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Rational Design and Real Time, In-Cell Detection of the Proapoptotic Activity of a Novel Compound Targeting Bcl-X_L

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Summary

Antiapoptotic Bcl-2-family proteins Bcl-2 and Bcl-X_L have been recently validated as drug discovery targets for cancer. Here, by using a combination of molecular modeling, NMR-based structural analysis, fluorescence polarization assays, and cell-based assays, we have designed and characterized a novel proapoptotic compound targeting these proteins. Our compound, Apogossypol, is capable of binding and inhibiting Bcl-2 and Bcl-X_L with high affinity and induces apoptosis of tumor cell lines. Mechanistic studies on the action of our compound were also performed via confocal microscopy that provided real-time detection of the interaction with Bcl-X_L in intact cells. Finally, preliminary data on cells freshly isolated from patients affected by chronic lymphocytic leukemia strongly suggest potential applications of Bcl-2 antagonists as chemosensitizers in cancer therapy.

Introduction

Programmed cell death plays an essential role in normal tissue homeostasis, ensuring a proper balance of cell production and cell loss. Defects in the regulation of programmed cell death promote tumorigenesis, and also contribute significantly to chemoresistance [1, 2]. Bcl-2-family proteins are central regulators of apoptosis [3–5]. Overexpression of antiapoptotic Bcl-2-family proteins Bcl-2 or Bcl-X_L occurs during the pathogenesis or progression of most cancers and leukemias. Among all genes studied to date in the NCI's panel of 60 human tumor cell lines, Bcl-X_L shows the strongest correlation with resistance to cytotoxic anticancer drugs [6]. Furthermore, a wide variety of gene transfer, antisense, peptide inhibitor, and other types of functional experiments have confirmed important roles for Bcl-2 and Bcl-X_L in preventing tumor cell apoptosis, validating these proteins as drug-discovery targets.

Already, antisense oligonucleotides targeting mRNAs encoding Bcl-2 have advanced into late-stage clinical trials, showing promising clinical activity [7–10]. However, Bcl-2 and Bcl-X_L represent challenging antisense targets, due to the long half-life of these proteins [11]. Also, redundancy among antiapoptotic Bcl-2-family proteins argues in favor of an approach that could nullify the actions simultaneously of both Bcl-2 and Bcl-X_L, ensuring restoration of apoptosis sensitivity in malignant cells.

We and others recently reported that Gossypol, a constituent of the cotton plant *Gossypium* [12], is a potent inhibitor of Bcl-2 and Bcl-X_L [13, 14]. The interaction of this natural product with Bcl-X_L was fully characterized by means of NMR-based methods and fluorescence polarization displacement assays [13]. Here, aided by a model of the docked structure of Gossypol into its target, Bcl-X_L [13], we were able to predict modifications of the natural product on a rational basis. We show here that such modifications lead to the analog Apogossypol, which lacks the reactive aldehydic groups and displays a proapoptotic activity comparable to Gossypol.

Results

Design, Synthesis, and Biophysical Characterization of Apogossypol

To solve the problems attributable to the presence of the two aldehydes in Gossypol, we synthesized and tested novel derivatives of Gossypol (Figure 1). We performed in silico docking studies with FlexX software using the three dimensional structure of Bcl-X_L and a small library of Gossypol derivatives in which the aldehyde functionalities are replaced. We docked eight different compounds and among these, Apogossypol, a devised analog of Gossypol missing the two aldehyde groups, gave the lowest binding energy. Although other derivatives were in the same energy range, the choice of Apogossypol was also driven by criteria of synthetic accessibility and novelty. In fact, many Gossypol analogs reported in literature are based on direct derivatization of the aldehyde functionalities to give Schiff-base compounds, which can retain certain reactivity. Apogossypol was predicted to dock with a binding energy comparable to Gossypol (-36 kcal/mol versus -30 kcal/mol of Gossypol) in the deep hydrophobic groove on the surface of Bcl-X_L that is known to be the same site targeted by endogenous antagonists of this protein (Figure 1C). The low energy conformations of Gossypol and Apogossypol are similar and the slight steric hindrance of the aldehydes in Gossypol accounts for the slightly lower binding energy of Apogossypol. In contrast, Apogossypol hexaacetate (Figure 1B) failed to bind Bcl-X_L in these modeling studies due to steric hindrance of the acetate groups and was used as a negative control in our studies.

