Discovery, Characterization, and Structure−Activity Relationships Studies of Proapoptotic Polyphenols Targeting B-Cell Lymphocyte/Leukemia-2 Proteins

Shinichi Kitada, Marilisa Leone, Sina Sareth, Dayong Zhai, John C. Reed,* and Maurizio Pellecchia*

The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, California 92037

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Among the most promising chemopreventive agents, certain natural polyphenols have recently received a great deal of attention because of their demonstrated inhibitory activity against tumorigenesis. In view of their anticancer properties, these compounds also hold great promise as potential chemotherapeutic agents. However, to translate these chemopreventive agents into chemotherapeutic compounds, their exact mechanisms of action must be delineated. By using a multidisciplinary approach guided by modern nuclear magnetic resonance spectroscopy techniques, fluorescence polarization displacement assays, and cell-based assays, we have begun to unravel the mechanisms of actions of certain polyphenols such as Gossypol (a compound from cotton seed extracts) and Purpurogallin (a natural compound extracted from *Quercus* sp. nutgall) and their derivatives. Our findings suggest that these natural products bind and antagonize the antiapoptotic effects of B-cell lymphocyte/leukemia-2 (Bcl-2) family proteins such as Bcl-x_L. Our in vitro and in vivo data not only open a window of opportunities for the development of novel cancer treatments with these compounds but also provide structural information that can be used for the design and development of novel and more effective analogues.

Introduction

Apoptosis is essential in multicellular organisms, where it regulates normal development and tissue homeostasis. Inhibition of apoptosis is implicated in virtually every known human malignancy.[1,2] This inhibition provides malignant cells with a selective growth advantage, allowing survival in the face of radiation or chemotherapy.[1,2] Proteins of the Bcl-2 (B-cell lymphocyte/leukemia-2) family are critical components of the intrinsic apoptotic pathway. Several homologues, as defined by sequence similarity to some or all of the four Bcl-2 homology (BH) domains in Bcl-2, are found in humans and function as either blockers or inducers of apoptosis. Antiapopototic and proapoptotic Bcl-2 family proteins dimerize, negating each other's function (reviewed in ref 3). Structural studies have elucidated a hydrophobic crevice on the surface of antiapoptotic Bcl-x_L proteins that binds the BH3 dimerization domain of proapoptotic family members. Thus, molecules that mimic proapoptotic BH3 domains represent a direct approach to overcoming the protective effects of antiapoptotic proteins such as Bcl-2 and Bcl-x_L. Antiapoptotic Bcl-2 proteins are overexpressed in many human cancers. Bcl-2 is overexpressed in 80% of B-cell lymphomas, 30−60% of prostate cancers, 90% of colorectal adenocarcinomas, and a wide variety of other cancers, and Bcl-x_L is overexpressed in breast[4] and lung cancers.[5]

These observations have instigated a number of recent studies involving a variety of approaches (including computational, combinatorial, and evolutionary strategies) aimed at the discovery of compounds targeting antiapoptotic Bcl-2 family proteins as potential therapeutic agents.[6−12] However, small-molecule inhibitors reported thus far display only moderate in vitro affinity and/or poor activity in vivo and, therefore, have provided only limited data for the validation of Bcl-2 and Bcl-x_L antagonists as anticancer agents. The discovery of more potent and cell-permeable compounds is therefore needed to validate Bcl-2-family proteins as anticancer targets and to provide further hope for the discovery of more effective anticancer therapies.

Results and Discussion

We screened a small library (n = 50) of natural products by using a combination of NMR-based binding assays and fluorescence polarization displacement assays (FPA). Two polyphenols were found that inhibited BH3 binding to Bcl-x_L: Gossypol and Purpurogallin (Figure 1). In contrast, other polyphenols such as pelagonidine, catechin, epigallocatechin, myricetin, caffeic acid, gallocatechin, quercetin, epicatechin, minocycline, and other nonnatural polyphenols did not show any inhibition in our FPA with Bcl-x_L.

