

Butein inhibits NF- κ B, AP-1 and Akt activation in adult T-cell leukemia/lymphoma

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Abstract. Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATLL) but there is no effective treatment for HTLV-1-associated diseases. Herein, we determined the effect of butein, a bioactive plant polyphenol, on cell growth, apoptosis and signaling pathways in HTLV-1-infected T-cell lines and on tumor growth in SCID mice. Treatment with butein caused a decrease in viability of HTLV-1-infected T-cell lines. T cells cultured with butein showed obvious apoptosis morphology, and cleavage of poly(ADP-ribose) polymerase with activation of caspase-3, -8 and -9. Pretreatment of cells with caspase inhibitor partially blocked butein-induced inhibition of cell viability. Butein also resulted in cell cycle arrest at G₁ phase. Butein markedly downregulated the protein expression levels of CDK4, CDK6, cyclin D1, cyclin D2, cyclin E, survivin, XIAP, c-IAP2 and phospho-pRb. Butein also inhibited i) total and phospho-protein levels of I κ B kinase (IKK) α and IKK β , ii) degradation and phosphorylation of I κ B α , iii) JunB and JunD, iv) total and phospho-protein levels of Akt, v) phosphorylation of RelA, vi) heat shock protein 90, and vii) DNA-binding activity of NF- κ B and AP-1. In mice harboring ATLL xenograft tumors, butein caused a significant inhibition of tumor growth and reduced serum levels of soluble interleukin-2 receptor α chain and soluble cluster of differentiation 30. Considered together, the results indicated that butein has antiproliferative and proapoptotic properties through the suppression of NF- κ B, AP-1 and Akt signaling in HTLV-1-infected T cells, both *in vitro* and *in vivo*, suggesting its therapeutic potential against HTLV-1-associated diseases including ATLL.

Introduction

The retrovirus, human T-cell leukemia virus type 1 (HTLV-1), is associated with adult T-cell leukemia/lymphoma (ATLL) and chronic inflammatory disorders such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), uveitis and arthritis (1). Globally, approximately 20 million people are estimated to be infected by HTLV-1, and 90% of them remain asymptomatic carriers during their lives (2). ATLL is a highly aggressive malignancy of mature CD4⁺ T cells and develops only after a long period of latency, usually several decades (1). Despite advances in the development of novel therapeutic agents, the prognosis of ATLL remains poor, and there is no definite cure for HAM/TSP (1). Many patients acquire resistance to chemotherapeutic agents, leading to treatment failures. A higher viral load in individuals with HTLV-1 infection increases their risk of developing ATLL and HAM/TSP (3,4). Therefore, reduction of the number of HTLV-1-infected T cells is crucial in the prevention and treatment of HTLV-1-associated diseases.

HTLV-1 induces oncogenesis through deregulation of selected cellular signaling pathways. In the virally infected T cells, activation of nuclear factor- κ B (NF- κ B), activator protein-1 (AP-1) and Akt results in upregulation of expression of a large number of cellular genes involved in cell proliferation, survival and metastasis (5,6). Thus, any new therapeutic agent must be designed to target multiple signal transduction pathways for maximum therapeutic benefits.

Natural phytochemicals, present in the human diet with cancer preventive and anticancer properties, are suitable alternatives (7). The plant polyphenol, butein (3,4,2',4'-tetrahydroxychalcone), has been traditionally used as an oriental medicine in Eastern Asia, and has various biological functions (8,9). Butein affects apoptosis, cell proliferation and the cell cycle in diverse cancers (8,9). The preliminary results of a clinical trial on the effects of butein-containing flavonoids in gastric cancer patients showed it was safe with good tolerability, and produced marked decrease in tumor size (10). Thus, butein seems to be a potent anticancer agent. Several previous reports have suggested the involvement of NF- κ B, Akt, extracellular signal-regulated kinase, p38 kinase and p53 in the induction of cell cycle arrest at G₁ or G₂/M and

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apoptosis, and inhibition of metastasis by butein in various cell lines (8,9,11-13). Therefore, we hypothesized that butein may act as a chemopreventive and/or chemotherapeutic agent against HTLV-1-associated diseases.

We report herein that butein inhibits cell growth and stimulates apoptosis of HTLV-1-infected T cells by inhibiting NF- κ B, AP-1 and Akt activities and signaling pathways. Importantly, in a xenograft model, butein inhibited HTLV-1-infected T cell-induced tumor growth. Our study revealed for the first time, butein effect on HTLV-1-infected T cells both *in vitro* and *in vivo*, and the molecular mechanism by which butein exhibited anti-ATLL effects.

Materials and methods

Reagents used. Butein (cat. no. B3803) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan), dissolved in dimethyl sulfoxide (DMSO) and used for the treatment of cells. The broad spectrum caspase inhibitor, Z-VAD-FMK (cat. no. G7232), was purchased from Promega Corp. (Madison, WI, USA). Antibodies against survivin (cat. no. 2808), Akt (cat. no. 9272), I κ B kinase (IKK) α (cat. no. 2682), IKK β (cat. no. 2684), phospho-Akt (Thr308) (cat. no. 13038), phospho-Akt (Ser473) (cat. no. 4060), phospho-I κ B α (Ser32 and 36) (cat. no. 9246), phospho-IKK α/β (Ser176/180 and Ser177/181) (cat. no. 2694), RelA (cat. no. 8242), phospho-RelA (Ser536) (cat. no. 3033), cleaved caspase-3 (cat. no. 9664), -8 (cat. no. 9496), -9 (cat. no. 9501) and poly(ADP-ribose) polymerase (PARP) (cat. no. 9541) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against Bcl-2 (cat. no. MS-597), CDK2 (cat. no. MS-617), CDK4 (cat. no. MS-299), CDK6 (cat. no. MS-398), cyclin E (cat. no. MS-870), retinoblastoma protein (pRb) (cat. no. MS-107) and actin (cat. no. MS-1295) were obtained from Neomarkers, Inc. (Fremont, CA, USA). Antibodies against XIAP (cat. no. M044-3), cyclin D1 (cat. no. K0062-3), heat shock protein (HSP)70 (cat. no. SR-812C), phospho-pRb (Ser780) (cat. no. M054-3S) were purchased from Medical & Biological Laboratories, Co. (Aichi, Japan). Antibodies against cyclin D2 (cat. no. sc-593), c-IAP2 (cat. no. sc-7944), I κ B α (cat. no. sc-371), JunB (cat. no. sc-46) and JunD (cat. no. sc-74), and NF- κ B subunits p50 (cat. no. sc-114X), p52 (cat. no. sc-298X), RelA (cat. no. sc-109X), c-Rel (cat. no. sc-70X) and RelB (cat. no. sc-226X), and AP-1 subunits c-Fos (cat. no. sc-52X), FosB (cat. no. sc-48X), Fra-1 (cat. no. sc-605X), Fra-2 (cat. no. sc-604X), c-Jun (cat. no. sc-45X), JunB (cat. no. sc-46X) and JunD (cat. no. sc-74X) for supershift assay were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody against HSP90 (cat. no. 610418) was from BD BioSciences (San Jose, CA, USA).

