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Novel metabolites from *Cunninghamella elegans* as a microbial model of the β-blocker carvedilol biotransformation in the environment

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ABSTRACT

Beta-adrenergic blocking agents like carvedilol are widely detected environmental pollutants. Its human metabolism is well known, while the fate of the carvedilol when metabolized by environmental strains is unknown. The aim of this study was to investigate the mechanism and pathways of carvedilol metabolism by the common environmental fungal strain *Cunninghamella elegans*. In this report the process of carvedilol biotransformation by *C. elegans* was described for the first time. A total of ten carvedilol derivatives were identified in *C. elegans* cultures. Similarly to mammalian metabolism of carvedilol, its fungal biotransformation proceeded through hydroxylation and conjugation reactions. However, in *C. elegans* cultures new products such as methyl-phenyl carvedilol and glucose-desmethyl carvedilol were identified, which had not been previously detected in human and animals. Moreover, an involvement of cytochrome P450 and cytochrome P450 reductase in carvedilol fungal metabolism was revealed.

1. Introduction

Beta-adrenergic receptors blocking agents (β-blockers) are one of the main classes of pharmaceuticals used in the treatment of hypertension, which affects about a billion people in the world (DiNicolantonio et al., 2015). Besides typical β_1 selective antagonists such as atenolol, metoprolol, bisprolol or nebivolol, carvedilol (CAR) deserves particular attention as a non-selective blocker. Carvedilol [(±)-1-[carbazolyl-4-oxy]-3-[(2-methoxy phenoxyethyl) amino]-2-propanol] is a new generation of β_1, β_2 and α_1 adrenergic receptors antagonists, which also presents antioxidative and M_2 muscarinic receptors regulating activities (DiNicolantonio et al., 2015; Chander et al., 2013). In recent years hepatic metabolism of carvedilol and its partial elimination by the renal route in mammals has been extensively investigated (Schaefer et al., 1998). Literature data indicate that cytochrome P450 (CYP450) is involved in the metabolism of carvedilol in human liver microsomes where the β-blocker is modified via oxidation and conjugation reactions (Horiuchi et al., 2010).

Pharmaceuticals and their human metabolites in the environment are under intensive scrutiny. Although drugs are designed to act on specific receptors or enzymes in humans, they may also interfere with metabolic functions of other vertebrates and invertebrates. Despite very low concentrations of pharmaceuticals in water, they can cause long-term effects on aquatic organisms because of their constant release, which makes drugs pseudo-persistent (Rand-Weaver et al., 2013; Olvera-Vargas et al., 2016). β-blockers are among the most frequently detected pharmaceuticals in surface water. Their way of action toward aquatic organisms has not been precisely established. Nevertheless, evidence for the presence of beta-adrenergic receptors in fish tissues has been demonstrated. Moreover, protein components of β-receptors have been found in fruit fly, yeast, molds and frogs. Stimulation of protozoans by isoproterenol suggests the presence of β-receptors also in these organisms (Huggett et al., 2002).

Environmental concentrations of pharmaceuticals depend on the extent of their release to ecosystems. Their persistence depends on physicochemical conditions such as pH, temperature or light and biotic agents including microorganisms. Bacteria and fungi in ecosystems use organic substances as sources of carbon, nitrogen and energy. The biodegradation activity of natural microbial communities plays a key role in mitigating the pollution, that is why an increasing interest in the understanding of the metabolic pathways of xenobiotic degradation has been observed in recent years (Caracciolo et al., 2015; Olvera-Vargas et al., 2016). Products of human as well as microbial drugs metabolism might be more active or more toxic than parent compounds. Moreover, a mixture of different drugs intermediates may exhibit a higher toxicity compared with each metabolite alone. *Cunninghamella* fungi are

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widespread in the environment. Their ability to transform pharmaceuticals by metabolic routes similar to those of mammalian drug metabolism can be used to evaluate the fate and impact of both parent compounds and of their metabolites (Olvera-Vargas et al., 2016). Furthermore, determination of the microbial metabolic pathways and enzymes involved in biodegradation of drugs can be used to develop new methods for their elimination like nanotube releasing the microbial enzymes which can dispose of organic pollutants (Chen et al., 2016).

Mammalian metabolism of carvedilol has been well documented, whereas its microbial bioconversion has not been studied in detail. Ettireddy et al. (2017) studied the ability of *Aspergillus niger*, *Escherichia coli*, *Streptomyces halstedii*, *Pseudomonas putida*, *Cunninghamella elegans* and *Sphingomonas paucimobilis* for the enantioselective resolution of racemic carvedilol. An enantioselective conversion of racemic carvedilol was noted during 10-day incubation of microorganisms and after that a decrease in the amount of the β-blocker with no further enantioselective resolution was observed. These results may suggest the ability of tested microorganisms to degrade carvedilol. Most of the tested biocatalysts showed poor efficiency in enantioselective resolution of racemic carvedilol to (S)-(-)-carvedilol, but only *A. niger* was found to possess high enantioselectivity (Ettireddy et al., 2017). On the other hand, the high potential of *Cunninghamella echinulata* to eliminate and detoxify carvedilol was shown in our previous study. Three carvedilol metabolites were identified in the *C. echinulata* cultures (Zawadzka et al., 2017). In the present study, metabolism of carvedilol by *C. elegans* was investigated in detail for the first time. The β-blocker intermediates were studied using LC-MS/MS techniques and compared with metabolites formed in mammals. Ten metabolites of carvedilol were detected in *Cunninghamella elegans* IM 1785/21Gp cultures. The fungal metabolism of carvedilol is more complex than previously suggested. Moreover, this is the first report about the involvement of cytochrome P450 and cytochrome P450 reductase in fungal degradation of carvedilol.

