

Full-length article

Biotransformation of indomethacin by the fungus *Cunninghamella blakesleeana*¹Peng ZHANG², Li-hong LIN⁴, Hai-hua HUANG³, Hai-yan XU², Da-fang ZHONG^{2,3,5}

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Key words

indomethacin; microbial transformation; *Cunninghamella blakesleeana*; liquid chromatography; mass spectrometry

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Abstract

Aim: To investigate the biotransformation of indomethacin, the first of the newer nonsteroidal anti-inflammatory drugs, by filamentous fungus and to compare the similarities between microbial transformation and mammalian metabolism of indomethacin. **Methods:** Five strains of *Cunninghamella* (*C. elegans* AS 3.156, *C. elegans* AS 3.2028, *C. blakesleeana* AS 3.153, *C. blakesleeana* AS 3.910 and *C. echinulata* AS 3.2004) were screened for their ability to catalyze the biotransformation of indomethacin. Indomethacin was partially metabolized by five strains of *Cunninghamella*, and *C. blakesleeana* AS 3.910 was selected for further investigation. Three metabolites produced by *C. blakesleeana* AS 3.910 were isolated using semi-preparative HPLC, and their structures were identified by a combination analysis of LC/MSⁿ and NMR spectra. These three metabolites were separated and quantitatively assayed by liquid chromatography-ion trap mass spectrometry. **Results:** After 120 h of incubation with *C. blakesleeana* AS 3.910, approximately 87.4% of indomethacin was metabolized to three metabolites: *O*-desmethyindomethacin (DMI, M1, 67.2%), *N*-deschlorobenzoylindomethacin (DBI, M2, 13.3%) and *O*-desmethyl-*N*-deschlorobenzoylindomethacin (DMBI, M3, 6.9%). Three phase I metabolites of indomethacin produced by *C. blakesleeana* AS 3.910 were identical to those obtained in humans. **Conclusion:** *C. blakesleeana* could be a useful tool for generating the mammalian phase I metabolites of indomethacin.

Introduction

Indomethacin is a non-steroidal anti-inflammatory agent with anti-pyretic and analgesic properties. The anti-pyretic effect is caused by a resetting of the hypothalamic temperature-regulating center, whereas the anti-inflammatory and analgesic effects are due to inhibition of prostaglandin synthesis. It is extensively used because of its excellent pharmaceutical properties. Indomethacin is used in the treatment of musculoskeletal disorders such as rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and also as an adjunct to periodontal therapy^[1-3].

A few investigations on the metabolism of indomethacin in humans have been reported. In humans, indomethacin is

metabolized by phase I metabolism to *O*-desmethyindomethacin (DMI), *N*-deschlorobenzoylindomethacin (DBI) and *O*-desmethyl-*N*-deschlorobenzoylindomethacin (DMBI). These metabolites are devoid of anti-inflammatory activity. Indomethacin and all phase I metabolites can be conjugated by phase II metabolism into glucuronide conjugates^[4-6].

An important factor in the evaluation of the efficacy and safety of a drug is the study of its mammalian metabolism. However, the identification of metabolites from animal sources and clinical samples may be hindered by insufficient quantities of material. Some micro-organisms can metabolize compounds in a similar manner to mammals, and have been used to isolate mammalian drug metabolites. With the low cost, ease of handling and scale-up capability,

selected micro-organisms, particularly fungi, have been used successfully as *in vitro* models to mimic and predict the metabolic fate of xenobiotics in mammalian systems^[7]. Using milder reaction conditions, the microbial system also provides an alternative to, or complement for, organic synthesis^[8].

Although the metabolism of indomethacin in humans has previously been studied, to our knowledge, no report on the potential of filamentous fungi to metabolize indomethacin has been published. Cultures of five strains of *Cunninghamella* were chosen for this investigation, because such fungi, in previous studies in our laboratory, have the ability to metabolize drugs, including naproxen^[9] and verapamil^[10], in a similar manner to mammals. Therefore, the aim of the current study was to investigate the metabolic fate of indomethacin in cultures of five strains of *Cunninghamella* and to further compare the similarities between microbial transformation and mammalian metabolism.