Cell culture. The HTLV-1-infected MT-4 and HUT-102, and ATLL-derived TL-Om1 T-cell lines, were cultured in RPMI-1640 medium (cat. no. 30264-56, Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 1% penicillin/streptomycin (cat. no. 09367-34, Nacalai Tesque, Inc.). Cells were maintained in a 37°C, 5% CO₂ humidified incubator. The study also included peripheral blood mononuclear cells (PBMCs) obtained from a healthy

donor. PBMC proliferation was induced with 20 μ g/ml of phytohemagglutinin (PHA) (cat. no. L8754, Sigma-Aldrich Co., St. Louis, MO, USA).

Cell proliferation and cytotoxic assay. The cell proliferative and toxic effects of butein were determined by the water-soluble tetrazolium (WST)-8 uptake method. Cells were plated (1 \times 10⁴ cells per well) in 96-well microtiter plates in triplicate and treated with various concentrations of butein. After incubation for 24-72 h, 10 μ l of the WST-8 reagent (cat. no. 07553-44, Nacalai Tesque, Inc.) was added to each well. After 4 h, WST-8 reduction was measured at 450 nm using a 680 XR microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Values were normalized to untreated control samples. The 50% growth inhibitory concentration (IC₅₀) value was calculated by dotting the data points to a logistic curve using the CalcuSym software (version 2.0; Biosoft, Cambridge, UK).

Analysis of cell apoptosis. Cells were treated with vehicle or butein for 24-72 h and then permeabilized by incubation on ice for 20 min with 100 μ g/ml of digitonin, and treated with phycoerythrin-conjugated APO2.7 antibody (cat. no. IM2088, Beckman Coulter, Inc., Marseille, France) for 15 min at room temperature. After staining with APO2.7 antibody, apoptosis was determined by Epics XL flow cytometry (Beckman Coulter, Inc., Brea, CA, USA). In addition, for analysis of morphological changes in nuclei, cells were stained by 10 μ g/ml of Hoechst 33342 (cat. no. 346-07951, Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and observed under a Leica DMI6000 microscope (Leica Microsystems, Wetzlar, Germany).

In vitro measurement of caspase Activity. Caspase activity was measured using Colorimetric Caspase Assay kits (cat. nos. 4800, 4805 and 4810, Medical & Biological Laboratories, Co.). Briefly, cell extracts were recovered using the cell lysis buffer supplied with the kit and assessed for caspase-3, -8 and -9 activities using colorimetric probes. The assay kits are based on detection of chromophore o-nitroanilide after cleavage from caspase-specific labeled substrates. Colorimetric readings were performed in an automated microplate reader.

Cell cycle analysis. Cells were stained with the CycleTEST Plus DNA Reagent kit (cat. no. 340242, Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA). The cell cycle distribution was analyzed for 10,000 collected cells by an Epics XL flow cytometry that uses the MultiCycle software (version 3.0; Phoenix Flow Systems, San Diego, CA, USA). The population of nuclei at each phase of the cell cycle was determined and apoptotic cells with hypodiploid DNA content were detected in the sub-G₀/G₁ region.

Western blot analysis. Whole cell extracts were prepared by subjecting butein-treated cells to lysis in lysis buffer [62.5 mM Tris-HCl (pH 6.8) (cat. no. 35434-21, Nacalai Tesque, Inc.), 2% sodium dodecyl sulfate (cat. no. 31607-65, Nacalai Tesque, Inc.), 10% glycerol (cat. no. 17045-65, Nacalai Tesque, Inc.), 6% 2-mercaptoethanol (cat. no. 21438-82, Nacalai Tesque, Inc.) and 0.01% bromophenol blue (cat. no. 021-02911, Wako

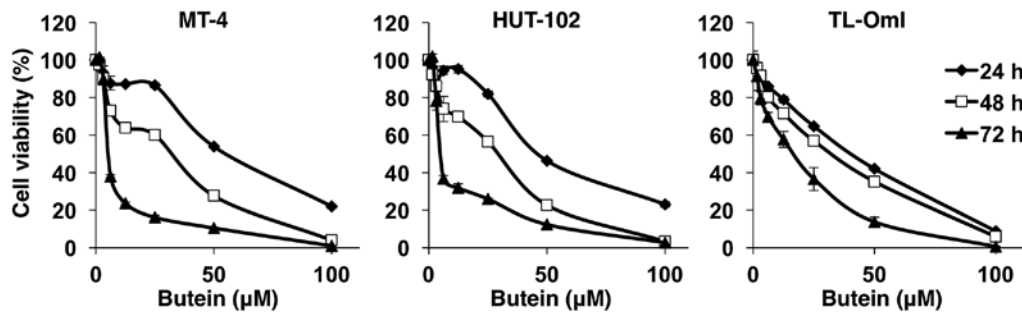


Figure 1. Effect of butein on cell viability. The indicated HTLV-1-infected T-cell lines were treated with butein (1.56-100 μ M) for 24-72 h, and the viability of cells was determined by the WST-8 assay. Data are mean \pm SD of triplicate cultures and represent the percentage of cell viability.

Pure Chemical Industries, Osaka, Japan)]. Lysates were spun to remove insoluble material. The supernatants were collected and kept at -80°C . Protein concentrations were determined using a commercial kit (DC Protein Assay, cat. no. 5000116JA, Bio-Rad Laboratories, Inc.). Equal amounts of protein extracts (20 μ g) were resolved on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). After electrophoresis, the proteins were electrotransferred onto polyvinylidene difluoride membranes (cat. no. IPVH00010EMD, Millipore, Darmstadt, Germany), blotted with the relevant antibodies. Finally, blots were hybridized with horseradish peroxidase-conjugated secondary anti-mouse (cat. no. 7076, Cell Signaling Technology, Inc.) or anti-rabbit IgG antibody (cat. no. 7074, Cell Signaling Technology, Inc.) and developed using an enhanced chemiluminescence reagent (cat. no. RPN2232, Amersham Biosciences Corp., Piscataway, NJ, USA).

RT-PCR. TRIzol reagent (cat. no. 15596026, Invitrogen Life Technologies, Carlsbad, CA, USA) was used to extract total RNA from cells. RNA (1 μ g) was reverse transcribed into cDNA using a PrimeScript RT-PCR kit (cat. no. RR014A, Takara Bio Inc., Otsu, Japan). The sequence-specific primers used for RT-PCR were as follows: for I κ B α , forward 5'-GCCTGGACTCCATGAAAGAC-3', reverse 5'-CAAGTG GAGTGGAGTCTGCTGCAGGTTGTT-3'; and for GAPDH, forward 5'-GCCAAGGTCATCCATGACAACTTTGG-3', reverse 5'-GCCTGCTTACCACCTTCTTGATGTC-3'. PCR amplifications were performed in a programmable thermal cycler. Results were analyzed at 30 cycles to obtain results at exponential levels of amplification.

Electrophoretic mobility shift assay (EMSA). To determine NF- κ B and AP-1 activation, we prepared nuclear extracts from butein-treated cells and performed EMSA, as previously described (14). Nuclear extracts were incubated with ^{32}P -labeled probes. The top strand sequences of the oligonucleotide probes or competitors were as follows: for a typical NF- κ B element of the *interleukin-2 receptor α chain (IL-2R α)* gene, 5'-GATCC GGCAGGGGAATCTCCCTCTC-3' and for the consensus AP-1 element of the *IL-8* gene, 5'-GATCGTGATGACTCA GGTT-3'. The above underlined sequences are the NF- κ B and AP-1 binding sites, respectively. In competition experiments, nuclear extracts were preincubated for 15 min with 100-fold excess of unlabeled oligonucleotides. For supershift assays, nuclear extracts were incubated with antibodies against NF- κ B or AP-1 subunits for 45 min at room temperature

before the complex was analyzed by EMSA. The dried gels were visualized.

