Oridonin, a diterpenoid purified from *Rabdosia rubescens*, inhibits the proliferation of cells from lymphoid malignancies in association with blockade of the NF-κB signal pathways

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Abstract

This study found that oridonin, a natural diterpenoid purified from *Rabdosia rubescens*, inhibited growth of multiple myeloma (MM; U266, RPMI8226), acute lymphoblastic T-cell leukemia (Jurkat), and adult T-cell leukemia (MT-1) cells with an effective dose that inhibited 50% of target cells (ED_{50}) ranging from 0.75 to 2.7 μg/mL. Terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling staining showed that oridonin caused apoptosis of MT-1 cells in a time-dependent manner. We explored effects of oridonin on antiapoptotic Bcl-2 family members and found that it down-regulated levels of Mcl-1 and BCL-x_L, but not Bcl-2 protein, in both MT-1 and RPMI8226 cells. Further studies found that oridonin inhibited nuclear factor-κB (NF-κB) DNA-binding activity in these cells as measured by luciferase reporter gene, ELISA-based, and electrophoretic mobility shift assays. Oridonin also blocked tumor necrosis factor-α– and lipopolysaccharide-stimulated NF-κB activity in Jurkat cells as well as RAW264.7 murine macrophages. Of note, oridonin decreased survival of freshly isolated adult T-cell leukemia (three samples), acute lymphoblastic leukemia (one sample), chronic lymphocytic leukemia (one sample), non-Hodgkin's lymphoma (three samples), and MM (four samples) cells from patients in association with inhibition of NF-κB DNA-binding activity. On the other hand, oridonin did not affect survival of normal lymphoid cells from healthy volunteers. Taken together, oridonin might be useful as adjunctive therapy for individuals with lymphoid malignancies, including the lethal disease adult T-cell leukemia. [Mol Cancer Ther 2005;4(4):578–86]

Introduction

We previously purified natural diterpenoid oridonin from *Rabdosia rubescens* by high-performance liquid chromatography and showed that it inhibited the proliferation of a wide variety of cancer cells, including those from prostate, breast, lung, and glioblastoma multiforme (1). However, the effect of oridonin against lymphoid malignancies and molecular mechanism by which oridonin inhibits the growth of cancer cells remain to be fully elucidated.

Adult T-cell leukemia is an aggressive malignancy of $CD4^+$ T lymphocytes in which the human T-cell lymphotropic virus type I (HTLV-I) has been recognized as the etiologic agent (2, 3). Despite the development of intensive combination chemotherapy regimens supported by granulocyte colony-stimulating factor, median survival time of individuals with adult T-cell leukemia is <13 months (4, 5). Therefore, development of new treatment strategies for adult T-cell leukemia is urgently needed.

Nuclear factor-κB (NF-κB) is a generic term for a dimeric transcription factor formed by the heterodimerization or homodimerization of a number of the rel family members (6). To date, five rel proteins have been identified: RelA (p65), RelB, and cRel, each having transactivation domains; and p50 and p52, which are expressed as the precursor proteins p105 (NF-κB1) and p100 (NF-κB2), respectively. These precursors require posttranslational processing and do not contain transactivation domains. The most abundant and active form of NF-κB is the dimeric complex of p50/RelA (p50/p65). NF-κB plays a pivotal role in immune and inflammatory responses through the regulation of genes encoding proinflammatory cytokines and inducible enzymes, such as cyclooxygenase 2 and inducible nitric oxide synthase (6). In addition, activated NF-κB is implicated in development, progression, as well as drug resistance of cancer cells (7). For example, adult T-cell leukemia cells possess constitutively activated NF-κB, which transcriptionally up-regulates levels of the antiapoptotic protein Bcl-x_L, resulting in prolonged cell survival and acquisition of drug resistance (8, 9). Other

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investigators showed that inhibition of NF-κB by proteasome inhibitor PS-341 or Bay 11-7082 induced growth inhibition and apoptosis of adult T-cell leukemia cells (10, 11). Also, NF-κB was constitutively activated in multiple myeloma (MM) cells and inhibition of NF-κB by PS-341 or forced-expression of IκBα, an inhibitor of NF-κB, sensitized MM cells to conventional chemotherapeutic agents, such as doxorubicin (12–14). Thus, NF-κB seems to be a promising molecular target of lymphoid malignancies, including adult T-cell leukemia and MM.

This study found that oridonin, a natural diterpenoid, induced growth arrest and apoptosis of cells from lymphoid malignancies in association with inhibition of NF-κB and down-regulation of Bcl-2 family proteins.

Materials and Methods

Cell Lines

HTLV-I–infected T-cell lines, MT-1 (2), and MT-2 (15) were kind gifts of I. Miyoshi (Kochi Medical School, Kochi, Japan) and were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (Life Technologies). U266, RPMI 8226, murine macrophage RAW264.7, and Jurkat cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 with 10% fetal bovine serum.

Oridonin

Oridonin was purified from *R. rubescens* by high-performance liquid chromatography and its structure is shown in Fig. 1. Oridonin was dissolved in DMSO (Burdick & Jackson, Muskegon, MI) at a stock concentration of 10 mg/mL and stored at −20°C. For *in vitro* use, oridonin was diluted in RPMI 1640. An aliquot was used only once.

Chemicals

Lipopolysaccharide (LPS) from *Esherichia coli* serotype 0111:B4 and tumor necrosis factor-α (TNF-α) were purchased from Sigma Chemical, Co. (St. Louis, MO).

