Mitochondrial-Mediated Apoptosis in Lymphoma Cells by the Diterpenoid Lactone Andrographolide, the Active Component of Andrographis paniculata

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Abstract

**Purpose:** Andrographolide is a diterpenoid lactone isolated from *Andrographis paniculata* (King of Bitters), an herbal medicine used in Asia. It has been reported to have anti-inflammatory, antihypertensive, antiviral, and immune-stimulant properties. Furthermore, it has been shown to inhibit cancer cell proliferation and induce apoptosis in leukemia and solid tumor cell lines.

**Experimental Design:** We studied the Burkitt p53-mutated Ramos cell line, the mantle cell lymphoma (MCL) line Granta, the follicular lymphoma (FL) cell line HF-1, and the diffuse large B-cell lymphoma (DLBCL) cell line SUDHL4, as well as primary cells from patients with FL, DLBCL, and MCL.

**Results:** We found that andrographolide resulted in dose- and time-dependent cell death as measured by MTT. Andrographolide significantly increased reactive oxygen species (ROS) production in all cell lines. To determine mechanism of cell death, we measured apoptosis by Annexin V/propidium iodide in the presence and absence of the antioxidant N-acetyl-L-cysteine (NAC), the glutathione (GSH)–depleting agent buthionine sulfoxamine (BSO), or caspase inhibitors. We found that apoptosis was greatly enhanced by BSO, blocked by NAC, and accompanied by poly(ADP-ribose) polymerase cleavage and activation of caspase-3, caspase-8, and caspase-9. We measured BAX conformational change and mitochondrial membrane potential, and using mouse embryonic fibroblast (MEF) Bax/Bak double knockouts (MEF<sup>Bax<sup>−/−</sup>/Bak<sup>−/−</sup>), we found that apoptosis was mediated through mitochondrial pathways, but dependent on caspases in both cell lines and patient samples.

**Conclusions:** Andrographolide caused ROS-dependent apoptosis in lymphoma cell lines and in primary tumor samples, which was enhanced by depletion of GSH and inhibited by NAC or the pan-caspase inhibitor Z-VAD-FMK. Further studies of diterpenoid lactones in lymphoma are warranted.

Andrographolide is a diterpenoid lactone isolated from *Andrographis paniculata* (King of Bitters; refs. 1–3), an important herbal medicine used in Asia to treat a range of diseases, such as respiratory infection, fever, bacterial dysentery, and diarrhea (4–6). It also has been studied in patients with HIV (7). The major bioactive component extracted from *A. paniculata* is andrographolide, and the three hydroxyls at C-3, C-19, and C-14 are responsible for its biological activity (8). Recently, the anticancer properties of andrographolide have been recognized, and some of its effects seem to proceed through redox-mediated pathways (9–12). We therefore hypothesized that andrographolide would lead to cell death in lymphoma cell lines and that the effect may be related to altered cellular redox state. We studied andrographolide in non–Hodgkin’s lymphoma cell lines as well as in primary malignant B cells from patients with diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), and follicular lymphoma (FL). We found that andrographolide induced reactive oxygen species (ROS) and caspase-dependent apoptosis in lymphoma cell lines and in primary tumor samples but not in normal lymphocytes, and that this was enhanced by depletion of glutathione (GSH) and inhibited by the antioxidant N-acetyl-l-cysteine (NAC) or the pan-caspase inhibitor Z-VAD-FMK. Furthermore, these effects seemed to proceed through BAX/BAK pathways.
Translational Relevance

The anticancer properties of the diterpenoid lactone andrographolide have recently been recognized, and the biological effects of this naturally occurring product derived from Andrographis paniculata are likely related to reactive oxygen species (ROS) signaling. Based on these observations, we hypothesized that andrographolide would be cytotoxic to lymphoma cells. In these studies, we examined lymphoma cell lines, including Ramos, Granta, SUDHL4, and HF-1, as well as primary lymphoma cells derived from patients. We show that andrographolide results in apoptosis in lymphoma cell lines and in primary lymphoma cells, that this is mediated through ROS-mediated caspase activation, and that these effects proceed through BAX/BAK mitochondrial pathways. These studies will provide the preclinical rationale to bring this novel natural compound to clinical trials for the treatment of lymphoma.

Materials and Methods

Reagents

Andrographolide (Supplementary Fig. S1), buthionine sulfoxamine (BSO), and NAC were purchased from Sigma Chemical Co. Z-VAD-FMK, Ac-DEVD-CHO, Ac-IETD-CHO, and Ac-LEHD-CHO were obtained from BioMol. Antibodies to caspase-3, caspase-9, and caspase-8 were purchased from Cell Signaling Technology, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon.

