

Cancer Prevention by Tea Polyphenols Is Linked to Their Direct Inhibition of Antiapoptotic Bcl-2-Family Proteins

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Abstract

Epidemiological data and *in vitro* studies on cancer chemoprevention by tea polyphenols have gained attention recently from the scientific community, nutritionists, the pharmaceutical industry, and the public. Despite the several efforts made recently to elucidate the molecular basis for the anticancer activity of these natural products, little correlation has been found thus far between the putative protein targets of compounds found in tea extracts and levels found in plasma after tea consumption. Here, by using a combination of nuclear magnetic resonance binding assays, fluorescence polarization assay, and computational docking studies, we found that certain green tea catechins and black tea theaflavins are very potent inhibitors (K_i in the nanomolar range) of the antiapoptotic Bcl-2-family proteins, Bcl-x_L and Bcl-2. These data suggest a strong link between the anticancer activities of these tea polyphenols and their inhibition of a crucial antiapoptotic pathway, which is implicated in the development of many human malignancies.

Introduction

Programmed cell-death (apoptosis) is critical for tissue homeostasis, for the physiological removal of unwanted cells during development, and in host defense mechanism (1). Inappropriate suppression of apoptosis is implicated in most human malignancies (2). Bcl-2-family proteins are important regulators of apoptosis. Antiapoptotic members of this family, such as Bcl-2 and Bcl-x_L, contain on the surface a hydrophobic groove in which they can bind the BH3 domain of the proapoptotic counterparts (3). This binding is crucial for the regulation of apoptosis *in vivo*, with pro- and antisurvival proteins neutralizing each other's function through dimerization. The observation that antiapoptotic Bcl-2 family members, such as Bcl-2 and Bcl-x_L, are generally overexpressed in many cancer cells (3) has stimulated a growing interest in the discovery of small molecules targeting such proteins, as potential anticancer therapeutics (4, 5).

We have investigated the role of several natural and non-natural polyphenols in inhibiting BH3 binding to Bcl-2 and Bcl-x_L, finding that gossypol (a natural compound from cottonseed extracts) and purpurogallin (an antioxidant compound used in edible oils) bind to Bcl-2 and Bcl-x_L, and induce cell-death in tumor cell lines (5). In this current work we focused our attention on polyphenols from green and black tea with known proapoptotic activity (6–11), as their chemical structures possess some similarities to those of gossypol and purpurogallin.

Materials and Methods

FPAs. FPAs were performed 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 of the stocks and samples was PBS. To each tube was added a solution containing 50 nM of Bcl-x_L and 10 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 of the assays were performed in quadruplicate, with blank wells receiving no compound. Then, the plate was read for total intensity, and polarization (in mP units) was measured. Controls included dose-response measurements in absence of the proteins, to assess any interactions between the compounds and the FITC-BH3 peptide.

NMR Spectroscopy. Two-dimensional [¹⁵N,¹H]-transverse relaxation optimized spectroscopy (TROSY) spectra were acquired using a 0.250 mM samples of ¹⁵N-labeled Bcl-x_L and different amounts of compounds. ¹⁵N-labeled and unlabeled Bcl-x_L were obtained and purified as described previously (12). The three-dimensional structure of Bcl-x_L in complex with Bak peptide (Ref. 12; Protein Data Bank code 1BXL) was used for chemical-shift mapping and docking studies.

$T_{1\rho}$ measurements (13) and saturation transfer experiments such as WaterLOGSY (13) experiments were also performed to additionally validate the binding of the compounds to Bcl-x_L (data not shown).

All of the experiments were performed with a 600 MHz Bruker Avance600 spectrometer, equipped with four rf channels and Z-axis pulse-field gradients or a 500 MHz Bruker Avance spectrometer, equipped with three 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 2.5s. $T_{1\rho}$ series were acquired using a spin-lock pulse of variable length. Measurements were then performed with a spin-lock duration of 1 ms, 100 ms, and 200 ms with 100 μM compounds in the absence and presence of 10 μM protein. In all of the 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 structures of the compounds were initially obtained by FlexX (14) as implemented in Sybyl. Two calculations were performed. In the first, all of the binding-site torsion angles were kept fixed, whereas 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 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.

