Inhibition of Anti-Apoptotic Bcl-2 Family Proteins by Natural Polyphenols: New Avenues for Cancer Chemoprevention and Chemotherapy

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Abstract: Amongst the most promising chemopreventive agents, certain natural polyphenols have recently received a great deal of attention from the scientific community, nutritionists, the pharmaceutical industry and the public, due to 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 mechanism of action must be delineated. The aim of this manuscript is to review recent findings suggesting that certain natural products bind and antagonize the anti-apoptotic effects of Bcl-2 family proteins such as Bcl-x_L and Bcl-2. We will summarize recent studies that were aimed at the identification of the molecular targets of natural polyphenols and at the characterization of their mechanism of action on a molecular level. We will emphasize the importance of these findings that resides not only in the opportunity for the development of novel cancer treatments with these compounds, but also in the structural information that can be used for the design and development of novel and more effective semi-synthetic analogues. The finding reviewed here should encourage the study of possible direct effects of other dietary compounds on Bcl-2 family proteins.

Key Words: Apoptosis; Bcl-2; Bcl-x_L; Polyphenols; Gossypol; Apogossypol; Purpurogallin; Catechins; Theaflavins; Chemoprevention; Combination therapy.

ANTI-APOPTOTIC BCL-2 FAMILY PROTEINS AS TARGETS FOR CANCER CHEMOPREVENTION AND CHEMOTHERAPY

Apoptosis is essential in multicellular organisms, where it regulates normal development and tissue homeostasis. Altered inhibition of apoptosis is implicated in virtually every known human malignancy [1, 2]. In cancer, 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. Anti-apopototic and pro-apoptotic Bcl-2 family proteins dimerize, negating each other's functions (reviewed in [3]). Structural studies have elucidated a hydrophobic crevice on the surface of anti-apoptotic Bcl-2 and Bcl-x_L proteins that binds the BH3 dimerization domain of pro-apoptotic family members [3].

Thus, molecules that mimic pro-apoptotic BH3 domains represent a direct approach to overcoming the protective effects of anti-apoptotic proteins such as Bcl-2 and Bcl-x_L. Anti-apoptotic Bcl-2 proteins are over-expressed in many human cancers. Bcl-2 is over-expressed in 80% of B-cell lymphomas, 30-60% of prostate cancers, 90% of colorectal adenocarcinomas and a wide variety of other cancers. Bcl-x_L is overexpressed in breast [4] and lung cancers [5], and is the single-best correlate with chemoresistance in the NCI's pending 60 human tumor cell lines [6]. 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 anti-apoptotic Bcl-2-family proteins as potential therapeutic agents [7-13]. These have been the subject of recent reviews and will not be discussed here [14, 15]. As general comment, however, small-molecule inhibitors reported thus far display either only moderate *in vitro* affinity and/or poor activity *in vivo* and, therefore, have provided only limited data so far for the validation of Bcl-2 and Bcl-x_L antagonists as anti-cancer agents.

In this review, we will report on recent findings from our laboratories on the inhibition of Bcl-x_L and Bcl-2 by natural dietary compounds [16-18]. As it will be shown, these natural products may represent interesting lead compounds for cancer chemoprevention and chemotherapy.

Anti-Apoptotic Bcl-2 Family Protein Antagonists: A Lesson from Mother Nature

During a screen of natural compounds against Bcl-x_L and Bcl-2 by using a combination of NMR-based binding assays and fluorescence polarization displacement assays (FPA), four classes of polyphenols were found that occupy the aforementioned hydrophobic crevice and inhibit BH3 binding to Bcl-x_L. These include compounds such as Gossypol, Purpurogallin and certain polyphenols from black and green tea extracts. Despite their diverse origins from natural sources, these compounds share some similarities in their chemical structures. These similarities permit generation of hypotheses about their binding mode, which are consistent

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with initial structure-activity data, and which thus should encourage the study of other natural and synthetic compounds that posses similar pharmacophoric substructures as starting points for generating novel potential lead compounds.

Gossypol and Purpurogallin

Gossypol (Fig. **1a**) is a natural product derived from cottonseed extracts that was originally extensively investigated in China as a natural male contraceptive agent [19]. 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 [20-26]. Recent studies aimed at the identification of the molecular targets of Gossypol have suggested its ability to induce apoptosis even in Bcl-2 or Bcl-x_L over-expressed cells, though the mechanism is unknown [27]. Based on *in vitro* displacement assays with a fluorescein-labeled BH3 peptide NLWAAQRYGRELRRMSD-K(FITC)-FVD [28], we was found that Gossypol directly interacts with Bcl-x_L and is able to displace BH3 peptides with a IC_{50} = 0.5 μM (Fig. **1b**). Similar results were also reported by Zhang *et al.* [29].

A second compound that inhibited Bcl-x_L is Purpurogallin (Fig. **1e**), an antioxidant compound used in edible oils. Purpurogallin has also been shown to inhibit tyrosine-specific protein kinase [30] and DNA synthesis in U-87 MG glioblastoma cells *in vitro* [31]. In our studies [16], Purpurogallin is a moderately potent inhibitor of Bcl-x_L, displacing the FITC-BH3 peptide with an IC_{50} of 2.2 μM (Fig. **1f**), again suggesting that its mechanism of antitumor action may be linked at least in part to its ability to inactivate Bcl-2 proteins.

Displacement studies were also supported by *in vitro* NMR-based binding assays using ^{15}N-labeled Bcl-x_L and chemical shift mapping techniques based on the known three-dimensional structure of Bcl-x_L [28], as well as by performing measurements of ^1H transverse relaxation rates ($T_{1\rho}$) of the compounds in presence and absence of sub-stoichiometric amounts of Bcl-x_L [16].

