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Cytotoxic Effect of Ergot Alkaloids in *Achnatherum inebrians* Infected by the *Neotyphodium gansuense* Endophyte

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Supporting Information

ABSTRACT: Ergonovine or ergonovinine was isolated from the aerial parts of endophyte (*Neotyphodium gansuense*) infected (E+) drunken horse grass (*Achnatherum inebrians*), neither of which existed in endophyte-free (E−) plants. Both of these ergot alkaloids had a cytotoxic effect on animal smooth muscle cells and increased cell growth inhibition with greater concentrations, in a significantly ($P < 0.05$) positive correlation. The median inhibitory concentrations (IC_{50}) for ergonovine and ergonovinine were 71.95 and 72.75 μg/mL, respectively. These results indicate that endophytic ergot alkaloids may be the cause of drunken horse grass poisoning.

KEYWORDS: *Neotyphodium gansuense, Achnatherum inebrians, ergot alkaloid, concentration, cytotoxicity, poisoning*

INTRODUCTION

Endophytic fungi that belong to the related genera *Epichloë* and *Neotyphodium* have been found in many cool-season grasses.[1,2] Published studies have focused mainly on the endophytes of *Lolium* and *Festuca* and are associated with increased host resistance to biotic[3−5] and abiotic stresses.[6,7]

Achnatherum inebrians (Hance) Keng (drunken horse grass) is a toxic perennial bunchgrass, which is so-named because it is associated with the narcosis of livestock that graze on native grasslands in northwestern China,[8] especially when forage is in short supply during the winter and spring. *A. inebrians* is distributed mainly throughout the harsh conditions of alpine or subalpine grasslands within Gansu, Xinjiang Uyghur Autonomous Region, Qinghai and Ningxia Hui Autonomous Regions as well as Inner Mongolia and Tibet.[9] This species is usually infected by the fungal endophyte *Neotyphodium gansuense*,[9,10] which apparently provides drunken horse grass with a strong competitive advantage by increasing its tolerance to drought,[5,11,12] salt,[12] cold,[13] heavy metals,[14,15] pests[5,16,17] and pathogenic fungi.[5,10]

Neotyphodium gansuense-infected (E+) drunken horse grass has been shown[18,19] to contain high levels of the ergot alkaloids ergonovine and ergine (i.e., lysergic acid amide), compared with endophyte-free (E−) specimens, and these compounds are probably the main cause of the aforementioned livestock narcosis.[20] Recently, it has been reported that mowing height of these plants, as well as cutting frequency, can influence the concentration of ergot alkaloids.[21] It was also found that salt and drought stresses can also influence levels of these alkaloids.[22]

One aim of the present study was to describe the isolation and structural elucidation of the main ergot alkaloids of E+ drunken horse grass. To further probe the poisoning mechanism of *A. inebrians* by the endophyte, an experiment was carried out to evaluate the cytotoxicity of these ergot alkaloids on animal smooth muscle cells.

MATERIALS AND METHODS

General. NMR spectra were recorded on Bruker AM-400 and Varian Mercury-600 BB NMR (600 MHz) spectrometers using TMS as an internal standard in $CDCl_3$. EIMS and FAB-MS were measured on an HP5988a GC-MS and a VG-ZAB-HS at 70 eV. Column chromatography was performed on 200−300 mesh silica gel (Qingdao Marine Chemical Inc., Qingdao, China), 75−150 μm CHP 20P MCI gel (Mitsubishi Chemical Corp., Tokyo, Japan), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Fractions were monitored by TLC and were visualized by heating the silica gel plates after being sprayed with 5% H_2SO_4 in EtOH.

Plant Materials. The aerial parts of *A. inebrians* were collected from the field in August 2008. The respective E+ and E− *A. inebrians* fields were established in May 2007 at the Yuzhong Campus (YZ) (104°09′ E, 35°89′ N; elevation = 1653 m) of Lanzhou University, China. Twenty-four plots (2 treatments × 12 replicates) were randomly built. The area of the plot was 24 m^2 (4 × 6) with 8 lines of 13 listed (40 cm apart), consisting of 104 plants of each plot.

