Preclinical studies of a nonpeptidic small-molecule inhibitor of Bcl-2 and Bcl-X_L [(−)-gossypol] against diffuse large cell lymphoma

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Abstract

Overexpression of Bcl-2/Bcl-X_L protein has been observed in more than 80% of B-cell lymphomas. Diffuse large cell lymphoma (DLCL) is the most common subtype of non-Hodgkin's lymphoma. (−)-Gossypol, a natural product isolated from cottonseeds, was discovered as a potent small-molecule inhibitor of Bcl-2 and Bcl-X_L proteins, with a K_i value in the nanomole per liter range for both. *In vitro*, (−)-gossypol showed significant growth inhibition effect against WSU-$DLCL_2$ lymphoma cell line and fresh cells obtained from a lymphoma patient with no effect on normal peripheral blood lymphocytes. As expected (−)-gossypol induced complete cytochrome *c* release from mitochondria, increased caspases-3 and -9 activity, and caused apoptotic death without affecting protein levels of Bcl-2, Bcl-X_L, Bax, and Bak. The addition of cyclophosphamide-Adriamycin-vincristine-prednisolone (CHOP) regimen to lymphoma cells preexposed to (−)-gossypol enhanced killing significantly. The maximum tolerated dose of (−)-gossypol in severe combined immunodeficient (SCID) mice was 40 mg/kg for three i.v. injections when given alone and 20 mg/kg × 3 when given in combination with CHOP. Using WSU-$DLCL_2$-SCID mouse xenograft model, the tumor growth inhibition, the tumor growth delay, and the $\log_{10}$ kill of mice treated with (−)-gossypol + CHOP were better than CHOP or (−)-gossypol alone. We conclude that adding Bcl-2/Bcl-X_L small-molecule inhibitor to standard chemotherapy may prove an effective strategy in lymphoma therapy. [Mol Cancer Ther 2005;4(1):13–21]

Introduction

Non-Hodgkin's lymphoma is a group of heterogeneous diseases resulting from a malignant proliferation of lymphocytes and 55,000 new cases were diagnosed in the United States alone in 2003 (1). Diffuse large-cell lymphoma (DLCL) is the most frequently occurring type of non-Hodgkin's lymphoma and accounts for 31% of all lymphomas (2). The four-drug combination cyclophosphamide-Adriamycin-vincristine-prednisone (CHOP) provides cure in 30% to 40% of unselected patients with DLCL (3). This regimen has become the ''standard'' treatment because newer regimens have not been found superior to CHOP (4, 5). Because the cytotoxic effect of CHOP is mediated through either direct or indirect induction of apoptosis in cancer cells, development of apoptosis resistance of DLCL cells to CHOP accounts for treatment failure in the majority of patients with DLCL. Hence, future efforts toward developing new therapies to improve survival and quality of life of DLCL patients must include strategies that specifically target apoptosis resistance of DLCL cells to chemotherapeutic agents.

Bcl-2 was originally identified at the chromosomal breakpoint of t(14;18)-bearing B-cell lymphomas (6) and belongs to a growing family of proteins that regulate apoptosis (7). The Bcl-2 family includes both death antagonists such as Bcl-2 and Bcl-X_L and death agonists such as Bax, Bak, Bid, and Bad (8–11). It is now recognized that overexpression of antiapoptotic proteins Bcl-2 and Bcl-X_L in cancer cells plays an important role in the resistance of cancer cells to current anticancer therapies. Indeed, overexpression of Bcl-2 and/or Bcl-X_L is found in 80% of non-Hodgkin's lymphoma (12). Bcl-2 and Bcl-X_L represent important molecular targets toward development of new therapeutic strategies for lymphoma.

We previously used the antisense oligonucleotide approach to down-regulate the expression of Bcl-2 (13). It was shown that although *bcl*-2 antisense oligonucleotide therapy is effective against systemic disease in severe combined immunodeficient (SCID) mice xenografts bearing human t(14;18) follicular lymphoma, it does not prevent disease dissemination into the central nervous system, causing subsequent animal death. Our results suggested that whereas inhibition of Bcl-2 is a promising therapeutic strategy for the treatment of lymphoma, oligonucleotide

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may have an intrinsic therapeutic limitation due to, for example, its inability to enter certain privileged organs such as the central nervous system. Hence, novel approaches to targeting antiapoptotic Bcl-2 proteins have to be sought.

The antiapoptotic function of Bcl-2/Bcl-X_L is attributed, at least in part, to their ability to heterodimerize with proapoptotic members such as Bak, Bax, Bid, and Bad. The experimental structures of Bcl-2 and Bcl-X_L showed that BH1 (Bcl-2 homology domain 1), BH2, and BH3 domains of Bcl-2 and Bcl-X_L form a hydrophobic binding groove (the BH3 binding groove) into which Bak or Bad BH3 domain binds. This binding pocket in Bcl-2/Bcl-X_L is essential for its antiapoptotic function. Therefore, it has been hypothesized that small molecules that bind to this BH3 binding site in Bcl-2/Bcl-X_L may be capable of blocking the heterodimerization of Bcl-2/Bcl-X_L with proapoptotic members in the Bcl-2 protein family, such as with Bad, Bak, Bid, and Bax. Such a blocking of antiapoptotic-proapoptotic protein heterodimerization in turn may inhibit the antiapoptotic function of Bcl-2/Bcl-X_L and induce apoptosis in cancer cells with Bcl-2/Bcl-X_L overexpression. Although it has been traditionally difficult to design nonpeptidic small-molecule inhibitors to block protein-protein interactions, several recent studies have shown that it is possible to discover and design potent, nonpeptidic small-molecule inhibitors that bind to the BH3 binding groove (14–17). For example, several small-molecule inhibitors of Bcl-2 were discovered through computational screening. Design of nonpeptidic small-molecule inhibitors of Bcl-2 and Bcl-X_L is a new and exciting research area.