Gossypol is a natural product derived from cottonseed extracts that was originally extensively investigated in China as a natural male contraceptive agent.[13] In recent studies, Gossypol exhibited inhibitory activity against a wide range of human carcinoma cell lines derived from breast (T47D), prostate (Du-145), cervix (HeLa), and pancreas (Miapaca, RwP-2), in culture and in tumor xenograft models.[14−20] Recent studies aimed at the identification of the Gossypol molecular target have suggested its ability to induce apoptosis even in Bcl-2 or Bcl-x_L overexpressed cells, though the mechanism is unknown.[21] On the basis of in vitro displacement assays with a fluorescein-labeled BH3 peptide NLWAAQRYGRELRRMSD-K(FITC)-FVD[22]

* To whom correspondence should be addressed. For J.C.R.: phone, 858-646-3140; fax, 858-646-3194; e-mail, jreed@burnham.org. For M.P.: phone, 858-646-3159; fax, 858-646-3195; e-mail, mpellecchia@burnham.org.

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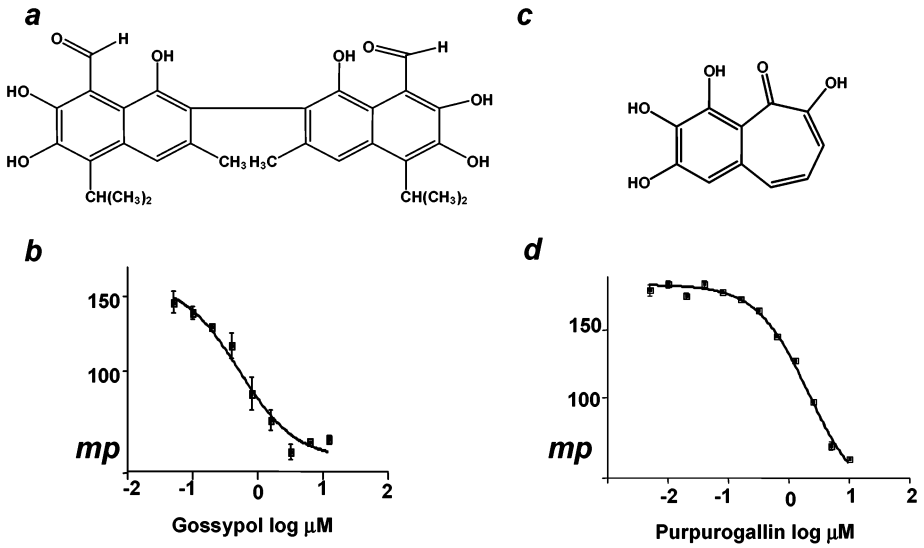


Figure 1. Gossypol and Purpurogallin compete for the BH3-binding pocket of Bcl-x_L. Shown are the chemical structures of Gossypol (a) and Purpurogallin (c). Results of fluorescence polarization based competitive binding assays (FPA) using a fluorescein-labeled Bad peptide (NLWAAQRYGRELRRMSD-K(FITC)-FVD) (Synpep Corporation, Dublin, CA) are shown in part c for Gossypol and part d for Purpurogallin. Gossypol did not show appreciable binding to BIR3 domains by NMR (see text) or by enzymatic assays.

(Synpep Corporation, Dublin, CA), we found that Gossypol directly interacts with Bcl-x_L and is able to displace BH3 peptides with $IC_{50} = 0.5\ \mu M$ (Figure 1). In contrast, Gossypol did not inhibit protein interactions or enzyme activity in other assays tested (including NMR-based binding to ^{15}N-labeled proteins such as BIR3 domain and BID, as well as FPA with BIR3 domain). As controls for our fluorescence polarization assays, we have tested several polyphenolic compounds as negative controls and we have tested a series of benzylidene-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid compounds (Chembridge Corp.) that were recently reported to bind Bcl-x_L in the micromolar range[7] as positive controls (not shown). Here, our data are in good agreement with the data reported in the literature.[7] As an additional control, we also tested a polyphenylurea (compound 1396-34), a specific small-molecule inhibitor of XIAP[23] developed in our laboratories, within its active dose range. This compound did not bind to Bcl-x_L in our FPA assays. Finally, we tested *p*-hydroxybenzaldehyde (PHBA) to verify that the activity shown is not merely due to Gossypol aldehyde groups. When tested within the same dose range used for Gossypol, PHBA did not show significant competition for BH3 peptides binding to Bcl-x_L in our FPA assays (not shown).

