

Preclinical Studies of TW-37, a New Nonpeptidic Small-Molecule Inhibitor of Bcl-2, in Diffuse Large Cell Lymphoma Xenograft Model Reveal Drug Action on Both Bcl-2 and Mcl-1

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Abstract **Purpose:** Overexpression of Bcl-2 protein has been observed in more than 80% of B-cell lymphomas, including diffuse large cell lymphoma (DLCL), the most common subtype of non-Hodgkin's lymphoma. We have previously employed the natural product (–)-gossypol to test its therapeutic potential as a small-molecule inhibitor of Bcl-2 for the treatment of B-cell lymphomas. **Experimental Design:** Recently, we have used a structure-based strategy to design a new class of potent small-molecule inhibitor acting on Bcl-2. One such lead compound is the benzenesulfonyl derivative TW-37, which was designed to target the BH3-binding groove in Bcl-2 where proapoptotic Bcl-2 proteins, such as Bak, Bax, Bid, and Bim bind. **Results:** In our fluorescence polarization–based binding assays using recombinant Bcl-2, Bcl-X_L, and Mcl-1 proteins, TW-37 binds to Bcl-2, Bcl-X_L, and Mcl-1 with *K_i* values of 290, 1,110 and 260 nmol/L, respectively. Hence, TW-37 is a potent inhibitor of Bcl-2 and has >3-fold selectivity over Bcl-X_L. *In vitro*, TW-37 showed significant antiproliferative effect in a *de novo* chemoresistant WSU-DLCL₂ lymphoma cell line and primary cells obtained from a lymphoma patient with no effect on normal peripheral blood lymphocytes. Coimmunoprecipitation experiments showed that TW-37 disrupted heterodimer formation between Bax or truncated-Bid and antiapoptotic proteins in the order Mcl-1 > Bcl-2 >> Bcl-X_L. As expected, TW-37 caused apoptotic death. Pre-exposure of lymphoma cells to TW-37 significantly enhanced the killing effect of cyclophosphamide-doxorubicin-vincristine-prednisone (CHOP) regimen. The maximum tolerated dose of TW-37 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-DLCL₂-SCID mouse xenograft model, the addition of TW-37 to CHOP resulted in more complete tumor inhibition compared with either CHOP or TW-37 alone. **Conclusions:** We conclude that the administration of TW-37, as a potent Bcl-2 and Mcl-1 inhibitor, to standard chemotherapy may prove an effective strategy in the treatment of B-cell lymphoma.

We have discovered new nonpeptidic small-molecule inhibitors (SMI) that bind and disarm antiapoptotic BCL2 family proteins, mimicking the natural proapoptotic proteins, such as Bid and Bax, which use their BH3 domain to bind to

antiapoptotic proteins such as Bcl-2 (1, 2). Bcl-2 overexpression is a key molecular feature of drug resistance of non-Hodgkin's lymphoma (NHL) cells to chemotherapy (3–5). NHL is a group of heterogeneous diseases resulting from a malignant proliferation of lymphocytes, which add up to 58,000 new cases diagnosed in the United States per year (6). NHL is now the fourth leading cause of death in males ages 20 to 39; NHL incidence has increased ~80% since the 1970s, and it is now the fifth most common cancer in the United States (6). Originally known as diffuse histiocytic lymphoma (7), diffuse large cell lymphoma (DLCL) is the most frequently occurring subtype of NHL (5, 8) and accounts for 31% of all lymphomas (9). We have established a severe combined immunodeficient (SCID) mouse xenograft model from cells taken from a patient with DLCL; this model enables analysis of efficacy and mechanism of action of BH3 mimetic SMIs *in vivo* (10, 11). The antiapoptotic function of Bcl-2 and other prosurvival BCL2 family members depends on the ability to heterodimerize with proapoptotic members such as Bid, Bak, Bax, and Bad and thus sequester these effectors away from permeabilization sites in the outer mitochondrial membrane (12). X-ray diffraction and nuclear magnetic resonance (NMR) studies of Bcl-X_L reveal an elongated hydrophobic

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binding groove into which the Bak or Bad BH3 domain binds (13, 14). A homologous binding groove has been defined in the prosurvival family members Bcl-2 and Mcl-1 (13, 15); the groove is essential to mediate the prosurvival functions of these Bcl-2 family members. The basic topology of this groove is conserved between Bcl-2, Bcl-X_L, and Mcl-1; there is a selectivity in binding defined by key amino acid side chains borne on the α 2, α 4, and α 5 helices, which vary (12). Because this groove normally accommodates the BH3 helix of proteins like Bid and Bax, it has been hypothesized that small molecules that bind to this BH3-binding groove in Bcl-2, Bcl-X_L, or Mcl-1 may be capable of blocking their heterodimerization with a subset of proapoptotic members in the Bcl-2 protein family, such as with Bax, Bid, and Bak (12, 16). Blockade of this heterodimerization by an SMI in turn would expand the pool of free proapoptotic effectors and thus induce apoptosis in cancer cells where overexpressed Bcl-2, Bcl-X_L, or Mcl-1 provide survival cues (16). Although it has been traditionally difficult to design SMI to block protein-protein interactions, several recent studies have shown that it is possible to discover and design potent SMI that bind to the BH3-binding groove (17–19). Design of such inhibitors of Bcl-2 and Bcl-X_L via structure-based three-dimensional database searching and computer-aided design, such as SAR by NMR (20), has resulted in the identification of several key drug leads (21). The newest compounds bind their targets in the nanomolar range, a dramatic improvement over the first compounds showing a K_i of ~ 10 $\mu\text{mol/L}$. None of the published compounds approach the goal of serving as “pan-BCL2” inhibitors, hitting Bcl-2, Bcl-X_L, and Mcl-1 with nanomolar dissociation constants. One would expect that treatment of patients with a BH3 mimetic SMI that “misses” an important target such as Mcl-1 might lead to the development of resistant tumors, which survive the treatment by virtue of their high expression of Mcl-1. We have thus aimed to develop such pan-BCL2 compounds and here report on the efficacy in lymphoma of the benzenesulfonyl derivative TW-37. Using multidimensional NMR methods such as heteronuclear single quantum coherence NMR spectroscopy using uniformly ¹⁵N-labeled Bcl-2 protein, TW-37 was conclusively shown to bind to the BH3-binding groove of Bcl-2, interacting with the same amino acid side chains in Bcl-2 as the natural peptide Bim (22).

The standard therapy for DLCL is the four-drug combination cyclophosphamide-doxorubicin-vincristine-prednisone (CHOP), which provides cure in 30% to 40% of unselected patients with DLCL (23, 24). 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. It is now recognized that overexpression of Bcl-2 family antiapoptotic proteins plays an important role in the resistance of lymphoma cells to current anticancer therapies. Indeed, overexpression of Bcl-2 and/or Bcl-X_L is found in 80% of NHL (25). Although first identified as a Bcl-2 family member overexpressed in myeloid leukemia (26), Mcl-1 is expressed in a variety of hematopoietic (27–30) and solid tumors (31), suggesting that Mcl-1 can provide a key new target for therapeutics. The level of Mcl-1 expression in chronic lymphocytic leukemia is also predictive (with $P < 0.016$) of the failure of response to the CD20-targeted antibody rituximab (32). In NHL, Michels et al. (33) found that high expression of Mcl-1 correlated

with unfavorable clinical outcome. Unfortunately, some of the newest drug candidates, such as ABT-737, bind poorly or not at all to Mcl-1 (34).

Materials and Methods

TW-37. Design, synthesis, purification, and chemical characterization of *N*-[(2-*tert*-butyl-benzenesulfonyl)-phenyl]-2,3,4-trihydroxy-5-(2-isopropyl-benzyl)-benzamide (TW-37) is described in detail by Wang et al. (22); in the inactive congener TW-37a (compound 6 in ref. 22), all three hydroxyl groups in the polyphenolic ring have been substituted with a methyl group, resulting in a 100-fold loss of binding (see Fig. 1A and Fig. 2D, inset for structures).

