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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

approved by the Animal Care and Use Committee of the University of the Ryukyus.

2.9. Statistical analysis

Data are expressed as mean ± SD. Statistical analyses were carried out using the Student's *t* test and Mann–Whitney's *U*-test as appropriate. A *p* value less than 5% denoted the presence of statistical significance.

3. Results

3.1. Honokiol reduces cell viability of HTLV-1-infected T-cell lines and primary ATL cells

To identify the therapeutic potential of honokiol, HTLV-1-infected T-cell lines were cultured with the indicated concentration of honokiol for 24 h, and cell viability was determined by WST-8 assay. Honokiol reduced the cell viability in a dose-dependent manner (Fig. 1A). The concentration of honokiol that caused 50% inhibition of cell viability (IC_{50}) ranged from 18 to 50 μM. Tax protein induces resistance to anticancer drugs [27]. However, there was no apparent correlation between sensitivity to honokiol and Tax expression. To compare the cytotoxicity of honokiol toward ATL cells with normal PBMC, viability was next measured in freshly isolated ATL cells from seven patients with acute ATL, two patients with chronic ATL or PBMC from two normal healthy volunteers incubated with different doses of honokiol for 24 h (Fig. 1B). Although honokiol was cytotoxic to normal PBMC, ATL cells showed higher susceptibility to the cytotoxic effects of honokiol; honokiol inhibited cell viability in both acute and chronic ATL cells, with an IC_{50} concentration of 22 to 53 μM, while up to 40 μM honokiol did not produce a significantly reduction of cell viability of normal PBMC. Collectively, treatment of these ATL cells with honokiol reduced cell survival compared with PBMC from normal healthy volunteers.

3.2. Honokiol causes G_1 phase cell cycle arrest

To gain insights into the mechanism of cell growth inhibition by honokiol, we determined the cellular DNA content distribution by flow cytometric analysis of treated cells. Cultivation of HTLV-1-infected T cell lines with 40 μM honokiol increased the population of cells in the G_1 phase, with marked reduction of cells in the S phase, relative to untreated cells (Fig. 1C). These changes were primarily the result of a G_1 phase cell cycle arrest in HTLV-1-infected T-cell lines.

3.3. Honokiol induces apoptosis of HTLV-1-infected T-cell lines

To further characterize the cytotoxicity of honokiol against HTLV-1-infected T-cell lines, we next analyzed the frequency of apoptotic cells by measuring APO2.7 staining. APO2.7-positive populations represent early apoptotic cells. Treatment with 80 μM honokiol for 24 h increased the proportion of cells positive for APO2.7 among all tested HTLV-1-infected T-cell lines (Fig. 2A). Although there was a slight increase in apoptosis at both the 20-μM and 40-μM concentrations, the honokiol-treated HTLV-1-infected T-cell lines exhibited apoptosis in a concentration-dependent manner (Fig. 2B). Taken together, these results implicated induction of apoptosis as mediating the inhibitory effect of honokiol on HTLV-1-infected T-cell viability.

3.4. Honokiol-induced apoptosis is caspase-dependent

We then investigated whether the observed honokiol-induced apoptosis was due to caspase activation. Cell extracts were obtained after