Materials and methods

Chemicals Indomethacin (purity 99.5%) was purchased from Dongyu Pharmaceutical (Shenyang, China). Methanol and acetonitrile purchased from Concord Technology (Tianjin, China) were of HPLC grade. Peptone and yeast extract were obtained from Aoboxing (Beijing, China). All other chemicals were of analytical grade.

Micro-organisms and medium *C. elegans* AS 3.156, *C. elegans* AS 3.2028, *C. blakesleeana* AS 3.153, *C. blakesleeana* AS 3.910, and *C. echinulata* AS 3.2004 were provided by the Institute of Microbiology, the Chinese Academy of Sciences (Beijing, China). Stock cultures were maintained on potato dextrose agar (Aoboxing, Beijing, China) slants at 4 °C and transferred every six months. First-stage fermentation was carried out in a medium consisting of 20 g of dextrose, 5 g of peptone, 5 g of yeast extract, 5 g of NaCl, 5 g of K₂HPO₄, and 1000 mL of distilled water. Second-stage fermentation was carried out in a wheat-bran medium with 1% wheat-bran in broth. The pH of two mediums were adjusted to 6.5 with 3.0 mol/L HCl, the mediums were then sterilized in Erlenmeyer flasks at 115 °C and 18 psi (pound per square inch) for 30 min, and cooled before incubation.

Fermentation procedures Microbial metabolism was facilitated by incubating the cultures with shaking on a rotary shaker at 28 °C. Fermentations followed a standard two-stage fermentation procedure. In general, for each of five strains of *Cunninghamella*, the first-stage of the fermentation was initiated by inoculating a 250-mL Erlenmeyer flask containing 50 mL broth with a loop of spores taken from a freshly growing agar slant. After incubation for 24 h, a 0.5

mL portion from the first-stage culture was used to inoculate a second-stage 100 mL flask containing 20 mL wheat-bran medium. The second-stage culture was incubated for 24 h, before the substrate in acetone-methanol (9:1, v:v) was added to a final concentration of 0.5 g/L. After 120 h of additional incubation, the cultures were centrifuged at 1500×g for 20 min, and the supernatant were decanted and kept at -20 °C until analysis.

Preparative-scale fermentation of indomethacin by *C. blakesleeana* AS 3.910 followed the same procedure as the screening experiments except that the volume of broth was increased. During the first stage, two flasks were prepared as described above, and 100 second-stage flasks of 100 mL capacity containing 20 mL of wheat bran medium were each inoculated with 0.5 mL of the first-stage culture. Indomethacin was added to a final concentration of 0.5 g/L, and the culture was incubated for an additional 120 h.

In order to determine whether indomethacin would chemically decompose or spontaneously transform under fermentation conditions, substrate controls were prepared by adding the substrate to sterile medium and then incubating without the presence of micro-organisms. Culture controls consisted of a fermentation blank, in which the micro-organisms were grown under identical conditions without the addition of the substrate.

Extraction of metabolites and liquid chromatography-mass spectrometry (LC/MSⁿ) assay A 0.1-mL aliquot of each sample was applied to a Bond Elute C₁₈ cartridge (Teda Fuji, Tianjin, China), preconditioned by washing with 4.0 mL of methanol and 4.0 mL of distilled water. The cartridge was washed with 1.0 mL of distilled water, and metabolites were eluted with 1.0 mL of methanol. The eluate was evaporated to dryness under a gentle stream of nitrogen at 40 °C, and the residue was reconstituted by addition of 200 μL of acetonitrile-water (7:3, v:v). A 20-μL aliquot was injected onto the LC/MSⁿ system.