Fluorescence polarization–based binding assay for recombinant Bcl-2, Bcl-X_L, and Mcl-1 protein. For this assay, we have employed the 21-residue BH3 peptide QEDIIRNIARHLAQVGDSDMR derived from Bid (35) labeled with 6-carboxyfluorescein succinimidyl ester (FAM-Bid) and recombinant proteins derived from human Bcl-2, Bcl-X_L, and Mcl-1 as described (22). It was determined that FAM-Bid has a K_i of 11 nmol/L to Bcl-2 protein, 25 nmol/L to Bcl-X_L protein, and 5.7 nmol/L to Mcl-1 protein (Fig. 1B–D). The competitive binding assay for Bcl-X_L was same as that for Bcl-2 with the following exceptions: 30 nmol/L Bcl-X_L protein and 2.5 nmol/L FAM-Bid peptide in the following assay buffer [50 mmol/L Tris-Bis (pH 7.4) and 0.01% bovine γ -globulin].

WSU-DLCL₂ cell line, patient-derived primary acute lymphoblastic leukemia cells, and normal peripheral blood lymphocytes. The DLCL cell line (WSU-DLCL₂) was established in our laboratory at Wayne State University's School of Medicine (36). WSU-DLCL₂ cells were plated in 24-well culture clusters (Costar, Cambridge, MA) at a density of 2×10^5 viable cells per mL per well. Triplicate wells were treated with 0.0 to 800 nmol/L TW-37 and CHOP [5.84 $\mu\text{mol/L}$ cyclophosphamide monohydrate (C₇H₁₄Cl₂N₂O₂P·H₂O; Sigma-Aldrich, St. Louis, MO), 1.5 $\mu\text{mol/L}$ doxorubicin, 260 $\mu\text{mol/L}$ vincristine, 1.0 $\mu\text{mol/L}$ prednisone]. Doses for CHOP were as determined previously (11). Cells were pre-exposed to TW-37 for 5 h before CHOP was added. Plates were incubated at 37°C in a humidified incubator with 5% CO₂. 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, Grand Island, NY) and a hemacytometer.

Fresh lymphoma cells obtained from acute lymphoblastic leukemia were used to assess the TW-37 cytotoxic effect on primary lymphoma cells. Similarly, normal peripheral blood lymphocytes obtained from a healthy donor were used as to assay the effect of TW-37 on normal human lymphocytes. Cells were plated in 24-well culture clusters at a density of 4×10^5 viable cells per mL per well. Triplicate wells were treated with TW-37. Plates were incubated at 37°C in a humidified incubator with 5% CO₂. All cultures were monitored throughout the experiment by cell count and viability every 24 h for 4 days using 0.4% trypan blue stain and a hemacytometer. Statistical analysis was done using the *t* test (two tailed) with 95% confidence intervals between treated and untreated samples. $P < 0.05$ was used to indicate statistical significance.

Coimmunoprecipitation of complexes and Western blot analysis. Primary antibodies specific for Bcl-2, Bcl-X_L, Mcl-1, Bid, and Bax were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lysates equivalent to 100 μg of protein were precleared with protein G-Sepharose and then immunoprecipitated over 24 h with an antibody specific for Bax (sc-20067) or truncated-Bid (t-Bid; sc-11423); immunoprecipitates were resolved using 12% SDS-PAGE and electroblotted to Hybond C-extra membranes (Amersham Life Science, Arlington Heights, IL). Membranes were subsequently immunoblotted with antibodies to human Mcl-1, Bcl-X_L, or Bcl-2 after blocking with 5% milk in PBS containing 0.05% Tween 20 (PBST) for 1 h at 25°C. Unlabeled primary antibodies to Mcl-1, Bcl-X_L, or Bcl-2 (1:1,000 dilution in PBST) were used to probe the membranes overnight at 4°C. Following this incubation, membranes were washed well in PBST and incubated with the

