

Apogossypol derivatives as antagonists of antiapoptotic Bcl-2 family proteins

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Abstract

Guided by a combination of nuclear magnetic resonance binding assays and computational docking studies, we synthesized a library of 5,5' substituted Apogossypol derivatives as potent Bcl-X_L antagonists. Each compound was subsequently tested for its ability to inhibit Bcl-X_L in an *in vitro* fluorescence polarization competition assay and exert single-agent proapoptotic activity in human cancer cell lines. The most potent compound BI79D10 binds to Bcl-X_L, Bcl-2, and Mcl-1 with IC₅₀ values of 190, 360, and 520 nmol/L, respectively, and potently inhibits cell growth in the H460 human lung cancer cell line with an EC₅₀ value of 680 nmol/L, expressing high levels of Bcl-2. BI79D10 also effectively induces apoptosis of the RS11846 human lymphoma cell line in a dose-dependent manner and shows little cytotoxicity against *bax*^{-/-}*bak*^{-/-} mouse embryonic fibroblast cells, in which antiapoptotic Bcl-2 family proteins lack a cytoprotective phenotype, implying that BI79D10 has little off-target effects. BI79D10 displays *in vivo* efficacy in transgenic mice, in which Bcl-2 is overexpressed in splenic B cells. Together with its improved plasma and microsomal stability relative to Apogossypol, BI79D10 represents a lead compound for the development of novel apoptosis-based therapies for cancer. [Mol Cancer Ther 2009;8(4):904–13]

Introduction

Programmed cell death (apoptosis) plays critical roles in normal tissue homeostasis, ensuring a proper balance of cell

production and cell loss (1, 2). Defects in the regulation of programmed cell death promote tumorigenesis and also contribute significantly to chemoresistance (3, 4). Bcl-2 (B-cell lymphoma/leukemia-2) family proteins are central regulators of apoptosis (5–7). In humans, six antiapoptotic members of the Bcl-2 family have been identified and characterized, including Bcl-2, Bcl-X_L, Mcl-1, Bfl-1, Bcl-W, and Bcl-B. Overexpression of antiapoptotic Bcl-2 family proteins occurs in many human cancers and leukemias, and therefore, these proteins are very attractive targets for the development of novel anticancer agents (8–11). Members of the Bcl-2 family proteins also include proapoptotic effectors, such as Bak, Bax, Bad, Bim, and Bid. Antiapoptotic and proapoptotic Bcl-2 family proteins dimerize and negate each other's functions (3). Structural studies have elucidated a hydrophobic crevice on the surface of antiapoptotic Bcl-2 family proteins that binds the BH3 dimerization domain of proapoptotic family members (10). Thus, molecules that mimic the BH3 domain of proapoptotic proteins induce apoptosis and/or abrogate the ability of antiapoptotic Bcl-2 proteins to inhibit cancer cell death.

We and others have reported that the natural product Gossypol (Fig. 1A) is a potent inhibitor of Bcl-2, Bcl-X_L, and Mcl-1, functioning as a BH3 mimic (12–15). (–)Gossypol is currently in clinical trials, displaying single-agent antitumor activity in patients with advanced malignancies (14). Given that Gossypol has toxicity problems likely due to two reactive aldehyde groups (16), we designed Apogossypol (Fig. 1A), a compound that lacks these aldehydes but retains activity against antiapoptotic Bcl-2 family proteins *in vitro* and in cells (17). Recently, we further compared the efficacy and toxicity in mice of Gossypol and Apogossypol. Our preclinical *in vivo* data show that Apogossypol has superior efficacy and markedly reduced toxicity compared with Gossypol (18). We also evaluated the single-dose pharmacokinetic characteristics of Apogossypol in mice. Apogossypol displayed superior blood concentrations over time compared with Gossypol due to slower clearance (19). These observations indicate that Apogossypol is a promising lead compound for cancer therapy. Recently, we reported the separation and characterization of Apogossypol atropoisomers (15). These studies revealed that the racemic Apogossypol is as effective as its individual isomers (15). In this current work, we focused our attention on preparing and evaluating activities of novel 5,5' substituted Apogossypol derivatives (Fig. 1B).

Materials and Methods

Molecular Modeling

Molecular modeling studies were conducted on a Linux workstation and a 64 3.2-GHz CPUs Linux cluster. Docking studies were done using the crystal structure of Bcl-X_L in

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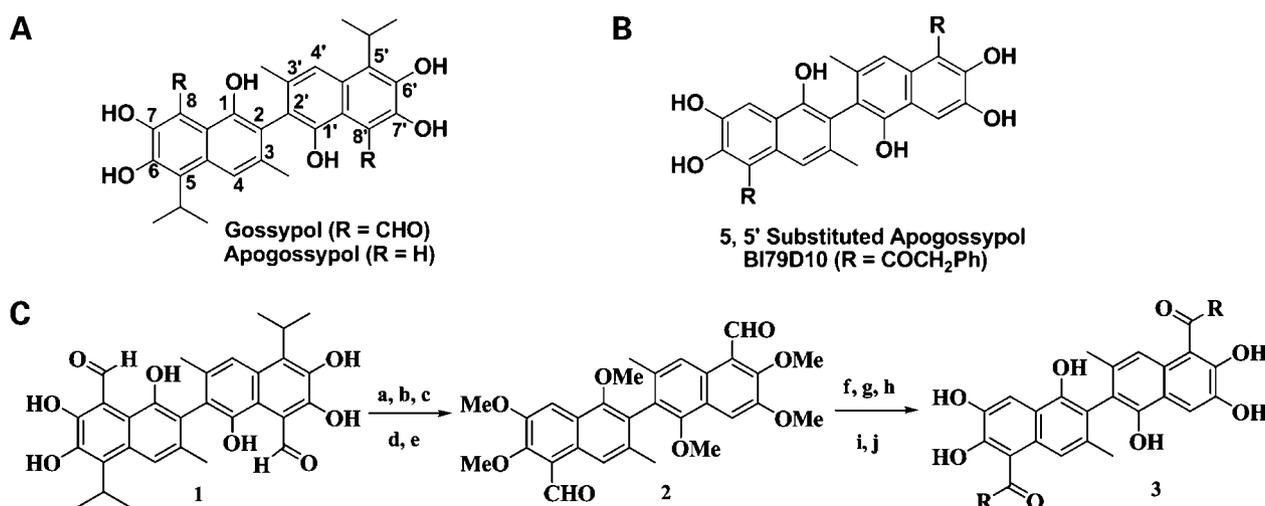