Xenograft tumor model. Five-week-old female C.B-17/Icr-severe combined immune deficient (SCID) mice were obtained from Kyudo, Co. (Tosu, Japan). To induce malignancy, 1×10^7 HUT-102 cells suspended in sterile RPMI-1640 medium (200 μ l) were inoculated subcutaneously into the postauricular region of SCID mice. The mice were divided at random into two treatment groups ($n=6$, each). Butein was solubilized in soybean oil (cat. no. 190-03776, Wako Pure Chemical Industries) and administered at a dose of 0.7 mg/kg intraperitoneally three times a week, and the treatment was continued for 27 days, beginning on the day after cell inoculation. The control group received the vehicle only. The tumor diameter was measured weekly with a shifting caliper and tumor volume was calculated. The mice were also weighed weekly, beginning on day 0. All mice were sacrificed on day 28. The tumors were excised and their weight measured. At the end of the study, blood samples were collected and the sera were separated by centrifuging blood and then stored at -80°C until assayed for secreted soluble IL-2R α [soluble cluster of differentiation 25 (sCD25)] and sCD30. This experiment was performed according to the Guidelines for Animal Experimentation of the University of the Ryukyus (Nishihara, Japan), and was approved by the Animal Care and Use Committee of the University of the Ryukyus (reference no. 5837).

Morphological analysis of tumor tissues and terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay. The tumor specimens were collected from the control mice group and 0.7 mg/kg butein group, fixed in formalin (Wako Pure Chemical Industries) solution, dehydrated through graded ethanol series (Japan Alcohol Selling Co., Tokyo, Japan) and embedded in paraffin (cat. no. 09620, Sakura Finetek Japan Co., Tokyo, Japan). The paraffin-embedded specimens of ATLL tumors were stained with hematoxylin and eosin (H&E, cat. nos. 234-12 and 1159350025, Merck, Darmstadt, Germany) and examined histologically. Analysis of DNA fragmentation by TUNEL testing was performed using a commercial kit (cat. no. 11684817910, Roche Applied Science, Penzberg, Germany). Cells were examined under a light microscope (Axioskop 2 Plus) with an Achroplan 40x/0.65 lens (both from Zeiss, Hallbergmoos, Germany). Images were acquired with an AxioCam 503 color and AxioVision LE64 software (Zeiss).

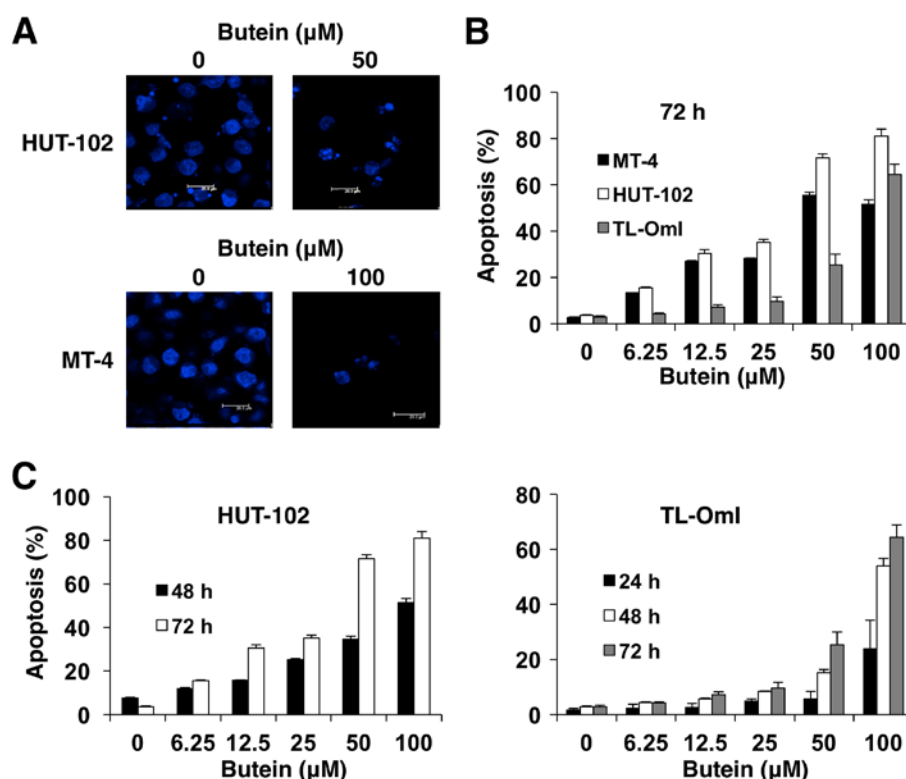


Figure 2. Butein induces apoptosis *in vitro*. (A) Fluorescence micrograph of cells stained with Hoechst 33342. HUT-102 and MT-4 cells were treated with 50 and 100 μ M of butein, respectively, for 48 h, and analyzed by microscopy after Hoechst 33342 staining. (B and C) Quantitative analysis of cell apoptosis by APO2.7 staining flow cytometry. HTLV-1-infected T-cell lines were treated with different concentrations of butein (6.25–100 μ M) for the indicated time periods and analyzed by flow cytometry after APO2.7 staining. Data are mean \pm SD values of triplicate cultures.

Biomarker analysis. Serum concentrations of human sCD25 (cat. no. DR2A00, R&D Systems, Inc., Minneapolis, MN, USA) and human sCD30 (cat. no. SK00582-01, Aviscera Bioscience, Inc., Santa Clara, CA, USA) were measured by enzyme-linked immunosorbent assay (ELISA), according to the protocol supplied by the manufacturer.

Statistical analysis. Values are shown as mean \pm standard deviation (SD). Data of two groups were compared by the Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Effects of butein on cell proliferation and cytotoxicity of HTLV-1-infected T-cell lines. To determine the *in vitro* effects of butein in ATLL cells, two HTLV-1-infected T-cell lines (MT-4 and HUT-102) and an ATLL-derived T-cell line (TL-Oml) were cultured in the presence of butein (1.56–100 μ M). As shown in Fig. 1, 24–72 h culture with butein significantly reduced cell proliferation and viability in a concentration- and time-dependent manner, with IC_{50} values at 72 h of 7.0 μ M for HUT-102, 7.9 μ M for MT-4 and 9.8 μ M for TL-Oml. In contrast, the IC_{50} was 27.8 μ M for normal PBMCs. Butein suppressed the proliferative response of PBMCs to the T cell mitogen PHA, with IC_{50} of 18.5 μ M. Although the IC_{50} values for HTLV-1-infected T-cell lines were lower than those for normal PBMCs, obvious selective cytotoxic activities of butein on HTLV-1-infected T-cell lines have not been demonstrated.

