(-)-Gossypol acts directly on the mitochondria to overcome Bcl-2- and Bcl-XL-mediated apoptosis resistance

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Abstract
Ablerrant overexpression of antiapoptotic members of the Bcl-2 protein family, including Bcl-2 and Bcl-XL, contributes to malignant transformation and subsequent resistance to traditional chemotherapeutics. Thus, these proteins represent attractive targets for novel anticancer agents. The small molecule, gossypol, was initially investigated as a contraceptive agent, but subsequently has been shown to possess anticancer properties in vitro and in vivo. Recently gossypol has been found to bind to Bcl-XL and, with less affinity, to Bcl-2. Here we investigate the ability of the (-) enantiomer of gossypol, (-)-gossypol, to overcome the apoptosis resistance conferred by Bcl-2 or Bcl-XL overexpression in Jurkat T leukemia cells. (-)-Gossypol potently induced cell death in Jurkat cells overexpressing Bcl-2 (IC50, 18.1 ± 2.6 μmol/L) or Bcl-XL (IC50, 22.9 ± 3.7 μmol/L). Vector-transfected control cells were also potently killed by (-)-gossypol (IC50, 7.0 ± 2.7 μmol/L). By contrast, the chemotherapeutics drug etoposide only induced efficient killing of vector-transfected cells (IC50, 9.6 ± 2.3 μmol/L). Additionally, (-)-gossypol was more efficient than etoposide at inducing caspase-3 activation and phosphatidylserine externalization in the setting of Bcl-2 or Bcl-XL overexpression. (-)-Gossypol-induced apoptosis was associated with Bak activation and release of cytochrome c from mitochondria, suggesting a mitochondrial-mediated apoptotic mechanism. Moreover, (-)-gossypol treatment of isolated mitochondria purified from Bcl-2-overexpressing cells also resulted in cytochrome c release, indicating a possible direct action on Bcl-2 present in the mitochondrial outer membrane. Taken together, these results suggest that (-)-gossypol is a potent and novel therapeutic able to overcome apoptosis resistance by specifically targeting the activity of antiapoptotic Bcl-2 family members. (-)-Gossypol may be a promising new agent to treat malignancies that are resistant to conventional therapies. [Mol Cancer Ther 2005;4(1):23–31]

Introduction
Apoptosis is a genetically defined mechanism of cell death common to multicellular organisms (1). This tightly regulated process disposes of detrimental or damaged cells through the activation of signaling pathways, which converge at effector proteases, particularly caspase-3 (2). Dysregulation of apoptotic mechanisms results in a myriad of pathologic conditions including autoimmune disorders, neurologic disorders, and malignancies (3–8). Defective apoptosis is common to many types of malignancies, both solid and hematologic, and is often due to overexpression of antiapoptotic members of the Bcl-2 protein family, most notably Bcl-2 and Bcl-XL. Overexpression of either of these related proteins serves to suppress the mitochondrial-mediated pathway of apoptosis, and contributes to the development of chemo- and radio-resistance (6, 7, 9). Therefore, the antiapoptotic activities of Bcl-2 and Bcl-Xl represent attractive targets for novel anticancer agents designed to overcome chemotherapy or radiotherapy resistance in cancer cells (7, 10, 11).

Functional inhibitors of Bcl-2 and Bcl-Xl include novel peptides and small molecules. Short (16–25 amino acids) BH3 domain peptides derived from proapoptotic members of the Bcl-2 protein family have been shown to bind Bcl-2 and Bcl-Xl (12–14) and induce apoptosis in various systems (14–17). In addition, a growing number of small-molecule inhibitors of Bcl-2 and Bcl-Xl have been identified using diverse screening approaches, including cell-based assays, high-throughput fluorescence polarization assays, and computer-based modeling studies (10, 18–23). Tetrocarcin A (Bcl-2) and Antimycin A3 (Bcl-Xl) were identified as Bcl-2 and Bcl-Xl inhibitors using cell-based assays (18, 19). A combination of computer modeling and fluorescence polarization assays have been used to identify additional molecules that bind to the BH3 binding pocket of Bcl-2 and Bcl-Xl and induce cell death in malignant cells (10, 20–22, 24). Most notable among these molecules are compound 6 (24), HA14-1 (22), and gossypol (21).

