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Full-length article

Phase II metabolites of etofesalamide in filamentous fungi[1]

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Key words

etofesalamide; *Cunninghamella;* metabolite; transformation; mass spectrum analysis

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Abstract

Aim: To study phase II metabolites of etofesalamide in filamentous fungi. **Methods:** Seven fungi were screened to transform etofesalamide. The metabolites of etofesalamide were assayed using liquid chromatography coupled to mass spectrometry. The major metabolite was subject to enzymatic hydrolysis to confirm its structure. **Results:** Etofesalamide was converted into two phase II metabolites: glucoside and riboside conjugates. Glucoside conjugate was the major product with a yield greater than 90%; no phase I metabolites were detected. **Conclusion:** Glucoside and riboside conjugations of etofesalamide in filamentous fungi differ from the phase II metabolism of glucuronidation in mammals.

Introduction

Etofesalamide [*N*-(4-ethoxyphenyl)-2-hydroxylbenzamide] is a new drug that has been developed by Shenyang Pharmaceutical University[1]. Etofesalamide is applied to acne, psoriasis, sensitization dermatitis, chronic eczema, and neurodermatitis in clinical use. The metabolism of etofesalamide in rabbits has been well studied[2, 3], and etofesalamide-2-glucuronide is the major metabolite.

Biotransformation of drugs by microbes has been examined extensively[4]. In recent years, a number of studies have shown that some fungi, particularly *Cunninghamella spp*, possess cytochrome P-450 monooxygenase systems analogous to those in mammals and phase II drug metabolism enzymes[5–7]. The present study uses etofesalamide as a substrate to explore the ability of fungi to convert etofesalamide into phase II metabolites, and compares these results with the results of etofesalamide metabolism in mammals to further investigate special characteristics of filamentous fungi in drug metabolism.

Materials and methods

Chemicals Etofesalamide was provided by the School of Pharmaceutical Engineering, Shenyang Pharmaceutical University (Shenyang, China) (purity >99%). β-*D*-glucosidase (EC 3.2.1.21) was purchased from Sigma (St Louis, MO, USA). All solvents used for assay were high performance liquid chromatography (HPLC) grade; the remaining chemicals were analytical grade or biochemical reagents.

Microorganisms *Aspergillus niger* and *Penicillium* were supplied by the Department of Microbiology, Shenyang Pharmaceutical University (Shenyang, China). *Mucor circinelloides* (AS 3.3421) was purchased from the Institute of Applied Ecology, Chinese Academy of Sciences (Shenyang, China). Other filamentous fungi, *Cunninghamella elegans* AS 3.156, *Cunninghamella elegans* AS 3.2028, *Cunninghamella echinulata* AS 3.2004, and *Cunninghamella blacksleana* AS 3.153, were purchased from the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China).

Cultures Microbial cultures were maintained on potato dextrose agar slants at 4 °C and transferred every 6 months to maintain viability.

The broth consisted of glucose 20 g, peptone 5 g, yeast extract 5 g, K_2HPO_4 5 g, and NaCl 5 g. These ingredients were mixed in 1 000 mL of distilled water, and the pH was adjusted to 6.0 with HCl (6.0 mol/L). The broth was autoclaved in individual Erlenmeyer flasks at 115 °C for 30 min and cooled before incubation.

Microbial transformation Special strains kept at 4 °C were transferred to respective solid cultures and incubated at 28 °C for 7 d to obtain well-grown mycelium with spores. In 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 °C for 24 h on a rotary shaker operating at 220 rpm to receive seed culture. 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, etofesalamide dissolved in acetone was added to yield a final concentration of 0.25 g/L and incubated for an additional 48 h. After microbial transformation, the flask contents were centrifuged at 1 500×g for 20 min, and 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 fungi control that was used to define and exclude the indigenous secondary metabolites generated by the fungi. The other was a blank substrate control (ie the culture with the etofesalamide but without the fungi) that was used to test whether etofesalamide would be chemically decomposed or spontaneously transformed under broth and microbial transformation conditions.

Enzymatic hydrolysis A 1.0 mL portion of each sample transformed by fungi was screened and incubated with β-*D*-glucosidase (100 kU/L) at 37 °C for 24 h after adjusting the pH to 5.0 using 50 mmol/L $NH_4H_2PO_4$ buffer (pH 3.0). Control experiments were carried out concurrently in the absence of α-*D*-glucosidase. Incubations were stopped by using solid-phase extraction (SPE) and analyzed using the liquid chromatography coupled to multistage mass spectrometry (LC/MS^n) method.

Extraction of metabolites and LC/MS^n assay After thawing, a 1.0 mL portion of each sample was applied to a Bond Elute C_{18} SPE column (Fuji Company, Tianjin, China) preconditioned with 2 mL methanol and 2 mL water. After loading the sample, the column was washed with 1 mL water and eluted with 2 mL methanol. The sample was evaporated to dryness at 35 °C under a gentle stream of nitrogen, and the residue was reconstituted with the addition of 100 μL of the mobile phase. A 20 μL aliquot of the solution was injected into the LC/MS^n system.

