0026-895X/02/6204-765–771$7.00
MOLECULAR PHARMACOLOGY
Vol. 62, No. 4
1773/1010923
Mol Pharmacol 62:765–771, 2002
Printed in U.S.A.

ACCELERATED COMMUNICATION

Inhibition of Bcl-X_L Phosphorylation by Tea Polyphenols or Epigallocatechin-3-Gallate Is Associated with Prostate Cancer Cell Apoptosis

ASLAMUZZAMAN KAZI, DAVID M. SMITH, QING ZHONG, and Q. PING DOU

Drug Discovery Program, H. Lee Moffitt Cancer Center & Research Institute (A.K., D.M.S., Q.Z., Q.P.D.), and Departments of Interdisciplinary Oncology (A.K., Q.Z., Q.P.D.) and Biochemistry & Molecular Biology (D.M.S., Q.P.D.), College of Medicine, University of South Florida, Tampa, Florida

Received April 3, 2002; accepted June 28, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Prostate cancer cells demonstrate slow growth kinetics and chemoresistance. Tea polyphenols have been shown to exert prostate cancer-preventative effects. Here we report that growth-arrested prostate cancer cells expressed high levels of a hyperphosphorylated Bcl-X_L in mitochondria. Treatment with tea polyphenols or the major tea component epigallocatechin-3-gallate blocked expression of the hyper-, but not hypophosphorylated Bcl-X_L in mitochondria, accompanied by cytochrome *c* release, caspase activation, and apoptosis. Studies using specific inhibitors suggest that tea inhibits p38 mitogen-activated protein kinase and the proteasome activities, leading to inhibition of Bcl-X_L phosphorylation and induction of prostate cancer cell death.

Epidemiological and animal studies have demonstrated the cancer preventative properties of green tea polyphenols (GTP) (Liao et al., 1995; Fujiki, 1999; Gupta et al., 1999; Yang, 1999). Four major green tea components are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC), all of which are also present in black and other teas. Among the tea polyphenols, EGCG has been the most extensively investigated because of its relative abundance and strong cancer preventative properties (Fujiki, 1999; Yang, 1999). Tea polyphenols have been found to affect numerous cancer-related proteins, including mitogen-activated protein kinase (Chung et al., 2001), matrix metalloproteinase (Demeule et al., 2000), the androgen receptor (Ren et al., 2000), EGF receptor (Liang et al., 1997), activator protein 1 (Chung et al., 1999), and nuclear factor-κB (Lin and Lin, 1997). Most recently, we have found that tea polyphenols containing ester bonds, such as EGCG or ECG, potently inhibit the proteasomal chymotrypsin-like, but not trypsin-like, activity in vitro and in vivo at concentrations similar to those found in the serum of green tea drinkers. In contrast, tea polyphenols without ester bonds, such as EGC or EC, are not proteasome inhibitors (Nam et al., 2001). Regardless of all the above findings, the detailed molecular mechanisms responsible for tea-mediated cancer prevention are still not established.

Under in vivo conditions, many human tumor cells contain an unduplicated DNA content, indicating growth arrest in the G_0/G_1 phase of the cell cycle (Cross et al., 1989; Pardee, 1989). Solid tumor cells are also often exposed to hypoxia and low-nutrient environment in vivo (Harrington et al., 1994; Dang and Semenza, 1999). Those nonproliferating tumor cells are resistant to many types of current anticancer drugs that are primarily effective against rapid dividing cancer cells (Kessel, 1994; Tomida and Tsuruo 1999; Smith et al., 2000). Indeed, human prostate cancer (PCa) cells demonstrate very slow growth kinetics and are resistant to current cancer therapies (Tang and Porter 1997; Ripple and Wilding,

This work was supported in part by research grants from the National Cancer Institute, National Institutes of Health, the United States Army Medical Research and Material Commend, and H. Lee Moffitt Cancer Center and Research Institute (to Q.P.D.) as well as by the Flow Cytometry Core Facility at Moffitt Cancer Center and Research Institute.

ABBREVIATIONS: GTP, green tea polyphenols; EGCG, (−)-epigallocatechin-3-gallate; EGC, (−)-epigallocatechin; ECG, (−)-epicatechin-3-gallate; EC, (−)-epicatechin; PCa, prostate cancer; PD169316, 4-(4-fluorophenyl)- 2-(4-nitrophe-nyl)-5-(4-pyridyl)1*H*-imidazole; PD98059, 2′-amino-3′-methoxyflavone; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; COX, cyclo-oxygenase.

1999). Thus, novel drugs need to be identified to either eradicate slow-growing/nonproliferating PCa cells or sensitize them to current chemotherapy. Understanding the molecular mechanism for the chemo-resistance of PCa cells should help us to achieve this goal.

Activation of the cellular apoptotic program is a current strategy for the treatment of human cancer. It has been demonstrated that radiation and standard chemotherapeutic drugs kill some tumor cells through induction of apoptosis (Fisher, 1994). Upon apoptosis stimulation, several key events occur in mitochondria, including the release of cytochrome *c* (Green and Reed, 1998; Gross et al., 1999). The mitochondrial cytochrome *c* release can be inhibited by expression of an antiapoptotic Bcl-2 family member (i.e., Bcl-2 or Bcl-X_L) and induced by expression of a proapoptotic member of Bcl-2 family, [i.e., Bax or Bid (Green and Reed, 1998; Gross et al., 1999)].

Here we report that growth-arrested human PCa cells express high levels of a hyperphosphorylated Bcl-X_L in mitochondria. Treatment with GTP or EGCG completely blocked the hyperphosphorylated, but not hypophosphorylated, Bcl-X_L expression, associated with cytochrome *c* release, caspase activation, and apoptosis induction. Further studies using specific pharmacological inhibitors demonstrate that tea may target both p38 MAP kinase- and the proteasome-mediated pathways, which are required for Bcl-X_L phosphorylation and PCa cell survival. Our study suggests that down-regulation of phosphorylated Bcl-X_L in mitochondria is at least one of the molecular mechanisms responsible for tea-mediated cancer-preventative function.