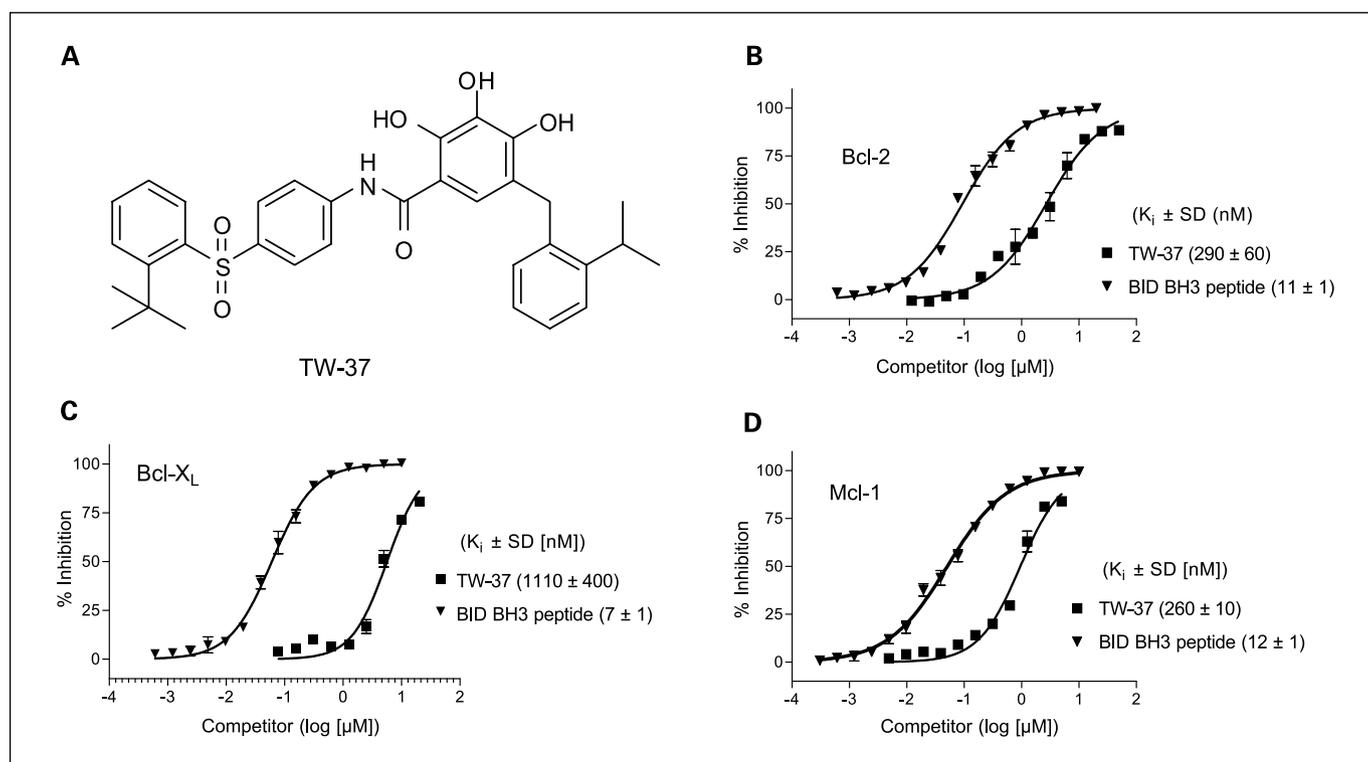


Fig. 1. *A*, chemical structure of TW-37 or *N*-[(2-*tert*-butyl-benzenesulfonyl)-phenyl]-2,3,4-trihydroxy-5-(2-isopropyl-benzyl)-benzamide. Using multidimensional NMR methods such as heteronuclear single quantum coherence NMR spectroscopy using uniformly ^{15}N -labeled Bcl-2 protein, TW-37 was conclusively shown to bind to the BH3-binding groove of Bcl-2, interacting with the same amino acid side chains in Bcl-2 as the natural peptide Bim. For example, invariant residues Asn¹⁴³ and Arg¹⁴⁶ in the $\alpha 5$ helix of Bcl-2 hydrogen bond to Bim residues Asp⁹⁹ and Asn¹⁰²; these Asn and Arg side chains in the $\alpha 5$ helix of Bcl-2 likewise hydrogen bond to the phenolic hydroxyl group on the polyphenolic ring of TW-37. The isopropyl-benzyl end of TW-37 interacts with helix $\alpha 2$, whereas the *tert*-butyl end of TW-37 nestles into the $\alpha 4$ helix of Bcl-2 (residues 133-137: VEELF; ref. 22). This helix ($\alpha 4$) is shorter in Bcl-X_L compared with Bcl-2 and Mcl-1 (see Fig. 1 in ref. 15), a feature that may explain the low affinity of the compound for Bcl-X_L. The amino acid sequence of Bcl-X_L from residues 120 to 132 (YQSFEQVVNELFR) folds into the $\alpha 4$ helix ending at its COOH-terminal residues with VVN. The homologous region in Mcl-1 (residues 224-236: VKSFSRVVMVHVFK) folds into $\alpha 4$ helix ending with MVHV. The folding of Mcl-1 in this region thus opens up a deeper hydrophobic pocket than Bcl-X_L, allowing the benzenesulfonyl moiety of TW-37 to be accommodated more easily in Mcl-1 (and Bcl-2) than by the homologous groove region of Bcl-X_L. *B* to *D*, Bid BH3 peptide (QEDIIRNIARHLAQVGDSDMR) is labeled fluorescently with FAM, while the compound TW-37 is unlabeled. The target for fluorescent Bid and TW-37 is a recombinant version of human Bcl-2, Bcl-X_L, or Mcl-1 described by Wang et al. (22).

horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology; 1:5,000 dilution in PBST) for 45 min to 1 h at 25°C. Proteins were visualized using an enhanced chemiluminescence assay (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Protein concentrations were determined using the Micro BCA protein assay (Pierce Chemical Co., Rockford, IL).

Evaluation of apoptosis: caspase fluorimetric activity assay. WSU-DLCL₂ cells exposed to 400 nmol/L TW-37 for 0 to 24 h were incubated on ice for 10 min in cell lysis buffer (BioVision Research Products, Palo Alto, CA). The clear supernatant after centrifugation at $2,000 \times g$ at 4°C was collected, and proteins were quantified according to the bicinchoninic acid protein assay methodology (Pierce Chemical). 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; 50 μL of $2\times$ Reaction Buffer containing 10 mmol/L DTT is added to each sample (BioVision Research Products); 50 $\mu\text{mol/L}$ final concentration of 7-amino-4-trifluoromethylcoumarin (AFC) conjugated substrates for caspase-3 (Ac-DEVD-AFC) and caspase-9 (Ac-LEHD-AFC; BioVision Research Products) is added to each sample for a total volume of 100 μL and incubated for 180 min at 37°C. AFC fluorescence, released by caspase activity, was measured on a fluorescence plate reader (Molecular Devices, Sunnyvale, CA), set at 400-nm excitation filter and 505-nm emission filter.

7-amino-actinomycin D staining and flow cytometry. 7-Amino-actinomycin D (Calbiochem-Novabiochem, La Jolla, CA) was diluted in PBS to a concentration of 200 mg/mL. As described previously using this stain (11), we were able to determine the percentage of viable,

apoptotic, and dead cells. TW-37-, CHOP-, and TW-37 + CHOP-treated and untreated WSU-DLCL₂ cells were harvested, washed with PBS, and stained with 7-amino-actinomycin D. Cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Data on 20,000 cells was acquired and processed using Lysis II software (Becton Dickinson). Scattergrams were generated by combining forward light scatter with 7-amino-actinomycin D fluorescence.

Morphology. Cells were cultured at 1.5×10^5 per mL in T-25 tissue culture plates. Cells then were exposed to 300 nmol/L TW-37 for 24 h. For light microscopic examination, WSU-DLCL₂ cells were seeded in 24-well culture plates as described above. Briefly, untreated (control) and cells treated with TW-37 were set in three replications. Aliquots from cell cultures were cytocentrifuged using a Cytospin II centrifuge. Cell smears were air-dried and 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. Features of apoptosis looked for included nuclear chromatin condensation and formation of membrane blebs and apoptotic bodies (37).

WSU-DLCL₂ xenografts. Four-week-old female ICR-SCID mice were obtained from Taconic Laboratory (Germantown, NY). The mice were adapted and WSU-DLCL₂ xenografts were developed as described previously (11). Each mouse received 10^7 WSU-DLCL₂ 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 TW-37, CHOP, and their combination. A dose-range finding study of three dose levels (20, 40, and 60 mg/kg) of the TW-37 plus a vehicle-only control given drug i.v. daily for five 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 min), then twice daily for the first 3 days (acute toxicity) then daily for 2 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 (11) for one injection (i.e., 40 mg/kg, i.v. cyclophosphamide; 3.3 mg/kg, i.v.

doxorubicin; 0.5 mg/kg, i.v. vincristine; and 0.2 mg/kg, orally prednisone) every day for 5 days. The MTD for TW-37/CHOP combinations were determined by administering TW-37 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-DLCL₂ xenograft were implanted s.c. and bilaterally into naive, similarly SCID adapted mice, as previously described. Mice were checked thrice per week for tumor development. Once transplanted WSU-DLCL₂ 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 TW-37, CHOP, and their combination was studied. Mice were observed the drugs; s.c. tumors were measured thrice per week. Tumor weight (mg) = $(A \times B^2) / 2$, where A and B are the tumor length and width (in mm), respectively. Animals were euthanized when their total tumor burden reached 2,000 mg to avoid discomfort. 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 (T_d) is the time (in days) required in order for the tumor to double its weight during the exponential growth phase.