LC/MS^n analysis was carried out using a Finnigan LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source system, a Shimadzu LC-10AD pump (Kyoto, Japan) and a data system (Version 1.2, Finnigan). The interface was adjusted to the following conditions: ion mode, positive; spray voltage, 4.5 kV; capillary temperature, 200 °C; sheath gas (nitrogen), 0.75 L/min; auxiliary gas (nitrogen), 0.15 L/min. MS/MS spectra were obtained by collision-induced dissociation (CID) using helium (He) as the collision gas. The HPLC conditions were as follows: the column was a Diamonsil C_{18} column (particle size 5 μm, 4.6 mm × 200 mm ID, Dikma Company, Beijing, China). The mobile phase consisted of methanol-water (80:20, v/v) at a flow rate of 0.5 mL/min.

Results

Fungal metabolite profile of etofesalamide *C blacksleana* AS 3.153 supported significant metabolism and was chosen to illustrate the identification of metabolites. The microbial transformation and control samples were extracted and analyzed as described above. The chromatograms of the blank fungi controls showed no metabolites or etofesalamide and the blank substrate controls revealed only the presence of etofesalamide and no metabolites of etofesalamide. Authentic etofesalamide could generate a pseudomolecular ion $[M+H]^+$ at *m/z* 258. Compared with the standards, two $[M+H]^+$ ions correlated with the metabolism of etofesalamide were observed in the total ion current (TIC) of the sample transformed by *C blacksleana* AS 3.153, including ions at *m/z* 420 (M1) and *m/z* 390 (M2) (Figure 1). The LC/MS^n data of etofesalamide and its two metabolites are shown in Table 1.

Table 1. Multistage mass spectrometry characteristics of metabolites and etofesalamide.

Metabolite and substrate	$[M+H]^+$ (*m/z*)	MS^2 fragment (*m/z*)	MS^3 fragment (*m/z*)	t_R (min)
M1	420	258	121, 138	9.59
M2	390	258	121, 138	11.20
Etofesalamide	258	121, 138	–	20.25

t_R, retention time.

The pseudomolecular ion of M1 was at *m/z* 420, 162 higher than that of etofesalamide, which suggests that the corresponding metabolite might be a glucoside conjugate. The MS/MS spectrum of the ion at *m/z* 420 yielded a single ion at *m/z* 258 similar to the $[M + H]^+$ ion of etofesalamide. In addition, the MS^3 spectrum of the M1 ion at *m/z* 258 yielded the same fragment ions as the MS/MS spectrum of etofesalamide, indicating that M1 could be the glucoside conjugate of etofesalamide.

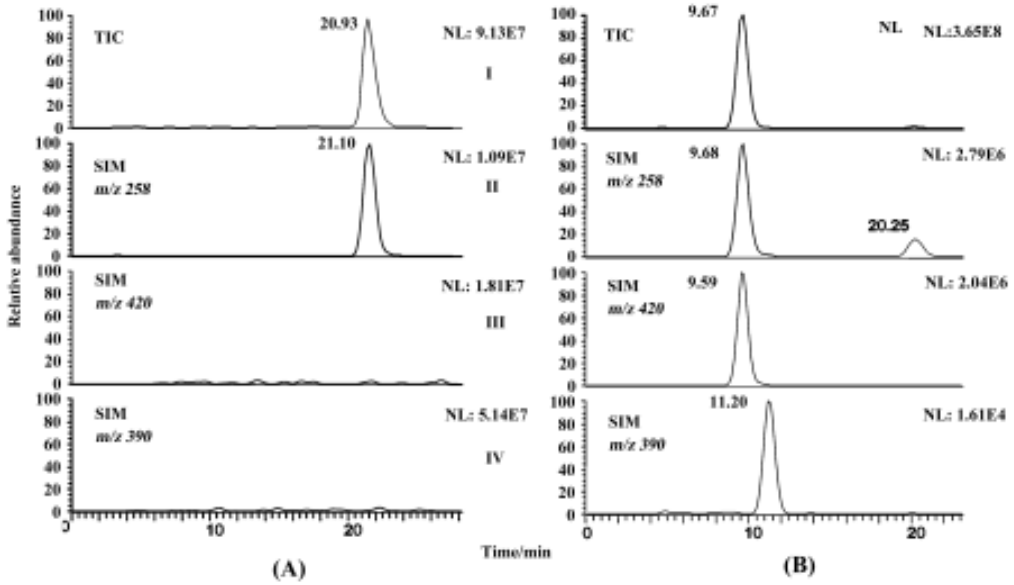

Figure 1. Chromatograms of total ion current (TIC, I) and selected ion monitoring (SIM, II, III, IV) scan modes in the LC/MS assay of authentic etofesalamide (A) and transformed etofesalamide (B).

According to the process described above, M2 could be a riboside conjugate of etofesalamide. Proposed metabolic pathways of etofesalamide in filamentous fungi are shown in Figure 2.