The surface representation was generated with MOLCAD (15) and color coded according to cavity depth (blue, shallow; yellow, buried).

Chemicals. Green tea extracts: (–)gallocatechin gallate, (–)gallocatechin, (–)EGC-3 gallate, (–)EGC, – catechin-3 gallate, (±)catechin, (–)ECG, and (+)EC were obtained from Sigma. Black tea extracts: theaflavin-3-3' digalate, theaflavin-3' gallate, theaflavin, and theaflavanin were obtained from Microsource Discovery Systems. Compounds were dissolved in DMSO at 100 mM concentration and stored at –20°C. NMR analysis was periodically performed on the compounds as a quality control, before additional dilution for binding assays.

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Notes: Drs. Leone and Zhai contributed equally to this work.

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¹ The abbreviations used are: FPA, fluorescence polarization assay; NMR, nuclear magnetic resonance; EC, epicatechin; ECG, epicatechin-3 gallate; EGC, epigallocatechin.

Table 1 Structure-activity relationships

A. Green tea extracts			
Compound	Structure	Bcl-x _L	Bcl-2
		K _i (nM)	K _i (nM)
GCG		315	235
GC		>10 ⁵	>10 ⁵
EGCG		490	335
EGC		>10 ⁵	>10 ⁵
CG		143	230
C		>10 ⁵	>10 ⁵
ECG		120	400
EC		>10 ⁵	>10 ⁵

Results and Discussion

Green tea is produced from the unfermented leaves of *Camelia sinensis*, and polyphenols, known as catechins, constitute its principal chemical components. EC, ECG, EGC, and EGC-3 gallate (Table 1A) are the major catechins contained in green tea. Black tea is made by extensive enzymatic oxidation of these polyphenols to lead to theaflavins. Theaflavin, theaflavin-3 gallate, theaflavin-3'gallate, and theaflavin-3-3' digallate are the principal theaflavins in black tea. Animal *in vivo* studies and human epidemiological observations indicated that green tea possesses inhibitory effects on the growth of tumor cell lines (7–11). Other tea constituents, such as the theaflavins (Table 1B) from black tea also possess antiproliferative or anticarcinogenic activities (6). We investigated the binding of different green

Table 1 Continued

B. Black tea extracts			
Compound	Structure	Bcl-x _L	Bcl-2
		K _i (nM)	K _i (nM)
Theaflavin digallate		>10 ⁵	>10 ⁵
Theaflavin-3' gallate		270	1230
Theaflavin		480	690
Theaflavanin		250	286

tea and black tea polyphenols (Table 1, A and B) to Bcl-x_L by NMR techniques, FPAs, and computational-docking studies. We found that epigallocatechin gallate (EGCG), GCG, ECG, and CG bind Bcl-x_L very tightly, whereas other green tea polyphenols, lacking the gallate group, such as GC, EGC, catechin (C), and EC do not interact with the protein. First we measured NMR ¹H T₁ρ (13) and saturation transfer effects on ligands by WaterLOGSY experiments (Ref. 13; data not shown) using samples containing 10 μM of protein and 100 μM of each compound. As shown in Fig. 1A for EGCG, this compound clearly binds to Bcl-x_L, as indicated by a remarkable decrease of its ¹H NMR signals intensity in the T₁ρ experiment acquired in presence of Bcl-x_L, when compared with the same spectrum measured in absence of protein. The striking effect is typical of binding in micro- to submicromolar range (13). Similar behavior was observed for the green tea polyphenols reported in Table 1A 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 a FPA in which binders were tested for their ability to displace the binding of a fluorescein-labeled BH3 peptide to Bcl-x_L or Bcl-2 (Fig. 1B; Fig. 2B). In agreement with the NMR binding data, several green tea extracts were able to inhibit Bcl-x_L (Table 1A) with the compounds ECG and CG representing the strongest inhibitors with K_i values of 120 and 143 nM, respectively. On the contrary, green tea polyphenols of which the 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. In contrast, these