Figure 1. **A**, structure of Gossypol and Apogossypol. **B**, structure of 5,5' substituted Apogossypol derivatives and BI79D10. **C**, synthesis of 5,5' substituted Apogossypol derivatives: *a*, NaOH, H₂O; *b*, H₂SO₄; *c*, DMS, K₂CO₃; *d*, TiCl₄, Cl₂CHOCH₃; *e*, HCl, H₂O; *f*, RMgBr or RL; *g*, NH₄Cl, H₂O; *h*, PCC, CH₂Cl₂; *i*, BBr₃; *j*, HCl.

complex with Bak-derived peptide (Protein Data Bank code 1BXL; ref. 20). The Bak-derived peptide was extracted from the protein structure and was used to define the binding site for small molecules. Apogossypol and its derivatives were docked into the Bcl-X_L protein by the GOLD (21) docking program using ChemScore (22) as the scoring function. The active site radius was set at 10 Å, and 10 GA solutions were generated for each molecule. The GA docking procedure in GOLD (21) allowed the small molecules to flexibly explore the best-fit conformations in the binding pocket whereas the protein structure was static. The binding poses of Apogossypol and its derivatives were compared to prioritize synthesis. The protein surface was prepared with the program MOLCAD (23) as implemented in Sybyl (Tripos, St. Louis) and was used to analyze the binding poses for studied small molecules.

Chemicals

The synthesis for 5,5' substituted Apogossypol derivatives is outlined in Fig. 1C. Briefly, Gossypol 1 was treated with NaOH solution followed by dimethyl sulfate to afford methyl Apogossypol. Reaction of methyl Apogossypol with TiCl₄ and dichloromethyl methyl ether resulted in loss of isopropyl groups and simultaneous bisformylation to give aldehyde 2 (24). The compound 2 was treated with different Grignard or lithium reagents to afford a secondary alcohol, which was oxidized to the phenone using pyridinium chlorochromate. Subsequent demethylation of the phenone afforded compound 3. Characterizations of the compound BI79D10 (Fig. 1B) are given as an example. The detail experiments and characterization of all other compounds are in the supplementary data. ¹H nuclear magnetic resonance (NMR) (CD₃OD) δ 7.61 (s, 2H), 7.30 (m, 8H), 7.22 (m, 2H), 6.97 (s, 2H), 4.40 (dd, *J*₁ = 15.6 Hz, *J*₂ = 22.8 Hz, 4H), 1.87 (s, 6H); ¹³C NMR (CD₃)₂SO δ 204.6, 149.4, 144.8, 144.5, 135.4, 134.2, 130.5, 128.6, 126.9, 126.3, 122.6, 119.4, 116.8, 115.0, 107.1, 51.0, 21.1; HRMS calculated for [C₃₈H₃₀O₈ +

H] 615.2019; Found 615.2013. High-performance liquid chromatography is 99% pure.

NMR Experiments

NMR-based binding assays have been conducted by acquiring one-dimensional ¹H experiments with 500 μL solution of Bcl-X_L at 25 μmol/L concentration in the absence and the presence of added compounds, each at 200 μmol/L concentration. By observing the aliphatic region of the spectra, binding could be readily detected due to chemical shift changes in active site methyl groups of Ile, Leu, Thr, Val, or Ala (region between -0.8 and 0.3 ppm). The binding mode was characterized by recording [¹⁵N, ¹H]-HSQC experiments with 500 μL solution of uniformly ¹⁵N-labeled Bcl-X_L (125 μmol/L concentration) in the absence and the presence of added compounds, each at 500 μmol/L concentration. ¹⁵N and unlabeled Bcl-X_L samples were prepared and purified as described previously (20). All experiments were done with a 600 MHz spectrometer Bruker Avance 600 equipped with four rf channels and z axis pulse-field gradients.

Fluorescence Polarization Assays

A Bak BH3 peptide (F-BakBH3; GQVGRQLAIGDDINR) was labeled at the NH₂ terminus with FITC (Molecular Probes) and purified by high-performance liquid chromatography. For competitive binding assays, 100 nmol/L GST-Bcl-X_L ΔTM protein was preincubated with the tested compound at varying concentrations in 47.5 μL PBS (pH 7.4) in 96-well black plates at room temperature for 10 min, then 2.5 μL of 100 nmol/L FITC-labeled Bak BH3 peptide was added to produce a final volume of 50 μL. The wild-type (WT) and mutant Bak BH3 peptides were included in each assay plate as positive and negative controls, respectively. After 30-min incubation at room temperature, the polarization values in millipolarization units (20) were measured at excitation/emission wavelengths of 480/535 nm with a multilabel plate reader (PerkinElmer).

