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Honokiol induces cell cycle arrest and apoptosis via inhibition of survival signals in adult T-cell leukemia

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ABSTRACT

Background: Honokiol, a naturally occurring biphenyl, possesses anti-neoplastic properties. We investigated activities of honokiol against adult T-cell leukemia (ATL) associated with human T-cell leukemia virus type 1 (HTLV-1).
Methods: Cell viability was assessed using colorimetric assay. Propidium iodide staining was performed to determine cell cycle phase. Apoptotic effects were evaluated by 7A6 detection and caspases activity. Expressions of cell cycle- and apoptosis-associated proteins were analyzed by Western blot. We investigated the efficacy of honokiol in mice harboring tumors of HTLV-1-infected T-cell origin.
Results: Honokiol exhibited cytotoxic activity against HTLV-1-infected T-cell lines and ATL cells. We identified two different effects of honokiol on HTLV-1-infected T-cell lines: cell cycle inhibition and induction of apoptosis. Honokiol induced G_1 cell cycle arrest by reducing the expression of cyclins D1, D2, E, CDK2, CDK4, CDK6 and c-Myc, while apoptosis was induced via reduced expression of cIAP-2, XIAP and survivin. The induced apoptosis was also associated with activation of caspases-3 and -9. In addition, honokiol suppressed the phosphorylation of IκBα, IKKα, IKKβ, STAT3, STAT5 and Akt, down-regulated JunB and JunD, and inhibited DNA binding of NF-κB, AP-1, STAT3 and STAT5. These effects resulted in the inactivation of survival signals including NF-κB, AP-1, STATs and Akt. Honokiol was highly effective against ATL in mice
Conclusions: Our data suggested that honokiol is a systemically available, non-toxic inhibitor of ATL cell growth that should be examined for potential clinical application.
General significance: Our findings provide a rationale for clinical evaluation of honokiol for the management of ATL.

1. Introduction

Adult T-cell leukemia (ATL) is a peripheral T-cell malignancy associated with human T-cell leukemia virus type 1 (HTLV-1) infection [1–3]. ATL is categorized into four types according to clinical phenotype: acute, chronic, smoldering and lymphoma [4]. Despite recent developments in intensive combination chemotherapy regimens, bone marrow transplantation and monoclonal antibody therapies, the prognosis of patients with acute or lymphoma ATL remains extremely poor [5]. This grave outcome is mainly due to the intrinsic resistance of leukemic cells to conventional chemotherapy, even in high doses, and to severe immunosuppression. Hence, innovative therapeutic strategies are still needed to prevent the progression of ATL and to develop curative treatments for this type of leukemia.

Recent advances have led to the identification of key molecules and cellular pathways involved in HTLV-1-mediated cellular transformation. HTLV-1 encodes the viral Tax oncoprotein whose expression confers pro-survival and pro-proliferative properties on cells [6]. Tax does not only transactivate viral genes, but also interferes with cell growth control pathways including NF-κB, AP-1 and Akt [6–10]. While Tax is required to initiate transformation, this viral oncoprotein is no longer expressed in many ATL cells, probably due to immune surveillance [8]. Signal transducer and activator of transcription (STATs) pathways in addition to NF-κB, AP-1 and Akt are activated in ATL cells that do not express Tax, although the mechanism of activation remains unknown [6–10].

Honokiol was initially described as a component of *Magnolia obovata*, a common component of Asian herbal teas [11]. It has also been used without noticeable side effects for many years in traditional Asian medicine [12]. Honokiol molecules contain two phenolic groups that confer antioxidant properties similar to vitamin E [13]

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or polyphenols such as flavonoids [14]. Recently, honokiol has been found to have anti-angiogenic, anti-inflammatory and anti-tumor properties in preclinical models, without appreciable toxicity [15]. However, the potential of honokiol in ATL treatment remains to be determined.

This study evaluated the therapeutic potential of honokiol against ATL in vitro and in vivo, and investigated possible mechanisms of action. Honokiol induced cell cycle arrest and apoptosis in HTLV-1-infected T-cell lines and inhibited the growth of HTLV-1-infected T cells in murine xenografts. Treatment of HTLV-1-infected T cell lines with honokiol in this study blocked NF-κB, AP-1, Akt and STATs activation, implying an upstream target of action.

2. Materials and methods

2.1. Reagents

Honokiol was extracted and purified from Magnolia as previously described [16]. It was also purchased from Wako Pure Chemical Industries (Osaka, Japan). Antibodies to cyclin D2, cIAP-2, IκBα, JunB and JunD were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while antibodies to Bax, Bcl-2, retinoblastoma protein (pRb), cyclin B1, cyclin E, CDK1, CDK2, CDK4, CDK6, p53, c-Myc and actin were purchased from NeoMarkers (Fremont, CA). Antibodies to XIAP, cyclin D1 and phospho-pRb (Ser780) were purchased from Medical & Biological Laboratories (Nagoya, Japan). Antibodies to cleaved poly (ADP-ribose) polymerase (PARP), caspase-9, cleaved caspase-9, cleaved caspase-3, survivin, IκB kinase (IKK)α, IKKβ, phospho-IKKα/β (Ser176 and Ser180 in IKKα and Ser177 and Ser181 in IKKβ), Akt, phospho-Akt (Ser473), phospho-Akt (Thr308), phospho-IκBα (Ser32 and Ser36), STAT3, phospho-STAT3 (Tyr705), phospho-STAT5 (Tyr694) and Bcl-x_L were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to STAT5 and cyclophilin D were obtained from BD Transduction Laboratories (San Jose, CA) and Calbiochem (San Diego, CA), respectively, and the antibody to Tax, Lt-4, was described previously [17].