Gossypol is a naturally occurring polyphenolic compound extracted from cotton plants and was initially
investigated as an antifertility agent (Fig. 1; refs. 25–27).

More recently, gossypol has been shown to possess antineoplastic activity against a variety of malignant cell types in vitro and in vivo (28–38). The ability of gossypol to bind to Bcl-2 and Bcl-XL was discovered using computer-assisted molecular modeling and fluorescence-polarization assays (21). Nuclear magnetic resonance imaging studies have shown gossypol binding to the BH3 binding pocket of Bcl-XL (21). Interestingly, the (−)-enantiomer of gossypol possesses higher affinity for Bcl-2 and Bcl-XL (21), reduced serum binding, and greater anticancer activity in vitro assays than the (+) enantiomer or racemic gossypol (37, 39–43). The precise molecular mechanism of gossypol action, however, is poorly defined, and it has remained unknown whether gossypol directly targets Bcl-2 and Bcl-XL in intact cells.

Here we report the ability of (−)-gossypol to overcome Bcl-2- and Bcl-XL-mediated apoptosis resistance in a Jurkat T cell leukemic model. In sharp contrast to the chemotherapeutic etoposide (VP-16), (−)-gossypol significantly inhibited cell growth and efficiently induced mitochondrial-mediated apoptosis in Jurkat cells overexpressing Bcl-2 and Bcl-XL. The effects of (−)-gossypol seem to be due to direct targeting of Bcl-2 family members in the outer mitochondrial membrane.

Materials and Methods

Cells and Reagents

Vector-transfected Jurkat T leukemic cells or Jurkat cells engineered to overexpress Bcl-2 were previously generated in our laboratory (44). Bcl-XL-overexpressing Jurkat T cells were a kind gift of Dr. Craig Thompson (University of Pennsylvania, PA). All transfected Jurkat cell lines were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 1% penicillin/streptomycin, 0.2% fungizone, and 0.5 mg/mL G418. Cells were grown at 37°C in an humidified 5% CO2 atmosphere. Media and supplements were obtained from Life Technology, Inc. (Life Technologies, Gaithersburg, MD). VP-16 (Sigma, St. Louis, MO) was dissolved in DMSO as a 20 mmol/L stock solution and stored at −20°C. Similarly, gossypol was dissolved in DMSO and stored at −20°C.

Figure 1. The chemical structure of (−)-gossypol.
gossypol was dissolved in DMSO as a 20 mmol/L stock and stored at -80°C. Monoclonal anti-cytochrome c was purchased from BD PharMingen (San Diego, CA), and anti-cytochrome c oxidase IV antibody was purchased from Molecular Probes (Eugene, OR). Monoclonal anti-caspase-3 antibody was purchased from Cell Signaling Technology (Beverly, MA). NH₂-terminal–specific anti-Bak monoclonal antibody was purchased from Oncogene Research Products (San Diego, CA). FITC-labeled anti-mouse secondary antibody was purchased from Sigma.

Growth Inhibition Assays

The cytotoxic effects of VP-16 and (-)-gossypol were assessed by MTS cell survival assays according to instructions provided by the manufacturer (Promega, Madison, WI). Briefly, cells were plated in 96-well plates at a density of 50,000 cells per well, in the absence or presence of varying concentrations of VP-16 or (-)-gossypol. As controls, additional cell cultures were treated with an equivalent concentration of the drug diluent DMSO (0.1%). Following a 12-hour treatment period, 50 μL of solution containing 20 μL of the CellTiter 96 Aqueous One Solution reagent (Promega) and 30 μL of RPMI 1640 were added to each well and allowed to incubate for 3 hours in 5% CO₂ at 37°C. The absorbance of each well was then determined at 490 nm using a Dynex Technologies MRX Revelation microplate reader (Chantilly, VA). MTS reagent-only readings were subtracted from all data and the data was analyzed using Dynex Revelation software. Absorbance readings for VP-16- and (-)-gossypol-treated wells were then plotted as a percentage of DMSO-treated controls. IC₅₀ values were extrapolated from trend line data.