Trypan Blue Exclusion Test

U266, RPMI 8226, Jurkat, MT-1, and MT-2 cells (5×10^5 cells/mL) were incubated with various concentrations of oridonin (0.5–3.0 μg/mL) for 2 days in 96-well plates (Flow Laboratories, Irvine, CA). Lymphoid malignant cells from patients as well as $CD19^+$ B cells and $CD3^+$ T cells from healthy volunteers were freshly isolated as previously described (16) and were cultured with either oridonin (1.0 μg/mL) or control diluent (0.01% DMSO) for 24 hours. After culture, cell numbers and viability were evaluated by staining with trypan blue and counting using light microscopy.

Thymidine Uptake Study

DNA synthesis was measured by tritiated thymidine uptake [^{3}H]dTh (Perkin-Elmer, Boston, MA). Cells (5×10^5 cells/mL) were cultured with various concentrations of oridonin (0.5–3.0 μg/mL) for 2 days in 96-well plates. Cells were pulsed with [^{3}H]dTh [0.5 μCi (0.185 MBq)/well] during the last 6 hours of the 48-hour culture, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA), and counted by using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). All experiments were done in triplicate.

Assessment of Apoptosis

Apoptotic cell death was examined by terminal deoxyribonucleotide transferase–mediated dUTP nick end labeling method using the *In situ* Cell Death Detection kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instruction. For quantification, three different fields were counted under the microscope and at least 300 cells were enumerated in each field. All experiments were done twice.

Western Blot Analysis

Lysates were made by standard methods as previously described (17). Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved on a 4% to 15% SDS polyacrylamide gel, transferred to an immobilon polyvinylidene difluoride membrane (Amersham Corp., Arlington Heights, IL), and probed sequentially with antibodies. Anti-IκBα (Imgenex, San Diego, CA), anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA), anti–Bcl-2 (Santa Cruz Biotechnology), anti–Mcl-1 (Santa Cruz Biotechnology), anti-Bax (Santa Cruz Biotechnology), anti–Bcl-x_L (Cell Signaling, Beverly, MA), and anti–β-actin (Santa Cruz Biotechnology) antibodies were used. The band intensities were measured using densitometry.

Degradation of IκBα and nuclear translocation of the p65 subunit of NF-κB were studied by Western blot analysis of cytoplasmic and nuclear extracts of TNF-α–treated Jurkat cells. Cells were suspended in ice-cold extraction buffer containing 20 mmol/L HEPES (pH 7.9), 20% glycerol, 10 mmol/L NaCl, 0.2 mol/LEDTA (pH 8.0), 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 1 mmol/L DTT, 100 μg/mL phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 1 μg/mL pepstatin, and 10 μg/mL leupeptin. After 10 minutes of incubation on ice, nuclei were collected by a short spin in a microcentrifuge. The supernatant was saved as a cytoplasmic fraction, and the

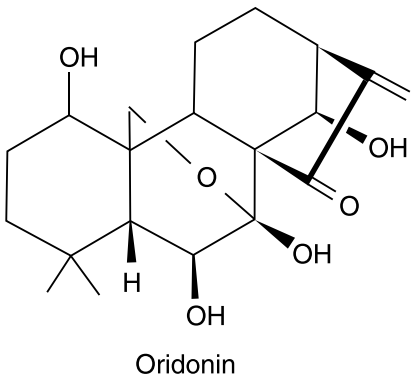

Figure 1. Structure of oridonin.

nuclei were resuspended in ice-cold extraction buffer containing 300 mmol/L NaCl. After 30 minutes of incubation, the supernatant was collected by centrifugation at 15,000 × *g* for 20 minutes at 4°C. Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad Laboratories). Proteins were resolved by 4% to 15% SDS polyacrylamide gel, transferred to an immobilon polyvinylidene difluoride membrane (Amersham), and probed sequentially with antibodies. Anti-IκBα (Imgenex) and anti-p65 (Santa Cruz Biotechnology) were used.

Transfections and Reporter Assay

The NF-κB reporter construct (pGL3-NF-κB) containing three copies of NF-κB site cloned into pGL3-basic plasmid (Promega, Madison, WI) was a generous gift from Dr. Moshe Arditi (Cedars-Sinai Medical Center, University of California at Los Angeles School of Medicine, Los Angeles, CA). RAW 264.7 cells (2×10^5 cells/mL) were plated on 24-well plates and incubated until 60% to 80% confluence, and then transfected with pGL3-NF-κB using the GenePORTER transfection reagent (Gene Therapy Systems, Inc., San Diego, CA). MT-1 cells (2×10^6) were transfected with pGL3-NF-κB (10 μg) by electroporation (100 V, 3 seconds). After 24 hours, cells were preincubated with either oridonin (0.5 μg/mL) or control diluent for 1 hour and exposed to LPS (100 ng/mL, 6 hours) or TNF-α (50 ng/mL, 6 hours). Luciferase activity in cell lysates was measured by the Dual Luciferase assay system (Promega), which was normalized by Renilla activities. All transfection experiments were done in triplicate wells and repeated separately at least thrice.

Evaluation of NF-κB DNA-Binding Activity by ELISA

The DNA-binding activity of NF-κB in lymphoid malignant cells was quantified by ELISA using the TransAM NF-κB p65 Transcription Factor Assay kit (Active Motif North America, Carlsbad, CA), as previously described (16).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was done as previously described (17). Briefly, 5 μg nuclear extracts