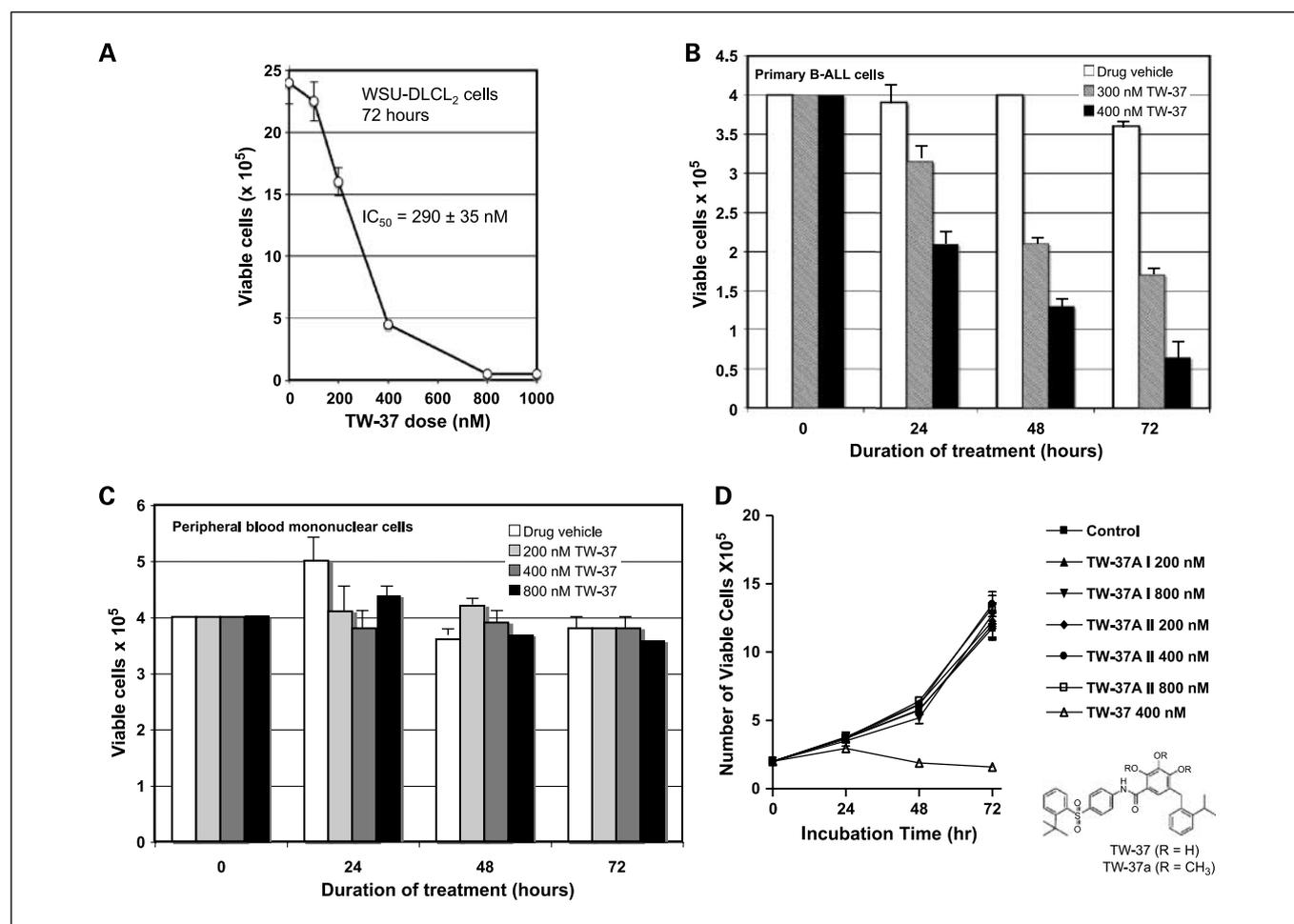


Fig. 2. Effect of TW-37 on an established DLCL, a primary B-cell acute lymphoblastic leukemia cell line, and on normal peripheral blood lymphocytes. **A**, we determine the effect of TW-37 on the established WSU-DLCL₂ lymphoma cell line in culture. Cells seeded at 2×10^5 per mL in a 24-well plate were exposed to 0 to 1,000 nmol/L TW-37 and incubated for 72 h. **B**, we determine the effect of TW-37 on primary cells derived from a patient with B-cell acute lymphoblastic leukemia (*B-ALL*). In this experiment, cells were cultured at 4.0×10^5 per mL in a 24-well plate; exposed to 0, 300, and 400 nmol/L TW-37; and incubated for up to 72 h. **C**, effect of TW-37 on normal peripheral blood lymphocytes (*PBL*). Cells were seeded at 4×10^5 per well and exposed to 200, 400, or 800 nmol/L TW-37. No major cell kill was noticed up to 72 h. Points/columns, average of two experiments; bars, SD. **D**, WSU-DLCL₂ cells are set up in culture as in (**A**), except that in (**D**), the inactive congener of TW-37 is used, a trimethylated version of TW-37 known as TW-37a (22). Inset, structure of TW-37 and TW-37a. In this series of experiments, we treated DLCL2 cells with TW-37 at 400 nmol/L (*open triangles*), drug diluent (*filled squares*), or the inactive congener TW-37a at 200 to 800 nmol/L. Two different batches of TW-37a were used (*I* or *II*).

Table 1. Binding affinity of TW-37 to Bcl-2 and Bcl-X_L and in comparison with Bid 21-residue BH3 peptide (residues 79-99: QEDIIRNIARHLAQVGDMSMDR)

Compound	$K_i \pm SD$ (nmol/L)		
	Bcl-2	Bcl-X _L	Mcl-1
TW-37	290 ± 60	1,110 ± 400	260 ± 10
TW-37a	~2,500	ND	ND
Bid BH3 peptide	11 ± 1	7 ± 1	12 ± 1

NOTE: K_i for TW-37a is determined in Supplementary Material of ref. 22, where it is referred to as compound 6. Abbreviation: ND, not determined.

Results

TW-37 binds to purified recombinant human Bcl-2, Bcl-X_L, and Mcl-1 targets with differential affinity. TW-37 is a new benzenesulfonyl derivative developed through computational screening and NMR focused on the antiapoptotic protein target Bcl-2 (22). This low molecular weight compound is an SMI targeted to the elongated groove of antiapoptotic proteins that normally bind the BH3 domain of proapoptotic effectors such as t-Bid, Bax, Bim, and others. The compound fits into the elongated hydrophobic groove mimicking natural BH3 peptides and interacts with amino acid side chains in this groove borne on the α_2 , α_4 , and α_5 helices first described for Bcl-X_L (13), as summarized in Fig. 1A. To measure the affinity of TW-37 for its pharmacologic targets, we used a fluorescence polarization assay recently described (22). In this assay, a 21-residue peptide derived from the BH3 domain of Bid was synthesized and labeled at the NH₂ terminus with FAM as the fluorescence tag (FAM-Bid). Competitive binding

experiments determined that the Bid BH3 peptide binds to recombinant Bcl-2, Bcl-X_L, and Mcl-1 proteins with K_i values of 11, 7, and 12 nmol/L (Fig. 1B-D), respectively. In comparison, TW-37 was determined to bind to Bcl-2, Bcl-X_L, and Mcl-1 proteins with K_i values of 290, 1,110, and 260 nmol/L, respectively (Fig. 1B-D and Table 1), under the same assay conditions (22). The mode of binding of TW-37 to Bcl-2 (and presumably to Bcl-X_L and Mcl-1) involves its ability to interact with some, but not all, of the amino acid side chains in Bcl-2, which mediate the binding of the α -helical BH3 domain of Bid (above) and other BH3-only proapoptotic proteins. These side chains in Bcl-2 protrude into an elongated hydrophobic cleft formed by the α_2 , α_4 , and α_5 helices in Bcl-2 (13, 15, 22). TW-37 presumably interacts with Bcl-X_L and Mcl-1 in much the same way it interacts with Bcl-2 analyzed by Wang et al. 22. These interactions are described in some detail in Fig. 1A legend.

Effect of TW-37 on cell growth of WSU-DLCL2 in vitro. The fact that TW-37 binds with high specificity and selectivity to purified antiapoptotic proteins Bcl-2, Bcl-X_L, and Mcl-1 prompted us to further investigate its possible utility as a chemotherapeutic drug in DLCL, which use these apoptotic proteins for their survival. To this end, we selected an established DLCL cell line developed and characterized in our laboratory (36) and compared its response with primary cells obtained from an acute lymphoblastic leukemia patient and with normal peripheral blood lymphocytes from a healthy donor. In each case, cells were exposed to TW-37 over 72 h, and cell viability was determined. In general, lymphoma cells exposed to TW-37 resulted in a dose- and time-dependent inhibition of cell proliferation. TW-37 inhibited the growth of the WSU-DLCL₂ line significantly at concentration of >100 nmol/L; at a concentration of 400 nmol/L (Fig. 2A), TW-37 inhibited the growth by >90%, and 800 nmol/L killed all WSU-DLCL₂ cells during the exposure period. This curve permits us to estimate an IC₅₀ for these conditions of

