Japonicone A suppresses growth of Burkitt’s lymphoma cells through its effect on NF-κB

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Running title: JA, an anticancer agent via targeting NF-κB

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Abstract

Purpose: Nuclear factor-κB (NF-κB), a transcriptional regulator of diverse genes involved in cell survival, proliferation, adhesion, and apoptosis, has been implicated in various malignancies. We discover a potent natural NF-κB inhibitor, Japonicone A (JA), from traditional herb Inula japonica Thunb, evaluate its preclinical pharmacology and therapeutic activity, and investigate the underlying mechanisms of action for its anti-tumor activity.

Experimental Design: Various types of cancer and normal cells were exposed to JA for cytotoxicity screening, followed by determination of cell apoptosis and cell cycle arrest. Western blotting, immunostaining and gene reporter assay were used to analyze NF-κB activity. Two xenograft models were for therapeutic efficacy evaluation.

Results: JA killed cancer cells but had low cytotoxicity to normal cells. Burkitt's lymphoma (BL) cells were particularly sensitive. JA inhibited the growth and proliferation of Raji, BJAB, and NAMALWA lymphoma cells and resulted in G2/M phase arrest and apoptosis. Further, exposure of cells to JA caused inactivation of the TNFα-TAK1-IKK-NF-κB axis and inhibition of TNFα-stimulated NF-κB activity and nuclear translocation, followed by down-regulation of NF-κB target genes involved in cell apoptosis (bcl-2, bcl-XL, XIAP, TRAF2) and in the cell cycle and growth (cyclin D, c-Myc). Moreover, JA inhibited local growth and dissemination of cancer cells to multiple organs in vivo.

Conclusion: JA exerts significant anticancer effects on BL cells in vitro and in vivo via targeting the NF-κB signaling cascade. These results highlight the potential of JA as a chemotherapeutic agent and warrant for development of it as a drug for therapy of lymphomas.
Statement of Translational Relevance:

Aberrant NF-κB activation, with increased expression of pro-proliferative and anti-apoptotic genes, is a characteristic of various human lymphoid malignances, indicating that NF-κB is a target for development of therapeutic drugs for lymphoid malignances. Traditional herbal medicines are claimed to have therapeutic efficacy with minimal adverse effects, providing sources and platforms for developing front-line drugs. Here, we report that Japonicone A (JA), a natural compound from *Inula japonica* Thunb, possesses marked *in-vitro* and *in-vivo* antitumor activity against Burkitt's lymphoma via inactivating the TAK1-IKK-NF-κB axis. JA significantly inhibits Burkitt's lymphoma cell growth, induces cell apoptosis and cell cycle arrest with less cytotoxicity to multiple normal cells. JA also inhibits lymphoma cells localized growth and dissemination to multiple organs and tissues. The results showed that JA could be a promising new chemotherapeutic agent though targeting the NF-κB signaling cascade. Therefore the potential of the test drug may be beyond the lymphoid malignances therapy.
Introduction

Burkitt's lymphoma (BL), an aggressive form of B-cell non-Hodgkin's lymphomas, is commonly diagnosed in children and young adults, and, rarely, in middle-aged adults (1). For BL, the World Health Organization has identified three clinical variants: endemic, sporadic, and immunodeficient. About 95% of the endemic variant is associated with malaria or Epstein-Barr virus (EBV) infection, common in Equatorial Africa and Papua New Guinea. The sporadic type is predominant in non-malarial areas, such as north America and Europe, and, in these areas, accounts for 1%-2% of lymphomas in adults and 40% of lymphomas in children (2). The immunodeficient form is common in patients with HIV infections. Only 5-15% of sporadic cases and 40% of cases of the immunodeficient form are associated with EBV infections (3, 4).

A characteristic of BL, a rapidly proliferating neoplasm, is the chromosomal translocation t(8;14)(q24;q32), which is present in 70-80% of patients. This translocation induces the over-expression and constitutive activation of the oncogene, c-Myc, and, by abnormal transcriptional regulation of downstream genes, results in cellular transformation, inhibition of cell cycle checkpoints, and resistance to apoptosis (1, 5, 6). Currently, intensive chemotherapy including cyclophosphamide, doxorubicin, vincristine, and cytarabine, and targeted therapy with rituximab, have improved treatment outcomes, especially in children; however, for adults, the prognosis remains poor (1,7-9). Therefore, development of more effective chemotherapeutic agents to improve therapy for lymphoma is urgent.