We synthesized Apogossypol in a one-step reaction by heating Gossypol in presence of 40% NaOH (Figure 1B) [15]. To verify and characterize the binding of Apogossypol to Bcl-X_L, we employed NMR-based binding

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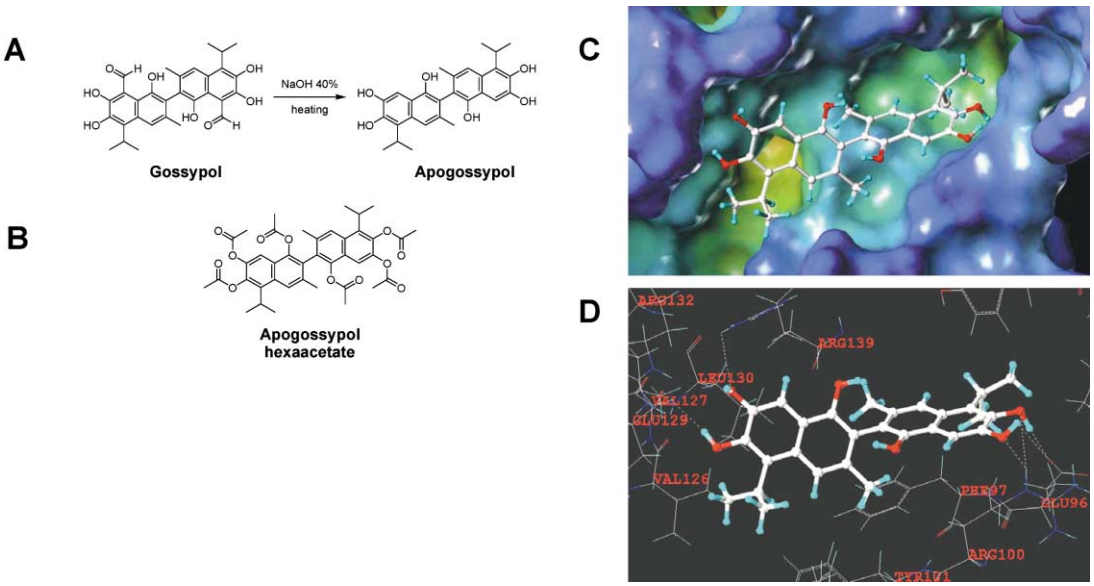

Figure 1. Molecular Modeling Studies and Analog Synthesis

(A) Chemical reaction for the conversion of Gossypol to Apogossypol.

(B) Chemical structure of Apogossypol hexaacetate.

(C) Surface representation of Bcl-x_L with the docked structure of Apogossypol obtained by FlexX.

(D) Detail of the docked structure of Apogossypol.

assays and fluorescence polarization displacement assays (FPAs). We first proved by NMR that Apogossypol binds to Bcl-X_L by measuring the changes in relaxation rates of our ligand in presence of recombinant purified Bcl-X_L protein. T1ρ experiments [16] of a sample containing 100 μM Apogossypol showed a complete loss of the protonic signals upon addition of 10 μM Bcl-X_L (Figure 2A), which is an indication of binding in the low micromolar range [17].

To identify the amino acids involved in the binding with Bcl-X_L, we produced uniformly ^{15}N-labeled Bcl-X_L protein, and performed 2D [^{15}N, ^{1}H]-TROSY NMR spectroscopy [18, 19]. This experiment permits mapping of the interactions upon addition of compound ligand via chemical shift perturbation. In particular, addition of Apogossypol to Bcl-X_L (from 1:2 to 2:1 molar ratios) resulted in chemical-shifts or disappearance of several resonances in the Bcl-X_L spectrum (Figure 2B). The residues most affected by Apogossypol binding are located in the same pocket of Bcl-X_L that is known to bind the BH3 peptides of endogenous Bcl-X_L antagonist proteins. Some changes however can also be observed for residues placed in a larger area surrounding this pocket (Figure 2D).

To evaluate the inhibitory properties of Apogossypol for Bcl-X_L, we employed a competitive fluorescence polarization assay (FPA), in which the displacement of a fluorescein-labeled BH3 peptide is monitored upon titration of compound [20]. Apogossypol was able to displace fluorescein-labeled BH3 peptide from Bcl-X_L with a Ki of 2.3 μM (96% confidence interval: 1.18 to 4.37; Figure 2C, solid squares). In our earlier studies of the parent compound, Gossypol, we found that the natural product displaced the same FITC-BH3 peptide with a Ki of $\sim$0.3 μM (96% confidence interval: 0.27 to 0.45). Thus, our modification of Gossypol reduced its affinity for Bcl-X_L, but still retained comparable affinity against the target compared to other synthetic compounds previously described [21–23].

