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Identification of the urinary metabolites of glionitrin A in rats using ultra-performance liquid chromatography combined with quadrupole time-of-flight mass spectrometry

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ABSTRACT

Glionitrin A (GN A) is a new diketopiperazine disulfide with an aromatic nitro group, which is isolated from the coculture of an *Aspergillus fumigatus* fungal strain and a Sphingomonas bacterial strain. After intravenous administration of GN A in rats, 13 urinary metabolites of GN A were identified using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectroscopy (UPLC–QTOP-MS) analysis in conjunction with data processing programs such as MetaboLynx™ and MassFragnent™. Reduction, nitro-reduction and hydration were the primary metabolic processes affecting GN A *in vivo*, followed by demethylation or oxidative deamination to alcohol, as well as cysteine, glycine, glucuronide or sulfate conjugation. The metabolite resulting from reduction was found to be a molecule with a dithiol group, and the metabolite made by nitro reduction was found to be an aromatic amine corresponding to GN A. Both of these products may have pharmacological or toxicological activity, which is valuable information in terms of using GN A as a lead compound. In addition, this work showed that UPLC–QTOP-MS analysis coupled with efficient data processing programs is useful for rapid and reliable characterization of GN A metabolites *in vivo*.

1. Introduction

Glionitrin A (GN A) (Fig. 1) is a new diketopiperazine disulfide isolated from the coculture of the *Aspergillus fumigatus* fungal strain KMC-901 and the Sphingomonas bacterial strain KMK-001 [1]. Its structure is similar to gliotoxin and dehydrogliotoxin, but it is unique in that it has a nitro aromatic ring. It showed promising antimicrobial activities against several microbes, including methicillin-resistant *Staphylococcus aureus*. It also displayed significant cytotoxic capacities toward some human cancer cell lines in an *in vitro* MTT cytotoxicity assay [1]. These features suggest that glionitrin A might be a potential drug candidate.

The identification of the metabolites of potential drug candidates provides essential information on drug efficacy and the toxicological profile and also provides information that may result in the generation of new, improved drug structures. Lately, metabolic studies performed in the early stage of drug discovery have been applied to judge whether new molecules are worth further development [2]. Liquid chromatography/mass spectrometry (LC/MS) is highly useful for this application. In particular, the use of a high-resolution MS system with excellent accuracy and stability coupled with a sophisticated data processing program has guaranteed the quality and productivity of metabolite acquisition processes [3]. Additionally, ultra-performance liquid chromatography (UPLC) provides improvement in separation and resolution in a shorter time compared with conventional high-performance liquid chromatography (HPLC) [4].

In the present study, the potential metabolites of GN A *in vivo* were determined through an analytical process based on UPLC–QTOF-MS, followed by a data search with a well-designed, automated data analysis program. This approach efficiently provided high-quality structural information on GN A metabolites excreted in the urine after intravenous administration, even though the biotransformed molecules occurred at trace levels concomitant with an excess of endogenous compounds.

2. Materials and methods

2.1. Chemicals and reagents

GN A was isolated from the coculture of an *Aspergillus fumigatus* fungal strain and a Sphingomonas bacterial strain. The crude

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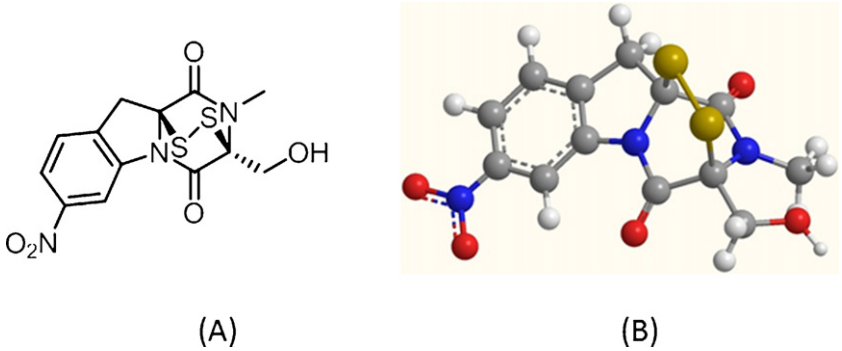

Fig. 1. Structure of GN A: (A) 2D structure and (B) 3D structure.

extract was fractionated by reverse-phase HPLC using gradient elution, and then GN A was purified by normal-phase HPLC followed by crystallization, which was precisely described in the previous report [1]. GN A crystals was dissolved in methanol at a concentration of 1 μg/ml and its purity was checked on chromatogram by UPLC–QTOF-MS analysis. Gliotoxin and acetonitrile (HPLC grade) and formic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Saline solution was purchase form Dai Han Pharm (Seoul, Korea). Ultrapure water (18.2 MΩ) was obtained using a Milli-Q apparatus from Millipore (Milford, USA).