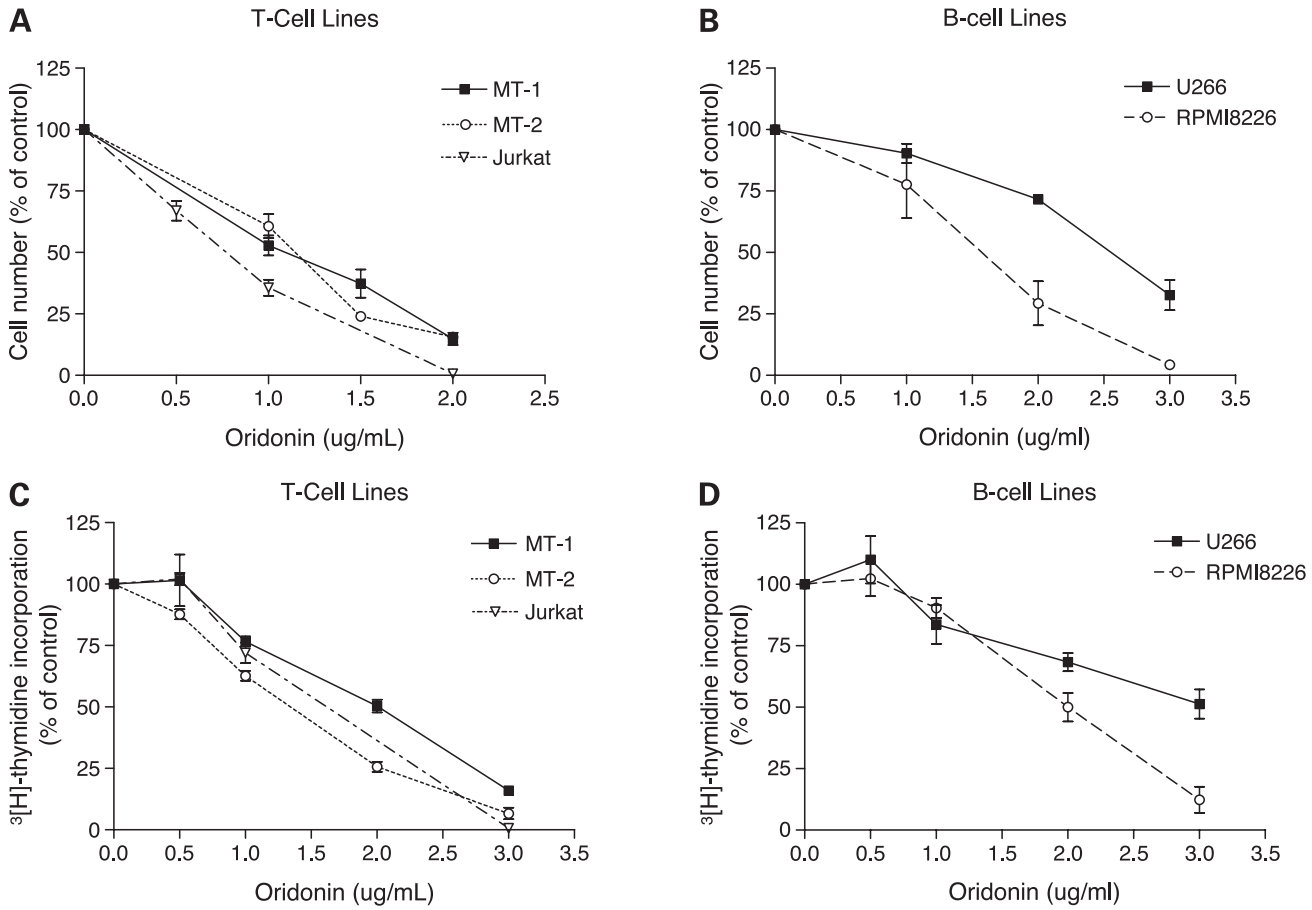

Figure 2. Trypan blue exclusion test: **A,** HTLV-1 – infected T cells (*MT-1*, *MT-2*); acute lymphoblastic T cells (*Jurkat*); **B,** MM (*U266*, *RPMI 8226*) cells were plated in 96-well plates and cultured with either oridonin (0.5 – 3.0 μg/mL) or diluent. After 2 d, cell number was counted by trypan blue exclusion test. Thymidine ^{3}H uptake: **C,** HTLV-1 – infected T cells (*MT-1*, *MT-2*); acute lymphoblastic T-cells (*Jurkat*); **D,** MM (*U266*, *RPMI 8226*) were plated in 96-well plates and cultured with either oridonin (0.5 – 3.0 μg/mL) or diluent. Cells were pulsed with [^{3}H]dTh [0.5 μCi (0.185 MBq)/well] during the last 6 h of the 48-h culture. *Points,* mean of three experiments done in triplicate; *bars,* SD.

were incubated with 16 fmol ^{32}P-end-labeled, NF-κB binding probe. The DNA-protein complex was separated from the free oligonucleotide on a 5% polyacrylamide gel. The specificity of NF-κB DNA binding was examined by competition with a double-stranded mutated oligonucleotide or unlabeled oligonucleotide. Gels were dried and exposed to Kodak XAR film (Eastman Kodak, New Haven, CT). The band intensity was measured by densitometry.

Statistical Analysis

Statistical analysis was done to assess the difference between two groups under multiple conditions by one-way ANOVA using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA).

Results

Oridonin inhibits the proliferation of lymphoid malignant cells. Cells were cultured in the presence of various concentrations of oridonin (0.5–3.0 μg/mL) for 2 days and cell number was enumerated by trypan blue exclusion test. Dose-response curves were drawn (Fig. 2A and B), and the effective dose that inhibited 50% of target cells (ED_{50}) was determined. All cell lines including those from HTLV-I–infected T-cells (MT-1, MT-2), MM (U266, RPMI8226), and acute lymphoblastic T-cell leukemia (Jurkat) were effectively inhibited in their proliferation in a dose-dependent manner with an ED_{50} of 1.2, 1.2, 0.8, 1.7, and 2.7 μg/mL, respectively. The effect of oridonin on DNA synthesis was assessed by

