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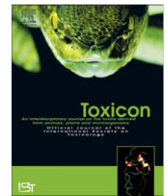

Apoptosis induced by enniatins H and MK1688 isolated from *Fusarium oxysporum* FB1501

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Enniatins are cyclic peptides isolated from bacteria, fungi, and plants, with numerous biological effects on animal systems. Recently, we have reported that certain enniatins (ENs), such as EN H and EN MK1688, have cytotoxic effects on several adenocarcinoma cell lines. In an effort to understand the mechanism behind their cytotoxicity, we investigated whether ENs can induce apoptosis in human colorectal carcinoma cells (HCT-15 cells). Treatment with the ENs H and MK1688 resulted in an alteration of cellular and nuclear morphology, leading to an increase in the number of the cells with apoptotic nuclei (seen as condensed or fragmented nuclei). Furthermore, it was observed that cellular DNA fragmentation increased in a dose-dependent manner in EN treated cells. These cells have elevated activity levels for caspase-3, the enzyme responsible for initiating cell death, compared with the untreated cells. Normal caspase-3 activity levels were observed when Z-VAD-FMK, a caspase inhibitor, was added simultaneously with the ENs. Based on our results, we propose that the new ENs H and MK1688 induce cytotoxicity via an apoptotic pathway.

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1. Introduction

Enniatins (ENs) are known to have various biological activities, acting as enzyme inhibitors, antifungal and antibacterial agents, and immunomodulatory substances (Fairlie et al., 1995; Fusetani et al., 1991). These cyclohexadepsipeptides, found in bacteria, fungi, and plants, have a molecular structure composed of an alternating sequence of three *N*–methyl–L-amino acids and three D-α-hydroxyisovaleric acids (Shemyakin et al., 1963; Strongman et al., 1988; Zhukhlistova et al., 1999). ENs were first described as phytotoxins, and were associated with plant diseases (Gaumann et al., 1960). For example, the ENs A, A1, B, and B1 have been reported as natural contaminants (Herrmann et al., 1996; Shemyakin et al., 1969), and several other EN analogues, such as ENs B2, B3, B4, D, E, F, and G, have also been identified and characterized (Lin et al., 2002; Tomoda et al., 1992).

Recently, multiple studies have indicated that ENs exert their inhibitory activity on animal systems via their activity as ionophores (Tomoda et al., 1992), changing ion transport across membranes and disrupting the ionic selectivity of cell walls (Logrieco et al., 2002). In the membrane, ENs form a dimeric structure, acting as ionophores to transport monovalent ions across the membranes. This effect is particularly debilitating in mitochondrial membranes, resulting in the uncoupling of oxidative phosphorylation (Benz, 1978; Ivanov et al., 1973). These basic mechanisms are responsible for diverse array of biological activities, such as the antibiotic activities on several Gram-positive bacteria (Hamill et al., 1969; Ovchinnikov et al., 1971), and insecticidal, herbicidal (Grove and Pople, 1980; Gupta et al., 1995; Hamill et al., 1969), and cholesterol acyltransferase-inhibiting properties (Tomoda et al., 1992). Furthermore,

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ENs were shown to inhibit one of the major multidrug efflux pumps (Pdr5p in *Saccharomyces cerevisiae*) at nontoxic levels via a different mechanism (Hiraga et al., 2005). As such, in combination with chemotherapeutic drugs, their pharmacological properties have the potential for many important clinical applications.

The novel ENs H, I, and MK1688 were isolated from the insect pathogenic fungus *Verticillium hemipterigenum*. Although they were previously known to have antiplasmodial and antimycobacterial activities (Nilanonta et al., 2003), their other biological activities remain unknown. Our group has recently reported the co-purification of the ENs H and MK1688 from a culture of *Fusarium oxysporum* KFCC 11363P (Song et al., 2006). These ENs have been shown to effectively inhibit cell growth in several human carcinoma cell lines at the micromolar level (Lee et al., 2008).

In order to understand their cytotoxic mechanisms, we investigated whether these ENs exert apoptotic effects by studying the morphological changes of nuclei, degree of DNA fragmentation and caspase activity in HCT-15 cells in this work.