Chemical shift mapping upon titration with Gossypol into the three-dimensional structure of Bcl-x_L revealed that the binding of the polyphenol mostly affected residues in the BH3-binding pocket, although the changes were rather widespread throughout a larger region surrounding this pocket (Fig. **1d**). $T_{1\rho}$ experiments with 10 μM Bcl-x_L and 100 μM Gossypol also confirm its binding to Bcl-x_L, with a complete loss in signals observed in presence of the protein (Fig. **1c**), which is characteristic of binding in the low- to sub-micromolar 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 (Fig. **1g**).

Catechins

The binding of different green tea polyphenols (Table **1**) to Bcl-x_L, was recently investigated by our laboratories [17] by means of nuclear magnetic resonance spectroscopy (NMR) techniques and fluorescence polarization assays (FPA), similarly to the work on Gossypol and Purpurogallin. We found that the compounds epigallocatechin gallate (EGCG), gallochatechin gallate (GCG), epicatechin gallate (ECG) and chatechin gallate (CG) bind Bcl-x_L very tightly, whereas other green tea polyphenols, lacking the gallate group, such as gallocatechin (GC), epigallochatechin (EGC), catechin (C), epicatechin (EC) did not interact with the protein (see Table **1** for chemical structures and stereochemistry). The binding was first shown by NMR ^1H $T_{1\rho}$ measurements. Fig. (**2a**) shows such data for CG. This compound binds to Bcl-x_L , as clearly indicated by a remarkable decrease of its ^1H NMR signals intensity in the $T_{1\rho}$ experiment acquired in presence of Bcl-x_L, when compared with the same spectrum measured in absence of protein. Such results are indicative of binding in micro- to sub-micromolar range. Similar behaviour was observed for the green tea polyphenols reported in Table **1** that have a gallate group, whereas the parent compounds lacking the gallate group did not bind. This is also illustrated in Fig. (**2a**) comparing the binding of CG and C, for Bcl-x_L by NMR spectroscopy. Inhibition constants of each compound were determined with the fluorescence polarization assay (Fig. **2b**). In agreement with the NMR binding data, several green tea extracts were able to inhibit Bcl-x_L (Table **1**) with the compounds ECG and CG representing the strongest inhibitors with Ki values of 120 and 143 nM, respectively. On the contrary, green tea polyphenols whose chemical structures were lacking the gallate group showed no inhibition at 100 μM, again in agreement with the NMR-based binding data. Similar findings were obtained in FPAs using antiapoptotic Bcl-2-family protein, Bcl-2. Finally, chemical shift mapping with EGCG and ^{15}N-labeled Bcl-x_L, corroborated the hypothesis of binding of the compound in the BH3-binding pocket [17].

Theaflavins

The binding of the black tea polyphenols theaflavin, theaflavin digallate, theaflavin-3' gallate and theaflavanin to Bcl-x_L, was similarly investigated. Based on the fluorescence polarization assays (FPA) (Fig. **3a**), it was found that compounds theaflavin, theaflavanin and theaflavin-3' gallate were strong Bcl-x_L inhibitors, whereas theaflavin digallate failed to bind. Inhibition constant determination (Table **2**) indicated that theaflavanin was the strongest binder of the series (Ki = 250 nM), followed by teaflavin-3' gallate (Ki = 270 nM) and finally by theaflavin (Ki = 480 nM). In contrast, no inhibition at 100 μM concentration was observed for theaflavin digallate. The active black tea compounds also suppressed BH3 peptide binding to Bcl-2 in FPAs (Table **2**).

NMR binding experiments were also performed with these polyphenols. In bi-dimensional heteronuclear NMR titration experiments with ^{15}N labeled Bcl-x_L, theaflavin, theaflavanin and theaflavin-3' gallate caused a wide broadening of the resonances. The observed broadening of the protein [^{15}N,^1H] resonances is characteristic of binding with intermediate exchange rates in the NMR time-scale, representative of low- to sub-micromolar binders (Fig. **3b**). Mapping of the observed changes in the NMR heteronuclear correlation spectra upon titration with these compounds into the three-dimensional structure of Bcl-x_L showed that many residues within and surrounding the BH3-binding pocket

