Genistein sensitizes diffuse large cell lymphoma to CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy

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Abstract

The incidence of non-Hodgkin's lymphoma (NHL) has been increasing and is now the leading cause of death in males aged 15–54. Diffuse large cell lymphoma (DLCL) is the most common subtype of NHL. These cells are notable for the high expression of the transcription factor nuclear factor kappa beta (NF-κB), raising the possibility that constitutive activation of the NF-κB pathway may contribute to the poor prognosis of DLCL patients. Soy isoflavone genistein promotes apoptosis by decreasing NF-κB activity. The combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) remains the standard therapy for DLCL with a cure rate of ~40%. The WSU-DLCL$_2$ cell line and its severe combined immunodeficient (SCID) xenograft have constitutively active NF-κB which provides us with an excellent model in which to study NF-κB modulation and CHOP sensitization by genistein. The antitumor activity of CHOP with or without a genistein was evaluated in our WSU-DLCL$_2$ model. *In vivo*, WSU-DLCL$_2$-bearing SCID mice received genistein alone (800 μg kg^{-1} day^{-1}, p.o. as gavages for 5 days), CHOP alone (''C'', 40 mg/kg, i.v.; ''H'', 3.3 mg/kg, i.v.; ''O'', 0.5 mg/kg, i.v.; and ''P'', 0.2 mg/kg, every day for 5 days, p.o.), or genistein for 5 days followed by CHOP. Tumor growth inhibition (T/C), tumor growth delay (T − C), and log$_{10}$ kill for genistein, CHOP, and genistein followed by CHOP were 33.6%, 19.2%, and 5.2%; 7, 8, and 17 days; and 1.0, 1.2, and 2.6, respectively. To begin elucidating the mechanism of genistein-induced sensitization of WSU-DLCL$_2$ cells to CHOP chemotherapy in this xenograft mouse model, we studied the *in vitro* effect of genistein on WSU-DLCL$_2$ growth inhibition, cell cycle, Bax:Bcl-2 ratio, NF-κB DNA binding, and apoptosis *in vitro*. At 30 μM, genistein inhibited the growth significantly, induced G$_2$-M arrest, increased Bax:Bcl-2 ratio, decreased NF-κB DNA binding, and induced apoptosis. Genistein also inhibited NF-κB DNA binding *in vivo*, whereas CHOP enhanced it. Our results show that genistein has growth modulatory effects on WSU-DLCL$_2$ cells and enhances the antitumor activity of CHOP. Because soy isoflavone genistein is a widely available nutritional supplement, its use in combination with CHOP chemotherapy should be further explored in a clinical trial in patients with NHL. (Mol Cancer Ther. 2003;2(12):1361–1368)

Introduction

Non-Hodgkin's lymphoma (NHL) is a group of heterogeneous diseases resulting from a malignant proliferation of lymphocytes. More than 55,000 cases were diagnosed in the US in 2002 (1). Diffuse large cell lymphoma (DLCL) is the most frequently occurring type of NHL accounting for 31% of all lymphomas (2). The four-drug combination CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) provides cure in 30–40% of unselected patients with DLCL (3). This regimen has become the ''standard'' treatment because more modern regimens have not been found to be superior to CHOP (4, 5). The newer regimens to CHOP include the use of additional drugs such as methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, and dexamethasone (m-BACOD) or prednisone, doxorubicin, cyclophosphamide, etoposide, cytarabine, bleomycin, vincristine, and methotrexate (ProMACE-CytaBOM).

The only widely accepted example of constitutively active (*i.e.*, found in the nucleus) nuclear factor kappa beta (NF-κB) is in B-cell lymphoma (which includes the DLCL type). B-lymphocytes develop by orderly rearrangement of immunoglobulin genes. One role for NF-κB in B cells is that of regulating the Ig*k* gene (6). Several groups have demonstrated that the constitutive form of NF-κB in mature B-cell lines is largely the p50-c-Rel heterodimer (6–8). The p50-p65 heterodimer is found at expected levels in the cytoplasm, but not in the nucleus of these cells. The mechanism accounting for nuclear levels of p50-c-Rel is not fully understood but may be explained by the instability of IκBα and by the increased transcription of the c-Rel gene (9–11).

