Microbial transformation of naproxen by Cunninghamella species

ZHONG Da-Fang, SUN Lu, LIU Lei, HUANG Hai-Hua

Laboratory of Drug Metabolism and Pharmacokinetics;
Department of Microbiology, Shenyang Pharmaceutical University, Shenyang 110016, China

KEY WORDS naproxen; microbial transformation; Cunninghamella; mass spectrum analysis

ABSTRACT

AIM: The metabolites of naproxen produced by Cunninghamella species were isolated and identified, and further to compare the similarities between microbial transformation and mammalian metabolism. METHODS: Naproxen was transformed by three strains of Cunninghamella species (Cunninghamella blakeslesna AS 3.153, Cunninghamella echinulata AS 3.2004, and Cunninghamella elegans AS 3.156). The metabolites of naproxen were separated and assayed by liquid chromatography-mass spectrometry method. Semi-preparative HPLC was used to isolate the major metabolite, and the structure was identified by nuclear magnetic resonance (NMR) and mass spectrometry. RESULTS: Naproxen was transformed into 2 metabolites, desmethylnaproxen and desmethylnaproxen-6-O-sulfate, both were the known mammalian metabolites. The conjugated metabolite was newly detected in microbial transformation samples. CONCLUSION: The microbial transformation of naproxen has some similarities with the metabolism of naproxen in mammals. The fungi belonging to Cunninghamella species could be used as complementary in vitro models for drug metabolism to predict and produce the metabolites of drugs in mammals.

INTRODUCTION

Naproxen [(S)-6-methoxy-α-methyl-2-naphthalene acetic acid] is a potent inhibitor of prostaglandin synthesis and prescribed for the treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and acute gouty arthritis. It also has antipyretic activity and analgesic effect, and is effective in the treatment of dysmenorrhea, post-operative pain, and migraine attacks\(^1\). The metabolism of naproxen in humans and animals has been well established. Naproxen is metabolized to desmethylnaproxen by 6-O-desmethylation, and the metabolite is 1 % as active as naproxen in an animal model of anti-inflammatory test\(^2\). The parent drug is excreted as unchanged form and an acyl glucuronide conjugate, whereas the glycine conjugate of naproxen has only been observed in animal studies\(^3,4\). Desmethylnaproxen is excreted unchanged as well as conjugated with sulfuric acid and glucuronic acid\(^4,5\).

Studies of drug metabolism and the toxicity of metabolites are important in drug development. However, identification of the metabolites from animal
sources and clinical samples can be hindered by insufficient quantity of material. Some microorganisms, especially the fungi belonging to Cunninghamella species, possess cytochrome P-450 monooxygenase systems analogous to those in mammals. So microbial transformation has been proposed as a complementary in vitro model for mammalian drug metabolism[6]. The advantage of using microbial models mainly lies in the ease that milligram quantity of phase I metabolites can be prepared by preparative-scale fermentation for complete structural elucidation and further toxicological test, especially when the metabolites are unavailable by chemical synthesis[7-9]. More recently, it has been shown that phase II metabolites of drugs may also be obtained by the same microbial models, including glucuronides, glucosides, and sulfate conjugates[10-13]. In this study, naproxen was used as a structural probe to identify its metabolites produced by microbial models, and further to investigate the similarities between microbial transformation and mammalian metabolism.

MATERIALS AND METHODS

Chemicals Naproxen was obtained from Xinan Pharmaceutical Factory (Chongqing, China). Methanol and acetonitrile were HPLC grade (Yuwang Co, Shandong, China). Peptone and yeast extract were biochemical reagents. All other chemicals were of analytical grade.

Microorganisms Cunninghamella blakesleleana AS 3.153, Cunninghamella echinulata AS 3.2004, and Cunninghamella elegans AS 3.156 were provided by the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). Microbial cultures were maintained on potato dextrose agar slants at 4 ºC and transferred every 6 months to maintain viability.

The broth consisted of glucose 20 g, peptone 5.0 g, yeast extract 5.0 g, K$_2$HPO$_4$ 5.0 g, and NaCl 5.0 g. The ingredients were mixed in 1000 mL of double-distilled deionized water, and pH was adjusted to 6.0 with HCl (6.0 mol/L). The broth was autoclaved in individual Erlenmeyer flask at 115 ºC for 30 min and cooled before incubation.

Microbial transformation conditions The spores and mycelia of each strain belonging to Cunninghamella species were aseptically transferred to potato dextrose agar slants and allowed to grow for 168 h. In the first-stage fermentation, a loop of fresh spores was inoculated with a 250-mL Erlenmeyer flask containing 50 mL broth. The cultures were incubated at 28 ºC for 24 h on a rotary shaker operating at 220 rpm. Then a 1.0-mL portion from the first-stage flask was inoculated with a second-stage 100-mL flask containing 20 mL broth. After 24-h incubation, naproxen dissolved in acetone was added to each flask to yield a final concentration of 250 mg/L. The incubation time required for maximum formation of metabolites was 96 h. After microbial transformation, the flask content was centrifuged at 1500×g for 20 min, the supernatant was transferred to tubes and kept at -20 ºC until analysis.