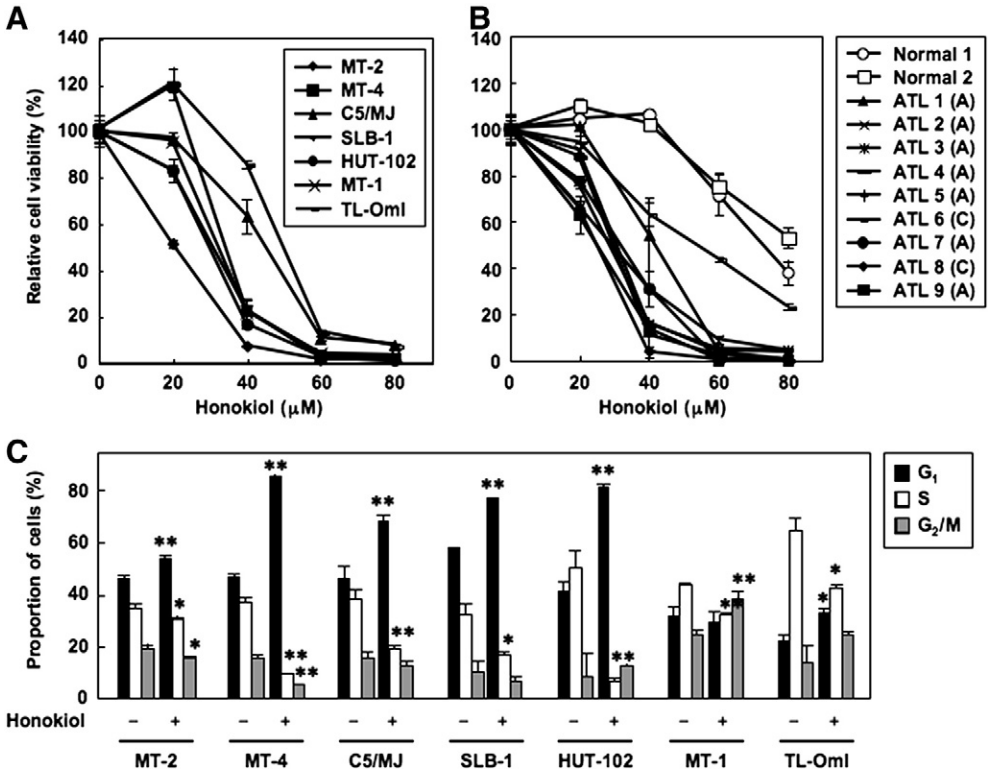

Fig. 1. Honokiol reduces cell viability in HTLV-1-infected T-cell lines and PBMC from ATL patients, and induces G_1 phase cell cycle arrest in HTLV-1-infected T-cell lines. HTLV-1-infected T-cell lines (A) and PBMC from ATL patients and healthy controls (B) were cultured with various concentrations of honokiol for 24 h. Viability was determined in triplicate cultures using the WST-8 assay. The results are expressed as percentage of control and represent mean ± SD of the results obtained. (C) Honokiol induces G_1 phase cell cycle arrest in HTLV-1-infected T-cell lines. Cells were incubated with or without 40 μM honokiol for 24 h. Cell cycle distribution was analyzed by flow cytometry following staining with propidium iodide. Data are expressed as the mean ± SD percentage of cells at various phases of the cell cycle (n = 3). $^*p<0.05$, $^{**}p<0.01$, compared with the control.

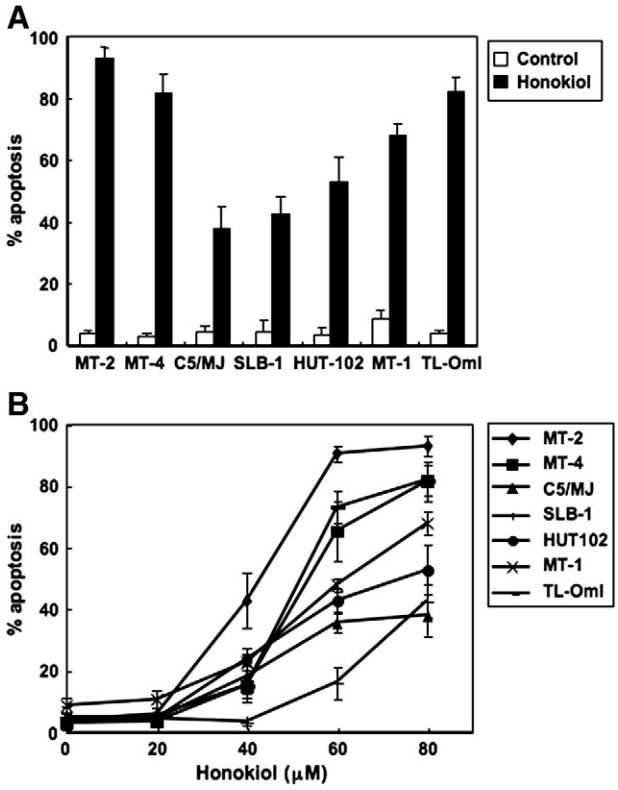

Fig. 2. Honokiol induces apoptosis of HTLV-1-infected T-cell lines. (A) HTLV-1-infected T-cell lines were cultured in the absence or presence of 80 μM honokiol. After 24 h, APO2.7 staining was analyzed by flow cytometry. Data are mean ± SD percentages of apoptotic cells for both untreated (open bars) and honokiol-treated (solid bars) cells (n = 3). (B) HTLV-1-infected T-cell lines were treated with various concentrations of honokiol for 24 h and then subjected to APO2.7 staining. Data are mean ± SD percentages of apoptotic cells (n = 3).