Analysis of Annexin V/Propidium Iodide Staining and Bak Activation

For determination of apoptosis, log-phase cell cultures were exposed for 12 hours to 20 μmol/L VP-16, 20 μmol/L (-)-gossypol, or 0.1% DMSO. Following treatment, cells were washed with PBS and stained with FITC-conjugated Annexin V antibody and propidium iodide according to instructions provided by the manufacturer (Roche Applied Science, Indianapolis, IN). Cells (50,000 per sample) were then analyzed using an Epics Coulter XL equipped with a 488-nm argon laser and Expo 32 software. Annexin V⁻/PI⁻ cells were scored as early apoptotic.

Bak activation was determined as previously described (45). Briefly, log-phase cultures of transfected Jurkat cells were treated for 4 hours with DMSO, VP-16, or (-)-gossypol. Cells were harvested by centrifugation, washed with PBS, and fixed in a 0.25% formaldehyde/PBS solution for 5 minutes. Samples were then washed with PBS thrice and resuspended in PBS containing 100 μg/mL of digitonin and 2 μg/mL anti-Bak monoclonal antibody (Oncogene Research Products). Following a 30-minute incubation at 4°C, cells were washed twice in PBS, then incubated for 30 minutes at 4°C in PBS containing FITC-conjugated, anti-mouse secondary antibody (1:2,000, Sigma). Samples were washed again and resuspended in 400 μL of PBS, after which, 10,000 cells per sample were analyzed using an Epics Coulter XL equipped with a 488-nm argon laser and Expo 32 software.

Detection of Cytochrome c Release in Whole Cells

The release of cytochrome c from mitochondria in intact cells was assessed as previously described (46).

Figure 3. (-)-Gossypol induces apoptotic cell death in Bcl-2- and Bcl-X₁-overexpressing cells. Vector/Jurkat, Bcl-2/Jurkat, and Bcl-X₁/Jurkat cells were plated at a density of 1 x 10⁶ cells/mL, then treated with 0.1% DMSO, 20 μmol/L VP-16, or 20 μmol/L (-)-gossypol. All cells were treated for 12 h. Flow cytometric analysis of Annexin V and PI staining was then done, as described in Materials and Methods, to identify the early apoptotic Annexin V⁻/PI⁻ population. The experiment was done twice, with similar results each time.
Vector/Jurkat, Bcl-2/Jurkat, and Bcl-X<sub>L</sub>/Jurkat cells were exposed to 50 μmol/L VP-16 or 50 μmol/L (−)-gossypol for 4 hours. The treated cells were then washed with PBS and resuspended in resuspension buffer A [20 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>, 1 mmol/L EDTA, 250 mmol/L sucrose, 1.5 mmol/L phenylmethylsulfonyl fluoride, 3 μg/mL leupeptin, and 20 μg/mL aprotinin] containing 700 μL of digitonin. Following a 2-minute incubation on ice, cells were centrifuged at 15,000 rpm for 30 minutes at 4°C. Protein concentrations of both the membrane (mitochondria) and cytosolic fractions were determined using the Bradford Assay (Bio-Rad, Hercules, CA). Proteins from pellet fractions (10 μg per lane) and cytosolic fractions (30 μg per lane) were then electrophoresed on 13% SDS/PAGE gels, and subjected to immunoblotting with anti-cytochrome c antibody (BD PharMingen).