Confocal Microscopic Studies

To determine whether Apogossypol binds Bcl-X_L in intact tumor cells, we devised a cell-based protein interaction assay using a green fluorescent protein (GFP)-tagged protein that contains a BH3 peptide (GFP-Bcl-Gs) [24]. Accordingly, low passage HeLa cells were transfected with plasmids encoding either wild-type Bcl-X_L or a mutant of Bcl-X_L designed to lack reactivity with Apogossypol, R139M-Bcl-X_L, together with GFP-Bcl-G_S at a ratio of 10:1. The resulting cells were treated 2 days later with Apogossypol, monitoring the spatial displacement of GFP-Bcl-Gs (a soluble protein) from its docking sites on mitochondrial Bcl-X_L by confocal microscopy using video time-lapse photography. The fluorescence intensity was quantified over regions of the transfected cells corresponding to mitochondria, where Bcl-X_L localizes, before and at various times after addition of Apogossypol (10 μM). As seen in Figure 3A, punctuate mitochondrial fluorescence was displaced by the compound within 2.5 min in cells expressing wild-type Bcl-X_L. In contrast, no displacement was observed with R139M-Bcl-X_L transfected cells (Figure 3B). Similarly, no GFP-Bcl-G_S displacement from wild-type-Bcl-X_L was observed with the control compound Apogossypol hexaacetate (not shown).

Cell-Based Viability Assays

To assess the effects of Gossypol and Apogossypol on apoptosis, we studied a leukemia cell line 380 that contains at (14;18) translocation activating the Bcl-2

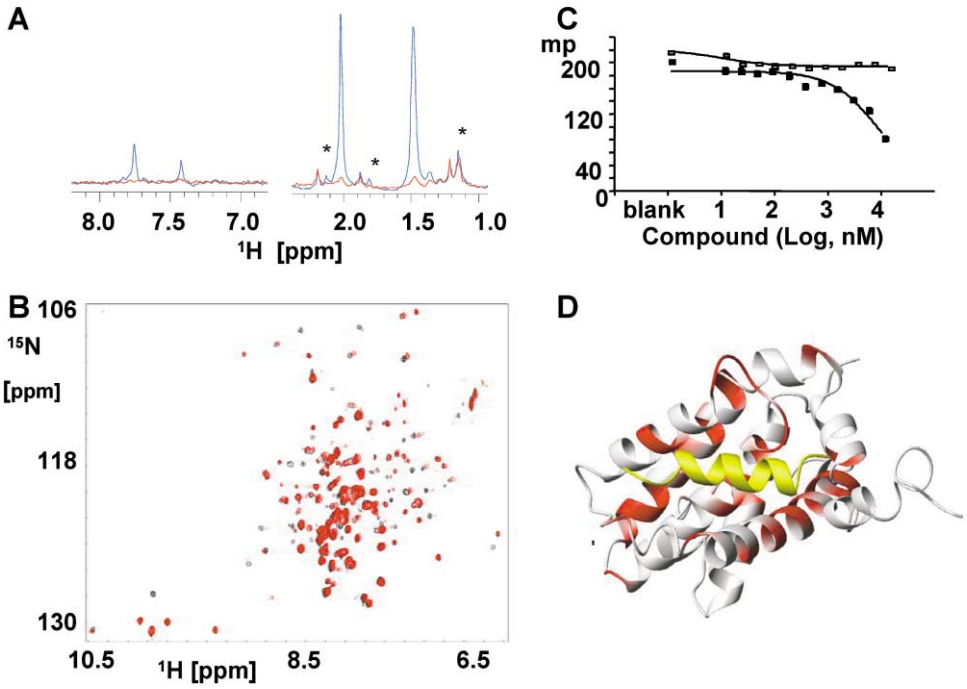

Figure 2. NMR Binding Studies and Fluorescence Polarization Assays

(A) T1ρ experiments (200 ms relaxation time) of free Apogossypol (blue) and Apogossypol in presence of Bcl-X_L (red). Asterisks indicate impurities from protein buffer.

