Microbial Transformation of Dextromethorphan by *Cunninghamella blakesleeana* AS 3.153

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The capability of *Cunninghamella blakesleeana* AS 3.153 to transform CYP2D6 probe drug dextromethorphan was investigated. Metabolites produced by strain AS 3.153 were detected by liquid chromatography-tandem mass spectrometry (LC-MS^n) and the metabolite dextrorphan was identified by reference to confirm its structure. The yield of dextrorphan produced by *C. blakesleeana* AS 3.153 was over 90%. Quinidine, a CYP2D6 selective inhibitor, was applied to investigate its effect on biotransformation. The concentration of quinidine was 4-folds higher than that of dextromethorphan and the yield of dextrorphan was reduced by 84%, which proved there was drug metabolism enzyme similar to CYP2D6 in *C. blakesleeana* AS 3.153. It is concluded that *C. blakesleeana* AS 3.153 can be used as the suitable model strain *in vitro* to mimic human CYP2D6 metabolism.

Key words *Cunninghamella blakesleeana*; microbial transformation; dextromethorphan; cytochrome P450 2D6; liquid chromatography-tandem mass spectrometry

In recent years, a number of studies have shown that some fungi, particularly *Cunninghamella* spp., possess cytochrome P450 monooxygenase systems analogous to those in mammals.[1—3] Compared with more traditional methods, there are clearly a number of practical advantages in using microbial transformation as a model for drug metabolism, such as its low cost, high yield and easiness to regulate. Therefore, it is a complementary tool in the investigation of drug metabolism in mammals.[4]

Most compounds (about 60%) are cleared metabolically by cytochrome P450 (CYPs) in the human liver, which is the major enzyme system often involved in the rate-limiting step of drug biotransformation processes.[5] CYP2D6 is a specific isoform of cytochrome P450 and although its relative content in hepatic CYPs is only 2% of the total amount, it oxidizes 30% of human drugs,[6] including antiarrhythmics, antidepressants, neuroleptics, β-blockers, opioids, among others.[7] Dextromethorphan, a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, is a well-known antitussive drug with negligible side effects.[8] In humans, dextromethorphan is reduced rapidly after oral absorption to dextrorphan (*O*-demethyl-dextromethorphan) by the polymorphic CYP2D6.[9] Dextromethorphan is also a widely used probe drug for the activity of CYP 2D6 *in vivo* as well as *in vitro*.[10]

In this study, *Cunninghamella blakesleeana* AS 3.153 transforming dextromethorphan to dextrorphan is investigated and the optimal transformation system is screened by orthogonal design. CYP2D6 selective inhibitor quinidine[11] is adapted to investigate its influence on the transformation potential of *C. blakesleeana* AS 3.153 and microbial model of dextromethorphan metabolism is established.

Experimental

Microorganisms *Cunninghamella blakesleeana* AS 3.153 was purchased from the Institute of Microbiology, Chinese Academy of Sciences in Beijing, China.

Cultures *Cunninghamella blakesleeana* AS 3.153 was maintained on potato slants at 4 °C and transferred every 6 months to maintain viability. There were four kinds of biotransformation media: (1) the common medium consisted of glucose 2.0 g, peptone 0.5 g, NaCl 0.5 g, K_2HPO_4 0.5 g, yeast extract 0.5 g; (2) wheatbran medium was the common medium with 1% wheatbran; (3) soybean medium consisted of glucose 2.0 g, soybean powder 0.5 g, NaCl 0.5 g, K_2HPO_4 0.5 g, yeast extract 0.5 g; (4) Sabouraud wheatbran medium (Sabouraud medium with 1% wheatbran, the Sabouraud medium consisted of glucose 4.0 g, peptone 1.0 g, NaCl 0.5 g). The medium (1) and (3) were generated by mixing their respective ingredients listed above with 100 ml of distilled water. These four kinds of medium were autoclaved in individual Erlenmeyer flasks at 115 °C for 30 min and cooled before incubation.

Chemicals Dextromethorphan with purity above 99% was provided by the Shenyang Huatai Institute of Pharmacy in Shenyang, China. Dextrorphan was synthesized in the School of Pharmaceutical Engineering, Shenyang Pharmaceutical University. Quinidine was purchased from Sigma Corporation (St Louis, MO, U.S.A.). All solvents used for assay were high performance liquid chromatography (HPLC) grade. The remaining chemicals were analytical grade and biochemical reagents.