IC₅₀ was determined by fitting the experimental data to a sigmoidal dose-response nonlinear regression model (SigmaPlot 10.0.1, Systat Software, Inc.). Data reported are mean of three independent experiments \pm SE. Performances of Bcl-2 and Mcl-1 fluorescence polarization assays (FPA) are similar. Briefly, 50 nmol/L of GST-Bcl-2 or GST-Mcl-1 were incubated with various concentrations of Apogossypol or its 5,5' substituted derivatives for 2 min, then 15 nmol/L FITC-conjugated Bim BH3 peptide (25) was added in the PBS buffer. Fluorescence polarization was measured after 10 min.

Isothermal Titration Calorimetry Assays

Titration were done by isothermal titration calorimetry (ITC) assays using a VP-ITC or ITC200 calorimeter from Microcal. Bcl-X_L was used at concentrations between 25 and 100 μ mol/L in 20 mmol/L sodium phosphate buffer (pH 7.4) and 5% to 10% DMSO. Titrants were used at concentrations of 10 \times to 15 \times of the protein in the same buffer. Titrations were carried out at 25°C. Data were analyzed using Microcal Origin software provided by the ITC manufacturer (Microcal).

Cell Viability Assays

The activity of the compounds against human cancer cell lines (PC3ML, H460, H1299, RS11846) were assessed by using the ATP-LITE assay (PerkinElmer). All cells were seeded in either F12 or RPMI 1640 with 5 mmol/L L-glutamine supplemented with 5% fetal bovine serum from Mediatech, Inc. and penicillin and streptomycin from Omega. For maintenance, cells were cultured in 5% fetal bovine serum. Cells were plated into 96-well plates at varying initial densities depending on doubling time. H460 and H1299 were plated at 2,000 cells per well, A549 and PC3 at 3000 cells per well, and RS118456S at 10,000 cells per well. Compounds were diluted to final concentrations with 0.1% DMSO. Before dispensing compounds onto cells, fresh 5% media were placed into wells. Administration of compounds occurred 24 h after seeding into the fresh media. Cell viability was evaluated using ATP-LITE reagent (PerkinElmer) after 72 h of treatment. Data were normalized to the DMSO control-treated cells using Prism version 5.01 (Graphpad Software).

The apoptotic activity of the compounds against RS11846 cells was assessed by staining with Annexin V and propidium iodide (PI). The lymphoma cell line RS11846 was cultured in RPMI 1640 (Mediatech, Inc.) containing 10% fetal bovine serum (Mediatech, Inc.) and penicillin/streptomycin (Mediatech, Inc.). Cells were cultured with various concentrations of 5,5' substituted Apogossypol for 1 to 2 d. The percentage of viable cells was determined by FITC-Annexin V and PI labeling, using an apoptosis detection kit (BioVision, Inc.), and analyzing stained cells by flow cytometry (FACSort; Bectin-Dickinson, Inc.). Cells that were Annexin V-negative and PI-negative were considered viable.

The apoptotic activity of the compound BI79D10 against mouse embryonic fibroblast (MEF) WT cells and MEF BAX/Bak double-knockout cells (DKO) was assessed by staining with Annexin V and PI. MEF WT and MEF DKO cells were seeded in 24-well plates at a seeding density of half a mil-

lion per well (in 1 mL DMEM supplemented by 10% FCS). On the next day, BI79D10 was added to WT and DKO cells at final concentration of 0, 2.5, 5.0, 7.5, and 10 μ mol/L. On the following day, floating cells were pooled with adherent cells harvested after a brief incubation with 0.25% Trypsin/EDTA solution (Life Technologies/In-Vitrogen, Inc.). Cells were centrifuged, supernatant was discarded, and cell pellet was resuspended with 0.2 mL of Annexin V binding buffer, followed by addition of 1 μ L Annexin-FITC and 1 μ L PI. The percentage of viable cells was determined by a three-color FACSort instrument, and data were analyzed by the Flow-Jo program, scoring Annexin V-negative, PI-negative as viable cells.

In vitro ADME Studies

Liver Microsomal Stability. Pooled rat liver microsomes (BD Biosciences) were preincubated with test compounds at 37.5°C for 5 min in the absence of NADPH. The reaction was initiated by addition of NADPH and then incubated under the same conditions. The final incubation concentrations were 4 μ mol/L test compound, 2 mmol/L NADPH, and 1 mg/mL (total protein) liver microsomes in PBS at pH 7.4. One aliquot (100 μ L) of the incubation mixture was withdrawn at 0, 15, 30, and 60 min and combined immediately with 200 μ L of ACN/methanol containing an internal standard. After mixing, the sample was centrifuged at \sim 13,000 rpm for 12 min. The supernatant was transferred into an autosampler vial, and the amount of test compound was quantified using the Shimadzu LCMS 2010EV mass spectrometer. The change of the AUC (area under the curve) of the parent compound as function of time was used as a measure of microsomal stability.

Plasma Stability. A 20- μ L aliquot of a 10-mmol/L solution in DMSO of the test compound was added to 2.0 mL heparinized rat plasma (Lampire, P1-150N) to obtain a 100- μ mol/L final solution. The mixture was incubated for 1 h at 37.5°C. Aliquots of 100 μ L were taken (0, 30 min, 1 h) and diluted with 200 μ L of methanol containing internal standard. After mixing, the sample was centrifuged at \sim 13,000 rpm for 12 min. The supernatant was transferred into an autosampler vial, and the amount of test compound was quantified using the Shimadzu LCMS-2010EV system. The change of the AUC of the parent compound as function of time was used as a measure of microsomal stability.