LC/MSⁿ analysis was performed on a Thermo Finnigan LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with an atmospheric-pressure ionization interface. The instrument was operated in positive electrospray ionization mode. The capillary voltage was fixed at 16 V, and the temperature was maintained at 200 °C. The spray voltage was set at 4.25 kV. The HPLC fluid was nebulized by using N₂ 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. A full-scan mass spectrum to obtain the protonated molecules [M+H]⁺ of each metabolite was collected. Multistage mass spectra were produced by collision-induced dissociation of the selected precursor ions with He present in the ion trap. The relative

collision energy was set at 25%–35%. Data were collected and analyzed with the Xcalibur software (version 1.2; Thermo Finnigan). The metabolites were separated using a mobile phase consisting of acetonitrile-water-formic acid (70:30:0.5, v:v:v) over 20 min at 0.5 mL/min with a Diamonsil C₁₈ column (200×4.0 mm inner diameter, 5 μm, Dikma) preceded by a Hypersil BDS-C₁₈ precolumn (10×4.6 mm inner diameter, 5 μm, Dikma). The concentrations of indomethacin and its metabolites in cultures were determined by a validated LC/MS/MS method using glyburide as internal standard. The sample preparation, chromatography and mass spectrometry system were described above. Indomethacin and its metabolites were monitored using the following mass transitions: indomethacin, *m/z* 358→174 (collision energy 35 eV); M1, *m/z* 344→160 (collision energy 30 eV); M2, *m/z* 220→174 (collision energy 30 eV); M3, *m/z* 206→160 (collision energy 25 eV). Indomethacin and M1–M3 were quantified by their peak area ratio relative to that of the internal standard (glyburide, *m/z* 494→369; collision energy 35 eV) by weighted ($w=1/c^2$) least-squares linear regression of calibration curves. The assay has been shown to be reproducible with precision below 15% and accuracy within ±15%. The lower limit of quantification was 50 ng/mL for the parent drug and all its metabolites.

Isolation and identification of metabolites The flask contents produced by the transformation of indomethacin using *C blakesleeana* AS 3.910 on a preparative scale were centrifuged at 1500×*g* for 20 min. The combined supernatant was extracted with three equal volumes of ethyl acetate. The organic extract was dried over sodium sulfate and evaporated to dryness under vacuum at 35 °C using a RE-52A rotary evaporator (Yarong Biology Instrument, Shanghai, China). In order to obtain sufficient quantities for the structural identification of metabolites, the methanol solution of extract was injected repeatedly onto a semi-preparative HPLC system (Shimadzu, Kyoto, Japan) consisting of two LC-6AD solvent delivery units, 2a DGU-14A degasser unit, an SCL-10A VP system controller, an SPD-10A VP UV-Vis detector, an FRC-10A fraction collector, and a CLASS-VP LC workstation. Separation was accomplished using a mobile phase consisting of methanol-water (42:58, v:v) at 9.5 mL/min on a Shim-Pack PRC-ODS column (250×20 mm inner diameter, Shimadzu, Kyoto, Japan) preceded by a GPRC-ODS precolumn (8×1.5 mm inner diameter, Shimadzu, Kyoto, Japan). The UV detector was set at 230 nm. Compounds with similar retention times were pooled, evaporated to dryness, and stored at 4 °C before structural analysis.

Each metabolite was dissolved in 0.5 mL of dimethyl-*d*₆-sulfoxide (99.96 atom% H) for nuclear magnetic resonance

(NMR) analysis. The NMR measurements were carried out at 600 MHz on a Bruker ARX 600 NMR spectrometer (Bruker, Faellanden, Switzerland). Chemical shifts were recorded on the δ scales in parts per million (ppm) relative to tetramethylsilane as an internal standard.

