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Spiruchostatin A and B, novel histone deacetylase inhibitors, induce apoptosis through reactive oxygen species-mitochondria pathway in human lymphoma U937 cells

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ABSTRACT

Spiruchostatin A (SP-A) and spiruchostatin B (SP-B) are the potent histone deacetylase inhibitors (HDACi), that has the potential for chemotherapy of leukemia but the exact mechanism of these compounds remains unclear. In the present study, the role of reactive oxygen species (ROS) production and the mechanism involved in the apoptosis was investigated in human lymphoma U937 cell. When the U937 cells were treated with SP-A and SP-B for 24 h at different concentrations, evidence of apoptotic features, including increase in DNA fragmentation and changes in nuclear morphology, were obtained. SP-B showed maximum potency to induce apoptosis, while SP-A was less potent. Apoptosis was also determined by increase in the fraction of sub-G1 cells and Annexin V-FITC staining cells. SP-A and SP-B induced apoptosis was accompanied by significant increase in the formation of intracellular reactive oxygen species (ROS). Pre-treatment with *N*-acetyl-L-cysteine (NAC), significantly inhibited the SP-A and SP-B mediated apoptosis, suggesting a vital role of ROS involved in the lethality of both agents. Moreover, SP-A and SP-B treatment resulted in the loss of mitochondrial membrane potential (MMP), and Fas, caspase-8 and caspase-3 activation. In addition Bid activation and the release of cytochrome-c to the cytosol was also observed. In this study, we suggest that a marked induction of intracellular ROS mediated mitochondrial pathway and the Fas plays a role in the SP-A and SP-B induced apoptosis. Taken together, our data provides further insights of the mechanism of action of SP-A and SP-B and their potential application as novel chemotherapeutic agents.

1. Introduction

Acetylation and deacetylation of histones play a crucial role to control transcriptional activity of different genes through affecting the interaction between DNA and histones [1,2]. Histones acetylation and deacetylation is regulated by a balance between two specific enzymes, histone acetyltransferases (HATs) and histone deacetylase (HDAC) [3,4]. Aberrant regulation of HATs and HDAC activity has been reported to play an important role in uncontrolled cancer growth [5,6]. Increased HDAC activity was reported in several human cancer cell lines and therefore HDAC inhibition is considered as the novel strategy in the cancer treatment [7,8]. Recently, histone deacetylase inhibitors (HDACi) have gained much attention and emerged as promising anti-cancer agents [9], because of their diverse mode of action which include disruption of co-repressor complexes, ROS induction, death receptors up-regulation, generation of lipid second messengers (e.g. ceramide), interference with chaperone protein function [10,11]. Until now, several HDACi have been identified and on the basis of their structural features they were divided into different classes [12]. FK228 (romidepsin) and suberoylanilide hydroxamic acid (SAHA) has

Abbreviations: SP-A, Spiruchostatin A; SP-B, Spiruchostatin B; ROS, reactive oxygen species; HDAC, histone deacetylase; MMP, mitochondrial membrane potential; NAC, *N*-acetyl-L-cysteine; FITC, fluorescein isothiocyanate; HATs, histone acetyltransferases; HDACi, histone deacetylase inhibitors; CTCL, cutaneous T-cell lymphoma; SAHA, suberoylanilide hydroxamic acid; SCLC, small cell lung cancer; DCFH-DA, dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HE, hydroethidine; pNA, chromophore p-nitroanilide; TCA, trichloroacetic acid; TMRM, tetramethylrhodamine methyl ester.

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already been approved by US Food and Drug administration (FDA), for the treatment of cutaneous T cell lymphoma (CTCL) [13], and others such as valproic acid and MS-275 are still in clinical trials [13,14].

In spite of the fact that HDAC inhibitors are promising anti-cancer agents, the molecular mechanism involved in the cell death after treatment with HDAC inhibitors remain unclear, but the induction of oxidative stress seems to be common theme involved in the cell death caused by HDAC inhibitors [15]. In solid tumor and leukemia cells, HDAC inhibitors have been reported to induce apoptosis through ROS generation [16]. Recently, Spiruchostatin A and B, the structurally similar 15-membered bicyclic depsipeptides, were successfully isolated from a culture broth of *Pseudomonas* sp. [17]. Both agents were found to possess potent HDAC inhibitory activity and have the potential for chemotherapy of leukemia [18,19]. Previously, the HDAC inhibitory activity of these agents were tested against the panel of 39 cancer cell line, in which SP-B was found to be the most potent inhibitor of HDAC1 and it showed the most potent inhibitory activity as compared to other HDACi including FK228 and SP-A [19]. Like other HDACi, SP-A and SP-B also characterized by the biological effects which include growth inhibition, cell cycle arrest and induction of p21 expression [3,20].

The purpose of this study is to determine the possible apoptosis inducing activity of SP-A and SP-B, and the detail molecular mechanism involved. To date, no report has regarded the effects of these compounds on ROS production and apoptosis in human lymphoma U937 cells. Therefore, this study was undertaken to further clarify the molecular mechanism involved in the apoptosis induced by SP-A and SP-B in human lymphoma U937 cells.