Although its molecular mechanism was not well understood previously, gossypol, a natural product isolated from cottonseeds and roots, has been studied as an anticancer agent since the 1980s in both *in vitro* and *in vivo* models and was recognized to have a different molecular mechanism from other anticancer agents. The racemic form of gossypol [(±)-gossypol] has been tested in several clinical trials and was well tolerated in patients, and it showed a low but measurable response in patients who had failed conventional therapy (18–20). Very recently, it was discovered that (±)-gossypol potently blocks the interaction between Bcl-X_L and the Bak peptide (21). It was then shown that *in vitro*, gossypol induces apoptosis in breast, colon, and prostate cancer cells that have high levels of Bcl-2 or Bcl-X_L. In addition, Kitada et al. (22) found that gossypol also binds and antagonizes the antiapoptotic effect of Bcl-X_L. Gossypol has two enantiomers, (−)-gossypol and (+)-gossypol, that have a similar binding affinity to Bcl-X_L and to Bcl-2 in competitive binding assays. However, the growth-inhibitory effect of (+)-gossypol on cancer cells *in vitro* was found to be greatly reduced in the presence of serum under culture conditions, whereas that of (−)-gossypol was not significantly affected (16, 21). As a result, (−)-gossypol has greater activity in inhibition of cell growth and induction of apoptosis in a variety of cancer cell lines. Using multidimensional nuclear magnetic resonance methods, (−)-gossypol was conclusively shown to bind at the BH3 binding groove of Bcl-2 and Bcl-X_L.

In this study, we investigated the antitumor activity of (−)-gossypol *in vitro* and in a SCID mouse xenograft model bearing the WSU-$DLCL_2$ cell line, which has high levels of Bcl-2 and Bcl-X_L protein expression.

Materials and Methods

WSU-$DLCL_2$ Cell Line, Patients, Lymphoma Cells, and Normal Peripheral Blood Lymphocytes

The chemoresistant DLCL cell line (WSU-$DLCL_2$) was established in our laboratory at Wayne State University's School of Medicine (23). The cell line was maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 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. No growth factors, mitogens, or EBV was added to the cell culture medium at any time. The cell line has a doubling time of 18 hours, and preserved the natural B-cell phenotype of the original tumor in the patient.

WSU-$DLCL_2$ cells were plated in 24-well culture clusters (Costar, Cambridge, MA) at a density of 2×10^5 viable cells/mL per well. Triplicate wells were treated with 0.0 to 10 μmol/L (−)-gossypol and CHOP (cyclophosphamide monophosphate 5.84 pmol/L, doxorubicin 1.5 pmol/L, vincristine 260 pmol/L, and prednisolone 1.0 μmol/L). Doses for CHOP were as determined previously (24). Cells were preexposed to (−)-gossypol for 5 hours before CHOP was added. 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 hours for 4 days using 0.4% trypan blue stain (Life Technologies, Inc., Grand Island, NY) and a hemacytometer.

Fresh lymphoma cells obtained from two patients, one with acute lymphoblastic leukemia and the other with DLCL, were compared with normal peripheral blood lymphocytes (PBL) obtained from a healthy donor. Cells were plated in 24-well culture clusters (Costar, Cambridge, MA) at a density of 4×10^5 viable cells/mL per well. Triplicate wells were treated with 0.0, 2.0, and 4.0 μmol/L (−)-gossypol. 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 hours for 4 days using 0.4% trypan blue stain (Life Technologies, Inc.) and a hemacytometer. Statistical analysis was done using a two-tailed t test, with 95% confidence intervals between treated and untreated samples. P values < 0.05 indicated statistical significance.

Western Blot Analysis

Proteins obtained from mitochondrial and cytosolic extracts were resolved using a 12% SDS-PAGE and transferred to Hybond C-extra membranes (Amersham Life Science, Arlington Heights, IL). Membranes were

blocked with 5% milk in PBS containing 0.05% Tween 20 (PBST) for 1 hour at 25°C and then incubated with unlabeled primary antibodies (1:1,000 dilution in PBST) overnight at 4°C. Following this incubation, membranes were washed well in PBST and incubated with the horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5,000 dilution in PBST) for 45 minutes to 1 hour at 25°C. Proteins were visualized using an enhanced chemiluminescence assay (ECL, Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Primary antibodies specific for cytochrome *c*, Bcl-2, Bcl-X_L, Bax, and Bak (Santa Cruz Biotechnology) were used. Blots were stripped between multiple probings by incubating the membranes in stripping buffer (100 mmol/L 2-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris-HCl, pH 6.7) for 25 minutes at 60°C with occasional agitation. Protein concentrations of the mitochondrial and cytosolic extracts were determined using the Micro bicinchoninic acid protein assay (Pierce Chemical Company, Rockford, IL).

7 Aminoactinomycin D Staining and Flow Cytometry

7aminoactinomycin D (7AAD, Calbiochem-Novabiochem, La Jolla, CA) was diluted in PBS to a concentration of 200 μg/mL. Using this stain as described previously (24), we were able to determine the percentage of viable, apoptotic, and dead cells. (−)-Gossypol-, CHOP-, and (−)-gossypol + CHOP–treated and untreated WSU-$DLCL_2$ cells were harvested, washed with PBS, and stained with 7AAD. Cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Data on 20,000 cells were acquired and processed using Lysys II software (Becton Dickinson). Scattergrams were generated by combining forward light scatter with 7AAD fluorescence.

Morphology

Cells were cultured at 1.5×10^5/mL in T-25 tissue culture plates. Cells were then exposed to 2.0 μmol/L (−)-gossypol for 24 hours. For light microscopic examination, WSU-$DLCL_2$ cells were seeded in 24-well culture plates as described above. Briefly, untreated cells (control) and cells treated with (−)-gossypol were set in three replications. Aliquots from cell cultures were cytocentrifuged in a Cytospin II centrifuge (Shandon Southern Instruments, Sewickley, PA). Cell smears were air-dried, stained with tetrachrome at full concentration for 5 minutes and then at 50% dilution with distilled water for another 5 minutes. Slides were analyzed under light microscopy (Nikon, Garden City, WY). Features of apoptosis looked for included nuclear chromatin condensation, formation of membrane blebs, and apoptotic bodies.