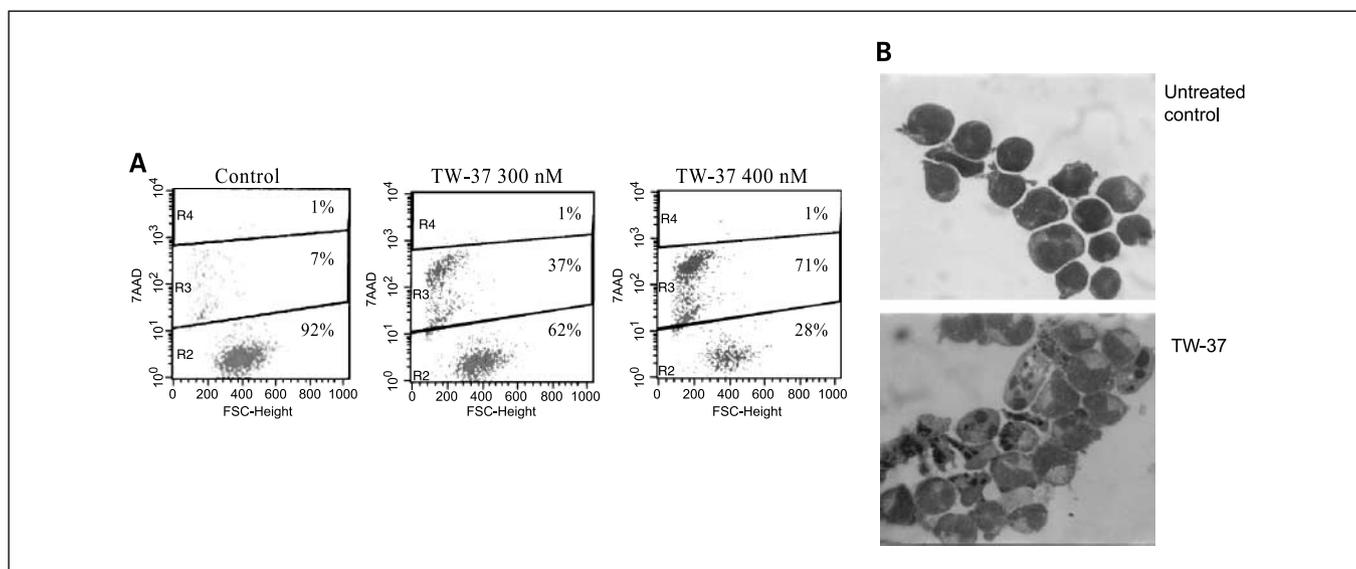


Fig. 3. Treatment of WSU-DLCL₂ cells induces aspects of apoptotic death determined by flow cytometry with 7-amino-actinomycin D and by microscopic assessment of morphology of stained cells. **A**, 7-amino-actinomycin D (7-AAD) flow cytometric analysis of apoptosis. Representative scattergrams generated from 7-amino-actinomycin D staining of WSU-DLCL₂ cells after 72 h of treatment with 0, 300, and 400 nmol/L TW-37. Top, dead cells; middle, apoptotic cells; bottom, live cells. The bold numbers represent the percentage of cells in the respective region. Heat-treated dead cells were used as a positive control (data not shown). **B**, morphologic assessment of apoptosis induction in cultured WSU-DLCL₂ cells. Photographs showing characteristic features of TW-37-exposed cells ($\times 1,000$). Bottom, WSU-DLCL₂ cells exposed to TW-37 (300 nmol/L for 24 h) reveal characteristic signs of apoptotic death. Smears were prepared and stained with tetrachrome as in ref. 37.

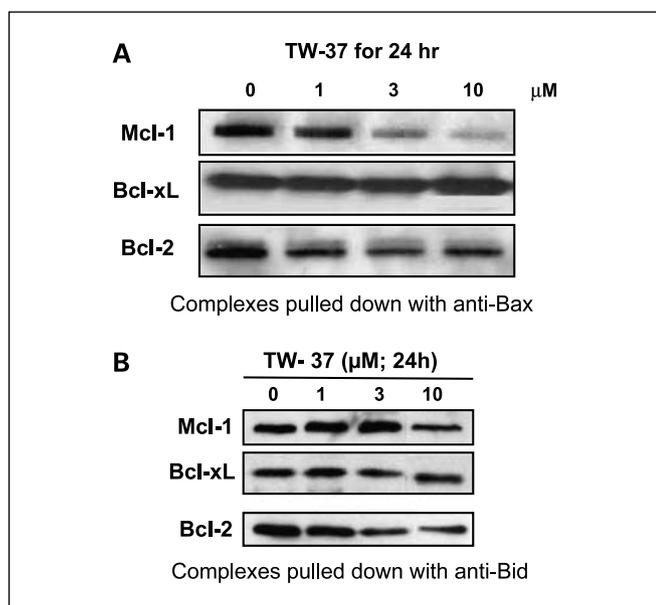


Fig. 4. Treatment of WSU-DLCL2 cells with TW-37 results in differential effects on the disruption of heterodimers between the proapoptotic Bax protein and three prosurvival drug targets. In this series of experiments, WSU-DLCL2 cells were exposed for 24 h to TW-37 provided at 0, 1, 3, or 10 $\mu\text{mol/L}$. Lysates equivalent to 100 μg of protein were precleared with protein G-Sepharose and then immunoprecipitated over 24 h with an antibody specific for (A) Bax (sc-20067) or (B) Bid (sc-11423). Immunoprecipitates were separated by SDS-PAGE and electroblotted to a membrane. Subsequent immunoblotting with Mcl-1, Bcl-X_L, or Bcl-2 antibodies reveals that complexes between Bax and Mcl-1 (*top*) or Bax and Bcl-2 (*bottom*) are more readily disrupted by TW-37 than complexes between Bax and Bcl-X_L (*middle*).

290 \pm 35 nmol/L. To learn whether this potent anti-lymphoma effect would extend to primary cells never cultured *in vitro*, we used cells derived from a pediatric case of acute lymphoblastic leukemia; TW-37 showed significant cytotoxic effect on these primary acute lymphoblastic leukemia cells at 300 and 400 nmol/L concentrations (Fig. 2B). On the other hand, exposing normal peripheral blood lymphocytes to 200 to 800 nmol/L TW-37, no major cell kill was noticed up to 72 h (Fig. 2C). In Fig. 2D, we use TW-37a, a structurally related but inactive congener of TW-37 on WSU-DLCL₂ cells; this compound showed no activity on the growth of these lymphoma cells in culture conditions where TW-37 shows an IC₅₀ of \sim 290 nmol/L.

Induction of apoptosis in WSU-DLCL2 by TW-37. Because TW-37 targets proteins in the apoptotic pathway, we investigated whether TW-37 is able to induce apoptotic cell death in WSU-DLCL₂. Cells were removed at different time points for cell count, viability, and evidence of apoptosis as assessed by morphology and 7-amino-actinomycin D (11). Following 72 h of exposure to TW-37 at 0, 300, and 400 nmol/L, flow cytometry revealed 7.9%, 37.3%, and 71.4% apoptotic cells, respectively (Fig. 3A). At 300 nmol/L, TW-37 elicited the formation of the distinct morphologic features of apoptosis by 24 h of treatment, including cell shrinkage, nuclear chromatin condensation, and formation of membrane blebs and pyknotic bodies (Fig. 3B).

Treatment of WSU-DLCL2 cells with TW-37 results in differential effects on the disruption of heterodimers between the proapoptotic Bax protein and three prosurvival drug targets. To learn whether the robust binding seen in Fig. 1 would translate into the key event in living cells of the disruption of

heterodimers, WSU-DLCL₂ cells were exposed for 24 h to TW-37 provided at 0, 1, 3, or 10 $\mu\text{mol/L}$. Lysates equivalent to 100 μg of protein were precleared with protein G-Sepharose and then immunoprecipitated over 24 h with an antibody specific for Bax (sc-20067); immunoprecipitates were separated by SDS-PAGE and electroblotted to a membrane. Subsequent immunoblotting with Mcl-1, Bcl-X_L, or Bcl-2 antibodies reveals that complexes between Bax and Mcl-1 or between Bax and Bcl-2 (Fig. 4A, *top* or *bottom*, respectively) are more readily disrupted by TW-37 than complexes between Bax and Bcl-X_L (Fig. 4A, *middle*). Bax is a proapoptotic protein featuring BH1, BH2, BH3, and BH4 motifs; thus, we wanted to learn whether TW-37 would also disrupt interactions with t-Bid, a BH3-only proapoptotic protein (38). In Fig. 4B, we pull down complexes using antibody to t-Bid in cells treated as in Fig. 4A; subsequent immunoblotting with Mcl-1, Bcl-X_L, and Bcl-2 antibodies was done as done in Fig. 4A. Like we observed with the Bax pulldown, the t-Bid pulldown shows little or no heterodimer disruption with Bcl-X_L. However, TW-37 treatment caused disruption of heterodimers between t-Bid and Mcl-1 or between t-Bid and Bcl-2.