Nuclear factor-κB (NF-κB), a transcription factor, is involved in lymphocyte development, activation, proliferation, and survival (10). It regulates genes involved in the activation of B and T lymphocyte cells, those involved in proliferation (cyclin D and c-Myc), and those involved in inhibition of apoptosis (Bcl-2, Bcl-XL, Bfl1/A1, cIAP, and XIAP) (10, 11). Aberrant NF-κB activation, with increased expression of pro-proliferative and anti-apoptotic genes, is a characteristic of various human lymphoid malignances (12, 13), such as Hodgkin’s lymphomas (HL) and...
non-Hodgkin’s lymphomas (NHL), including diffuse large B-cell lymphoma and BL. Constitutive NF-κB activation is also evident in lymphoid cells transformed by oncogenic viruses, such as EBV and human T-cell lymphotrophic virus-1 (HTLV-I) (14). Constitutive activation of NF-κB is involved in the anti-apoptotic mechanism of BL (15). c-Myc exerts its immune inhibitory activities via the NF-κB activation pathway and causes immune evasion of BL (16). Moreover, in BL, NF-κB appears to be necessary for the constitutive activation of translocated c-Myc and for increasing the expression of c-Myc via binding its recognition element on the Ig heavy-chain region (17). These results indicate that NF-κB is a target for development of therapeutic drugs for lymphoid malignances.

Traditional herbal medicines, containing various biologically active natural compounds, are claimed to have therapeutic efficacy with minimal adverse effects, providing sources and platforms for developing front-line drugs (18-21). Japonicone A (JA) is a natural product that we recently isolated from the aerial part of *Inula japonica* Thunb (22), a traditional medicine used to treat bronchitis, digestive disorders, diabetes, and inflammation. Some preparations from *Inula japonica* Thunb exhibit potent inhibition of NF-κB activity and thereby to exert an anti-inflammatory effect (23). We have previously evaluated the growth inhibitory effects of compounds isolated from *Inula japonica* Thunb on various cell lines (24). Here, we show that JA exhibits selective killing of cancer cells of various types but has more toxicity to BL cells. We further demonstrate that JA arrests lymphoma cells at the G2/M phase of the cell cycle and induces apoptosis mainly through inhibiting activation of the TNFα-TAK1-IKK-NF-κB signaling cascade. This leads to the inhibition of growth of cultured cells and the reduction of localized growth and dissemination of tumors in mice, demonstrating promising preclinical activity of JA for therapy of BL.
Materials and methods

Compounds and reagents
JA, with a purity >97% was isolated from *Inula japonica* Thunb in the Natural Products Laboratory at the Second Military Medical University, Shanghai, China. Cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). DC protein assay kits (500-0113) were obtained from Bio-Rad (Hercules, CA, USA), and the ECL plus system was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All cell culture supplies were from Invitrogen Gibco Co. (Grand Island, NY, USA)

Cell lines and cell cultures
Human BL cells, Raji, BJAB, and NAMALWA, and human Embryonic Kidney (HEK) 293T cell were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Raji, BJAB, and NAMALWA cells were cultured in RPMI1640 and 293T in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere with 5% CO₂. The details of cell lines used in this study are available in Supplementary Table S1. JA was dissolved in DMSO and diluted in cell culture media to a final concentration of ≤0.1%.

Cell viability assay
Cell viability was determined with Cell Counting Kit-8 (CCK-8), as instructed by the manufacturer. Briefly, cancer cells were seeded in 96-well plates and were either treated for 48 h with JA at serial concentrations, or were treated for various times (0, 24, 48 or 72 h). After treatment, CCK-8 solution (10 µl) was added to each well, followed by 3 h of incubation. The absorbance was recorded at an optical density of 450 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) to calculate the percentages of cell survival.
**Cell cycle and apoptosis analysis**

Propidium iodide (PI) staining was used to analyze DNA content and cell cycle distribution. After exposure to different concentrations of JA for 24 h, cells were harvested and fixed with 70% ethanol, followed by centrifugation (3000 rpm, 5 min), incubation with RNase (100 μg/ml) at 37°C for 30 min, and staining with PI (50 μg/ml in phosphate-buffered saline). The DNA content of cells and cell cycle distribution were analyzed by a Cell Lab Quanta™ SC flow cytometer (Beckman Coulter, USA).

Analyses for apoptosis were performed with an Annexin V-FITC Apoptosis Detection Kit (BioVision, CA, USA). Cells (5 × 10⁵) were exposed to different concentrations of JA for 24 h. They were collected by centrifugation and re-suspended in 500 μl of 1× Binding Buffer. Annexin V-FITC (5 μl) and PI (5 μl) were added to the cells. After incubation at room temperature for 5 min in the dark, cells were analyzed by fluorescence-activated cell sorting (FACS) using a FACSCan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Cells that stained positive for early apoptosis (Annexin V-FITC stained only) and for late apoptosis (Annexin V-FITC and PI stained) were combined for analysis.