(B) Superposition of [^{15}N, ^{1}H]-TROSY spectra of free Bcl-X_L (250 μM) (black) and Bcl-X_L after addition of Apogossypol (125 μM) (red).

(C) Fluorescence polarization-based competitive binding assays (FPA) using a fluorescein-labeled BH3 peptide from the Bad protein (NLWAAQ RYGRELRRMSD-K(FITC)–FVD) (Synpep Corporation, Dublin, CA) in complex with Bcl-X_L are shown for Apogossypol (solid squares) and Apogossypol hexaacetate (open squares).

(D) Chemical-shift mapping of Apogossypol into the three-dimensional structure of Bcl-X_L in complex with Bak peptide. The peptide is displayed in yellow. Regions affected by the binding of Apogossypol are shown in red.

gene. The effect of Apogossypol and Gossypol on leukemia cell viability was evaluated by staining with Annexin V-FITC/propidium iodide (PI), followed by flow-cytometry analysis. Both Apogossypol and Gossypol induced apoptosis of 380 cells in a dose-dependent manner (Figure 4A). In contrast, Apogossypol hexaacetate was inactive in these apoptosis assays, in agreement with our docking studies and fluorescence polarization assay results for this compound.

To further explore anticancer activities of Apogossypol, we tested its cytotoxicity against primary leukemic cells freshly isolated from 12 different patients affected by chronic lymphocytic leukemia (CLL). Among them, 9 patients were untreated, while 3 patients had been treated with conventional chemotherapeutic agents, developing refractory disease (Rai stage 0: 3 cases, Rai stage 1: 2 cases, and Rai stage 2: 7 cases).

Considerable variability in apoptotic responses to Apogossypol was observed, reflecting heterogeneity of this disease. Apogossypol induced apoptosis of 6 of the 9 treatment naïve CLL samples, with an LD_{50} of approximately 16 μM. However, when used in combination with a conventional cytotoxic anticancer drug, F-ara-A (the active metabolite of fludarabine), Apogossypol displayed synergistic effects in a subset of CLL patients, including 2 of the 3 fludarabine-refractory CLL specimens. Thus, while neither Apogossypol nor F-ara-A individually induced apoptosis of these CLL cells, apoptosis was induced in a dose-dependent manner by the combination of these agents (Figure 4B shows representative data).

Discussion

The recent discovery of small-molecule chemical compounds capable of binding Bcl-2 and Bcl-X_L at micromolar concentrations in vitro and overcoming their protective effects in the cultured tumor cells has suggested a new approach to cancer treatment [13, 21–23]. Among all small-molecule inhibitors described to date, Gossypol binds Bcl-X_L with the highest affinity, suggesting that it may provide an attractive path forward for design of chemical antagonists of Bcl-2 and Bcl-X_L.

Gossypol has been shown to suppress tumor cell growth and promote apoptosis of human tumor cell lines tested in vitro [25–29]. This natural product displays antitumor activity in tumor xenograft studies in mice, and has been tested in human clinical trials involving patients with advanced malignancies [27, 30–33]. Gossypol has also been used as a male contraceptive agent in China for centuries. However, several side-effects of Gossypol limit its usefulness [27], probably due to the presence of two reactive aldehydes in its structure. Thus, it is reasonable to speculate that these highly reactive groups, which are known to participate in Schiff's base-type reactions with primary amines of pro-