Detection of Cytochrome c Release from Isolated Mitochondria
Bcl-2/Jurkat cells (3 × 10<sup>6</sup> cells) were harvested by centrifugation, washed once with PBS, and resuspended in resuspension buffer B [30 mmol/L sucrose, 75 mmol/L KCl, 3 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 0.5 mmol/L MgCl<sub>2</sub>, 10 mmol/L HEPES (pH 7.4), 1.5 mmol/L phenylmethylsulfonyl fluoride, 3 μg/mL leupeptin, and 20 μg/mL aprotinin]. The resuspended cells were then homogenized by 25 strokes in a Type B tight-fit Dounce homogenizer. Resultant homogenates were centrifuged at 3,000 rpm for 5 minutes to clear nuclear fragments, following which, cytoplasmic and mitochondrial fractions were isolated by centrifugation at 15,000 rpm for 20 minutes at 4°C. The mitochondria-enriched pellet fraction was then resuspended in resuspension buffer B to achieve a protein concentration of 2 μg/mL. The resuspended mitochondria (100 μg per sample) were treated with DMSO (0.1%) or (−)-gossypol for one hour at 37°C. Following treatment, mitochondria were centrifuged for 20 minutes at 15,000 rpm at 4°C. The supernatants, containing released cytochrome c, were removed and subjected to immunoblotting with anti-cytochrome c.

Caspase-3 Cleavage Analysis
Log-phase cell cultures were treated with 20 μmol/L VP-16, 20 μmol/L (−)-gossypol, or 0.1% DMSO for 0, 4, 8, or 12 hours, then harvested by centrifugation. The cell pellets were washed with PBS, and resuspended in cell lysis buffer [50 mmol/L Tris (pH 8.0), 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP40, 1.5 mmol/L phenylmethylsulfonyl fluoride, 3 μg/mL leupeptin, and 20 μg/mL aprotinin]. Protein concentrations were determined using the Bradford assay. Following a 2-minute incubation on ice, cells were centrifuged at 15,000 rpm for 5 minutes at 4°C. Protein concentrations of both the membrane (mitochondria) and cytosolic fractions were determined using the Bradford Assay (Bio-Rad, Hercules, CA). Proteins from pellet fractions (10 μg per lane) and cytosolic fractions (30 μg per lane) were then electrophoresed on 13% SDS/PAGE gels, and subjected to immunoblotting with anti-cytochrome c antibody (BD PharMingen).
assay (Bio-Rad) and proteins (30 µg per lane) were electrophoresed on 13% SDS/PAGE gels, transferred to nitrocellulose membranes, and probed with anti-caspase-3 monoclonal antibody (Cell Signaling Technology).

**Immunoblotting Analysis of Bcl-2, Bcl-XL, and Bax Expression**

Whole cell lysates from Vector/Jurkat, Bcl-2/Jurkat and Bcl-XL/Jurkat cell cultures treated for 12 hours with DMSO, VP-16 or (−)-gossypol were prepared in lysis buffer containing 50 mmol/L Tris (pH 8.0), 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP40, 1.5 mmol/L phenylmethylsulfonyl fluoride, 3 µg/mL leupeptin, and 20 µg/mL aprotinin. Equal quantities of protein (25 µg/sample, determined by Bradford assay) were electrophoresed on 13% SDS/PAGE gels, transferred to nitrocellulose membranes, and analyzed for Bcl-2, Bcl-XL and Bax expression. Hamster anti-mouse Bcl-2 monoclonal antibody was obtained from BD Biosciences (San Diego, CA). Anti-human Bcl-X L monoclonal antibody and anti-mouse Bax polyclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Results**