A second compound that inhibited Bcl-x_L is Purpurogallin, an antioxidant compound used in edible oils.[24] Purpurogallin has also been shown to inhibit tyrosine-specific protein kinase[25] and DNA synthesis in U-87 MG glioblastoma cells in vitro.[26] In our studies, Purpurogallin is a moderately potent inhibitor of Bcl-x_L, displacing the FITC-BH3 peptide with an IC_{50} of 2.2 μM, strongly suggesting that its mechanism of antitumor action may be linked at least in part to its ability to inactivate Bcl-2 proteins.

To provide further insights into the mechanism of action of these polyphenols at the structural level, we studied the binding of Gossypol and Purpurogallin to Bcl-x_L by heteronuclear NMR spectroscopy techniques.[27] Use of recombinant ^{15}N-labeled Bcl-x_L allowed us to map the chemical-shift differences upon binding into the three-dimensional structure of Bcl-x_L.[22] The binding of Gossypol and Purpurogallin to ^{15}N-labeled Bcl-x_L induces similar large variations in the [^{15}N,^{1}H] correlation spectrum of the protein. At a 1:1 molar ratio of Gossypol/Bcl-x_L, many resonances are broadened beyond detection, characteristic of binding with intermediate exchange rates on the NMR time scale, which translates to a dissociation constant in the low micromolar to submicromolar range (Figure 2a). A higher concentration of Purpurogallin is required to obtain a similar effect (3:1 ratio; not shown), indicating that at NMR concentrations used (millimolar) the binding of Purpurogallin to Bcl-x_L appears somehow weaker than Gossypol, in agreement with our fluorescence polarization displacement assays.

Mapping of the observed changes in the NMR spectra upon titration with Gossypol and Purpurogallin into the three-dimensional structure of Bcl-x_L reveals that the binding of the polyphenols mostly affects residues in the BH3-binding pocket, although the changes are rather widespread throughout a larger region surrounding this pocket (Figure 2b). As controls, Gossypol did not bind to the XIAP BIR3 domain[23] in a similar NMR binding assay with ^{15}N-labeled protein and to the proapoptotic Bcl-2 member BID (not shown). Protein-based experiments require generally high micromolar to millimolar concentrations of protein and ligand, which could result in nonspecific binding interactions. For this reason, we have also measured ligand-based NMR binding assays by observing changes in ligand nuclear spin relaxations properties in the presence of a substoichiometric amount of Bcl-x_L.[27] We measured the $T_{1\rho}$[28] and saturation transfer effects on ligands using WaterLOGSY experiments.[29] $T_{1\rho}$ experiments with 10 μM Bcl-x_L and 100 μM Gossypol showed a complete loss in signals (Figure 2c), which is characteristic of binding in the low micromolar to submicromolar range. The broadening effects on Purpurogallin are less dramatic, but binding is clearly appreciable in $T_{1\rho}$ experiments with a nearly complete loss of signal intensity at 300 ms (Figure 2d).