Isolation of Cytosol and Mitochondria Fractions

Cells were collected and then washed with ice-cold 1× PBS. The cells were then suspended in 5 volumes of chilled buffer A [250 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 17 μg/mL phenylmethylsulfonylfluoride, 8 μg/mL aprotinin, 2 μg/mL leupeptin (pH 7.4)] and left on ice for 15 minutes. Cell fractionation was done as previously described (25, 26). Briefly, cells were homogenized using ice-cold cylinder cell homogenizer (20–25 strokes). Cellular and nuclear debris were removed by centrifuging the homogenates twice at 750 × *g* spin for 10 minutes at 4°C. The supernatants were pelleted again at 10,000 × *g* for 25 minutes at 4°C. The resultant mitochondrion pellets were resuspended in 1 volume ice-cold buffer A. Supernatants were further cleared at 100,000 × *g* for 1 hour and these supernatants contained the cytosolic fraction.

Caspase Fluorometric Activity Assay

WSU-$DLCL_2$ cells exposed to 2.0 μmol/L (−)-gossypol for 0 to 24 hours were incubated on ice for 10 minutes in cell lysis buffer (BioVision Research Products, Palo Alto, CA). The clear supernatant after centrifugation at 2,000 × *g* at 4°C was collected and proteins quantified according to the bicinchoninic acid protein assay methodology (Pierce Chemical Company). A total of 100 μg protein in a volume of 50 μL cell lysis mixture was resuspended on ice in triplicates in a 96-well plate. Fifty microliters of 2× reaction buffer containing 10 mmol/L DTT was added to each sample (BioVision Research Products). A 50-μmol/L final concentration of 7-amino-4-trifluoromethyl coumarin (AFC)–conjugated substrates for caspase-3 (Ac-DEVD-AFC) and caspase-9 (Ac-LEHD-AFC; BioVision Research Products) was added to each sample for a total volume of 100 μL and incubated for 180 minutes at 37°C. AFC fluorescence, released by caspase activity, was measured on a fluorescence plate reader (Molecular Devices) set at 400-nm excitation filter and 505-nm emission filter.

WSU-$DLCL_2$ Xenografts

Four-week-old female ICR-SCID mice were obtained from Taconic Laboratory (Germantown, NY). The mice were adapted and WSU-$DLCL_2$ xenografts were developed as described previously (24). Each mouse received 10^7 WSU-$DLCL_2$ cells (in serum-free RPMI 1640) s.c. in each flank area. When s.c. tumors developed to ~1,500 mg, mice were euthanized and tumors dissected and mechanically dissociated into single-cell suspensions. Mononuclear cells were separated by Ficoll-Hypaque density centrifugation and washed twice with RPMI 1640. These cells were subjected to phenotypic analysis for comparison with the established tumor cell line to insure the human origin and its stability. After formation of s.c. tumors, serial propagation was accomplished by excising the tumors, trimming extraneous material, and cutting the tumors into fragments of 20 to 30 mg that are transplanted s.c., using a 12-gauge trocar, into the flanks of a new group of mice.

Maximum Tolerated Dose, Efficacy Trial Design for (−)-Gossypol, CHOP, and Their Combination

A dose-range finding study of three dose levels (20, 40, and 60 mg/kg) of the (−)-gossypol plus a vehicle-only control drug given i.v. daily for 5 consecutive days was conducted in SCID mice. Animal survival was monitored for 3 weeks. The maximum tolerated dose (MTD) is defined as the dose that will lead to no deaths of any of the animals

and no more than 10% loss of body weight during treatment followed by weight gain. MTD studies were done on non-tumor-bearing SCID mice. Animal groups were ear-tagged and observed for immediate toxicity (first 30 to 60 minutes), twice daily for the first 3 days (acute toxicity), then daily for 3 weeks. Animals were weighed daily and monitored for activity, skin changes indicating dehydration (secondary to diarrhea), and any other physical or behavioral abnormalities. CHOP MTD in SCID mice was previously determined in our laboratory (24) for one injection (i.e., cyclophosphamide 40 mg/kg i.v., doxorubicin 3.3 mg/kg i.v., vincristine 0.5 mg/kg i.v., and predisone 0.2 mg/kg p.o.) every day for 5 days. The MTD for (−)-gossypol/CHOP combinations were determined by administering (−)-gossypol at 20, 40, and 60 mg/kg i.v. daily for 3 days, plus CHOP at its MTD.

For the subsequent drug-efficacy trials, 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 thrice per week for tumor development. Once transplanted WSU-$DLCL_2$ fragments developed into palpable tumors (60–100 mg), groups of five animals were removed randomly and assigned to different treatment groups. Using this model, the efficacy of (−)-gossypol, CHOP, and their combination was studied. Mice were observed for measurement of s.c. tumors, changes in weight, and side effects of the drugs. S.c. tumors were measured thrice per week.

Assessment of Tumor Response

The end points for assessing antitumor activity were according to standard procedures used in our laboratory and are as follows: tumor weight (mg) = $(A \times B^2)/2$, where A and B are the tumor length and width (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: $\log_{10}$ gross = $(T - C) - /(3.32)(\text{Td})$.

All studies involving mice were done under Animal Investigation Committee–approved protocols. Tumor weights in SCID mice were plotted against time on a semilog sheet with the growth pattern resembling an S shape. Tumor doubling (Td) is the time (in days) required for the tumor to double its weight during the exponential growth phase.

Statistical Analysis

For the comparison of tumor weight, the power to detect differences in the mean tumor weight at the completion of treatment between treatment and control groups has been calculated based on a sample of five mice per 10 xenografted tumors per group. Power calculations assume that the use of a two-sided, two-sample t test with equal variance and assume the difference between means to be a proportion of the SD of the outcome measurement. For example, a 1-unit difference between groups represents a difference of 1 SD between groups. The study has at least 90% power to detect differences larger than 1.6 units of SD between groups.