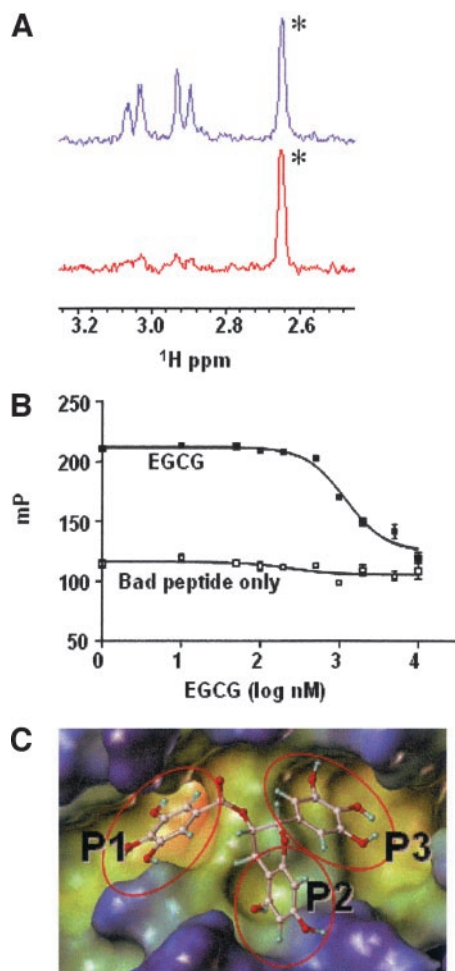


Fig. 1. EGCG binds Bcl- x_L . **A**, $T_{1\rho}$ experiments (200 ms relaxation time) with EGCG before (*blue*) and after (*red*) addition of 10 μ M Bcl- x_L . Peaks shown in (**A**) represent the protons 4 α and 4 β of the catechin group, the * indicates residual methyl resonances from deuterated DMSO (Sigma). **B**, fluorescence polarization-based competitive binding assay for EGCG and for FITC-Bad peptide alone (in absence of protein) used as negative control. **C**, surface representation of Bcl- x_L with the docked structure of EGCG obtained by FlexX. The three subpockets (P1, P2, and P3) occupied by the ligand are indicated.

natural products did not show inhibition in other protein interactions assays (XIAP plus Smac peptide) or enzymatic assays (caspase-8) up to 100 μ M (data not shown).

To gain additional insights on the mechanism of action of these catechins at the structural level, we have performed docking studies with FlexX (11) software implemented in Sybyl (TRIPOS). For this analysis, we used the Bcl- x_L conformation when in complex with Bak-peptide. We found that GCG, EGCG, CG, and ECG docked quite well in the BH3-binding pocket (docking energy approximately -45 kJ/mol). For the sake of illustration, the binding region on the surface of Bcl- x_L can be subdivided into three subpockets (P1, P2, and P3). The gallate moiety of GCG, EGCG, and CG mainly fits to the less lipophilic (P1) pocket, with the exception of ECG in which the gallate was predicted to be located in the opposite pocket (P3). In contrast compounds without the gallate group, such as GC, EGC, EC, and C, did not dock well (docking energy approximately -30 kJ/mol). The docked structures of CG and C, superimposed, show that the latter lacks the gallate ring and, thus, occupies just one subpocket (P3; Fig. 2C), suggesting that the occupation of all three of the subpockets is necessary for tight binding.