Enzymatic hydrolysis To further investigate the structure of M1, the sample of etofesalamide transformed by *C blacksleana* AS 3.153 was subjected to enzymatic hydrolysis. The results are expressed as the percentage of metabolite that was hydrolyzed with β-*D*-glucosidase compared to the control, which was hydrolyzed spontaneously. Treatment with β-*D*-glucosidase led to a greater than 50% reduction in M1. This result provides further evidence that M1 is the metabolite conjugated with glucoside because β-*D*-glucosidase is substrate selective.

Microbial transformation of etofesalamide by the screened fungi Table 2 shows the percentages of etofesalamide metabolized by different strains of fungi. In the seven fungi screened, *Aspergillus niger* and *Penicillium* had no ability to transform etofesalamide; four strains of *Cunninghamella spp* resulted in almost complete meta-bolism, particularly the *C blacksleana* AS 3.153 strain. *Mucor circinelloides* AS 3.3421 had a similar ability to *Cunninghamella spp* to transform etofesalamide. Etofesalamide was converted into two phase II metabolites by these fungi, glucoside and riboside conjugates, in which glucoside conjugate was the major product with the highest yield (94.5%). In addition, no phase I metabolites were detected, and *C blacksleana* AS 3.153 supported significant metabolism, which may be useful in the study of phase II metabolism of etofesalamide *in vitro*.

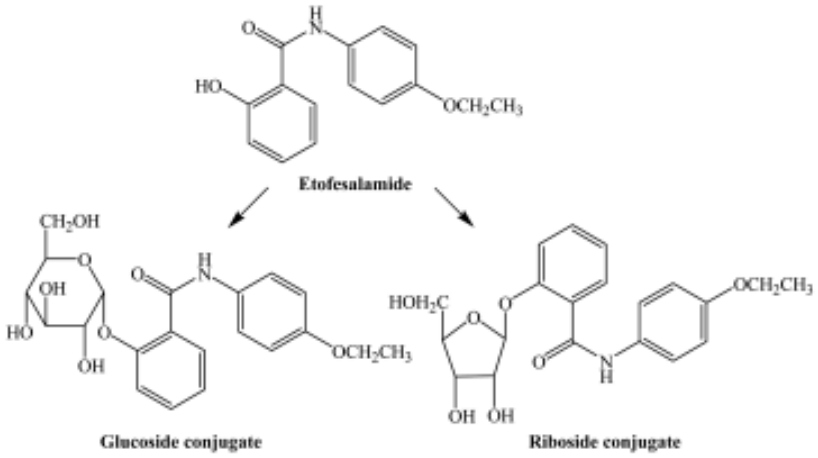

Figure 2. Proposed metabolic pathways of etofesalamide in filamentous fungi.

Discussion

In this study, seven filamentous fungi were screened for their abilities to metabolize etofesalamide. Four strains of *Cunninghamella* species showed a strong ability to convert etofesalamide into the phase II metabolites, glucoside and riboside

Table 2. Yields of transformation products and remaining etofesalamide by different fungal strains.

Strain	Total phase I metabolites* (%)	Glucoside* (%)	Riboside* (%)	Remaining etofesalamide (%)
C. blacksleana AS 3.153	0	94.5	1.5	4.0
C. echinulata AS 3.2004	0	90.8	2.1	7.1
C. elegans AS 3.2028	0	74.6	7.9	17.5
C. elegans AS 3.156	0	93.2	0	6.8
Mucor circinelloides AS 3.3421	0	38.8	9.2	52.0
Aspergillus niger	0	0	0	100
Penicillium	0	0	0	100

*The percentage of one metabolite compared to the total percentage of metabolites and remaining substrates. n=3.

conjugates. No phase I metabolites were observed. The high potential of certain fungal strains to produce phase II metabolites was illustrated by these results.

Our results show that a number of filamentous fungi transform etofesalamide by phase II reactions to excretable sugar conjugates. Glucoside and riboside conjugations differ from the phase II metabolism observed in mammals. In mammalian species, such as rabbits, glucuronide conjugation rather than glucoside conjugation is the major metabolic pathway for etofesalamide[2,3]. Conjugations with glucuronic acid are considered to be a dominant metabolic pathway in humans for detoxifying and eliminating lipophilic chemicals from the body[8]. In comparison with glucuronide conjugation, there is only limited information in the literature regarding the glucoside conjugation process in humans. At present, apart from N-linked glucoside drug metabolites, only two *O*-linked glucosides of mycophenolic acid and morphine have been identified in humans[9,10]. In addition, glucuronide conjugation, which is a characteristic conjugation in humans, is rarely found in microorganisms, and only two examples of glucuronide conjugation have been reported[11]. Compared with the phase II reactions in humans, glucoside conjugation is the major metabolic pathway in microorganisms, and it is used extensively to study the detoxification of cytotoxins and carcinogens[12-14]. Riboside conjugation is obviously another potential phase II reaction in biotransformation in filamentous fungi, but only one such conjugate has been reported recently[15].

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