Microbial Transformation The strain AS 3.153 kept at 4 °C was aseptically transferred to potato slants and incubated at 28 °C for 6 d to obtain well-grown mycelium with spores. The fermentation has two stages. In the first-stage of fermentation, a loop of fresh spores was inoculated to a 250 ml Erlenmeyer flask containing 50 ml common medium. The cultures were incubated at 28 °C for 24 h on a rotary shaker operating at 220 rpm. At the second-stage, a 1.0 ml portion from the first-stage flask was inoculated to a 100 ml flask containing 20 ml biotransformation medium. After 24 h of incubation, dextromethorphan dissolved in acetone–water (5 : 1, v/v) (0.2 mol/l) was added to each flask to yield a certain final concentration and incubated for an additional 120 h. After microbial transformation, the flask contents were centrifuged at 1500×*g* for 20 min. The supernatant was transferred to tubes and kept at −20 °C until analysis.

Two types of controls were run synchronously with the fermentation and worked-up using the same method. One was a blank strain control that was used to define and exclude the indigenous secondary metabolites generated by the strains. The other was a blank substrate control (*i.e.* the culture with the dextromethorphan but without the strain) that was used to test whether dextromethorphan would be chemically decomposed or spontaneously transformed under medium and microbial transformation conditions.

Liquid Chromatography-Mass Spectrometry (LC-MS^n) Assay For qualitative analysis, a 0.1 ml aliquot of each sample was applied to a Bond Elute C_{18} 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 water, and metabolites were eluted with 1.0 ml of methanol. After the eluate was evaporated to dryness under a gentle stream of nitrogen at 40 °C, its residue was reconstituted by addition of 200 μl of methanol : water (1 : 1, v/v). A 20 μl aliquot was injected onto the LC-MS^n system.

For quantitative analysis, a liquid chromatography-tandem mass spectrometric method was applied to simultaneously determine the yield of dextromethorphan and dextrorphan in transformation culture. To a 500 μl aliquot of microbial transformation samples, 100 μl of methanol–water (50 : 50, v/v), 50 μl of the I.S. solution (chlorphenamine 100 ng/ml) and

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100 μl NH_3–NH_4Cl buffer (1 mol/l, pH 11) were added. The mixed samples were then extracted with 3 ml of *n*-hexane–dichloromethane–isopropanol (300 : 150 : 15, v/v/v). The mixture was vortex-mixed for approximately 1 min, then shaken on a mechanical shaker for 15 min. After centrifugation at 2000×***g*** for 5 min, the upper organic layer was removed and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 200 μl of the mobile phase, then vortex-mixed. A 20 μl aliquot of the resulting solution was injected onto the LC-MS^n system for analysis.

LC-MS^n analysis was performed on a Thermo Finnigan LCQ ion trap mass spectrometer (San Jose, CA, U.S.A.) equipped with an atmospheric-pressure ionization interface. The instrument was operated in positive electrospray ionization (ESI) mode. A full-scan mass spectrum was collected to obtain the protonated molecules $[M+H]^+$ of each metabolite. Multistage mass spectra (MS^2) were produced by collision-induced dissociation (CID) of the selected precursor ions with He present in the ion trap. The relative collision energy was set at 30—40%. The metabolites were separated using a mobile phase consisting of methanol : water : formic acid (47 : 53 : 0.5, v/v/v) over 10 min at 0.5 ml/min with a Diamonsil C_{18} column (200×4.6 mm i.d., 5 μm, Dikma Co., Beijing) preceded by a Hypersil BDS-C_{18} precolumn (10×4.6 mm i.d., 5 μm, Dikma Co., Beijing).

Establish the Microbial Model of Dextromethorphan Metabolism Dextromethorphan was added to biotransformation system to form the concentration of 1.0 mmol/l and incubated for an additional 120 h at 28 °C on a rotary shaker operating at 220 rpm. The metabolites were identified by liquid chromatography-tandem mass spectrometry. The yields were calculated to transform dextromethorphan.

An orthogonal design $L_9(3^4)$ was used to optimize microbial model. Optimal system for maximal productivity of dextrorphan produced by *C. blakesleeana* AS 3.153 was determined based on three parameters at three levels. The substrate concentration, transformation medium and its initial pH were optimized.