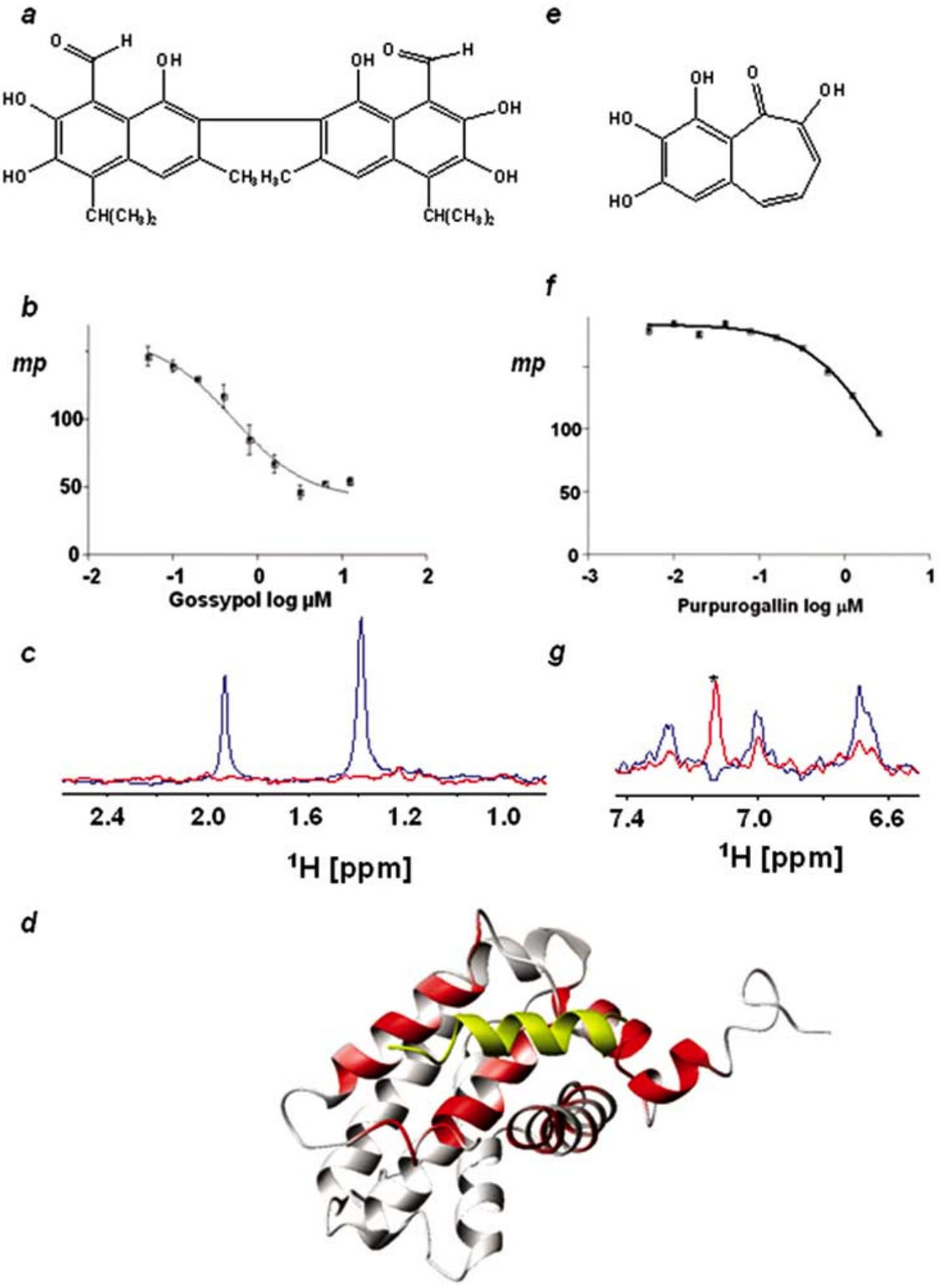
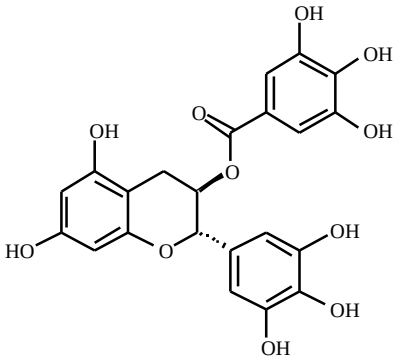
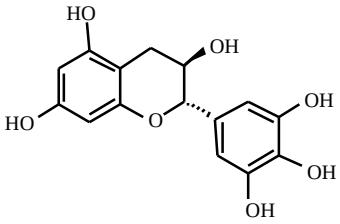
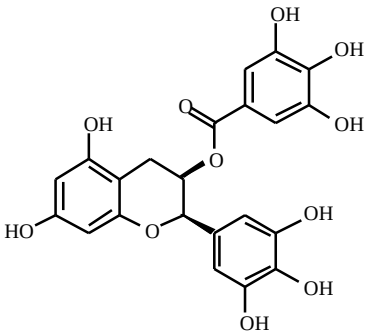
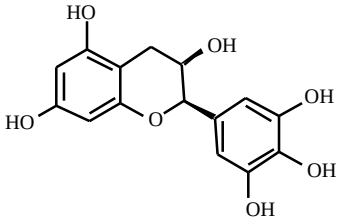
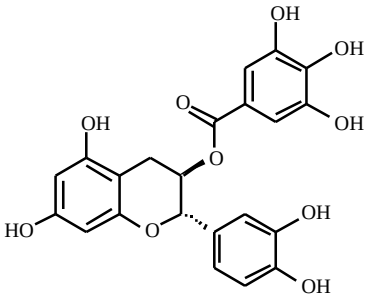
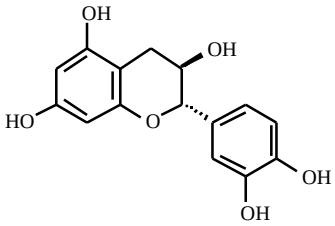
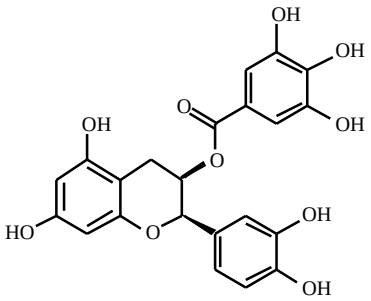
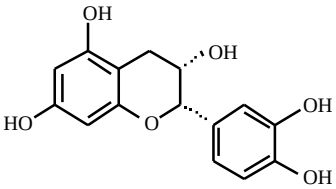


Fig. (1). *Gossypol and Purpurogallin*. a) Chemical structure of Gossypol; b) Fluorescence polarization assay (FPA) in which Gossypol is displacing the binding of a fluorescently labeled BH3 peptide to Bcl-x_L; c) $T_{1\rho}$ measurements with Gossypol (100 μM) in presence (red) and absence (blue) of 10 μM Bcl-x_L; d) Mapping chemical shift changes (in red) on the surface of Bcl-x_L upon binding of Gossypol. The BH3 peptide is displayed in yellow; e) Chemical structure of Purpurogallin; f) Fluorescence polarization assay (FPA) in which Purpurogallin is displacing the binding of a fluorescently labeled BH3 peptide to Bcl-x_L; g) $T_{1\rho}$ measurements with Purpurogallin (100 μM) in presence (red) and absence (blue) of 10 μM Bcl-x_L. Adapted from Ref. [16].