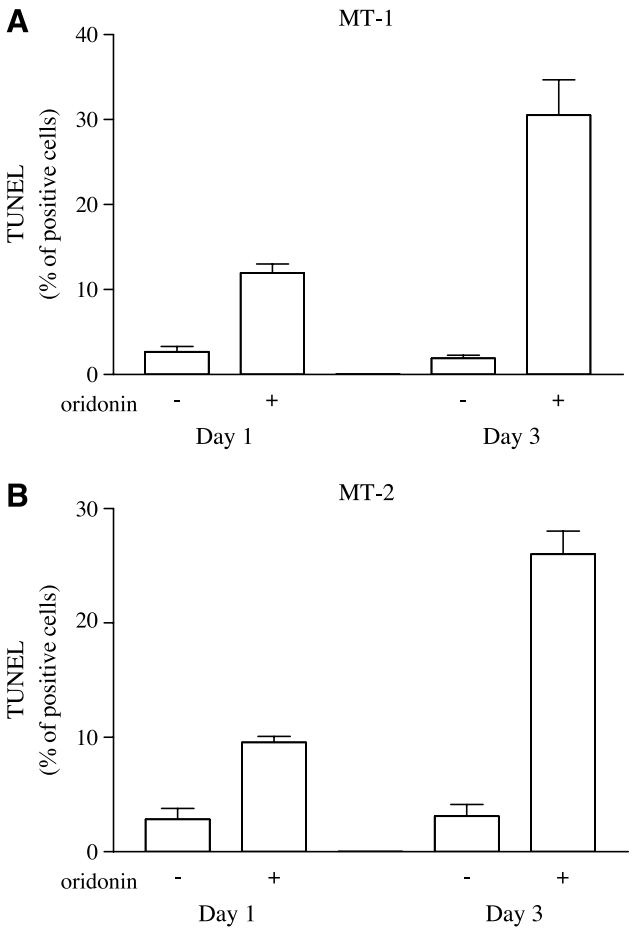

Figure 3. Terminal deoxyribonucleotide transferase–mediated dUTP nick end labeling assay. MT-1 (**A**) and MT-2 (**B**) cells were plated on 96-well plates and cultured with either oridonin (1.0 μg/mL) or control diluent; 1 or 3 d later, apoptosis was measured by terminal deoxyribonucleotide transferase–mediated dUTP nick end labeling assay. *Columns,* mean of two experiments done in triplicate; *bars,* SD.

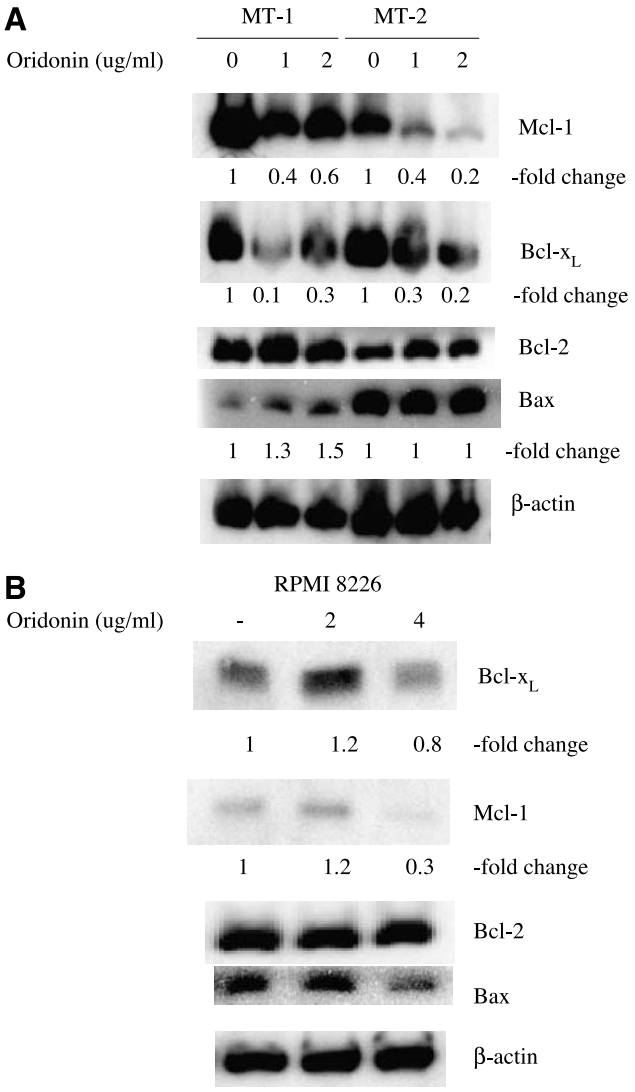

Figure 4. Effect of oridonin on Bcl-2 family of proteins. MT-1 (**A**), MT-2 (**A**), and RPMI 8226 (**B**) cells were cultured with either oridonin (1 or 2 μg/mL for MT-1 as well as MT-2 cells, and 2 or 4 μg/mL for RPMI 8226 cells) or diluent. After 24 h, cells were harvested and proteins were extracted and subjected to Western blot analysis. The polyvinylidene fluoride membrane was sequentially probed with anti-Mcl-1, anti-Bcl-x_L, anti-Bcl-2, anti-Bax, and anti-β-actin antibodies, and band intensities were measured using densitometry.

tritiated thymidine uptake. Consistent with the results of trypan blue exclusion test, oridonin effectively inhibited DNA synthesis of lymphoid malignant cells (Fig. 2C and D).

Oridonin Induces Apoptosis of HTLV-I–Infected T cells

We studied whether oridonin possessed proapoptotic effects against HTLV-I–infected T cells by utilizing the terminal deoxyribonucleotide transferase–mediated dUTP nick end labeling assay. It caused apoptosis of both MT-1 and MT-2 cells in a time-dependent manner. For example, oridonin (1.0 μg/mL) induced a mean of 12 ± 2% (±SD) of MT-1 cells to become apoptotic on the first day and 30 ± 9% on the third day of culture (Fig. 3).