Butein simultaneously induces cell apoptosis and cell cycle arrest. First, the effect of butein on cell morphology was examined using microscopy. Cells cultured with butein for 48 h demonstrated morphologies characteristic of apoptosis, such as chromatin condensation and nuclear fragmentation (Fig. 2A). To further investigate the mode of butein-induced cell death, HTLV-1-infected T-cell lines were treated with 6.3–25 μ M butein for 24 h. Butein induced concentration-dependent increases in the sub- G_0/G_1 population (Fig. 3A and B). We also assessed the expression of 38-kDa mitochondrial membrane antigen APO2.7 during apoptosis (15). Apoptotic cells (APO2.7-positive) increased after treatment with butein concentration- and time-dependently (Fig. 2B and C). Collectively, these results indicate that butein induces apoptosis of HTLV-1-infected T-cell lines. In addition to apoptosis, the same experiment also showed that butein dose-dependently increased the percentage of cells in the G_0/G_1 phase and also reduced the percentage of cells in the S phase (Fig. 3A and B). Considered together, the results showed the arrest of more cells at the G_0/G_1 phase of the cell cycle.

Butein induces caspase activation. The apoptotic process is executed by a member of the highly conserved cysteine proteases, caspases. Initiator caspase-8 and -9 or effector caspase-3 are synthesized as inactive proenzymes and become finally activated by proteolytic cleavage (16). To identify the mechanism underlying butein-induced apoptosis of HTLV-1-infected T cells, we investigated cleavage of caspase-3, -8 and -9, and also PARP, a substrate for caspase-3. Exposure

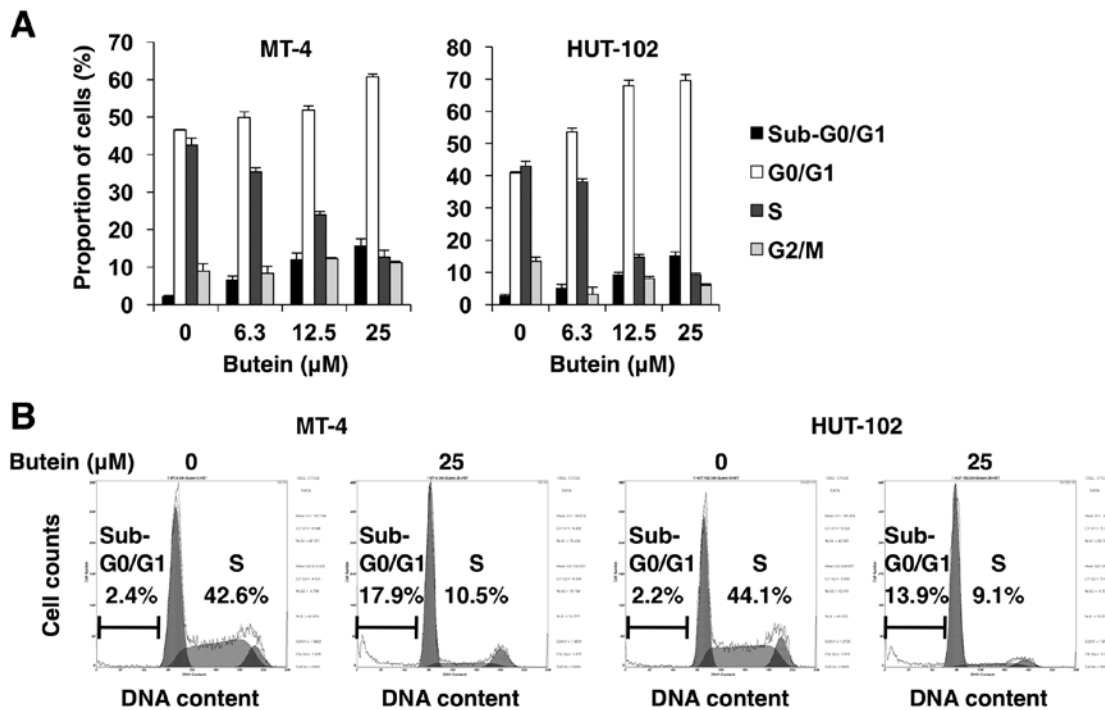


Figure 3. Cell cycle analysis. (A) MT-4 and HUT-102 cells were treated with different concentrations of butein (6.3–25 μ M) for 24 h, and flow cytometry was used to detect the cell cycle phase distribution and cell death in the sub- G_0/G_1 phase. The percentage of each cell cycle fraction was quantified by MultiCycle software. Data are mean \pm SD values of triplicate cultures. (B) Representative results of an experiment conducted in triplicate with similar results.

of MT-4 and HUT-102 cells to butein (12.5–100 μ M for 48 h) caused concentration-dependent increase in the cleavage of caspase-3, -8 and -9, and PARP (Fig. 4A), as well as increase in activities of caspase-3, -8 and -9 (Fig. 4B). To further explore the significance of the latter activation, a broad spectrum caspase inhibitor, Z-VAD-FMK, was used to suppress the effect of butein. Pretreatment of cells with Z-VAD-FMK partially attenuated butein-induced inhibition of cell viability (Fig. 4C), suggesting that butein-mediated cytotoxic effect is mediated, at least in part, through a caspase-dependent apoptosis pathway.

Effects of butein on regulatory proteins involved in cell cycle regulation and apoptosis. Cell cycle progression is tightly regulated by interaction between CDKs and cyclins (17). To identify the molecular mechanism underlying butein-induced G_0/G_1 arrest, cells were cultured with butein (12.5–100 μ M) for 48 h. They were harvested for protein extraction and western blot analysis. As shown in Fig. 5A, levels of CDK4, CDK6, cyclin D2 and cyclin E proteins were significantly decreased in butein-treated MT-4 and HUT-102 cells, compared to DMSO-treated cells. Butein also inhibited the expression of cyclin D1 in HUT-102 but not in MT-4 cells. However, butein had no significant effect on CDK2 protein level. Furthermore, butein induced dephosphorylation of pRb protein (Fig. 5A, top panels) and changed the hyperphosphorylated form to a hypophosphorylated form of pRb (Fig. 5A, second panels). The deregulation of apoptosis is often studied by the expression of anti-apoptotic proteins. There was a dose-dependent decrease in the protein expression of survivin and XIAP, but not Bcl-2, in butein-treated MT-4 and HUT-102 cells. Butein also inhibited the expression of c-IAP2 in HUT-102 but not

in MT-4 cells (Fig. 5B). The effects of butein seem to slightly differ dependent on cell lines.

Butein inhibits NF- κ B in HTLV-1-infected T-cell lines. NF- κ B forms a family of transcription factors that regulate numerous biological processes, including immune response, inflammation, cell growth, survival and development. The NF- κ B proteins are usually sequestered in the cytoplasm by a family of inhibitors, including I κ B α . I κ B α degradation is mediated through its phosphorylation by IKK, a trimeric complex composed of two catalytic subunits, IKK α and IKK β , and a regulatory subunit, IKK γ (18). Incubation of MT-4 and HUT-102 cells with butein resulted in significant dose-dependent inhibition of phosphorylation and upregulation of I κ B α protein (Fig. 6A). To determine whether butein-mediated upregulation of I κ B α is due to transcriptional activation of I κ B α , we examined the expression of I κ B α mRNA in cells treated with butein by RT-PCR. As shown in Fig. 6B, butein treatment had no influence on I κ B α mRNA, suggesting that butein induces the inhibition of degradation of I κ B α protein. Next, we measured total and phospho-protein levels of IKK α and IKK β to evaluate the possible inhibitory mechanism of butein on I κ B α protein phosphorylation. Immunoblot analysis demonstrated butein dose-dependent suppression of phospho-protein levels of IKK α and IKK β in MT-4 and HUT-102 cells. Total protein levels of both IKK α and IKK β were reduced in HUT-102 cells, while total protein level of IKK β alone was reduced in MT-4 cells (Fig. 6A). Thus, the effects of butein seem to slightly differ dependent on cell lines.