**Western immunoblotting**

Immunoblotting was accomplished as described (25). In brief, the boiled protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to methanol pre-activated-PVDF membranes (Millipore). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 hr at room temperature and incubated with primary antibodies against corresponding proteins overnight at 4°C. Blots were washed three times in TBS-Tween 80 buffer, followed by incubation with the appropriate horseradish peroxidase-linked secondary antibodies for 1 h at room temperature. The proteins in the blots were visualized using the ECL plus system (Amersham Pharmacia Biotech, Buckinghamshire UK). The antibodies used in this study are listed in **Supplementary Table S2**.
Immunoprecipitation assay
Raji cells were treated with JA (1.0 μM) with or without TNFα (5 ng/ml) for 12 h. The cells were collected and lysed with RIPA lysis buffer (Millipore) and microcentrifuged for 10 min at 14,000×g, 4°C. A portion (200 μl) of the cell lysate was incubated with primary antibody overnight at 4°C. The indicated primer antibody was added, and the preparations were incubated with rocking for 1-3 hr at 4°C. After microcentrifugation, the pellets were collected, and the immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting to establish their interactions with other proteins.

Immunofluorescence analysis
Cytoplasmic-nuclear translocation of NF-κB p65 was analyzed by confocal microscopy according to the method described previously (26). Briefly, cells were grown on glass coverslips overnight, incubated with JA for 12 h, and then stimulated by TNFα for another 30 min. Treated cells were fixed and incubated with anti-NF-κB p65 primary antibody, followed by fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody (Invitrogen, Eugene, OR, USA) and 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and examined with a Zeiss Confocal Laser Scanning Microscope. Analyses for cytoplasmic-nuclear translocation of NF-κB p65 were accomplished with Thermo Scientific ArrayScan VTI HCS software.

Therapeutic efficacy of JA on localized and disseminated lymphoma xenografts
For the localized model, 2 ×10⁶ NAMALWA cells were injected subcutaneously into the right flanks of 4-week old female Balb/c nude mice (obtained from Shanghai Slac Laboratory Animal Co., Shanghai, China). Mice bearing tumors about 0.5 cm in diameter were randomized into control and treatment groups (n=6). For two weeks, these animals were dosed intraperitoneally with JA (30 mg/kg/day) or with vehicle. Tumor growth and body weights of the mice were monitored every other day. Tumor mass (weight in “g”) was determined by caliper measurements using the formula
“1/2a × b²”, where “a” is the long diameter, and “b” is the short diameter (in cm). For dissemination, 2 ×10⁶ NAMALWA cells were injected intravenously into the tail veins of 4-6 week old female NOD/SCID mice (Beijing HFK Bioscience Co., Ltd., Beijing, China). After 3 days, the mice were randomly separated into control and treatment groups (n=5) and dosed intraperitoneally with JA (30 mg/kg/day) or with vehicle for 3 weeks. In these models, tumors grew systemically and mice become paralyzed when tumor cells infiltrated the spinal cord, resulting in hind-leg paralysis. Mice were sacrificed at onset of paralysis. The spines and femoral bone were collected and fixed in Bouin’s solution (Sigma, USA) for 24 h. The animal use and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute for Nutritional Sciences.

HE staining

The spines and femoral bone were embedded in paraffin and sectioned into 4-μm slices. Sections were stained with hematoxylin and eosin. Separate sections containing three different areas in each tumor were examined whether dissemination of human lymphoma was present in a double-blinded manner by a skilled pathologist.

Statistical analysis

All of the values were recorded as the means ± s.e.m. from at least three independent experiments. Statistical significance was determined through a two-tailed Student’s t test, with a P value of <0.05 being considered statistically significant.
Results

For cells in culture, JA has an extensive tumor-killing effect and exhibits the most potent cytotoxicity to lymphoma cells.

JA is a natural product isolated from traditional herbal medicine, *Inula japonica* Thunb (Fig. 1A). Modern pharmacological evaluations have established that this herb medicine has potent cytotoxicity. We conducted a cell-based screening, examining the effects of JA on the viability of various tumor cells and normal cells (Fig. 1B; Supplementary Fig. S1 and Table S1). Nineteen human cancer cell lines and eight normal cell lines were exposed to various concentrations of JA (0-50 μM) for 48 h. Cell viability was determined by the CCK-8 assay. JA produced marked growth inhibition of human lymphoma, leukemia, colon, esophageal, gastric, liver, prostate, and ovarian carcinoma cells, with IC50 values of about 400 nM to 15 μM. Of these, lymphoma cells were most sensitive to the cytotoxic effect of JA (IC50 values: Raji, 800 nM; BJAB, 700 nM; NAMALWA, 400 nM) (Fig. 1B). Normal ovarian epithelial cells (IOSE) and normal liver cells (7702 and LO2), incubated with JA for 48 h, exhibited less cytotoxicity, indicating that JA selectively kills cancer cells.

JA inhibits cell growth, induces arrest in the G2/M phase of the cell cycle, and causes apoptosis in cultured human lymphoma cells.