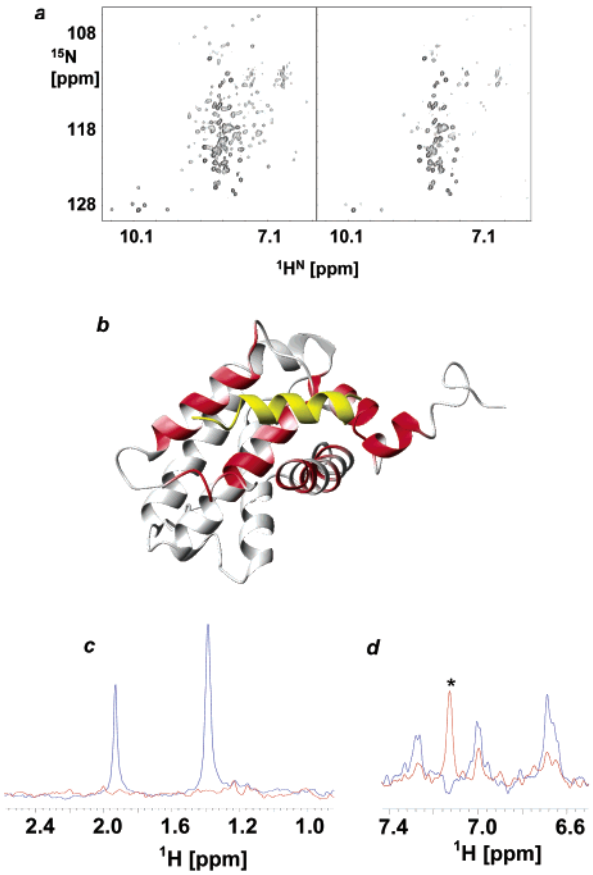


Figure 2. NMR binding studies. (a) 2D [^{15}N,^{1}H] transverse relaxation-optimized spectra for Bcl-x_L in the apo (left) and Gossypol-bound (right) forms. (*b*) Chemical-shift mapping of Gossypol into the three-dimensional structure of Bcl-x_L in complex with Bak peptide (PDB code 1BXL). The peptide is displayed in yellow. Regions affected by the binding of Gossypol are in red. (c, d) $T_{1\rho}$ experiments (300 ms relaxation time) with Gossypol (c) and Purpurogallin (d): blue, without protein; red, with 10 μM Bcl-x_L. Peaks shown in part c represent the isopropyl and the methyl groups in Gossypol. In part d, the peak marked with an asterisk represents residual imidazole present in the protein preparation.

Similar conclusions were reached from WaterLOGSY experiments (not shown).

Docking studies with FlexX software[30] implemented in Sybyl (Tripos) using the Bcl-x_L conformation found in the complex with Bak peptide showed an optimal location for Gossypol in the deep hydrophobic cleft normally occupied by the Bak helical BH3 peptide in the complex (Figure 3a). We docked both the (+)- and the (−)-stereoisomers of Gossypol because these exhibited different activity in previous cell-based assays that showed that (−)-Gossypol is 10 times more effective than (+)-Gossypol as a cytotoxic agent.[31] The goodness of the fit as measured by a scoring function,[32] and the intermolecular energy after minimization with the DOCK routine of Sybyl, was considerably better for (−)-Gossypol (−32.7 kcal/mol) versus (+)-Gossypol (−25 kcal/mol), in agreement with these observations. The structure of (−)-Gossypol is shown (Figure 3), but the overall positioning of both stereoisomers of Gossypol is very similar. In the proposed model, the naphthalene moieties work as scaffolds to position the two isopropyl groups and some of the hydroxyl groups to make favorable contacts with hydrophobic and polar side