2.2. Stability of GN A in saline and urine

Stabilities of GN A in saline and urine were tested according to the methods precisely explained in supplement materials. Here is brief description as follows: For urine stability test, 100 μl of quality control (QC) samples were prepared at lower (0.01 μg/ml), low (0.1 μg/ml), medium (1 μg/ml) and high (10 μg/ml) of GN A by spiking an appropriate amount in urine. Five replicates of each QC samples were evaluated in every condition of stability tests. To test short-term stability, QC samples were stored at room temperature for 0, 1, 2, 4 and 8 h as well as at 4 °C for 24 h. To evaluate the long-term stability, QC samples were stored at −80 °C for 2 and 4 weeks. The stability of GN A through the freeze (−80 °C) – thaw (room temperature) cycles were tested with QC samples that underwent the freeze and thawing process three times. For evaluation of stability in IV dosing solution, 100 μl of test sample was prepared at the concentration of 1 μg/ml of GN A by adding in saline. Five replicates were stored at room temperature for 0, 15, 30, 60 and 120 min. Each stability test was stopped by adding 200 μl of methanol containing gliotoxin (250 ng/ml; internal standard). Test samples were analyzed using LC–MS/MS and the percentages of GN A remaining at each time point relative to sample at the starting point were calculated. A compound was considered stable if the percentages at each time point were within ±15% of the sample at starting point.

2.3. Animal experiments

Eight-week-old male Sprague–Dawley rats (weighing 270–300 g) were purchased from Orientbio Korea (Seoul, Republic of Korea). The rats were housed in a room with an ambient temperature of 23 ± 2 °C, 12-h light/dark cycles, and a relative humidity of 55 ± 10% for 7 days. Rats were fasted for 12 h before drug injection and for a further 4 h after dosing. Water was available *ad libitum* during the experiments. For the drug injection, the jugular vein of each rat was cannulated using polyethylene tubing (PE50) under anesthesia with a 2:1 mixture of Zoletil® and Rompun®. Rats were administered a single intravenous (IV) dose of GN A dissolved in saline (10 mg/kg). Blank urine samples were collected before drug administration over 8 h and urine samples were collected for 8 h after IV injection. All urine samples were stored at −80 °C until analyzed. The animal experimental protocol was approved by the institutional animal care and use committee of Korea Institute of Science and Technology.

2.4. Sample preparation

A 200 μL aliquot of each urine sample was centrifuged at 10,000 × *g* for 15 min at 4 °C to remove particulates and was then diluted with the same volume of water. 200 μL of diluted urine was transferred to an autosampler vial. Five microliters of the prepared urine sample were injected into the UPLC-QTOF-MS system for analysis.

2.5. UPLC/ESI-QTOF-MS analysis

UPLC–QTOF-MS was used to identify GN A metabolites in rat urine. An ACQUIRY UPLC® (Waters, Milford, MA, USA) was directly connected to a QTOF-MS (SYNAPT™ G2, Waters, Milford, MA, USA). Separation was achieved using an Acquity UPLC BEH C18 column (1.7 μm particle size, 2.1 mm inner diameter, 100 mm length; Waters) at 40 °C. The gradient elution was performed using a mixture of solvent A (0.1% formic acid in 1% acetonitrile) and solvent B (0.1% formic acid in 99% acetonitrile) at a flow rate of 0.4 ml/min. The starting conditions were 100% A for 1 min, changing to 0% A at 17 min, and the solvent composition was then held at 100% B for 1 min. Re-equilibration of the system with 100% A (v/v) for 2 min was conducted prior to the next injection. All samples were kept at 4 °C during the analysis.

Mass spectrometry was performed in negative ionization mode with an electrospray ionization source (ESI) interface. The capillary voltage was set to 2500 V, and the cone voltage was 40 V. Nitrogen was used as the desolvation and cone gas at a flow rate of 600 L/h and 60 L/h, respectively. The source temperature was 120 °C, and the dissolution temperature was 350 °C. Leucine-enkephalin (0.2 μg/L in 50% methanol) was utilized as the lock mass (mass-to-charge ratio (*m*/*z*) 554.2615) at a flow rate 20 μL/min. Full scan data were collected at a range of *m*/*z* 50–1200 over a period of 15 min with a scan time of 0.5 s and an interscan delay of the 0.1 s. *m*/*z* values in resolution mode, and all of the acquired spectra were automatically corrected during acquisition based on the lock mass. The mass spectrometric data were collected into two separate data channels, using collision energy alternating between 0 (low-energy scans) and 30 eV (high-energy scans) in the centroid mode. Before analysis, the mass spectrometer was calibrated with 0.2 mM sodium formate solution.