Results

Baseline Expression of Bcl-2 and Bcl-X_L in WSU-$DLCL_2$ and the Effect of (−)-Gossypol on Cell Growth *In vitro*

The chemical structure of gossypol is presented in Fig. 1*A*. Gossypol is a naturally occurring polyphenolic constituent extracted from the cotton plant (*Gossypium*). WSU-$DLCL_2$ cell lines, fresh cells obtained from two lymphoma patients, as well as normal PBLs from a healthy donor, were exposed to (−)-gossypol over 72 hours. Cell viability was determined by trypan blue exclusion assay. Cells exposed to (−)-gossypol resulted in a dose- and time-dependent inhibition of cell proliferation (Fig. 1*B*). (−)-Gossypol inhibited the growth of the WSU-$DLCL_2$ line at concentrations greater than 1.0 μmol/L. At a concentration of 4.0 μmol/L, (−)-gossypol completely inhibited the growth as early as 24 hours after initial exposure, and a concentration of 10 μmol/L killed all WSU-$DLCL_2$ cells. Moreover, (−)-gossypol showed significant cytotoxic effect on fresh lymphoma cells at 2.0 and 4.0 μmol/L concentrations (Fig. 1*C*). On the other hand, on exposing normal PBLs to 2.0 or 4.0 μmol/L (−)-gossypol, no major cell kill was noticed up to 72 hours (Fig. 1*D*).

Induction of Apoptosis in WSU-$DLCL_2$ by (−)-Gossypol

Next we investigated whether (−)-gossypol is able to induce apoptotic cell death in WSU-$DLCL_2$. Cells were removed at different time points for cell count, viability, and evidence of apoptosis as assessed by morphology and 7AAD (24). After 24 hours, results indicate that (−)-gossypol, at 1.0, 2.0, 4.0, and 10 μmol/L, induced 10%, 21%, 35%, and 65% apoptosis, respectively (Fig. 2). For morphology, cytospin preparations from (−)-gossypol-treated WSU-$DLCL_2$ cells and control were prepared using a Cytospin II centrifuge. The smears were air-dried, stained with tetrachrome for 5 minutes, and analyzed using light microscopy. Features of apoptosis that were looked for included cell shrinkage, nuclear chromatin condensation, formation of membrane blebs, and pyknotic bodies. (−)-Gossypol at 2.0 μmol/L caused the formation of the distinct morphologic features of apoptosis (Fig. 3). Next, we investigated whether (−)-gossypol is capable of inducing apoptosis in a patient-derived non-Hodgkin's lymphoma sample. Indeed (−)-gossypol at a concentration of 2.0 μmol/L was able to induce apoptosis on 64% of the treated fresh lymphoma cells after 24 hours compared with untreated control (figure not shown).

(−)-Gossypol Releases Cytochrome *c* in WSU-$DLCL_2$ Cells

We hypothesized that (−)-gossypol-induced apoptosis may be mediated by the release of cytochrome *c* from mitochondria into the cytosol. Release of cytochrome *c*

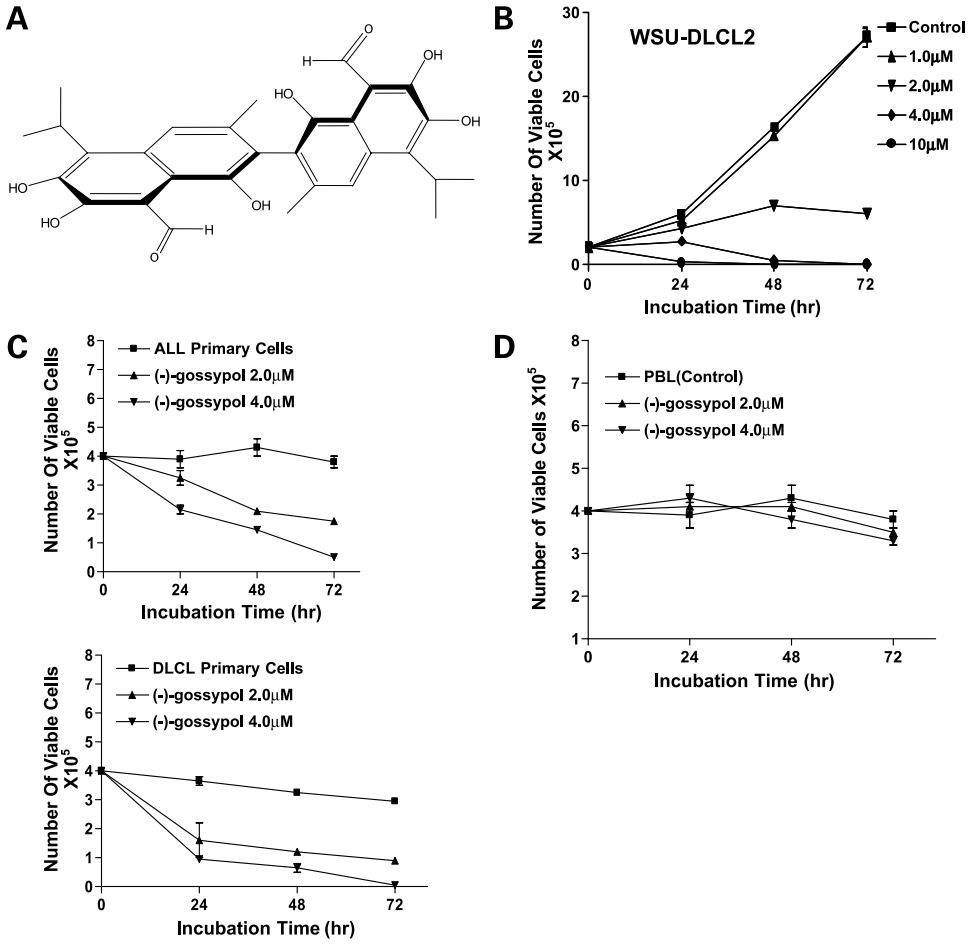

Figure 1. **A,** chemical structure of gossypol. Gossypol is a naturally occurring polyphenolic constituent extracted from the cotton plant (*Gossypium*). **B,** effect of (−)-gossypol on cell proliferation of WSU-$DLCL_2$. Cells were cultured at 1.5×10^5 cells/mL in a 24-well plate, exposed to 0.0, 1.0, 2.0, 4.0, and 10 μmol/L (−)-gossypol, and incubated for up to 72 h. **C**, effect of (−)-gossypol on fresh B-cell acute lymphoblastic leukemia (*ALL*) and diffuse large B-cell lymphoma (*DLCL*) cells obtained from patients. Cells were cultured at 4.0×10^5 cells/mL in a 24-well plate, exposed to 0.0, 2.0, and 4.0 μmol/L (−)-gossypol, and incubated for up to 72 h. **D,** effect of (−)-gossypol on normal PBLs. Cells were seeded at 4×10^5 cells/well and exposed to 2.0 or 4.0 μmol/L (−)-gossypol; no major cell kill was noticed up to 72 h. *Points,* average of two experiments; *bars,* SD.