Materials and Methods

Materials. Highly purified tea polyphenols [EGCG (>95%), ECG (>98%), EGC (>98%), and EC (>98%)] and green tea polyphenols (Polyphenon 100) were purchased from Sigma (St. Louis, MO) and used directly without further purification. Lambda (λ) protein phosphatase was obtained from New England BioLabs (Beverly, MA). The selective inhibitors to p38 MAP kinase (PD169316), MAP kinase kinase/MEK (PD98059), and phosphatidylinositol 3-kinase (Wortmannin) as well as the fluorogenic peptide substrate *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin (for the caspase-9 activity) were purchased from Calbiochem (San Diego, CA). The specific proteasome inhibitor lactacystin was from Biomol (Plymouth Meeting, PA). Polyclonal antibodies to a sequence of amino acids 201 to 216 of human Bcl-X_L (Ab-1; the carboxyl or C-terminal antibody) was from Oncogene Research Products (Cambridge, MA); to the amino terminus of human Bcl-X_L (M-125), to Bax (N20) and to actin (C-11) were from Santa Cruz Biotechnology (Santa Cruz, CA); to human poly(ADP-Ribose) polymerase (PARP) was from Roche Applied Science (Indianapolis, IN). Monoclonal antibodies to the Bcl-X_L N terminus and to cytochrome *c* were from BD PharMingen (San Diego, CA); to Bcl-2 from DAKO Co. (Glostrup, Denmark); to cytochrome oxidase unit II (COX) from Molecular Probes (Eugene, OR).

Cell Culture and Treatment. Human PCa cell lines LNCaP and PC-3 were grown in RPMI 1640 and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cell cultures were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2. To induce G_1 arrest, 80 to 90% confluent cells were incubated in serum-free medium for 72 h. The growth-arrested cells were then treated with GTP, a purified tea polyphenol or a pharmacological inhibitor, as described in legends of figures.

Whole Cell Extract, Subcellular Fractionation, and Western Blot Assay. A whole-cell extract was prepared as described previously (An and Dou, 1996). Both cytosolic and mitochondrial fractions were isolated at 4°C using a previous protocol (Gao and Dou, 2000). Western blot assay with the enhanced chemiluminescence system was performed as we described previously (An and Dou, 1996; Gao and Dou, 2000). For densitometric analysis, intensities of interested protein bands detected in Western blotting were scanned, and ratios of these proteins to the loading control protein (such as actin or p48) were calculated (Nam et al., 2001).

In Vitro Phosphatase Treatment. After 72 h of serum starvation, prostate cancer cells were harvested, washed with PBS, and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). In vitro phosphatase treatment was performed according to a protocol provided by the manufacturer (New England Biolabs). Briefly, a protein extract aliquot (40 μg) was incubated with either λ protein phosphatase (400 units) or the control buffer at 30°C for 4 h in a phosphatase reaction buffer containing 2 mM $MnCl_2$. After incubation, protein samples were analyzed by Western blot assay.

Flow Cytometry and Cell-Free Caspase Activity Assay. Cell cycle analysis based on DNA content was performed as we described previously (Nam et al., 2001). The cell-cycle distribution is shown as the percentage of cells containing G_1, S, G_2, and M DNA judged by propidium iodide staining. The apoptotic population (Ap) is determined as the percentage of cells with sub-G_1 DNA content. To measure caspase-9 activity, a protein extract (20 μg) was incubated for 2 h at 37°C with 20 μM of a fluorogenic peptide substrate, *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin, in a 96-well plate. After incubation, the hydrolyzed AFC groups were measured by a Wallac Victor^2 1420 Multilabel counter (Turku, Finland) with 405/535 nM filters.

Results

Increased Expression of a Hyperphosphorylated Form of Bcl-X_L in G_1 Prostate Cancer Cells. When human PCa LNCaP cells were serum-starved for 72 h, their G_0/G_1 population was increased by ~30% (Fig. 1A). We determined changes in Bcl-X_L protein levels during serum starvation process. A specific polyclonal antibody to the C terminus of human Bcl-X_L protein detected doublet bands with a molecular mass of 34 to 36 kDa (Fig. 1B, a), which was later found to be a hyperphosphorylated form of Bcl-X_L (named as Bcl-X_L-*hyper*; see Fig. 1C). The levels of the Bcl-X_L-*hyper* were low in growing LNCaP cells, but increased by 6-fold after 24-h serum starvation and by 10- to 11-fold after 48 or 72 h (Fig. 1B, a), as determined by densitometric analysis. The same Bcl-X_L C-terminal antibody also detected doublet band(s) of ~48 kDa with unknown nature, whose expression was relatively unchanged in LNCaP cells during serum starvation and therefore used as a loading control (Fig. 1B, a; see also Fig. 1C, a).

A specific polyclonal antibody to the N terminus of human Bcl-X_L protein did not detect the Bcl-X_L-*hyper* in LNCaP cells; instead, it detected another Bcl-X_L band with a molecular mass of 31 kDa, a hypophosphorylated form of Bcl-X_L (named as Bcl-X_L-*hypo*; see Fig. 1C, b). In contrast to Bcl-X_L-*hyper*, levels of Bcl-X_L-*hypo* were relatively unchanged during serum starvation (Fig. 1B, b). Constitutive levels of Bcl-X_L-*hypo* were also observed when a monoclonal Bcl-X_L N-terminal antibody was used (data not shown). When a mixture of both C- and N-terminal polyclonal antibodies was applied in Western blotting, increased levels of Bcl-X_L-*hyper*, but not Bcl-X_L-*hypo*, were again detected in growth-arrested LNCaP cells (Fig. 1B, b). In this experiment, constitutive

levels of actin protein were used as a control (Fig. 1B, c). When PCa PC-3 cells were serum-starved, Bcl-X_L-*hyper* expression was again increased, whereas Bcl-X_L-*hypo* levels were unchanged (data not shown).