Cell culture

Ramos (Burkitt lymphoma) cell line was obtained from the American Type Culture Collection, HF-1 (FL) was from Dr. Richard Miller (Stanford University, Palo Alto, CA), SUDHL4 (DLBCL) was from Dr. Ron Gartenhaus (University of Maryland, Baltimore, MD), and Granta (MCL) was a kind gift from Dr. Steven Bernstein (University of Rochester, Rochester, NY). Malignant cells from patients with FL, DLBCL, and MCL were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) and 1% sodium pyruvate (Granta only) and in the presence of penicillin/streptomycin/glutamine at 37°C in a humidified 5% CO2 incubator. Cell viability was measured using the trypan blue or propidium iodide (PI) exclusion method or MTT assay (see below). Cells were treated with andrographolide or andrographolide + BSO where indicated. Fifty micromoles in DMSO or 100 mmol/L in water stock solution were prepared for both substances. Before treatment, PBS was used to dilute andrographolide stock solution, and PBS/andrographolide solution was added to the medium to achieve the desired working concentration. The control groups used the same amount of DMSO and PBS in the medium as the treatment groups.

Incubations

Cells were incubated with the following drugs: 0 to 100 μmol/L andrographolide (10), 100 μmol/L BSO (13), 10 mmol/L NAC (14), and 50 μmol/L caspase inhibitors (Z-VAD-FMK, Ac-DEVD-CHO, Ac-IETD-CHO, and Ac-LEHD-CHO; ref. 10).

Primary MCL, DLBCL, and FL cells

Following written consent approved by the Northwestern University Institutional Review Board, peripheral blood was drawn from three patients with leukaemia-phase FL, one with MCL, and two with transformed DLBCL. The three FL patients had bulky abdominal adenopathy (>12 cm) and a rapidly rising lymphocyte count (absolute lymphocyte count, 238.5 K/μL) with fluorescence in situ hybridization (FISH) confirmation of t(14;18) in 95% of nuclei. The patient with MCL had newly diagnosed MCL with a WBC of 28,000/μL, with 96% malignant cells, and had t(11:14) by FISH in the blood and bone marrow. The two patients with DLBCL had transformed lymphoma with >100,000/μL circulating large cells. The peripheral blood was diluted 1:1 with PBS (Ca2+- and Mg2+-free) and layered over Ficoll-Paque Plus (Sigma). Samples were then centrifuged at 150 × g for 20 minutes at room temperature; theuffy coat layer was removed and centrifuged again. Isolated peripheral blood mononuclear cells (all malignant cells in the six samples) were then resuspended in RPMI 1640 + 10% FBS to 1 × 10⁶ cells/mL.

MTT assay

The effects of andrographolide on cell viability were measured by MTT assay in Ramos, Granta, HF-1, and SUDHL4 cells according to the instructions of an improved detection kit provided by the manufacturer (CellTiter 96 AQeuous One Solution Cell Proliferation Assay, Promega). Briefly, 2.5 × 10⁴ cells/90 μL were seeded in 96-well microtiter plates. After incubating with different concentrations of andrographolide (10 μL) for the designated times, 20 μL MTT solution was added to each well and the plates were incubated for an additional 1 to 4 hours at 37°C. The absorbance was read at 490 nm using a microplate reader (MRX Revelation; DYNEX Technologies). The absorbance values were expressed as % over the control group. Because reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

ROS measurement

ROS accumulation in treated and untreated cells was measured by fluorescence-activated cell sorting (FACS; refs. 15, 16). Cells were incubated in 5 μmol/L 2′,7′-dichlorofluorescin diacetate (H2DCFDA) for 30 minutes at 37°C in the dark. After washing, cells were suspended in 1 mL of cold PI (200 ng/mL)/PBS for 5 minutes to test ROS in living cells. ROS were measured by oxidation of H2DCFDA to dichlorofluorescin (DCF). Fluorescence intensity was read by flow cytometry using the
Beckman Coulter EPICS XL-MCL Cytometer on the FL1 channel. Results were analyzed and calculated by FCS Express V3 software (De Novo Software) and Excel (Microsoft).

Quantitation of apoptosis
After incubations and washing, $1 \times 10^6$ cells were labeled with Annexin V–FITC and PI reagent in the binding buffer according to the Annexin V–FITC apoptosis detection kit instruction provided by the manufacturer (Invitrogen). The fluorescent signals of FITC and PI were detected at 518 and 620 nm, respectively, on a Beckman Coulter FACS machine. For each analysis, 30,000 events were recorded. Results were analyzed and calculated by FCS Express V3 software and Excel. The % apoptosis was the sum of (Annexin V–FITC+/PI−) and (Annexin V–FITC+/PI+) cells.

Immunofluorescent staining
After washing and cytospin, cells were fixed for 20 minutes by formaldehyde (4% in PBS). Then, cells were blocked in blocking buffer (1% bovine serum albumin with 0.02% Triton X-100) for 1 hour and further incubated with anti-Bax 6A7 monoclonal antibody (Sigma) overnight at 4°C. After washing with PBS (+0.02% Triton X-100 in PBS) and rebloking with blocking buffer for 1 hour, cells were incubated with anti-mouse Alexa Fluor 633 or Cy3 secondary antibody for another 1 hour. After washing, coverslips were mounted onto slides using ProLong antifade mounting reagent (Invitrogen). Cells were visualized under Nikon C1Si confocal or UV LSM510 Meta confocal microscopes.