Soy isoflavones are among the most promising potential anticarcinogenic compounds in the diet. Epidemiological studies indicate that consumption of soy-containing diets is associated with a lower incidence of certain human cancers (12–14). The typical daily isoflavone intake by an average Japanese person is approximately 50 mg/day (14). Numerous biological activities for the major soy isoflavone genistein have been demonstrated *in vitro*. For example,

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genistein, a known tyrosine kinase inhibitor (15), inhibits topoisomerase I and II (16) and angiogenesis (17). Isoflavones have been shown to inhibit free radical formation (18), reduce lipid oxidation (19), and stimulate antioxidant enzymes (20). A group of investigators (21) has demonstrated that genistein inhibits activation of NF-κB in cancer cells *in vitro*. Another report from the same group (22) showed that blood lymphocytes from healthy male subjects receiving 50 mg soy isoflavone mixture twice daily for 3 weeks are protected from tumor necrosis factor α (TNF-α)-induced NF-κB activation. Recently, inhibition of NF-κB activation by genistein resulted in induction of apoptosis in the PC3 cell line (23).

NF-κB transcription factor has long been known to play a central role in the immune system by regulating the expression of key genes that encode cytokines, adhesion factors, chemokines, and inhibitors of apoptosis (IAPs). Activation of this transcription factor can prevent cancer cells from undergoing apoptosis caused by chemotherapeutic agents, such as those included in the CHOP regimen. NF-κB has emerged as a major contributor to resistance in lymphoma, mainly because of its ability to protect cells from apoptosis (24). NF-κB is well established as a transcription factor (25–27) that is activated during cellular defense mechanisms against damaging stimuli such as radiation, hypoxia, and the fight against infectious microorganisms where pro-inflammatory cytokines play a major role. Therefore, we hypothesized that genistein given in conjunction with CHOP chemotherapy would improve the therapeutic efficacy of the regimen in an animal model we developed as chemoresistant to CHOP (28). In this study, we investigated the antitumor effect of CHOP regimen alone and when genistein is given before CHOP, in a severe combined immunodeficient (SCID) mouse xenograft model bearing the WSU-DLCL$_2$ cell line.

Materials and Methods

WSU-DLCL$_2$ Cell Line

The human DLCL cell line (WSU-DLCL$_2$) was established in our laboratory at Wayne State University's School of Medicine (28). The cell line was maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cells were incubated in a humidified 5% CO_2 atmosphere at 37°C.

Small fragments of the WSU-DLCL$_2$ xenograft were implanted s.c. and bilaterally into naive, similarly adapted mice, as previously described. Mice were checked 3 times per week for tumor development. Once transplanted, WSU-DLCL$_2$ fragments developed into palpable tumors, groups of five animals were removed randomly, and assigned to different treatment groups. Using this model, the efficacy of genistein given at 800 μg kg^{-1} day^{-1}, p.o., as gavages alone for 5 days was compared with CHOP alone and genistein for 5 days followed by CHOP on day 5. CHOP was given at MTD for each agent in the SCID mice as previously determined in our laboratory (29) for one injection, that is, cyclophosphamide ''C'', 40 mg/kg, i.v.; ''H'', 3.3 mg/kg, i.v.; ''O'', 0.5 mg/kg, i.v.; and ''P'', 0.2 mg/kg, p.o. every day for 5 days (29). Mice were observed for measurement of SC tumors, changes in weight, and side effects of the drugs. SC tumors were measured 3 times per week. Animals were euthanized when their total tumor burden reached 1500 mg to avoid discomfort.

Assessment of Tumor Response

The endpoints for assessing antitumor activity were according to standard procedures used in our laboratory (29) and are as follows: Tumor weight (mg) = $(A \times B^2)/2$, where A and B are the tumor length and width (in millimeters), respectively; Tumor growth inhibition (T/C) is calculated by using the median tumor weight in the treated group (T) when the median tumor weight in the control group (C) reached approximately 900 mg; Tumor growth delay (T − C) is the difference between the median time (in days) required for the treatment group tumors (T) to reach 700 mg and the median time (in days) for the control group tumors (C) to reach the same weight; and Tumor cell kill net ($\log_{10}$) = (T − C) − (duration of treatment in days)/(3.32)(Td).

Cell Growth

WSU-DLCL$_2$ cells were plated in 24-well culture clusters (Costar, Cambridge, MA) at a density of 2×10^5 viable cells ml^{-1} well^{-1}. Triplicate wells were treated with genistein at 20 and 30 μM, (C) cyclophosphamide monophosphate [5.84 pM], (H) doxorubicin [1.5 pM], (O) vincristine [260 pM], (P) Prednisone [1.0 μM], or diluent (control). Plates were incubated at 37°C in a humidified incubator with 5% CO_2. All cultures were monitored throughout the experiment by cell count and viability every 24 h for 4 days using 0.4% trypan blue stain (Life Technologies, Inc., Grand Island, NY) and a hemacytometer.