NF- κ B is a complex of proteins, in which various combinations of NF- κ B proteins constitute active NF- κ B heterodimers that bind to specific DNA sequences. Thus, to show that

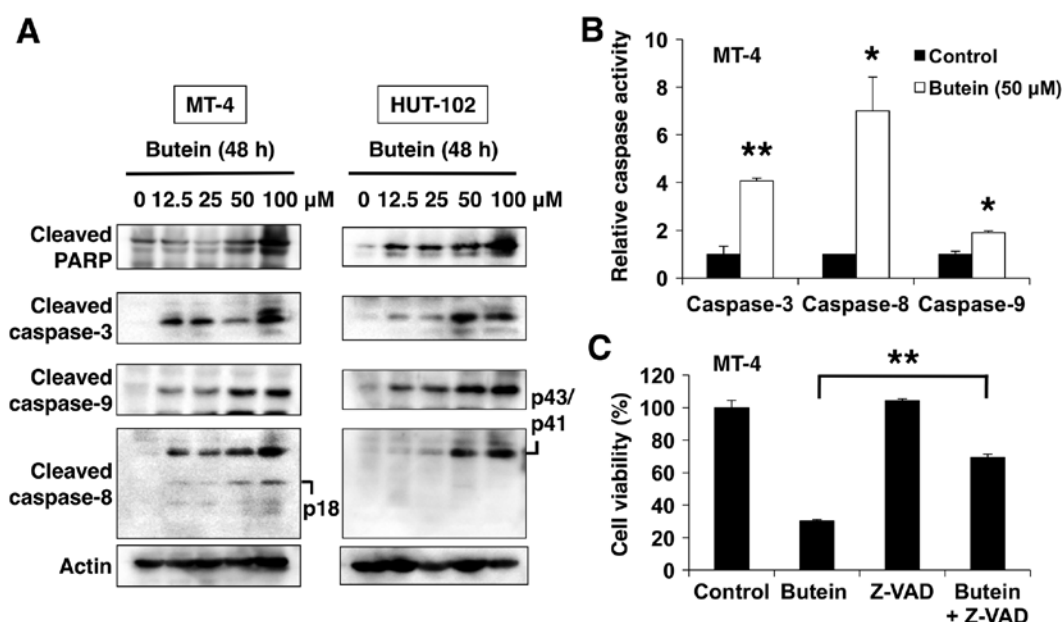


Figure 4. Effects of butein on activation of caspases. (A) Effects of butein on protein expression of cleaved caspase-3, -8, -9 and PARP. MT-4 and HUT-102 cells were incubated with different concentrations of butein (12.5–100 μ M) for 48 h and then harvested. Total cell lysates were prepared, and protein was subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for actin. (B) Caspase-3, -8 and -9 activities were determined using relevant caspase colorimetric assay kits. MT-4 cells were treated with 50 μ M of butein for 48 h and then lysed for protein extraction. Quantitative results of caspase-3, -8 and -9 activity levels, which were expressed as multiples of induction beyond each respective control. Data are mean \pm SD values of triplicate cultures. * P <0.05 and ** P <0.01, vs. vehicle-treated control. (C) Effect of pan-caspase inhibitor on butein-induced cell death. MT-4 cells were incubated with 20 μ M of Z-VAD-FMK for 1 h, followed by addition of butein (25 μ M; 48 h). Cell viability was determined by the WST-8 assay. Data are mean \pm SD of triplicate cultures. ** P <0.01 vs. butein-treated control.

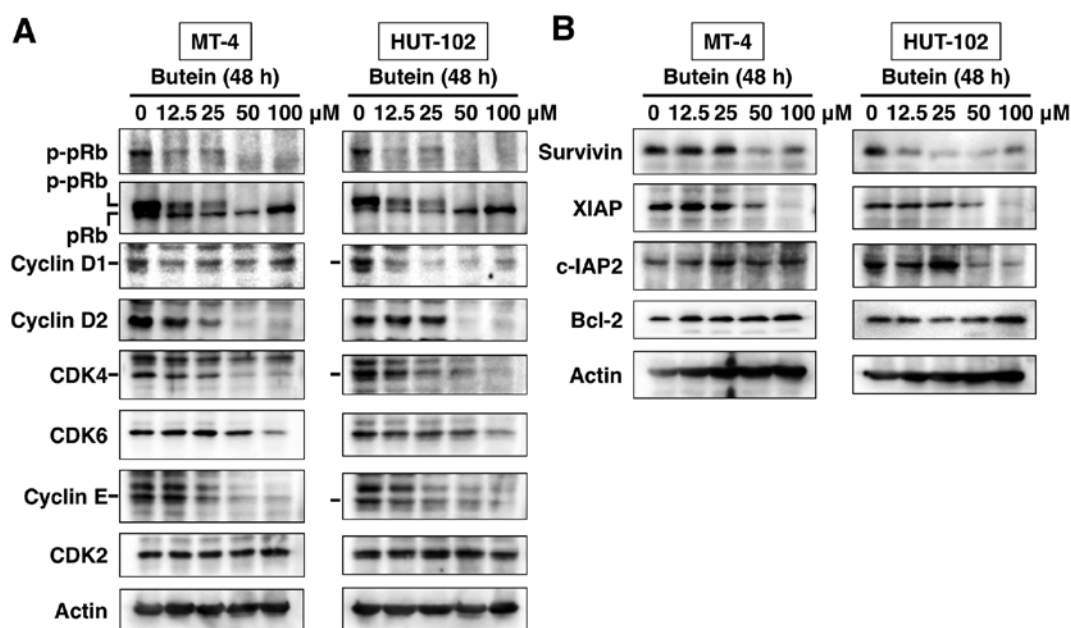


Figure 5. Effect of butein on the expression of various cell cycle regulatory proteins and anti-apoptotic proteins. (A) Effects of butein on protein expression of phosphorylated pRb, cyclins and CDKs in MT-4 and HUT-102 cells. The cells were cultured with the indicated concentrations of butein for 48 h. (B) Effects of butein on the expression of various anti-apoptotic proteins in MT-4 and HUT-102 cells. The cells were treated with butein (12.5–100 μ M; 48 h) and then harvested. Total cell lysates were prepared, and protein was subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection. Actin was used as the internal control.

the band visualized by EMSA in HTLV-1-infected T-cell lines was indeed NF- κ B, nuclear extracts from MT-4 and HUT-102 cells were incubated with antibodies to the NF- κ B subunits and analyzed by EMSA. The results showed shifts of the bands to higher molecular masses (Fig. 6C), suggesting

that the activated complex consisted of p50, RelA and RelB. The addition of excess unlabeled NF- κ B caused complete disappearance of the band, whereas the addition of AP-1 oligonucleotide had no effect on the DNA binding. We also investigated whether butein could inhibit constitutive NF- κ B