from the mitochondrial intermembraneous space into the cytosol is a prominent manifestation of apoptosis (25, 26). Figure 4 indicates that all the cytochrome *c* was accumulated in the mitochondria of the untreated cells and undetectable in the cytosol. Exposing cells to (−)-gossypol for 24 hours revealed the release of cytochrome *c* into the cytosol. By 48 hours, cytochrome *c* was translocated completely in the cytosol. It is worth mentioning that in WSU-$DLCL_2$ cells, Bax protein was detected at a low level in the cytosolic extract.

(−)-Gossypol Induction of Caspase Activity

Apoptosis is associated with the activation of specific cysteine proteases referred to as caspases (27, 28). We assessed whether (−)-gossypol activated specific caspases during apoptosis of WSU-$DLCL_2$ cells. Treatment of WSU-$DLCL_2$ with 2.0 μmol/L (−)-gossypol for 0, 4, 6, 8, and 24 hours resulted in increase in activities of caspase-9 and caspase-3 as early as 4 hours (Fig. 5). The maximum increase in caspase-9 and caspase-3 activity was seen at 24 hours and 8 hours, respectively.

Effect of (−)-Gossypol on Bcl-X_L, Bcl-2, Bax, and Bak

We assessed whether (−)-gossypol modulated levels of protein expression of some Bcl-2 family members (Bcl-2, Bcl-X_L, Bax, and Bak) in WSU-$DLCL_2$ cells. We observed no significant changes in protein expression of treated WSU-$DLCL_2$ cells compared with control (Fig. 6). The decrease in Bak at 48 and 72 hours, compared with the bands of loading controls, was not significant. These findings are consistent with our hypothesis that (−)-gossypol interferes with the antiapoptotic function of Bcl-2 and Bcl-X_L, rather than expression levels of these proteins.

(−)-Gossypol Sensitizes WSU-$DLCL_2$ to CHOP Chemotherapy *In vitro*

Previously, we have investigated the effect of CHOP on our WSU-$DLCL_2$ cells and determined the IC_{50} *in vitro* (24). Here we studied the effects of (−)-gossypol alone at 1.0 and 1.5 μmol/L, CHOP alone at its IC_{50}, and their combination against WSU-$DLCL_2$ cells *in vitro*. As shown in Fig. 7*A*, when (−)-gossypol was added 5 hours before CHOP, there was complete and sustained growth inhibition, in contrast to CHOP or (−)-gossypol alone in which there was less cell growth inhibition.

MTD of (−)-Gossypol in SCID Mice

The MTD for (−)-gossypol was determined to be 120 mg/kg, given in three divided dosages daily of 40 mg/kg per i.v. injection. Animals at this dose experienced weight loss of <5% and had scruffy fur, however, with full recovery 48 to 72 hours after completion of treatment.

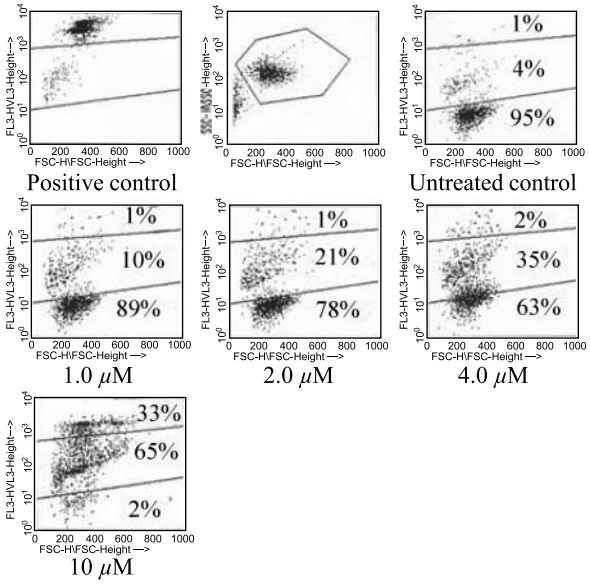

Figure 2. 7AAD flow cytometric analysis of apoptosis. Representative scattergrams generated from 7AAD staining of WSU-DLCL$_2$ cells after treatment with 0.0, 1.0, 2.0, 4.0, and 10 μmol/L (−)-gossypol for 24 h, compared with controls. *Top,* live cells; *middle,* early apoptotic cells; *bottom,* late apoptotic and dead cells. *Bold numbers,* percentage of cells in the respective region. *First box,* heat-treated dead cells used as a positive control.

Animals that received daily injections of 40 mg/kg for 4 days died by the 5th day after treatments were completed. A dose of 60 mg/kg per i.v. injection given daily for 3 days was toxic. The MTD of CHOP in SCID mice was previously determined in our laboratory (24) for one injection (i.e., cyclophosphamide 40 mg/kg iv, doxorubicin 3.3 mg/kg iv, vincristine 0.5 mg/kg iv, and predisone 0.2 mg/kg p.o.)