Effect of Oridonin on Bcl-2 Family of Proteins

Oridonin induces growth arrest and apoptosis of HTLV-I–infected T-cells, which prompted us to examine whether oridonin modulated levels of antiapoptotic Bcl-2 family of proteins in these cells. Both MT-1 and -2 cells constitutively expressed Mcl-1, Bcl-xL, and Bcl-2 proteins (Fig. 4A). Exposure of these cells to oridonin (1 or 2 μg/mL, 24 hours) dramatically down-regulated levels of Mcl-1 and Bcl-xL, but not Bcl-2 proteins (Fig. 4A). For example, oridonin (2 μg/mL, 24 hours) decreased levels of Mcl-1 and Bcl-xL protein by 40% and 70%, respectively (Fig. 4A). The effect of oridonin on Bcl-2 family proteins were also studied using MM cells; it down-regulated levels of Mcl-1 and Bcl-x_L but not Bcl-2 proteins in RPMI 8226 cells (Fig. 4B). In addition, we explored the effects of oridonin on proapoptotic protein, Bax, in these cells; oridonin slightly increased levels of Bax in MT-1 cells, but not in MT-2 and RPMI 8226 cells (Fig. 4A and B).

Effect of Oridonin on NF-κB in MT-1 Cells

We previously showed that eight herbal mixture PC-SPES blocked LPS-stimulated NF-κB activity in murine macrophages and rescued mice from LPS-induced septic shock (17). Oridonin from *R. rubescens* is one of the components of PC-SPES. We, therefore, explored whether oridonin possessed anti–NF-κB activity in adult T-cell leukemia cells. We transiently transfected MT-1 cells with a NF-κB reporter construct and cultured these cells with either oridonin or control diluent. MT-1 cells possessed measurable NF-κB transcriptional activity, and oridonin (5 μg/mL, 18 hours) inhibited this activity by ~50% (Fig. 5A). The effect of oridonin on NF-κB activity was further confirmed using an ELISA-based assay. MT-1 cells possessed measurable NF-κB DNA-binding activity, and treatment of MT-1 cells with oridonin (5 μg/mL, 18 hours) inhibited the NF-κB binding activity by 50% compared with untreated control cells (Fig. 5B). As control, 100× molar excess of the wild-type NF-κB consensus oligonucleotides was added to the assay of control lysate. Binding was inhibited by at least 80%; however, mutated NF-κB consensus oligonucleotides at the same molar excess was unable to inhibit binding (Fig. 5B), ascertaining specificity of binding of NF-κB to its consensus binding site. Furthermore, we did EMSA to assess the effect of oridonin on NF-κB DNA-binding activity. Oridonin (5 μg/mL, 18 hours) almost completely disrupted formation of NF-κB DNA-binding complex in MT-1 cells (Fig. 5C). The NF-κB/DNA complex was competed with 100× molar excess of unlabeled oligonucleotides (*lane 3*), but not with same molar excess of mutated oligonucleotides (*lane 4*), confirming the specificity of NF-κB band.

Effect of Oridonin on TNF-α– or LPS-stimulated NF-κB Activity

Furthermore, we explored whether oridonin blocked TNF-α–stimulated NF-κB activity in Jurkat cells. The cells were transiently transfected with the NF-κB reporter construct and then exposed to TNF-α (50 ng/mL, 6 hours),

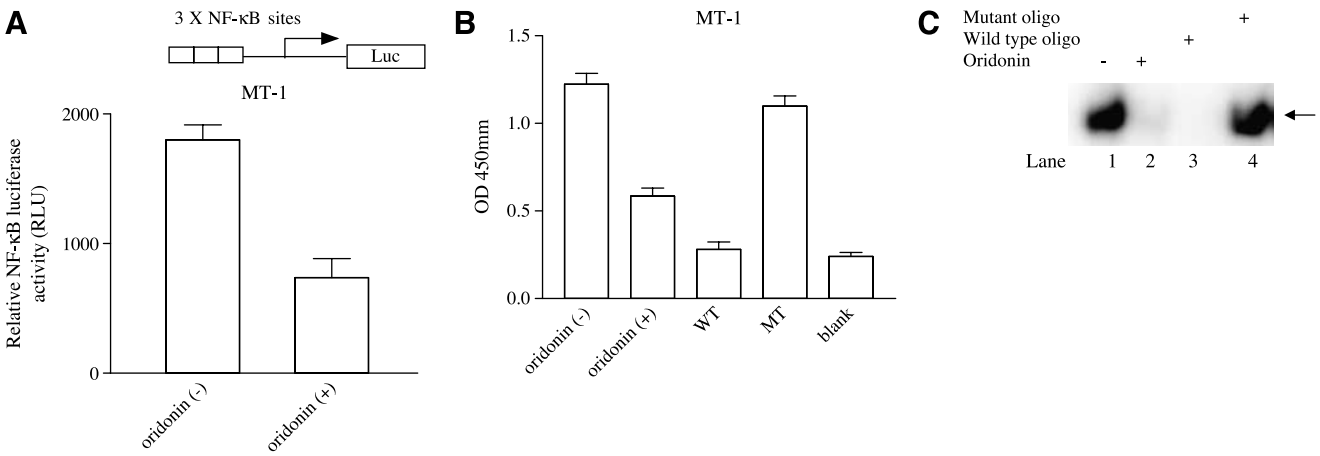

Figure 5. Effect of oridonin on NF-κB in MT-1 cells. **A**, NF-κB luciferase reporter assay. The construct (pGL3-NF-κB) containing three copies of the NF-κB–binding site cloned into pGL3-basic plasmid is shown (*top*). MT-1 cells were transfected with pGL3-NF-κB. After 48 h, cells were exposed to either oridonin (5 μg/mL, 18 h) or diluent. pRL-SV40-Luciferase (Renilla luciferase) vector was cotransfected for normalization. *Columns,* mean of three experiments done in triplicate; *bars,* SD. **B**, NF-κB ELISA. MT-1 cells were cultured with either oridonin (5 μg/mL) or diluent. After 18 h, nuclear protein was extracted and subjected to NF-κB ELISA for measurement of NF-κB DNA-binding activity. *Columns,* mean of two experiments done in duplicate; *bars,* SD. *WT,* wild-type oligonucleotides; *MT,* mutated oligonucleotide. **C**, EMSA. MT-1 cells were cultured with either oridonin (5 μg/mL) or diluent. After 18 h, nuclear protein was extracted and subjected to EMSA. *Arrow,* gel location of NF-κB bound to DNA.