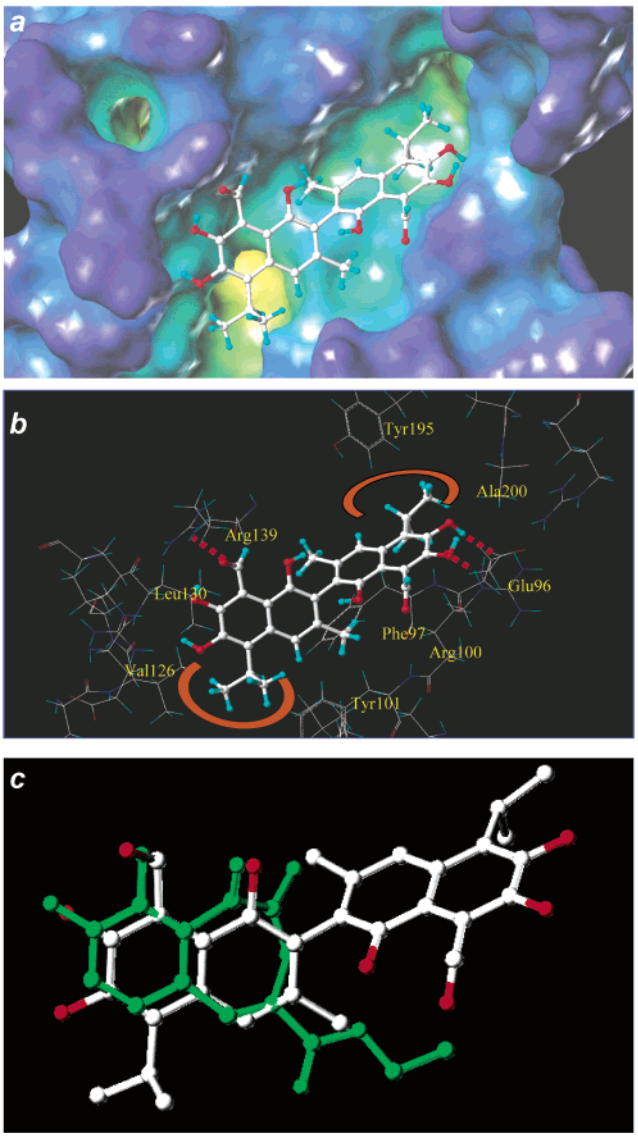
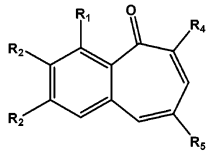


Figure 3. Molecular modeling studies. (a) Surface representation of Bcl-x_L with the docked structure of Gossypol obtained by FlexX. The surface is depicted according to cavity depth (blue, surface exposed; yellow, buried) representation. (b) Detailed interactions between (−)-Gossypol (white and red sticks) and Bcl-x_L. Only selected amino acids are shown and labeled for clarity. Hydrogen bonds are shown as dashed lines, and van der Waals interactions involving the isopropyl groups are highlighted. (c) Superposition of compound 5D1 (green) and (−)-Gossypol (white with red for oxygen atoms).

chains, respectively, as highlighted in Figure 3b. We are currently testing the validity of this model by synthesizing some analogues in which the aldehyde functional groups, which do not seem to be deeply involved in the interaction with the protein, are either replaced or derivatized. We also plan to produce single point mutants of Bcl-x_L that, on the basis of the model, are predicted to abolish binding to Gossypol.

Docking studies with Purpurogallin produced ambiguous results, given the large surface area of the BH3-binding pocket of Bcl-x_L, compared with the small size of Purpurogallin, thus leading to several possible solutions. However, placement of Purpurogallin into the binding pocket of Bcl-x_L could be attempted by using the (−)-Gossypol structure as a template structure. To this end, we also further studied the inhibitory proper-

Table 1. Structure−Activity Relationships of Purpurogallin Derivatives



compd	R_1	R_2	R_3	R_4	R_5	IC_{50} (μM) Bcl-x_L [a]	IC_{50} (μM) HeLa
Purpurogallin	−OH	−OH	−OH	−OH	−H	2.2	6.5
5D1	−H	−OH	−OH	−OH	$-COOC_2H_5$	73	51.5
1163	−H	−OH	−OH	−OH	$-COOCH_3$	2.6	∼30
1142	−H	−OH	−OH	−OH	−COOH	7.4	22.9
6A1	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	−H	>100	>100
6A7	$-OCH_3$	$-OCH_3$	−OH	$-OCH_3$	−H	>100	>100

[a] IC_{50} values against Bcl-x_L were derived from dose−response curves in a fluorescence polarization assay with a FITC-labeled BH3 peptide.