Results

Screening of cultures Five strains of *Cunninghamella* were screened for the ability to catalyze the biotransformation of indomethacin. The results indicated that indomethacin was partially metabolized, and the percentages of transformation were *C elegans* AS 3.156 (38.76%), *C elegans* AS 3.2028 (69.29%), *C blakesleeana* AS 3.153 (51.90%), *C blakesleeana* AS 3.910 (87.41%) and *C echinulata* AS 3.2004 (39.52%). Because the highest proportion of the indomethacin was transformed by *C blakesleeana* AS 3.910, this organism was selected for further investigation.

Identification of indomethacin metabolites The LC/MSⁿ chromatograms of the culture control showed no metabolites or indomethacin present, and those of the substrate control showed only the presence of indomethacin but no metabolites. As shown in Figure 1, three metabolites and indomethacin were observed in the *C blakesleeana* AS 3.910 culture sample.

Following preparative-scale transformation of indomethacin by *C blakesleeana* AS 3.910, three metabolites (M1–M3) were isolated by semi-preparative HPLC and their structures were identified by a combination analysis of LC/MSⁿ (Table 1) and NMR (Table 2 and Table 3) spectra. The identity of M0 was confirmed by comparison of the LC retention times and mass spectra (Table 1) with the synthetic references. The structures of indomethacin and its metabolites are shown in Figure 2.

Table 1. Chromatographic and mass spectral data for indomethacin and its metabolites produced by *Cunninghamella blakesleeana* AS 3.910 and metabolic yields.

Compound	LC Retention time (min)	Yield (%)	MS [M+H] ⁺	MS/MS ^a (<i>m/z</i>)
M0	13.98	12.6	358	174 (100), 139 (37), 312 (7)
M1	7.81	67.2	344	160 (100), 139 (43), 298 (8)
M2	5.35	13.3	220	174 (100)
M3	4.54	6.9	206	160 (100)

^a Only the most abundant ions (>5%) are shown. The numbers in parentheses are relative abundance values, expressed as percentages.

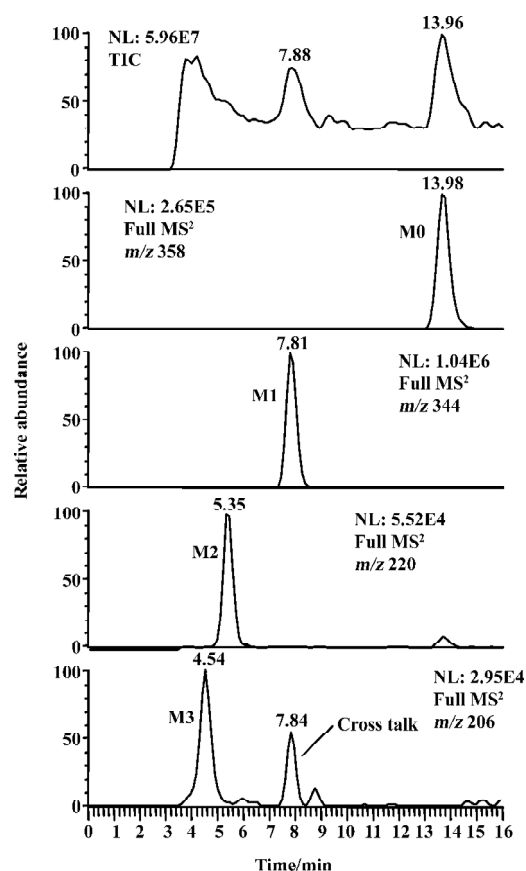


Figure 1. Total ion current (TIC) and full MS² scan chromatograms of indomethacin and its metabolites in the sample of *Cunninghamella blakesleeana* incubated with 0.5 g/L of indomethacin for 120 h. NL, normalized y-axis normalization mode, in which the instrument automatically sets the vertical scale equal to the height of the largest peak; M0, indomethacin; M1, *O*-desmethyindomethacin; M2, *N*-deschlorobenzoylindomethacin; M3, *O*-desmethyl-*N*-deschlorobenzoylindomethacin.

Table 3. ¹³C NMR data of indomethacin and its metabolites produced by *Cunninghamella blakesleeana* AS 3.910^a.