The Bcl-X_L-*hyper* Is a Phosphorylated Form of Bcl-X_L. Bcl-X_L protein is phosphorylated in vivo, which leads to a gel mobility shift (Poruchynsky et al., 1998; Fan et al., 2000). We hypothesized that the Bcl-X_L-*hyper* observed under our experimental conditions is a phosphorylated form of Bcl-X_L. To test this hypothesis, PC-3 and LNCaP cells were serum-starved and then used for protein extraction. Aliquots of the protein extracts were treated with either λ protein phosphatase or the control buffer, followed by measurement of levels of Bcl-X_L-*hyper* and Bcl-X_L-*hypo* in Western blot assay. The phosphatase treatment significantly decreased the expression of Bcl-X_L-*hyper* band, as detected by the C-terminal antibody, which was associated with appearance of a new band of ~28 kDa, which should be the unphosphorylated Bcl-X_L (named as Bcl-X_L-*unphos*; Fig. 1C, a). The increased intensity of the Bcl-X_L-*unphos* band was probably due to a strong interaction of this form of Bcl-X_L with the antibody. Associated with decreased levels of Bcl-X_L-*hyper*, the levels of Bcl-X_L-*hypo* were slightly increased after the phosphatase treatment (Fig. 1C, b), indicating a conversion of Bcl-X_L-*hyper* to Bcl-X_L-*hypo* by dephosphorylation. The Bcl-X_L N-terminal antibody also detected the appearance of a similar Bcl-X_L-*unphos* band (Fig. 1C, b). When a mixture of both antibodies was used, decreased Bcl-X_L-*hyper* expression and slightly increased Bcl-X_L-*hypo* levels, as well as the new Bcl-X_L-*unphos* band, were again detected (Fig. 1C, c). In this experiment, levels of the LNCaP-specific p48 protein remained unaffected and served as a control (Fig. 1C, a and c).

Down Regulation of Bcl-X_L-*hyper*, but not Bcl-X_L-*hypo*, by Green Tea Polyphenols in the Mitochondria of G_1 Prostate Tumor Cells. It has been suggested that Bcl-X_L plays a key role in survival and chemo-resistance of PCa cells (Green and Reed, 1998; Gross et al., 1999) and that green tea has chemo-preventative effects on prostate cancer (Liao et al., 1995; Fujiki, 1999; Gupta et al., 1999; Yang, 1999). We hypothesized that green tea-mediated cancer preventative function is related to inhibition of Bcl-X_L expression. If so, treatment of prostate tumor cells with GTP should be able to decrease Bcl-X_L protein expression. Indeed, when serum-starved LNCaP cells were treated with GTP for 3 h, expression of Bcl-X_L-*hyper* was decreased to an undetectable level, whereas the p48 levels were unaffected (Fig. 2A, a, lanes 3 versus 1). The decreased Bcl-X_L-*hyper* expression was caused by effects of GTP, because when the same LNCaP cells were treated with the vehicle H_2O, the Bcl-X_L-*hyper* levels were not decreased (Fig. 2A, a, lanes 2 versus 1). In contrast to the dramatic reduction of Bcl-X_L-*hyper* expression, levels of Bcl-X_L-*hypo* were only slightly decreased in the LNCaP cells treated with GTP (Fig. 2A, b). Furthermore, the GTP treatment had no inhibitory effect on expression of Bax protein (Fig. 2A, c). Down-regulation of Bcl-X_L-*hyper* expression was found to be GTP concentration-dependent: the lowest GTP concentration needed in LNCaP cells was between 10 and 25 μg/ml (Fig. 2B).

Treatment of serum-starved PC-3 cells with GTP, but not the vehicle, also inhibited expression of Bcl-X_L-*hyper*, but not Bcl-X_L-*hypo* (Fig. 2C, a versus b). In this experiment, expression of Bcl-2 protein was only slightly inhibited by GTP (Fig. 2C, c). To determine whether GTP was able to down-regulate Bcl-X_L-*hyper* expression in other tumor or transformed cells, human breast cancer MCF-7 (data not shown) and simian virus-transformed human fibroblast VA-13 cells (Fig. 2D) were serum-starved and then treated with GTP. Again, this treatment completely inhibited expression of Bcl-X_L-*hyper*, but not Bax (Fig. 2D). Taken together, it seems that GTP is able to selectively inhibit expression of Bcl-X_L-*hyper* in prostate and other tumor or transformed cells.

We next determined where Bcl-X_L-*hyper* was localized and where GTP exerted the inhibitory effect. After serum-starved LNCaP cells were treated with the vehicle H_2O, most of the Bcl-X_L-*hyper* doublets were found in the mitochondrial fraction, whereas a small portion of the top (but not the bottom)

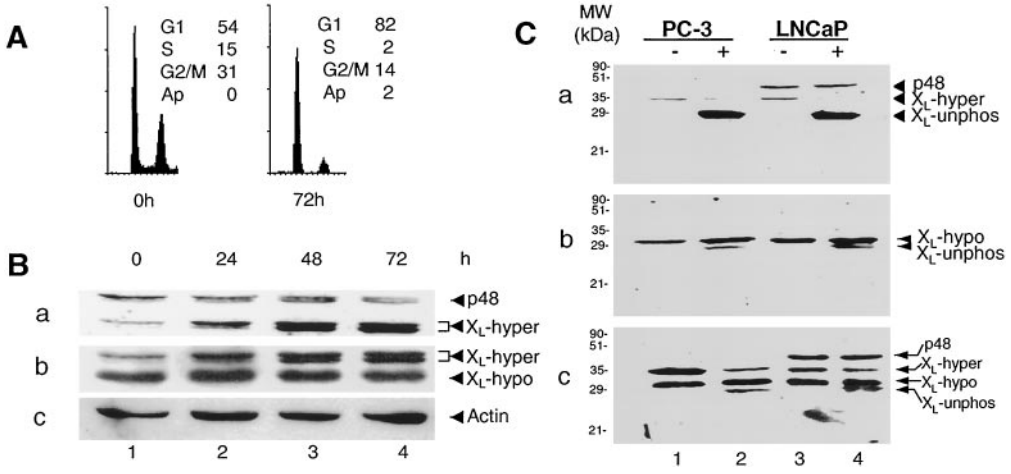

Fig. 1. Increased hyperphosphorylation of Bcl-X_L in serum-starved PCa cells. A, growing LNCaP cells (0 h) were incubated in serum-free medium for 72 h, followed by flow cytometry analysis. B, LNCaP were serum-starved for up to 72 h, followed by whole-cell extraction and Western blot assay using specific antibodies to the Bcl-X_L C terminus (a), both C and N terminus of Bcl-X_L (b), or actin (c). Molecular masses: Bcl-X_L-*hyper* (X_L-*hyper*), 34 to 36 kDa; Bcl-X_L-*hypo* (X_L-*hypo*), 31 kDa; actin, 43 kDa. The p48 band, an unknown protein detected in LNCaP cells by the Bcl-X_L C-terminal antibody and used as a control. C, protein extracts, prepared from PC-3 and LNCaP cells serum-starved for 72 h, were incubated with λ protein phosphatase (+) or the control buffer (−) at 30°C for 4 h, followed by Western blotting using antibodies to C (a) or N terminus (b) of Bcl-X_L or both (c). X_L-*unphos*, 28 kDa, is unphosphorylated form of Bcl-X_L. Protein molecular mass markers (in kDa) are shown at left; the range of 110 kDa contained no bands and was not shown due to the space limit. Similar results were obtained in three independent experiments.