Cell Cycle

WSU-DLCL2 cells were seeded in 25-T tissue culture flasks and treated with or without 20, 30, and 40 μM genistein for 24, 48, and 72 h. The cells were centrifuged and washed in PBS and subjected to flow cytometric analysis on FACStar Plus (Becton Dickinson, San Jose, CA) after propidium iodide labeling.

Apoptosis

For light microscopic examination, WSU-DLCL$_2$ cells were seeded in 24-well culture plates as described above. Briefly, untreated (control) and cells treated with genistein, CHOP, and genistein followed by CHOP were set in three replications. Aliquots from cell cultures were cytocentrifuged using a Cytospin II centrifuge (Shandon Southern Instruments, Sewickley, PA). Cell smears were air-dried, stained with tetrachrome at full concentration for 5 min and then at 50% dilution with distilled water for another 5 min. Slides were analyzed under light microscopy (Nikon, Garden City, WY).

Electrophoretic Mobility Shift Assay

Activation of transcription factor was assayed following a procedure described previously (30). Briefly, after treatment, cells ($1–2 \times 10^6$) were lysed in 0.2 ml of hypotonic lysis buffer containing protease inhibitors. The homogenate was centrifuged, and the pellet was resuspended in ice-cold

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nuclear extraction buffer. After 30 min, the lysates were centrifuged in a microcentrifuge, and the supernatant (nuclear extract) was collected and stored at −22°C until needed. Electrophoretic mobility shift assay (EMSA) was performed by incubating 10 μg of nuclear protein with ^{32}P end-labeled oligo probes in 20 μl binding reaction buffer for 30 min at room temperature. The oligos contain a 5′OH blunt ends, which can be labeled to high specific activity with T4 polynucleotide kinase. The DNA-protein complex formed was separated from free oligos on 7% native PAGE. The gel was dried using a Bio-Rad gel drier and subjected to autoradiography for 24–48 h.

Western Blot Analysis

WSU-DLCL$_2$ cells from genistein-treated or controls were washed twice with 1× PBS, resuspended in Triton X-100 lysis buffer [300 mM sodium chloride, 50 mM Tris-HCl (pH 7.6), 5% Triton X-100, and protease inhibitors] and kept at 4°C for at least 45 min. Cells were centrifuged at 14,000 × *g* for 10 min and the supernatant containing the cytosolic extract was saved. The protein concentration was determined using the Micro BCA protein estimation kit (Pierce, Rockford, IL). Membranes were then incubated in 1:1000 concentration of Bax, Bcl-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and/or poly-ADP-ribose-polymerase (PARP) (Travigen, Gaithersburg, MD). The proteins were visualized using the ECL chemiluminescence reagents (Amersham International Ltd., Buckinghamshire, England).

Results

Therapeutic Effects in the SCID Mouse Xenograft

The efficacy of genistein alone, CHOP alone, and genistein before CHOP was studied *in vivo* using the WSU-DLCL$_2$-SCID model. As shown in Fig. 1, mice in all treatment groups developed SC tumors. There were no statistical differences in tumor weights of the four experimental groups up to day 9. On day 14, the mean tumor weight in the genistein-CHOP combination decreased significantly ($P < 0.001$). Three mice in the genistein-CHOP combination were tumor-free; however, on day 17, their tumors began to grow again. These results were obtained after one cycle of genistein-CHOP combination.

Results shown in Table 1 indicate the antitumor activity of genistein alone, CHOP alone, or genistein-CHOP combination, against WSU-DLCL$_2$-bearing SCID mice. T/C, T − C, and $\log_{10}$ kill were 33.6%, 19.2%, and 5.2%; 7, 8, and 17 days; and 1.0, 1.2, and 2.6, respectively. T/C values are used to determine tumor response. All experimental groups, except the control, were considered active against WSU-DLCL$_2$ tumor (T/C values of ≤42%). However, if $\log_{10}$ kill values are added as a criterion, only the genistein-CHOP combination is considered clinically highly active. An activity rating score of +++ (active) or ++++ (highly active) is needed to effect partial or complete tumor regression. Thus, a score of ''+'' or ''++'' is not considered active by usual clinical criteria (29). The mean and range of tumor weight varied among genistein, CHOP, and their

