)
3	126.1, d	7.42, dd (8.2, 1.4)	126.6, d	7.37, d (8.4)
4	133.8, s	–	134.3, s	–
5	105.7, d	7.12, brs	108.9, d	7.14, s
6	157.7, s	–	155.2, s	–
7	118.3, d	7.15, dd (8.2, 2.3)	118.3, d	7.09, brd (8.7)
8	129.3, d	7.70, d (8.8)	129.7, d	7.72, d (8.7)
9	128.9, s	–	128.8, s	–
10	126.2, d	7.69, s	126.2, d	7.67, s
6-OMe	55.3, q	3.92, 3H, s	–	
1′	180.8, s	–	180.5, s	–
2′	45.3, d	3.88, q (6.9)	45.1, d	3.83, q (6.8)
3′	18.1, q	1.60, 3H, d (6.9)	18.4, q	1.49, 3H, d (6.9)

[a] In $CDCl_3$-CD_3OD (9.5–0.5) at 400 MHz for ^1H- and 100 MHz for ^{13}C NMR
Carbon multiplicities were determined by DEPT 135 experiments; s: quaternary, d: methine, q: methyl carbons. Coupling constants (*J*) are in Hz.



	R
S-naproxen (**1**)	CH_3
S-demethylnaproxen (**2**)	H

al antitumor agent 9-methoxyellipticine using *C. echinulata*, is reported, the same as happened in mammals [14]. Lomatiol, the mammalian metabolite of the natural antitumor agent lapachol, was the major microbial metabolite of lapachol using *C. echinulata* [15]. Microbial metabolism of the antihistamine drugs pyrilamine maleate and triprolidine by *C. elegans* and the correlation of the resulted metabolites with those of mammalian were discussed [16, 17]. Similar results were also obtained for β-ionone and α-ionone by using *C. blakesleeana* [18]. Both *C. echinulata* and *C. blakesleeana* metabolized retinoic acid to several known mammalian metabolites along with two minor new metabolites [19]. A multiple pathway modeling of warfarin metabolism by *C. elegans*, its use as a possible model for mammalian metabolism and the conversion of warfarin to the known mammalian metabolite 4′-hydroxywarfarin using *C. bainieri* were also studied [20–22]. Five mammalian metabolites of propranolol were detected after its microbial transformation in *C. bainieri* [23]. Out of ten microbial metabolites of the antimalarial drug artemether using *C. elegans* and other organisms, four were detected in a rat liver microsome preparation [24]. *O*-Dealkylation reactions similar to those of mammals were reported for the analgesic drug phenacetin and its *O*-alkyl homologs using *C. elegans* [25]. Two hydroxylated metabolites of the diterpene sclareol using *Cunninghamella* species and were expected to be similar to the mammalian metabolites of this compound [26]. *C. echinulata* generated two major metabolites (carbazole and *N*-hydroxymethyl-carbazole) identical to those of mammalian metabolites of *N*-methylcarbazole, in addition to other two minor metabolites [27, 28]. The diuretic drug, furosemide, was metabolized by *C. elegans* to 4-chloro-5-sulfamoyl anthranilic acid and furosemide acyl glucoside, which are also present in mammalian systems [29, 30]. Quinine was metabolized, similar to its mammalian fate, to 3-hydroxy-quinine using *C. echinulata* [31]. *C. elegans* reported to metabolize chlorpromazine and methdilazine to several metabolites (including their sulfoxide derivatives) which were found in animal studies [32]. Besipirdine (HP 749) is an indole derivative that holds potential to treat the memory impairment associated with Alzheimer's disease [33, 34]. The fungi *C. elegans* hydroxylated and *N*-dealkylated the aromatic ring in HP 749, which mimic the hepatic metabolism of this new drug [33]. Biotransformation of antipyrine with *C. echinulata* and *C. elegans* afforded the known mammalian metabolites 3-hydroxy-methylantipyrine, 4-hydroxyantipyrine and antipyrine-3-carboxylic acid, respectively [35]. Metabolism of 2-nitrofluorene by *C. elegans* and rat liver microsomes reported in parallel and resulted in the formation of phenolic and ring-hydroxylated products [36, 37]. The reported mammalian metabolites 3,4-methylenedioxybenzyl methyl ketoxime, 3,4-methylenedioxybenzyl methyl ketone and *N*-acetyl-3,4-methylenedioxy-amphetamine were isolated as a result of microbial transformation of 3,4-methylenedioxy-*N*-methyl-amphetamine and 3,4-methylenedioxy-amphetamine using *C. echinulata* [38]. Six mammalian metabolites of the tricyclic antidepressant, cyclobenzaprine were isolated after its microbial transformation in *C. elegans* [39].