band of the Bcl-X_L-*hyper* doublets was in the cytosol (Fig. 2E, a, lanes 1 and 3). Similar levels of Bcl-X_L-*hypo* were found in both mitochondrial and cytosolic fractions, and more of the LNCaP-specific p48 band was observed in the cytosolic fraction (Fig. 2E, a, lanes 1 and 3). Treatment with GTP completely inhibited expression of the Bcl-X_L-*hyper* doublets in mitochondria and also decreased the top band of Bcl-X_L-*hyper* doublets in the cytosol (Fig. 2E, a, lanes 2 versus 1 and 4 versus 3). Similar to the result using a whole-cell extract (Fig. 2A), GTP had no inhibitory effects on the levels of either Bcl-X_L-*hypo* or the p48 in both mitochondrial and cytosolic fractions (Fig. 2E, a). As controls, the mitochondria-specific COX protein was detected only in the mitochondrial fraction (Fig. 2E, b), and more actin protein was observed in the cytosol than in the mitochondrial fraction (Fig. 2E, c). GTP treatment did not affect levels of either COX or actin in the isolated cellular fractions (Fig. 2E, b and c). Therefore, it seems that GTP selectively inhibits expression of Bcl-X_L-*hyper* in the mitochondria of prostate cancer LNCaP cells.

Down-Regulation of Bcl-X_L-*hyper* by GTP Is Associated with Prostate Cancer Cell Apoptosis. We then tested whether decreased level of mitochondrial Bcl-X_L-*hyper* by GTP treatment in PCa cells was associated with cytochrome *c* release, caspase activation, and apoptosis. Treatment of serum-starved LNCaP cells with GTP for 3 h induced cytochrome *c* release from the mitochondria to the cytosol (Fig. 3A). In addition, after down-regulation of Bcl-X_L-*hyper* (Figs. 2A and 3B) and cytochrome *c* release (Fig. 3A) at 3 h, caspase-9 was activated by GTP, as measured by cell-free activity assay (Fig. 3B). The activity of caspase-9 was increased by ~2-fold at 6 h and by ~7-fold at 12 h (Fig. 3B). Furthermore, the apoptosis-specific cleavage fragment p85 of PARP was first detected after 6 h of GTP treatment and its levels increased significantly at 12 h (Fig. 3C). Associated with that, the pre-G_1 apoptotic population was increased by ~10% at 6 h and by ~35% at 12 h (Fig. 3D). All the apoptotic events, including cytochrome *c* release, caspase-9 activation, PARP cleavage, and pre-G_1 population increase were not observed in the vehicle-treated LNCaP cells (Fig. 3, A-D). The apoptosis-specific PARP cleavage was also observed in GTP- but not vehicle-treated PC-3 cells (Fig. 2C, e, lane 3), further demonstrating that induction of PCa cell death by GTP is tightly associated with down-regulation of Bcl-X_L-*hyper* expression (compare Figs. 3 and 2).

EGCG among Tea Polyphenols Has the Greatest Potency to Down-Regulate Bcl-X_L-*hyper* Expression and Induce Prostate Cancer Cell Apoptosis. To determine which component(s) of GTP is responsible for their ability to down-regulate Bcl-X_L-*hyper* expression, we first compared effects of purified EGCG and EGC. Treatment of growth-arrested LNCaP or PC-3 cells with EGCG at 50 μM for 3 h completely blocked Bcl-X_L-*hyper* expression, which mimics the effect of GTP (in Figs. 2, A and C, a, and 4, A and B). In contrast, EGC had no effects under the same experimental conditions (Fig. 2, A and C, and Fig. 4, A and B). EGCG at 5, 10, 25, and 50 μM inhibited 15, 85, 93, and 100% of Bcl-X_L-*hyper* expression, respectively (as determined by densitometric analysis), in serum-starved LNCaP cells, indicating a concentration-dependent effect (Fig. 4C). Both EGCG and EGC had little or no effect on expression of Bcl-X_L-*hypo* (Fig. 2, A and C, b). In addition, neither EGCG nor EGC affect levels of Bax or Bcl-2, compared with the vehicle (Fig. 2, A and C, c, lanes 4 and 5). Importantly, apoptosis-specific PARP cleavage was induced by only EGCG but not EGC in LNCaP, PC-3, and DU145 cells (Fig. 2C, e, and data not shown). When LNCaP and PC-3 cells were treated with 50