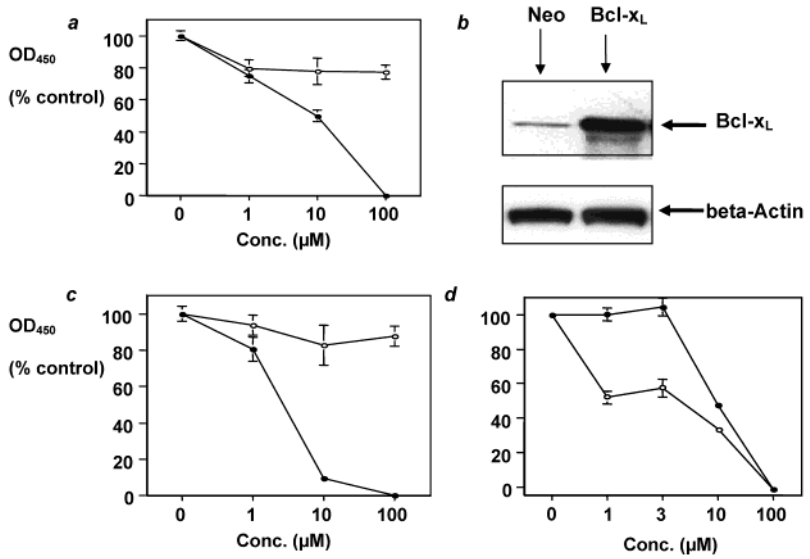


Figure 4. Inhibitory effect of compounds on cancer cell survival. The effects of Gossypol on viability of tumor cells in culture were monitored by using XTT assays with (a) MCF7 and (c) ZR75-1 cell lines (black circles). As a negative control, a generic polyphenolic compound was also tested (open circles). Low-passage HeLa cells (between passage number 10 and 20) were transfected with pcDNA3-Bcl-x_L (black circles) or control pcDNA3 plasmids (open circles). (b) Immunoblot analysis confirmed overexpression of Bcl-x_L in the cells transfected with pcDNA3-Bcl-x_L compound to pcDNA3-control transfectants. Cell lysates were normalized for total protein content, 25 μg per lane. (d) HeLa transfectants were treated with various doses of Gossypol (0, 1, 3, 10, and 100 μM). Data shown represent the mean ± standard deviation ($n = 4$).

ties of several Purpurogallin analogues (Table 1) in an attempt to identify the essential pharmacophoric substructures common to Gossypol and Purpurogallin. From these studies (Table 1), we concluded that (a) only two hydroxyl phenols groups are essential for binding, (b) substitutions in position R_5 in Purpurogallin are tolerated only by small groups, given the steric hindrance from the methyl of the methyl esters, and (c) the hydrogen bond donor properties of the phenols are important. On the basis of these data, we propose a model of the superposition of Purpurogallin and its derivatives to Gossypol that reflects the observed SAR studies (Figure 3c). For example, in this model, Purpurogallin fits rather well into the binding pocket of Bcl-x_L with a docking energy of −21 kcal/mol, whereas docking of compound 5D1 required its ethyl group to be folded into a gauche conformation to be accommodated into the Bcl-x_L binding pocket, which may reflect its weaker affinity (Table 1).

To evaluate the cytotoxic activity of our compounds on human tumors cells, we tested their biological activities using XTT dye reduction assays using two breast cancer cell lines: MCF7 (high expressor of Bcl-2/Bcl-x_L) and ZR75-1 (low expressor of Bcl-2/Bcl-x_L). Gossypol is a cytotoxic agent for MCF7 and ZR75-1 cells (Figure 4a,c), reducing cell viability in a dose-dependent manner, with IC_{50} values of 13.2 and 8.4 μM, respectively. Purpurogallin, however, did not show appreciable activity in these assays, potentially because of its hydrophilic character (ClogP ≈ 0.7). Consistent with this observation, a Purpurogallin derivative 5D1 that is predicted to have better cell-membrane permeability properties (based on its ClogP of ∼2.5) reduced cell viability in a dose-dependent manner, with an IC_{50} value of ∼50 μM in the ZR75-1 cell line (not shown). For these reasons, we further evaluated the cellular activity of our compounds in HeLa cells (Table 1), which are known to be less selective for compound uptake. The inhibition data obtained with HeLa cell viability assays parallel the in vitro binding data with Bcl-x_L (Table 1), with a correlation coefficient of $r = 0.9$ ($p = 0.001$).