Western blotting
Following the various incubations, cells were washed with PBS and centrifuged, and cell pellets were treated...
with lysis buffer containing protease and phosphatase inhibitors (Roche; Sigma). Protein concentrations were determined with the Bio-Rad protein assay kit. An aliquot of each cell lysate was used for protein assay (17). Total protein samples (25-50 μg) were subjected to 12% SDS-PAGE electrophoresis. Proteins were electrophoretically transferred to a nitrocellulose membrane. After incubating with blocking buffer containing 5% nonfat milk for 1 hour, the membranes were incubated with the primary antibody overnight at 4°C, washed with TBS–Tween 20 (TBST) thrice, incubated with secondary antibody for 1 hour at room temperature, and washed thrice with TBST. Immune complexes were visualized by enhanced chemiluminescence kit and film (Amersham Biosciences, Millipore, or Denville Scientific, Inc.).

Preparation of cytosolic and mitochondrial fractions

Cells were treated with andrographolide for 0, 3, 18, and 24 hours. Mitochondrial and cytosolic fractions were prepared using a mitochondria isolation kit for cultured cells from Pierce according to the manufacturer’s instructions. The mitochondrial pellet was resuspended in sample buffer for SDS-gel electrophoresis and analyzed by Western blotting for BAX antibody (Cell Signaling Technology). COX IV (Cell Signaling Technology) is used as an internal control for the mitochondrial fraction, and GAPDH (Chemicon) for the cytosolic fraction. Cytosolic fractions were also subjected to Western blotting for BAX.

**MEF**<sup>wt</sup> and **MEF**<sup>Bax<sup>-/-</sup>/Bak<sup>-/-</sup></sup> experiments

Mouse embryonic fibroblasts (MEF) were a kind gift from Dr. Craig Thompson (University of Pennsylvania, Philadelphia, PA). Cells were cultured in DMEM containing 10% FBS and in the presence of penicillin/streptomycin/glutamine at 37°C in a humidified 5% CO<sub>2</sub> incubator. Cell viability was measured using the trypan blue or PI exclusion method. Cells were treated with andrographolide at

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**Fig. 2.** Andrographolide-induced ROS accumulation and Δψ<sub>m</sub> change in lymphoma cell lines were dose dependent. Cells were treated with andrographolide at the indicated dose and time period. A, representative histograms show that andrographolide induced ROS production in Ramos, Granta, HF-1, and SUDHL4 cell lines. A shift to the right (red) indicates ROS production. B, quantification of ROS accumulation in four cell lines. The data represent percentage increase compared with control. Columns, mean of three experiments completed in triplicate; bars, SD. *, P < 0.05, control versus andrographolide. C, quantification of Δψ<sub>m</sub> in three cell lines. The data represent percentage decrease compared with control. Points, mean of three experiments completed in triplicate; bars, SD. *, P ≤ 0.05, control versus andrographolide.
40 and 60 μmol/L for 24 or 30 hours. Cell morphology was observed by microscope. Further, Annexin V–FITC/PI apoptosis assay was performed as described above.

Mitochondrial membrane potential (Δψm) measurement
Tetramethylrhodamine (Invitrogen) was added to the culture medium to a final concentration of 250 nmol/L just before the end of the incubation time. Cells were further incubated for 30 minutes at 37°C. Cells were washed in PBS twice and resuspended in FACS buffer containing 20 nmol/L tetramethylrhodamine and 2% FBS. Fluorescence intensity was measured on the FL-2 channel of a flow cytometer. Results were analyzed and calculated by FCS Express V3 software and Excel.

Statistics
Data are expressed as the mean ± SD. Comparisons between two values were performed by unpaired Student’s t test. For multiple comparisons among different groups of data, the significant differences were determined by the Bonferroni method. Significance was defined at P ≤ 0.05.
Results

Andrographolide inhibits cell viability

To evaluate the effect of andrographolide on cell viability, we treated Ramos, Granta, HF-1, and SUDHL4 cells with 0 to 100 μmol/L andrographolide for 24 or 48 hours. Andrographolide resulted in loss of cell viability (by MTT assay) in all four lymphoma cell lines in a dose- and time-dependent manner (Fig. 1). IC50 (defined herein as that concentration that achieved 50% inhibition of cell viability) at 48 hours was 20 μmol/L for Ramos, 40 μmol/L for Granta, 15 μmol/L for HF-1, and 30 μmol/L for SUDHL4. These data indicate that andrographolide inhibits cell viability in Ramos, Granta, HF-1, and SUDHL4 lymphoma cell lines in a dose- and time-dependent manner.