various treatments and processed for immunoblotting. As shown in Fig. 3A, treatment of MT-2 and HUT-102 cells resulted in cleavage of the caspase-3-specific substrate PARP into the characteristic 89 kDa fragments. In addition, the immunoblot analysis clearly demonstrated the cleaved products of caspases-3 and -9 in honokiol-treated cells and that the production was dose dependent. Immunoblotting allowed us to examine caspase processing, but it could not indicate whether the cleavage products were enzymatically active. Therefore, caspases-3 and -9 activities were determined by cleavage of caspase-specific labeled substrates in colorimetric assays. Honokiol induced the activation of caspases-3 and -9 in MT-2, MT-4 and HUT-102 cells, respectively (Fig. 3B), confirming that honokiol-induced apoptosis of HTLV-1-infected T-cell lines is mediated through caspase activation.

3.5. Honokiol modulates the regulators of cell cycle and apoptosis

To clarify the molecular mechanisms underlying the observed honokiol-induced inhibition of cell growth and apoptosis, we next investigated the expression of several intracellular regulators of the cell cycle and apoptosis by Western blot analysis. As shown in Fig. 4, honokiol had no effect on the expression levels of cell cycle regulatory proteins cyclin B1, CDK1 and p53 or the pro-apoptotic protein Bax, which targets p53. In contrast, honokiol significantly reduced the expression of cell cycle regulatory proteins cyclins D1, D2, E, CDK2, CDK4, CDK6 and c-Myc, as well as anti-apoptotic proteins cIAP-2, XIAP and survivin in MT-2 cells in a time-dependent manner. D-type CDKs (CDK4 and CDK6) are responsible for phosphorylation of pRb. In addition, cyclin E expressed at G_1/S boundary is also involved in phosphorylation of pRb through activation of CDK2 [28]. As shown in Fig. 4, honokiol treatment caused a suppression of pRb phosphorylation in MT-2 cells. Because cyclins D1, D2, E, CDK2, CDK4, CDK6, c-Myc, cIAP-2 and XIAP are encoded by Tax-responsive genes [28–34], we also examined the level of Tax expression. However, immunoblot analysis of MT-2 cells treated by honokiol revealed no significant changes in the

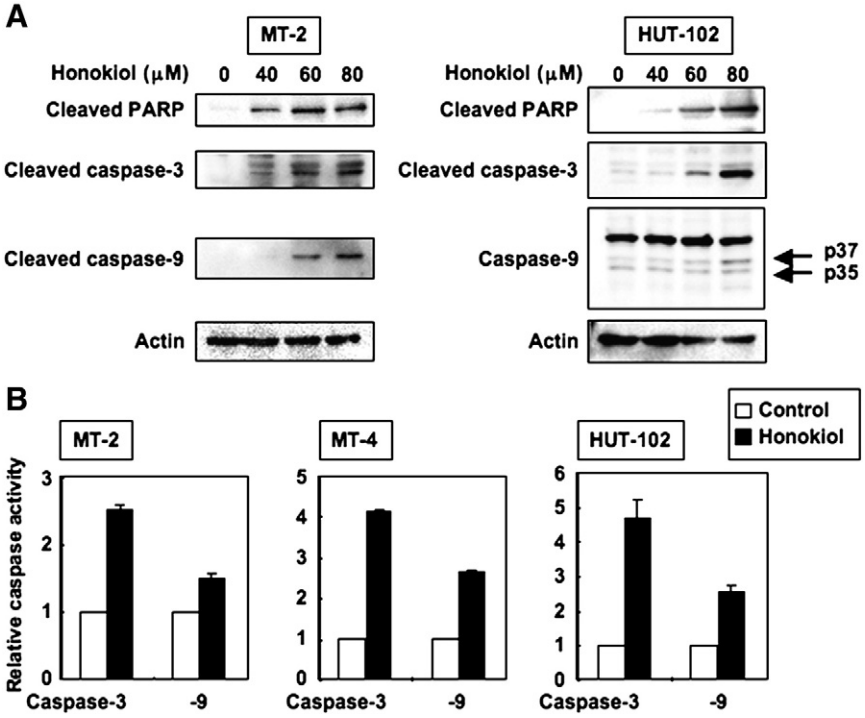

Fig. 3. Honokiol induces caspase-dependent apoptosis of HTLV-1-infected T-cell lines. (A) MT-2 and HUT-102 cells were incubated with the indicated concentrations of honokiol for 12 h. Cellular proteins were resolved by SDS/polyacrylamide gel electrophoresis, and caspase activity was determined by the production of PARP, caspases-3 and -9 cleavage products using immunoblot analysis. As loading controls, actin protein expression was included. (B) Honokiol-induced apoptosis is caspase dependent, based on treatment of the indicated cells with or without 60 μM honokiol. After 24 h, cell lysates were prepared and incubated with the labeled caspase substrates, and caspase activity was measured using an automated microplate reader. Caspase activity is expressed relative to untreated cells, which were assigned an arbitrary value of 1. Data are mean ± SD (n = 3).