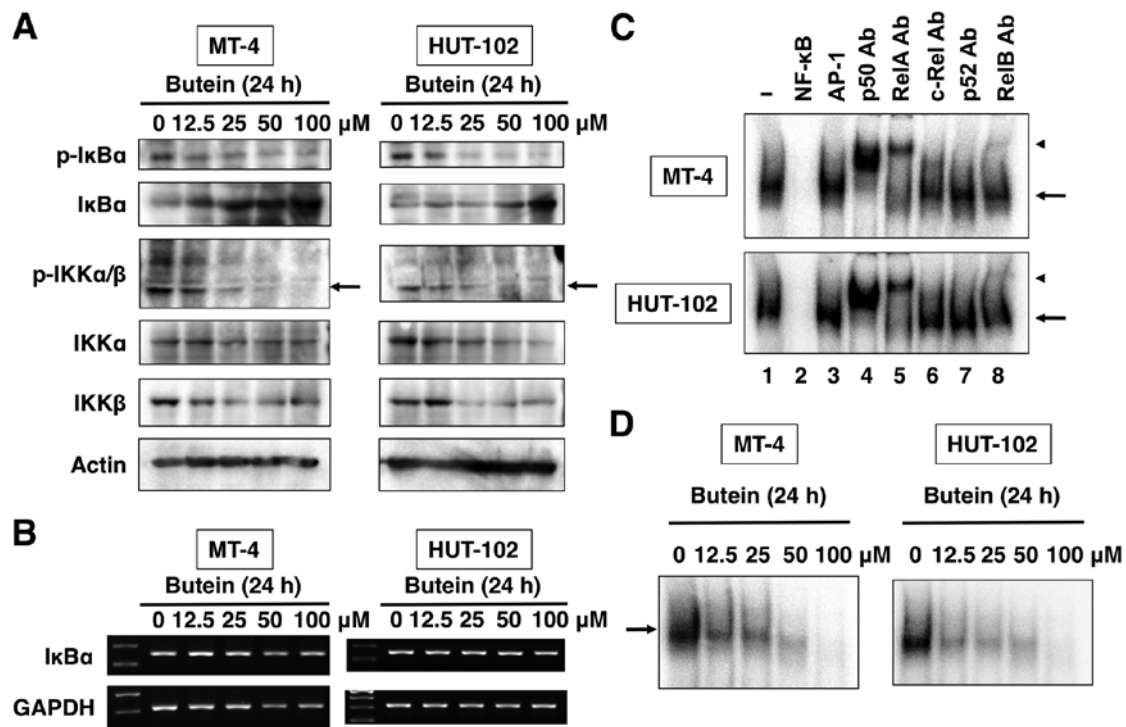


Figure 6. Butein inhibits constitutively active NF- κ B in HTLV-1-infected T-cell lines. (A) Butein inhibits total and phospho-protein levels of IKK α / β and suppresses phosphorylation of I κ B α . MT-4 and HUT-102 cells were treated with the indicated concentrations of butein for 24 h, after which the cell extracts were prepared, and protein was resolved on SDS-PAGE. (B) Effects of butein on I κ B α mRNA expression in HTLV-1-infected T-cell lines. MT-4 and HUT-102 cells were cultured with the indicated concentrations of butein for 6 h, and total RNA extracted from cells was subjected to RT-PCR. GAPDH as internal control. (C) NF- κ B is composed of several NF- κ B subunits. Nuclear extracts from untreated cells were incubated with the indicated antibodies, an unlabeled NF- κ B or AP-1 oligo probe. They were then assayed for NF- κ B activation by EMSA. Ab, antibody. Arrow and arrowhead show a specific complex and a supershifted band, respectively. (D) MT-4 and HUT-102 cells were treated with the indicated concentrations of butein for 24 h. The nuclear extracts were assayed for NF- κ B activation by EMSA. Arrow shows a specific complex.

in HTLV-1-infected T-cell lines. MT-4 and HUT-102 cells were cultured in the presence of different concentrations of butein for 24 h and then analyzed for NF- κ B activation. Butein dose-dependently inhibited constitutive NF- κ B activation in HTLV-1-infected T-cell lines (Fig. 6D). These results indicate that butein can suppress constitutively active NF- κ B in HTLV-1-infected T cells.

Butein suppresses AP-1 activity. AP-1 is a group of dimeric transcription factor complexes composed of members of the Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun (c-Jun, JunB and JunD) families, which play central roles in the proliferation and transformation of T cells (6). EMSA showed supershifted band for AP-1, as detected with specific antibodies to JunB and JunD in MT-4 and HUT-102 cells (Fig. 7A). As shown in Fig. 7B, butein produced a significant and dose-dependent suppression of AP-1 activation in HTLV-1-infected T-cell lines. As shown in Fig. 7A, JunB and JunD are functional components of AP-1 transcription factor in HTLV-1-infected T-cell lines. The experiment demonstrated that butein inhibited AP-1 signaling pathway through dose-dependent suppression of JunB and JunD protein levels (Fig. 7C).

Butein inhibits Akt and HSP90 protein expression in HTLV-1-infected T-cell lines. Akt is a component of an essential pathway for cell survival and growth during development and carcinogenesis. It regulates cell survival by directly targeting XIAP and by indirectly modulating survivin levels (19,20).

Akt also regulates cell cycle and proliferation by indirectly modulating the levels of cyclin D1, cyclin D2 and cyclin E (21). Since butein decreased the levels of XIAP, survivin, cyclin D1, cyclin D2 and cyclin E in the treated cells, we determined its effect on Akt protein. Butein at 50-100 μ M for MT-4 and 12.5-100 μ M for HUT-102 cells, caused a dose-dependent inhibition of Akt protein expression. Treatment of MT-4 cells with butein at 50-100 μ M and HUT-102 cells with butein at 12.5-100 μ M caused a dose-dependent inhibition in Akt phosphorylation at Thr308. Butein at 12.5-100 μ M for MT-4 and 25-100 μ M for HUT-102 cells, also inhibited the phosphorylation of Akt at Ser473 (Fig. 8). RelA is phosphorylated at Ser536 by Akt and such phosphorylation enhances RelA transactivation potential (22). Butein also caused inhibition of RelA phosphorylation (Fig. 8). The molecular chaperone HSP90 is involved in the stabilization and conformational maturation of many signaling proteins that are deregulated in cancers. HSP90 client proteins include signaling proteins (IKKs and Akt) and cell cycle regulators (CDKs) (23). Therefore, we investigated the effect of butein on the expression of HSP90. As shown in Fig. 8, butein reduced HSP90 protein levels, but increased HSP70 levels.