Since lymphoma cells were most sensitive to JA, the antitumor efficacy of JA was evaluated, and its molecular mechanism of action in lymphoma cells was determined. In a dose- and time-dependent manner, JA had a strong inhibitory effect to Raji, BJAB, and NAMALWA lymphoma cells (Fig. 2A and B). To determine if JA inhibited cell cycle progression, Raji, BJAB, and NAMALWA cells were exposed to various concentrations of JA for 24 h, and the distribution of cells in the cycle was determined by PI staining and flow cytometric analysis. For all three types of lymphoma cells, JA induced cell cycle arrest in the G2/M phase in a concentration-dependent manner (Fig. 2C). Moreover, for all three types of cells, JA induced a dose-dependent increase in
cells undergoing apoptosis (Fig. 2D). The decline in mitochondrial membrane
potential is a characteristic of apoptosis. By staining with the fluorescent,
membrane-permeant JC-1 dye and performing flow cytometric analysis, which can be
used to estimate integrity and changes in membrane potential, a dose-dependent
dissipation of potential was found in the mitochondrial membranes after a 24-h
incubation of cells with JA (Supplementary Fig. S2), further establishing JA-induced
apoptosis. These results confirmed that the cytotoxicity of JA to lymphoma cells is
accomplished through arrest of cell cycle progression and induction of apoptosis.

JA inhibits NF-κB activity and TNFα-induced nuclear translocation.

In many types of tumors, NF-κB signaling has a critical role in cancer development
and progression (12, 27). Various NF-κB transcriptional target genes are involved in
cell apoptosis, cell cycle progression, and growth. Some sesquiterpene dimers isolated
from *Inula japonica* Thunb exhibit potent inhibitory activity on NF-κB activation (23).
To determine the effect of JA on the NF-κB signaling cascade, an NF-κB gene
reporter assay was conducted with 293T cells stably transfected with the
NF-κB-dependent luciferase reporter vector in the presence or absence of TNFα
stimulation. Cells exposed to JA showed inhibition of the background and
TNFα-induced NF-κB activity (Fig. 3A). To determine if JA reduced nuclear
translocation of NF-κB, laser scanning confocal microscopy was performed for 293T
cells stimulated with TNFα (Fig. 3B). The increase in nuclear translocation of NF-κB
p65 after stimulation with TNFα was reduced after exposure of cells to JA at serial
concentrations (Fig. 3B and C). These data confirmed the inhibitory effect of JA on
NF-κB activation.

JA inhibits the TNFα-TAK1-NF-κB signaling cascade in lymphoma cells.

As a mediator of the activated NF-κB signaling cascade, TAK1 (transforming growth
factor β–activated kinase 1) binds to the adaptor protein, TAB1, and subsequently
activates downstream signaling kinases, such as IKKα/β, MAPK, and JNK and
modulates the NF-κB dependent genes (28-30). Since disturbances in TAK1-NF-κB signaling are implicated in lymphoma progression, inhibition of the TAK1-NF-κB cascade could activate the intrinsic caspase pathway and lead to the apoptosis (31). To determine if TNFα-TAK1-NF-κB signaling is involved in the JA-induced inhibition of lymphoma cells, co-immunoprecipitation experiments were performed to examine the TNFα-induced binding activity between TAK1 and TAB1, an intermediate event in activation of the TNFα-NF-κB signaling cascade (30, 32). The increase in binding affinity of total TAK1 and phosphorylated TAK1 with TAB1 stimulated by TNFα was reduced after treatment of cells with JA (Fig. 4A). A further experiment confirmed that JA inhibited TNFα-induced TAK1 and IκBα phosphorylation in a concentration dependent manner among three BL cells (Fig. 4B). These results suggest that JA blocks the TNFα-induced interaction of TAB1 with TAK1 and inhibits recruitment of the TAK1/TAB1 complex, thereby mediating the inactivation of TAK1 and downstream signaling.

To evaluate the downstream signaling cascade after TAK1 inhibition, cells were exposed to JA and then stimulated with TNFα for various times. In JA-treated Raji cells, the TNFα-induced phosphorylations of IKKα/β, IκBα, NF-κB p65, ERK, JNK/SAPK, and p38 were reduced relative to that in cells without JA exposure (Fig. 4C). Moreover, the nuclear and cytoplasmic extraction assay (Fig. 4D) and immunostaining assay (Fig. 4E) were both demonstrated that JA prevented the nuclear translocation of p65 in lymphoma cells, a characteristic of NF-κB inactivation. These data suggest that JA inhibits TNFα-induced NFκB activity via impairment of the TAK1/TAB1 complex and inactivation of downstream IKKα/β, MAPK, and JNK signaling.