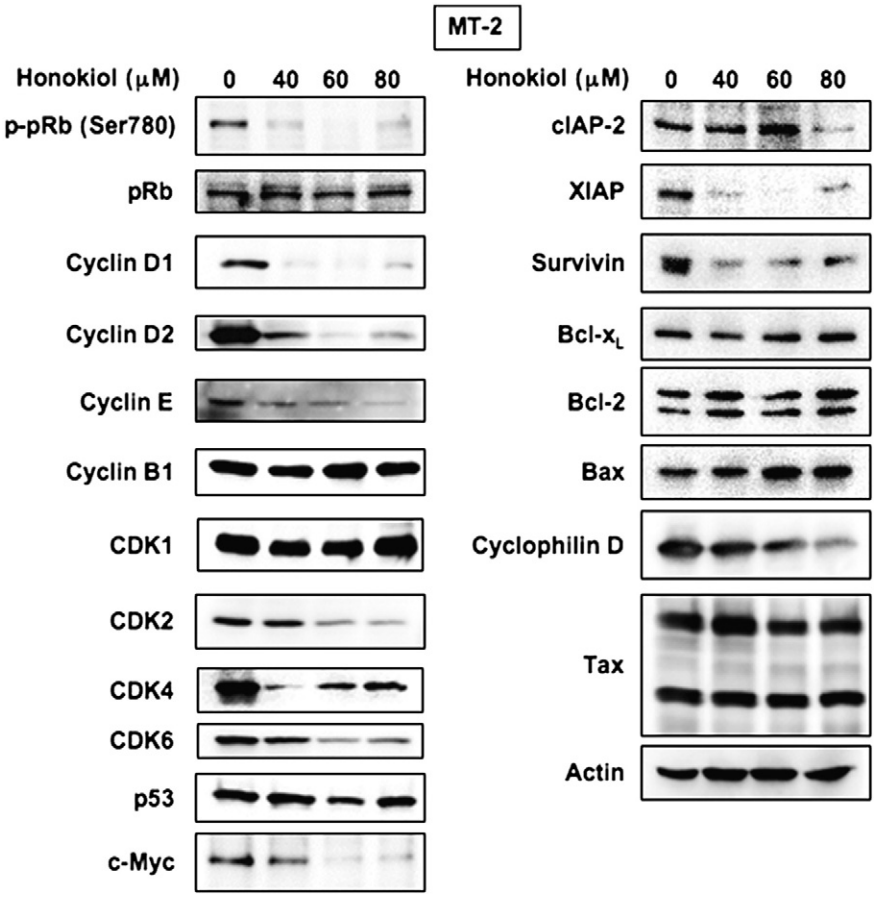

Fig. 4. Effects of honokiol on the expressions of cell cycle and apoptosis regulatory proteins. MT-2 cells were treated with the indicated concentrations of honokiol for 12 h. Whole cell extracts were prepared and immunoblotted with specific antibodies against regulators of cell cycle and apoptosis. Antibody to Tax detected a 40 kDa molecule (p40) and p68 (a fusion between the envelope and the Tax-coding sequence). As loading controls, actin protein expression was included.

level of Tax expression compared to untreated cells (Fig. 4). These observations suggested that honokiol treatment does not target the Tax oncoprotein, and the altered expression of cyclins D1, D2, E, CDK2, CDK4, CDK6, c-Myc, cIAP-2 and XIAP proteins did not result from Tax down-regulation. Although honokiol was reported to induce expression of the mitochondrial protein cyclophilin D [35], honokiol inhibited its expression in MT-2 cells (Fig. 4).