Butein suppresses growth of HTLV-1-infected T-cell line in SCID mice. Butein significantly inhibited tumor growth in SCID mice inoculated with HTLV-1-infected HUT-102 cells (Fig. 9A, left panels). In the control group, the average tumor volume of 1,545 mm³ was reached in 28 days after

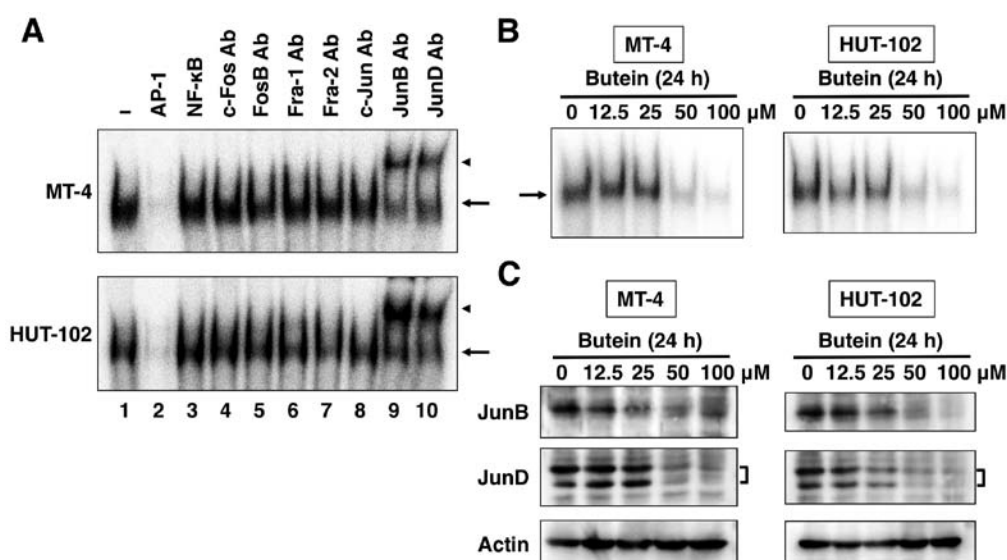


Figure 7. Butein inhibits constitutively active AP-1 in HTLV-1-infected T-cell lines. (A) AP-1 is composed of JunB and JunD subunits. Nuclear extracts from untreated cells were incubated with the indicated antibodies, unlabeled AP-1 or NF-κB oligo probe. They were then assayed for AP-1 activation by EMSA. Ab, antibody. Arrow and arrowhead show a specific complex and a supershifted band, respectively. (B) MT-4 and HUT-102 cells were treated with the indicated concentrations of butein for 24 h. The nuclear extracts were assayed for AP-1 activation by EMSA. Arrow shows a specific complex. (C) Butein downregulates the expression of JunB and JunD proteins. Cells were treated with the indicated concentrations of butein for 24 h. The total protein extracts were prepared for immunoblot analysis. Actin was used as the loading control.

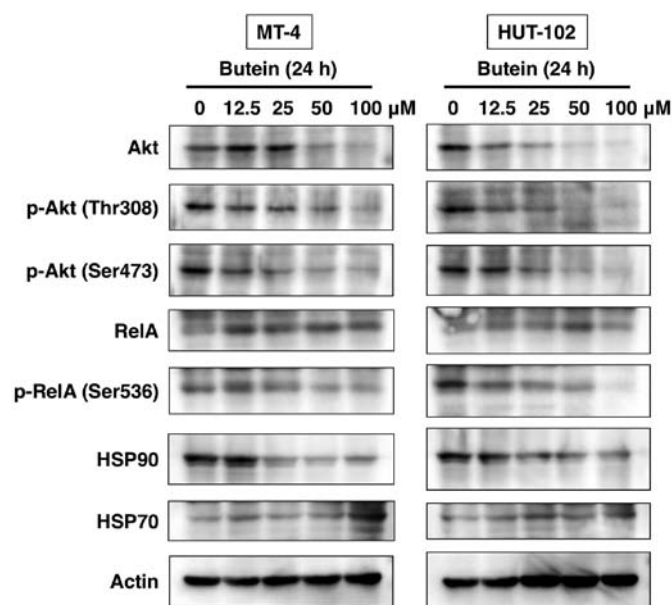


Figure 8. Effects of butein on Akt signaling pathway and HSP proteins. Cells were treated with the indicated concentrations of butein for 24 h. Cell lysates were separated by SDS-PAGE and protein levels of phosphorylated and total Akt and RelA, and HSPs were analyzed with immunoblotting. Actin was used as the internal control.

HUT-102 cell inoculation (Fig. 9B, left panel). At the same time point, the average tumor volume in the butein-treated group was 727 mm³. Tumor tissues were removed, photographed (Fig. 9A, right panel), and weighed. The mean tumor weight of butein-treated mice was significantly lower than that of vehicle-treated mice (Fig. 9B, middle panel). In addition, the body weight of mice remained stable with no significant differences between the two groups (Fig. 9B, right panel). At post-inoculation day 28, quantitative ELISA

used to determine the circulating levels of surrogate tumor markers sCD25 (24) and sCD30 (25) secreted by HUT-102 tumor xenografts showed 42% and 59% decrease in their levels, respectively, in butein-treated mice, compared with the control group (Fig. 9C). Considered together, our results showed that treatment with butein resulted in significant decreases in serum sCD25 and sCD30 of SCID mice.

Finally, H&E and TUNEL staining assays were performed to assess whether the inhibitory effect on tumor growth induced by butein was due to an increase in apoptotic cell death. H&E staining showed a significant increase in apoptotic tumor cells in butein-treated mice, compared with the control group, which were characterized by cytoplasmic condensation, chromatin hyperchromatism and condensation, and nuclear fragmentation. TUNEL assays confirmed these findings and demonstrated increased number of TUNEL-positive tumor cells in butein-treated mice (Fig. 9D).

Discussion

ATLL is classified into four clinical subtypes based on the clinical manifestations: acute, lymphoma, chronic and smoldering (26). Acute, lymphoma and unfavorable chronic types are considered the aggressive forms of ATLL, while favorable chronic and smoldering types are indolent ATLL (26). Various dietary and lifestyle factors may be involved in the development and progression of ATLL. Aggressive ATLL carries poor prognosis, with only 24% for 3-year overall survival rate in patients on standard combination chemotherapy (27). Therefore, there is a great need for novel therapeutic approaches for ATLL. Chemotherapeutic agents are usually highly toxic to normal tissues. To reduce the number of HTLV-1-infected T cells, natural products are suitable alternatives since they have minimal side-effects compared to synthetic drugs.