It should be noted in these experiments that we are treating cells with doses of drug 10- to 30-fold elevated over either the K_i in Fig. 1B to D (300-1,100 nmol/L) or the IC₅₀ in Fig. 2A (290 nmol/L). One explanation for this discrepancy is that the IC₅₀ probably reflects the mechanism suggested by Hinds et al. (39) in which the hydrophobic groove of natural antiapoptotic proteins in the living cell is probably not unliganded even when it is not filled by a BH3-helix but partially occupied by the hydrophobic COOH-terminal 24-amino-acid residues of Bcl-2, Mcl-1, or Bcl-X_L. This hydrophobic tail is absent from the recombinant constructs tested in Fig. 1B to D.

TW-37 induction of caspases activity. Apoptosis is associated with the activation of specific cysteine proteases referred to as

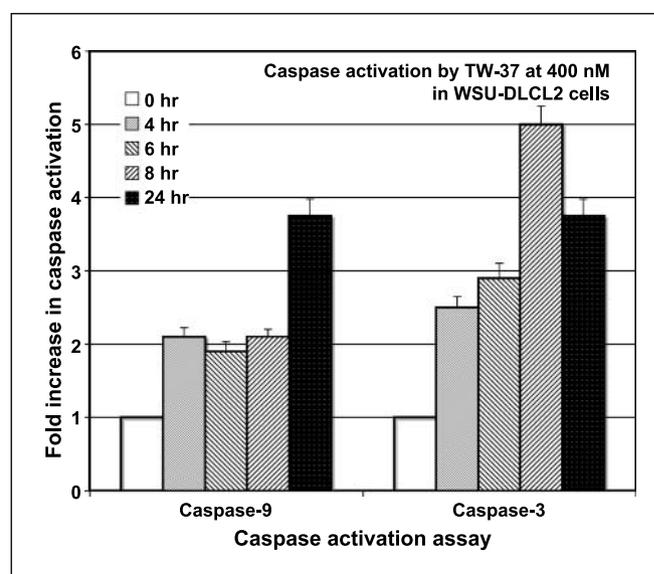


Fig. 5. Caspase-3 and caspase-9 fluorimetric activity assay reveal treatment with 400 nmol/L TW-37 progressively induces apoptosis characteristic caspases in WSU-DLCL₂ over a 24-h treatment period. One hundred micrograms of proteins from cell lysates were incubated in triplicates with the corresponding substrates for caspase-3 (Ac-DEVD-AF) and caspase-9 (Ac-LEHD-AFC). Fluoromethylcoumarin fluorescence released by caspase activity was measured and analyzed.

caspsases (11, 40, 41). We assessed whether TW-37 activated specific caspsases during apoptosis of WSU-DLCL₂ cells. Treatment of WSU-DLCL₂ with 400 nmol/L for 0, 4, 6, 8, and 24 h resulted in increase in activities of caspase-9 and caspase-3 as early as 4 h (Fig. 5). The maximum increase in caspase-9 and caspase-3 activity was seen at 24 and 8 h, respectively.

TW-37 enhances CHOP chemotherapy in vitro. Previously, we have investigated the effect of CHOP on our WSU-DLCL₂ cells and determined the IC₅₀ and IC₂₅ *in vitro* (11). Here, we studied the effects of TW-37 alone at 300 nmol/L, CHOP alone at its IC₂₅, and their combination against WSU-DLCL₂ cells *in vitro*. As shown in Fig. 6, when TW-37 was added 5 h before CHOP, there was growth inhibition, which was significant compared with either CHOP or TW-37 alone.

MTD of TW-37 in SCID mice and determination of efficacy. The MTD for TW-37 was determined to be 120 mg/kg given in three divided dosages daily of 40 mg/kg per injection, *i.v.* Animals at this dose experienced weight loss of <5% and had scruffy fur, however, with full recovery 48 to 72 h after completion of treatment. However, daily injections of 40 mg/kg for four consecutive days was toxic, as shown by a loss of >20% body weight. In addition, 60 mg/kg per injection, *i.v.* injected daily for 3 days was toxic. CHOP MTD in SCID mice was previously determined in our laboratory (37) for one injection (*i.e.*, 40 mg/kg, *i.v.* cyclophosphamide; 3.3 mg/kg, *i.v.* doxorubicin; 0.5 mg/kg, *i.v.* vincristine; and 0.2 mg/kg, orally prednisone) every day for 5 days. The MTD of the TW-37/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 TW-37 at its MTD (*i.e.*, 120 mg/kg divided in three injections for three consecutive days) plus CHOP at its MTD was toxic to all SCID mice due to weight loss of >20% of animal body weight. Therefore, we reduced the TW-37 dose to 20 mg/kg per day for three consecutive days for the combination treatments shown in Fig. 7.

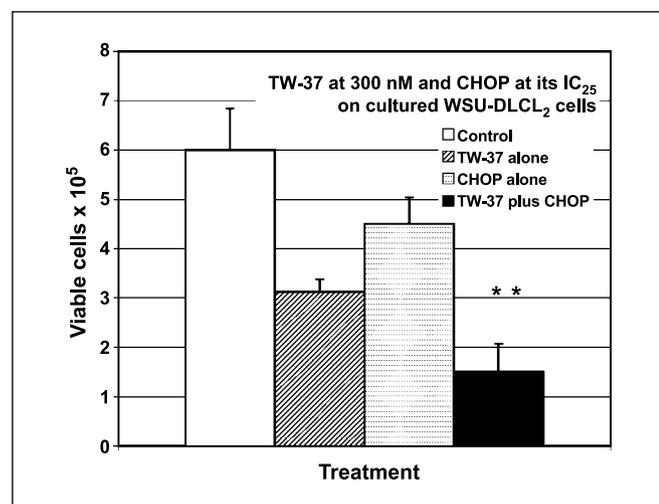


Fig. 6. Near its IC₅₀ (290 nmol/L), TW-37 synergizes in cultured WSU-DLCL₂ cells with the four-drug combination CHOP at IC₂₅ of CHOP. In these experiments, cells seeded at 1.5×10^5 per mL in a 24-well plate were treated with drug vehicle, TW-37 at 300 nmol/L, CHOP alone at its IC₂₅ (*i.e.*, 5.84 pmol/L cyclophosphamide monohydrate, 1.5 pmol/L doxorubicin, 260 pmol/L oncovin, 1.0 μmol/L prednisone), or TW-37 at 300 nmol/L + CHOP at its IC₂₅. Columns, average of two experiments; bars, SD. WSU-DLCL₂ cells were pre-exposed to TW-37 for 5 h before CHOP was added; in all cases, viable cells were measured after a 72-h period of culture.

Figure 7A shows the tumor weight of mice treated with TW-37, CHOP, and their combination, compared with control. Mice in all treatment groups developed *s.c.* tumors. Tumor weights in the TW-37 + CHOP combination decreased significantly ($P < 0.01$) compared with either TW-37 or CHOP group, an impressive log₁₀ reduction of 1.8. Antitumor activity of TW-37 alone, CHOP alone, or TW-37 + CHOP combination against WSU-DLCL₂-bearing SCID mice as measured by T/C, T-C, and log₁₀ kill were 57%, 19%, and 11%; 4, 8, and 12 days; and 0.6, 1.2, and 1.8, respectively (Table 2). T/C values are used to determine tumor response. CHOP alone and TW-37 + CHOP were considered active against WSU-DLCL₂ tumor (T/C < 42%). The dose and schedule of TW-37 alone and in combination with CHOP against WSU-DLCL₂ xenograft tumor merits refinement, planned for future work.

In Fig. 7B, we weighed the mice over 17 days of treatment using the same treatment dose and scheduling as in Fig. 7A. After 12 days, mice treated with CHOP lost ~9% of their body weight compared with initial weight; the curve for CHOP alone overlaps the curve for the combination, showing that addition of TW-37 to CHOP did not cause any additional toxicity.