Andrographolide induces ROS accumulation in lymphoma cell lines

To examine whether andrographolide affects the oxidative function of the cell, we quantified ROS at different time points by measuring the fluorescent signal of DCF using FACS. Cells were treated with andrographolide at the indicated dose and time period (Fig. 2). We showed significant (P ≤ 0.05) ROS accumulation at 48 hours for Ramos, 1 to 6 hours for Granta, 0.5 to 3 hours for HF-1, and 4 hours for SUDHL4 (Fig. 2A). A shift to the right (red) indicates ROS accumulation. Figure 2B shows quantification of ROS accumulation in all four cell lines. The data shown represent percentage increase compared with control and represent the mean ± SD. Taken together, these data show that andrographolide-induced ROS accumulation is dose and time dependent in lymphoma cell lines.

Andrographolide induces disruption of mitochondrial membrane potential

Disruption of the mitochondrial membrane potential (Δψm) is one of the earliest intracellular events that occur in apoptosis. It has been shown that andrographolide results in loss of matrix metalloproteinase (MMP) in a hepatocellular carcinoma cell line (12). To investigate whether andrographolide affects Δψm in lymphoma cell lines, we measured Δψm in Ramos (19 hours), HF-1

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Fig. 4. Mechanism of andrographolide-induced apoptosis. Cells were treated with andrographolide in the presence or absence of NAC (10 mmol/L) or Z-VAD-FMK (50 μmol/L) at the indicated dose and time period, and Western blot was performed. A, andrographolide resulted in cleavage of PARP and activation of caspase-3, caspase-8, or caspase-9 in Ramos. B, andrographolide-induced PARP cleavage started at 16-h incubation. NAC and Z-VAD-FMK inhibited andrographolide-induced PARP cleavage in Granta. C and D, andrographolide resulted in cleavage of PARP and activation of caspase-3, caspase-8, or caspase-9. E and F, andrographolide-induced cleavage of PARP, caspase-3, caspase-8, and caspase-9 was seen at 18 h (HF-1) or 16 h (SUDHL4) and completely inhibited by NAC and by the pan-caspase inhibitor Z-VAD-FMK.
(16 hours), and SUDHL4 (16 hours; Fig. 2C). For Ramos, at lower concentrations of andrographolide (20–40 μmol/L), we start to see the change of Δψm at 19 hours (Fig. 2C). Similar results were seen in HF-1 and SUDHL4 cells. These data show that andrographolide results in dose-dependent loss of Δψm.

Andrographolide induces NAC-reversible, caspase-dependent apoptosis in Ramos, Granta, HF-1, and SUDHL4 cell lines

To determine whether andrographolide-induced loss of cell viability was related to apoptosis, we quantified apoptosis by FACS after staining with Annexin V–FITC and PI (Fig. 3). We found that andrographolide resulted in significant (P < 0.05) dose-dependent apoptosis in Ramos at 72 hours and in Granta, HF-1, and SUDHL4 at 48 hours. The AC50 (defined herein as that concentration that achieved 50% apoptosis) was 40 μmol/L for Ramos at 72 hours, 40 μmol/L for Granta or HF-1 at 48 hours, and 30 μmol/L for SUDHL4 at 48 hours.

To determine if apoptosis was related to ROS, we coinubated cells with the antioxidant NAC. In all cell lines, NAC completely abrogated andrographolide-induced apoptosis (Fig. 3A; P < 0.05). These data suggest that apoptosis by FACS after staining with Annexin V–FITC and PI (Fig. 3) and the time course of caspase activation (Fig. 5) in malignant cells from patients with FL, DLBCL, and MCL, as well as the lack of significant apoptosis in normal human lymphocytes compared with the control group (Fig. 5).
Fig. 6. Andrographolide resulted in BAX conformational changes in cell lines (HF-1 and SUDHL4) and patient samples (FL and DLBCL), and in BAX mitochondrial translocation. Andrographolide induced BAX conformational changes in (A) SUDHL4 cell line, (B) primary DLBCL cells (n = 1 patient sample), (C) HF-1 cell line, and (D) primary FL cells (n = 1 patient sample). All BAX conformational changes were blocked by NAC or Z-VAD-FMK. SUDHL4 (E) or HF-1 (F) cells were treated with 30 and 40 μmol/L andrographolide, respectively, for the indicated period of time. Mitochondrial and cytosolic fractions were separated as described in Materials and Methods and analyzed by Western blotting for proapoptotic BAX protein. COX IV and GAPDH were used as internal controls for mitochondrial and cytosolic extracts, respectively. Andrographolide-induced BAX translocation from cytoplasm to mitochondria was time dependent. Mito BAX, mitochondrial-related BAX; Cyto BAX, cytosolic-related BAX.
Fig. 7. Andrographolide-induced cell death was dependent on the BAX/BAK pathway. Apoptosis in wild-type (WT) MEFs (MEFwt) and in MEFs obtained from Bax/Bak DKO mice (MEF<sup>Bax</sup><sup>−/−</sup>/Bak<sup>−/−</sup>) after treatment with andrographolide was examined. Andrographolide significantly increased cell death in MEF<sup>wt</sup> (P < 0.05), but in MEF<sup>Bax</sup><sup>−/−</sup>/Bak<sup>−/−</sup>, there was minimal cell death. A, the progressive detachment and rounding up of cells after 24-h andrographolide exposure was dose dependent in MEF<sup>wt</sup>, and that was not seen in MEF<sup>Bax</sup><sup>−/−</sup>/Bak<sup>−/−</sup> cells under the microscope. B, apoptosis (Annexin V–FITC and PI) was performed after 30-h andrographolide treatment. The bar graph shows that there was a significant difference (*, P < 0.05) in apoptosis between MEF<sup>wt</sup> and MEF<sup>Bax</sup><sup>−/−</sup>/Bak<sup>−/−</sup> at 60 μmol/L andrographolide, but no change from baseline in MEF<sup>Bax</sup><sup>−/−</sup>/Bak<sup>−/−</sup> cells compared with MEF<sup>wt</sup>. C, similarly, there was a significant decrease in MMP (Δψ<sub>m</sub>) after 4-h andrographolide exposure in MEF<sup>wt</sup> (*, P < 0.05) but not in MEF<sup>Bax</sup><sup>−/−</sup>/Bak<sup>−/−</sup> cells. D, Western blots confirmed that MEF<sup>Bax</sup><sup>−/−</sup>/Bak<sup>−/−</sup> had no BAX and BAK proteins.
was related to the cellular redox state, perhaps as a consequence of depletion of the endogenous antioxidant GSH, because NAC restores intracellular GSH.