Table 1. Structure-Activity Relationships (SAR) for Green Tea Extracts

GREEN TEA	STRUCTURE	Bcl-x_L	Bcl-2
COMPOUND		Ki (nM)	Ki (nM)
(-)Gallocatechin-3 gallate (GCG)		315	235
(-)Gallocatechin (GC)		$>10^5$	$>10^5$
(-)Epigallocatechin-3 gallate (EGCG)		490	335
(-)Epigallocatechin (EGC)		$>10^5$	$>10^5$
(-)Catechin-3 gallate (CG)		143	230

(Table 1) contd….

GREEN TEA	STRUCTURE	Bcl-x_L	Bcl-2
COMPOUND		Ki (nM)	Ki (nM)
(-)Catechin (C)		$>10^5$	$>10^5$
(-)Epicatechin-3 gallate (ECG)		120	400
(+)Epicatechin (EC)		$>10^5$	$>10^5$

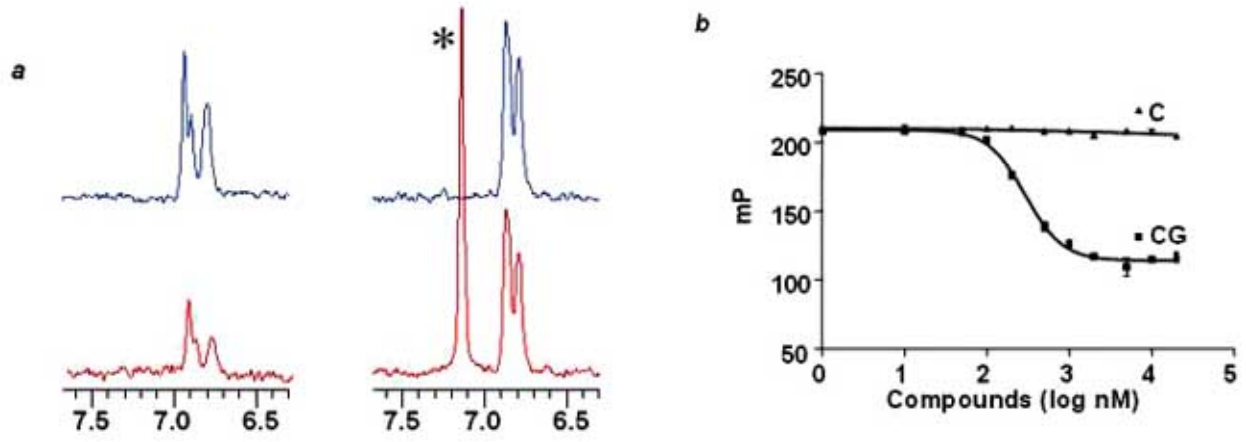
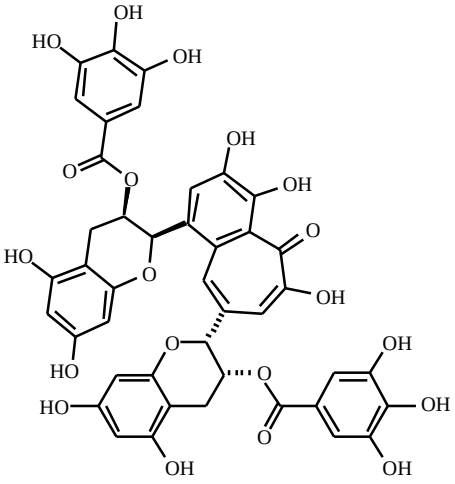
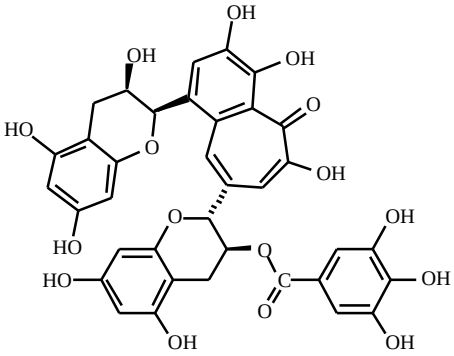
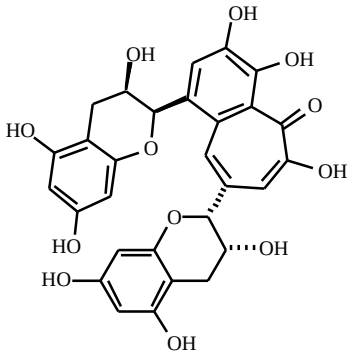
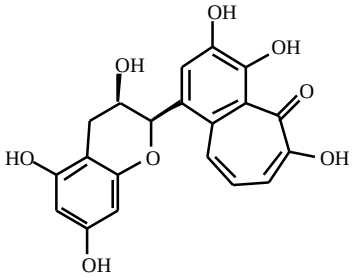


Fig. (2). *Catechin and Catechingallate*. a) $T_{1\rho}$ measurements with catechin at 100 μM (left) and catechingallate at 100 μM (right) in presence (red) and absence (blue) of 10 μM Bcl-x_L; b) Fluorescence polarization assay (FPA) in which catechingallate (CG) is displacing the binding of a fluorescently labeled BH3 peptide to Bcl-x_L. Catechin (C) has no effect at the same concentration range. Adapted from ref. [17].