2. Materials and methods

2.1. Chemicals

Spiruchostatin A and B were kindly provided by Prof. Tadashi Katoh (Faculty of Pharmaceutical Sciences, Tohoku Pharmaceutical University, Sendai, Japan). Both agents were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. The agents were diluted with culture media to prepare serial concentrations just before the use. The Annexin V-FITC kit was from Immunotech (Marseille, France). RNase A was purchased from Nacalai Tesque (Kyoto, Japan). DCFH-DA and HE was from molecular probes (Eugene). The FLICE/caspase-8 colorimetric protease assay kit was obtained from MBL (Nagoya, Japan). Antibodies against caspase-3, Bid, Bcl-2, Ac-H3, Ac-H4 and secondary horseradish peroxide (HRP)-conjugated anti-rabbit or anti-mouse IgG were obtained from Cell Signaling technology (Danvers M.A.). Anti-β-actin was from Sigma Aldrich (Saint Louis) and anti-cytochrome-c pAb, anti Bax and Fas were purchased from Santa Cruz Biotechnology Inc (CA). Anti caspase-8 was from R&D systems (Minneapolis, USA). All other reagents were of analytical grade.

2.2. Cell culture

A human myelomonocytic lymphoma cell lines, U937 and Molt-4 were obtained from Human Sciences Research Resource Bank (Japan Human Sciences Foundation, Tokyo, Japan). HL-60 cells were kindly provided as a gift by Emeritus Prof. Yoshisada Fujiwara, Kobe University. The cells were grown in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in humidified air with 5% CO_2.

2.3. Cell cycle analysis

For flow cytometry, cells were fixed with 70% ice cold ethanol at least for 2 h or stored overnight at −20 °C, and subsequently treated with 0.25 mg/ml RNase A (Nacalai Tesque, Kyoto, Japan) and 50 μg/ml PI to obtain the distribution of PI-based cell-cycle phases. The samples were finally run on an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA) [21].

2.4. DNA fragmentation assay

For the detection of apoptosis the percentage of DNA fragmentation was assessed up till 24 h post treatment using the method of Sellins and Cohen [22], with minor modifications. Briefly, approximately 3×10^6 cells were lysed using 200 μl of lysis buffer (10 mM Tris, 1 mM EDTA and 0.2% Triton X-100, pH 7.5) and centrifuged at 13,000*g* for 10 min. Subsequently, each DNA sample in the supernatant and the resulting pellet was precipitated in the 25% trichloroacetic acid (TCA) at 4 °C overnight and quantified using a diphenylamine reagent after hydrolysis in 5% TCA at 90 °C for 20 min. The percentage of fragmented DNA in each sample was calculated as the amount of DNA in the supernatant divided by total DNA for that sample (supernatant plus pellet).

2.5. Morphological detection of apoptosis

The morphological changes in the cells were examined by Giemsa staining. To identify the apoptotic cells after treatment with SP-A and SP-B, cells were harvested after 24 h of incubation at 37 °C, were washed with PBS and collected by centrifugation. Then the cells were fixed with methanol and acetic acid (3:1) and spread on the glass slides. After drying, staining was performed with 5% Giemsa solution (pH 6.8) for 5 min [23].

2.6. Detection of apoptosis using Annexin V-FITC/PI staining

To determine early apoptosis and secondary necrosis, phosphatidylserine (PS) externalization of apoptosis was determined by analysis of propidium iodide (PI) and fluorescein isothiocyanate (FITC)-labeled Annexin V (Immunotech, Marseille, France) using Flow cytometry (Epics XL, Beckman-Coulter, Miami, FL) [24] according to the instructions of the manufacturer. Briefly, cells were treated with SP-A and SP-B at different concentrations for 24 h at 37 °C, cells were collected, washed with cold PBS at 4 °C and centrifuged at 1200 rpm for 3 min. The resulting pellet was mixed with the binding buffer of the Annexin V-FITC kit. FITC-labeled Annexin V (5 μl) and PI (5 μl) were added to the 490 μl suspension and mixed gently. After incubation at 4 °C for 20 min in the dark, the cells were analyzed with a flow cytometry.

2.7. Assessment of intracellular reactive oxygen species (ROS)

Evaluation of intracellular H_2O_2 was performed by flow cytometry using the fluorescence generate in the cells loaded with the peroxide-sensitive fluorescent probe, a H_2O_2 sensitive dye, dichlorofluorescein diacetate (DCFH-DA) (Molecular probes, Eugene, OR). DCFH-DA rapidly diffuses in to the cytosol of cells where it is hydrolyzed to the non-fluorescent, oxidation sensitive DCFH. In the presence of cytosolic peroxide, DCFH is rapidly oxidized to the non-diffusible, fluorescent DCF.

Briefly, after treatment with SP-A and SP-B for different time periods, the cells were collected and washed with PBS, and were stained with 10 μM of DCFH-DA at 37 °C for 30 min. The fraction of DCF fluorescence positive cells was measured by flow cytometry as the proportion of cells containing intracellular H_2O_2 [25].