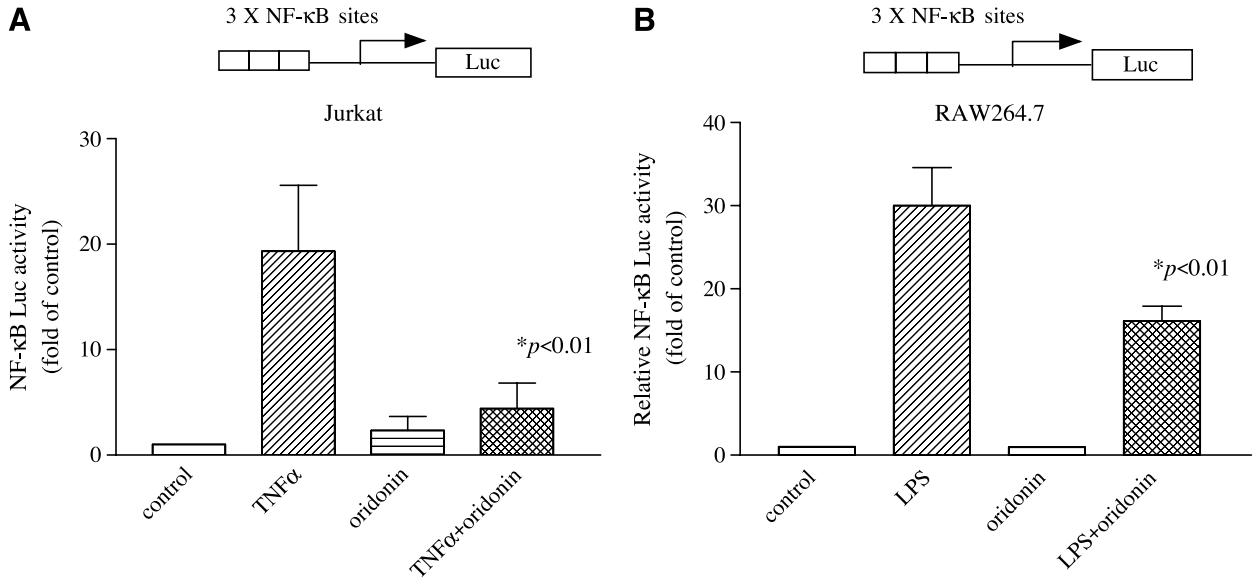

Figure 6. **A**, effect of oridonin on TNF-α–stimulated NF-κB transcriptional activity in Jurkat cells. The construct (NF-κB-Luc) containing three copies of the NF-κB–binding site attached to pGL3 luciferase reporter plasmid is shown (*top*). Jurkat cells were transfected with this reporter construct. These cells were cultured with either oridonin (5 μg/mL) or control diluent for 1 h. At the end of the treatment, cells were washed twice with PBS and treated either with or without TNF-α (50 ng/mL) for 6 h at which time luciferase activity was measured. **B**, effect of oridonin on LPS-stimulated NF-κB transcriptional activity in RAW264.7 cells. RAW264.7 cells were transfected with the NF-κB luciferase reporter construct. These cells were cultured with either oridonin (5 μg/mL) or diluent for 1 h. At the end of the treatment, cells were washed twice with PBS and treated either with or without LPS (100 ng/mL) for 6 h at which time luciferase activity was measured. pRL-SV40-Luciferase (Renilla luciferase) vector was cotransfected for normalization. *Columns,* mean of three experiments done in triplicate; *bars,* SD. Statistical significance was determined by one-way ANOVA.

which increased reporter activity by 30-fold compared with untreated control cells (Fig. 6A). When cells were preincubated with oridonin (5 μg/mL, 1 hour), TNF-α–stimulated NF-κB activity was decreased by 80% ($P < 0.01$; Fig. 6A). In addition, we utilized murine macrophage RAW264.7 cells to study whether oridonin blocked LPS-stimulated NF-κB activity. Exposure of RAW264.7 cells to LPS (100 ng/mL, 6 hours) increased reporter activity by 30-fold compared with control cells, and preincubation of these cells with oridonin (5 μg/mL, 1 hour) blunted LPS-stimulated NF-κB activity by 48% ($P < 0.01$; Fig. 6B).

Effect of Oridonin on TNF-α–Induced Phosphorylation and Degradation of IκBα and Nuclear Translocation of NF-κB (p65) in Jurkat Cells

Activation of NF-κB involves two important steps: (*a*) phosphorylation and subsequent degradation of IκBα caused by IκB kinase, resulting in release of NF-κB, and (*b*) the nuclear translocation of the activated NF-κB. To elucidate the effect of oridonin on these steps, control and oridonin-treated Jurkat cells were exposed to TNF-α (50 ng/mL) for various durations. The kinetics of IκBα phosphorylation and degradation were studied by Western blot analysis using cytoplasmic extracts. As shown in Fig. 7A and B, no significant difference in the pattern of IκBα phosphorylation and degradation after treatment with oridonin was observed. Next, to study the translocation of the activated NF-κB into the nucleus, levels of the p65 subunit of NF-κB in the nuclear extracts of control and oridonin-treated Jurkat cells were measured. Jurkat cells constitutively expressed NF-κB and exposure of Jurkat cells to TNF-α (50 ng/mL) for 15 minutes increased levels of NF-κB by 1.3-fold compared with control cells (Fig. 7C). These results were nearly identical when the experiments were repeated with oridonin-treated Jurkat cells.