Extraction and Isolation of Ergot Alkaloid. The air-dried leaves and stems of *A. inebrians* (17.4 kg) were ground to pass though a 0.5 mm sieve and then extracted three times (each for 7 days) with 95% ethanol at room temperature. The resulting 3.75 kg of concentrated crude extract (fraction 1) was first acidified (pH 2) with hydrochloric acid (fraction 2), and the insoluble deposit was removed. Subsequently, the solution was alkalinized (pH 11) with sodium hydroxide (fraction 3) and extracted into chloroform (fraction 4) and then *n*-butyl alcohol (fraction 5). The extracted residue was marked as fraction 6. Fraction 4 (34.4 g) contained the alkaloids detected by

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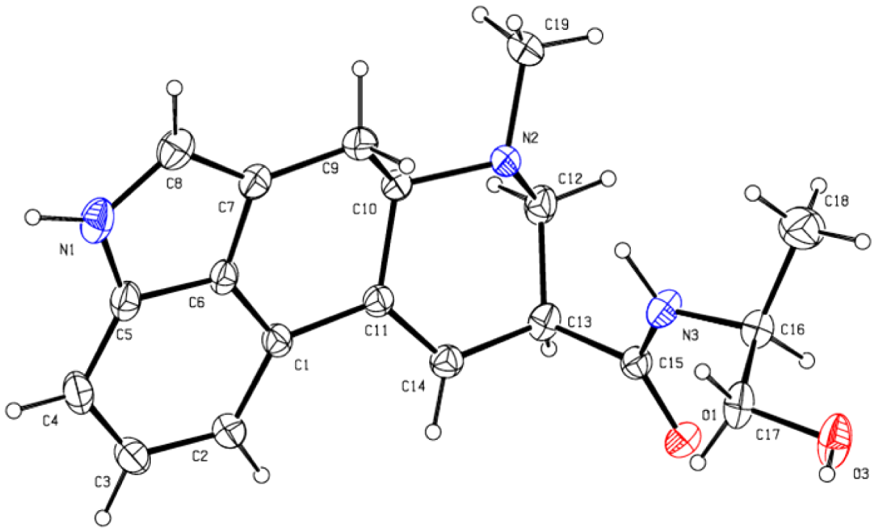

Figure 1. Single-crystal X-ray diffraction analysis for ergonovinine.

TLC. The chloroform extract (fraction 4) was further purified by silica gel column chromatography (CC; ⌀ 46 × 630 mm, 200−300 mesh, 270 g) and eluted with a step gradient of chloroform/methanol (100:1, 50:1, 30:1, 20:1, 10:1, 5:1, 2:1, 1:1, 9.7 L), followed by repeated CC ($CHCl_3$/MeOH, 3:1) on silica gel, Sephadex LH-20, and preparative TLC. Compound **1** (ergonovinine, 23.2 mg) was produced by recrystallization and further Sephadex LH-20 CC ($CHCl_3$/MeOH, 1:1). Compound **2** (ergonovine, 17.8 mg) was fully purified and obtained by repeated CC on silica gel eluted with a step gradient of chloroform/methanol (20:1, 10:1).

Cytotoxicity Assay of the Ergot Alkaloids. The 96-well nanoculture plates with a microsquare pattern [NCP-L-MS (96)] were provided by NUNC Corp. (Roskilde, Denmark).

Vascular smooth muscle cells (VSMC) from cattle (*Bos taurus*) were purchased from the Shanghai cell bank of the Chinese Academy of Sciences and maintained at 35 °C under an atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, VA, USA) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin.

The cytotoxicity of the ergot alkaloids was determined by measuring the cell viability of cultured cells after exposure to different concentrations of extract from the grass. The cytotoxicity of ergot alkaloids was assessed according to the methyl thiazolyl tetrazolium assay (MTT) method.[23] To initiate cell culture, VSMC cells were seeded at a density of 1×10^4 cells/well on a conventional monolayer 96-well plate for expanding propagation. Each of the two ergot alkaloids (1 mg) was dissolved in 0.1% dimethyl sulfoxide (DMSO) (10 μL) and then diluted to concentrations of 125, 100, 75, 50, 12.5, 6.25, and 3.175 μg/mL with the medium and filtered using 0.22 μm syringe filters. These were then added to 24 h cultured cells in 96-well tissue culture plates. Logarithmic phase VSMC cells with a density of 1×10^5 cells/well were inoculated into 96-well plates for 18 h, and then the various concentrations of ergot alkaloids were added as the treatments. Media with 0.1% DMSO in DMEM was used as the blank group, and the cell cultures without added ergot served as the control, each treatment being repeated eight times, independently. The cells were then exposed to different concentrations of the above alkaloids. The cells in the control wells received medium containing the same volume of DMSO (0.1%). Twenty-four hours after the incubation, 20 μL of MTT reagent (5 mg/mL in PBS) was added and cells were incubated for an additional 4 h. The formazan produced by the viable cells was solubilized by the addition of 100 μL of DMSO. The suspension was placed on a microvibrator for 15 min, and absorbance at 490 and 570 nm was quantitated after subsequent addition of MTT solution and DMSO and was also read using a Versamax + Multiwash III ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The experiment was performed three times for each alkaloid concentration treatment. The percentage of growth inhibition was calculated with respect to vehicle control using the following formula:

$$\text{inhibition rate } (\%) = (A_{\text{control}} - A_{\text{test}})/A_{\text{control}} - A_{\text{blank}} \times 100\%$$

Statistical Analysis. All values are expressed as the mean ± SE. Analysis of variance (ANOVA) using SPSS software (SPSS 13.0 Inc., Chicago, IL, USA) was conducted for the cell inhibition rate that resulted from various concentrations of the two ergot alkaloids. Analysis of regression was also carried out between the cell inhibition rate and concentrations of the ergot alkaloids.