Table 2. Structure-Activity Relationships (SAR) for Black Tea Extracts

BLACK TEA	STRUCTURE	Bcl-x_L	Bcl-2
COMPOUND		K_i (nM)	K_i (nM)
Theaflavin digallate		$>10^5$	$>10^5$
Theaflavin-3' gallate		270	1230
Theaflavin		480	690
Theaflavanin		250	286

were affected by the compound binding, although the changes extended to a larger region surrounding this pocket [17].

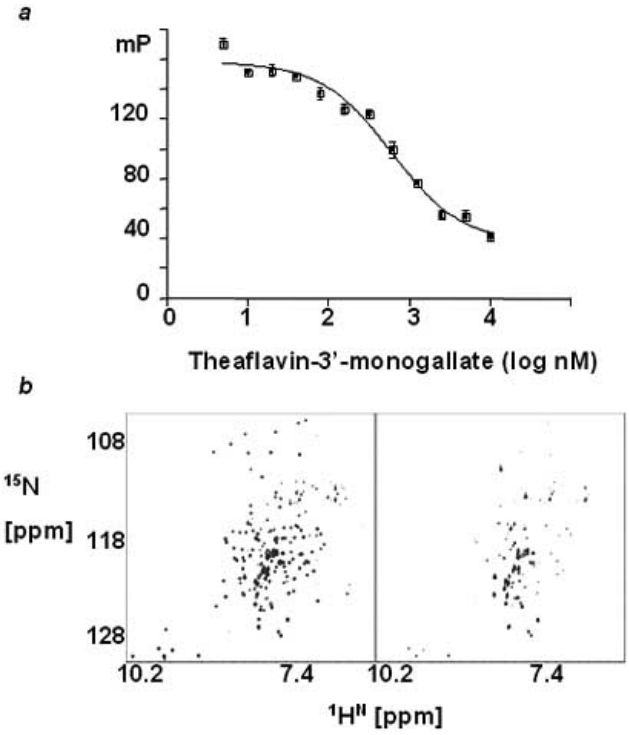


Fig. (3). *Theflavin-3' gallate*. (*a*) FPA results for theaflavin-3' gallate. (*b*) 2D [^{15}N,^{1}H]-TROSY spectra for Bcl-x_L (0.250 mM) before (left) and after addition of theaflavin-3' gallate (1 mM) (right). Adapted from ref. [17].

Molecular Docking Studies

Molecular docking studies have provided some insights into the binding mode of the polyphenols into the three-dimensional structure of Bcl-x_L, although requiring experimental confirmation by X-ray crystallography or NMR. These studies were performed using the Bcl-x_L conformation found in the complex with Bak-peptide, and the software FlexX as implemented in Sybyl. Docking studies with Gossypol showed an optimal location of the compound in the deep hydrophobic cleft normally occupied by the Bak helical BH3 peptide in the complex (Fig. **4a**). In the proposed model, the naphthalene moieties work as scaffolds to position the two isopropyl groups and some of the hydroxyl groups to make favourable contacts with hydrophobic and polar side chains, respectively, as highlighted in Fig. **4b**. The validity of this model could be tested by either synthesizing analogues that are predicted to interact more or less favourably with the protein and/or to produce single point mutants of Bcl-x_L that, based on the model, are predicted to abolish binding to Gossypol. An example of such studies will be given later in the manuscript.

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, superposition of the structures of a Purpurogallin derivative and Gossypol (Fig. **4c**) reveals similarities can be used to interpret some of the SAR studies, as it will shown later in the manuscript.

Docking studies with green tea polyphenols were quite interesting. We was found that GCG, EGCG, CG, ECG docked quite well in the BH3-binding pocket with the gallate moiety of GCG, EGCG, and CG mainly fitting to the less liphophilic sub-pocket, with the exception of ECG in which the gallate was predicted to be located in the opposite pocket. In contrast compounds without the gallate group, such as GC, EGC, EC, C, did not dock well. The docked structures of CG and C superimposed, in Fig. **4d**, showed that the latter lacks the gallate ring and thus occupies only partially the large binding groove of Bcl-x_L, indicating a rather good correlation between the docking studies with NMR binding and displacement assay data. Similarly, docking studies with EGCG (Fig. **4e**) and theaflavin-3' gallate (Fig. **4f**) showed a quite good fit into the Bcl-x_L-binding site, again suggesting that compounds capable of occupying the large site on the surface of Bcl-x_L exhibit the strongest binding and inhibition [17].

CORRELATING POLYPHENOL-BASED INHIBITION OF BCL-2/X_L WITH CYTOTOXICITY IN CELLS

Cytotoxic activity of Gossypol and Purpurogallin on human tumors cells was evaluated by 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 was found to be a cytotoxic agent for MCF7 and ZR75-1 cells, (Fig. **5a,b**), reducing cell viability in a dose-dependent manner, with IC_{50} values of 13.2 μM and 8.4 μM, respectively. Purpurogallin, however, did not show appreciable activity in these assays, potentially due to 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 IC_{50} value of ~ 50 μM in the ZR75-1 cell line. For these reasons, the cellular activity of these compounds were further evaluated in HeLa cells (Table **3**), which are known to be less selective for compound uptake. The inhibition data obtained with HeLa cells by cell viability assays parallel the *in vitro* binding data with Bcl-x_L (Table **3**), with a correlation coefficient of r = 0.9 (p = 0.001).