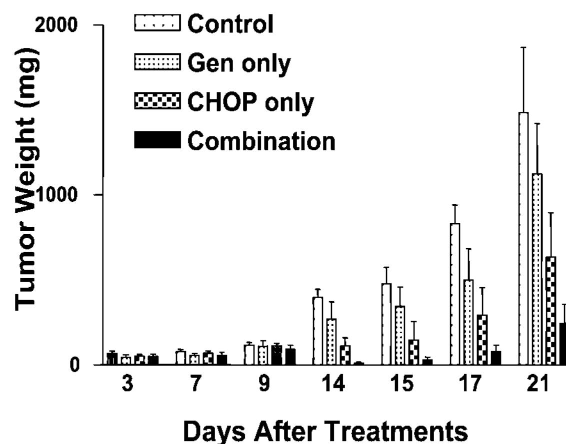

Figure 1. Tumor weight (in milligrams; mean ± SD) of WSU-DLCL$_2$ in control (Diluent), genistein alone, CHOP alone, and their combination. Tumor weight decreased significantly in mice that received a combination of genistein followed by CHOP compared with genistein alone, CHOP alone, or control. Genistein was given at 800 μg/kg, p.o., QD × 5 days. CHOP was given at MTD × 1 injection, that is, C = cyclophosphamide (40 mg/kg, i.v.), H = doxorubicin (3.3 mg/kg), O = vincristine (0.5 mg/kg), and P = prednisone (0.2 mg, p.o.) for 5 days. In the combination, genistein was given 5 days before CHOP. All animals were treated 5 days after transplantation.

combination. Animals treated with genistein-CHOP combination showed the most consistent reduction in tumor weight and range. The smallest tumor size was seen in the genistein-CHOP group, with a range of 14–235 mg.

Genistein Inhibits NF-κB DNA Binding in WSU-DLCL$_2$-SCID Model *in Vivo*

We conducted a gel mobility shift assay (EMSA) on WSU-DLCL$_2$ SC tumors removed from SCID mice that were treated with genistein alone, CHOP, and genistein followed by CHOP and compared it with untreated control. Data from the autoradiography indicate that genistein alone inhibited NF-κB DNA binding compared to control (Fig. 2). The tumors from CHOP-treated mice expressed high NF-κB, which may contribute to resistance to CHOP-induced killing. In addition to the observation that genistein pretreatment down-regulated constitutive NF-κB binding, genistein completely inhibited the CHOP-induced increase in NF-κB binding.

In another experiment, serum concentrations of SCID mice treated with 0.0–10,000 μg/kg genistein were measured using on-line solid-phase extraction and liquid chromatography electrospray mass spectrometry (LC/ES-MS) (Table 2). The 0.23 μM concentration of active genistein detected in the serum of mice treated with 800 μg/kg was adequate to sensitize CHOP and to decrease the activity of NF-κB *in vivo*.

Effect of Genistein on Cell Growth of WSU-DLCL$_2$ *in Vitro*

WSU-DLCL$_2$ lymphoma cells were exposed to 0–40 μM genistein over 72 h and cell viability was determined by trypan blue exclusion assay. Genistein treatment resulted in a dose- and time-dependent inhibition of cell

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Table 1. Antitumor activity of genistein, CHOP, and their combination in WSU-DLCL$_2$-bearing SCID mice

Agent[a]	No. of Animals	T/C (%)	T − C (days)	Log_{10} Kill (Gross)	Activity Score[b]	Mean (mg)	Range (mg)
Control	10	0.0	0	0.0	–	746	626–1068
Genistein	5	33.6	7	1.0	+	259	216–1192
CHOP	5	19.2	8	1.2	++	148	14–914
Genistein-CHOP	5	5.2	17	2.6	++++	40	14–235

[a]Genistein was given at 800 μg/kg, p.o., QD × 5 days. CHOP was given at MTD × 1 injection, that is, C = cyclophosphamide (40 mg/kg, i.v.), H = doxorubicin (3.3 mg/kg), O = vincristine (0.5 mg/kg), and P = prednisone (0.2 mg, p.o.) for 5 days. In the combination, genistein was given 5 days before CHOP. All animals were treated 5 days after transplantation.
[b]Rating score of +++ (active) or ++++ (highly active) is needed to effect partial or complete tumor regression; + or ++ is not considered active by usual clinical criteria (29).

proliferation (Fig. 3). Genistein inhibited the growth at concentration greater than 3 μM. The IC_{50} for WSU-DLCL$_2$ cell line at 72 h was 30 μM, whereas at 40 μM, genistein completely inhibited the growth as early as 24 h following initial exposure.