(−)-Gossypol Promotes Cell Death in Bcl-2- and Bcl-XL-Overexpressing Cells

Overexpression of Bcl-2 or Bcl-XL is known to inhibit chemotherapy-induced apoptosis (6, 7, 9). To determine the impact of (−)-gossypol on cell lines overexpressing these antiapoptotic proteins, we employed Jurkat T leukemia cells engineered for overexpression of Bcl-2 (Bcl-2/Jurkat) or Bcl-XL (Bcl-XL/Jurkat), as well as vector-transfected control cells (Vector/Jurkat). Cells were treated for 12 hours with varying concentrations of (−)-gossypol or the chemotherapy drug VP-16, then assayed for viability in MTS assays. As shown in Fig. 2, a dramatic loss in viability was seen in Vector/Jurkat cells in response to either (−)-gossypol (IC50, 7.0 ± 2.7 µmol/L) or VP-16 (IC50, 9.6 ± 2.3 µmol/L). On the other hand, Bcl-2/Jurkat and Bcl-XL/Jurkat cells were resistant to VP-16. VP-16 failed to cause a 50% loss in viability in these cell lines even when used at concentrations as high as 1 mmol/L (data not shown). By contrast, (−)-gossypol was highly effective at promoting loss of viability in Bcl-2/Jurkat and Bcl-XL/Jurkat cells, with IC50 values of 18.1 ± 2.6 and 22.9 ± 3.7 µmol/L, respectively. Immunoblotting revealed that (−)-gossypol-induced cell death was not associated with a loss of exogenous Bcl-2 or Bcl-XL in the transfected cell lines (Fig. 2C). These findings show that (−)-gossypol is capable of causing death in cells rendered chemoresistant by virtue of Bcl-2 or Bcl-XL overexpression.

(−)-Gossypol Induces Apoptotic Cell Death in Bcl-2/Jurkat and Bcl-XL/Jurkat Cells

To determine whether (−)-gossypol was promoting apoptotic cell death in the Bcl-2- and Bcl-XL-overexpressing cell lines, we done flow cytometric analysis of...
Annexin V and propidium iodide (PI) staining. As shown in Fig. 3, treatment of Vector/Jurkat cells for 12 hours with 20 μmol/L VP-16 caused a substantial increase in the percentage of Annexin V+/PI- early apoptotic cells, as compared with cells treated with 0.1% DMSO alone (58.3% and 6.1%, respectively). Significantly fewer Annexin V+/PI- cells were seen following a 12-hour VP-16 treatment of Bcl-2/Jurkat and Bcl-XL/Jurkat cultures, underscoring the chemoresistance of these cell lines. (−)-Gossypol treatment (20 μmol/L for 12 hours) caused 54.2% of Vector/Jurkat cells to become Annexin V+/PI-, compared with 6.1% in DMSO-treated cells. Importantly, high percentages of apoptotic cells were also seen in (−)-gossypol-treated Bcl-2/Jurkat and Bcl-XL/Jurkat cells (40.9% and 72.9%, respectively) when compared with DMSO- or VP-16-treated cells.

(−)-Gossypol Promotes Caspase-3 Activation and Cytochrome c Release in Bcl-2- and Bcl-XL-Overexpressing Cells

To verify that (−)-gossypol could promote apoptosis signaling in Bcl-2- and Bcl-XL-overexpressing cells, we investigated activation of the major cellular executioner caspase, caspase-3. Caspase-3 is synthesized as a 32-kDazymogen form, which becomes processed during apoptosis to yield active subunits of 17 and 10 kDa. Immunoblotting revealed that, contrary to the action of VP-16, (−)-gossypol efficiently induced caspase-3 processing in both Bcl-2/Jurkat and Bcl-XL/Jurkat cells (Fig. 4).

The activation of caspase-3 is known to occur downstream from mitochondrial cytochrome c release during drug-induced apoptosis (47, 48). Moreover, Bcl-2 and Bcl-XL prevent caspase activation and subsequent apoptotic cell death by preventing the release of cytochrome c into the cytosol (49, 50). Agents that act to inhibit Bcl-2 or Bcl-XL might, therefore, be expected to promote cytochrome c release. In Fig. 5, cells were treated with VP-16 or (−)-gossypol, followed by subcellular fractionation and immunoblotting for cytochrome c. Consistent with previous results, VP-16 was able to provoke cytochrome c release in Vector/Jurkat cells, but not in Bcl-2/Jurkat or Bcl-XL/Jurkat cells. By contrast, (−)-gossypol promoted potent cytochrome c release into the cytosol in all three cell lines. The appearance of cytochrome c in cytosolic fractions was concurrent with a loss of cytochrome c from the mitochondria-enriched pellet fraction. Additionally, no significant changes were detected in the cytosolic or mitochondrial levels of cytochrome c oxidase IV, indicating a specific release of cytochrome c protein. Thus, taken together with results from Figs. 2 to 4, these data show that (−)-gossypol can effectively overcome the inhibitory effects of Bcl-2 or Bcl-XL overexpression to promote apoptosis signaling and cell death in the Jurkat model system.