**JA suppresses NF-κB transcriptional target genes involved in cell growth, apoptosis, and cell cycle progression.**

Evasion of apoptosis is an indispensable contribution of NF-κB in the pathogenesis of human tumors, including hematologic malignancies, by triggering expression of
transcriptional target anti-apoptotic proteins, such as BCL-2, BCL-XL, cIAPs, and XIAP, (33, 34). It was apparent that, for lymphomas, JA induced cell cycle arrest and apoptosis via inhibiting NF-κB signaling. To test this concept, the expression of NF-κB p65 and its main target proteins involved in apoptosis and cell cycle progression were measured in JA-treated lymphoma cells by Western blot assays. JA inhibited the total p65 expression, especially that of NAMALWA cells, and decreased the expression of bcl-2 (BJAB with bcl-2 negativity) and bcl-XL, as well as XIAP (Fig. 4F), and further triggered the intrinsic caspase pathway via releasing cytochrome c, decreasing inactivated caspase-3 (pro-caspase 3) and cleaving PARP (Fig. 4F).

TRAF2, an NF-κB target gene involved in anti-apoptosis signaling through interacting with the IAP family members (29), was also down-regulated in all three types of lymphoma cells treated with JA (Fig. 4F).

After activation, NF-κB is translocated to the nucleus and activates transcription of genes responsible for cell growth, such as cyclin D1 and c-Myc, which are over-expressed and required for malignant transformation and growth of BL (6, 35). JA treatment of Raji, BJAB, and NAMALWA lymphoma cells also resulted in a reduction of cyclin D1 and c-myc (Fig. 4F).

Entry into cell mitosis is blocked by G2/M checkpoint mechanisms when NF-κB signaling is inhibited through RNAi-mediated NF-κB p65 silencing or by chemical inhibitors (36-39). Regulation of the G2/M transition is dependent on activation of the CDK1/cyclinB1 complex (40). Western blotting showed that JA treatment resulted in a reduction of cyclin B1 and CDK1 in a dose-dependent manner (Fig. 4F), suggesting that JA disturbs the CDK1/cyclin B1 complex to trigger arrest in the G2/M phase of cell cycle, as aforementioned.

Constitutive activation of NF-κB through IκBα down-regulation eliminates the inhibitory effect of JA on cell growth.

Down-regulation of the NF-κB inhibitor, IκBα, is required for canonical NF-κB
activation (41, 42). Thus, constitutive activation of NF-κB through RNAi-mediated IκBα down-regulation should reduce the inhibitory effect of JA on cell growth. Considering the reduction of NF-κB activity and nuclear translocation in 293T cells (Fig. 3) and their sensitivity to lentivirus infection, we selected 293T cells to examine this concept. Efficient knockdown of IκBα resulted in an increase of NF-κB p65 expression and phosphorylation (Fig. 5A) as well as in the expression of the NF-κB target gene, cyclin D1 (Fig. 5A), showing activation of the NF-κB signal cascade. In 293T cells, constitutive activation of NF-κB by either of two independent lentivirus-mediated shRNAs conferred resistance to JA (Fig. 5B). These results indicate that JA exerts an inhibitory effect on cell growth via inactivation of NF-κB signaling.

JA has therapeutic efficacy for B-cell NHL xenografts.

The effects of JA on growth of xenografts of human NAMALWA tumors and dissemination of these cells were evaluated. This cell line was selected, as it was more sensitive to JA. BALB/c nude mice bearing subcutaneous xenografts of NAMALWA cells were treated with JA (30 mg/kg body weight) for two weeks. JA inhibited tumor growth by 65.4% relative to control mice treated with the vehicle (p< 0.001) (Fig. 6A). Autopsies at the end of therapy showed that JA-treated mice had much smaller tumor masses (Fig. 6B; Supplementary Fig. S3A). The protein expression profiles of NAMALWA tumor tissues from 4 individual animals treated with JA revealed down-regulation of NF-κB p65 expression, increased cleavage of PARP and decreased expression of the NF-κB-initiated genes, XIAP and c-Myc, as well as a slightly reduced expression of bcl-2 (Fig. 6C), which is consistent with results derived for cell cultures. This compound caused no observable toxic effects on mice, and body weights were not changed (Supplementary Fig. S3B).

Human lymphomas exhibit a wide spectrum of growth patterns, from limited to widespread dissemination and invasion (1). With NAMALWA cells, these observations were extended by investigating the therapeutic efficacy of JA on tumor
dissemination and progression. In the dissemination model, immunocompromised NOD/SCID mice, injected intravenously with NAMALWA cells, received JA (30 mg/kg) for 3 weeks. During therapy, four of five mice with vehicle treatment developed severe posterior paralysis; only two mice treated with JA exhibited a modest posterior paralysis (Fig. 6D). Mice were killed after therapy and analyzed, by gross tissue and histological analyses, for hematogenous dissemination of tumors and for invasion to secondary sites. In mice treated with the vehicle, there was, in the spinal core, ganglion spinale, peripheral nerve tract, spatium intermusculare around spine and femoral bone, a diffuse infiltration of atypical lymphoid cells that were round and medium-sized (Fig. 6D and E; Supplementary Fig. S3D-G). Mice treated with JA developed little or no lymphoma dissemination to these tissues and organs (Fig. 6D and E; Supplementary Fig. S3D-G), confirming the therapeutic effect of JA on relieving limb paralysis and hematogenous dissemination and invasion. Moreover, vehicle and JA-treated mice were analyzed for lymphoma invasion of major organs such as lung, ovary, liver, kidney and adrenal gland. In these mice, ovarian metastases were present (Fig. 6F), and such metastases are found in lymphomas and in gastric, colorectal, and breast cancers (43-45). In contrast, mice treated with JA exhibited fewer ovarian metastases (Fig. 6F; Supplementary Fig. S3C). Moreover, JA prevented lymphoma invasion of the adrenal glands (Data not shown). No other metastases were evident.