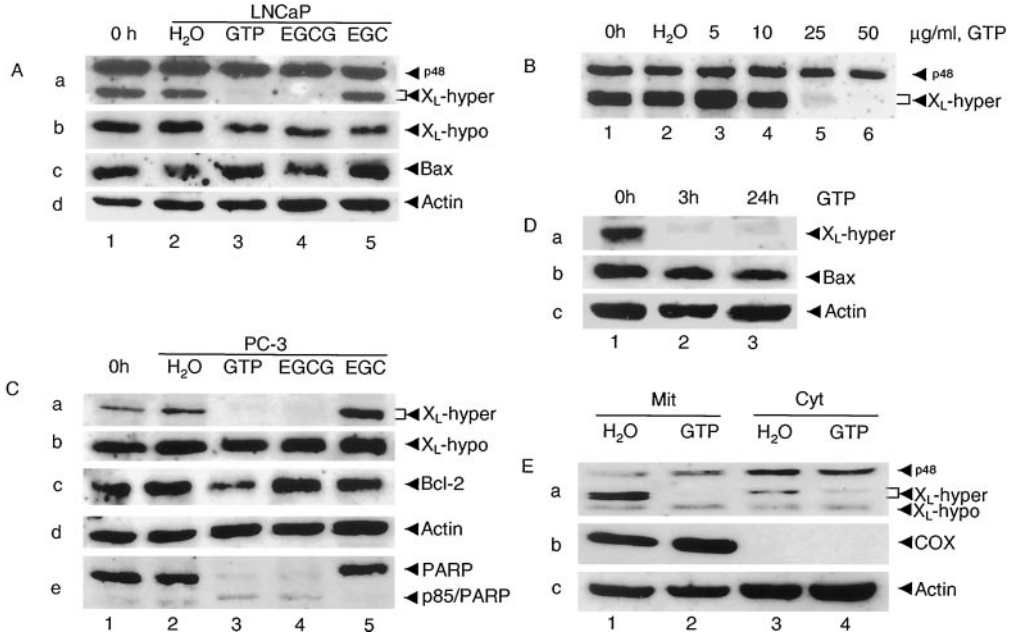

Fig. 2. Down-regulation of Bcl-X_L-*hyper* expression by GTP or EGCG in G_1 PCa cells. LNCaP (A) or PC-3 (C) cells were serum-starved for 72 h (0 h), followed by a 3-h treatment with the solvent (H_2O), 50 μg/ml GTP, 50 μM EGCG, or 50 μM EGC. B, 72-h serum-starved LNCaP cells were treated with increasing concentrations of GTP (5, 10, 25, and 50 μg/ml) for 12 h. D, 72-h serum-starved VA-13 cells were treated with 50 μg/ml GTP for 3 or 24 h. E, 72-h serum-starved LNCaP cells were treated with either the solvent H_2O or 50 μg/ml GTP for 3 h, followed by isolation of cytosolic and mitochondrial fractions. Samples prepared in each experiment were used in Western blot assay using each indicated antibody (a mixture of both Bcl-X_L C- and N-terminal antibodies were used in E, a). Molecular masses of Bcl-2, Bax, PARP, and COX are 26, 21, 116, and 26 kDa, respectively. The apoptosis-specific PARP cleavage fragment p85 is also indicated in C. Similar results were obtained in at least three independent experiments.

μM ECG, a ~55% inhibition of Bcl-X_L-*hyper* expression was observed (Fig. 4, A and B, lanes 4 versus 1). In contrast, EC, similar to EGC, was inactive (Fig. 4, A and B, lane 5). Taken together, these results demonstrate that EGCG is the major green tea polyphenol that is responsible for down-regulating Bcl-X_L-*hyper* and inducing prostate cancer cell apoptosis.

It has been shown that c-Jun NH_2-terminal protein kinase pathway plays a role in Bcl-X_L phosphorylation in vivo (Fan et al., 2000) and that EGCG is able to directly inhibit Erk activity in vitro (Chung et al., 2001). To determine whether EGCG targets a Bcl-X_L kinase pathway in PCa cells, we examined effects of different kinase inhibitors on Bcl-X_L-*hyper* expression. PD169316, a specific inhibitor of p38 MAP kinase (Kummer et al., 1997), was found to inhibit Bcl-X_L-*hyper* expression in a concentration-dependent manner: 55% at 10 μM and nearly 100% at 25 μM, with potency similar to that of EGCG (Fig. 4D, lanes 4 and 5 versus 1; compare also to Fig. 4C). In contrast, the specific MEK inhibitor PD98059 and the phosphatidylinositol 3-kinase inhibitor Wortmanin had no or very little inhibitory effects (Fig. 4D, lanes 2, 3, 6). These data indicate that p38 MAP kinase is involved in Bcl-X_L phosphorylation in PCa cells.

We have also reported that EGCG and ECG, but not EGC and EC, potently and specifically inhibit the chymotrypsin-like activity of the proteasome in vitro and in vivo (Nam et al., 2001). To determine whether inhibition of the proteasome activity is required for down-regulation of Bcl-X_L-*hyper* by tea polyphenols (Fig. 4, A and B), we tested effects of the specific proteasome inhibitor lactacystin. Lactacystin potently inhibited Bcl-X_L-*hyper* expression by 80% at 10 μM and by 100% at 25 μM (Fig. 4D, lanes 7–9), whose potency was comparable with those of EGCG and PD169316 (Fig. 4, D versus C). It seems that the proteasome-mediated pathway also regulates Bcl-X_L phosphorylation in vivo in prostate cancer cells.

Discussion

Our current study has reported that treatment with GTP or EGCG can down-regulate expression of Bcl-X_L-*hyper*, but not Bcl-X_L-*hypo*, protein in PCa cell mitochondria, associated with cytochrome *c* release and apoptosis induction. This novel mechanism may contribute to the previously demonstrated cancer-preventative properties of green tea (Liao et al., 1995; Fujiki 1999; Gupta et al., 1999; Yang, 1999).

