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Metarhizin A suppresses cell proliferation by inhibiting cytochrome c oxidase activity

Yasuhiro Katou [a], Naoya Endo [b], Toshiyuki Suzuki [b], Jiang Yu [a], Haruhisa Kikuchi [a], Yoshiteru Oshima [a], Yoshimi Homma [b,*]

[a] Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8678, Japan
[b] Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan

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ABSTRACT

Aims: Metarhizin A was originally isolated from *Metarhizium flavoviride* as a potent inhibitor of the growth of insect and mammalian cells. In this study, we aimed to understand the molecular targets of metarhizin A involved in its anti-proliferative activity against human cells.
Main methods: Cell cycle regulators and signaling molecules were examined by immunoblotting using specific antibodies. A mitochondria-enriched fraction was prepared from mouse liver, and mitochondrial activity was monitored using an oxygen electrode. Enzyme activity was measured using purified cytochrome c oxidase and permeabilized cells.
Key findings: Metarhizin A inhibits the growth of MCF-7 cells with an IC_{50} value of ~0.2 μM and other cells in a similar manner; a cell cycle-dependent kinase inhibitor, p21, is selectively induced. Significant amounts of reactive oxygen species (ROS) are generated and ERK1/2 is activated in cells treated with metarhizin A. Metarhizin A completely suppresses oxygen consumption by mitochondria, and potently inhibits the activity of cytochrome c oxidase. It induces cell death when MCF-7 cells are cultured under limiting conditions.
Significance: Metarhizin A is a potent inhibitor of cytochrome c oxidase and activates the MAPK pathway through the generation of ROS, which induces growth arrest of cells, and, under some conditions, enhances cell death. The cytochrome c oxidase system is a possible molecular target of metarhizin A.

Introduction

Entomopathogenic fungi infect insects through the cuticle, grow as hyphal bodies, and cause host death by nutritional destruction of tissues and the production of toxic metabolites and pathogenic enzymes (Hajek and St Leger, 1994; Griesch and Vilcinskas, 1998). The fruiting bodies of some of these fungi have been extensively examined in attempts to develop novel bioactive compounds including insecticidal, antitubercular, and immunosuppressive agents (Muzuno, 1999; Liu et al., 2001; Isaka et al., 2005; Kodaira, 1962; Hamill et al., 1969; Fujita et al., 1994). *Metarhizium flavoviride* has been used in insect pest control, and viridoxins A and B have been isolated from this organism as potent toxins against Colorado potato beetle (Gupta et al., 1993). Recently, two novel compounds with anti-proliferative effects on Drosophila S2 cells were isolated from *M. flavoviride*, metarhizins A and B (Kikuchi et al., 2009). These viridoxins and metarhizins possess a pyrone diterpene frame that is unique to this organism, and all suppress the proliferation of insect S2 cells.

In studies using human breast cancer MCF-7 cells, it has been reported that another pyrone diterpene-type compound, sesquicillin, induces growth arrest at G1 phase in association with the induction of the CDK inhibitor protein, $p21^{Waf1/Cip1}$ (Jeong et al., 2002). The p21 protein is a well-characterized Cip/Kip family CDK inhibitor (Sherr and Roberts, 1999) that induces cell growth arrest via the inactivation of CDK activity and the inhibition of proliferating cell nuclear antigen (PCNA) (Xiong et al., 1993; Waga et al., 1994). A variety of transcriptional factors are thought to be involved in the transcription of the *p21* mRNA, and reactive oxygen species (ROS) are also known to be factors in the up-regulation of p21 expression (Shen et al., 2006; Russo et al., 1995). Preliminary results have shown that metarhizin A also suppresses the proliferation of MCF-7 cells and other mammalian cells. Metarhizin A is structurally unique, and its use as a leading compound for the development of new drugs is the subject of interest. The molecular targets of this compound have not yet been identified. Thus, it is important to understand the molecular mechanism underlying the anti-proliferative activity of metarhizin A against human cells. In the present study, we examined the effects of metarhizin

* Corresponding author at: Department of Biomolecular Research, Fukushima Medical University School of Medicine, 1 Hikariga-oka, Fukushima 960-1295, Japan. Tel.: +81 24 547 1660; fax: +81 24 548 3041.
E-mail address: yoshihom@fmu.ac.jp (Y. Homma).

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A on cell cycle and signal transduction pathways using MCF-7 cells. We further investigated the effects of metarhizin A on mitochondrial function and found that it suppresses mitochondrial activity by inhibiting the cytochrome c oxidase system.

Materials and methods

Cell culture and proliferation assay

MCF-7 (human breast carcinoma cell line) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in a humidified incubator under 5% CO_2 at 37 °C. For cell proliferation analysis, cells were plated in 96-well plates (5×10^3 cells/well) and incubated in the presence or absence of drugs. After incubation for various periods, cell proliferation was evaluated using Cell Count Reagent SF (Nacali Tesque, Tokyo) according to the manufacturer's instructions. Alternatively, cell growth was examined by measuring DNA labeling with 5-ethynyl-2′-deoxyuridine (EdU) (Invitrogen). MCF-7 cells (2×10^4 cells/well) were cultured in the presence of vehicle (DMSO) or 1 μM metarhizin A in 6-well plates at 37 °C overnight. Cells were further incubated with EdU for 30 min, and observed under a fluorescence microscope after conjugation with a fluorescence probe to EdU by a click reaction. Cytotoxicity was examined by determining lactate dehydrogenase (LDH) activity in culture supernatants using a CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega).