(−)-Gossypol Induces Bak Activation

To further investigate (−)-gossypol-induced apoptotic signaling, we examined activation of the proapoptotic protein Bak. Bak resides in the mitochondrial outer membrane and normally exists in an inactive conformation in stabilizing complex with the VDAC2 protein (51). In response to apoptotic stimuli, Bak undergoes conformational changes which reveal an “active conformer-specific” NH2-terminal epitope (45, 52). Active conformer Bak oligomerizes in the mitochondrial outer membrane, directly resulting in the impairment of mitochondrial integrity. Mitochondrial Bcl-2 and Bcl-XL are able to bind and sequester Bak that is in the active conformation, inhibiting Bak oligomerization and preventing mitochondrial dysfunction (45, 53). The conformational change in the Bak protein can be detected using an antibody that specifically recognizes the NH2-terminal active conformer epitope (Oncogene Research Products). In Fig. 6, we used flow cytometry to quantify the percent of cells exhibiting Bak activation following 4 hour treatment with DMSO, VP-16, or (−)-gossypol. As depicted in Fig. 6A, (−)-gossypol treatment increased Bak activation in all three cell lines. The greatest degree of (−)-gossypol-induced Bak activation was seen in Vector/Jurkat cells (69.3%), followed by Bcl-2/Jurkat (30.0%) and Bcl-XL/Jurkat (42.6%), compared with a maximum of 5.3% activation in response to DMSO. The expression of total Bak protein, however, was not affected by drug treatment in any of the three cell lines (Fig. 6C).

(−)-Gossypol Promotes Cytochrome c Release in Isolated Mitochondria from Bcl-2-Overexpressing Cells

The ability of (−)-gossypol to bind Bcl-2 and Bcl-XL in vitro, and to induce cytochrome c release and Bak activation in intact Jurkat cells suggests that the major site of (−)-gossypol action may be at the level of the mitochondria. Demonstration that (−)-gossypol acts directly on the mitochondria would support the hypothesis that a Bcl-2 family member(s) is a direct intracellular target of (−)-gossypol. To investigate this possibility, we examined the impact of (−)-gossypol on purified mitochondria isolated from Bcl-2-overexpressing cells. Mitochondria were isolated as described in Materials and Methods and treated for 1 hour with (−)-gossypol. Supernatants were then subjected to immunoblotting to detect released cytochrome c. As shown in Fig. 7, a dose-dependent release of cytochrome c was observed upon treatment with 10 to 50 μmol/L of (−)-gossypol. Treatment with 0.1% DMSO or VP-16 (data not shown) did not lead to a substantial release of cytochrome c. Amido black staining showed equivalent loading in all lanes (data not shown). The observed release of cytochrome c in response to (−)-gossypol at concentrations paralleling those required to induce cell death indicates that in intact cells (−)-gossypol acts directly on the mitochondria, the known site of several Bcl-2 family members, including Bcl-XL, Bcl-2, and Bak.

Discussion

Overexpression of antiapoptotic members of the Bcl-2 protein family is commonly observed in variety of human malignancies. Among hematologic cancers, overexpression of Bcl-2 or Bcl-XL has been shown to directly correlate with resistance to chemotherapy drugs in...
Gossypol was first investigated as an antifertility agent but has subsequently been reported to possess antitumor activities in vitro and in vivo (28–38). More recently, gossypol was identified as a potential Bcl-2/Bcl-X\(_L\) binding molecule using computer-assisted modeling techniques (21, 24). Fluorescence polarization binding studies and nuclear magnetic resonance structural analyses have revealed that racemic gossypol binds to the BH3 binding pocket of Bcl-X\(_L\) with a \(K_i\) of \(\sim 0.3\) \(\mu\)mol/L (21, 66), and to the BH3 binding pocket of Bcl-2 with a \(K_i\) of \(\sim 10\) \(\mu\)mol/L (66). Additional modeling and binding studies have shown higher binding affinity for the \((-)\) enantiomer of gossypol relative to the \((+)\) enantiomer (21). Furthermore, the \((-)\) enantiomer of gossypol is a more potent inhibitor of cell growth when compared with either \((+)\) gossypol or racemic gossypol (37, 39, 41, 42).