2.2. Cells

HTLV-1-infected T-cell lines, MT-2 [18], MT-4 [19], C5/MJ [20], SLB-1 [21], HUT-102 [1], MT-1 [22] and TL-Oml [23], were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. MT-2, MT-4, C5/MJ and SLB-1 are HTLV-1-transformed T-cell lines established initially by an in vitro coculture protocol. MT-1 and TL-Oml are T-cell lines of leukemic cell origin established from patients with ATL. HUT-102 was also established from a patient with ATL and constitutively expresses viral genes, but its clonal origin is unclear. Peripheral blood mononuclear cells (PBMC) from healthy volunteers, 7 patients with acute ATL and 2 patients with chronic ATL were also analyzed. All patients supplied informed consent to participate in the study.

2.3. Assays for cell viability and apoptosis

In these assays, 1×10^5/ml (cell lines) or 1×10^6/ml (PBMC) cells were cultured with various concentrations of honokiol in 96-well plates. After 24 h, cell viability was evaluated by measuring the mitochondria-dependent conversion of water-soluble tetrazolium (WST)-8 (Wako Pure Chemical Industries, Osaka, Japan) to a colored formazan product. Apoptotic events in cells were detected by staining with phycoerythrin-conjugated APO2.7 monoclonal antibody (Beckman Coulter, Marseille, France), which specifically detects the 38-kDa mitochondrial membrane antigen 7A6 [24] and analysis by flow cytometry on a Coulter EPICS XL (Beckman Coulter, Fullerton, CA).

2.4. Cell cycle analysis

Cell cycle analysis was performed with the CycleTEST PLUS DNA reagent kit (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cell suspensions were analyzed on a Coulter EPICS XL using EXPO32 software. The population of cells in each cell cycle phase was determined with MultiCycle software.

2.5. In vitro measurement of caspase activity

Caspase activity was measured using colorimetric caspase assay kits from Medical & Biological Laboratories. Cell extracts were recovered using the cell lysis buffer supplied with the kit and assessed for caspases-3 and -9 activities using colorimetric probes. The assay kits are based on detection of chromophore *p*-nitroanilide after cleavage from caspase-specific labeled substrates. Colorimetric readings were performed in an automated microplate reader.

2.6. Western blot analysis

Cells were lysed in a buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (20 μg) were subjected to electrophoresis on SDS-polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with specific antibodies. The bands were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained as described by Antalis and Godbolt [25] with modifications, and EMSA was conducted as described previously [26]. Briefly, 5 μg of nuclear extract was incubated with ^{32}P-labeled probes. The DNA–protein complex was then separated from free oligonucleotides on a 4% polyacrylamide gel. The probes used were prepared by annealing the sense and antisense synthetic oligonucleotides as follows: a typical NF-κB element from the interleukin-2 receptor α chain (IL-2Rα) gene (5′-gatcCGGCAGGGGAATCTCCCTCTC-3′), AP-1 element of the IL-8 gene (5′-gatcGTGATGACTCAGGTT-3′), STAT3 consensus binding motif (SIE) derived from the c-fos gene (5′-gatcGACATTTCCCGTAAATCG-3′) and the STAT5 consensus binding motif (β-casein) derived from the β-casein gene (5′-gatcAGATTTCTAGGAATTCAAATC-3′). The oligonucleotide 5′-gatcTGTCGAATGCAAATCACTAGAA-3′, containing the consensus sequence of the octamer binding motif, was used to identify specific binding of the transcription factor Oct-1, which regulates the transcription of a number of so-called housekeeping genes. The above underlined sequences represent the NF-κB, AP-1, STAT3, STAT5 and Oct-1 binding sites, respectively.

2.8. In vivo therapeutic effect of honokiol

Five-week-old female C.B-17/Icr-SCID mice were obtained from Ryukyu Biotec Co. (Urasoe, Japan). Mice were engrafted with 5×10^6 HUT-102 cells by subcutaneous injection in the postauricular region and then randomly placed into two groups of seven mice each; one received the vehicle only, while the other was treated with honokiol. Treatment was initiated on the day after cell inoculation. The honokiol was dissolved in 10% ethanol and 90% Intralipid (Terumo, Tokyo, Japan) at a concentration of 11 mg/ml, and 135 mg/kg body weight of honokiol was administered intraperitoneally every day for 28 days. Control mice received the same volume of the vehicle only for 28 days. Tumor size was monitored once a week. All mice were sacrificed on day 28, and then the tumors were dissected out and weighed. This experiment was performed according to the Guidelines for Animal Experimentation of the University of the Ryukyus, and was