The Influence of Quinidine on the Transformation Potential of *C. blakesleeana* AS 3.153 Dextromethorphan and quinidine were added to the transformation system synchronously. The concentration of quinidine was 1.0 mmol/l, 2.0 mmol/l, 3.0 mmol/l, 4.0 mmol/l and 5.0 mmol/l. The concentration of dextromethorphan was 1.0 mmol/l. The blank substrate control was run synchronously with only 1.0 mmol/l dextromethorphan to determine the yield of dextrorphan. Triplicate samples were collected. After 120 h incubation, the flask contents were centrifuged at 1500×***g*** for 20 min. Then the supernatant was transferred to tubes and kept at −20 °C until analysis.

Results

Identification of Dextromethorphan and Dextrorphan by LC-MS^n The microbial transformation samples and the control samples were extracted and analyzed as described in "Microbial transformation" and "LC-MS^n assay." It is found that the chromatograms of the blank culture controls showed no metabolites of dextromethorphan or parent drug, and the blank substrate control showed only the presence of dextromethorphan (Fig. 1A). Compared with the controls, two pseudo-molecular ions ($[M+H]^+$) correlated with the metabolism of dextromethorphan were observed in the total ion current (TIC) of microbial transformation samples, including ions at *m*/*z* 272 and *m*/*z* 258.

M1 eluting at 7.74 min possessed the same pseudo-molecular ion, full scan MS/MS spectrum, and chromatographic behavior as those of the authentic dextromethorphan (Fig. 1B). So M1 was identified as the unmetabolized dextromethorphan. The full scan mass spectrum of dextromethorphan showed a prominent protonated molecular ion at *m*/*z* 272, which yielded a base peak at *m*/*z* 215 in the MS-MS spectrum.

M2 had a retention time of 4.44 min and possessed the same pseudo-molecular ion, full scan MS-MS spectrum, chromatographic behavior as the authentic dextrorphan. In positive electrospray ionization spectrum, the pseudo-molecular ion of M2 was at *m*/*z* 258, 14 u lower than that of dextromethorphan, which suggested that it was a desmethylated metabolite. The product ion spectrum of the ion at *m*/*z* 258 gave a prominent ion at *m*/*z* 201, which was also 14 u lower than the product ion of M1. These mass spectra of M2 were consistent with those of the authentic dextrorphan. Therefore, M2 was identified as dextrorphan.

Establish the Microbial Model of Dextromethorphan Metabolism Metabolites of dextromethorphan were detected by the liquid chromatography-tandem mass spectrometry. *C. blakesleeana* AS 3.153 has high potential to transform dextromethorphan, from which the yield of transformed

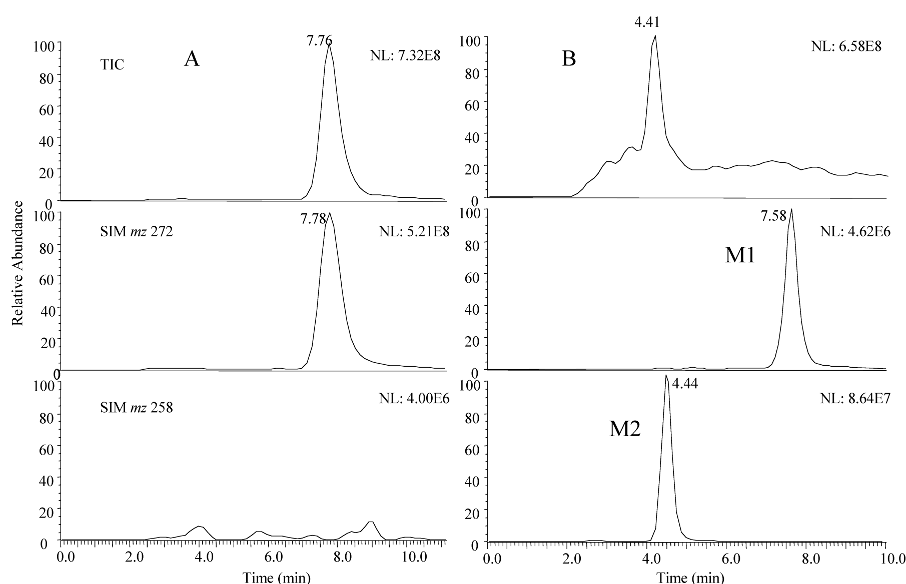

Fig. 1. The LC-MS^n Chromatograms of Dextromethorphan Drug Control Sample (A) and Transformed Sample (B) in Total Ion Current (TIC) Scan Mode and Selected Ion Monitoring (SIM) Scan Mode

NL is an abbreviation for the normalized *y*-axis normalization mode, in which the instrument automatically sets the vertical scale equal to the height of the largest peak; M1, dextromethorphan; M2, dextrorphan.

