Japonicone A Suppresses Growth of Burkitt Lymphoma Cells through Its Effect on NF-κB

Xiaoguang Li, Xinying Yang, Yanling Liu, Nuoxi Gong, Wenbo Yao, Peizhan Chen, Jiangjiang Qin, Huizi Jin, Jingquan Li, Ruiai Chu, Lei Shan, Ruiwen Zhang, Weidong Zhang, and Hui Wang

Abstract

**Purpose:** NF-κB, a transcriptional regulator of diverse genes involved in cell survival, proliferation, adhesion, and apoptosis, has been implicated in various malignancies. We discovered a potent natural NF-κB inhibitor, Japonicone A, from the traditional herb *Inula japonica* Thunb, evaluated its preclinical pharmacology and therapeutic activity, and investigated the underlying mechanisms of action for its antitumor activity.

**Experimental Design:** Various types of cancer and normal cells were exposed to Japonicone A for cytototoxicity 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 used for therapeutic efficacy evaluation.

**Results:** Japonicone A killed cancer cells but had low cytocytotoxicity to normal cells. Burkitt lymphoma cells were particularly sensitive. Japonicone A inhibited the growth and proliferation of Raji, BJAB, and NALM-6 lymphoma cells and resulted in G2–M phase arrest and apoptosis. Furthermore, exposure of cells to Japonicone A caused inactivation of the TNF-α–TAK1–IKK–NF-κB axis and inhibition of TNF-α–stimulated NF-κB activity and nuclear translocation, followed by downregulation 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, Japonicone A inhibited local growth and dissemination of cancer cells to multiple organs in vivo.

**Conclusion:** Japonicone A exerts significant anticancer effects on Burkitt lymphoma cells in vitro and in vivo through targeting of the NF-κB signaling cascade. These results highlight the potential of Japonicone A as a chemotherapeutic agent and warrant its development as a therapy for lymphomas. *Clin Cancer Res; 19(11); 2917–28. ©2013 AACR.*

Introduction

Burkitt lymphoma, an aggressive form of B-cell non-Hodgkin lymphomas (NHL), is commonly diagnosed in children and young adults, and, rarely, in middle-aged adults (1). For Burkitt lymphoma, the World Health Organization has identified 3 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 nonmalarial areas, such as north America and Europe, and, in these areas, accounts for 1% to 2% of lymphomas in adults and 40% of lymphomas in children (2). The immunodeficient form is common in patients with HIV infections. Only 5% to 15% of sporadic cases and 40% of cases of the immunodeficient form are associated with EBV infections (3, 4).

A characteristic of Burkitt lymphoma, a rapidly proliferating neoplasm, is the chromosomal translocation t (8;14)(q24;q32), which is present in 70% to 80% of patients. This translocation induces the overexpression 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,
Translational Relevance

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

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.

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; refs. 10, 11). Aberrant NF-κB activation, with increased expression of proproliferative and antiapoptotic genes, is a characteristic of various human lymphoid malignances (12, 13), such as Hodgkin lymphomas (HL) and NHL, including diffuse large B-cell lymphoma and Burkitt lymphoma. 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; ref. 14). Constitutive activation of NF-κB is involved in the antiapoptotic mechanism of Burkitt lymphoma (15). c-Myc exerts its immune inhibitory activities via the NF-κB activation pathway and causes immune evasion of Burkitt lymphoma (16). Moreover, in Burkitt lymphoma, NF-κB seems to be necessary for the constitutive activation of translocated c-Myc and for increasing the expression of c-Myc via binding of its recognition element on the immunoglobulin (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 first-line drugs (18–21). Japonicone A 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 Japonicone A exhibits selective killing of cancer cells of various types but has more toxicity to Burkitt lymphoma cells. We further show that Japonicone A arrests lymphoma cells at the G2–M phase of the cell cycle and induces apoptosis mainly by inhibiting activation of the TNF–α–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, showing promising preclinical activity of Japonicone A for therapy for Burkitt lymphoma.