Discussion

Third-generation BH3 mimetic SMIs bind antiapoptotic Bcl-2 family members with greater specificity and selectivity. Beginning with the pioneering studies of Wang et al. (42) and Degtrev et al. (17), more than a dozen nonpeptidic SMI BH3 antagonists have been identified since 2000 belonging to at least eight different chemical classes (21, 43). These compounds include Huang's HA14-1 (a chromene derivative), BH3I-1 (a thiazolidin derivative), and BH3I-2 (a benzenesulfonyl derivative), which are the oldest known BH3 SMIs with IC₅₀ and K_i in the micromolar range. The indole derivative GX015-070 (Obatoclax) has advanced into clinical trials for late-stage chronic lymphocytic leukemia (44). Gossypol, a natural product extracted from cotton seeds and roots, was used to treat patients with metastatic adrenal cancer (45) and breast cancer (46) before it was discovered as a BH3 mimetic. It is now known that (–)-gossypol, the active enantiomer of gossypol, binds to Bcl-2 family proteins (Bcl-2, Bcl-xL, and Mcl-1) with good affinities (22) and has recently advanced into clinical trials for the treatment of patients with advanced malignancies (47). In addition, promising new analogues of gossypol, such as apogossypolone (ApoG2; ref. 1) have been designed and evaluated as inhibitors of these antiapoptotic Bcl-2 proteins. Recently, ABT-737 was reported as a potent nonpeptidic inhibitor with low nanomolar binding affinities to Bcl-2, Bcl-xL, and Bcl-w proteins but devoid of significant binding affinity to Mcl-1 protein in *in vitro* biochemical binding assays (34). Interestingly, the benzenesulfonamide derivative ABT-737 does not perform as well in tumors as would be expected from its *in vitro* affinity for Bcl-2. Oltersdorf et al. (34) report an IC₅₀ of 0.855 ± 0.14 μmol/L for ABT-737 for the established diffuse lymphoma cell line SuDHL-4 (cell line described in ref. 47). More recently, Konopleva et al. (48) have tested ABT-737 in acute myeloid leukemia and report wide variation in IC₅₀ (1.4–103.2 nmol/L). Data presented here in Fig. 2A reveal that our new compound TW-37 shows an IC₅₀ against cultured WSU-DLCL₂ cells of 290 ± 25 nmol/L, which is 7-fold better than

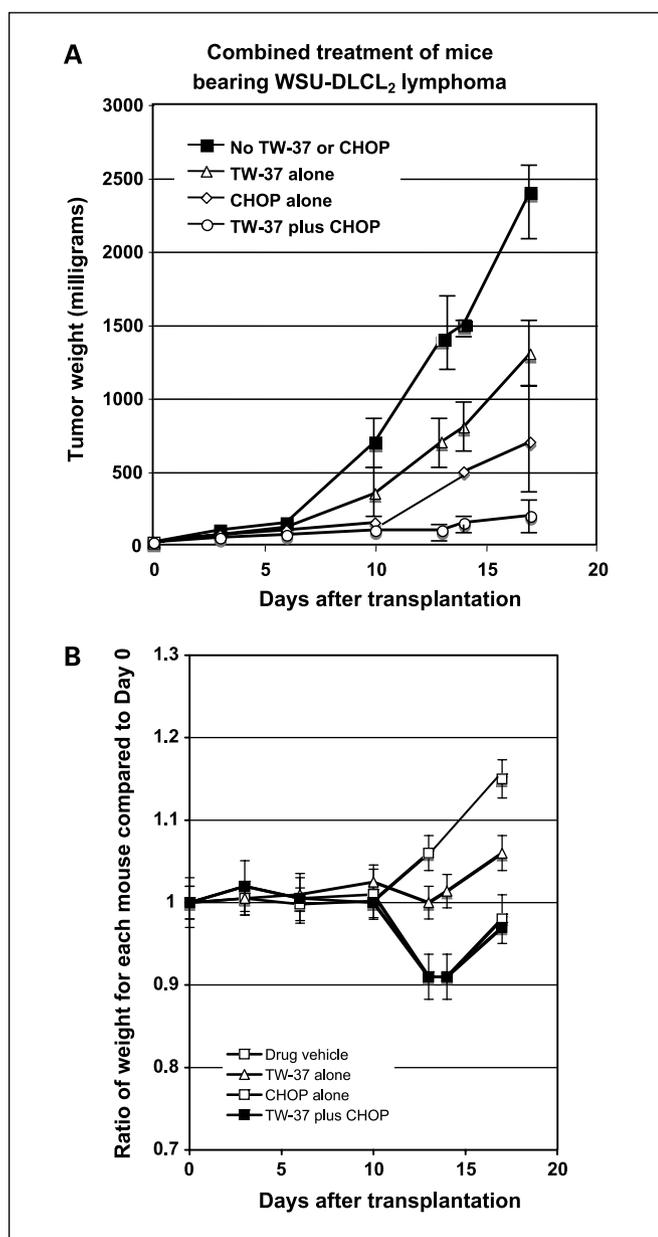


Fig. 7. Tumor reduction resulting from the four-drug regimen CHOP in combination with the BH3 mimetic SMI TW-37 in a mouse xenograft model of diffuse lymphoma. *A*, tumor weight (mg) of WSU-DLCL₂-bearing SCID in control (diluent), TW-37, CHOP, and TW-37/CHOP. Points, mean; bars, SD. The TW-37 treatment group was 20 mg/kg given i.v. for three consecutive d. The CHOP treatment group was at its MTD. The combined treatment group was at the MTD of CHOP + 20 mg TW-37 per kg (i.v.) for three consecutive d. Tumor weight decreased significantly in mice that received TW-37/CHOP combination compared with either treatment group alone (TW-37 alone or CHOP alone), with $P < 0.01$. All animals were treated 5 d after tumor transplantation. Together, combination treatment with CHOP and TW-37 lead to a \log_{10} 1.8 reduction of tumor mass during the treatment period (Table 2). *B*, we weighed the mice over 17 d of treatment using the same treatment dose and scheduling as in (*A*). After 12 d, mice treated with CHOP alone or the TW-37 + CHOP combination lost ~9% of their body weight compared with initial weight at day 0.

what we reported for (–)-gossypol on the same cells. We have not yet done a head-to-head comparison of TW-37 and ABT-737 in any diffuse lymphoma cell line. However, we would predict based on the *in vitro* binding that the performance of the two drugs in a head-to-head comparison would chiefly

reflect the expression of Mcl-1, given the fact that the K_i s for this BCL2 family member differ by a factor of ~3.

The apparent efficacy of BH3 mimetic SMIs to induce apoptosis may be determined by their target specificity on Bcl-2, Bcl-X_L, Mcl-1 and how these proteins serve in the Korsmeyer rheostat set points for that cell type. We show here that TW-37 binds avidly to both Bcl-2 and Mcl-1 and can disrupt heterodimers between these antiapoptotic pro-tumor cues and their natural proapoptotic antitumor partners. How can we reconcile the efficacy of TW-37 on diffuse lymphoma cells compared with ABT-737, considering that ABT-737 binds to Bcl-2 *in vitro* with ~1 nmol/L affinity versus 370 nmol/L affinity of TW-37 for Bcl-2? We think that the answer lies in the other Bcl-2 antiapoptotic family members found in diffuse B-lymphoma large cell lymphomas such as WSU-DLCL₂ and SuDHL-4 (47). Two prominent antiapoptotic family members found in a variety of DLCLs (49) are Bcl-X_L and Mcl-1. Although it binds extremely tightly to Bcl-2, the Abbott compound ABT-737 binds poorly to Mcl-1, with $K_i > 1,000$ nmol/L (34). Our data for TW-37 (Fig. 1C) show that TW-37 binds to recombinant Mcl-1 *in vitro*, with $K_i \sim 300$ nmol/L, at least thrice greater than the affinity of ABT-737 for Mcl-1. Moreover, treatment of living lymphoma cells with the drug TW-37 is able to disrupt heterodimers between Mcl-1 and Bax more potently than this treatment disrupts Bcl-X_L:Bax heterodimers (Fig. 4), consistent with the superior affinity of the drug for Mcl-1 over Bcl-X_L.