Parallel Artificial Membrane Permeation Assay. A 96-well microtiter plate (Millipore) was completely filled with aqueous buffer solution (pH 7.2) and covered with a microtiter filterplate (Millipore). The hydrophobic filter material was impregnated with a 10% solution of hexadecane in hexane, and the organic solvent was allowed to completely evaporate. Permeation studies were started by the transfer of 200 μ L of a 100- μ mol/L test compound solution on top of the filter plate. In general, phosphate buffer at pH 7.2 was used. The maximum DMSO content of the stock solutions was <5%. In parallel, an equilibrium solution lacking a membrane was prepared using the exact concentrations and specifications but lacking the membrane. The concentrations of the acceptor and equilibrium solutions were

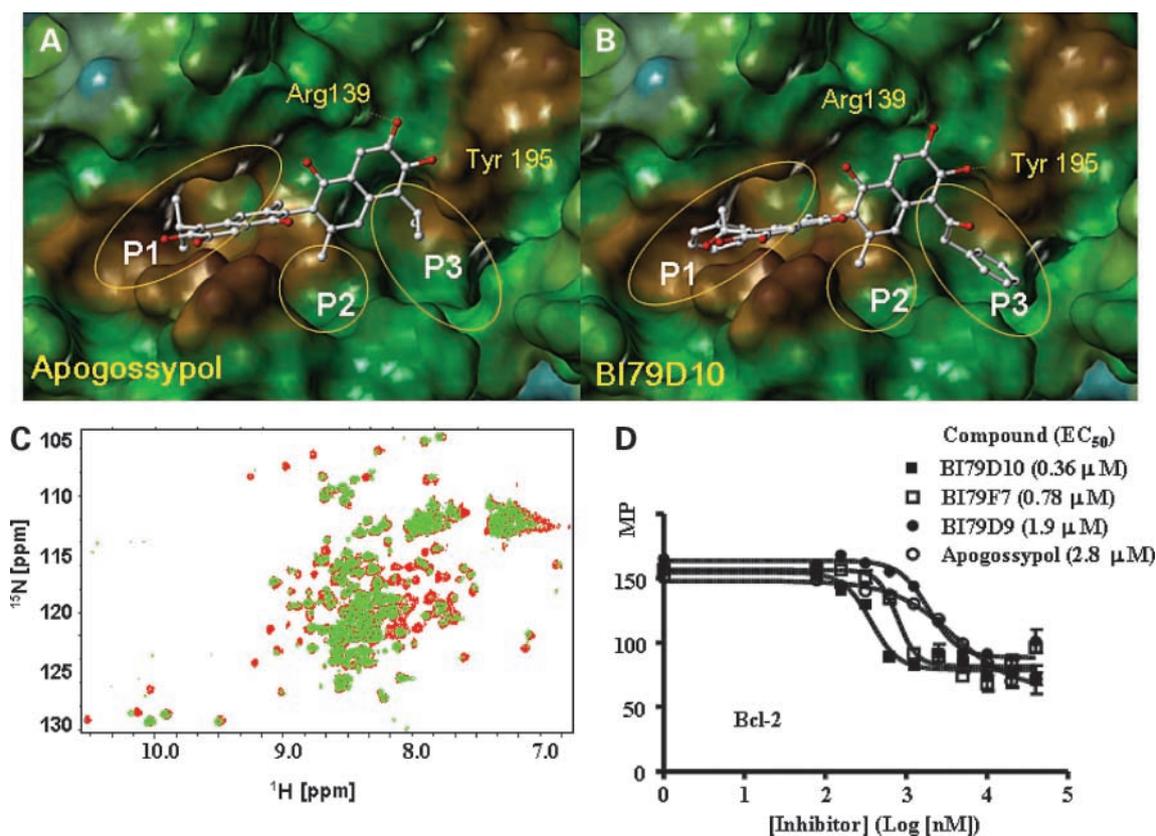


Figure 2. Molecular docking studies. Stereo views of docked structures of Apogossypol (A) and BI79D10 (B) into the BH3 peptide binding groove in Bcl-X_L. C, superposition of [¹⁵N, ¹H]TROSY spectra of free Bcl-X_L (125 μmol/L; red) and spectra after addition of BI79D10 (500 μmol/L; green). D, fluorescence polarization-based competitive binding curves of BI79D10 (solid squares), BI79F7 (hollow squares), BI79D9 (solid dots), and Apogossypol (hollow dots) to Bcl-2.

determined using the Shimadzu LCMS-2010EV and AUC methods. The permeation of a compound through the membrane layer is described by the percentage permeation (percentage of flux). The flux values were calculated considering the concentration of the acceptor compartment after 8 h and that of a reference well with the same concentration containing no membrane barrier.

Bcl-2 Transgenic Mice Studies

Transgenic mice expressing Bcl-2 have been described as the B6 line (26). The BCL-2 transgene represents a mini-gene version of a t(14;18) translocation, in which the human BCL-2 gene is fused with IgH locus and associated IgH enhancer. The transgene was propagated on the BALB/c background. These mice develop polyclonal B-cell hyperplasia with asynchronous transformation to monoclonal aggressive lymphomas beginning at ages of ~6 mo, with ~90% of mice undergoing transformation by ages of 2 to 24 mo. All animals used here had not yet developed aggressive lymphoma.