RESULTS AND DISCUSSION

Isolation and Identification of the Alkaloids from E+ Plant Compounds. Single-crystal X-ray diffraction for ergonovinine is shown in Figure 1, and ^1H and ^{13}C NMR spectral data for ergonovine and ergonovinine are shown in the Supporting Information. Two ergot alkaloids, ergonovinine and ergonovine, were identified (Figure 2).

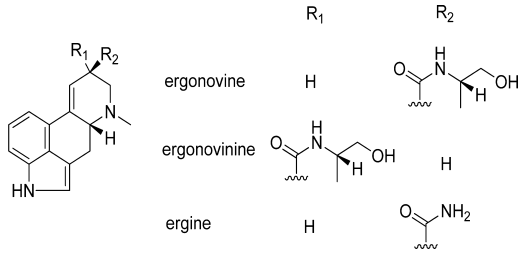

Figure 2. Structures of ergonovinine, ergonovine, and ergine and their C-8 epimers.

Ergonovine (25 mg from 2 kg) and ergonovinine (30 mg from 7 kg) were first isolated from dry powdered drunken horse grass, but without studying the biological activity.[24] Ergonovine and ergine (i.e., lysergic acid amide) were the major ergot alkaloids in drunken horse grass from Xinjiang province.[18] Previous research also investigated the ergonovine and ergine levels and their temporal variation within E+ and E− drunken horse grass grown in Gansu province.[19] Furthermore, seven alkaloids were also detected from drunken horse grass growing naturally near the Jinqiang River, Tianzhu county, Gansu province.[25] Ergine was reported as the main alkaloid, but we did not isolate it during this experiment because of its instability

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under high temperature. Ren[26] first reported that animals produced symptoms of intoxication after grazing on drunken horse grass. However, sheep were not significantly intoxicated[27,28] when gavaged with drunken horse grass powder, contrary to equines and rabbits (*Oryctolagus cuniculus*).[29] Research showed that the *Neotyphodium* endophyte was the cause of drunken horse grass toxicity in rabbits.[20] Recently, it was also reported that the *Neotyphodium* endophyte was apparently responsible for the toxicity of drunken horse grass on sheep (*Ovis aries*), which was especially harmful to kidney and liver function.[30]

Cytotoxicity of the Two Ergot Alkaloids. Each of the two ergot alkaloids exhibits cytotoxicity on animal smooth muscle cells, the cell inhibition rate increasing with alkaloid concentration in a significantly ($P < 0.05$) positive correlation (Figure 3). The regression equations for these two alkaloids

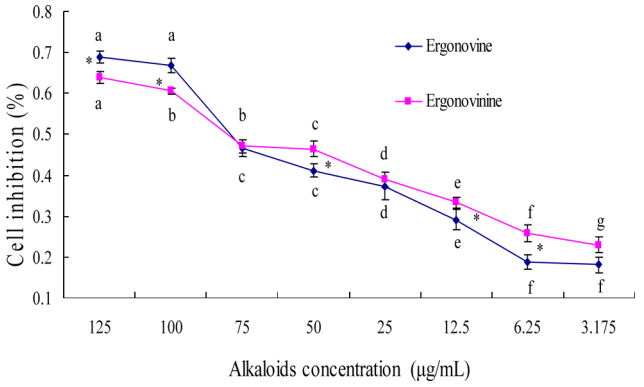

Figure 3. Cytotoxicity of the two ergot alkaloids on VSMC.

were $Y_{ergonovine} = 0.0041x + 0.205$ ($R^2 = 0.945$, $P < 0.05$) and $Y_{ergonovinine} = 0.0032x + 0.2672$ ($R^2 = 0.9411$, $P < 0.05$). The median inhibitory concentrations (IC_{50}) for ergonovine and ergonovinine were 71.95 and 72.75 μg/mL, respectively.

In conclusion, to the best of our knowledge, the present work represents the first study on the cytotoxic effect on animal smooth muscle cells of the pure ergot alkaloids isolated from E + drunken horse grass. These alkaloids may be the cause of E+ drunken horse grass poisoning and elicit its clinical symptoms. The absence of detection of these chemicals could indicate plants that are safe for grazing animals.

■ ASSOCIATED CONTENT

Supporting Information

Tables 1 and 2, ^{1}H NMR and ^{13}C NMR spectroscopy of ergonovine and ergonovinine. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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