Genistein Induces G$_2$-M Arrest in WSU-DLCL$_2$ Cells

Genistein has been shown to induce G$_2$-M cell cycle arrest in human solid tumors (31). We show a similar effect of genistein on the WSU-DLCL$_2$ lymphoma cell line at a lower concentration (30 μM *versus* 50 μM in solid tumors). Flow cytometry analysis of WSU-DLCL$_2$ cells treated with 0, 20, 30, and 40 μM genistein resulted in an increased G$_2$-M arrest (Fig. 4).

The percentage of cells in the G$_2$-M phase was increased to >80% at 48 h in cells exposed to 30 and 40 μM genistein. These results demonstrate that genistein causes a G$_2$-M cell cycle arrest in the lymphoma cell line which may account for the growth inhibition observed earlier in the cell proliferation studies.

Genistein Sensitizes WSU-DLCL$_2$ to CHOP Chemotherapy *in Vitro*

As shown in Fig. 5, when genistein at IC_{50} was added to CHOP at IC_{50}, there was significant and sustained growth inhibition, in contrast to CHOP or genistein alone where there was little growth inhibition compared to G + CHOP.

Induction of Apoptosis by Genistein

Next we investigated whether genistein is able to induce apoptotic cell death in WSU-DLCL$_2$. Features of apoptosis that were looked for included cell shrinkage, nuclear chromatin condensation, formation of membrane blebs, and pyknotic bodies. Genistein at 30 μM caused the formation of the distinct morphological features of apoptosis (Fig. 6). At 48 h, genistein induced apoptosis on more than 30% of the WSU-DLCL$_2$ cells. To explore the mechanisms by which genistein induces apoptosis, we investigated the expression of Bax and Bcl-2.

Bax:Bcl-2 Ratio in Genistein-Exposed WSU-DLCL$_2$ *in Vitro*

We assessed whether genistein modulated protein levels of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2. WSU-DLCL$_2$ cells were treated with 0 and 30 μM genistein over 3 days. Whole cell extracts were harvested and subjected to Western blot analysis for the detection of Bax and Bcl-2. The calculated ratio of Bax:Bcl-2 protein expression is shown in Fig. 7. The Bax:Bcl-2 ratio initially decreased then increased on days 2 and 3.

Genistein Inhibits NF-κB DNA Binding *in Vitro*

To demonstrate that genistein can inhibit the activity of NF-κB *in vitro*, we exposed the WSU-DLCL$_2$ cells to 30 μM genistein for 24, 48, and 72 h. NF-κB binding was inhibited as early as 24 h in WSU-DLCL$_2$ cells exposed to 30 μM genistein (Fig. 8). NF-κB bands were undetectable at 48 and 72 h (this experiment was repeated 3 times).

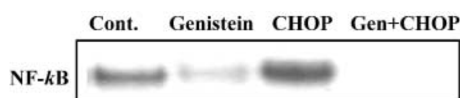

Figure 2. Gel mobility shift assay (EMSA) showing inhibition of NF-κB DNA binding by genistein and activation by CHOP in SCID-bearing WSU-DLCL$_2$ tumors. Mice were treated with diluent (*Cont.*), genistein at 800 μg/kg, p.o. by gavages, QD × 5 days, and CHOP was given at MTD × 1 injection, that is, cyclophosphamide (40 mg/kg, i.v.), doxorubicin (3.3 mg/kg), vincristine (0.5 mg/kg), and prednisone (0.2 mg, p.o.). Subcutaneous tumors were removed and nuclear extracts were prepared and analyzed for NF-κB binding by EMSA.

Table 2. Serum concentrations of genistein in SCID mice using on-line solid-phase extraction and LC/ES-MS

Treatment[a] (μg/kg)	Genistein Concentration in Mouse Serum (μM)[b]
0.0	0.05
0.0	0.05
400	0.09
800	0.23
800	0.21
800	0.23
1,000	0.20
1,000	0.19
5,000	0.22
5,000	0.23
10,000	0.54
10,000	0.50

[a]Genistein was administered to animals p.o. as gavages for 5 days.
[a]Serum was collected 5 h post-last treatment.