Materials and Methods

Compounds and reagents

Japonicone A, with a purity more than 97% was isolated from *Inula japonica* Thunb in the Natural Products Laboratory at the Second Military Medical University (Shanghai, PR China). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories. DC protein assay kits (500–0113) were obtained from Bio-Rad, and the enhanced chemiluminescence (ECL) plus system was purchased from Amersham Pharmacia Biotech. All cell culture supplies were from Invitrogen Gibco Co.

Cell lines and cell cultures

Human Burkitt lymphoma cells, Raji, BJAB, and NAMALWA, and human embryonic kidney (HEK) 293T cell were obtained from the American Type Culture Collection. Raji, BJAB, and NAMALWA cells were cultured in RPMI-1640 and 293T in Dulbecco’s modified Eagle medium supplemented with 10% FBS, 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. Japonicone A was dissolved in dimethyl sulfoxide (DMSO) and diluted in cell culture media to a final concentration of 0.1% or less.

Cell viability assay

Cell viability was determined with CCK-8, as instructed by the manufacturer. Briefly, cancer cells were seeded in 96-well plates and were either treated for 48 hours with Japonicone A at serial concentrations, or were treated for various times (0, 24, 48, or 72 hours). After treatment, CCK-8 solution (10 μL) was added to each well, followed by 3 hours of incubation. The absorbance was recorded at an optical density of 450 nm using a SpectraMax190 microplate reader (Molecular Devices) 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 Japonicone A for 24 hours, cells were harvested and fixed with 70% ethanol, followed by centrifugation (3,000 rpm, 5 minutes), incubation with RNase (100 µg/mL) at 37°C for 30 minutes, and staining with PI (50 µg/mL in PBS). The DNA content of cells and cell-cycle distribution were analyzed by a Caliper Quanta SC flow cytometer (Beckman Coulter).

Analyses for apoptosis were conducted with an 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 previously (25). In brief, the boiled protein extracts were separated by SDS-PAGE, and transferred to methanol-precipitated-polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked with 5% nonfat dry milk in TBS–Tween 80 buffer, then incubated with primary antibodies against corresponding proteins overnight at 4°C. Blots were washed 3 times in TBS–Tween 80 buffer, followed by incubation with the appropriate horseradish peroxidase–linked secondary antibodies for 1 hour at room temperature. The proteins in the blots were visualized using the ECL plus system (Amersham Pharmacia Biotech). The antibodies used in this study are listed in Supplementary Table S2.

Immunoprecipitation assay

Raji cells were treated with Japonicone A (1.0 µmol/L) with or without TNF-α (5 ng/mL) for 12 hours. The cells were collected and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Millipore) and microcentrifuged for 10 minutes 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 to 3 hours 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 Japonicone A for 12 hours, and then stimulated by TNF-α for another 30 minutes. Treated cells were fixed and incubated with anti-NF-κB p65 primary antibody, followed by FITC-labeled goat anti-rabbit immunoglobulin G (IgG) antibody (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories), 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 Japonicone A on localized and disseminated lymphoma xenografts

For the localized model, 2 × 10^6 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.). Mice bearing tumors about 0.5 cm in diameter were randomized into control and treatment groups (n = 6). For 2 weeks, these animals were dosed intraperitoneally with Japonicone A (30 mg/kg/d) 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 × b^2”, where “a” is the long diameter and “b” is the short diameter (in cm). For dissemination, 2 × 10^6 NAMALWA cells were injected intravenously into the tail vein of 4- to 6-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Beijing HFK Bioscience Co., Ltd.). After 3 days, the mice were randomly separated into control and treatment groups (n = 5) and dosed intraperitoneally with Japonicone A (30 mg/kg/d) 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) for 24 hours. 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.