Carbon	M0 ^b	M1 ^b	M2 ^b	M3 ^b
C-2	135.2	134.8	133.7	134.0
C-3	101.8	103.5	100.3	102.8
C-4	111.4	112.1	109.5	110.5
C-5	155.6	153.4	153.1	150.9
C-6	113.5	113.3	110.9	111.2
C-7	130.3	129.5	128.9	129.8
C-8	114.7	114.6	104.3	104.1
C-9	130.8	130.9	130.2	130.2
C-10	13.3	13.24	11.5	12.0
C-11	29.6	29.8	30.3	31.0
C-12	172.2	172.1	173.5	174.1
C-13	55.5		55.4	
C-14	168.0	167.9		
C-15	134.3	134.4		
C-16	131.2	131.1		
C-17	129.1	129.1		
C-18	137.7	137.5		
C-19	129.1	129.1		
C-20	131.2	131.1		

^a Chemical shifts are expressed in ppm down field from the signal for tetramethylsilane, and coupling constants in Hz are in parentheses.

^b NMR spectrum measurements were carried out at 600 MHz and dissolved in dimethyl-*d*₆-sulfoxide.

Parent drug (M0) The compound eluting at 13.98 min by LC/MSⁿ analysis were identified as indomethacin (M0), because the protonated molecules, full scan MS/MS spectra, and LC retention time of the compound were identical to those of the corresponding synthetic reference.

Metabolite M1 The retention time of M1 ([M+H]⁺ *m/z*

Table 2. ¹H NMR data of indomethacin and its metabolites produced by *Cunninghamella blakesleeana* AS 3.910.

Proton	M0 ^b	M1 ^b	M2 ^b	M3 ^b
H-4	7.04 <i>d</i> (2.39)	6.82 <i>d</i> (2.27)	6.88 <i>d</i> (1.41)	6.70 <i>d</i> (1.87)
H-6	6.72 <i>dd</i> (8.99, 2.39)	6.55 <i>dd</i> (8.84, 2.27)	6.61 <i>dd</i> (8.55, 1.41)	6.47 <i>dd</i> (8.48, 1.87)
H-7	6.93 <i>d</i> (8.99)	6.86 <i>d</i> (8.84)	7.10 <i>d</i> (8.55)	7.00 <i>d</i> (8.48)
H-10	2.22 <i>s</i>	2.19 <i>s</i>	2.28 <i>s</i>	2.26 <i>s</i>
H-11	3.67 <i>s</i>	3.58 <i>s</i>	3.49 <i>s</i>	3.42 <i>s</i>
H-12	12.36 <i>s</i>	12.34 <i>s</i>	10.61 <i>s</i>	10.44 <i>s</i>
H-13	3.76 <i>s</i>		3.71 <i>s</i>	
H-(16,17,19,20)	7.66	7.65		

^a Chemical shifts are expressed in ppm down field from the signal for tetramethylsilane, and coupling constants in Hz are in parentheses.

^b NMR spectrum measurements were carried out at 600 MHz and dissolved in dimethyl-*d*₆-sulfoxide.