Table 1. Experimental Conditions and Mean Yield of Dextrorphan Produced with Orthogonal Design L_9 (3^4)

Number	Factors			Yield (%)
	Substrate (mM) A	Transformation medium B	Initial pH C	
1	1 (1.0)	1 (Common medium)	1 (4.5)	5.43
2	1 (1.0)	2 (Wheatbran medium)	2 (6.5)	94.00
3	1 (1.0)	3 (Soybean medium)	3 (8.5)	84.88
4	2 (2.0)	1 (Common medium)	2 (6.5)	83.06
5	2 (2.0)	2 (Wheatbran medium)	3 (8.5)	99.14
6	2 (2.0)	3 (Soybean medium)	1 (4.5)	2.32
7	3 (4.0)	1 (Common medium)	3 (8.5)	51.25
8	3 (4.0)	2 (Wheatbran medium)	1 (4.5)	62.28
9	3 (4.0)	3 (Soybean medium)	2 (6.5)	55.57

dextrorphan was 82.7%. Therefore, *C. blakesleeana* AS 3.153 was the suitable model strain to metabolize dextromethorphan.

Table 1 Summarizes the mean yield of dextrorphan obtained from the experiment in different treatment conditions. According to an analysis of the range of the results after 120 h fermentation with an orthogonal design, the important factors determining the production of dextrorphan and the impact of changing treatment levels on production were described.

Using the orthogonal design L_9 (3^4) approach, the relationship between transformation conditions and yield of dextrorphan was calculated. The factors effecting the transformation could be ranked in importance (magnitude in parentheses): initial pH (8.5)>transformation medium (wheatbran medium)>drug concentration (2.0 mmol/l). As the suitable environment of *C. blakesleeana* AS 3.153 was the level of partial acidity and the yields of dextrorphan with pH 6.5 or pH 8.5 were almost the same, pH 6.5 was selected to be optimal level. Therefore, the optimal system of dextromethorphan transformed by *C. blakesleeana* AS 3.153 was dextromethorphan at a concentration of 2.0 mmol/l, in wheatbran medium, with the initial pH at 6.5. Confirmatory experiment of the optimal model conditions was performed and the yield of dextrorphan was over 90%.

The Influence of Quinidine on the Transformation Potential of *C. blakesleeana* AS 3.153 Figure 2 shows the inhibitory effect of quinidine on the transformation of dextromethorphan by *C. blakesleeana* AS 3.153. After transformation of 120 h, the yield of dextrorphan reached 90% without the addition of inhibitor (substrate control). The yield of dextrorphan decreased obviously when selective inhibitor quinidine was added. Quantitatively, it decreased to 17.93% when the concentration of quinidine was 1.0 mmol/l, and decreased to only 6% when quinidine was added to 5.0 mmol/l. Compared with the control samples, the yield of dextrorphan displayed an 84% reduction. From the above data, it can be concluded that the transformation potential of *C. blakesleeana* AS 3.153 was significantly depressed by CYP2D6 selective inhibitor quinidine, which proved that there was drug metabolism enzyme similar to CYP2D6 in *C. blakesleeana* AS 3.153.

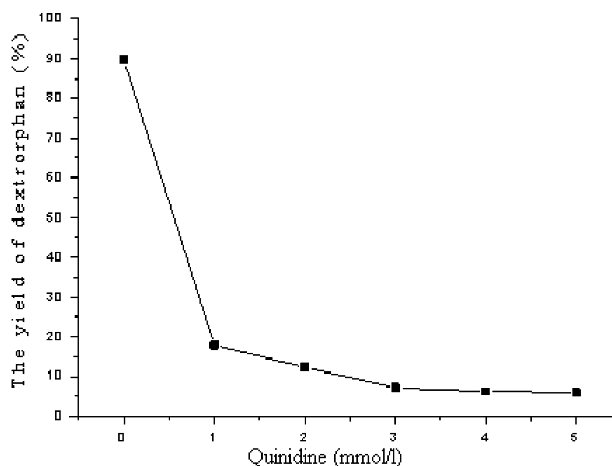

Fig. 2. The Inhibitory Effect of Quinidine on the Transformation of Dextromethorphan by *C. blakesleeana* AS 3.153