Toward the development of a pan-BCL2 drug: the key role of Mcl-1 in the clinical outcome of lymphomas and leukemias. Mcl-1 is frequently overexpressed in B-cell chronic lymphocytic leukemia (50); moreover, higher levels of Mcl-1 are associated with failure to achieve complete remission of B-cell chronic lymphocytic leukemia following chemotherapy (51). The role of Mcl-1 expression in follicular lymphoma has recently been explored using immunocytochemistry; results show that Mcl-1 expression in the follicle is highest in centroblasts (33). Centroblasts are characteristic of a stage in B-lymphocyte development where hypermutation of the *IgV* gene regions occurs. The *c-myc* gene is often rearranged in DLCL arising from these centroblastic cells (3), leading to its overexpression. High-level expression of *c-myc* is thought to lead to dramatic effects on cell phenotype because *myc* acts as a hub of a gene-regulatory

Table 2. Antitumor activity of TW-37, CHOP, and their combination in WSU-DLCL₂-bearing SCID mice

Agent	No. animals	T/C (%)	T-C (d)	Log ₁₀ kill (gross)
Control	5	100	0	0.0
TW-37	5	57	4	0.6
CHOP	5	19	8	1.2
TW-37 + CHOP	5	11	12	1.8

NOTE: TW-37 was given at 20 μg/kg, QD × 3 d. CHOP was given at MTD × 1 injection (i.e., 40 mg/kg, i.v. cyclophosphamide; 3.3 mg/kg doxorubicin; 0.5 mg/kg vincristine; 0.2 mg, orally prednisone) for 5 d. In the combination, TW-37/CHOP combination was given 60 mg/kg (i.v. via tail vein, divided in three injections for 3 d) plus CHOP at its MTD. Combination of TW-37 at its MTD (i.e., 120 mg/kg divided in three injections for three consecutive days) plus CHOP at its MTD was toxic to all SCID mice.

network (52). Surprisingly, expression of the prosurvival *c-myc* gene in these cells is often low as is the expression of Bcl-2 (53), and we speculate that the *Mcl-1* gene may provide surrogate survival cues to cells during this period. Likewise, Mcl-1 overexpression may provide key survival cues for diffuse lymphoma cells, tumor cells thought to arise from centroblasts. In contrast to mice null for the *Bcl-2* gene, which surprisingly are born alive without the benefit of the *Bcl-2* gene during embryonic and fetal development, mice null for both *Mcl-1* alleles die at embryonic day 4, suggesting an essential role for Mcl-1 in the regulation of embryonic apoptosis (54). Solution structure of Mcl-1 reveals that Mcl-1 features a hydrophobic binding groove like Bcl-2 and Bcl-X_L, but in a conformation intermediate between the open structures characterized by peptide complexes and the closed state observed in unliganded structures (15). Much like its better-studied cousins Bcl-2 and Bcl-X_L, the Mcl-1 protein sequesters proapoptotic regulators, whose release leads to mitochondrial membrane permeabilization, release of cytochrome *c* into the cytosol, and activation of caspase-9 (55). Pharmacologic agents unrelated to BH3 mimetic SMIs can induce apoptosis in tumor cells by indirect action on Bcl-2 family members; recent studies of the mechanism of action of the compound SU-9516 in the histiocytic lymphoma cell line U-937 (56) show that this 3-substituted indolinone cyclin-dependent kinase 2 inhibitor kills leukemia cells through a transcriptional down-regulation of Mcl-1, which tips the Korsmeyer rheostat (57, 58) in leukemia cells towards cell death (59). The new BH3 mimetic drug described here potently disrupts heterodimers between Mcl-1 and both multidomain (Bax) and BH3-only (t-Bid) proapoptotic effectors (Fig. 4A and B), but at concentrations about 1 order of magnitude higher than either the *K_i* or *IC₅₀* would predict (i.e., 3,000 versus 300 nmol/L). This 10-fold discrepancy between heterodimer dissociation and *IC₅₀* suggests that the mechanism of action of TW-37 is not the disruption of heterodimers only. The heteronuclear single quantum coherence NMR studies (22) clearly delineate drug interaction with residues in the hydrophobic pocket, the site where the α -helical domain of BH3 proteins like Bid bind to Bcl-2, Bcl-X_L, and Mcl-1. This pocket may not be unliganded in the absence of proapoptotic partners. Instead, studies suggest that this pocket can naturally be occupied by the hydrophobic COOH terminus that is removed from the recombinant forms of Bcl-2 and Bcl-X_L, which have been used in crystallographic studies and fluorescence polarization studies of drug binding. This COOH terminus is not a classic BH3 domain; nevertheless, its hydrophobicity drives interaction with the pocket not only in several studied antiapoptotic proteins, such as Bcl-2 and Bcl-w, but also in the proapoptotic protein Bax (39, 60–62).

The importance of Mcl-1 in apoptosis is also highlighted in a very recent study of Van Delft et al. in which they deliberately overexpress Mcl-1 in a mouse E μ /bcl-2 lymphoma model and show that such overexpression significantly shortens the survival

of tumor-bearing mice treated with ABT-737 (63). These remarkable results thus support our suggestion that Mcl-1 overexpression might provide a Bcl-2–positive, Bcl-X_L–positive lymphoma cell a mechanism to escape the action of ABT-737.

TW-37 adds to the efficacy of the “standard” four-drug cocktail CHOP. Most studies (17–19) have used *in vitro*, cell-based assays to show that SMI of Bcl-2 or Bcl-X_L are potential molecularly targeted agents. However, the main challenge in developing a novel therapeutic agent is that it needs to show therapeutic efficacy *in vivo*. Many SMIs, 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 they are toxic. Here, we tested the toxicity of TW-37 in our WSU-DLCL₂-SCID model. The MTD of TW-37 in SCID mice was 40 mg/kg for three i.v. injections when given alone and 20 mg/kg \times 3 when given in combination with CHOP regimen. In addition, our results show that TW-37 by itself was effective in decreasing tumor weight; however, when 60 mg/kg TW-37 was administered in conjunction with CHOP, it achieved a significantly longer tumor growth delay ($P = 0.01$) compared with either CHOP or TW-37 alone (Table 2). In addition, administration of TW-37 with CHOP did not increase CHOP toxicity (Fig. 7B). It should be emphasized that WSU-DLCL₂-SCID is a model of resistant lymphoma (37). Moreover, results presented in Table 2 and Fig. 6 are those following one cycle of therapy, whereas in a clinical setting, lymphoma is treated with multiple cycles of CHOP chemotherapy. Multiple cycles is particularly an attractive option because one cycle did not eradicate the tumors. Studies over the past few decades have shown that more complicated cytotoxic regimens were not superior to CHOP, which remains the “gold standard” (23, 24, 37, 65, 66). The efficacy of this regimen in lymphoma has been significantly enhanced recently by the addition of an anti-CD20 antibody (rituximab; ref. 67). Bcl-2/Mcl-1 SMI can be another innovative way to enhance CHOP activity by antagonizing a major resistance mechanism to apoptosis. Our study suggests that TW-37 represents a promising new agent that should be developed for the treatment of NHLs in the clinic. Our findings provide compelling evidence that TW-37 acts as a small-molecule BH3 mimetic on a well-defined diffuse lymphoma model in culture and grown as a xenograft in mice. Moreover, the compound acts at *IC₅₀* of \sim 300 nmol/L in this lymphoma cell line (Fig. 2A) and also in freshly isolated lymphoma cells direct from the patient (Fig. 2B). Although this group is limited, we feel that these findings warrant further preclinical investigation of TW-37 in a wider sampling of not only diffuse lymphoma but other types of lymphoma.

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