3. Experimental

3.1. General experimental procedure

The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, on a Bruker AMX-NMR spectrometer operating at 400 for ^{1}H, and 100 MHz for ^{13}C-NMR. The HRMS spectra were measured on a Bioapex FT-ICR MS with electrospray ionization. TLC analyses were carried out on precoated Si gel G_{254} 500 μm, with the following developing system: $CHCl_3$-MeOH (90 : 10) or C18-reversed phase plates, 200 μm using CH_3CN–H_2O (50 : 50). For Si gel 60, 40 μm or LiChroprep RP-18, 25–40 μm were used.

3.2. Chemicals

Six naproxen-Na tablets (Aleve®, Procter & Gamble, each equivalent to naproxen 200 mg) were grinded and extract with 5% ethanolic HCl. The ethanolic extract was concentrated and partitioned between $CHCl_3$ and H_2O (200 ml). The organic layer was washed with 5% $NaHCO_3$, NaCl solutions and finally with H_2O and then evaporated under reduced pressure to afford **1** (1150 mg).

3.3. Organisms

Preliminary microbial metabolism studies were conducted as previously reported [40]. Twenty-eight microbial cultures, obtained from the University of Mississippi, Department of Pharmacognosy culture collection were used for screening. The microbes used were reported earlier [40], in addition to: *Gongronella butleri* ATCC 22822, *Mucor mucedo* UI 4605 and *Mucor ramannianus* 1839 (sih) which were obtained from Dr. Charles Sih, Department of Pharmaceutical Biochemistry, University of Wisconsin, Madison, Wisconsin, U.S.A. Stock cultures were maintained on agar slants of media recommended by the ATCC and were stored at 4 °C.

3.4. Microbial metabolism of naproxen (1) by Cunninghamella elegans

C. elegans ATCC 9245 was grown in 8 1 l culture flasks, both containing 250 ml of compound medium α. A total of 400 mg of **1** was dissolved in 1 ml EtOH, equally divided between the eight flasks and distributed among the 24 h old stage II cultures. After 7 days, the incubation mixtures were pooled and filtered. The filtrate (1.8 l) was exhaustively extracted with EtOAc (3 × 600 ml), which was then dried over anh. Na_2SO_4 and evaporated under reduced pressure. The residue (890 mg) was flash chromatographed over 100 g silica gel 60 starting with (100%) $CHCl_3$ and gradient eluted with increasing proportions of MeOH. Polar fractions were subjected to repeated RP C18 flash chromatography using a H_2O–CH_3CN gradient to afford **2** (360 mg, R_f normal phase, 0.36),

S-naproxen (**1**) (2-(6-methoxy-2-naphthyl)propionic acid): Colorless needles from acetone, m.p. 155 °C, $[\alpha]_D^{25}$ +55.5 (*c* 1.0, $CHCl_3$); UV λ_{max} (log ε) (MeOH) 209 (4.50), 240 (4.75), 280 (3.82), 318 (3.60) nm; IR υ_{max} ($CHCl_3$) 3460 (OH), 3020–2910, 1707 (C=O), 1602, 1450, 1260, 1145, 1020, 860 cm^{-1}; ^{13}C- and ^{1}H NMR, see Table. FT-ICR MS *m/z* calculated for $C_{14}H_{14}O_3Na$ $[M + Na]^+$ 253.0841, found 253.0847.

S-demethylnaproxen (**2**) (2-(6-hydroxy-2-naphthyl)propionic acid): Colorless needles from MeOH, mp 182–184 °C, $[\alpha]_D^{25}$ +35.5 (*c* 0.1, $CHCl_3$) (literature values: mp 188 to 191 °C, $[\alpha]_D^{25}$ +94, pyridine) [41]; UV λ_{max} (log ε) (MeOH) 208 (4.52), 240 (4.75), 278 (3.85), 315 (3.66) nm; IR υ_{max} ($CHCl_3$) 3596, 3478 (OH), 3024–2930, 1708 (C=O), 1608, 1456, 1262, 1145, 1026, 862 cm^{-1}; ^{13}C- and ^{1}H NMR, see Table; FT-ICR MS *m/z* calculated for $C_{13}H_{12}O_3Na$ $[M + Na]^+$ 239.0684, found 239.0647.

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