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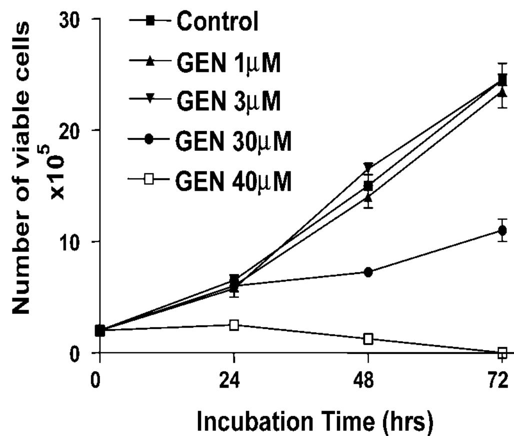

Figure 3. The effect of genistein on cell proliferation of WSU-DLCL$_2$ cells. Cells were cultured at 1.5×10^5/ml in a 24-well plate with or without the agents and incubated for up to 72 h. *Points,* average of two experiments; *bars,* SD.

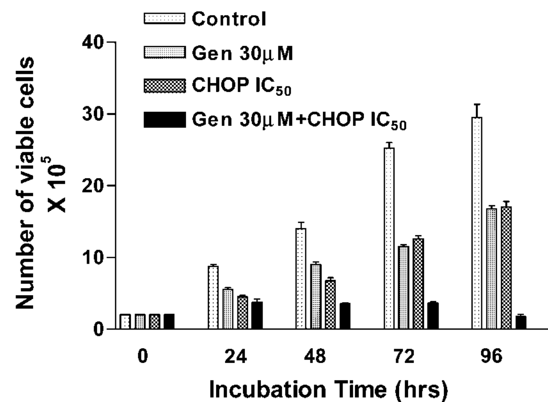

Figure 5. Effect of genistein at IC$_{50}$ (30 μM), CHOP at IC$_{50}$ (cyclophosphamide monophosphate, C = 5.84 pM; doxorubicin H = 1.5 pM, oncovin O = 260 pM, P = 1.0 μM), G (IC$_{50}$) + CHOP (IC$_{50}$), or diluent (*Control*) on the growth of WSU-DLCL$_2$ cells *in vitro*. Cells were cultured at 1.5×10^5/ml in a 24-well plate with or without the agents and incubated for up to 96 h.

Discussion

DLCL cells are notable for the high expression of the transcription factor NF-κB, raising the possibility that constitutive activation of the NF-κB pathway may contribute to the poor prognosis of DLCL patients. Soy isoflavone genistein is a known agent that promotes apoptosis by decreasing NF-κB activity. CHOP remains the standard therapy for DLCL with a cure rate of ~40% (2–5). The WSU-DLCL$_2$ cell line and its SCID xenograft have constitutively active NF-κB which provides us with an excellent model in which to study NF-κB modulation and CHOP sensitization by genistein. The antitumor activity of CHOP with or without a genistein was evaluated in our WSU-DLCL$_2$ model. In this report, we show that genistein given to SCID mice bearing DLCL tumors exhibits antitumor activity. Moreover, administering it before CHOP resulted in significantly higher antitumor activity compared to that achieved with CHOP administered alone.

Genistein is one of the principal biologically active isoflavones found in soy products. Its lack of toxicity makes it an attractive agent for use in conjunction with chemotherapy. We have designed this experiment based on the premise that genistein may work as a chemo-sensitizing agent; therefore, it was given before CHOP chemotherapy. We have also examined the toxicity of genistein in our DLCL$_2$-SCID model and found that it had no toxicity up to 3000 μg/kg. In the animal efficacy experiment, genistein was given p.o. as gavages (800 μg/kg) every day, for a total of 5 days before CHOP. CHOP was given at its MTD, in one injection [CHO; given i.v., P (prednisone); given p.o. for 5 days as in the clinic] (29). Tumor weight patterns in mice assigned to the four treatment groups, control, genistein, CHOP, and genistein followed by CHOP, are shown in Fig. 1. Genistein followed by CHOP showed significantly ($P = 0.01$) longer tumor growth delay compared to all other treatments. Table 1 shows the antitumor activity of genistein, CHOP, or genistein followed by CHOP given at