H&E staining

The spines and femoral bone were embedded in paraffin and sectioned into 4-μm slices. Sections were stained with hematoxylin and eosin (H&E). Separate sections containing 3 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 mean ± SEM from at least 3 independent experiments. Statistical significance was determined through a two-tailed Student t test, with a P value of less than 0.05 being considered statistically significant.
**Results**

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

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

**Japonicone A inhibits cell growth, induces arrest in the G₂-M phase of the cell cycle, and causes apoptosis in cultured human lymphoma cells**

Because lymphoma cells were most sensitive to Japonicone A, the antitumor efficacy of Japonicone A was evaluated, and its molecular mechanism of action in lymphoma cells was determined. In a dose- and time-dependent manner, Japonicone A had a strong inhibitory effect to Raji, BJAB, and NAMALWA lymphoma cells (Fig. 2A and B). To determine if Japonicone A inhibited cell-cycle progression, Raji, BJAB, and NAMALWA cells were exposed to various concentrations of Japonicone A for 24 hours, and the distribution of cells in the cycle was determined by PI staining and flow-cytometric analysis. For all 3 types of lymphoma cells, Japonicone A induced cell-cycle arrest in the G₂–M phase in a concentration-dependent manner (Fig. 2C). Moreover, for all 3 types of cells, Japonicone A 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 conducting 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-hour incubation of cells with Japonicone A (Supplementary Fig. S2), further establishing Japonicone A–induced apoptosis. These results confirmed that the cytotoxicity of Japonicone A to lymphoma cells is accomplished through arrest of cell-cycle progression and induction of apoptosis.

**Japonicone A inhibits NF-κB activity and TNF-α–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 Japonicone A 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 TNF-α stimulation. Cells exposed to Japonicone A showed inhibition of the background and TNF-α–induced NF-κB activity (Fig. 3A). To determine if Japonicone A reduced nuclear translocation of NF-κB, laser scanning confocal
microscopy was conducted for 293T cells stimulated with TNF-α (Fig. 3B). The increase in nuclear translocation of NF-κB p65 after stimulation with TNF-α was reduced after exposure of cells to Japonicone A at serial concentrations for 24 hours. B, viability of Raji cells after exposure to Japonicone A at various concentrations for 24, 48, and 72 hours. C, effects of Japonicone A at various concentrations on the cell cycle of lymphoma cells (Raji, BJAB, and NAMALWA) treated for 24 hours. The results are presented as the percentage distributions for specific phases. D, apoptosis of Raji, BJAB, and NAMALWA cells exposed to different concentrations of Japonicone A for 24 hours. Apoptotic cells were determined by flow cytometry using Annexin V. The data shown are representative of values from at least 3 independent experiments with similar results (mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001 vs. the control).

**Figure 2.** Inhibition of lymphoma cell growth and induction of dose-dependent G2–M cell-cycle arrest and cell apoptosis by Japonicone A. A, viability of Burkitt lymphoma cells, Raji, BJAB, and NAMALWA, after exposure to Japonicone A at various concentrations for 48 hours. B, viability of Raji cells after exposure to Japonicone A at various concentrations for 24, 48, and 72 hours. C, effects of Japonicone A at various concentrations on the cell cycle of lymphoma cells (Raji, BJAB, and NAMALWA) treated for 24 hours. The results are presented as the percentage distributions for specific phases. D, apoptosis of Raji, BJAB, and NAMALWA cells exposed to different concentrations of Japonicone A for 24 hours. Apoptotic cells were determined by flow cytometry using Annexin V. The data shown are representative of values from at least 3 independent experiments with similar results (mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001 vs. the control).