We further investigated the involvement of ROS in andrographolide-induced apoptosis by pretreating cells with the reduced GSH-depleting agent BSO (100 μmol/L) and then added andrographolide or andrographolide + NAC at the indicated dose and time periods (Fig. 3B). We found that BSO greatly enhanced andrographolide-induced apoptosis, and apoptosis was again completely inhibited by the antioxidant NAC. With the addition of BSO, the AC_{50} was significantly lower in all cell lines (72 hours, 10 μmol/L in Ramos; 48 hours, 40 μmol/L in Granta; 48 hours, 5 μmol/L in HF-1; and 48 hours, 20 μmol/L in SUDHL4) compared with andrographolide alone. Further, BSO, when added to andrographolide in Ramos cells, shows a striking increase in ROS production compared with andrographolide alone (data not shown).

Next, to determine if andrographolide-induced apoptosis was caspase dependent, the caspase inhibitors Z-VAD-FMK, Ac-LEHD-CHO, Ac-IETD-CHO, and Ac-DEVD-CHO were preincubated with Ramos, Granta, HF-1, and SUDHL4 cell lines, and then andrographolide was added at the indicated doses and time periods (Fig. 3C). In all four cell lines, the pan-caspase inhibitor Z-VAD-FMK resulted in complete inhibition, whereas the caspase-3 inhibitor Ac-DEVD-CHO significantly (P < 0.05) inhibited apoptosis in Ramos and Granta. Further, the caspase-8 inhibitor and the caspase-9 inhibitor (Ac-IETD-CHO and Ac-LEHD-CHO) also resulted in significant inhibition (P < 0.05) in Ramos and Granta (Fig. 3C). Together, these data show that andrographolide-induced apoptosis in lymphoma cell lines is ROS and caspase dependent, and that both the intrinsic and the extrinsic caspase pathways are relevant.

**Andrographolide induces poly(ADP-ribose) polymerase and caspase cleavage**

To further investigate the mechanism of apoptosis, we examined caspase activation and poly(ADP-ribose) polymerase (PARP) cleavage by andrographolide using immunoblotting (Fig. 4). After 24 hours of andrographolide exposure in Granta cells, we found that cleavage of PARP, caspase-3, and caspase-8 was dose dependent and completely inhibited by NAC (Fig. 4A). PARP cleavage was also time dependent, as shown in Fig. 4B. We begin to see PARP cleavage after 16 hours, and it is inhibited by NAC and Z-VAD-FMK. In Fig. 4C and D, after 24 hours of andrographolide exposure, we observed dose-dependent, NAC-inhibitable cleavage of PARP, caspase-8, caspase-9, and caspase-3 in Ramos cells. In Fig. 4E and F, we also show time-dependent cleavage of PARP, caspase-8, caspase-9, and caspase-3 at 40 and 30 μmol/L, respectively, in HF-1 and SUDHL4 cells at concentrations that have been shown to induce apoptosis (Fig. 3). As in Granta cells, NAC and Z-VAD-FMK also inhibited caspase and PARP cleavage induced by andrographolide in HF-1 and SUDHL4. These data provide further evidence that andrographolide-induced apoptosis is ROS and caspase dependent and may proceed by both intrinsic and extrinsic pathways.