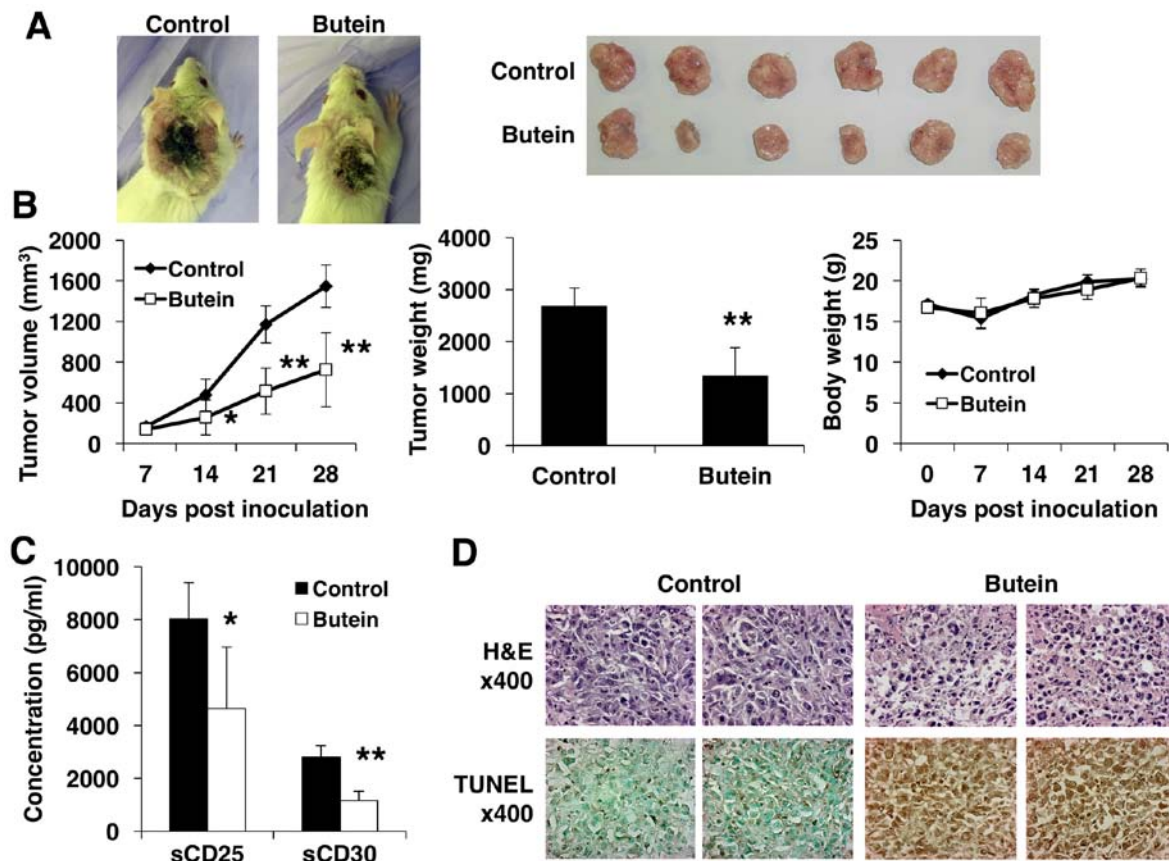


Figure 9. Antitumor effects of butein in HUT-102 tumor-bearing SCID mice. (A) HUT-102 cells were transplanted subcutaneously into the postauricular region of SCID mice (n=6). Mice were treated with butein (0.7 mg/kg) or vehicle three times a week (left panels). Visible HUT-102 xenograft tumors were harvested before sacrifice (right panel). (B) Serial changes in tumor volume (left panel) and body weight of mice (right panel) during the experiment. Weight of tumor tissues from the butein and control groups (middle panel). Data are mean \pm SD values. * $P < 0.05$ and ** $P < 0.01$, vs. vehicle-treated control. (C) Mean \pm SD values of serum concentrations of sCD25 and sCD30 in mice. * $P < 0.05$ and ** $P < 0.01$, vs. vehicle-treated control. (D) Cell apoptosis in tumor tissues determined by H&E and TUNEL staining. Magnification, $\times 400$.

In this study, we showed that butein decreased cell viability of HTLV-1-infected T-cell lines. The ability of the cells to divide is regulated by two classes of molecules, CDKs, a family of serine/threonine kinases and their binding partners, cyclins. The progress of the cells division process occurs through the interaction of various cyclins with their respective CDK subunits (28). Quiescent cells enter the cell cycle after mitogenic stimulation and upregulation of cyclins D and E during the G_1 phase of the cell cycle (29). Cyclin D is associated with CDK4 and CDK6 (30), while cyclin E is associated with CDK2 (31). Thus, cyclins and CDKs play important roles in DNA synthesis and cell division. In contrast, pRb controls cell cycle progression at the G_1 to S transition in response to certain signals for growth inhibition (32). pRb is phosphorylated by catalytic subunits of CDK4/CDK6 and CDK2 complexed with specific regulatory subunits of cyclins D and E, respectively. Treatment of HTLV-1-infected T-cell lines with butein reduced the expression levels of cyclin D1, D2, E, CDK4, CDK6 and phospho-pRb. Based on these findings, it is highly possible that butein causes G_1 arrest by blocking cyclin D-CDK4/6- and cyclin E-CDK2-mediated pRb phosphorylation, which is crucial to the progression of the cell cycle at the G_1 to S transition.

The process of apoptosis is controlled by various inter-related pathways, which ensure that caspases, the proteolytic

initiators, and executioners of apoptosis, are triggered only in cells requiring termination. We have shown in the present study the activation of caspase-3, -8 and -9 during the application of butein and that the addition of a general caspase inhibitor partially decreased butein-mediated cell death. These data provide evidence that butein-induced apoptosis was at least in part caspase-dependent in HTLV-1-infected T-cell lines. Butein also downregulated the protein expression of survivin, XIAP and c-IAP2, which are possible upstream factors of caspase proteins.

NF- κ B can modulate the transcriptional activation of genes associated with cell proliferation, metastasis, tumor promotion and suppression of apoptosis (33,34). Hence, agents capable of suppressing NF- κ B activation are therapeutically promising in inhibiting carcinogenesis. Our results suggest that the effects of butein on NF- κ B were mediated through inhibition of phosphorylation and the associated subsequent proteolysis of I κ B α since butein blocked phosphorylation and degradation of I κ B α . Furthermore, butein inhibited total and phospho-protein levels of IKK α and IKK β , and phosphorylation of RelA. Treatment of HTLV-1-infected T-cell lines with butein further inhibited NF- κ B-regulated gene products, survivin, XIAP, c-IAP2, cyclin D1, cyclin D2, CDK4 and CDK6.

Akt occupies a key regulatory node in the phosphoinositide 3-kinase pathway, below which the pathway branches

significantly to influence a wide range of cellular processes that promote cell cycle progression, cell growth and resistance to apoptosis by modulating the levels of survivin, XIAP, cyclin D1, cyclin D2 and cyclin E (19-21). Our results demonstrated that butein decreased total and phospho-protein levels of Akt in HTLV-1-infected T-cells. Akt is known to phosphorylate RelA at Ser536 through an IKK α - or IKK β -dependent mechanism (35). Butein may inhibit phosphorylation of RelA through inactivation of Akt and IKKs. In addition, AP-1 regulates the expression and function of various cell cycle regulators, such as cyclin D1 and cyclin E (36). JunB is also controlled by NF- κ B (37). Thus, NF- κ B and AP-1 can also collaborate in ATLL, depending on a variety of factors. Butein inhibited AP-1 signaling pathway via reduction of JunB and JunD. Considered together, these findings suggest that the effects of butein are mediated through a diverse range of molecular targets, which collectively result in inhibition of cell growth and survival.

Of note, butein profoundly inhibited HSP90 protein. Previous studies showed that HSP90 inhibitors simultaneously inactivated and destabilized several oncoproteins (e.g., IKKs, Akt and CDKs) in cancer cells (23,38). On the other hand, inhibition of HSP90 alone induces upregulation of another chaperone, HSP70 (38). Our results also showed that butein increased the expression of HSP70. HSP70 is known to be anti-apoptotic, and therefore the induction of HSP70 may ultimately limit the efficacy of HSP90 inhibitors (38). Collectively, these results suggest that butein also inhibits HSP90, thereby affecting various signaling pathways involved in the regulation of cell growth and survival.

To establish the relevance of these *in vitro* findings to the intact animal, we implanted SCID mice with HTLV-1-infected T cell line HUT-102. Treatment with butein significantly decreased HUT-102 growth in these mice and significantly reduced serum levels of sCD25 and sCD30 without affecting body weight. The results also showed upregulation of TUNEL-positive cells undergoing early apoptosis in tissues of mice treated with butein, relative to the control mice.

In conclusion, we demonstrated in the present study the effects of butein, a plant polyphenol, on the growth of HTLV-1-infected T-cell lines in both *in vitro* and in an *in vivo* animal model. Although this study warrants further investigation on ATLL primary samples, the results suggest that butein is a potentially valuable chemopreventive and/or chemotherapeutic agent against ATLL.

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