These data also provide a framework to identify the essential pharmacophoric substructures common to Gossypol and Purpurogallin. From the data obtained in Table **3** it was concluded that: (a) only two hydroxyl phenols groups are essential for binding; (b) substitutions in position $\mathbf{R_5}$ 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. These data seemed to be in agreement with the molecular docking studies of compound 5D1 (Fig. **4c**) that required its ethyl group be folded into a gauche conformation to be accommodated into the Bcl-x_L binding pocket, reflecting its weaker affinity (Table **3**).

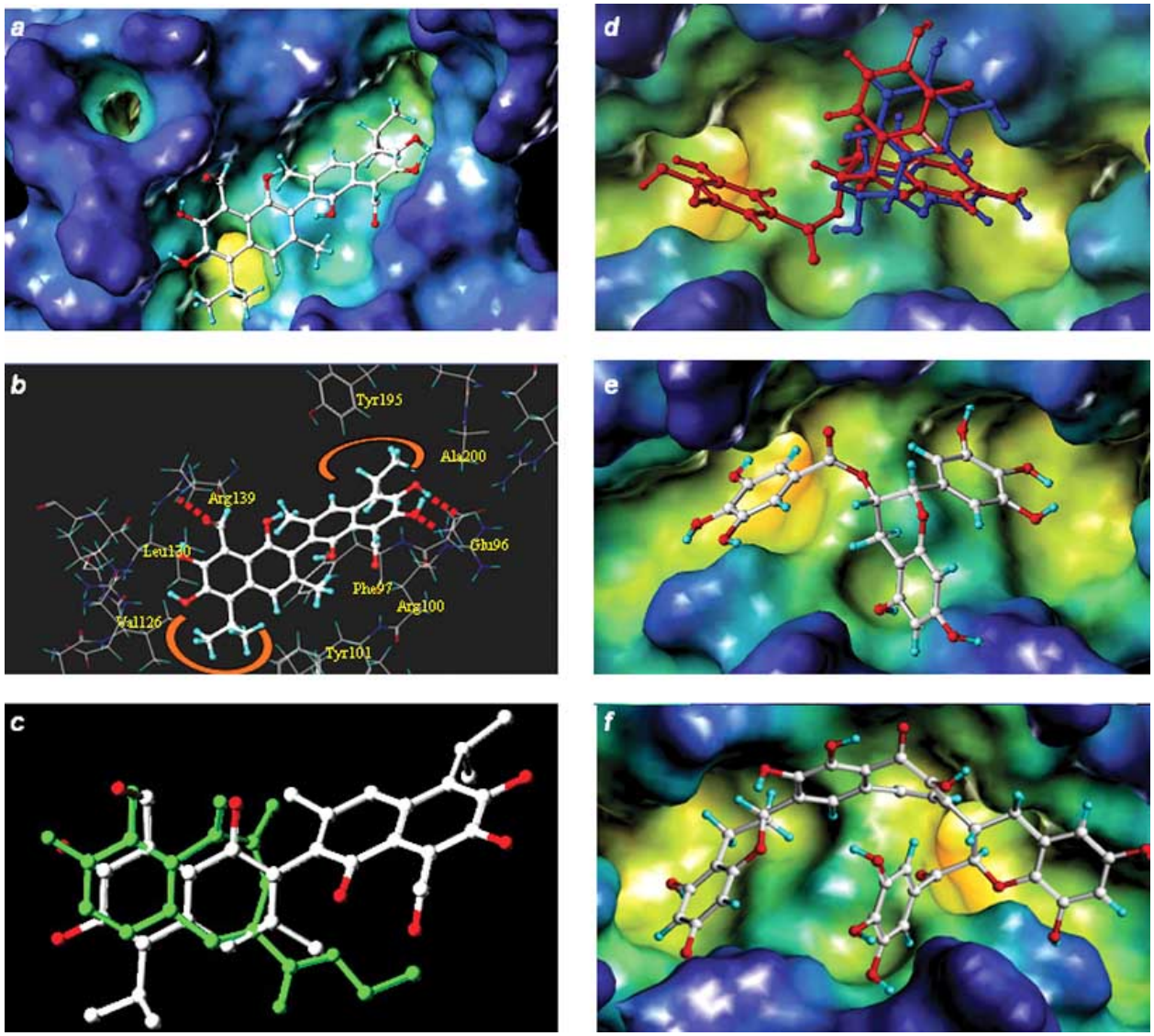


Fig. (4). *Molecular docking studies with the three-dimensional structure of Bcl-x_L.* In the surface representation of Bcl-x_L the color code is according to cavity depth (blue, shallow; yellow, deep). a) Docked structure of Gossypol; b) detail of the interactions between Gossypol and the side-chains of Bcl-xL; c) Superposition of the chemical structures of Gossypol and Purporogallin; d) Docked structures of catechin (C, blue sticks) and catechingallate (CG, red sticks); e) and f) docked structures and EGCG and theaflavin-3' gallate, respectively. Adapted from refs. [16, 17].

Is the Inhibition of Bcl-2/x_L by Catechins and Theaflavins Correlated with the Chemopreventive Activity of Tea?

So far multiplicities of mechanisms have been proposed to explain the cancer chemoprevention effect of tea consumptions. However many proposed mechanisms were criticised for a) implying inhibition of crucial physiological molecules that would result in high toxicity of tea; b) for the deficiency of universality of the target involved in the process of tumorigenesis, or c) simply because the effective concentration of catechins used in the *in vitro* assays seemed to be too high (micromolar range and higher) for the anticancer chemoprevention observed by tea *in vivo* [32-36].