**Japonicone A inhibits the TNF-α–TAK1–NF-κB signaling cascade in lymphoma cells**

As a mediator of the activated NF-κB signaling cascade, TAK1 (TGF-β–activated kinase 1) binds to the adaptor protein, TAB1, and subsequently activates downstream signaling kinases, such as inhibitor of IκB kinase (IKK)α/β, mitogen-activated protein kinase (MAPK), and c-jun-NH2-kinase (JNK) and modulates the NF-κB–dependent genes (28–30). Because 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 TNF-α–TAK1–NF-κB signaling is involved in the Japonicone A–induced inhibition of lymphoma cells, coimmunoprecipitation experiments were carried out to
examine the TNF-α–induced binding activity between TAK1 and TAB1, an intermediate event in activation of the TNF-α–NF-κB signaling cascade (30, 32). The increase in binding affinity of total TAK1 and phosphorylated TAK1 with TAB1 stimulated by TNF-α was reduced after treatment of cells with Japonicone A (Fig. 4A). A further experiment confirmed that Japonicone A inhibited TNF-α–induced TAK1 and IκBα phosphorylation in a concentration-dependent manner among 3 Burkitt lymphoma cells (Fig. 4B). These results suggest that Japonicone A blocks the TNF-α–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 Japonicone A and then stimulated with TNF-α for various times. In Japonicone A–treated Raji cells, the TNF-α–induced phosphorylations of IKKα/β, IκBα, NF-xB p65, ERK, JNK/SAPK, and p38 were reduced relative to that in cells without Japonicone A exposure (Fig. 4C). Moreover, the nuclear and cytoplasmic extraction assay (Fig. 4D) and immunostaining assay (Fig. 4E) both showed that Japonicone A prevented the nuclear translocation of p65 in lymphoma cells, a characteristic of NF-κB inactivation. These data suggest that Japonicone A inhibits TNF-α–induced NF-κB activity via impairment of the TAK1/TAB1 complex and inactivation of downstream IKKα/β, MAPK, and JNK signaling.

**Japonicone A 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 antiapoptotic proteins, such as Bcl-2, Bcl-xL, cIAPs, and XIAP, (33, 34). It was apparent that, for lymphomas, Japonicone A induced cell-cycle arrest and apoptosis via inhibition of 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 Japonicone A–treated lymphoma cells by Western blot analyses. Japonicone A inhibited the total p65 expression, especially that of NAMALWA cells, and decreased the expression of Bcl-2 (Bcl-2 protein) and XIAP (33, 34). It was apparent that, for lymphomas, Japonicone A induced cell-cycle arrest and apoptosis via inhibition of 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 Japonicone A–treated lymphoma cells by Western blot analyses. Japonicone A inhibited the total p65 expression, especially that of NAMALWA cells, and decreased the expression of Bcl-2 (Bcl-2 protein) and XIAP (33, 34). It was apparent that, for lymphomas, Japonicone A induced cell-cycle arrest and apoptosis via inhibition of NF-κB signaling.
Figure 4. Suppression of the NF-κB signaling cascade in Japonicone A (JA)–induced growth inhibition and apoptosis of lymphoma cells. A, the effect of Japonicone A on the binding affinity of total and phosphorylated TAK1 and TAB1 stimulated with TNF–α. Raji cells were exposed to Japonicone A (1 μmol/L) for 12 hours, then stimulated with TNF–α (5 ng/mL) for 30 minutes. Immunoprecipitation assays were conducted to evaluate the binding affinity of endogenous TAK1–TAB1. B, dose-dependent effect of Japonicone A 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 Japonicone A (2.5 μmol/L) for 2 hours and TNF–α (10 ng/mL) for different time intervals. The effect of Japonicone A on the NF-κB signaling cascade was determined by Western blot analyses of phosphorylated and total IKKα/IKKβ, IκBα, p38, JNK, ERK, and p65 with β-actin as loading control. D, NF-κB p65 gene expression in the cytoplasm and nucleus of Japonicone A–treated Raji and NAMALWA cells. Cells were pretreated with the indicated concentrations of Japonicone A for 16 hours and stimulated with TNF–α (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 Japonicone A (1 μmol/L) for 16 hours, followed by stimulation with or without TNF–α (10 ng/mL) for 30 minutes. 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 Japonicone A for 24 hours and NF-κB–related and target genes were analyzed by Western blot analyses with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Hsp90 as loading control.
involved in antiapoptosis signaling through interaction with the inhibitor of apoptosis family members (29), was also downregulated in all 3 types of lymphoma cells treated with Japonicone A (Fig. 4F).

After activation, NF-kB is translocated to the nucleus and activates transcription of genes responsible for cell growth, such as cyclin D1 and c-Myc, which are overexpressed and required for malignant transformation and growth of Burkitt lymphoma (6, 35). Japonicone A 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-kB signaling is inhibited through RNA interference (RNAi)–mediated NF-kB p65 silencing or by chemical inhibitors (36–39). Regulation of the G2–M transition is dependent on activation of the CDK1/cyclin B1 complex (40). Western blotting showed that Japonicone A treatment resulted in a reduction of cyclin B1 and CDK1 in a dose-dependent manner (Fig. 4F), suggesting that Japonicone A disturbs the CDK1/cyclin B1 complex to trigger arrest in the G2–M phase of cell cycle, as aforementioned.