2. Materials and methods

2.1. Reagents

The ENs H and MK1688 were purified from *F. oxysporum* KFCC 11363P, as previously described (Lee et al., 2008). Hoechst 33258, paraformaldehyde, and camptothecin were purchased from Sigma Chemical Co. Dubellco's Minimal Essential Medium (DMEM) and fetal bovine serum were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). All other chemicals unless indicated were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture

The human colorectal cancer cell line HCT-15 was purchased from American Type Culture Collection (Rockville, MD, USA). HCT-15 cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO_2 in humidified air. At the onset of experiments, cells were transferred into either culture dishes or 8-well plates via treatment with trypsin.

2.3. Analysis of nuclear morphology by fluorescence staining

Changes in the nuclear morphology of cells treated with enniatins were investigated with fluorescence staining as previously (Li et al., 2005). Cells (4×10^4 cells/well) were seeded onto an 8-well plate (Nunc, Roskilde, Denmark). Five hours later, drugs were added to the cells to a final concentration of 5 μM. At 24 h post-drug treatment, the culture medium was removed, and the cells rinsed with PBS and fixed with 4% paraformaldehyde/PBS for 15 min. The cells were then washed for 15 min with 0.1% Trition X-100/PBS, and incubated in the dark with Hoechst 33258, at 10 μg/ml, for 30 min. Apoptotic nuclei with condensed, new moon-type or fragmented chromatins were easily distinguished from normal nuclei using a Nikon fluorescence microscope (TE 2000 u; Tokyo, Japan). A minimum of 300 cells from at least three different microscopic fields were analyzed to see nuclear morphology in one independent experiment. In order to obtain reliable estimates of apoptotic cells, the same experiment was repeated three times independently.

2.4. Determination of DNA fragmentation

Low-molecular weight DNA was extracted from approximately 2.0×10^6 cells as described by Hinshaw et al. (1994), with slight modifications. Briefly, cells were plated on a 60 mm Petri dish (Nunc, Roskilde, Denmark). The next day morning, the cells were treated with drugs for 24 h, then rinsed with PBS, and detached from the plate. The cells were resuspended in ice-cold lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA, 0.2% Triton X-100), and incubated on ice for 30 min. The lysates were centrifuged at $10{,}000 \times g$ at 4 °C for 10 min, and the supernatants were extracted once with buffered phenol, once with buffered phenol–chloroform, and once with chloroform–isoamyl alcohol (24:1, vol/vol). The DNA was ethanol-precipitated and resuspended in 10 mM Tris (pH 7.5) and 1 mM EDTA, treated with RNase A for 30 min at 37 °C, and then analyzed using gel electrophoresis on a 2% agarose gel.

2.5. Analysis of caspase-3 activity

The caspase-3 activity of the drug-treated cells was determined using a commercial kit (CaspACE Assay System, Promega). First, approximately 1.5×10^6 cells were plated in a 60 mm Petri dish. Drugs were added to the cells the following morning, and for apoptosis-inhibited samples, Z-VAD-FMK was also added to the cells at a final concentration of 50 μM. At 24 h post-drug treatment, the culture medium was removed and the cells were harvested into a microcentrifuge tube with a rubber policeman. The cell density was adjusted to 10^8 cells/ml in PBS, and an equal number of cells for each sample were resuspended with the cell lysis buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA) for 5 min, and lysed by freeze–thawing three times. The cell lysates were centrifuged at 15,000 rpm for 30 min at 4 °C and the supernatants were collected for the caspase assay. The caspase activity in the cell extracts was measured according to the manufacturer's guide. Briefly, the supernatants collected from the cell lysates were mixed with caspase assay buffer, DMSO, and DEVD-pNA substrate in a 96-well plate with equal amounts of cell extract protein being added to all reactions. The plate was covered with a plate sealer and incubated at 37 °C for 4 h. Color development was measured with a Spectra Max 190 (Molecular Device; Sunyvale, CA, USA) at a wavelength of 405 nm.

2.6. Data analysis

All the data represented the average values (the mean ± standard deviation) from three independent experiments with at least three wells or microscopic fields each time. Statistical analyses were performed using Student's *t*-test. Differences were considered significant at $P < 0.01$ or $P < 0.05$.

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