It was suggested that EGCG from green tea and its related compounds could act as anticancer agents by inducing G_1 arrest of cycling cells [37, 38], inhibiting growth factor-mediated proliferation [39-41], and by inhibiting carcinogen-induced mutagenesis [42]. It was also proposed that the cancer chemoprevention activity of EGCG could be directly linked to the inhibition of telomerase [43] or to the inhibition of angiogenesis [44]. However many studies related green tea anticancer properties to inhibition of apoptosis [38-49].

For example, Pan & coworkers [50] suggested that certain black tea polyphenols can induce apoptosis through

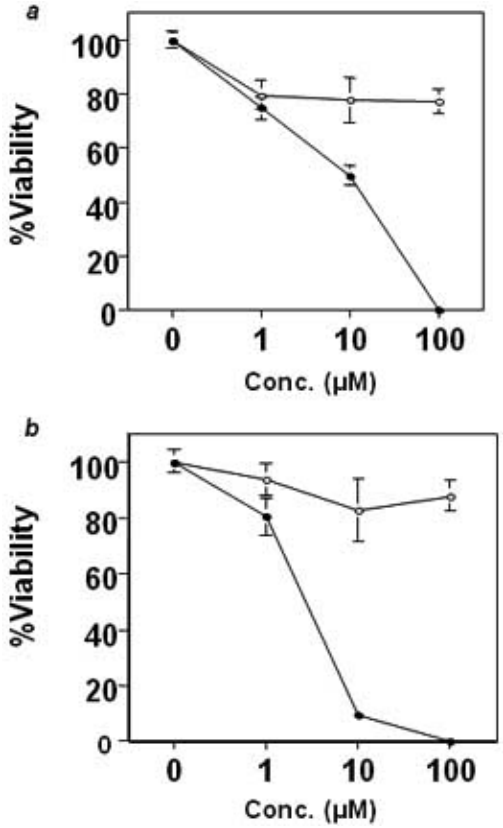


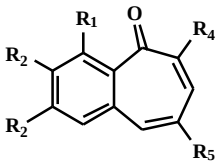
Fig. (5). *Inhibitory effect of Gossypol on cancer cell survival.* The effects of Gossypol on viability of tumor cells in culture were monitored by using XTT assays with (***a***) MCF7 and (***b***) ZR75-1 cell lines (black circles). As a negative control, a generic polyphenolic compound was also tested (open circles). Adapted from ref. [16].

activation of caspase 9 and 3 in human U937 cells and through cytochrome *c* release. Masuda & coworkers [51] studied the antitumoral effects of EGCG in the human head and neck squamous cell carcinoma lines. The authors pointed out that apoptosis induced by EGCG in these cell lines was also associated with an increase in the level of the pro-apoptotic protein Bax, a decrease in the level of the anti-apoptotic proteins Bcl-2 and Bcl-x_L and with the activation of caspase 9. These results suggested that apoptosis induced by green tea catechins in these cell lines was mediated by a mitochondrial pathway. The decrease in the level of anti-apoptotic proteins Bcl-x_L and Bcl-2 was correlated with the inhibition of the transcription factor Stat3 [52-54], which was a n inducer of the expression of the two proteins, but the mechanism by which EGCG could increase the level of Bax protein remained unclear.

In addition Lee and Lin [55] explained that EGCG and theaflavins could block protein Kinase C (PKC) activation. Very recently it has been suggested that PKC-alpha could actually play an important role in initiating caspase-dependent apoptosis by modulating Bcl-x_L expression. In fact, a decrease in PKC-alpha was correlated with a decline of Bcl-x_L levels and induction of apoptosis in hepatic epithelial cells [56].

By linking the latter observations with the findings reviewed here, it can be concluded that tea polyphenols could act as cancer antagonists, not only inducing apoptosis by decreasing the levels of Bcl-x_L (and potentially other Bcl-2 family proteins), but also through direct inhibition of this protein (as well as Bcl-2 and potentially other family members) [58]. However, considering that the plasma level of tea catechins reached after drinking a few cups of this beverage was demonstrated to be less than 300 nM [33, 57] and recognizing that this value is comparable to the effective concentrations of theaflavins and catechins showed be relevant for Bcl-x_L inhibition, it is more reasonable to assume that direct inhibition of the Bcl-2/x_L mechanism could represent the principal means by which tea polyphenols exert their anticancer activity.

Table 3. Structure Activity Relationships (SAR) of Purpurogallin Derivatives



CMPD	R1	R2	R3	R4	R5	IC_{50} (μM) (Bcl-x_L)	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

From Natural Products to Semi-Synthetic Compounds: Improving the Natural Killers via Structure Based-Design

As shown in the previous section, the pharmacophore models and docked structures developed based on experimental NMR binding data and structure activity relationships and molecular modelling provide an important framework on which to advance the design of even more potent and selective anticancer drugs based on natural products targeting Bc1-2-family proteins.

An example of this is the compound Apogossypol. Aided by a model of the docked structure of Gossypol into its target, Bcl-X_L reported in Fig. **4a,b**, we were able to predict modifications of the natural product on a rational basis and showed that such modifications lead to the analogue Apogossypol, which lacks the reactive aldehydic groups and displays a pro-apoptotic activity comparable to Gossypol [18].