3.6. Inhibitory effects of honokiol on constitutively active NF-κB and AP-1

NF-κB and AP-1 family members are implicated as regulators of apoptosis and proliferation. These transcription factors can modulate the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis, tumor promotion, inflammation and suppression of apoptosis [36,37]. NF-κB can regulate the expression of several gene products linked with tumorigenesis factors such as tumor cell survival or inhibition of apoptosis (cIAP-2, XIAP and survivin) and proliferation (cyclins D1, D2, E, CDK2, CDK4, CDK6 and c-Myc) [28,30–34,36,38–43]. AP-1 also regulates the expression and function of cell cycle regulators such as cyclin D1 [44]. In addition, the cyclin D2 promoter contains NF-κB- and AP-1-binding sites [45]. NF-κB and AP-1 are constitutively active in Tax-expressing and HTLV-1-infected T-cell lines as well as in primary ATL cells [7–10], and Tax activates the NF-κB and AP-1 pathways [6–10]. We therefore investigated whether honokiol modulates NF-κB and AP-1 activation in our system.

To study the DNA-binding activity of NF-κB and AP-1, we performed EMSA with radiolabeled double-stranded NF-κB and AP-1 oligonucleotides. MT-2 cells were treated with different concentrations of honokiol for 12 h and then analyzed. The results showed that honokiol suppresses constitutively active NF-κB and AP-1 in a dose-dependent manner in MT-2 cells. The inhibitory effect also appeared specific to NF-κB and AP-1, and not related to cell death, because no significant change in binding activity of Oct-1 was observed after the treatment with honokiol (Fig. 5A, left).

The activity of NF-κB is regulated by its interaction with the family of NF-κB inhibitors known as IκB. Inhibition of NF-κB results in the formation of inactive NF-κB–IκB complexes in the cytoplasm [46]. In response to various stimuli including Tax, the IKK complex then phosphorylates IκB bound to the NF-κB complex as a substrate [10,46]. The subsequent proteasome-mediated degradation of IκB exposes the nuclear localization signal of NF-κB, thus releasing NF-κB proteins to be translocated to the nucleus, where they regulate the transcription of specific genes [46]. We therefore also examined whether honokiol affects such IκBα phosphorylation. Western blot analysis using an antibody that detects only the serine-phosphorylated form of IκBα indicated that MT-2 cells exhibit constitutive IκBα phosphorylation and that honokiol completely suppresses this effect (Fig. 5B). IKK is part of a multiprotein complex that contains IKKα and IKKβ subunits, and active IKKα and IKKβ are phosphorylated on serine residues within the activation loop of the kinase domain [46]. Honokiol suppressed IKKα and IKKβ phosphorylation in a dose-dependent manner (Fig. 5B). Thus, honokiol inhibited constitutively active NF-κB by inhibiting IKK activity.

AP-1 is a group of basic leucine zipper transcription factors consisting of the Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun (c-Jun, JunB