These results demonstrate that JA has anti-tumor activity and therapeutic efficacy in both localized and disseminated lymphoma xenografts models.
Discussion

Although traditional chemotherapy remains the mainstay in human cancer treatment, the response rates to most chemotherapeutic agents are low, and clinical improvement is marginal. Additionally, severe toxicities and drug resistance often occur, reducing the quality of life for patients and hindering the effective application of these agents. Because of their rich structural diversity and promising therapeutic applications, natural products and their derivatives have caught the attention of pharmacologists and chemists (18, 19). We have been interested in developing new, effective, and safe drugs from natural products for cancer therapy (25, 46-48). *Inula japonica* Thunb is a traditional medicinal herb used for treatment of bronchitis, digestive disorders, diabetes, and inflammation. We have reported that some constituents from this herb exhibit anticancer activity, although their underlying mechanisms of action remain to be elucidated (24).

Herein, we reported that JA, a natural product isolated from *Inula japonica* Thunb, exhibited potent anticancer activity, with less cytotoxicity to normal cells. BL cells showed the most sensitivity to this compound, with IC50 values of 400-800 nM. JA exerted potent anti-cancer effects on Raji, BJAB, and NAMALWA lymphoma cells by inhibiting cell growth and proliferation, arresting cells in the G2/M phase of cell cycle by interrupting the CDK1/cycling B complex, and inducing cell apoptosis in a dose-dependent manner. JA, a newly discovered dimeric sesquiterpene lactone, contains a lactone ring conjugated with an exomethylene group. Many natural NF-κB inhibitors contain a similar chemical structure, which reacts with biological nucleophiles, especially the sulfhydryl group of cysteine residues via a Micheal-type reaction (49). We also reported that JA inhibits TNFα-induced NF-κB activation and nuclear translocation by interrupting the upstream TAK1/TAB1 complex (40) and suppressing the IKKa/β-IkBα-NF-κB signaling axis, leading to the reduction of NF-κB-initiating genes, such as bcl-2, bcl-xL, XIAP, and TARF2 and cyclin D and c-Myc, which are involved, respectively, in anti-apoptosis and cell growth (12). These results indicated that JA exerts its antitumor effect through targeting the canonical
NF-κB pathway. Indeed, constitutive activation of NF-κB via silencing the expression of the NF-κB inhibitor, IκBα, resulted in the elimination of growth inhibition by JA in 293T cell. Thus, these results reveal the function of JA as an NF-κB inhibitor. Although NF-kB can be inhibited in both cancer cells and normal cells, the consequence of such inhibition may be significantly different between cancer cells and normal cells, which may be related to the addiction of NF-kB in cancer cells (with stronger effects on cancer cells). Fig. 6G depicted our proposed model for the effects of JA on BL cells and the mechanisms of action, based on the aforementioned in vitro and in vivo findings.

The capacity of the transcription factor NF-κB to regulate pro-survival and anti-apoptosis signaling makes it a promising target for cancer therapy. In various human lymphoid malignances, there is constitutive activation of NF-κB, which leads to uncontrolled growth, anti-apoptosis, and immune evasion. Activation of the IKK-NF-κB signaling axis by a variety of pathways, ranging from inflammation cytokines to DNA damage induce its target gene expression to promote either the growth and survival of cancer cells or to enhance apoptosis via a tumor suppressor mechanism (12). This alternative phenomenon is also found in lymphoma cells (31, 50). Other genes or pathways, such as the tumor suppressor genes, p53 and PTEN, are involved in the determination of which effects dominate and the fate of cells after NF-κB activation or inactivation (12).

Human lymphomas, including BL, are characterized by rapid growth and widespread invasion. The extranodal spread occurs in a variety of organs, particularly the bone marrow, gastrointestinal tract, and central nervous system (CNS) (1). Through blocking NF-κB activation, JA inhibited localized growth and extranodal dissemination to multiple organs and tissues, such as spinal core, spium intermusculare around spine, bone marrow and ovaries, and prevented the development of hind-leg paralysis in two mouse models of lymphoma. The therapeutic efficacy of JA in B-cell NHL xenografts merits its evaluation for the treatment for lymphoid malignancies. Currently, clinical effective treatment of Burkitt’s lymphoma is usually based on intensive chemotherapy in varying
combinations of DNA damage agents such as cyclophosphamide and cytarabine and tubulin inhibitor such as vincristine (1,7-9). This enlightens us to test the effects of JA on microtubule dynamics and DNA integrity, and improve the therapeutic regimes through possible combination chemotherapy with these agents.