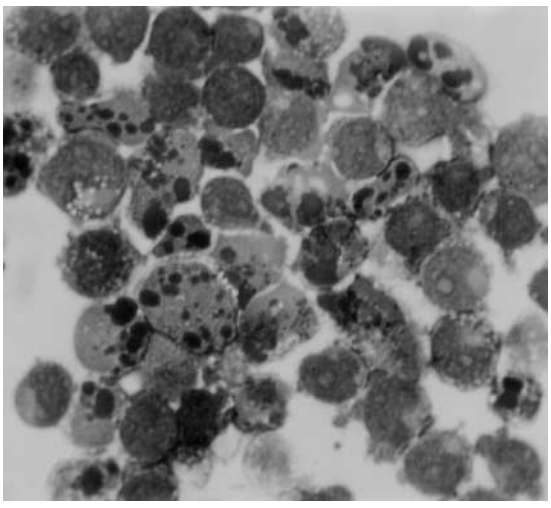

Figure 3. Apoptosis induction in WSU-DLCL$_2$ cells *in vitro*. Photographs showing characteristic features of (−)-gossypol-exposed cells (×1,000). The photograph shows WSU-DLCL$_2$ cells exposed to (−)-gossypol, 2.0 μmol/L for 24 h, go through apoptotic death.

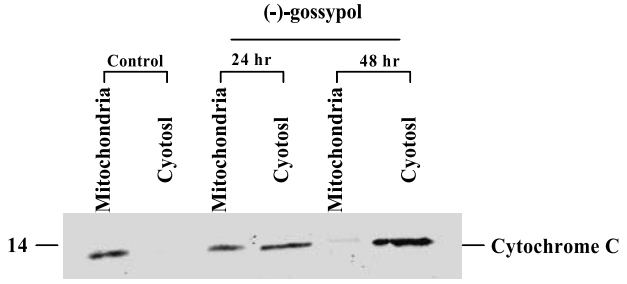

Figure 4. Cytochrome *c* protein expression in WSU-DLCL$_2$ cells exposed to (−)-gossypol. Cells (2×10^5/mL) were exposed to 2.0 μmol/L (−)-gossypol for 0.0, 24, and 48 h. Proteins obtained from mitochondria and cytosolic extracts were separated by 12.0% SDS-PAGE. Cytochrome *c* specific protein was detected using an anti–cytochrome *c* monoclonal antibody.

daily for 5 days. The MTD of the (−)-gossypol/CHOP combination was determined to be 60 mg/kg (i.v. via tail vein, divided in three injections for 3 days) plus CHOP at its MTD. Combination of (−)-gossypol at its MTD (i.e., 120 mg/kg divided in three injections for 3 consecutive days) plus CHOP at its MTD was toxic to all SCID mice.

Antitumor activity of (−)-gossypol alone, CHOP alone, or (−)-gossypol + CHOP combination against WSU-DLCL$_2$-bearing SCID mice as measured by T/C were 56%, 19%, and 10%; $T - C$, 3, 8, and 10 days; and log kill, 0.5, 1.2, and 1.5, respectively (Table 1). T/C values are used to determine tumor response. CHOP alone and (−)-gossypol + CHOP were considered active against 10 WSU-DLCL$_2$ tumor (T/C values of <42%). Figure 7 *B* shows the tumor weight of mice treated with (−)-gossypol, CHOP, and their combination, compared with control. Tumor weights in the (−)-gossypol + CHOP combination decreased significantly ($P < 0.01$) compared with either (−)-gossypol or CHOP group.

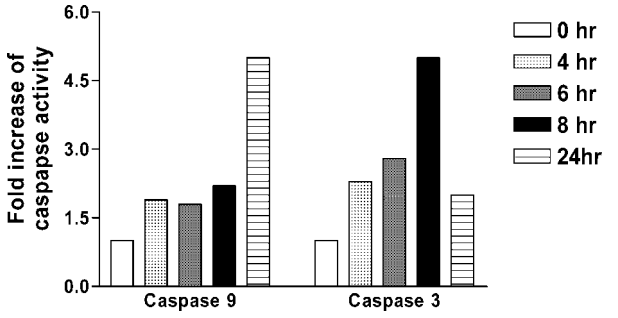

Figure 5. Caspases = 3 and = 9 fluorometric activity assay on WSU-DLCL$_2$ cells exposed to 2 μmol/L (−)-gossypol for 0 to 24 h. Proteins (100 μg) from cell lysates were incubated in triplicates with the corresponding substrates for caspase = 3 (Ac-DEVD-AF) and caspase-9 (Ac-LEHD-AFC). Fluoromethylcoumarin fluorescence released by caspase activity was measured and analyzed.

Discussion

Only 30% to 40% of advanced-stage DLCL is curable by multiagent chemotherapy, such as CHOP regimen (3, 5, 29). Although many attempts to improve cure rate have been made by using other modern regimens, progress has been limited (4, 5). There is increasing evidence that high expression of Bcl-2, Bcl-X_L, or both may play a critical role in lymphoma progression and resistance to a wide spectrum of chemotherapeutic agents. Hence, Bcl-2 and Bcl-X_L have become attractive targets for designing new antilymphoma drugs (9). Nonpeptidic small-molecule inhibitors that are capable of antagonizing the activity of Bcl-2 and Bcl-X_L may have great therapeutic potential as an entirely new class of antilymphoma drugs and may be used as modulators in conjunction with cytotoxic agents, namely, CHOP regimen.

Bcl-2 was originally identified in B-cell lymphoma. Furthermore, cells obtained from patients with B-cell lymphoma were found to have high levels of Bcl-2 and Bcl-X_L (6, 12, 30). This observation is clinically important in the development of new therapeutic strategies for lymphoma and other B-cell tumors (31, 32). Our WSU-$DLCL_2$ cell line was established from a relapsed DLCL patient who was clinically resistant to chemotherapy (23, 24). The established cell line expresses high levels of Bcl-2 and Bcl-X_L and is resistant to standard chemotherapeutic agents. We have also documented that the WSU-$DLCL_2$ SCID-xenograft model is resistant to various chemotherapeutic agents including the CHOP regimen (24).