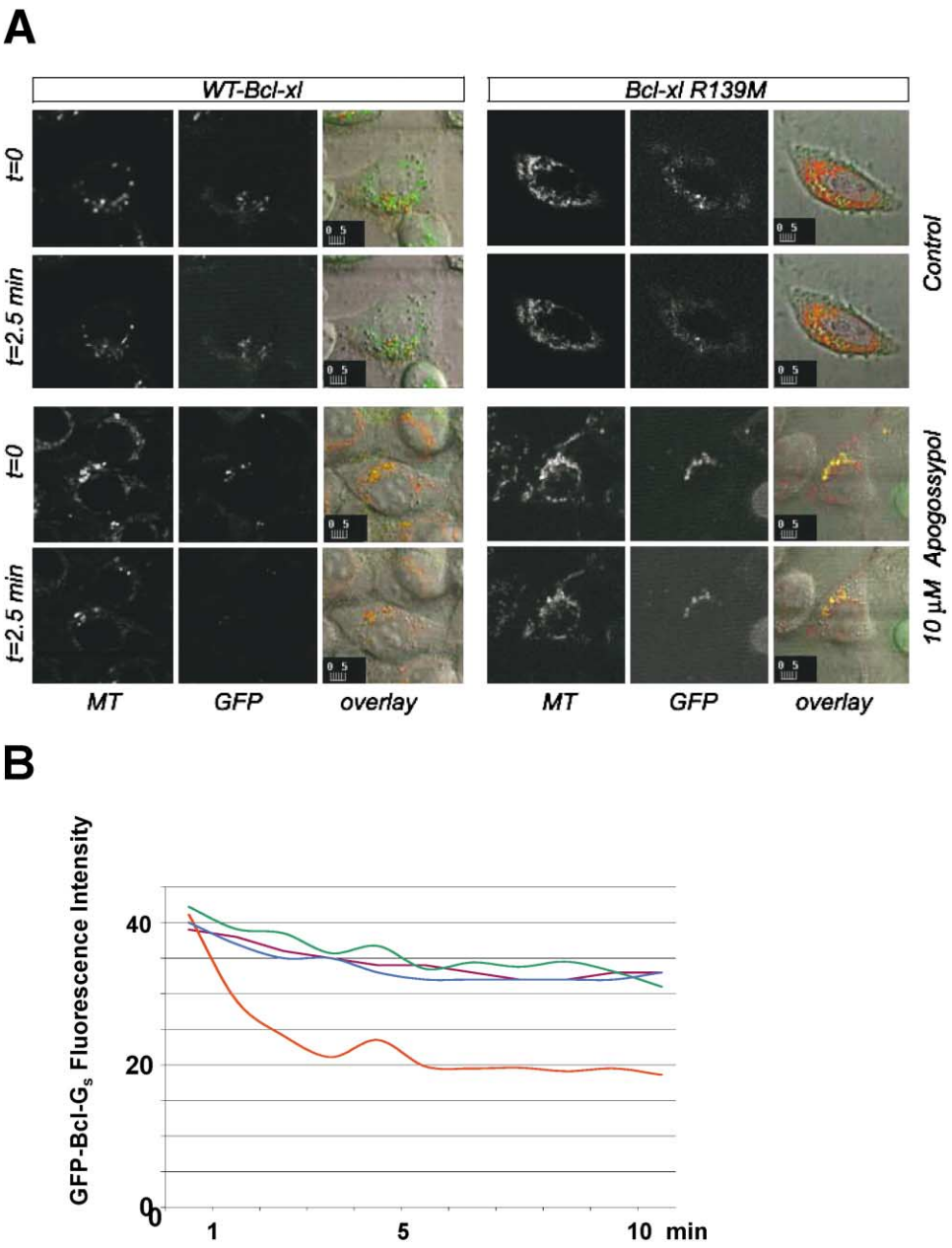

Figure 3. Confocal Microscopy Studies

(A) HeLa cells were transfected with plasmids encoding GFP-Bcl-G_S and either wild-type Bcl-X_L (left) or mutant Bcl-X_L (R139M) (right). After two days, cells were stained with Mitotracker Red to locate mitochondria. Cells were imaged before (top) and 2.5 min after (bottom) Apogossypol addition. Panels show fluorescence data collected with red-specific (left) or green-specific (middle) filters, and the overlay of those images (right). Note that Apogossypol caused displacement of GFP-Bcl-G_S from mitochondria in Bcl-X_L-expressing but not Bcl-X_L (R139M)-expressing cells.

(B) Fluorescence decay in control and treated wild-type Bcl-X_L and R139M-Bcl-X_L cells. The measurements revealed the exponential character of the fluorescence decay after Apogossypol addition in Hela cells expressing Bcl-X_L but not the R139M mutant of Bcl-X_L. (Purple, wt-Bcl-X_L control; red, wt-Bcl-X_L + 10 μM Apogossypol; green, R139M-Bcl-X_L control; and blue, R139M-Bcl-X_L + 10 μM Apogossypol).

teins and nucleic acids, interfere with the compound's stability in vivo. Thus, these aldehyde functionalities are likely to be a major cause of the adverse side-effects of Gossypol in vivo, causing it to react indiscriminately with nucleic acids and proteins in tissues.