Two kinds of controls were run synchronously with the fermentation and worked-up in the same way. One was the blank culture control in order to define and exclude the indigenous secondary metabolites generated by the microorganisms. The other was the blank substrate control, ie, naproxen was added to the sterile broth without microorganism, to test whether naproxen would be chemically decomposed or spontaneously transformed under broth and fermentation conditions.

Extraction of metabolites and LC/MS$^1$ assay A 1.0-mL portion of each sample was filtered through precut membrane (0.45 µm). The filter was applied to a preconditioned 1.5-mL Sep-Pak C$_18$ cartridge (JT Baker Phillipsburg, USA). The column was washed with water, and eluted with 2.0 mL methanol. A small aliquot of the methanol solution (20 µL) was injected into the column.

Liquid chromatography-mass spectrometry (LC/MS) analysis was performed using a Finnigan LCQ ion trap mass spectrometer (San Jose, USA) equipped with an atmospheric pressure ionization interface. The instrument was operated in a negative electrospray ionization mode. The capillary voltage was fixed at -16 V, and its temperature was maintained at 180 ºC. The spray voltage was set at -4.25 kV. The HPLC fluid was nebulized using N$_2$ as both the sheath gas at a flow rate of 0.75 L/min, and the auxiliary gas at a flow rate of 0.15 L/min. Collision-induced dissociation (CID) experiments were conducted using helium as the collision gas, and the relative collision energy was set at 30 %-35 %. Data were collected and analyzed by the Navigator software (version 1.2, Finnigan). Liquid chromatography was performed with a Shimadzu LC-10AD solvent delivery system (Kyoto, Japan). Samples were separated on Diamonsil C$_18$ column (partical size 5 µm, 4.6 mm×200 mm ID, Dikma Co, Beijing, China). The mobile phase consisted of acetonitrile - ammonium acetate 10 mmol/L (30:70, v/v) at a flow rate of 0.5 mL/min.
Isolation and identification of the metabolite

A preparative scale fermentation of Cunninghamella elegans allowed to isolate sufficient quantity of the putative fungal metabolite for structural elucidation. After microbial transformation, the flask content was centrifuged at 1500 \( \times g \) for 20 min. The combined supernatant was adjusted to pH 1.0 with HCl (6.0 mol/L) and extracted with three equal volumes of diethyl ether. The organic layer was dried over sodium sulfate and evaporated to dryness in vacuum. The residue was dissolved in 5.0 mL methanol for HPLC analysis.

The semipreparative HPLC was performed with a Hewlett-Packard 1100 system (Hewlett-Packard, USA) consisting of a quaternary pump, a vacuum degasser, a variable wavelength detector and an autosampler. Separation was achieved using an Inertsil C18 column (partical size 10 \( \mu \)m, 10.7 mm×250 mm, Gasukuro Kogyo, Japan). The mobile phase was methanol-ammonium acetate 20 mmol/L (80:20, v/v) at a flow rate of 3.0 mL/min. The UV detection wavelength was 254 nm. To isolate the metabolite, the extract was injected repeatedly and the compound with the same retention time at 4.0 min was pooled, evaporated to dryness, and stored at 4 °C before structural elucidation.

The purified metabolite and naproxen were dissolved in 0.5 mL Me3SO (99.9 atom % \(^1\)H) for nuclear magnetic resonance (NMR) analysis. The \(^1\)H-NMR measurements were carried out at 300 MHz on a Bruker ARX 300 spectrometer (Bruker, Faellanden, Switzerland). Chemical shifts were reported on the \( \delta \) scale relative to tetramethylsilane as an internal standard.

RESULTS

Microbial metabolites of naproxen

The microbial transformation samples and the control samples were extracted and analyzed as described above. The chromatograms of the blank culture controls showed no metabolites or naproxen, and the blank substrate control showed only the presence of naproxen. Compared with the controls, three pseudo-molecular ions ([M-H]) correlated with the metabolism of naproxen were observed in the total ion current (TIC) of microbial transformation samples, including ions at \( m/z \) 229 (M1), \( m/z \) 215 (M2), and \( m/z \) 295 (M3) (Fig 1).

M1 eluting at 16.4 min possessed the same pseudo-molecular ion, full scan MS/MS spectrum, and chromatographic behavior with authentic naproxen. So M1 was identified as unmetabolized naproxen. The full scan mass spectrum of naproxen showed a prominent deprotonated molecular ion at \( m/z \) 229, which yielded a base peak at \( m/z \) 185 in MS/MS spectrum (Fig 2A). The ion at \( m/z \) 185, 44 u lower than the precursor ion, was proposed to arise via the loss of a neutral molecule of CO₂ from the carboxyl group of naproxen.