Finally, to further explore whether Bcl-x_L is the

primary target of Gossypol, we used HeLa transfectants overexpressing Bcl-x_L. Low-passage HeLa cells were transfected with pcDNA3-Bcl-x_L or control pcDNA3 plasmids (Figure 4b,d) and then treated with various doses of compounds (0, 1, 3, 10, and 100 μM). The dose−response curve of the Bcl-x_L transfectants was significantly shifted to the right ($p < 0.01$, two-way ANOVA), compared with the neocontrol transfectants, indicating that a higher amount of Gossypol is required to induce apoptosis of Bcl-x_L overexpressing cells, corroborating our findings that Gossypol targets Bcl-x_L. Also consistent with these observations are the measured IC_{50} values of Gossypol for MCF-7 and ZR75-1 cells (Figure 4), with MCF-7 expressing higher levels of endogenous Bcl-x_L than ZR75-1 and consequently tolerating a higher amount of Gossypol.

Conclusions

Bcl-2 proteins are known to induce chemoresistance in cancer patients, and Bcl-2/Bcl-x_L-mediated chemoresistance has been a subject of intense investigation. Our finding that certain naturally occurring polyphenols are potent Bcl-x_L inhibitors provides for the first time a plausible molecular explanation for the anticancer activity of these natural products. Our observations also constitute a first example of a small-molecule compound (Gossypol) targeting a Bcl-2 family protein that has been tested in human clinical trials of cancer patients.[19,20] The pharmacophore model developed here, based on experimental NMR binding data and structure−activity relationships, provides an important framework on which to advance the design of even more potent and selective anticancer drugs targeting Bc1-2 family proteins.

Experimental Section

Fluorescence Polarization Assays (FPA). FPA assays were conducted with a fluorescein-labeled Bad peptide (NL-WAAQRYGRELRRMSD-K(FITC)-FVD) (Synpep Corporation, Dublin, CA) using a LJL Analyst HT (Molecular Devices Co., Sunnyvale, CA). Dilution buffer for all stocks and samples was 50 mM Tris-Bis, pH 7.4, 0.01% bovine γ-globulin. A series of 2-fold dilutions of Gossypol were prepared, i.e., 100, 50, and 0.1 μM in dilution buffer. To each tube was added a solution containing 30 nM Bcl-x_L and 4 nM fluoresceinated peptide. The tubes were incubated for 5 min at room temperature, and 20 μL of each of reaction mixture was transferred to a 96-well black PS, HE microplate (LJL Biosystems Co.). All assays were performed in quadruplicate, with blank wells receiving no Gossypol. Then the plate was read for total intensity and polarization (in mP units) was measured. Controls included dose−response measurements in the absence of the proteins to assess any interactions between the compounds and the FITC-BH3 peptide. Eventual effects were taken into account by subtraction.

NMR Spectroscopy. 2D [^{15}N,^{1}H] transverse relaxation-optimized spectra[27,32] for Bcl-x_L were obtained with 0.5 mM samples of ^{15}N-labeled Bcl-x_L. ^{15}N-labeled and unlabeled Bcl-x_L samples were prepared and purified as described in ref 22. For chemical-shift mapping and docking studies, we used the three-dimensional structure of Bcl-x_L in complex with Bak peptide (PDB code 1BXL). In addition to chemical-shift mapping with labeled proteins, $T_{1\rho}$ measurements[28] and saturation transfer experiments such as WaterLOGSY experiments[29] were also performed to further validate the binding of the studied compounds to Bcl-x_L. All experiments were performed with a 500 MHz Varian Unity+ spectrometer or a 600 MHz Bruker Avance600 spectrometer, both equipped with four rf channels and *z*-axis pulse-field gradients. Selective water saturation was performed with a train of selective IBURP2 pulses of 7 ms durations spaced by a 10 ms delay. Total saturation time used was 2.5 s. The $T_{1\rho}$ series were measured with a spin-lock pulse of variable length. Measurements were then performed with 1, 10, 50, 150, 200, 250, and 300 ms spin-lock time with 100 μM compounds in the absence and presence of 10 μM protein. In all experiments, dephasing of residual water signals was obtained with a WATERGATE sequence.