Mouse Experiments

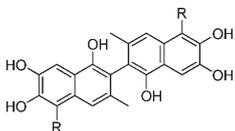
Compounds dissolved in 500 μL of solution (ethanol/cremophor EL/saline, 10:10:80) were injected i.p. to age-matched and sex-matched B6Bcl2 mouse, whereas control mice were injected i.p. with 500 μL of the same formulation

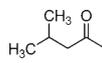
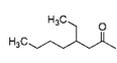
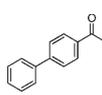
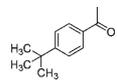
without compound. After 24 h, B6Bcl2 mice were sacrificed by i.p. injection of lethal dose of Avertin. Spleen was removed and weighed. The spleen weight of mice is used as an end-point for assessing activity, as we determined that spleen weight is highly consistent in age-matched and sex-matched Bcl-2 transgenic mice in preliminary studies (18). Variability of spleen weight was within ±2% among control-treated, age-matched, sex-matched B6Bcl2 mice. Spleen tissue was fixed in z-FIX for 3 d, rinsed in PBS, and saved for histologic analysis of spleen (H&E staining and TUNEL assay).

Results and Discussion

We have recently reported that Apogossypol is a promising inhibitor of Bcl-X_L with improved *in vivo* efficacy and reduced toxicity compared with Gossypol (17, 18). Molecular docking studies of Apogossypol into the BH3 binding groove in Bcl-X_L (Fig. 2A) suggest that Apogossypol forms two hydrogen bonds with residues Arg¹³⁹ and Tyr¹⁹⁵ in Bcl-X_L through adjacent sixth and seventh hydroxyl groups on the right naphthalene ring. The isopropyl group on the left naphthalene ring inserts into the first hydrophobic pocket (P1) in Bcl-X_L (Fig. 2A), whereas the methyl group and

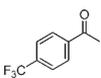
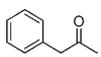
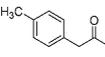
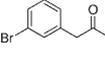
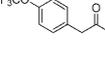
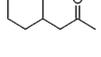
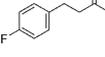
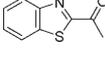
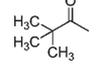
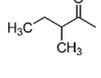
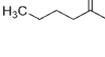
Table 1. Evaluation of 5,5' substituted Apogossypol derivatives using a combination of one-dimensional ¹H-NMR binding assays, competitive FPAs, and cell viability assays



Compound	R	One-dimensional ¹ H-NMR binding assay*, Bcl-X _L	FPA IC ₅₀ (μmol/L), Bcl-X _L	PC3ML ⁺ EC ₅₀ (μmol/L)	H460 ⁺ EC ₅₀ (μmol/L)	H1299 ⁺ EC ₅₀ (μmol/L)	RS11846 ⁺ EC ₅₀ (μmol/L)	RS11846 ⁺ EC ₅₀ (μmol/L)
Gossypol		++	2.72	3.1	3.0	6.0	2.2	4.23
Apogossypol		++	3.69	10.3	5.8	3.4	5.0	8.6
BI79F5	-H	+	ND	12.6	10.1	13.4	10.0	24.7
BI79D1		++	ND	3.9	1.5	4.8	15	14.7
BI79D3		+	1.30	7.5	1.1	3.6	10	13.7
BI79D11		+	1.29	3.0	1.5	3.0	2.8	6.6
BI79D8		+	0.45	3.4	1.1	3.1	4.0	4.5
BI79D6		+	2.9	3.6	0.31	4.2	ND	18.3
BI79D9		+	0.16	3.0	0.59	2.4	1.8	4.2
BI79D12		-	ND	7.7	8.2	9.6	2.8	25.9
BI79F3		-	ND	2.8	3.6	4.8	2.3	13.4

(Continued on the following page)

Table 1. Evaluation of 5,5' substituted Apogossypol derivatives using a combination of one-dimensional ¹H-NMR binding assays, competitive FPAs, and cell viability assays (Cont'd)

Compound	R	One-dimensional ¹ H-NMR binding assay*, Bcl-X _L	FPA IC ₅₀ (μmol/L), Bcl-X _L	PC3ML [†] EC ₅₀ (μmol/L)	H460 [†] EC ₅₀ (μmol/L)	H1299 [†] EC ₅₀ (μmol/L)	RS11846 [‡] EC ₅₀ (μmol/L)	RS11846 [‡] EC ₅₀ (μmol/L)
BI79F4		+	0.25	2.9	2.2	2.0	2.5	3.8
BI79D10		+++	0.19	4.6	0.68	3.5	2.6	4.9
BI79F7		++	0.32	2.5	0.82	1.7	2.2	3.0
BI79F11		++	1.31	3.1	2.7	2.6	8.4	5.3
BI79F12		++	1.30	1.9	3.3	3.9	1.8	6.2
BI79F8		+	ND	1.9	1.8	2.1	2	5.2
BI79F6		++	0.14	2.8	1.5	2.2	2.3	3.1
BI79D7		+	0.39	5.2	1.4	5.8	2.9	7
BI79D2		++	ND	ND	ND	ND	ND	14.7
BI79D5		+	ND	ND	ND	ND	ND	17.1
BI79D4		+	ND	ND	ND	ND	ND	11.7

Abbreviation: ND, not determined.

*Four-point rating scale: +++, very active; ++, active; +, mild; -, weak.