In parallel, we have also investigated the binding of the black tea polyphenols theaflavin, theaflavin digallate, theaflavin-3' gallate, and

theaflavin to Bcl- x_L . On the basis of the FPAs, we found that theaflavin, theaflavinin, and theaflavin-3' gallate were strong Bcl- x_L inhibitors, whereas theaflavin digallate failed to bind. K_i determination (Table 1B) indicated that theaflavin was the strongest binder of the series ($K_i = 250$ nM), followed by theaflavin-3' gallate ($K_i = 270$ nM; Fig. 3B), and finally by theaflavin ($K_i = 480$ nM). In contrast, no inhibition at 100 μ M concentration was observed for theaflavin digallate. The active black tea compounds also suppressed BH3 peptide binding to Bcl-2 in FPAs but not display inhibitory effects in various other *in vitro* assays (data not shown).

We performed NMR binding experiments and docking studies with these polyphenols. In bidimensional heteronuclear NMR titration experiments (13) with 15 N-labeled Bcl- x_L , theaflavin, theaflavinin, and theaflavin-3' gallate caused a wide broadening of the resonances (Fig. 3A). This result is typical of tight binding, although a high compound to protein ratio was needed, probably due to the limited solubility of the compounds at concentrations needed for NMR (500 μ M). The observed broadening of the protein [15 N, 1 H] resonances is characteristic of binding with intermediate exchange rates in the NMR time scale, representative of low- to submicromolar binders. Mapping of the observed changes in the NMR heteronuclear correlation spectra on titration with these compounds into the three-dimensional structure of Bcl- x_L showed that many residues within and surrounding the BH3-

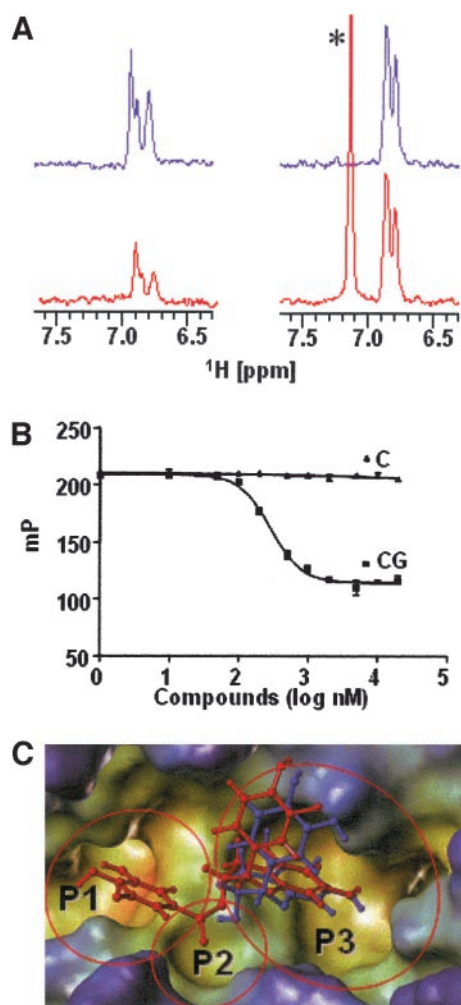


Fig. 2. Comparison between CG and C. **A**, $T_{1\rho}$ experiments (200 ms relaxation time) of CG (*left*) and C (*right*); spectra recorded in absence and presence of protein are reported in *blue* and *red*, respectively; an * indicates residual imidazole signal from the protein purification buffer. **B**, superposition of FPA results for CG and C. **C**, surface representation of Bcl- x_L binding pocket with the docked structures of CG (*red*) and C (*blue*).