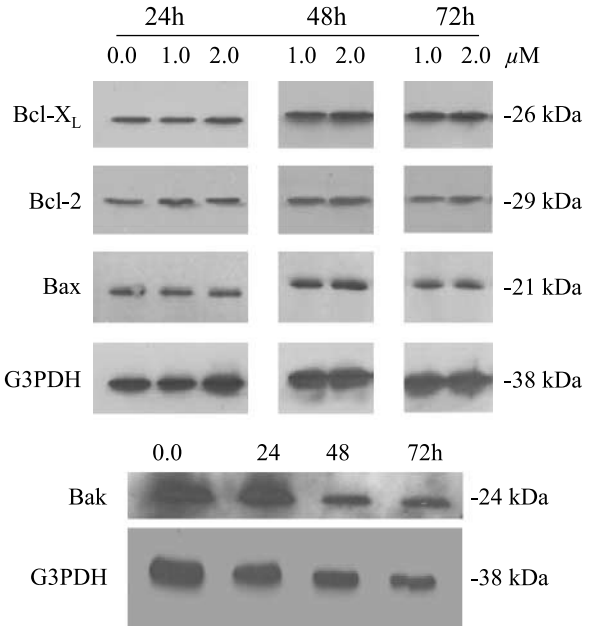

Figure 6. Effect of (−)-gossypol on the protein expression of Bcl-X_L, Bcl-2, Bax, Bak, and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) in WSU-$DLCL_2$ cells. WSU-$DLCL_2$ cells were exposed to 0.0, 1.0, and 2.0 μmol/L (−)-gossypol for 24, 48, and 72 h. Whole-cell extracts (20 μg per well) were analyzed with Western blots. Data shown are after 24, 48, and 72 h.

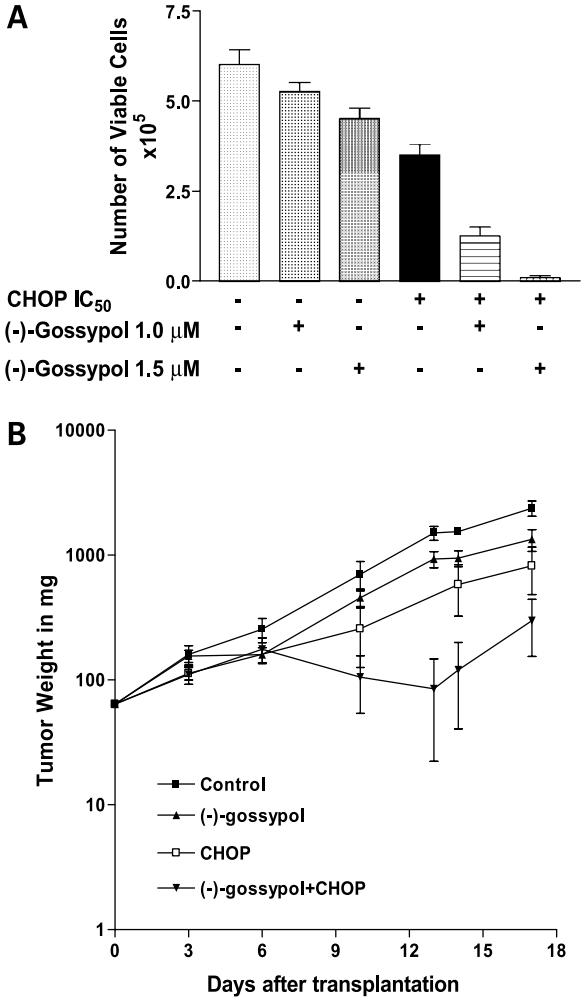

Figure 7. **A,** effect of (−)-gossypol at 1.0 and 1.5 μmol/L, CHOP at IC_{50} (cyclophosphamide monophosphate, 5.84 pmol/L; doxorubicin, 1.5 pmol/L; oncovin, 260 pmol/L; prednisolone, 1.0 μmol/L), their combinations, or diluent (*Control*) on the growth of WSU-$DLCL_2$ cells *in vitro* after 24 h. Cells were cultured at 1.5×10^5/mL in a 24-well plate with or without the agents. *Points,* average of two experiments; *bars,* SD. WSU-$DLCL_2$ cells were preexposed to (−)-gossypol for 5 h before CHOP was added. **B,** $\log_{10}$ tumor weight (in milligrams, mean ± SD) of WSU-$DLCL_2$-bearing SCID in control (diluent), (−)-gossypol, CHOP, and (−)-gossypol + CHOP. Tumor weight decreased in mice that received (−)-gossypol/CHOP combination. All animals were treated 5 d after tumor transplantation.

Antiapoptotic proteins are very promising targets for suppression and/or inhibition of lymphoma cell survival and resistance to cytotoxic chemotherapies. Many groups have been developing strategies to block the activity of Bcl-2 and trigger apoptosis. Methods include the use of peptides or small molecules to the Bcl-2 binding pocket, preventing its heterodimerization with and sequestration of proapoptotic molecules. Design of nonpeptidic, druglike, cell-permeable, potent small-molecule inhibitors that bind to the BH3 binding groove in Bcl-2/Bcl-X_L and block their anti-apoptotic function (9, 14–17) is a new and very exciting area of research for treating t(14;18)-carrying

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

Agent	No. of animals	T/C (%)	$T - C$ (days)	Log_{10} kill (gross)
Control	5	0.0	0	0.0
(−)-Gossypol	5	56	3	0.5
CHOP	5	19	8	1.2
Gossypol + CHOP	5	10	10	1.5

Note: (−)-Gossypol was given at 20 μg/kg daily for 3 days. CHOP was given at MTD ×1 injection (cyclophosphamide, 40 mg/kg i.v.; doxorubicin 3.3 mg/kg, vincristine, 0.5 mg/kg; and predisone, 0.2 mg p.o.) for 5 days. (−)-Gossypol + CHOP combination was given at 60 mg/kg (i.v. via tail vein, divided in three injections for 3 days) plus CHOP at its MTD. Combination of (−)-gossypol at its MTD (i.e., 120 mg/kg divided into three injections for 3 consecutive days) plus CHOP at its MTD was toxic to all SCID mice.

lymphomas. We have discovered (−)-gossypol (Fig. 1*A*) through structure-based computer screening as a potent, cell-permeable, small-molecule inhibitor of Bcl-2 and Bcl-X_L proteins (16, 21, 22). Our results from fluorescence polarization method indicate that (−)-gossypol has a K_i value of 464 ± 128 nmol/L to Bcl-2 and 570 ± 82 nmol/L to Bcl-X_L, compared with Bak 16-mer, which showed a K_i value of 2,085 nmol/L to Bcl-2 and 225 nmol/L to Bcl-X_L. However, efficacy of small-molecule inhibitor (−)-gossypol needs to be proven in a biological system in which antitumor activity against human lymphoma can be measured.