Discussion

In humans, dextromethorphan and dextrorphan can undergo *N*-demethylation to give 3-methoxymorphinan and 3-hydroxymorphinan respectively, and CYP3A is known to be involved in those pathways (Fig 3).[7,9] For the *O*-demethylation of 3-methoxymorphinan to 3-hydroxymorphinan, CYP2D6 is involved. Finally, dextrorphan and 3-hydroxymorphinan are glucuronidated to give detrorphan glucuronide and 3-hydroxymorphinan glucuronide, which are eliminated by urine. Although dextromethorphan is mainly excreted as glucuronides conjugates in humans, and previous studies using *Cunninghamella* species as models of mammalian drug metabolism indicated that these fungi were efficient at producing glucuronides[12] and sulfate conjugates[3] of drugs, we did not detect the conjugates of dextromethorphan or dextrorphan in the *C. blakesleeana* AS 3.153 culture samples by LC-MS^n analysis.

Dextrorphan is the main metabolite in *C. blakesleeana* AS 3.153. Meanwhile, a small amount of 3-methoxymorphinan and 3-hydroxymorphinan can also be detected in model strain. According to the peak area, these two metabolites accounted for no more than 1% of the total metabolites, which were not investigated in this study. By orthogonal design, an optimal transformation system was established and the yield of dextrorphan was over 90%. Under optimal conditions, 3-methoxymorphinan was not detected. However, the yield of dextrorphan clearly decreased when selective inhibitor quinidine was added. Compared with the control sample, the yield of dextrorphan displayed an 84% reduction. Meanwhile, when quinidine was added to 5 mmol/l, 3-methoxymorphinan can be detected in the culture. It's presumed that quinidine

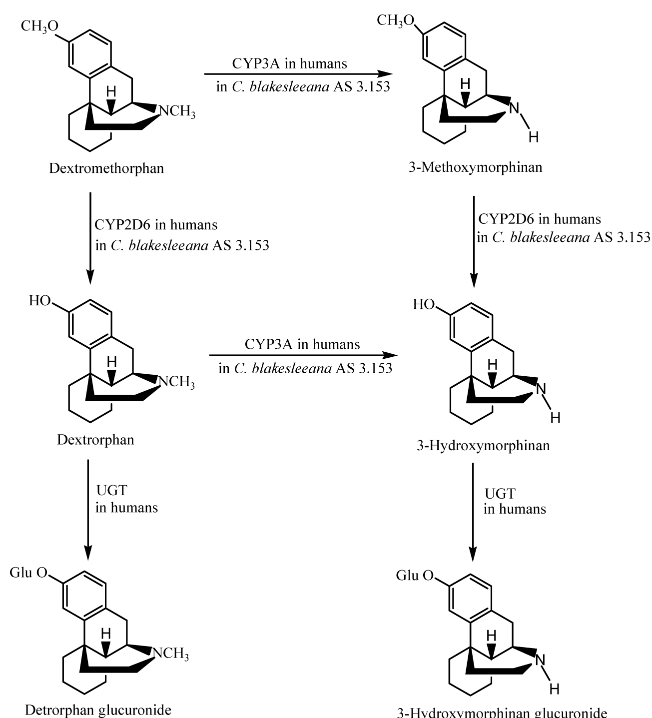

Fig. 3. Proposed Main Metabolic Pathways of Dextromethorphan in *C. blakesleeana* AS 3.153 and Comparison with Those of Humans

inhibited the *O*-demethylation pathway in model strain and the transformation of dextromethorphan partly changed to *N*-demethyl pathway. In our study, after sucking filtration, the wet mycelia weights of samples with inhibitors and control samples were all ranged from 48.0 mg to 49.5 mg/ml, which showed that quinidine didn't interfere with the growth of mycelia. Therefore, it can be concluded that at cellular level, there exists drug metabolism enzyme similar to human CYP2D6 in *C. blakesleeana* AS 3.153. The microbial model of dextromethorphan is established based on this finding.

As one of the advantages of the microbial model, it can be used to predict potential routes of mammalian metabolism in the early phase of drug development.[13] The ability of *Cunninghamella* species to mimic mammalian metabolism and to perform novel biotransformation clearly indicates that microbial systems represent an attractive alternative to the use of mammalian systems or synthesis of metabolites.[14] By this means, sufficient quantity of metabolites can be isolated and identified, then used as standard for metabolite identification and to investigate its pharmacokinetic, pharmacological and toxicological properties.

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