Effect of Oridonin on TNF-α–Induced NF-κB DNA-Binding Activity

We next utilized EMSA to explored the effect of oridonin on TNF-α–induced NF-κB DNA-binding activity in Jurkat cells. Jurkat cells possessed the measurable NF-κB DNA-binding activity and TNF-α further increased this binding by 5-fold (Fig. 7D). Oridonin completely blocked both basal and TNF-α–stimulated NF-κB DNA-binding activity in these cells (Fig. 7D). The TNF-α–induced NF-κB/DNA complex was competed with 100× molar excess of unlabeled oligonucleotides (*lane 5*), but not with same molar excess of mutated oligonucleotides (*lane 6*), confirming the specificity of NF-κB band. Taken together, oridonin probably inhibited NF-κB transcriptional activity via inhibition of the ability of NF-κB to bind to DNA of the target genes.

Effect of Oridonin on Freshly Isolated Cells from Patients with Lymphoid Malignancies

We explored the effect of oridonin on survival of freshly isolated lymphoid malignant cells from patients. Oridonin (1.0 μg/mL, 24 hours) decreased survival of freshly isolated cells from adult T-cell leukemia (three samples), acute lymphoblastic leukemia (one sample), chronic lymphocytic leukemia (one sample), non-Hodgkin's lymphoma (three

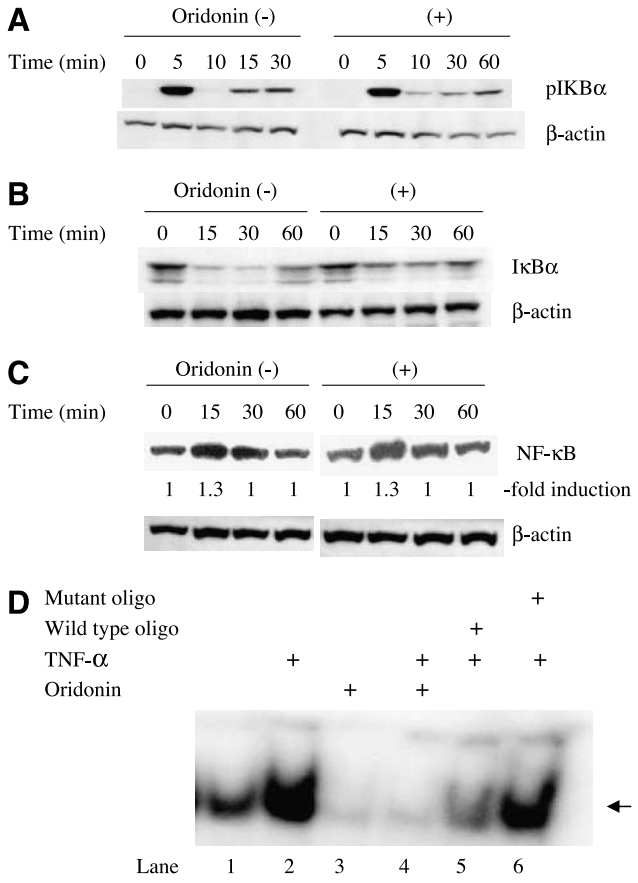

Figure 7. Effect of oridonin on TNF-α–induced phosphorylation (**A**) degradation of IκBα (**B**) and nuclear translocation of NF-κB (p65; *C*) in Jurkat cells. Jurkat cells were pretreated with either oridonin (5 μg/mL) or diluent for 1 h. At the end of the treatment, cells were washed twice with PBS and treated with TNF-α (50 ng/mL) for the indicated time periods. The cytoplasmic (**A**, **B**) and nuclear extracts (**C**) of these cells were prepared and subjected to Western blot analysis to measure the level of IκBα and p65 of NF-κB, respectively. **D**, EMSA. Jurkat cells were cultured with either oridonin (5 μg/mL) or control diluent for 1 h. At the end of the treatment, cells were washed twice with PBS and treated either with or without TNF-α (50 ng/mL). After 6 h, cells were harvested and nuclear protein was extracted and subjected to EMSA. *Arrow,* gel location of NF-κB bound to DNA.

samples), and MM (four samples) patients by >40% compared with diluent-treated control cells (Table 1). On the other hand, oridonin did not affect survival of $CD19^+$ B cells and $CD3^+$ T cells from healthy volunteers (data not shown). We also examined the effect of oridonin on NF-κB activity in these cells. Nuclear protein was available from nine cases. NF-κB DNA-binding activity was measurable in all cases as monitored by an ELISA-based assay (Fig. 8); oridonin (1 μg/mL, 24 hours) decreased NF-κB DNA-binding activity by >50% in seven of nine cases (Fig. 8).