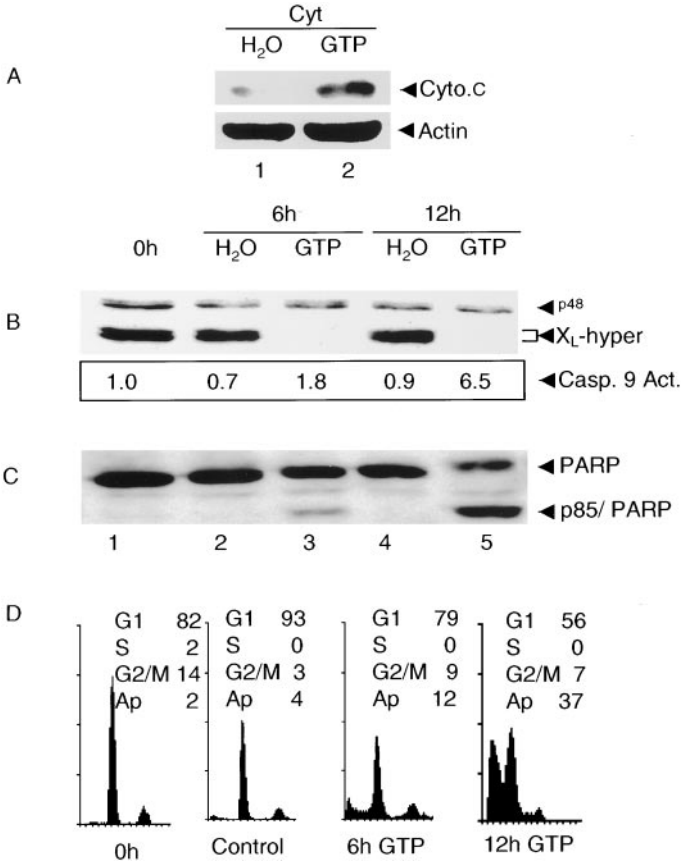

Fig. 3. Induction of PCa cell apoptosis by GTP is associated with inhibition of Bcl-X_L-*hyper* expression. LNCaP cells, after serum starvation for 72 h (0 h), were treated with either 50 μg/ml GTP or the control vehicle (H_2O) for 3 (A), 6, or 12 h (B-D). A, cytosolic fraction was prepared and immunoblotted for levels of cytochrome *c* (molecular mass, 17 kDa) and actin. B and C, whole cell extracts were used to determine levels of Bcl-X_L-*hyper* (with the C-terminal antibody) or PARP cleavage as well as cell-free caspase-9 activity. D, flow cytometry analysis. Ap, the apoptotic cell population with $<G_1$ DNA content. Control, 12-h H_2O treatment (6 h H_2O treatment gave similar results). Similar results were obtained in three independent experiments.

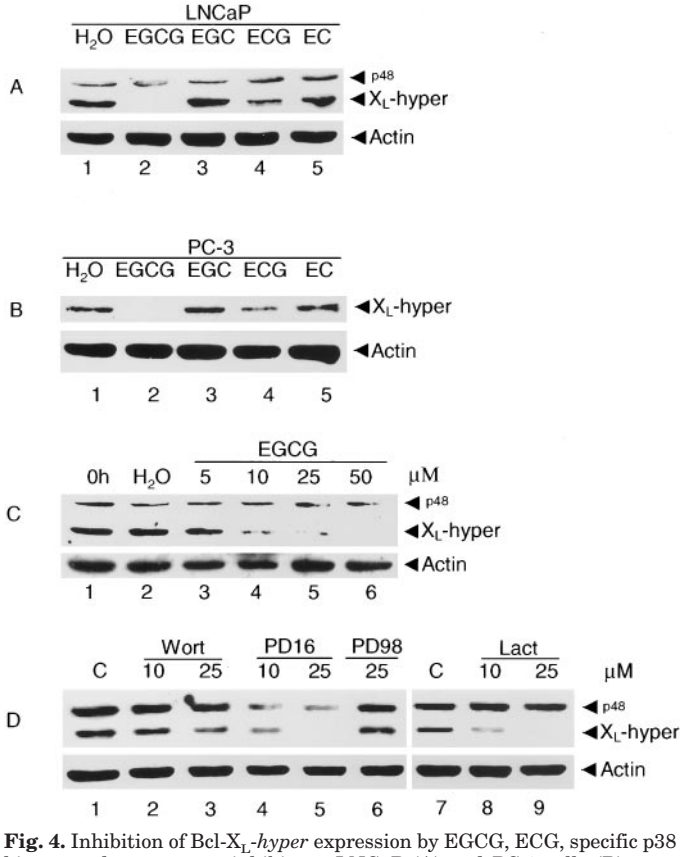

Fig. 4. Inhibition of Bcl-X_L-*hyper* expression by EGCG, ECG, specific p38 kinase and proteasome inhibitors. LNCaP (A) and PC-3 cells (B) were serum-starved for 72 h, followed by treatment with either the solvent H_2O or purified EGCG, EGC, ECG, or EC at 50 μM for 3 h. C, 72-h serum-starved LNCaP cells were treated for 12 h with either solvent H_2O or EGCG at indicated concentrations. D, 72-h serum-starved LNCaP cells were treated for 3 h with either control solvent dimethyl sulfoxide (C) or pharmacological inhibitors, Wortmannin (Wort), PD169316 (PD16), PD98059 (PD98), or lactacystin (Lact), at indicated concentrations. Samples prepared in each experiment were used in Western blot assay using specific antibody to the Bcl-X_L C terminus or actin. Similar results were obtained in two to four independent experiments.

The following arguments support that the Bcl-X_L band, we named Bcl-X_L-*hyper*, is a phosphorylated form. First, several groups have shown that Bcl-X_L protein is phosphorylated in vivo which leads to a mobility shift (Poruchynsky et al., 1998; Fan et al., 2000). Second, EGCG has been found to directly inhibit activities of several kinases, including IκB kinase (Yang et al., 2001), p70 S6 kinase (Nomura et al., 2001), and Erk (Chung et al., 2001) under cell-free conditions. Third, the Bcl-X_L-*hyper* band seems to be selectively recognized by a specific polyclonal antibody to the C terminus of human Bcl-X_L protein (Fig. 1C). Only the Bcl-X_L-*hyper* band was detected in PC-3 cell extracts by the antibody (Fig. 1C, a, lane 1), although another p48 band was also detected in LNCaP cell extracts (Fig. 1C, a, lane 3). Fourth, the mobility of the Bcl-X_L-*hyper* band is slower than those of the Bcl-X_L-*hypo* and Bcl-X_L-*unphos* (Fig. 1C). Finally, in vitro phosphatase treatment significantly decreased the level of Bcl-X_L-*hyper*, associated with appearance of a new band with faster mobility that should be unphosphorylated form of Bcl-X_L (Fig. 1C, a). The phosphatase treatment did not affect levels of p48 expression (Fig. 1C), demonstrating specificity on phosphorylated proteins. We plan to look further into the involved molecular mechanism by developing an in vitro Bcl-X_L phosphorylation assay.

Our results also indicated that phosphorylation of Bcl-X_L is associated with G_1 arrest (Fig. 1). We have found increased levels of Bcl-X_L-*hyper* during serum starvation. This starvation arrested 82% of LNCaP cells in G_1 phase of the cell cycle (Fig. 1A). We hypothesized that the increased Bcl-X_L-*hyper* in G_1 phase contributes to resistance of PCa cells to apoptosis induction. Indeed, some studies have shown that under serum-deprived condition Bcl-X_L expression is increased, protecting cells from apoptosis (Zhang et al., 2000; Takehara et al., 2001). In addition, in vivo many human tumor cells (including prostate cancer) contain high percentages of G_0/G_1 DNA content (Cross et al., 1989; Pardee 1989) and are hypoxic and low-nutrient (Harrington et al., 1994; Dang and Semenza, 1999). Many tumor cells also overexpress the antiapoptotic proteins Bcl-X_L and Bcl-2 and are resistant to chemotherapy and radiotherapy (Green and Reed, 1998; Gross et al., 1999). It should be noted that although serum deprivation is commonly used to synchronize cell lines in the G_0/G_1 phase of the cell cycle, there are other differences between serum-starved conditions and in vivo tumor microenvironments.