Our studies were aided by *in silico* docking using the three dimensional structure of Bcl-x_L and a small library of Gossypol derivatives in which the aldehyde functionalities are replaced and among these, Apogossypol, a devised analog of Gossypol missing the two aldehyde groups, gave the lowest binding energy. We synthesized and tested Apogossypol *in vitro* by NMR and fluorescence polarization as described in the previous sections. Apogossypol was able to displace fluorescein-labeled BH3 peptide from Bcl-x_L with a Ki of 2.3 μM, thus showing a somehow reduced affinity. However, apoptosis assays with cell lines indicate that Apogossypol elicits tumor cell death at similar concentrations compared to parent compound Gossypol (Fig. **6a**). Thus, while Apogossypol is less potent than Gossypol at inhibition of the isolated Bcl-x_L protein, the non-reactive, more drug-like characteristics of Apogossypol presumably result in more effective delivery of the compound to the target molecule in intact cells. Consequently, the relatively small loss of inhibitory activity of Apogossypol *in vitro* is largely compensated by its improved chemical physical properties and selectivity for Bcl-x_L. Consistent with this hypothesis, *in vitro* NMR studies that 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. Further supporting evidences that the principal cellular target of Apogossypol is Bcl-x_L, introduction of mutations into the Bcl-x_L protein by site-directed mutagenesis (namely R139M), predicted on the basis of the molecular modeling to abolish or decrease the interaction with Apogossypol, prevented Apogossypol-mediated displacement of BH3-containing a Green Fluorescent Protein (GFP)-tagged Bcl-Gs protein from Bcl-x_L in intact cells as via time-lapsed confocal microscopy [18]. In contrast, Apogossypol successfully displaced the GFP-tagged BH3 domain protein from wild type Bcl-x_L in tumor cells, demonstrating that this semi-synthetic derivative of Gossypol antagonizes its intended target in cells. Because Bcl-x_L localizes principally to the surface of mitrochondria in tumor cells, the data reported showed that punctuate mitochondrial fluorescence due to GFP-Bcl-Gs binding to Bcl-x_L 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 [18].

Bcl-2/x_L Antagonists as Chemosensitizers in Cancer Therapy

The proof that suppression of Bcl-2 expression sensitizes cancer cells to chemotherapy has been provided by the antisense nucleotide Genasense™ that reduces the levels of Bcl-2 in cells. This approach is currently in phase III clinical trial (Genta).

To further explore chemosensitization effects induced by a small molecular compounds targeting Bcl-2/x_L proteins, we tested the cytotoxicity of Apogossypol against primary leukemic cells freshly isolated from 12 different patients affected by chronic lymphocytic leukemia (CLL) [18]. 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 (Fig. **6b**). The cytotoxicity data of Apogossypol against primary leukemic cells from patients affected by 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 [1, 2], representing the most common form of adult leukemia in North America and Europe. Over-expression of Bcl-2 protein is one of the most consistent and prominent etiological factors associated with this disease that often progresses to chemo-refractory disease, indicating a need for novel approaches. The data shown here support the idea that small-molecules inhibitors of Bcl-2 and Bcl-x_L, such as Apogossypol, are capable of neutralizing the cytoprotective effects of Bcl-2, acting as chemosensitizers. Indeed Apogossypol and F-ara-A can act in a synergistic manner, whereby Apogossypol reverses chemoresistance, presumably through its effects on Bcl-2.

Future Perspectives

Although additional experimental studies at the structural level and with respect to cell-based activity are necessary to fully elucidate the mechanism of action of certain polyphenols, the studies reviewed here provide a clear link between their anticancer properties and the direct inhibition of Bcl-2, Bcl-x_L and potentially other anti-apoptotic Bcl-2 proteins. In addition to the polyphenols reported here, other dietary factors and other naturally occurring substances may emerge as potential therapeutic or preventative agents in the battle against many types of cancer. However, much of the current support for these agents is epidemiologically based. In this respect, studies such as those reported in this review, aimed at revealing molecular targets and mechanisms of

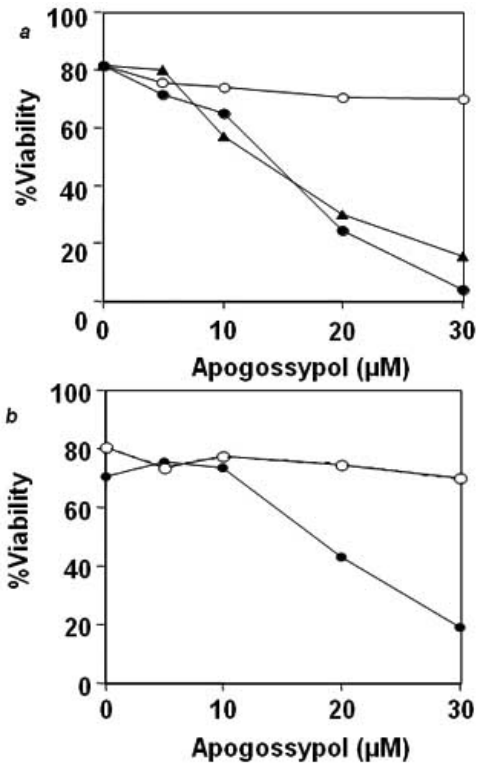


Fig. (6). *Apogossypol*. (a) The effect of Apogossypol (solid circles), Gossypol (solid triangles), and control (open circles) on cell viability of 380 cell line. (c) Cytotoxicity of Apogossypol against cultured CLL cells when tested alone (open circles) and in combination with 10 μM F-ara-A (solid circles). Adapted from ref. [18].

action for such compounds may help in the translation of their use in cancer therapy. Promising chemopreventive drugs could therefore be developed from other dietary pholyphenols, such as Resveratrol, a plant-derived polyphenolic compound that induces apoptosis as well as modulate the function of the androgen receptor in prostate cancer cell lines, or from other dietary substances such as soy isoflavones, curcumin, phenethyl isothiocyanate, sulforaphane, lycopene, perillyl alcohol, and potentially many others that are presently unknown. Given the studies reported in this review, it is likely that in some cases the mechanism of action for such compounds could include the direct inhibition of anti-apoptotic Bcl-2 proteins.

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