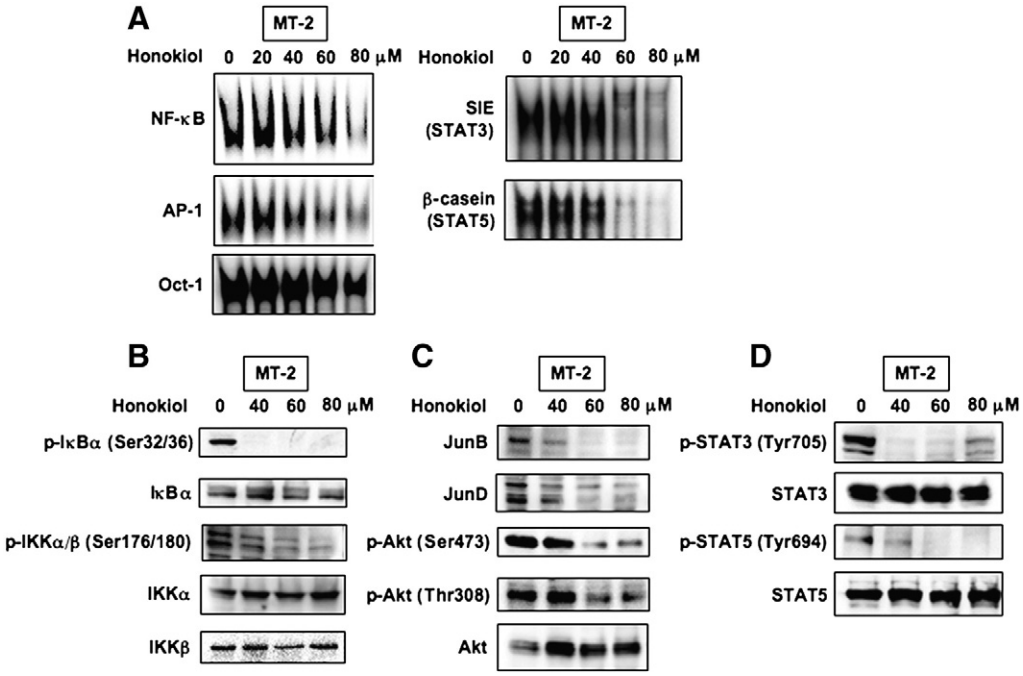

Fig. 5. Inhibition of NF-κB, AP-1, Akt and STATs activities by honokiol. MT-2 cells were incubated with the indicated concentrations of honokiol for 12 h. (A) Honokiol suppresses constitutively active NF-κB, AP-1 and STATs. Nuclear extracts were prepared and analyzed for DNA binding of NF-κB, AP-1, STATs and Oct-1 by EMSA using the specific probes. (B–D) Honokiol inhibits the phosphorylation of IκBα, IKKα, IKKβ, Akt, STAT3 and STAT5, and the expression of JunB and JunD. Cell extracts were immunoblotted with the indicated antibodies.

and JunD) families [37]. Activated AP-1 DNA-binding activity in HTLV-1-infected T cells involves JunB and JunD [9,47], the expression of which was also decreased dose dependently by honokiol (Fig. 5C). These findings suggested that honokiol suppresses JunB and JunD expression, resulting in AP-1 inactivation.

3.7. Inhibitory effects of honokiol on constitutively active Akt and STATs

Another cell signaling pathway modulated by Tax is Akt, a pro-survival serine/threonine kinase that is constitutively activated in HTLV-1-infected T-cell lines and primary ATL cells [7]. Akt was phosphorylated on Ser473 and Thr308 in untreated MT-2 cells, while honokiol inhibited Akt phosphorylation in a dose-dependent manner in MT-2 cells (Fig. 5C).

In addition to activating the NF-κB, AP-1 and Akt pathways, STAT3 and STAT5 are constitutively activated in HTLV-1-infected T-cell lines and primary ATL cells [6,7,9]. The STAT proteins are a family of transcription factors essential for cytokine-regulated processes including cell growth and proliferation via the activation of downstream genes [48]. Cyclin D1 and XIAP contain STAT5-binding site in their promoter regions, and the activities of these genes are mediated by STAT5 [49,50]. The STATs are in turn activated by the JAKs, a group of receptor-associated enzymes with tyrosine phosphorylation activity. Tyrosine-phosphorylated STATs dimerize and then translocate to the nucleus, where they interact with specific DNA response elements to induce or repress transcription. In MT-2 cells, honokiol inhibited the phosphorylation of STAT3 and STAT5 on tyrosine residues for activation (Fig. 5D), without affecting the overall abundance of STAT3 and STAT5 (Fig. 5D). Thus, when added to MT-2 cells, honokiol converts active, tyrosine-phosphorylated STAT3 and STAT5 to inactive, non-tyrosine-phosphorylated forms. Nuclear extracts were examined for STAT3 and STAT5 DNA-binding activities by EMSA using radiolabeled oligonucleotide probes containing STAT3 and STAT5 DNA-binding elements, respectively. Compared with control cells, the STAT3 and STAT5 DNA binding activities were lower in cells receiving honokiol, and the effect was dose dependent (Fig. 5A, right). Collectively, these data showed that honokiol blocks STAT3 and STAT5 activation when added to MT-2 cells.

3.8. Honokiol exhibits anti-ATL activity in mice

To determine whether honokiol exhibits anti-ATL activity in vivo, SCID mice were inoculated subcutaneously with 5×10^6 HUT-102 cells and treated with honokiol or vehicle on the next day after cell inoculation. After 28 days, the mean tumor volume (Fig. 6A) and weight (Fig. 6B) were significantly lower than in untreated mice. There was no significant difference in body weight gain between days 0 and 28 among the untreated and treated groups (data not shown). During the same period, mice treated with honokiol appeared generally healthy and no other major side effects were observed. These results suggested that honokiol has anti-ATL effects in vivo.

4. Discussion

ATL is characterized by the accumulation of apoptosis-resistant HTLV-1-transformed T cells [27]. Although the basis for this resistance is not fully understood, it has been reported that HTLV-1-infected T cell lines and primary ATL cells overexpress anti-apoptotic factors such as cIAP-2 [33] and survivin [51]. The natural product honokiol was reported to induce apoptosis in a variety of tumor cells [15]. Therefore, we investigated whether honokiol could overcome the apoptotic resistance inherent in ATL cells. We found that honokiol acts directly on HTLV-1-infected T-cell lines to induce cytotoxicity in a manner that causes caspase-dependent apoptosis within 24 h. Honokiol induces cyclophilin D, thus potentiating the mitochondrial permeability transition pore, and causing necrosis in cells with wild-type p53 [15]. HTLV-1-transformed T-cell lines including MT-2, MT-4 and HUT-102 have wild-type p53, but the p53 protein is stabilized, which represents its functional inactivation [52]. Tax can inactivate p53 transactivation function [53].