Another important finding in the present study is the tight association between inhibition of Bcl-X_L phosphorylation by GTP and EGCG and induction of PCa cell apoptosis. Previous animal and human epidemiological studies have suggested that the polyphenols present in green tea have protective effects against a variety of cancers including prostate cancer (Liao et al., 1995; Fujiki, 1999; Gupta et al., 1999; Yang, 1999). Different molecular mechanisms have been suggested for tea polyphenols' anticancer activity but none of them have been shown to be directly responsible for the cancer-preventative properties of tea (see *Introduction*). We hypothesized that GTP and EGCG might inhibit Bcl-X_L phosphorylation and consequently induce PCa cell apoptosis, which contributes to green tea-mediated cancer preventative function. Indeed, we observed that GTP and EGCG selectively down-regulated the expression of Bcl-X_L-*hyper*, but not Bcl-X_L-*hypo* in preparations of PCa cell extracts and mitochondria (Fig. 2, A, C, and E). This reduction in Bcl-X_L-*hyper* expression by GTP or EGCG was time- and concentration-dependent (Figs. 2–4) and found in prostate cancer (Figs. 2–4), breast cancer (data not shown) and simian virus 40-transformed cells (Fig. 2D). Furthermore, GTP or EGCG had little effect on expression of Bcl-2 and Bax proteins (Fig. 2), indicating selectivity to Bcl-X_L in the phosphorylated form. Importantly, reduction of the mitochondrial Bcl-X_L-*hyper* by GTP (Fig. 2E) was associated with induction of cytosolic cytochrome *c* release, caspase-9 activation, PARP cleavage, and apoptosis (Fig. 3). Our results are consistent with other studies that showed that the mitochondrial Bcl-X_L prevents apoptosis via inhibition of cytochrome *c* release (Green and Reed, 1998; Gross et al., 1999).

The direct target of tea and EGCG that regulates Bcl-X_L phosphorylation is currently unknown. It is possible that tea and EGCG can directly inhibit the Bcl-X_L kinase activity in vivo, resulting in decreased level of the Bcl-X_L-*hyper*. Consistent with this argument, it has been shown that the c-Jun NH_2-terminal protein kinase pathway plays a role in Bcl-X_L phosphorylation in vivo (Fan et al., 2000) and that EGCG is able to directly inhibit Erk activity in vitro (Chung et al., 2001). Our results also showed that the specific p38 MAP kinase inhibitor PD169316 could inhibit expression of the Bcl-X_L-*hyper* in a concentration-dependent manner (Fig. 4D). In contrast, the MEK and phosphatidylinositol 3-kinase inhibitors had no or very little inhibitory effects (Fig. 4D). These data at least suggest that p38 kinase is involved in the Bcl-X_L phosphorylation pathway.

We also found that EGCG and ECG, both of which contain an ester bond, inhibited expression of Bcl-X_L-*hyper*, but EGC and EC without ester bond had no effect (Fig. 4, A and B). We have reported that EGCG and ECG, but not EGC and EC, potently and specifically inhibit the chymotrypsin-like activity of the proteasome in vitro and in vivo (Nam et al., 2001). These results suggest that the proteasome may regulate the Bcl-X_L phosphorylation pathway in PCa cells. Indeed, lactacystin, a specific proteasome inhibitor, also inhibited expression of Bcl-X_L-*hyper* in a concentration-dependent manner (Fig. 4D). Identification of the Bcl-X_L kinase, establishment of an in vitro Bcl-X_L phosphorylation assay, and use of synthetic EGCG analogs (Smith et al., 2002) will help to uncover the target of tea and EGCG.

Although the present studies focused on the level of Bcl-X_L phosphorylation, it should be noted that Bcl-X_L transcription can be up-regulated by signal transducer and activator of transcription 3 (Stat 3) or Stat 5, which are regulated by various kinase pathways (Catlett-Falcone et al., 1999; Horita et al., 2000; Sevilla et al., 2001). EGCG might also be able to inhibit Stat 3-mediated Bcl-X_L transcription (Masuda et al., 2001). However, the novel aspect of our investigation is the demonstration of the inhibition of Bcl-X_L phosphorylation by tea polyphenols and EGCG in prostate cancer cells. These studies have implied that inhibition of Bcl-X_L phosphorylation in mitochondria may contribute to the prostate cancer preventative properties of tea polyphenols.

Our future studies will focus on characterization of the Bcl-X_L-*hyper* phosphorylation sites, how Bcl-X_L-*hyper* inhibits the mitochondrial cytochrome *c* release, how p38 MAP kinase and the proteasome regulate Bcl-X_L phosphorylation, and the detailed molecular mechanisms for EGCG-mediated inhibition of p38 kinase and the proteasome. Synthetic ana-

logs of natural polyphenols (Smith et al., 2002) should help to identify the tea target(s) regulating Bcl-X_L phosphorylation and create more potent and specific compounds for the prevention and treatment of human prostate and other cancers. Many of the current chemotherapeutic drugs were originally developed from natural products. We believe that the research presented here is the initial step for further developing such novel cancer-preventative agents.

References

An B and Dou QP (1996) Cleavage of retinoblastoma protein during apoptosis: an interleukin 1 beta-converting enzyme-like protease as candidate. *Cancer Res* **56:** 438–442.

Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, et al. (1999) Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* **10:**105–115.

Chung JY, Huang C, Meng X, Dong Z, and Yang CS (1999) Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in *H-ras*-transformed cells: structure-activity relationship and mechanisms involved. *Cancer Res* **59:**4610–4617.

Chung JY, Park JO, Phyu H, Dong Z, and Yang CS (2001) Mechanisms of inhibition of the Ras-MAP kinase signaling pathway in 30.7b Ras 12 cells by tea polyphenols (−)-epigallocatechin-3-gallate and theaflavin-3,3′-digallate. *FASEB J* **15:**2022–2024.