**Andrographolide induces apoptosis in primary malignant cells from patients with FL, MCL, and DLBCL**

Based on our observations in lymphoma cell lines, we examined whether andrographolide also induced apoptosis in primary lymphoma patient samples and normal human lymphocytes and, if so, by which cell death pathways. First, we treated fresh FL, MCL, and DLBCL malignant cells with andrographolide at the indicated doses and time periods. (Fig. 5 shows a representative sample. Similar results were seen in all patient samples.) Interestingly, we found that primary malignant cells from patients were more sensitive to andrographolide than the cell lines. Andrographolide induced significant dose-dependent apoptosis (P < 0.05) in primary malignant cells at a lower AC_{50}, and earlier time points (5 μmol/L, 24 hours for FL; 10 μmol/L, 18 hours for DLBCL; 10 μmol/L, 14 hours for MCL). Similar to the cell lines, NAC completely prevented andrographolide-induced apoptosis (P < 0.05) in FL, DLBCL, and MCL (Fig. 5A). By contrast, andrographolide did not cause significant apoptosis in normal human lymphocytes compared with malignant cells at the same andrographolide concentrations (Fig. 5A).

Next, we pretreated with the caspase inhibitors (50 μmol/L each) to determine if andrographolide-induced apoptosis was caspase dependent. The inhibitors of caspase-8, caspase-9, and pan-caspase inhibitor (Ac-IETD-CHO, Ac-LEHD-CHO, and Z-VAD-FMK) significantly inhibited andrographolide-induced apoptosis (P < 0.05) in primary FL cells. Z-VAD-FMK also significantly inhibited andrographolide-induced apoptosis (P < 0.05) in MCL primary cells. The inhibition patterns were similar to the results we observed in the cell lines.

In Fig. 5B, we show that in fresh FL cells, NAC inhibited PARP cleavage starting at concentrations of andrographolide as low as 5 μmol/L, and that BID cleavage was seen at 10 μmol/L and also inhibited by NAC. Similarly, caspase-3, caspase-8, and caspase-9 were cleaved at concentrations as low as 10 μmol/L, and this was inhibited by NAC. In Fig. 5C, after 24-hour treatment of andrographolide in the presence or absence of NAC, we showed dose-dependent cleavage of PARP, caspase-8, and caspase-9 in malignant cells from a patient with DLBCL. In Fig. 5D and E, we show cleavage of PARP and caspase-9 starting at 16 hours of incubation with 5 μmol/L andrographolide in primary MCL cells. BID cleavage was also time dependent and was first seen at 4 to 8 hours of exposure. NAC and Z-VAD-FMK inhibited the activation of PARP and caspase-9 in these cells. Taken together, these data show that as in cell lines, andrographolide-induced apoptosis in primary lymphoma cells is dose, ROS, and caspase dependent. It occurs at lower concentrations of andrographolide and does not affect normal human lymphocytes.
Andrographolide-mediated apoptosis depends on mitochondrial pathways

To investigate the involvement of mitochondrial pathways in lymphoma cell death, we determined the role of BAX/BAK in andrographolide-induced apoptosis. BAX is a "multidomain" proapoptotic protein of the Bcl-2 family that is triggered by BID to undergo homo-oligomerization with BAK, resulting in release of cytochrome c from the mitochondria (18). To explore the role of Bcl-2 family proteins in andrographolide-induced apoptosis in lymphoma, we first performed whole-cell protein Western immunoblotting. We found that total BAX protein expression did not change after exposure to andrographolide (data not shown). However, we then investigated conformational change of BAX following mitochondrial translocation during apoptosis (10, 12, 19). Using the BAX (6A7) monoclonal antibody, which specifically binds the BAX protein with conformational change (10, 12), andrographolide induced BAX conformational change in the SUDHL4 (DLBCL cell line) and HF-1 (FL cell line) cells (Fig. 6A and C, respectively) and, similarly, in primary DLBCL and FL samples (Fig. 6B and D, respectively). These data show an increase of BAX fluorescent staining at 16 to 18 hours in andrographolide-treated cells (Fig. 6A-D). Furthermore, BAX conformational change was ROS and caspase dependent, as treatment with NAC or pretreatment with the pan-caspase inhibitor Z-VAD-FMK eliminated the andrographolide-induced BAX conformational change in cell lines (Fig. 6A and C) and primary patient samples (Fig. 6B and D). These data indicate that andrographolide-induced apoptosis in both cell lines and primary lymphoma cells depends in part on Bcl-2 family proteins and is ROS and caspase dependent.

To further investigate mitochondrial events during andrographolide-induced apoptosis, we extracted cytosolic and mitochondrial fractions for immunoblotting. In the SUDHL4 and HF-1 cell lines, we found BAX accumulation in the mitochondrial fractions of both cell lines by 3 hours, whereas BAX in the cytoplasm was reduced (Fig. 6E and F). Thus, andrographolide induces BAX conformational change and mitochondrial translocation from the cytoplasm in lymphoma, leading to cellular apoptosis.