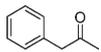
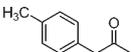
[†]Compounds against RS11846 cell line using ATP-LITE assay.

[‡]Compounds against RS11846 cell line using Annexin V-FITC and PI assay.

the isopropyl group on the right naphthalene ring insert into the adjacent two hydrophobic pockets, P2 and P3, respectively (Fig. 2A). Analysis of the predicted binding models indicates that whereas the overall core structure of Apogossypol fits rather well into BH3 binding groove of Bcl-X_L, the two isopropyl groups do not apparently fully

occupy the hydrophobic pockets P1 and P3. Therefore, a library of 5,5' substituted Apogossypol derivatives (Fig. 1B) that replace the isopropyl groups with larger hydrophobic substituents was designed with the aim of deriving novel molecules that could occupy the hydrophobic pockets on Bcl-X_L more efficiently.

Table 2. Cross-activity of selected 5,5' substituted Apogossypol derivatives against Bcl-X_L, Bcl-2, and Mcl-1

Compound	R	EC ₅₀ (μmol/L)			K _d (μmol/L)
		FPA			ITC
		Bcl-X _L	Bcl-2	Mcl-1	Bcl-X _L
Apogossypol		3.69	2.80	2.60	1.70
BI79D10		0.19	0.36	0.52	0.17
BI79F7		0.32	0.78	1.10	0.04
BI79D9		0.16	1.90	2.20	2.75

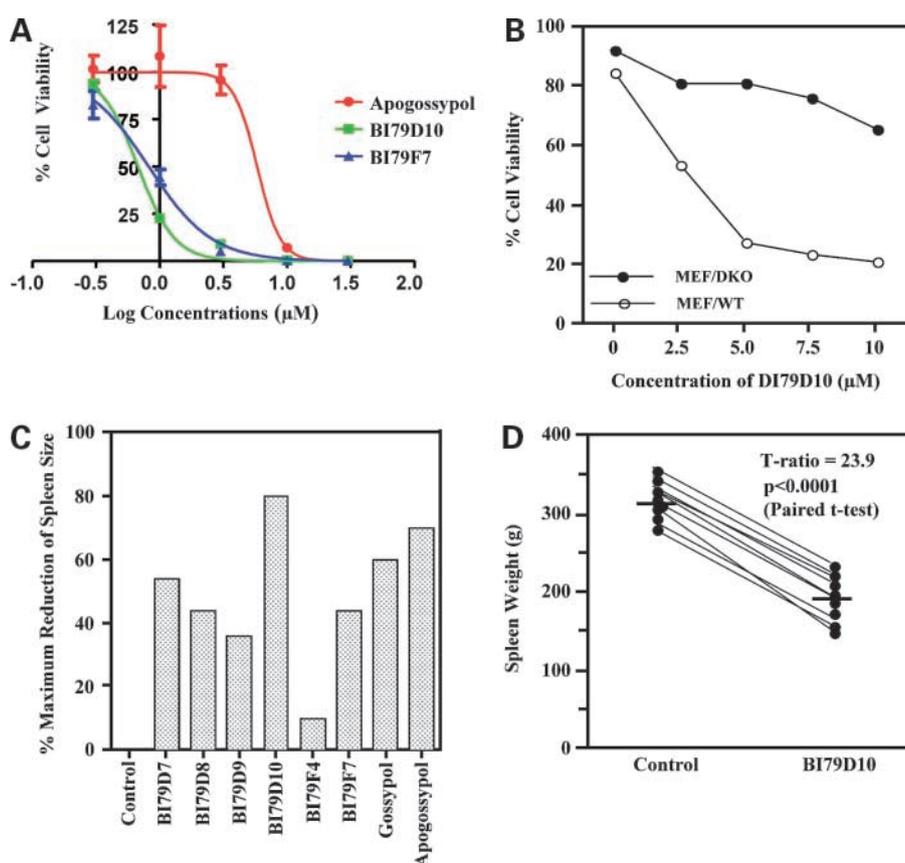
The designed 5,5' substituted Apogossypol derivatives were synthesized (Fig. 1C and Supplementary Data 1 and 2)³ and evaluated by NMR spectroscopy binding assays, competitive FPAs, and cell viability assays (Table 1). Compound BI79D10 (Fig. 1B) displayed high affinity for Bcl-X_L in these assays. It induced significant chemical shift changes in active site methyl groups (region between -0.3 and 0.8 ppm) in the one-dimensional ¹H-NMR spectra of Bcl-X_L (Supplementary Fig. S3A)³ and also has an IC₅₀ value of μmol/L in FPAs, which is almost 20 times more effective than Apogossypol (Table 1; Supplementary Fig. S3B).³ To confirm the result from the one-dimensional ¹H-NMR binding assay, we also produced uniformly ¹⁵N-labeled Bcl-X_L protein and measured two-dimensional [¹⁵N, ¹H]-TROSY correlation spectra in the absence and the presence of compound BI79D10 (Fig. 2C). BI79D10 displayed strong binding with Bcl-X_L, as qualitatively evaluated by the nature of the shifts at the ligand/protein ratio of 4:1. A group of compounds, such as BI79D7, BI79D8, BI79D9, BI79F4, BI79F6, and BI79F7 also displayed high binding affinity to Bcl-X_L in the FPAs with IC₅₀ values ranging from 0.14 to 0.45 μmol/L and induced chemical shift changes in the one-dimensional