In view of the recently described Bcl-2/Bcl-X\(_L\) binding activity of gossypol, we investigated the impact of the \((-)\) enantiomer of this compound on chemoresistant leukemia cell lines that overexpress Bcl-2 or Bcl-X\(_L\). We found that \((-)\)-gossypol was effective at inhibiting the growth of chemoresistant Jurkat cell lines overexpressing Bcl-2 or Bcl-X\(_L\). Similar results were recently described by Kitada et al. (21) after treatment of Bcl-X\(_L\)-overexpressing HeLa cells with racemic gossypol. We also observed that \((-)\)-gossypol, in contrast to VP-16, induced the translocation of cytochrome \(c\) from the mitochondria into the cytosol in Bcl-2- or Bcl-X\(_L\)-overexpressing cells. Furthermore, we showed that \((-)\)-gossypol was able to induce robust release of cytochrome \(c\) from isolated mitochondria derived from Bcl-2-overexpressing cells. Taken together, these observations indicate a direct action of \((-)\)-gossypol on mitochondria, and suggest direct targeting of Bcl-2 family members in the outer mitochondrial membrane in intact cells.

Two potential models of \((-)\)-gossypol action are consistent with our results and current understanding of the regulation of cell death by the Bcl-2 protein family. In the first model, \((-)\)-gossypol may act by directly binding to Bcl-2 or Bcl-X\(_L\) and inhibiting their antiapoptotic activity by displacing bound proapoptotic factors such as Bak, Bax, or “BH3-domain only” proteins. Structural data and molecular modeling studies support the direct interaction of \((-)\)-gossypol with Bcl-2 and Bcl-X\(_L\) and have identified the BH3 binding pocket of Bcl-X\(_L\) as the specific site of interaction with gossypol (21).\(^6\) In the second possible model, \((-)\)-gossypol may interact directly with proapoptotic Bcl-2 family members such as Bak to promote apoptotic activity. Bak resides in the mitochondrial outer membrane and has been shown to contain a homologous BH3 binding pocket critical for interactions with the Bak-activating, “BH3-domain only” protein Bid (67). Once activated, Bak oligomerizes in the mitochondrial outer membrane and induces defects in membrane integrity and the release of apoptogenic factors, including cytochrome \(c\) (68). Whereas neither model can be excluded by our data, it is also possible that \((-)\)-gossypol may bind to the BH3 binding pocket of various Bcl-2 family members to directly activate multidomain proapoptotic members (Bak or Bax), simultaneous with direct inhibition of antiapoptotic members (Bcl-2 or Bcl-X\(_L\)).

Traditional anticancer therapeutics act by diverse mechanisms to induce cancer cell apoptosis primarily through mitochondrial-mediated mechanisms (69). Overexpression of Bcl-2 and Bcl-X\(_L\) has been found to stabilize the outer mitochondrial membrane and prevent the release of cytochrome \(c\) following a variety of insults, including treatment with chemotherapeutic agents (70–72). Employing a model system designed to mimic a clinically common scenario, namely malignant cell overexpression of Bcl-2 or Bcl-X\(_L\), we found \((-)\)-gossypol was able to efficiently overcome Bcl-2 and Bcl-X\(_L\) activity and induce cell death in leukemic cells refractory to traditional chemotherapy. These observations point to \((-)\)-gossypol as an attractive candidate molecule for clinical investigation of apoptosis resistant malignancies.

References


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