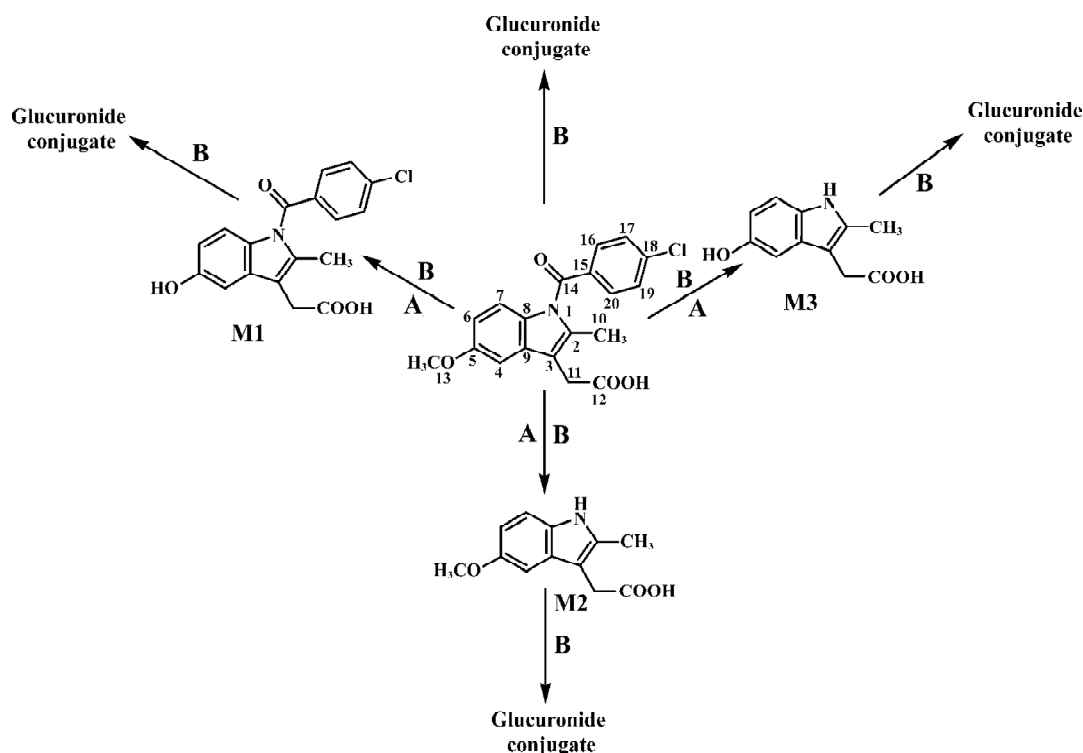


Figure 2. Proposed metabolic pathways of indomethacin produced by *Cunninghamella blakesleeana* and comparison with those of humans. (A) *Cunninghamella blakesleeana*; (B) Humans.

344) obtained by LC/MSⁿ analysis was 7.81 min. The protonated molecule of M1 was 14 Da lower than that of M0. The diagnostic ions at *m/z* 160 for M1 from the parent ion at *m/z* 344 were 14 Da lower than the main fragment ion in the MS/MS spectrum of M0 (parent ion at *m/z* 358) (Table 1). This suggested that M1 was a monodesmethylated derivative. Comparisons of the NMR spectra of M0 and M1 indicated that M1 was *O*-desmethyindomethacin, based on the disappearance of the proton signal of 5-methoxy (Table 2) and the carbon signal of 5-methoxy (C-13, Table 3). Hence, M1 were deduced to be *O*-desmethyindomethacin.

Metabolite M2 The retention time of M2 ([M+H]⁺ *m/z* 220) obtained by LC/MSⁿ analysis was 5.35 min. The protonated molecule of M2 ([M+H]⁺ *m/z* 220) was 138 Da lower than that of M0. The diagnostic ions at *m/z* 174 for M2 from the parent ion at *m/z* 220 were identical with the main fragment ion in the MS/MS spectrum of M0 (parent ion at *m/z* 358) (Table 1). This suggested that M2 was *N*-deschlorobenzoylindomethacin, which was further confirmed by NMR spectra of M2. Comparisons of the NMR spectra of M0 and M2 indicated that M2 was *N*-deschlorobenzoylindomethacin, based on the disappearance of the signal of the aromatic proton H-15 to H-20 (Table 2) and the disappearance of the

signal of the aromatic carbon of chlorobenzoyl moiety (Table 3). Based on these MS and NMR data, metabolite M2 was identified as *N*-deschlorobenzoylindomethacin.