¹H-NMR spectra of Bcl-X_L (Table 1; Supplementary Fig. S3C and D).³ To confirm results of the NMR binding data and FPAs, we further evaluated the binding affinity of BI79D10 and other compounds for Bcl-X_L using ITC (Table 2). In agreement with NMR binding and FPA data, BI79D10 and its paramethyl substituted derivative BI79F7 displayed potent binding affinity to Bcl-X_L with K_d values of 0.17 and 0.04 μmol/L, respectively, which is 10 and 40 times more potent than Apogossypol (K_d = 1.7 μmol/L) in the same assay. Molecular docking studies of BI79D10 in the BH3 binding groove of Bcl-X_L (Fig. 2B) showed that 5,5' benzyl groups insert deeper into hydrophobic pockets (P1 and P3) in Bcl-X_L, hence occupying these regions more efficiently compared with isopropyl groups of Apogossypol (Fig. 2B). The chemscore value for BI79D10 was 33.9, improved from 24 of Apogossypol. Consistent with NMR binding, FPA, and ITC data, compounds such as BI79D10 and BI79F7 display significant efficacy in inhibiting cell growth in PC3ML cells, which express high levels of Bcl-X_L. Their EC₅₀ values ranged from 1.9 to 4.6 μmol/L, hence 2-fold to 5-fold more potent than Apogossypol (EC₅₀, 10.3 μmol/L).

To evaluate the binding properties and specificity of 5,5' substituted Apogossypol derivatives to other antiapoptotic Bcl-2 family proteins, we evaluated selected Bcl-X_L active compounds against Bcl-2 and Mcl-1 using FPAs (Table 2; Fig. 2D). These potent Bcl-X_L inhibitors also displayed strong binding affinity to Bcl-2 and Mcl-1. The most potent compound, BI79D10, binds to Bcl-2 and Mcl-1 with EC₅₀ values of 0.36 and 0.52 μmol/L, respectively, which are ~8-fold and 5-fold more potent than Apogossypol (EC₅₀, 2.8 μmol/L). The compound BI79F7 is slightly less active than the compound BI79D10, whereas the compound BI79D9 has similar activity as Apogossypol. Because BI79D10 and BI79F7 displayed strong binding affinities to Bcl-2 and Mcl-1 in FPA, we further evaluated all 5,5' substituted Apogossypol derivatives against H460 and H1299 cell lines, which express high levels of Bcl-2 and Mcl-1, respectively (Table 1). In agreement with FPA data, compounds BI79D10 and BI79F7 potently inhibited growth of the H460 cell line with EC₅₀ values of 0.68 and 0.82 μmol/L, respectively, which are approximately seven to eight times more potent than Apogossypol (EC₅₀, 5.8 μmol/L; Fig. 3A and Supplementary Fig. S3E).³ Compounds BI79D6 and BI79D9 having similar structure as BI79D10 also strongly inhibited cell growth in the H460 cell line with EC₅₀ values of 0.30 and 0.59 μmol/L, respectively. Most of the tested 5,5' substituted Apogossypol derivatives also showed potent cell activity in the H460 and H1299 cell lines with EC₅₀ values ranging from 1 to 4 μmol/L. In contrast, the compound BI79F5 (Table 1), the negative control compound with only hydrogen atoms on 5,5' positions, displayed weak cell growth inhibition activity in both H460 (EC₅₀, 10.1 μmol/L) and H1299 (EC₅₀, 13.4 μmol/L) cell lines, indicating that 5,5' substituted groups are necessary for strong inhibition. This observation is in agreement with reports for the potent Bcl-X_L antagonist ABT-737 (27), which is not effective against Mcl-1 (27) and is consequentially not effective in killing Mcl-1 overexpressing cell lines, such as H1299 (28).

³ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Figure 3. **A**, inhibition of cell growth by Apogossypol derivatives in the H460 human lung cancer cell line. Cells were treated for 3 d, and cell viability was evaluated using ATP-LITE assay. **B**, MEF cells with WT (*MEF/WT*; white symbols) or *bax*^{-/-}*bak*^{-/-} DKO (*bax*^{-/-}*bak*^{-/-} DKO (black symbols) genotypes were treated with BI79D10 at various concentrations. **C**, effects of 5,5' substituted Apogossypol derivatives on shrinkage of Bcl-2 mouse spleen at a single i.p. injection dose of 0.06 mmol/kg. All shrinkage data are percentages of maximum reduction of mice spleen size. **D**, effects of BI79D10 on shrinkage of spleen of six Bcl-2 mice at a single i.p. injection dose of 0.06 mmol/kg.



We further tested 5,5' substituted Apogossypol derivatives for their ability to induce apoptosis of the human lymphoma RS11846 cell line, which expresses high levels of Bcl-2 and Bcl-X_L. For these assays, we used Annexin V-FITC and PI double staining, followed by flow cytometry analysis (Table 1). Most of synthesized Apogossypol derivatives effectively induced apoptosis of the RS11846 cell line in a dose-dependent manner (Table 1; Supplementary Fig. S4).³ In particular, compounds BI79D10, BI79D9, BI79F4, and BI79F7 are most effective with EC₅₀ values ranging from 3.0 to 5.5 μmol/L, which is consistent with previous results in human cancer PC3ML and H460 cell lines. Again, the negative control compound BI79F5 induced weak apoptosis (EC₅₀, 24.7) of the RS11846 cell line, consistent with its poor anti-Bcl-2 activity.