Discussion

Oridonin, a natural diterpenoid, inhibited the proliferation of lymphoid malignant cells, including those from

Table 1. Effect of oridonin on freshly isolated malignant lymphoid cells

Patient no.	Age/sex	Disease type	Reduction in cell number (%control)
1	45/F	ATL (lymphoma)	57
2	63/M	ATL (acute)	72
3	81/F	NHL (diffuse large, T)	62
4	25/F	ALL (B)	62
5	53/M	NHL (diffuse large, B)	60
6	64/M	MM (IgA-κ)	56
7	53/F	MM (IgA-κ)	59
8	66/M	ATL (lymphoma)	39
9	53/F	CLL (B)	60
10	70/F	MM (IgG-κ)	49
11	37/F	NHL (follicular, B)	80
12	75/F	MM (IgA-κ)	75

NOTE: Viable, freshly isolated lymphoid cells were enumerated by trypan blue exclusion after incubation for 2 days in enriched culture media either with or without oridonin (1 μg/mL).
Abbreviations: ATL, adult T-cell leukemia; ALL, acute lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; T, T cell; B, B cell; M, male; F, female.

adult T-cell leukemia, MM, and lymphoblastic T-cell leukemia. It induced apoptosis of these cells in conjunction with down-regulation of antiapoptotic proteins, Bcl-x_L and Mcl-1. In addition, we found that the compound possessed anti–NF-κB activity. It inhibited the NF-κB DNA-binding activity; however, it did not interfere with nuclear translocation of NF-κB (Fig. 7). The X-ray structure of RelA showed that it possessed cysteine residues in its DNA-binding site, which was critical for optimal protein/DNA interaction (18, 19). Oridonin might affect cysteine residues and disrupt NF-κB protein/DNA interaction. Our results are reminiscent of the mode of action of several other compounds with terpene structure. For example, avicin (20), a family of triterpenoid saponins from *Acacia victoricae*, and kamebakaurin (21),

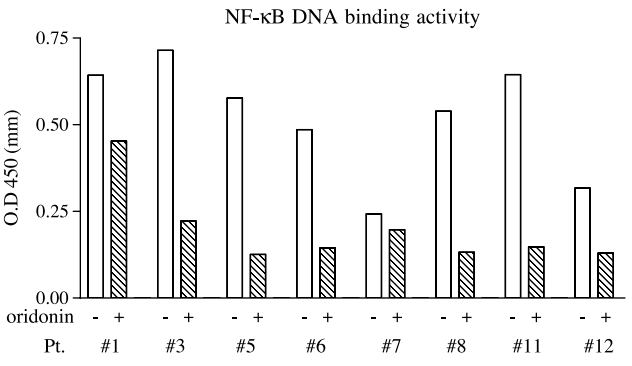

Figure 8. Effect of oridonin on NF-κB DNA-binding activity in freshly isolated cells from patients with lymphoid malignancies. The cells from patients with lymphoid malignancies (listed in Table 1) were cultured with either oridonin (1.0 μg/mL) or control diluent. After 24 h, nuclear protein was prepared and subjected to NF-κB ELISA for measurement of NF-κB DNA-binding activity. *Pt,* patient.

from *Isodon japonicus*, inhibited NF-κB transcriptional activity by inhibition of binding of NF-κB to specific DNA sequences; DTT treatment, which was able to prevent alkylation of free sulfhydryls, reversed this inhibition. Moreover, kamebakaurin failed to inhibit the binding of NF-κB when Cys^{62} in the DNA-binding site of NF-κB was mutated (21), suggesting that kamebakaurin inhibited NF-κB transcriptional activity by modifying Cys^{62} of NF-κB. Furthermore, recent studies showed that the synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, induced apoptosis of a wide variety of cancer cells including those from MM and acute myeloid leukemia (22–24). 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid blocked NF-κB signaling without affecting TNF-α–stimulated nuclear translocation of NF-κB in human myeloid leukemia cells (24). The inhibition of NF-κB at a level after nuclear translocation of activated NF-κB may be a common feature of compounds with terpene structure.

Recently, other investigators showed that oridonin induced apoptosis in association with up-regulation of proapoptotic protein Bax in murine fibrosarcoma L929 cells (25). In the present study, oridonin slightly increased levels of Bax in MT-1 cells, but not in MT-2 and RPMI8226 cells. The effect of oridonin may vary in different cell types.

Importantly, oridonin decreased survival of freshly isolated lymphoid malignant cells from patients with MM, adult T-cell leukemia, non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and acute lymphocytic leukemia (Table 1) in conjunction with inhibition of NF-κB DNA-binding activity (Fig. 8). Oridonin was not able to inhibit NF-κB DNA-binding activity in two of nine cases. In one MM case (case 7), NF-κB DNA-binding activity was extremely low compared with other cases, and oridonin did not further decrease this activity (Fig. 8). Concerning adult T-cell leukemia (case 1) sample, oridonin decreased survival of adult T-cell leukemia cells by 57% compared with diluent-treated cells, although inhibition of NF-κB DNA-binding activity mediated by oridonin was <20% in these cells. Probably, other cell survival transcription factors, such as activator protein-1 and/or signal transducers and activators of transcription, are active, and oridonin might block these signal pathways. The NF-κB activity in Jurkat cells was weaker than that in MT-1 cells (Fig. 5 and 7), although both cells were similarly sensitive to growth inhibition mediated by oridonin (Fig. 2). These observations augment our hypothesis that oridonin might function by affecting multiple signaling pathways in different cell types. Further studies are warranted to clarify other biological functions of oridonin.

The activated NF-κB plays a pivotal role in autoimmune diseases through overproduction of proinflammatory cytokines and inducible enzymes, such as cyclooxygenase 2 and inducible nitric oxide synthase in lymphocytes, macrophages, and synovial cells (26). This study found that oridonin blocked both TNF-α– and LPS-stimulated NF-κB activity in human T cells and murine macrophages (Fig. 6). In addition, we have recently found that oridonin inhibited NF-κB activity in inflamed synovial cells from patients with rheumatoid arthritis (Ikezoe et al., data not shown). Oridonin may have potential therapeutic application in autoimmune diseases including rheumatoid arthritis.

Taken together, oridonin might be useful for the treatment of individuals with lymphoid malignancies, including adult T-cell leukemia and other types of cancers in which NF-κB is activated. Also, our preliminary results suggest potential applications of oridonin in inflammatory diseases.

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