Cross F, Roberts J, and Weintraub H (1989) Simple and complex cell cycles. *Annu Rev Cell Biol* **5:**341–396.

Dang CV and Semenza GL (1999) Oncogenic alterations of metabolism. *Trends Biochem Sci* **24:**68–72.

Demeule M, Brossard M, Page M, Gingras D, and Beliveau R (2000) Matrix metalloproteinase inhibition by green tea catechins. *Biochim Biophys Acta* **1478:**51–60.

Fan M, Goodwin M, Vu T, Brantley-Finley C, Gaarde WA, and Chambers TC (2000) Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. *J Biol Chem* **275:**29980–2998.

Fisher DE (1994) Apoptosis in cancer therapy: crossing the threshold. *Cell* **78:**539–42.

Fujiki H (1999) Two stages of cancer prevention with green tea. *J Cancer Res Clin Oncol* **125:**589–597.

Gao G and Dou QP (2000) N-terminal cleavage of Bax by calpain generates a potent pro-apoptotic 18 kDa-fragment that promotes Bcl-2-independent cytochrome *c* release and apoptotic cell death. *J Cell Biochem* **80:**53–72.

Green DR and Reed JC (1998) Mitochondria and apoptosis. *Science (Wash DC)* **281:**1309–1312.

Gross A, McDonnel JM, and Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* **13:**1899–1911.

Gupta S, Ahmad N, Mohan RR, Husain MM, and Mukhtar H (1999) Prostate cancer chemoprevention by green tea: in vitro and in vivo inhibition of testosterone-mediated induction of ornithine decarboxylase. *Cancer Res* **59:**2115–2120.

Harrington EA, Fanidi A, and Evan GI (1994) Oncogenes and cell death. *Curr Opin Genet Dev* **4:**120–129.

Horita M, Andreu EJ, Benito A, Arbona C, Sanz C, Benet I, Prosper F, and Fernandez-Luna JL (2000) Blockade of the Bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of Bcl-xL. *J Exp Med* **191:**977–984.

Kessel D (1994) Modes of resistance to antitumor agents. *In Vivo* **8:**829–834.

Kummer JL, Rao PK, and Heidenreich KA (1997) Apoptosis induced by withdrawal of trophic factors is mediated by *p38* mitogen-activated protein kinase. *J Biol Chem* **272:**20490–20494.

Liang YC, Lin-shiau SY, Chen CF and Lin JK (1997) Suppression of extracellular signals and cell proliferation through EGF receptor binding by (−)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J Cell Biochem* **67:**55–65.

Liao S, Umekita Y, Guo J, Kokontis JM, and Hiipakka RA (1995) Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate. *Cancer Lett* **96:**239–243.

Lin YL and Lin JK (1997) (−)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-κB. *Mol Pharmacol* **52:**465–472.

Masuda M, Suzui M, and Weinstein IB (2001) Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression and chemosensitivity in human head and neck squamous cell carcinoma cell lines. *Clin Cancer Res* **7:**4220–4229.

Nam S, Smith DM, and Dou QP (2001) Ester bond-containing tea polyphenols potently inhibit proteasome activity *in vitro* and *in vivo*. *J Biol Chem* **276:**13322–13330.

Nomura M, Kaji A, He Z, Ma WY, Miyamoto K, Yang CS, and Dong Z (2001) Inhibitory mechanisms of tea polyphenols on the ultraviolet B-activated phosphatidylinositol 3-kinase-dependent pathway. *J Biol Chem* **276:**46624–46631.

Pardee AB (1989) G1 events and regulation of cell proliferation. *Science (Wash DC)* **246:**603–608.

Poruchynsky MS, Wang EE, Rudin CM, Blagosklonny MV, and Fojo T (1998) Bcl-xL is phosphorylated in malignant cells following microtubule disruption. *Cancer Res* **58:**3331–3338.

Ren F, Zhang S, Mitchell SH, Butler R, and Young CY (2000) Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. *Oncogene* **19:**1924–1932.

Ripple GH and Wilding G (1999) Drug development in prostate cancer. *Semin Oncol* **26:**217–226.

Sevilla L, Zaldumbide A, Pognonec P, and Boulukos KE (2001) Transcriptional regulation of the bcl-x gene encoding the anti-apoptotic Bcl-xL protein by Ets, Rel/NFkappaB, STAT and AP1 transcription factor families. *Histol Histopathol* **16:**595–601.

Smith DM, Gao G, Zhang X, Wang G, and Dou QP (2000) Regulation of tumor cell apoptotic sensitivity during the cell cycle. *Int J Mol Med* **6:**503–507.

Smith DM, Wang Z, Kazi A, Li LH, Chan TH, and Dou QP (2002) Synthetic analogs of green tea polyphenols as proteasome inhibitors. *Mol Med,* in press.

Takehara T, Liu X, Fujimoto J, Friedman SL, and Takahashi H (2001) Expression and role of Bcl-xL in human hepatocellular carcinomas. *Hepatology* **34:**55–61.

Tang DG and Porter AT (1997) Target to apoptosis: a hopeful weapon for prostate cancer. *Prostate* **32:**284–293.

Tomida A and Tsuruo T (1999) Drug resistance mediated by cellular stress response to the microenvironment of solid tumors. *Anticancer Drug Des* **14:**169–177.

Yang CS (1999) Tea and health. *Nutrition* **15:**946–949.

Yang F, Oz HS, Barve S, de Villiers WJ, McClain CJ, and Varilek GW (2001) The green tea polyphenol (−)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal epithelial cell line IEC-6. *Mol Pharmacol* **60:**528–533.

Zhang XJ, Yan J, Cuttle L, Endre Z, and Gobe G (2000) Escape from apoptosis after prolonged serum deprivation is associated with the regulation of the mitochondrial death pathway by Bcl-x_l. *Biochem Biophys Res Communun* **277:**487–493.

Address correspondence to: Dr. Q. Ping Dou, Drug Discovery Program, H. Lee Moffitt Cancer Center and Research Institute, MRC 1259C, 12902 Magnolia Dr., Tampa, FL 33612-9497. E-mail: douqp@moffitt.usf.edu