Metabolite M3 The retention time of M3 ([M+H]⁺ *m/z* 206) obtained by LC/MSⁿ analysis was 4.54 min. The protonated molecule of M3 ([M+H]⁺ *m/z* 206) was 152 Da lower than that of M0. The MS/MS spectrum of M3 (parent ion at *m/z* 206) produced diagnostic ions at *m/z* 160, which was consistent with the main fragment ion in the MS/MS spectrum of M1 (parent ion at *m/z* 344) (Table 1). This suggested that M3 was *O*-desmethyl-*N*-deschlorobenzoylindomethacin, which was further confirmed by NMR spectra of M3. The disappearance of signal of aromatic protons in the chlorobenzoyl moiety and of the signal of 5-methoxy protons indicated that M3 was *O*-desmethyl-*N*-deschlorobenzoylindomethacin, which was also confirmed by the disappearance of signal of aromatic carbons in the chlorobenzoyl moiety and of the signal of 5-methoxy carbon. On the basis of these MS and NMR data, M3 was unambiguously identified as *O*-desmethyl-*N*-deschlorobenzoylindomethacin.

Microbial transformation profiles and comparison with mammalian metabolism This study has shown that *C*

blakesleeana transformed indomethacin to the following metabolites: *O*-desmethylindomethacin, *N*-deschlorobenzoylindomethacin and *O*-desmethyl-*N*-deschlorobenzoylindomethacin. In Figure 2, the structures of these metabolites and the proposed biotransformation pathways in micro-organism are compared to those identified in humans in previous studies^[4-6]. After 120 h of incubation by *C blakesleeana*, approximately 87.4% of indomethacin was metabolized to three metabolites. The yields of those metabolites were M1 (67.2%), M2 (13.3%), and M3 (6.9%) (Table 1).

Discussion

We report here the successful biotransformation of indomethacin by *C blakesleeana*. As shown in Figure 2, After 120 h of incubation with *C blakesleeana* AS 3.910, approximately 87.4% of indomethacin was metabolized to three metabolites: *O*-desmethylindomethacin (DMI, M1, 67.2%), *N*-deschlorobenzoylindomethacin (DBI, M2, 13.3%) and *O*-desmethyl-*N*-deschlorobenzoylindomethacin (DMBI, M3, 6.9%). Three phase I metabolites of indomethacin produced by *C blakesleeana* AS 3.910 were identical to those obtained in humans.

It is well recognized that conjugations are important metabolic pathways of many compounds both in micro-organisms and in mammals. Conjugated metabolites (glucuronides, glucosides and sulfate conjugates) of drugs can be formed by microbial models^[8-12]. Although indomethacin is excreted as glucuronides conjugates in humans^[4-6], and previous studies using *Cunninghamella* species as models of mammalian drug metabolism indicated that these fungi were efficient at producing glucuronides^[11] and sulfate conjugates^[9] of drugs, we did not detect the glucuronide or sulfate conjugates of the above metabolites in the *Cunninghamella* species culture samples by LC/MSⁿ analysis.

In conclusion, three metabolites of indomethacin were formed by *C blakesleeana* with the 67.2%, 13.3%, and 6.9% yields, respectively. The microbial transformation of indomethacin has some similarities with the metabolism of indomethacin in humans (Figure 2). Metabolites M1–M3 are phase I metabolites of indomethacin in both microbial models and in humans. In humans, *O*-desmethylindomethacin formation is critical to the elimination of indomethacin and represents 40%–55% of total drug eliminated in the urine^[5]. As in humans, *O*-desmethylindomethacin was the major metabolite in the current microbial model. The microbial transformation of indomethacin further demonstrated the high

potential of *Cunninghamella* to produce primary metabolites of a drug. The ability of *Cunninghamella* to mimic mammalian metabolism and to perform novel biotransformations clearly indicates that microbial systems represent an attractive alternative to the use of mammalian systems or chemical synthesis of metabolites. The quantities of drugs metabolites produced at low cost were sufficient for complete structural identification and for further use in investigating their pharmacokinetic, pharmacological and toxicological properties.

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