In summary, we have demonstrated that JA, derived from *Inula japonica* Thunb, has potent and selective anti-lymphoma activity by inducing cell apoptosis and cell cycle arrest through inactivating the TAK1-IKK-NF-κB axis. Although additional pharmacological and toxicological studies are needed, our results highlight the potential of JA as a chemotherapeutic agent targeting lymphomas and other NF-κB associated tumors.
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Conflict-of-interest disclosure:

The authors declare no actual or potential competing financial interests.
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Figure legends

Figure 1. Selective killing effect of JA on cancer cells. (A) Chemical structure of JA. (B) 50% inhibitory concentrations (IC50) of JA for various cancer cells and normal cells. Normal cells, including human ovarian epithelial cells (IOSE144), human hepatic immortal cells (HL-7702 and LO2), immortalized human bronchial epithelial cells (BEAS-2B), human gastric epithelial cells (GES-1), human umbilical vein endothelial cells (HUVEC), mouse embryonic fibroblasts (MEF), and a variety of human cancer cell lines were treated with JA (0-50 μM) for 48 h. Cytotoxicity was assessed with a CCK-8 assay, and IC50 values for JA on multiple cell lines were calculated. The results are representative of at least three independent experiments.

Figure 2. Inhibition of lymphoma cell growth and induction of dose-dependent G2/M cell cycle arrest and cell apoptosis by JA. (A) Viability of BL cells, Raji, BJAB, and NAMALWA, after exposure to JA at various concentrations for 48 h. (B) Viability of Raji cells after exposure to JA at various concentrations for 24 h, 48 h, and 72 h. (C) Effects of JA at various concentrations on the cell cycle of lymphoma cells (Raji, BJAB, and NAMALWA) treated for 24 h. The results are presented as the percent distributions for specific phases. (D) Apoptosis of Raji, BJAB, and NAMALWA cells exposed to different concentrations of JA for 24 h. Apoptotic cells were determined by flow cytometry using Annexin V. The data shown are representative of values from at least three independent experiments with similar results (mean ± s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001 versus the control).

Figure 3. JA suppression of TNFα-induced NF-κB activation. (A) NF-κB reporter assay for 293T cells exposed to JA. The Renilla luciferase control vector was co-expressed with the NF-κB reporter luciferase construct in 293T cells. Later (24 h), cells were incubated with JA (2.5 μM) for 16 h followed by treatment with or without TNFα (10 ng/ml), and luciferase activity was measured and normalized by use of a dual luciferase reporter assay (mean ± s.e.m. in three separate experiments, * P < 0.05,
*** P < 0.001). (B) Immunofluorescent staining of NF-κB p65 in 293T cells after treatment with the indicated concentrations of JA for 16 h, followed by stimulation with or without TNFα (10 ng/ml). Green signaling represents p65 staining and blue indicates nuclei stained with DAPI. Scale bar, 50 μm. Data are representative of three or more experiments with similar results. (C) Analysis of cytoplasmic-nuclear translocation of NF-κB p65 through Thermo Scientific ArrayScan VTi HCS software (mean ± s.e.m., ** P < 0.01, versus the control, respectively).

Figure 4. Suppression of the NF-κB signaling cascade in JA-induced growth inhibition and apoptosis of lymphoma cells. (A) The effect of JA on the binding affinity of total and phosphorylated TAK1 and TAB1 stimulated with TNFα. Raji cells were exposed to JA (1 μM) for 12 h, then stimulated with TNFα (5 ng/ml) for 30 min. Immunoprecipitation assays were performed to evaluate the binding affinity of endogenous TAK1-TAB1. (B) Dose-dependent effect of JA on activation of genes in the NF-κB signaling cascade in lymphoma cells. Hsp90 was used as loading control. (C) Expression of genes in the NF-κB signaling cascade in Raji cells exposed to JA (2.5 μM) for 2 h and TNFα (10 ng/ml) for different time intervals. The effect of JA on the NF-κB signaling cascade was determined by western blot analyses of phosphorylated and total IKKα/β, IκBα, p38, JNK, ERK, and p65 with β-actin as loading control. (D) NF-κB p65 gene expression in the cytoplasm and nucleus of JA-treated Raji and NAMALWA cells. Cells were pretreated with the indicated concentrations of JA for 16 h and stimulated with TNFα (20 ng/ml). Proteins were extracted from the cytoplasm and nucleus, with β-tubulin and histone 3, respectively, as the internal standards. (E) Immunostaining of NF-κB p65 in Raji cells after treatment with JA (1 μM) for 16 h, followed by stimulation with or without TNFα (10 ng/ml) for 30 min. Scale bar, 20 μm. (F) Expression of NF-κB p65 and its main target genes mediating cell growth, the cell cycle, and apoptosis. Raji, BJAB, and NAMALWA cells were exposed to various concentrations of JA for 24 h and NF-κB-related and target genes were analyzed by western blot assays with GAPDH and Hsp90 as loading control.
Figure 5. NF-κB activation through IκB silencing confers JA resistance in 293T cell. (A) Constitutive activation of NF-κB signaling via lentivirus-mediated silencing of IκBα. Immunoblots show expression of IκBα and NF-κB signaling proteins in 293T cells with knocked-down IκBα. (B) Viability of 293T cells with scramble or IκBα shRNA transfection after being treated with various concentrations of JA. Data are shown as means ± s.e.m. for three separate experiments.