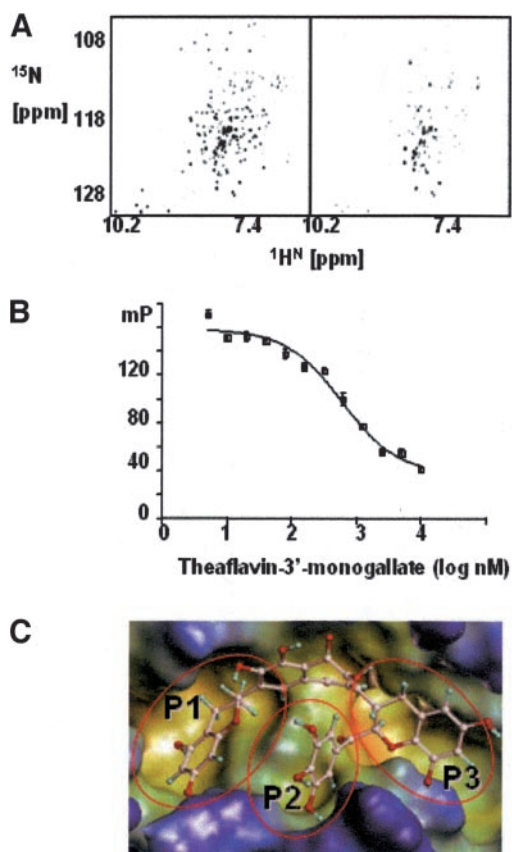


Fig. 3. Theaflavin-3' gallate binds Bcl-x_L. A, two-dimensional [¹⁵N,¹H]-TROSY spectra for Bcl-x_L (0.250 mM) before (left) and after addition of theaflavin-3' gallate (1 mM; right). B, FPA results for theaflavin-3' gallate. C, surface representation of Bcl-x_L binding pocket with the docked structure of theaflavin-3' gallate, the three subpockets (P1, P2, and P3) occupied by the ligand are circled.

binding pocket were affected by the compound binding, although the changes extended to a larger region surrounding this pocket. In contrast with Bcl-x_L, theaflavin-3' gallate did not associate with the proapoptotic protein Bid in a similar NMR binding experiment using ¹⁵N-labeled Bid, thus confirming the specificity of these results. Similar results were obtained with EGCG and ¹⁵N-labeled Bcl-x_L, corroborating the binding of the compound in the BH3-binding pocket (data not shown).

Docking studies showed that theaflavin and theaflavin-3' gallate docked quite well into the Bcl-x_L-binding site (docking-energy approximately -60 kJ/mol) occupying all three of the subpockets in which the binding region could be subdivided (Fig. 3C), again suggesting that compounds capable of occupying all three of the subpockets of Bcl-x_L exhibit the strongest binding and inhibition.

Several mechanisms have been proposed to explain the cancer chemopreventive effects of tea consumption. However, most proposed mechanisms can be criticized for: (a) implying inhibition of crucial physiological targets that would cause toxicity; (b) the lack of expression of the putative target in responsive tumor lines; or (c) the effective concentrations of catechins used in the *in vitro* assays are too high (1–10 mM) for the anticancer activity observed *in vivo* (16). Whereas tea polyphenols conceivably could affect apoptosis through multiple mechanisms (6–11, 16–19), our findings indicate that tea polyphenols may act as apoptosis-promoting cancer antagonists by binding to and suppressing Bcl-2-family proteins. Considering that the plasma level of tea catechins after drinking a few cups of tea has been demonstrated to be <300 nM (20), and that this concentration is

comparable with the effective concentrations of theaflavins and catechins we found to be relevant for Bcl-x_L and Bcl-2 inhibition, we conclude that Bcl-2-family proteins likely represent one of the physiologically relevant targets of tea polyphenols linked to their anticancer activity.

Because Bcl-2-family proteins are not expressed in plants, we speculate that plants may have developed certain polyphenols to act as natural pesticides by targeting Bcl-2-family proteins in insects and nematodes and, thus, inducing cell death in these animals. As corroborated by our recent studies on other polyphenols (5), these natural products may represent interesting lead compounds for cancer chemoprevention and chemotherapy.

In conclusion, our findings provide unprecedented insights into the mechanism of action of the major natural products present in the most widely consumed beverage, next to water. The data presented should encourage the development of novel clinical uses for these natural compounds in the prevention and treatment of many types of cancer. Finally, our structural docking studies may suggest ways to improve the potency and selectivity of these compounds using medicinal synthetic chemistry for more effective suppression of Bcl-2-family proteins.

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