**Constitutive activation of NF-kB through IκBα downregulation eliminates the inhibitory effect of Japonicone A on cell growth**

Downregulation of the NF-kB inhibitor, IκBα, is required for canonical NF-kB activation (41, 42). Thus, constitutive activation of NF-kB through RNAi-mediated IκBα downregulation should reduce the inhibitory effect of Japonicone A on cell growth. Considering the reduction of NF-kB 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-kB p65 expression and phosphorylation (Fig. 5A) as well as in the expression of the NF-kB target gene, cyclin D1 (Fig. 5A), showing activation of the NF-kB signal cascade. In 293T cells, constitutive activation of NF-kB by either of 2 independent lentivirus-mediated short hairpin RNAs (shRNA) conferred resistance to Japonicone A (Fig. 5B). These results indicate that Japonicone A exerts an inhibitory effect on cell growth via inactivation of NF-kB signaling.

**Japonicone A has therapeutic efficacy for B-cell NHL xenografts**

The effects of Japonicone A 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 Japonicone A. BALB/c nude mice bearing subcutaneous xenografts of NAMALWA cells were treated with Japonicone A (30 mg/kg body weight) for 2 weeks. Japonicone A 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 Japonicone A–treated mice had much smaller tumor masses (Fig. 6B and Supplementary Fig. S3A). The protein expression profiles of NAMALWA tumor tissues from 4 individual animals treated with Japonicone A revealed downregulation of NF-kB p65 expression, increased cleavage of PARP, and decreased expression of the NF-kB—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 Japonicone A on tumor dissemination and progression. In the dissemination model, immunocompromised NOD/SCID mice, injected intravenously with NAMALWA cells, received Japonicone A (30 mg/kg) for 3 weeks. During therapy, 4 of 5 mice with vehicle treatment developed severe posterior paralysis; only 2 mice treated with Japonicone A exhibited a modest posterior paralysis (Fig. 6D). Mice were killed after therapy and analyzed, by gross tissue and histologic 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, splatium intermusculare around spine, and femoral bone, a diffuse infiltration of atypical lymphoid cells that were round and medium-sized (Fig. 6D and E and Supplementary Fig. S3D–S3G). Mice treated with Japonicone A developed little or no lymphoma dissemination to these tissues and organs (Fig. 6D and E and
Figure 6. Japonicone A (JA) inhibition of localized growth and dissemination to multiple organs of NAMALWA cells. A, Japonicone A inhibition of localized growth of NAMALWA tumors. NAMAWA cells were transplanted into the right sides of nude mice, which were intraperitoneally dosed daily with Japonicone A (30 mg/kg) for 2 weeks. Tumor volumes were measured (mean ± SEM; n = 6; †, P < 0.05; ‡, P < 0.01; ‡‡, P < 0.001 vs. the control treatment). B, the weights of tumors harvested from mice treated with the vehicle or Japonicone A. 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 Japonicone A (30 mg/kg) or vehicle (n = 5). H&E staining of the parafin 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 shown by invasion of lymphoma cells in spinal cord, ganglion spinale, spatium intermusculare, bone marrow, and femoral bone and disruption of bone trabecula in spine. The black arrow indicates the invading lymphoma cells (scale bar in black and red color, respectively 50 μm and 200 μm). No evidence of human lymphoma dissemination was observed in Japonicone A-treatment group. F, the gross ovarian metastasis of lymphoma was also determined. G, schematic form of the proposed mechanisms of the effect of Japonicone A on Burkitt lymphoma cells. Japonicone A exerts its anticancer activity on lymphoma cells by inhibiting cell growth and proliferation, arresting cells in the G2–M phase of the cell cycle, inducing apoptosis by interrupting the upstream TAK1/TAB1 complex, and suppressing the IKKα/β–IκBα–NF-κB signaling axis.
Supplementary Fig. S3D–S3G), confirming the therapeutic effect of Japonicone A on relieving limb paralysis and hematogenous dissemination and invasion. Moreover, vehicle and Japonicone A–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 Japonicone A exhibited fewer ovarian metastases (Fig. 6F and Supplementary Fig. S3C). Moreover, Japonicone A prevented lymphoma invasion of the adrenal glands (data not shown). No other metastases were evident.