Figure 6. JA Inhibition of localized growth and dissemination to multiple organs of NAMALWA cells. (A) JA Inhibition of localized growth of NAMALWA tumors. NAMALWA cells were transplanted into the right sides of nude mice, which were intraperitoneally dosed daily with JA (30 mg/kg) for two weeks. Tumor volumes were measured (mean ± s.e.m., n=6; * P< 0.05, ** P< 0.01, *** P< 0.001 versus the control treatment). (B) The weights of tumors harvested from mice treated with the vehicle or JA. (C) Western blotting of NF-κB signaling cascade proteins involved in cell growth and apoptosis in tumor tissues. The Arabic numbers indicate individual tumors. (D) NOD/SCID mice engrafted intravenously with NAMALWA cells and treated with JA (30 mg/kg) or vehicle (n=5). HE staining of the paraffin sections of abdominal vertebra was analyzed for the disseminated lymphoma cells (scale bar, 400 μm). Numbers and condition of mice with posterior paralysis were shown. The black asterisk indicted areas of lymphoma invasion. (E) Lymphoma dissemination was observed in several organs and tissues of vehicle-treatment mice as demonstrated by invasion of lymphoma cells in spinal cord, ganglion spinale, spatium intermusclare, bone marrow and femoral bone and disruption of bone trabecula in spine. The black arrow indicted the invade lymphoma cells (Scale bar in black and red color, respectively 50 μm and 200 μm). No evidence of human lymphoma dissemination was observed in JA-treatment group. (F) The gross ovarian metastasis of lymphoma was also determined. (G). Cartoon of the proposed mechanisms of the effect of JA on BL cells. JA exerts its anti-cancer activity on lymphoma cells by inhibiting cell growth and proliferation, arresting cells in the G2/M phase of the cell cycle, inducing
apoptosis through interrupting the upstream TAK1/TAB1 complex, and suppressing the IKKα/β-IκBα-NF-κB signaling axis.
Figure 1

A

B

IC 50

- Normal cells
- Cancer cells

Concentration (μM)
Figure 4

Panel A: Western blot analysis of TAK1 and TAB1 expression in response to TNFα and JA treatment.

Panel B: Comparison of TAK1, p-TAK1, and IκBα expression in Raji, BJAB, and NAMALWA cells treated with TNFα and JA.

Panel C: Time course of NF-κB p65, IκBα, and β-actin expression in response to TNFα and JA.

Panel D: Cytoplasmic and nuclear localization of NF-κB p65, Histone H3, β-tubulin, and Hsp90 in Raji and NAMALWA cells treated with TNFα and JA.

Panel E: Metabolic activity of cells treated with TNFα and JA as assessed by MTT assay.

Panel F: Western blot analysis of pro-caspase 3, PARP, Bcl-2, Bcl-xL, XIAP, TRAF2, cyclin D1, cyclin B1, CDK1, c-myc, Hsp90, and GAPDH expression in Raji, BJAB, and NAMALWA cells treated with TNFα and JA.
Figure 5

B

Cell growth (% of control)

Concentration (μM)

293T

3

2

1

0

120 100 80 60 40 20 0

IKBα shRNA Ctrl shRNA

IKBα sh1

IKBα sh2

NF-kB p65

NF-kB p65

Cyclin D1

Hsp90

A

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Figure 6

A. Tumor mass (mg) vs. Treatment days (d)

B. Tumor weight graph

C. Western blot analysis of control and JA treatments

D. Normal, Control, JA treatment posterior paralysis number: 4/5 (severe) 2/5 (modest)

E. Histology images of spinal cord, ganglion spinalis, bone trabecula, intermusculare, bone marrow, femoral bone under control and JA treatment conditions

F. Image showing ovarian metastatic tumor under control and JA treatment conditions

G. Schematic diagram illustrating the interaction between Japonic one A and NF-κB pathways, highlighting cell cycle regulation and apoptosis.
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Japonicone A suppresses growth of Burkitt's lymphoma cells through its effect on NF-κB

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