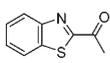
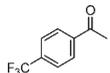
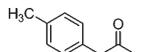
We next explored whether the compound BI79D10, the most potent 5,5' substituted Apogossypol derivative had cytotoxic activity against Bak/Bak DKO MEF cells, in which antiapoptotic Bcl-2 family proteins lack a cytoprotective phenotype (29, 30). The compound BI79D10 only displayed slight toxicity in Bak/Bak DKO MEFs while it kills 80% WT MEF cells at 10 μmol/L using FITC-Annexin V/PI assays (Fig. 3B), implying that the compound BI79D10 only displayed slight off-target effects.

To test the pharmacologic properties of 5,5' substituted Apogossypol derivatives, we determined their *in vitro* plasma stability, microsomal stability, and cell membrane per-

meability (Table 3). From these studies, we could conclude that our synthesized compounds displayed superior plasma stability and, overall, are more stable than Apogossypol. BI79D10 only degraded 15% after a 1-hour incubation in rat plasma. In addition, BI79D10 and BI79F7 showed similar or improved microsomal stability compared with Apogossypol, whereas BI79D2 and BI79D8 degraded faster than Apogossypol in rat hepatocytes microsomal preparations. BI79D10 and BI79F7 also displayed improved cell membrane permeability compared with Apogossypol.

Hence, using a combination of one-dimensional ¹H-NMR binding assays, FPAs, ITC assays, cytotoxicity assays, and preliminary *in vitro* ADME data, compounds such as BI79D10 and BI79F7 were selected for further *in vivo* studies using B6Bcl-2 transgenic mice. B cells of the B6Bcl-2 transgenic mice overexpress Bcl-2 and accumulate in the spleen of mice. The spleen weight is used as an end point for assessing *in vivo* activity, as we have determined that spleen weight is highly consistent in age-matched and sex-matched Bcl-2 transgenic mice and variability was within ±2% among control-treated age-matched, sex-matched B6Bcl2 mice (18). We first screened the *in vivo* activities of compounds such as BI79D10 and BI79F7, side by side with Apogossypol and Gossypol in a single Bcl-2 transgenic mouse at 60 μmol/kg. All tested compounds induced spleen weight reduction of mice (Fig. 3C), and BI79D10 displayed best efficiency causing

Table 3. Plasma stability, microsomal stability, and cell permeability of selected 5,5' substituted Apogossypol derivatives

Compound	R	Plasma stability (T = 1 h)	Microsomal stability (T = 1 h)	Cell permeability
Apogossypol		53%	75%	Low
BI79D7		90%	68%	Medium
BI79D8		79%	27%	Low
BI79D9		62%	52%	Low
BI79D10		85%	64%	Medium
BI79F4		ND	41%	Low
BI79F7		72%	92%	Medium
BI79D2		90%	30%	Medium

Abbreviation: ND, not determined.

40% reduction in spleen weight and showed mild gastrointestinal toxicity (Supplementary Data 5).³ Because the maximum spleen shrinkage would be no more than 50% in this experimental model, the *in vivo* effect of BI79D10 induced near maximal (80%) biological activity at 60 $\mu\text{mol/kg}$ (36 mg/kg). To confirm the result from a single mouse experiment, we next evaluated the *in vivo* activity of BI79D10 in groups of six mice each. In agreement with the single mouse experiment, BI79D10 treatment of these mice resulted in a significant (~40%) reduction of spleen weight ($P < 0.0001$) compared with the control group of six mice (Fig. 3D). All mice tolerated the treatment well with no macroscopic toxicity; the maximal weight loss was 4% during the course of study of BI79D10.

Conclusions

In summary, a library of 5,5' substituted Apogossypol derivatives was synthesized and evaluated in a variety of *in vitro* and *in vivo* assays. The most potent compound,

BI79D10, was found to bind to Bcl-X_L, Bcl-2, and Mcl-1 with IC₅₀ values of 190, 360, and 520 nmol/L, respectively, in FPA and also potently inhibited growth in culture of the PC3ML, H460, and H1299 cancer cell lines, which express Bcl-X_L, Bcl-2, and Mcl-1, respectively, with EC₅₀ values in the submicromolar to nanomolar range. BI79D10 effectively induced apoptosis of the RS11846 human lymphoma cell line in a dose-dependent manner and show little cytotoxicity against Bax/Bak DKO MEF cells, in which antiapoptotic Bcl-2 family proteins lack a cytoprotective phenotype, implying that BI79D10 has little off-target effects. Finally, BI79D10 showed favorable *in vitro* ADME properties and superior *in vivo* efficacy in Bcl-2 transgenic mice, in which Bcl-2 is overexpressed in B cells compared with Apogossypol. Considering the critical roles of antiapoptotic Bcl-2 family proteins in tumorigenesis, chemoresistance, and the potent inhibitory activity of BI79D10 against antiapoptotic Bcl-2 family proteins, BI79D10 represents a lead compound for the development of novel apoptosis-based cancer therapies.

Disclosure of Potential Conflicts of Interest

The authors are affiliated with Coronado Biosciences and have received grant support from CSRA. No other potential conflicts of interest were disclosed.

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Correction

Apogossypol derivatives as Bcl-2 antagonists

In the article on Apogossypol derivatives as Bcl-2 antagonists in the April 2009 issue (1), Hong-Gang Wang's name should have appeared in the list of authors between Tyler A. Reed and Nicholas Lawrence. Dr. Wang's affiliation is Moffitt Cancer Center, Tampa, Florida.

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Apogossypol derivatives as antagonists of antiapoptotic Bcl-2 family proteins

Jun Wei, Shinichi Kitada, Michele F. Rega, et al.

Mol Cancer Ther 2009;8:904-913.

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