On the other hand, MT-1 possesses missense mutation at codon 176 and expresses high levels of p53 [54]. Thus, functionally inactive

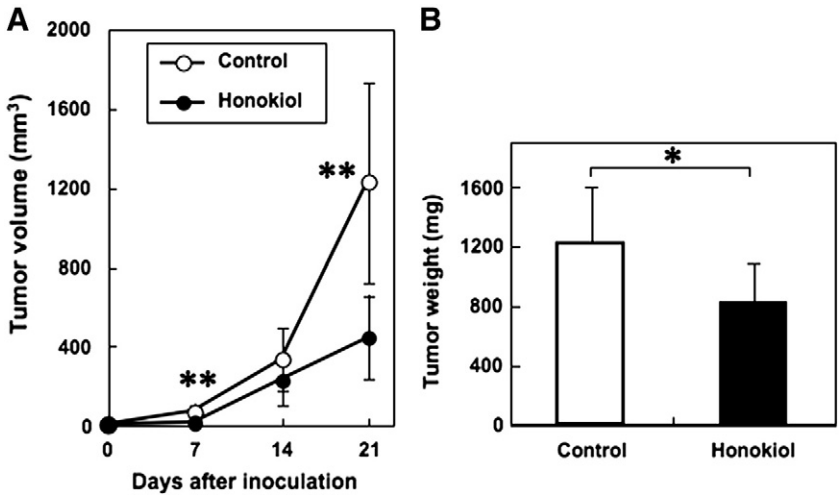

Fig. 6. Effect of honokiol on in vivo growth of HUT-102 cells in SCID mice. (A) Growth of the tumors after subcutaneous inoculation of HUT-102 cells with honokiol. The mice were monitored for tumor volumes. (B) Weight of tumors removed from honokiol-treated mice and untreated mice at day 28 after cell inoculation. Data are expressed as mean ± SD of seven mice in each group. $^{*}p<0.05$, $^{**}p<0.01$, compared with controls.

p53 is not an ideal target for honokiol in HTLV-1-infected T cells. In MT-2 cells with a high endogenous level of cyclophilin D, honokiol did not induce cyclophilin D and necrosis.

Further, our data in primary ATL cells confirmed the activity of honokiol in cell lines and tumor models. Indeed, honokiol induced cytotoxicity toward ATL cells at concentrations that are minimally toxic to normal PBMCs. Exposure of HTLV-1-infected T-cell lines to honokiol modulated the expression of key apoptotic regulatory proteins, including inhibitor of the apoptosis protein family member described above. Our results also indicated a potent anti-proliferative effect of honokiol on HTLV-1-infected T cell lines, which was linked to the G_1 arrest. Its administration was associated with down-regulation of cyclins D1, D2, E, CDK2, CDK4, CDK6 and c-Myc.

To determine the mechanisms underlying the honokiol activity, we examined its effect on the expression and phosphorylation of key signal transduction proteins. Phosphorylated IκBα, IKKα, IKKβ, Akt, STAT3 and STAT5 levels were severely reduced. We also found that inhibition of the AP-1 pathway was characterized by a dose-dependent decrease in JunB and JunD. Constitutive activation of NF-κB, AP-1, Akt and STATs pathways is important for ATL genesis and development [6–10]. A detailed mechanism by which honokiol inhibits these four signals probably lies upstream of these pathways.

NF-κB, AP-1, Akt and STATs can collaborate in ATL, dependent on a variety of factors. Among those, frequent concomitant activations of NF-κB, AP-1 and STATs have been noted, based on the vicinity of many NF-κB-, AP-1- and STATs-responsive DNA motifs in gene promoters such as cyclins D1, D2 and XIAP [38,40,41,44,45,49,50]. JunB is also controlled by NF-κB [38]. In addition, Akt, NF-κB and AP-1 can cross-regulate their activations in HTLV-1-infected T cells. Tax promotes Akt phosphorylation by interacting with and activating the upstream phosphatidylinositol-3-kinase [55]. Activated Akt is important in the activation of pro-survival pathways in HTLV-1-transformed T cells, possibly through the downstream activation of NF-κB and AP-1 [55,56]. In addition, Tax down-regulates the expression of phosphatidylinositol 3,4,5-triphosphate inositol phosphatases through NF-κB activation, leading to overactivation of the phosphatidylinositol-3-kinase signaling cascade [57]. These findings suggested that honokiol has a diverse range of molecular targets, supporting the concept that it acts in numerous biochemical and molecular cascades (Fig. 7).