M2 had a retention time of 7.2 min. In electrospray ionization negative ion spectrum, the pseudo-molecular ion of M2 was at \( m/z \) 215, 14 u lower than that of naproxen, suggesting that it was a desmethylated metabolite. The product ion spectrum of the ion at \( m/z \) 215 gave a prominent ion at \( m/z \) 171 (Fig 2B), which was also 44 u lower than the precursor ion, indicating that the carboxyl group was unaltered. These mass spectra of M2 were consistent with O-desmethylnaproxen. This identification was further substantiated by \(^1\)H-NMR analysis. The NMR data of the metabolite differed from those of naproxen in that the signal of 6-methoxy proton at \( \delta \) 3.86 was lost, and a signal of free phenolic proton at \( \delta \) 9.71 appeared, indicating that M2 was the 6-O-desmethylated metabolite of naproxen, desmethylnaproxen.

M3 had a retention time of 5.0 min. The full scan mass spectrum of M3 showed a deprotonated molecular ion at \( m/z \) 295. The MS/MS spectrum of the ion at \( m/z \) 295 yielded major ions at \( m/z \) 251 and \( m/z \) 215 (Fig 2C). The ion at \( m/z \) 251 also corresponded to the loss of CO₂, similar to the parent drug and M2. The ion at \( m/z \) 215 produced by the loss of 80 u from the precursor ion, possessed the same mass to charge ratio with the deprotonated molecular ion of M2. Additionally, the MS³ spectrum of the product ion at \( m/z \) 215 (Fig 2D) yielded the same fragment ions as the MS/MS spectrum of M2 (Fig 2B), indicating that M3 was a sulfate conjugate of desmethylnaproxen\(^{14,15}\).

Difference in the microbial transformation profiles

There was minor difference among the transformation profiles of naproxen by three strains of Cunninghamella species (Tab 1). Both Cunninghamella blakesleean and Cunninghamella elegans transformed naproxen completely into metabolites, and the concentration of unmetabolized naproxen was negligible (below 1 % of naproxen added). But in the sample of Cunninghamella echinulata, naproxen still accounted for about 29 % of the total of parent drug and two metabolites, suggesting that the capability of Cunninghamella echinulata to transform naproxen was weaker than that of the other two strains. Since Cunninghamella elegans transformed naproxen into only one metabolite...
(desmethylnaproxen), it was selected as the model microorganism to prepare sufficient quantity of the metabolite for structural elucidation. Both *Cunninghamella blakesleana* and *Cunninghamella echinulata* were able to further transform desmethyl-naproxen into a phase II metabolite, desmethylnaproxen-6-O-sulfate. The ratio of desmethylnaproxen to its sulfate was about 7:2 in the sample of *Cunninghamella blakesleana*, and about 8:1 in the sample of *Cunninghamella echinulata*.

**DISCUSSION**

In the present study, naproxen was transformed by *Cunninghamella* species into two metabolites, desmethylnaproxen and its sulfate. It was reported\(^1\) that naproxen was completely transformed into desmethylnaproxen by *Cunninghamella elegans* ATCC 9245, and that desmethylnaproxen was the major metabolite of naproxen using *Cunninghamella blakesleana* ATCC 8688a. Conjugated metabolites were not observed in that research, although naproxen is mainly excreted as conjugates of naproxen and desmethylnaproxen in animals and humans. In this study, we first found a phase II metabolite of naproxen, desmethylnaproxen-6-O-sulfate, in the samples of *Cunninghamella blakesleana* AS 3.153 and *Cunninghamella echinulata* AS 3.2004.

The microbial transformation of naproxen (Fig 3)
has some similarities with the metabolism of naproxen in mammals. Desmethylnaproxen was the only phase I metabolite of naproxen in both microbial models and mammals. And one of the known mammalian conjugated metabolites, desmethylnaproxen-6-O-sulfate, was also produced by microbial transformation. Though previous studies using *Cunninghamella* species as models of mammalian drug metabolism indicated that these fungi were efficient to produce glucuronide conjugates of drugs\[10,11\], we did not observe glucuronide conjugates of naproxen or desmethylnaproxen in the samples of *Cunninghamella* species by LC/MS analysis, which was partly due to the lack of some phase II enzymes in the fungi used.

In conclusion, the microbial transformation of naproxen further demonstrated the high potential of *Cunninghamella* species to produce not only primary (phase I) metabolites, but also conjugated (phase II) metabolites of drugs. And the advantage of microbial models includes low cost, easy of handling, and scale-up capability. Therefore, these microbial models could be used to predict potential routes of mammalian metabolism of drug candidates in the early phase of drug
development. By this means, sufficient quantity of putative metabolites could be isolated and identified, then used as standards for metabolite identification and determination in mammalian studies. As the actual mammals could be partly substituted by microbial models, the number of laboratory animals used in preclinical study might be decreased significantly.

REFERENCES