Andrographolide-mediated cell death is regulated by a BAX/BAK protein–dependent cell pathway

BAX and BAK and their complexes are known to play a central role in facilitating the release of mitochondrial intermembrane proteins during apoptosis (9). To further investigate the role of BAX and BAK in andrographolide-induced cell death, we examined apoptosis in wild-type MEFs (MEFwt) and in MEFs obtained from Bax/Bak double-knockout (DKO) mice (MEF<sup>Bax<sup>−/−</sup>/Bak<sup>−/−</sup></sup>) following treatment with andrographolide. We found that andrographolide significantly increased cell death in MEF<sup>wt</sup> (P < 0.05), but in MEF<sup>Bax<sup>−/−</sup>/Bak<sup>−/−</sup></sup>, there was minimal cell death (Fig. 7). Figure 7A shows that progressive detachment and rounding up of cells after andrographolide exposure is dose dependent in MEF<sup>wt</sup> and not seen at 40 μmol/L and minimally at 60 μmol/L in MEF<sup>Bax<sup>−/−</sup>/Bak<sup>−/−</sup></sup> (Fig. 7A). In Fig. 7B, apoptosis (Annexin V–FITC and PI) was performed after 30 hours of andrographolide treatment. The bar graph shows that there was a significant difference in apoptosis between MEF<sup>wt</sup> and MEF<sup>Bax<sup>−/−</sup>/Bak<sup>−/−</sup></sup> at 60 μmol/L andrographolide. Moreover, after 4 hours, 60 μmol/L andrographolide caused significant loss of Δψ<sub>M</sub> in MEF<sup>wt</sup> (P < 0.05), whereas in MEF<sup>Bax<sup>−/−</sup>/Bak<sup>−/−</sup></sup> it was not seen (Fig. 7C). To further confirm that MEF<sup>Bax<sup>−/−</sup>/Bak<sup>−/−</sup></sup> had no BAX and BAK proteins, we performed Western blots as shown in Fig. 7D. These results suggest that BAX and/or BAK are necessary for andrographolide-mediated cell death, as their absence attenuated cell death in response to andrographolide.

Discussion

We have shown that andrographolide, the active component derived from the plant <i>A. paniculata</i>, causes redox-dependent apoptosis in several non–Hodgkin’s lymphoma cell lines and in primary lymphoma cells. It seems that the mechanism is redox dependent, is mediated through caspase activation, depends on BAX conformational change, and is accompanied by translocation of BAX from the cytoplasm to the mitochondria.

The fundamental molecular mechanisms of the biological effects of lactone diterpenoids have been explored. Andrographolide targets NF-κB for its anti-inflammatory activity (20, 21). The pharmacokinetics of andrographolide in human plasma also have been evaluated by oral administration of 200 mg andrographolide (22), and adducts of andrographolide in human urine have been documented after oral administration (23). The extract (>10% or ≥10% andrographolide) of <i>A. paniculata</i> did not affect the reproductive and fertility ability in male Wistar rats at >1,000 mg/kg per day (24). Recently, andrographolide has also been shown to inhibit cancer cell proliferation and induce apoptosis in cancer cell lines, including leukemia (HL-60), prostatic adenocarcinoma (PC-3), breast cancer (MDA-MB-231 and MCF 7), liver cancer (HepG2 and Hep3B), cervical cancer (HeLa), and colorectal cancer (HTCT16 and HT-29; refs. 9–11, 25–31), but not previously in lymphoma cell lines.

We hypothesized that andrographolide might inhibit cell proliferation and cause apoptosis in lymphoma cell lines and in primary lymphoma cells by mechanisms that involved cellular redox systems, caspase activation, and mitochondrial pathways. Indeed, we found that the effects of andrographolide were dose and time related, and were accompanied by ROS generation (Fig. 2). That ROS played an important role in apoptosis of lymphoma cell lines Ramos, Granta, HF-1, and SUDHL4 was shown by near-complete abrogation of apoptosis by the antioxidant NAC and by enhancement of apoptosis by the GSH-depleting agent BSO. These observations
were not restricted to cell lines, as we also found that andrographolide, at lower concentrations, resulted in ROS- and caspase-dependent inhibition in primary patient samples from patients with FL, MCL, and DLBCL.

We further postulated that andrographolide induced apoptosis in patient samples and cell lines through intrinsic caspase pathways, and that Bcl-2 family proteins and mitochondrial regulation would be required. We found BID cleavage in patient samples (Fig. 5B and D). We also found that andrographolide induced Δψm change in Ramos, HF-1, and SUDHL4 (Fig. 2C). Further, we showed that andrographolide induced conformational change of BAX in the SUDHL4 and HF-1 cell lines and in malignant cells from patients with DLBCL and FL. This was inhibited by NAC, indicating that BAX conformational change depended on oxidant mechanisms (Fig. 6). We also found that andrographolide induces BAX mitochondrial translocation in HF-1 and SUDHL4 lymphoma cell lines (Fig. 6), results similar to those in HepG2 cells as described by Zhou et al. (10). Additionally, BAX and BAK and their complexes are known to play a central role in facilitating the release of mitochondrial intermembrane proteins during apoptosis (9). To further characterize the mechanism, we used Bax/Bak MEF DKOs (MEF<sup>Bax−/−/Bak−/−</sup>; Fig. 7) and found that MEF<sup>Bax−/−/Bak−/−</sup> had reduced Δψm and were killed by similar concentrations of andrographolide, but MEF<sup>Bax−/−/Bak−/−</sup> were not, suggesting that apoptosis proceeds through the BAX/BAK pathway. We also found (Fig. 6) that caspase inhibition blocked BAX conformational change, suggesting that caspases are required for this step in andrographolide-induced apoptosis.