In this study, we hypothesized that (−)-gossypol as a small-molecule inhibitor of Bcl-2 and Bcl-X_L has therapeutic potential in lymphoma. The small-molecule inhibitor binds directly to Bcl-2/Bcl-X_L groove and blocks their subsequent heterodimerization with proapoptotic member, which results in the initiation of downstream apoptotic events. Indeed, (−)-gossypol displayed significant activity against WSU-DLCL$_2$ cell line and fresh lymphoma cells obtained from patients. At 2.0 μmol/L, (−)-gossypol was capable of inducing significant apoptosis on WSU-DLCL$_2$ as detected by 7AAD and flow cytometry. Exposing patient-derived B-cell lymphoma cells to 2.0 μmol/L (−)-gossypol resulted in 64% apoptosis. Moreover, (−)-gossypol had only very little effect on normal PBLs isolated from a healthy donor at 2 or even 4 μmol/L. These results show that blocking Bcl-2/Bcl-X_L function by itself is sufficient to trigger apoptosis in lymphoma cells overexpressing these molecules. The finding is consistent with results of down-regulation of Bcl-2 by other means such as antisense Bcl-2 oligonucleotides (13, 33).

Next, we investigated selected steps in the Bcl-2-dependent pathway of apoptosis. These include release of cytochrome *c* and activation of caspases-9 and -3. It is well known that the release of cytochrome *c* from the mitochondria to cytosol is an important upstream manifestation of the initiation of apoptotic cell death, with links to both caspases-9 and -3 (27, 28). After exposure of WSU-DLCL$_2$ cells to (−)-gossypol, cytochrome *c* is virtually completely released from the mitochondria into the cytosol by 48 hours. Five-fold increase in the activity of caspase-9 and caspase-3 was noticed at 24 and 8 hours, respectively. The apoptosome allows the recruitment of procaspase-9 molecules, causing the activation of caspase-9 and the caspase cascade that leads to apoptosis. Small-molecule inhibitors disrupt the function of their target proteins, in this case Bcl-2 and Bcl-X_L, and therefore should not affect protein levels. Our results indicated no significant changes in protein expression of Bcl-2, Bcl-X_L, Bax, or Bak (Fig. 6). These findings are consistent with our hypothesis that (−)-gossypol interferes with the function, rather than expression levels, of the Bcl-2 family of proteins.

Antiapoptotic proteins are very promising targets for suppression and/or inhibition of lymphoma cell survival and resistance to cytotoxic chemotherapies. Because our WSU-DLCL$_2$ cell line is resistant to chemotherapy, we hypothesized that the small-molecule inhibitor (−)-gossypol is capable of sensitizing it to CHOP chemotherapy through inhibition of the function of antiapoptotic proteins, Bcl-2 and Bcl-X_L. We showed that the addition of (−)-gossypol, at a concentration lower than its IC_{50}, to CHOP resulted in complete and sustained growth inhibition. This was in contrast to CHOP or (−)-gossypol alone that resulted in less cell growth inhibition (Fig. 7*A*). This confirms the notion that inhibition of antiapoptotic proteins may sensitize cancer cells to the killing effect of cytotoxic agents.

Most studies (14–17) have used *in vitro* cell-based assays to show that small-molecule inhibitors of Bcl-2/Bcl-X_L are potential molecularly targeted agents. However, the main challenge in developing a novel therapeutic agent is that one needs to show the therapeutic efficacy *in vivo*. Many small-molecule inhibitors, despite their excellent *in vitro* cytotoxicity, fail to make their way to clinical trials. This is because they either fail to achieve significant antitumor activity *in vivo* or are toxic. Therefore, we tested the toxicity of (−)-gossypol in our WSU-DLCL-SCID model. The MTD of (−)-gossypol in SCID mice was 40 mg/kg for three i.v. injections when given alone and 20 mg/kg for three injections when given in combination with CHOP regimen.

Our results show that (−)-gossypol was effective in decreasing tumor weight when used alone. However, when 60 mg/kg (−)-gossypol was administered in conjunction with CHOP, it achieved a significantly longer tumor growth delay ($P = 0.01$) compared with either CHOP or (−)-gossypol alone (Fig. 7*B*). Antitumor activity of (−)-gossypol, CHOP, or (−)-gossypol + CHOP combination as measured by T/C were 56%, 19%, and 10%; $T - C$, 3, 8, and 10 days; and log_{10} kill, 0.5, 1.2, and 1.5, respectively.

Studies over the past few decades have shown that more complicated cytotoxic regimens were not superior to CHOP, which remains the ''gold standard'' (3–5). The efficacy of this regimen in lymphoma has been significantly enhanced recently by the addition of anti-CD20 antibody (Rituximab; ref. 35). Bcl-2/Bcl-X_L small-molecule inhibitor can be another innovative way to enhance CHOP activity by antagonizing a major resistance mechanism to apoptosis.

Our results in this study show the superiority of the (−)-gossypol + CHOP combination over either (−)-gossypol or CHOP alone. Usually, in a lymphoma clinical setting, CHOP regimen is given to patients with lymphoma for more than one cycle. Therefore, we plan to give multiple cycles of (−)-gossypol + CHOP. Although (−)-gossypol was able to inhibit tumor growth *in vivo*, no cures were achieved. We think that giving (−)-gossypol in combination with CHOP for more than one cycle will achieve cures in mice.

Our study suggests that (−)-gossypol represents a promising new agent that should be developed for the treatment of non-Hodgkin's lymphomas in humans.

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