Chemicals

Metarhizin A (Fig. 1A) was obtained from the culture broth of *M. flavoviride* F-778 (Kikuchi et al., 2009). The strain F-778 was isolated from the field cricket, *Teleogryllus emma* Ohmachi et Matsuura (Orthoptera: Gryllidae) collected at Tsukuba, Ibaraki on October 19, 1989. Based on its conidiogenous structures and conidial morphology, the fungus was identified as *Metarhizium flavoviride* Gams & Rozsypal *var. flavoviride*. The structure of metarhizin A was confirmed by its ^1H and ^{13}C NMR spectra and high resolution mass spectra. ^1H NMR spectra indicated a purity of greater than 98%. Reduced glutathione (GSH), vitamin E and cytochrome c oxidase were purchased from Sigma-Aldrich. Anti-actin antibody was purchased from Abcam, anti-p21, anti-p27, and anti-p53 were from MBL (Nagoya, Japan), anti-p16 was from Santa Cruz Biotechnology, anti-phospho-p53 (phospho S15) was from Abnova, anti-c-Myc, anti-phospho-p44/42 ERK1/2, anti-phospho-SAPK/JNK, and anti-phospho-p38 MAPK were from Cell Signaling Technology, and HRP (horseradish peroxidase)-conjugated goat anti-mouse IgG (H + L), and HRP-conjugated anti-rabbit IgG (H + L) were from Bio-Rad Laboratories.

Flow cytometric analysis

Cells were suspended in trypsin, washed with PBS, and fixed in 75% ethanol for 1 day at 4 °C. The fixed cells were washed with PBS, treated with RNase A for 30 min at 37 °C, and then stained with propidium

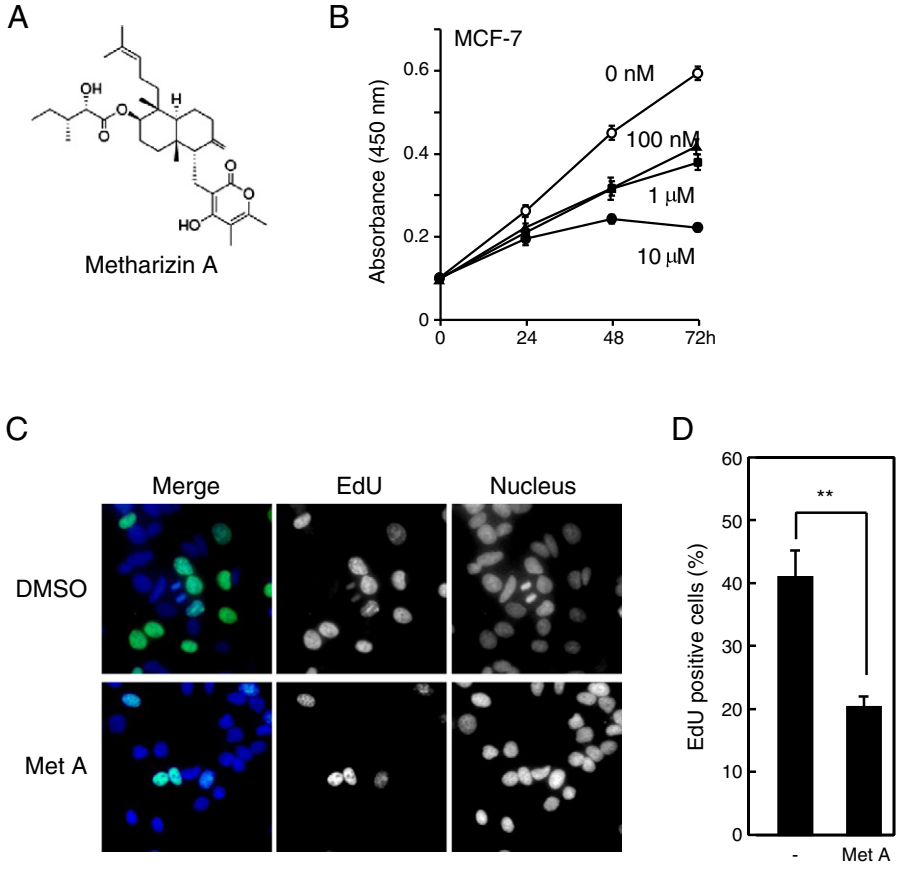

Fig. 1. Effect of metarhizin A on cell growth. (A) Structure of metarhizin A. (B) Growth inhibition of MCF-7 cells treated with metarhizin A. Cells were treated with vehicle (0.5% DMSO) or various concentrations of metarhizin A (10 nM, filled diamonds; 100 nM, filled triangles; 1 μM, filled squares; 10 μM, filled circles). Results are expressed as percentage (mean and SD). (C) Effect of metarhizin A on DNA labeling. MCF-7 cells were cultured in the presence of vehicle or 1 μM metarhizin A overnight, incubated with EdU, and observed under a fluorescence microscope after conjugation with a fluorescence probe to EdU by a click reaction. (D) Metarhizin A decreased the proportion of DNA-replicated cells from 40.9% to 20.3% (mean and SD, n = 3; **P < 0.01).