The concentrations that achieved 50% growth inhibition (IC<sub>50</sub>) and 50% apoptosis (AC<sub>50</sub>; Figs. 1 and 3) are similar or even lower than concentrations in other cancer cell lines [HL-60 cell line (29), SMMC-7721 human carcinoma cell line (12), and HepG2 human hepatoma cell line (10)] and are clinically relevant. There are data that suggest that serum concentrations of between 1.9 and 3.8 μmol/L can be achieved with doses of andrographolide commonly used in China (32) and that 20-fold higher doses can be given safely (7). This is well within the range that resulted in biological effects and apoptosis in patient samples in our studies. Further, based on our data (Fig. 5A), andrographolide did not cause significant apoptosis in normal lymphocytes compared with patient samples or cell lines. Further, there was no hematologic toxicity seen in a phase I trial in HIV and non-HIV patients (7).

The mechanism of ROS-dependent cell death related to andrographolide is not clear. Woo et al. (33) have reported that andrographolide upregulates cellular-reduced GSH in neonatal rat cardiomyocytes. However, andrographolide has been found to react with reduced thiols (33), so that initially there is a reduction in GSH, which is then followed by activation of glutamine cysteine ligase and its modifier subunit as an endogenous antioxidant cellular defensive response to GSH reduction. However, Ji et al. (34) have reported that andrographolide initially increased intracellular GSH levels, followed by a decrease, whereas inhibition of cellular GSH synthesis by BSO augmented andrographolide-induced cytotoxicity and apoptosis in Hep3B cells. Our observation that NAC reverses andrographolide-induced apoptosis and ROS generation may be explained by the ability of NAC to restore GSH levels depleted by andrographolide. It is possible that the inherent cellular defense mechanism in neonatal rat cardiomyocytes to increase GSH after insult is not present in lymphoma cells or lymphoma cell lines, or that andrographolide may have a mechanism in lymphoma cell lines and patient samples similar to the liver cancer cell line (Hep3B). We have previously found that GSH levels in lymphoma cell lines are >5-fold higher than in primary tumors (data not shown). This may explain our observations that the AC<sub>50</sub> of andrographolide is much lower in patient samples than in cell lines.

We found that andrographolide induced BAX conformational change in lymphoma cell lines and in fresh patient samples from patients with FL and DLBCL (Fig. 6). BAX conformational change is known to follow caspase-8 activation and is accompanied by pore formation in the outer mitochondrial membrane and precedes the release of cytochrome c from mitochondria, an important early step in mitochondrial-mediated apoptosis (18, 19, 35). Zhou et al. (10) have shown that andrographolide induced BID cleavage and BAX conformational change in HepG2 cells without upregulation of total BAX protein level. Similarly, we found no increase in total BAX (data not shown) but found NAC-inhibitable conformational change in lymphoma cell lines and fresh patient samples. These data suggest the potential clinical relevance to lymphoma biology and suggest that lactone diterpenoids may have antitumor activity in patients with lymphoma. Recently, radiation-sensitizing effects of andrographolide have been published in an in vitro and in vivo model of Ras-transformed cells (36).

It is interesting that we see both caspase-8 and caspase-9 activation with andrographolide, suggesting that both intrinsic and extrinsic caspase pathways are involved in andrographolide-induced apoptosis in lymphoma. Zhou et al. (37) have found that andrographolide may enhance TRAIL (tumor necrosis factor–related apoptosis-inducing ligand)–induced apoptosis through death receptor 4 upregulation, and that this is mediated through p53. Our data suggesting that the pan-caspase inhibitor was required to block andrographolide-induced apoptosis are consistent with a process that may involve caspase pathways and/or other pathways. Currently, studies of death receptor pathways in andrographolide-induced apoptosis of lymphoma cells are ongoing.

It is also intriguing that our data with andrographolide are extant for cell lines from B-cell lymphomas with very different biological and molecular signatures and, indeed, hold true for samples from patients with the corresponding clinicopathologic subtypes of lymphoma. Although there are marked differences in the clinical...
behavior of these three types of lymphoma that would argue against categorizing them as a single entity, it is possible that the activity of this natural diterpenoid lactone operates through biological pathways that are independent of the heretofore established biology of these lymphomas, and thereby will open new pathways to a common approach to treatment.

We have shown that andrographolide, the active component of the plant *A. paniculata*, causes cell death in lymphoma cell lines and in fresh malignant cells from lymphoma patients. The mechanism is related to the redox state of the cells, as it is blocked completely by NAC, and it is caspase dependent. Furthermore, apoptosis is mediated through mitochondrial pathways in both cell lines and primary patient samples. This novel natural lactone diterpenoid deserves further preclinical and clinical investigation in lymphoma.

### Disclosure of Potential Conflicts of Interest

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