Several attempts were made in the last decade to overcome such undesirable side effects [26, 34–36], but these structure-activity relationship (SAR) studies were severely limited by lack of knowledge of the cancer-relevant molecular target of Gossypol. In the present study, we were aided by the knowledge of the molecular target(s) of Gossypol and by the model of our docked structure to perform virtual modifications of the compound. The simplest modification that is predicted to

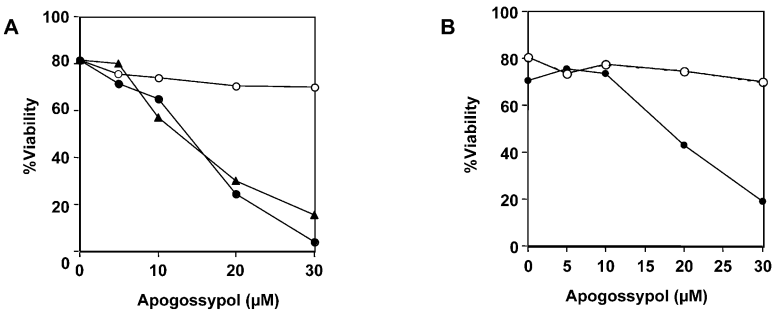

Figure 4. Cell-Based Assays

(A) The effect of Apogossypol (solid circles), Gossypol (solid triangles), and Apogossypol hexaacetate (open circles) on cell viability of the 380 cell line.

(B) Cytotoxicity of Apogossypol against cultured CLL cells when tested alone (open circles) and in combination with 10 μM F-ara-A (solid circles). For all points a mean value is shown. The standard error was ± 2%.

retain the inhibitory activity of Gossypol is the removal of its aldehyde functionalities to give Apogossypol. When synthesized and subsequently tested, Apogossypol retained inhibitory activity in vitro against Bcl-X_L, although with a somehow reduced affinity. However, our apoptosis assays with cell lines indicate that Apogossypol elicits cell death at similar concentrations compared to parent compound Gossypol. Thus, while Apogossypol is less potent than Gossypol at inhibition of the isolated Bcl-X_L protein, the nonreactive, more drug-like characteristics of Apogossypol presumably result in more effective delivery of the compound to the target molecule in intact cells. Consequently, the relative loss of inhibitory activity of Apogossypol in vitro is largely compensated by its improved chemical physical properties and selectivity for Bcl-X_L. In fact, in vitro NMR studies conducted in our laboratory show that while Gossypol is not stable in buffered solutions for more than one or two days, Apogossypol is stable at the same conditions for many days. In this context, sperm lactate dehydrogenase (LDH) is a known target of Gossypol, linked to its utility as a male contraceptive. We found that Apogossypol is much less active compared to Gossypol against sperm LDH (not shown), further supporting our hypothesis that Apogossypol is a more selective inhibitor of Bcl-X_L.

To gain further insight into the mechanism of action of our compound we introduced mutations in the Bcl-X_L protein by site-directed mutagenesis (namely R139M), which was predicted on the basis of our model to abolish the interaction with Apogossypol. For this purpose we devised a cell-based assay using a GFP-tagged Bcl-Gs protein and monitored the displacement by Apogossypol via time-lapsed confocal microscopy. The data clearly show that punctuate mitochondrial fluorescence was displaced by Apogossypol in cells expressing wild-type Bcl-X_L, whereas no displacement was observed with R139M-Bcl-X_L transfected cells, as predicted. Finally, fluorescence polarization displacement data with this mutant indicate a reduction in activity for Apogossypol, although affinity for the FITC-BH3 peptide is also decreased, thus making a quantitative comparison unattainable with this method.

The cytotoxicity data of Apogossypol against primary leukemic cells freshly isolated from patients affected by chronic lymphocytic leukemia (CLL) strongly suggest potential applications of selective Bcl-2/X_L antagonists as chemosensitizers. CLL is a quintessential example of a human malignancy caused by defective programmed cell death [3], representing the most common form of adult leukemia in North America and Europe. Overexpression of Bcl-2 protein is one of the most consistent and prominent etiological factors associated with this disease. CLL often progresses to chemorefractory disease, indicating a need for novel approaches. Our data support the idea that Apogossypol is capable of neutralizing cytoprotective effects of Bcl-2, acting as chemosensitizer. Indeed Apogossypol and F-ara-A can act in a synergistic manner, whereby Apogossypol reverses chemoresistance through its effects on Bcl-2.

Thus, taken together, our data strongly suggest that Apogossypol may be a useful therapeutic agent for the treatment of CLL and other malignancies linked to overexpression of Bcl-2 or Bcl-X_L, where chemorefractory states represent a barrier to successful eradication of cancer.