Drinking green tea and one of its components, polyphenol epigallocatechin-3-gallate prevents angiogenesis in animals [58]. Honokiol, an active component of the Asian herbal teas is a small-molecule polyphenol [11] and exerts anti-angiogenesis activity [15]. HTLV-1-transformed cells induce angiogenesis, which is associated with the adhesion of HTLV-1-transformed cells to endothelial cells and gap junction-mediated heterocellular communication between the two cell types [59]. Therefore, honokiol could inhibit the development of ATL through the suppression of angiogenesis.

Natural products have been the source of many medically beneficial drugs, and their importance in the prevention and treatment of cancer is becoming increasingly apparent. It has been suggested that local cofactors such as food culture play an important role in ATL occurrence. Particularly in Japan, it takes approximately 50 years after infection for ATL to develop and this duration is comparatively longer than in other areas of the world. Natural products, including polyphenols found in green tea [60], resveratrol found in grapes and wine [61], capsaicin found in red pepper [62], fucoidan found in brown seaweed [63], isoflavones including genistein found in soybean [64,65], and deguelin found in African plants [66] and their synthetic derivatives have demonstrated activity against HTLV-1-infected T-cell lines and ATL cells. Indeed, some of these compounds have entered clinical trials for HTLV-1 carriers [67] and patients with HTLV-1-associated neurological disease [68]. Daily intake of the capsulated green tea and fucoidan significantly diminished the HTLV-1 proviral load compared with controls [67,68]. Thus, the use of medicinal botanicals and other natural compounds, perhaps in combination with

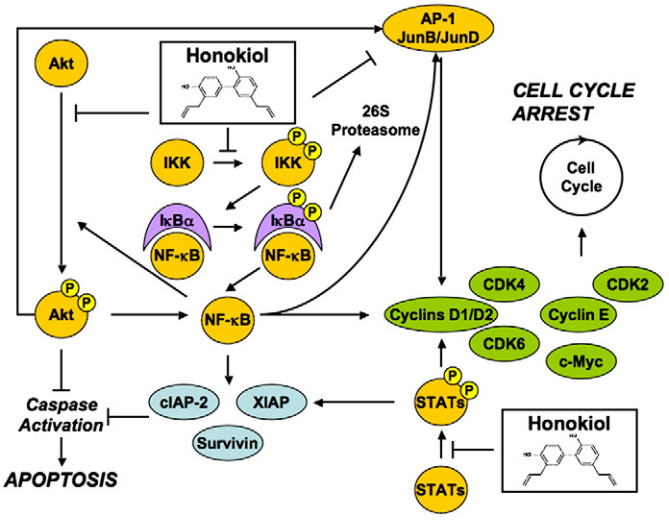

Fig. 7. Proposed model of the action of honokiol.

existing therapies, is gaining credibility and promise for the treatment and prevention of ATL.

Pharmacokinetic studies in mice demonstrated that honokiol is readily absorbed and maintained in the plasma for more than 10 h [69]. The plasma concentration attainable in mice intraperitoneally administered with 250 mg/kg honokiol was 1000 μg/ml (i.e., 3750 μM) [69]. In HUT-102-incubated tumor-bearing mice, honokiol at 135 mg/kg significantly inhibited the tumor growth. The expected honokiol levels correspond to concentrations that approximate or exceed cytotoxicity levels for ATL cells in vitro. Further investigation is needed to determine whether honokiol can be applied clinically for the treatment of ATL; however, our studies in mice and studies from clinical trials investigating Japanese herbal remedies that contain honokiol [70] suggested that honokiol may be a safe and potent chemotherapeutic agent.

In conclusion, we have shown that honokiol administered at pharmacological doses induces apoptosis via a caspase-dependent pathway and slows the cell cycle in HTLV-1-infected T cells. We thus characterized its mechanism of action. Honokiol is also systematically active against ATL in vivo. Importantly, honokiol was well tolerated by the host animal in therapeutically beneficial doses. These results suggest that honokiol, used either alone or in combination with other drugs, represents a promising targeted approach to improve patient outcome in ATL.

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