Molecular Modeling. Molecular modeling studies were conducted on several R12000 SGI Octane workstations with the software package Sybyl, version 6.9 (Tripos). The docked structure of Gossypol was initially obtained by FlexX[30] as implemented in Sybyl. Two calculations were performed. In the first, all binding-site torsion angles were kept fixed, while in the second side chain torsion angles were free to change. The average scoring function for the 30 best solutions was only slightly lower when the side chains were free to rotate. The position of the side chains in the model did not change substantially from the initial values. The scoring function for (+)-Gossypol was inferior to that of (−)-Gossypol, but the overall positioning of both steroisomers was very similar. The resulting best scoring structures were subsequently energy-minimized by using the routine DOCK of Sybyl, keeping the site rigid. The energy of the ligands after the DOCK minimization was within 5 kcal/mol from their global minimum of energy. Superposition of compounds was obtained by the routine MULTIFIT of Sybyl. Color figures showing three-dimensional structures were prepared with the programs Sybyl and MOLMOL.[33]

Inhibitory Effect of Compounds on Cancer Cell Survival. The effects of the compounds studied in this paper on viability of tumor cells in culture were monitored by using XTT[34] assays with MCF7 and ZR75-1 cell lines. MCF7 cells were grown in DMEM containing 10% fetal bovine serum, penicillin/streptomycin, supplemented with 10^{-10} M insulin, 1 mM sodium pyruvate, and glutamine. ZR75-1 cells were grown in RPMI containing 10% fetal bovine serum, penicillin/streptomycin, supplemented with HEPES buffer, 1 mM sodium pyruvate, and glutamine. Cells were regularly tested for mycoplasma contamination. Cells were seeded in triplicate at an initial cell density of 1000 cells per well. Blank wells received no cells. Gossypol, Purpurogallin, and compound 5D1 were added at final concentrations of 0, 1, 10, and 100 μM and incubated for 3 days. Relative numbers of viable cells were determined by XTT assay. Briefly, in a 96-well plate, we added 50 μL of a mixture of 1 mg/mL XTT[34] (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide) (Polysciences, Washington, PA) containing 0.025 mM PMS (phenazine methosulfate) to each well. The 96-well plates were reincubated for an additional 4 h to allow for XTT formazan production. Then the contents of each plate were mixed and optical densities were determined at a wavelength of 450 nm (OD_{450}). Net OD_{450} was determined after subtracting the OD_{450} of blank wells. Low-passage HeLa cells (between passage number 10 and 20) were transfected with pcDNA3-Bcl-x_L or control pcDNA3 plasmids using Lipofectamine Plus reagent (Invitrogen) and selected in medium containing 800 μg/mL G418. Immunoblot analysis of Bcl-x_L was accomplished as described by Krajanski in *Cancer Research*, 1996. HeLa transfectants were treated with various doses of Gossypol, Purpurogallin, and their derivatives (0, 1, 3, 10, and 100 μM).

Chemicals. Pure polyphenols were obtained from Sigma (Gossypol and Purpurogallin) and/or from Microsource Discovery Systems (Purpurogallin derivatives). Reference compounds were obtained from Chembridge Corp. (San Diego, CA). Gossypol was tested as a racemic mixture of (+)- and (−)-isomers. Compounds were dissolved in DMSO at 100 mM and stored at −20 °C. NMR analysis was periodically performed on the compounds as a quality control prior to further dilution for binding and displacement assays. Reactivity of Gossypol was tested with a ^{15}N-labeled test protein (BIR3 domain of XIAP). A solution containing 1 mM Gossypol and 200 μM ^{15}N-labeled BIR3 was incubated for 2 h, and the [^{15}N,^{1}H] correla-

tion spectrum was recorded and compared with the spectrum of the apo-Bir3. No appreciable differences in the spectra were observed.

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