Significance

Altered expression of Bcl-2-family proteins plays a central role in apoptosis dysregulation in cancer and leukemia, promoting malignant cell expansion and contributing to chemoresistance. Previously, the natural polyphenol Gossypol was found to have cytotoxic activity against several carcinoma cell lines. We recently discovered that Bcl-2 and Bcl-X_L are targets of Gossypol, providing a potential molecular basis for the observed proapoptotic activity of this natural product and setting the stage for design of analogs with improved properties. Here, we describe the design, synthesis, and characterization of a nonreactive compound analog lacking the two highly reactive aldehydes, and thus having better drug-like properties. Our compound analog, Apogossypol, induces apoptosis of tumor cell lines at concentrations comparable to parent compound Gossypol and demonstrates the potential applications of Bcl-2 antagonists in cancer chemotherapy.

Experimental Procedures

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 Apogossypol was initially obtained by FlexX [37] as implemented in Sybyl. 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 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. Color figures showing

three-dimensional structures were prepared with the programs SYBYL and MOLCAD [38].

Chemistry

Apogossypol was obtained by heating racemic Gossypol acetic acid (SIGMA, 150 mg, 0.26 mmol) and 1.5 ml 40% NaOH at 90°C for 4 hr. The reaction mixture was poured into ice containing concentrated H_2SO_4 and the aqueous phase was extracted with Et_2O. The organic phase was dried and the solvent evaporated to give crude Apogossypol (170 mg, 81% yield) as a racemic mixture of (+) and (−) isomers. ^{1}H NMR (DMSO-*d*, 500 MHz): 7.41 (s, 2H), 7.38 (s, 2H), 3.38 (m, 2H), 1.94 (s, 6H), and 1.45 (t, J = 6.8, 12H). ^{13}C NMR (DMSO-*d*, 500 MHz): 149.7, 144.6, 145.0, 132.6, 128.5, 124.7, 119.5, 115.9, 103.3, 21.5, 20.5, and 15.6 [39]. Proton chemical shifts in D_2O buffer were slightly different.

NMR Spectroscopy

$T_1\rho$ experiments and 2D [^{15}N, ^{1}H]-TROSY spectra were performed with a 500 MHz Bruker Avance spectrometer, equipped with three rf channels and z-axis pulse-field gradients. $T_1\rho$ series were performed with a spin-lock pulse of variable length (1 ms, 100 ms, and 200 ms) with 100 μM compounds in the absence and presence of 10 μM protein. 2D [^{15}N, ^{1}H]-TROSY was measured by titrating Apogossypol to 250 μM ^{15}N-labeled Bcl-X_L dissolved in H_2O/D_2O (9:1) phosphate buffer (40 mM) (pH = 7.5). Apogossypol was soluble in aqueous buffer up to 500 μM and its solubility is comparable to that of Gossypol. ^{15}N-labeled and unlabeled Bcl-X_L were expressed and purified as previously described [40]. The three-dimensional structure of Bcl-X_L in complex with Bak peptide (PDB code 1BXL) [40] was used for chemical shift mapping and docking studies. In all experiments, dephasing of residual water signals was obtained with a WATERGATE sequence.

Fluorescence Polarization Assays

Fluorescence polarization assays (FPA) were conducted with a fluorescein-labeled Bad peptide (NLWAAQRYGRELRRMSD-K(FITC)–FVD) (Synpep Corporation, Dublin, CA) using an LJL Analyst HT (Molecular Devices Co., Sunnyvale, CA). Dilution buffer for all stocks and samples was 50 mM phosphate buffer (pH 7.4). A series of 2-fold dilutions of Apogossypol were prepared, i.e., 100 μM, 50 μM, down to 0.1 μM in dilution buffer. To each tube was added a solution containing 120 nM of Bcl-X_L and 4 nM fluoresceinated peptide. The tubes were incubated for 5 min at room temperature,and 20 μl each of reaction mixture was transferred to 96-well black PS, HE Microplate (LJL Biosystems Co.). All assays were performed in quadruplicate, with blank wells receiving no Apogossypol. Then, the plate was read for total intensity and polarization (in mP units) was measured. Controls included dose-responses measurements in absence of the proteins, to assess any interactions between the compounds and the FITC-BH3 peptide and the compound Apogossypol hexaacetate, predicted not to interact with Bcl-X_L. Approximate K_i values were obtained according to the following equation: $K_i = IC_{50}/(1 + [\text{Bcl-}X_L]/K_d)$, where K_d is the dissociation constant for the FITC-BH3 peptide derived from a titration of Bcl-X_L in the same FP assay. Two-way ANOVA analysis was performed to evaluate the differences between Gossypol and Apogossypol: F ratio for curve effect = 5.658417, $p < 0.05$.