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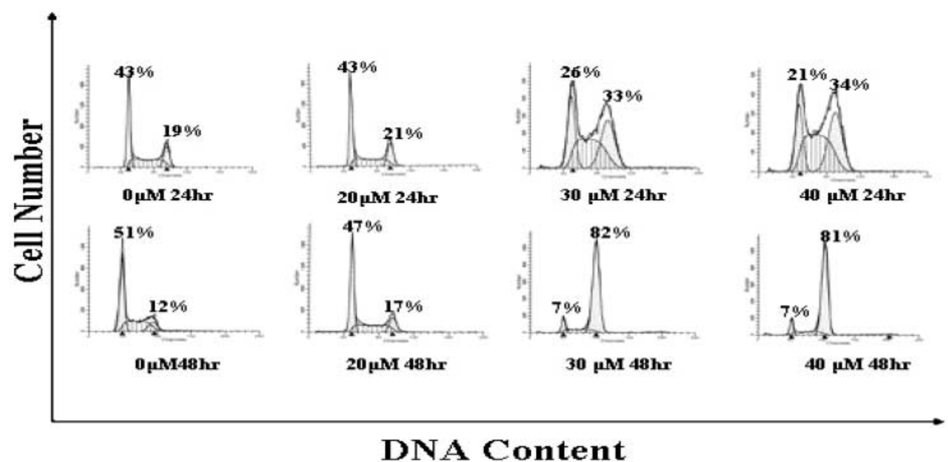
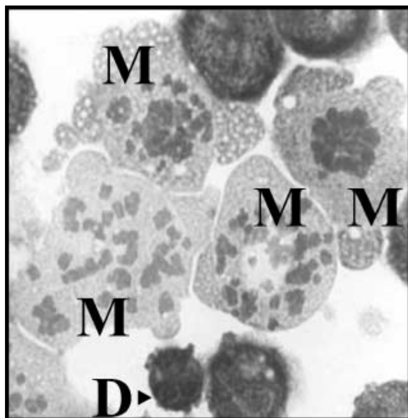


Figure 4. Genistein induces G$_2$-M arrest. Flow cytometric analysis of cell cycle distribution of control and 20, 30, and 40 μM genistein-treated WSU-DLCL$_2$ cells. *Bold % numbers,* percentage of cells in G$_1$ and G$_2$-M phases of the cell cycle, respectively.



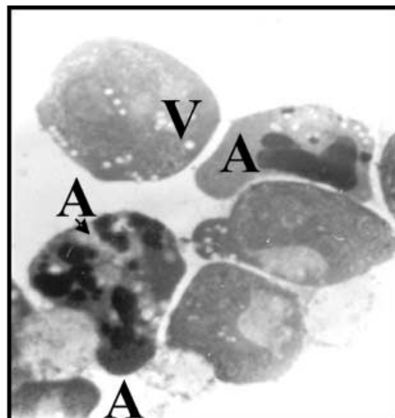


Figure 6. Apoptosis induction in WSU-DLCL$_2$ cells *in vitro*. Photographs showing characteristic features of genistein-exposed cells (×1000). The photographs to the right show WSU-DLCL$_2$ cells exposed to genistein 30 μM for 72 h. Cells labeled *M* = mitotic, *D* = dead, *V* = viable, and *A* = apoptotic.

MTD, against WSU-DLCL$_2$-bearing SCID mice. The endpoints of this experiment were tumor growth inhibition (T/C), tumor growth delay (T − C), and log$_{10}$ kill. When tumor responses are determined by the T/C value, genistein alone and CHOP alone are considered active (T/C = 33.6% and 19.2%, respectively). A T/C value of ≤42% is indicative of antitumor activity.

The WSU-DLCL$_2$ cell line and its SCID xenograft have constitutively active NF-κB which provides us with an excellent model in which to study NF-κB modulation and chemotherapy sensitization by genistein. Overexpression of NF-κB protects cells from apoptosis and inhibition of NF-κB sensitizes cells to apoptosis-inducing agents (32, 33). Genistein may promote apoptosis by decreasing NF-κB activity (24). Therefore, we assessed whether genistein inhibits NF-κB activation in the WSU-DLCL$_2$ s.c. tumors in animals. Our *in vivo* results show that genistein inhibited NF-κB DNA binding dramatically compared with control (Fig. 2). The tumors from CHOP-treated mice expressed high NF-κB, which may in turn contribute to resistance to CHOP-induced tumor killing. In addition to the observation that genistein pretreatment down-regulated constitutive NF-κB binding, genistein completely inhibited the CHOP-induced increase in NF-κB binding. It is worth to mention that genistein by itself has antitumor activity in addition to being a potent inhibitor of NF-κB. The serum concentrations of biologically active genistein of mice treated with 0.0–10,000 μg/kg genistein showed a range of 0.05–0.54 μM. The serum concentration was measured using on-line solid-phase extraction and LC/ES-MS. The 0.23-μM concentration of active genistein detected in the serum of mice treated with 800 μg/kg p.o. as gavages seems to be adequate to decrease the activity of NF-κB *in vivo* (Fig. 2). It is noteworthy that there are no studies to date documenting the effects of genistein on a specific target *in vivo*. Our study clearly shows, for the first time, that a specific target, NF-κB, is significantly inactivated in tumors obtained from genistein-treated animals. Although, inactivation of NF-κB in cell cultures studies requires higher concentration of genistein, the significance of our *in vivo* data in light of low serum concentration of genistein needs further in-depth investigation.