These results show that Japonicone A has antitumor 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. In addition, 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 Japonicone A, a natural product isolated from Inula japonica Thunb, exhibited potent anticancer activity, with less cytotoxicity to normal cells. Burkitt lymphoma cells showed the most sensitivity to this compound, with IC_{50} values of 400 to 800 nmol/L. Japonicone A exerted potent anticancer effects on Raji, BJAB, and NAMALWA lymphoma cells by inhibiting cell growth and proliferation, arresting cells in the G_{2}−M phase of cell cycle by interrupting the CDK1/cyclin B complex, and inducing cell apoptosis in a dose-dependent manner. Japonicone A, 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 biologic nucleophiles, especially the sulfhydryl group of cysteine residues via a Micheal-type reaction (49). We also reported that Japonicone A inhibits TNF-α–induced NF-κB activation and nuclear translocation by interrupting the upsteam TAK1/TAB1 complex (40) and suppressing the IκBα/β–IκBz–NF-κB signaling axis, leading to the reduction of NF-κB–initiating genes, such as bcl-2, bcl-xl, XIAP, TAR2, cyclin D, and c-Myc, which are involved, respectively, in antiapoptosis and cell growth (12). These results indicated that Japonicone A exerts its antitumor effect through targeting of the canonical NF-κB pathway. Indeed, constitutive activation of NF-κB via silencing of the expression of the NF-κB inhibitor, IκBz, resulted in the elimination of growth inhibition by Japonicone A in 293T cell. Thus, these results reveal the function of Japonicone A as an NF-κB inhibitor. Although NF-κB 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-κB in cancer cells (with stronger effects on cancer cells). Figure 6G depicted our proposed model for the effects of Japonicone A on Burkitt lymphoma 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 prosurvival and antiapoptosis signaling makes it a promising target for cancer therapy. In various human lymphoid malignancies, there is constitutive activation of NF-κB, which leads to uncontrolled growth, antiapoptosis, and immune evasion. Activation of the IκK−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 Burkitt lymphoma, 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; ref. 1). Through blocking NF-κB activation, Japonicone A inhibited localized growth and extranodal dissemination to multiple organs and tissues, such as spinal core, spatium intermusculare around spine, bone marrow, and ovaries, and prevented the development of hind-leg paralysis in 2 mouse models of lymphoma. The therapeutic efficacy of Japonicone A in B-cell NHL xenografts merits its evaluation for the treatment of lymphoid malignancies. Currently, clinical effective treatment of Burkitt 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 Japonicone A on microtubule dynamics and DNA integrity, and improve the therapeutic regimens through possible combination chemotherapy with these agents.

In summary, we have shown that Japonicone A, derived from Inula japonica Thunb, has potent and selective antilymphoma activity by inducing cell apoptosis and cell-cycle arrest through inactivation of the TAK1–IκK−NF-κB axis. Although additional pharmacologic and toxicologic studies are needed, our results highlight the potential of Japonicone A as a chemotherapeutic agent targeting lymphomas and other NF-κB–associated tumors.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: X. Li, X. Yang, Y. Liu, W. Yao, R. Zhang, W. Zhang, H. Wang.

Development of methodology: X. Li, W. Yao.

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Li, Y. Liu, N. Gong, P. Chen, H. Jin, W. Zhang, H. Wang.

Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): X. Li, X. Yang, Y. Liu, W. Yao, J. Li, W. Zhang, H. Wang.

Writing, review, and/or revision of the manuscript: X. Li, X. Yang, Y. Liu, P. Chen, R. Zhang, W. Zhang, H. Wang.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Qin, J. Li, R. Chu, L. Shan, W. Zhang.

Study supervision: W. Zhang, H. Wang.

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Xiaoguang Li, Xinying Yang, Yanling Liu, et al.


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