2. Materials and methods

2.1. Chemicals

Carvedilol, proadifen, 1-aminobenzotriazole, metyrapone, anhydrous magnesium sulfate, sodium chloride, trisodium citrate dihydrate and disodium hydrogencitrate sesquihydrate were purchased from Sigma-Aldrich (Poland). The other reagents with a high analytical purity grade were obtained from Sigma-Aldrich (USA) and POCH (Poland). All PCR reagents came from Applied Biosystems (USA).

2.2. Cultivation of C. elegans

C. elegans IM 1785/21Gp was sourced from the microbial collection of the Department of Industrial Microbiology and Biotechnology, University of Lodz (Poland). Spores of *C. elegans* from 10-day-old cultures on ZT slants according to Długoński et al. (1984) were used for the preparation of fungal inoculum in Sabouraud medium (Difco, USA) and cultivated on a rotary shaker at 180 rpm at 28 °C. Cultures for the evaluation of carvedilol degradation and its elimination in the presence of chosen cytochrome inhibitors, and the expression of cytochrome P450 and cytochrome P450 reductase genes were prepared in modified Czapek-Dox medium (40 g L^{-1} $C_6H_{12}O_6$; 3 g L^{-1} $NaNO_3$; 1 g L^{-1} KH_2PO_4; 0.5 g L^{-1} KCl; 0.5 g L^{-1} $MgSO_4 \times 7$ H_2O; 0.01 g L^{-1} $FeSO_4 \times 7$ H_2O). Stock solutions of carvedilol were prepared in sterile DMSO. 4 mL of *C. elegans* preculture was introduced into 16 mL pure medium (control samples) or medium with the addition of carvedilol (5; 20 mg L^{-1}). Abiotic controls were performed without the addition of fungal inoculum. The cultures used for the evaluation of carvedilol metabolism in the presence of CYP450 inhibitors were additionally supplemented with proadifen, metyrapone or 1-aminobenzotriazole (ABT) at the concentrations 1.5, 2 and 1 mM, respectively. CYP450 inhibitors stocks were prepared in ethanol. The cultures were prepared in triplicate and incubated for 4 days at 28 °C on a rotary shaker (140 rpm).

2.3. Sample preparation for determining the growth of microorganism

The mycelium of *C. elegans* was filtered, washed twice with distilled water and dried at 95 °C to achieve a constant weight.

2.4. Sample preparation for carvedilol quantitation and quantification

C. elegans cultures were disintegrated using a Mixer Mill MM400 (Retsch, Germany) after incubation. In the next step, 10 mL of the homogenates was shaken for 3 min on a vortex mixer with the same volume of ACN. Then, anhydrous magnesium sulfate (2 g), sodium chloride (0.5 g), trisodium citrate dihydrate (0.5 g) and disodium hydrogencitrate sesquihydrate (0.25 g) were added to each sample and vortexed again for 3 min according to the modified QuEChERS protocol. Extraction efficiency was 85 ± 3.1%. The samples were frozen in Eppendorf tubes for 24 h at −70 °C, then they were thawed on ice and centrifuged at 4 °C for 20 min at 10000 × *g*. A solution of water:ACN (98:2; v/v) was used for dilution of samples to prepare the calibration curve within the working range of 0.1–25 ng/mL (r = 0.995). The limits of detection (LOD) and quantification (LOQ) of carvedilol were 0.0178 and 0.0592 ng/mL, respectively.

2.5. LC-MS/MS methods

All LC–MS/MS analyses of carvedilol biotransformation by *C. elegans* were carried out using an AB Sciex 4500 QTRAP mass spectrometer (AB Sciex, USA) and an Eksigent microLC 200 System (Eksigent, USA). An Eksigent C18 (0.5 mm × 50 mm × 3 μm, 120 Å) column at 50 °C was used for the chromatographic separation of carvedilol and its fungal metabolites. Water (A) and acetonitrile (B) with 0.1% formic acid were used as eluents in quantitative and qualitative LC-MS/MS methods.

2.5.1. Quantitative analysis of carvedilol elimination

The quantitative analysis was conducted with a constant flow rate of the mobile phases at 50 μL/min. The gradient of eluent A was started at 98% and maintained for 0.1 min, then its content decreased from 98% to 2% during 1.1 min, was maintained for 0.9 min and returned to the initial conditions in 2.1 min and was maintained for 0.4 min to stabilize the chromatographic column. Each sample was injected at a volume of 5 μL. Carvedilol was detected based on the 407.1/222.1 and 407.1/100.1 ions as a quantifier and a qualifier, respectively, in the multiple-reaction monitoring mode (MRM). The analyses were carried out using the microESI ion source in the positive ionization mode with the parameters IS: 5000 V, CUR: 25, GS1: 20, GS2: 30, and TEM: 300 °C. The declustering potential (DC), entrance potential (EP) and collision energy (CE) were set to 91 V, 10 V and 35 V, respectively.

2.5.2. Qualitative analysis of carvedilol metabolites formed in C. elegans cultures

Two methods of qualitative analysis of carvedilol metabolites produced by *C. elegans* were developed. The first was based on predicted multiple reaction monitoring (pMRM) prepared using LightSight™ software. The equipment, the chromatographic column, the injection volume and the mobile phases were the same as in the quantitative analysis of the β-blocker. The gradient of eluent A with a constant flow 15 μL/min was started at 98% for 0.2 min; then its content decreased from 98% to 2% for 7.8 min and was then maintained unchanged to 9 min. The gradient of eluent A was subsequently returned to the initial conditions in 9.1 min and maintained for 0.4 min. The microESI ion source parameters were optimized to CUR: 25, IS: 5000 V, TEM: 300 °C, GS1: 30, GS2: 20. Two variants of collision energy settings were used

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