In the *in vitro* study, the WSU-DLCL$_2$ cell line was exposed to 0–40 μM genistein over 72 h. Genistein

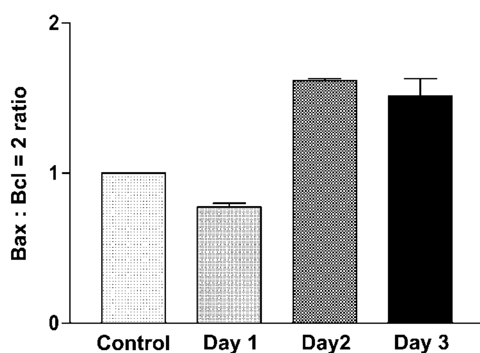


Figure 7. Genistein effect on the Bax:Bcl-2 ratio. Densitometric analysis of Bax and Bcl-2 was determined, normalized to G3PDH control, and plotted as the ratio of Bax:Bcl-2. WSU-DLCL$_2$ cells were treated with 0 and 30 μM genistein over 72 h.

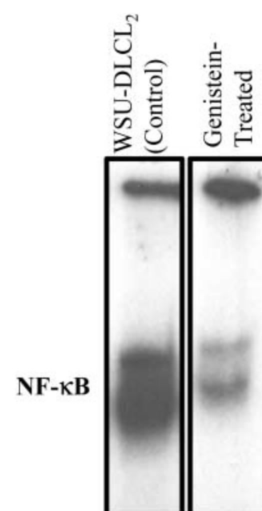


Figure 8. Genistein inhibits NF-κB DNA binding. WSU-DLCL$_2$ cells were treated with 30 μM genistein for 24, 48, and 72 h. Nuclear extracts were analyzed for NF-κB DNA binding using EMSA. NF-κB bands were undetectable at 48 and 72 h (this experiment was repeated 3 times).

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treatment resulted in a dose- and time-dependent inhibition of cell proliferation (Fig. 3). Genistein inhibited the growth of tumor cells at concentrations greater than 3 μM. It is worth to note that the concentration of genistein that caused complete inhibition in this lymphoma cell line is lower than that in solid tumors (34). Flow cytometric analysis of WSU-DLCL$_2$ cells treated with genistein showed an increase in G$_2$-M arrest (Fig. 4). The percentage of cells in the G$_2$-M phase was increased to >80% at 48 h in cells exposed to 30 and 40 μM genistein. These results demonstrate that genistein causes a G$_2$-M cell cycle arrest in the lymphoma cell line which may account for the growth inhibition observed in the cell proliferation studies.

To investigate the possible potentiation of the cytotoxic effect of CHOP regimen, we have investigated the effects of genistein alone at IC$_{50}$, CHOP alone at IC$_{50}$, and their combination against WSU-DLCL$_2$ cells *in vitro*. As shown in Fig. 5, when genistein at IC$_{50}$ was added to CHOP at IC$_{50}$, there was complete and sustained growth inhibition, in contrast to CHOP or genistein alone where there was cell growth recovery. Next we investigated whether genistein was able to induce apoptotic cell death in WSU-DLCL$_2$. Genistein at 30 μM caused the formation of the distinct morphological features of apoptosis (Fig. 6). We also observed an increase of Bax and a decrease of Bcl-2 protein expression.

To investigate if genistein could inhibit the activity of NF-κB *in vitro*, we exposed the WSU-DLCL$_2$ cells to 30 μM genistein for 24, 48, and 72 h. NF-κB binding was decreased at 24 h and as long as 72 h in WSU-DLCL$_2$ cells exposed to 30 μM genistein (Fig. 8).

Collectively, the results demonstrate the superiority of administering genistein before CHOP, over CHOP, or genistein alone. We also begin to elucidate some of the mechanisms of genistein's chemotherapy-potentiating effects. The results from this study should prove useful in guiding translational research on the clinical application of soy isoflavone genistein in the treatment of NHLs.

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