Confocal Microscopy

Low-passage HeLa cells (between 10th and 12th passages) were transfected with wild-type Bcl-X_L or mutant Bcl-X_L R139M plasmid and GFP-Bcl-Gs at a ratio of 10: 1 (typically, 2.7 μg of Bcl-X_L plasmid and 0.3 μg GFP-Bcl-Gs) using Lipofectamine plus reagent (Invitrogen Cat. No. 11514-015). These cells were chosen because they are highly transfectable and contain only low levels of endogenous Bcl-X_L. Briefly, HeLa cells were plated in 6-well plates the day before transfection so that they were 50%–80% confluent on the day of transfection. The DNA and the Plus reagent were precomplexed: a total of 3 μg of plasmid DNA was diluted into 100 μl of Dulbecco's Modified Eagle's Medium (DMEM) without serum. Plus reagent was mixed well before use and added to diluted DNA, mixed again, and incubated at room temperature for 15 min. Lipofectamine reagent (6 μl, InVitrogen Cat. No. 18324) was diluted into 100 μl of DMEM without serum in a second tube. Precomplexed DNA and diluted Lipofectamine reagent were mixed and incubated for 15 min. The medium was replaced with 0.8 ml of OPTIMEM without serum. Then, the DNA-plus-Lipofectamine reagent complexes were added to each well of cells containing fresh medium, mixed gently, and incubated at 37°C at 5% CO_2 for 4 hr. After 4 hr, an equal volume of DMEM + 10% FCS was added to each well. On the following day, HeLa cells were transferred to glass bottom MatTek dishes (Part # P35GC-1.5-10-C, γ-irradiated, poly-d-lysine coated, MatTek Corporation, Ashland, MA).

The time-lapsed imaging was performed using laser-scanning confocal microscope MRC 1024-MP (Bio-Rad) equipped with thermostage (Warner Instruments). The images were collected with the 60×, 1.4 N.A., oil immersion objective lens. The intensity of fluorescence of mitochondrial GFP-Bcl-Gs excited with a 488 nm laser line was registered with emission filter 525/35 and measured with the MetaMorph, v.6.1.2 software (Universal Imaging Co.). All measurements were taken from sites carrying colocalized signals from the GFP-BCL-Gs and the Mitotracker red CMXRos (Molecular Probes) excited with a 568 nm laser line. Confocal images were acquired every 30 s up to 10 min. HeLa cells were chosen for this experiment because endogenous levels of Bcl-X_L are extremely low, allowing us to detect the effects of transfected Bcl-X_L wild-type versus mutant Bcl-X_L in response to Apogossypol.

Cell Culture and Apoptosis Assay

380 lymphoma cells were cultured at 1×10^6/ml in RPMI containing 10% fetal calf serum (FCS), 1 mM L-glutamine and antibiotics. At least 5×10^5 cells were recovered by centrifugation for evaluation of percentage apoptotic cells using double staining with Annexin V-FITC/PI, followed by flow-cytometry analysis using the FL-1 and FL-3 channels of a flow cytometer (Becton Dickinson; FACSort; San Jose, CA). B-CLL cells were cultured at 2×10^6/ml in IMDM containing 20% fetal calf serum (FCS), 1 mM L-glutamine, and antibiotics. At least 5×10^5 cells were recovered by centrifugation for evaluation of percentage apoptotic cells using double-staining with Annexin V-FITC/PI, followed by flow-cytometry analysis using the FL-1 and FL-3 channels of a flow cytometer (Becton Dickinson; FACSort; San Jose, CA). Experiments were performed three times and FACS data were acquired in duplicate. Experiments were reproducible with deviations of ± 2%.

Patient Specimens

Heparinized pheripheral blood was obtained from patients diagnosed with B-CLL according to standard criteria [41]. Lymphocytes were isolated by Ficoll density-gradient centrifugation and verified by immunofluorescence flow cytometry to be composed of >95% CD5/CD19/CD23 triple-positive B cells. Patient's informed consent was obtained prior to initiation of this study. The study protocol was approved by IRB at UCSD.

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