Synergistic Cell Death by EGCG and Ibuprofen in DU-145 Prostate Cancer Cell Line

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Abstract. *Background: One of the green tea components epigallocatechin-3-gallate (EGCG) significantly prevented the growth of prostate cancer cells. In this study, synergistic effect of EGCG and ibuprofen (EGCG+ibuprofen) was investigated to determine their anti-proliferative and pro-apoptotic action in DU-145 prostate cancer cells. Materials and Methods: Cell death analysis, immunoblotting, RT-PCR analysis, and caspase activity assay were used. Results: EGCG+ibuprofen treatment resulted in 90% growth inhibition, while ibuprofen or EGCG alone reduced cell numbers by 25% and 20%, respectively. EGCG+ibuprofen induced MAPK activation, caspase activation and the inhibition of Bfl-1 expression, all of which were blocked by the antioxidant, N-acetyl-L-cysteine (NAC). Moreover, addition of ceramide rescued the NAC-inhibited MAPK activation and pretreatment with the ceramide synthase inhibitor, fumonisin B1, reduced cell death. Conclusion: Our results suggest that in DU-145 prostate cancer cells: (i) EGCG+ibuprofen treatment has a synergistic effect on apoptosis, and (ii) oxidative stress, directly or indirectly via ceramide synthesis mediates pro-apoptotic signaling.*

In the United States, prostate cancer (PCA) is the most common non-skin malignancy in men over 65 years of age and the second biggest cause (after lung) of cancer related death in males (1). The American Cancer Society estimates that there will be 218,890 new cases and 27,050 deaths in 2007 (2).

Epigallocatechin-3-gallate (EGCG) is the epicatechin component found at highest concentration in green tea leaves and has recently been studied intensively as an anticarcinogenic and antiangiogenic agent (3-5). Numerous studies have demonstrated that EGCG treatment of prostate carcinoma cells, both hormone-sensitive LNCaP cells and hormone-insensitive DU-145 and PC-3 cells, results in the induction of apoptosis (5, 6). EGCG treatment of androgen-sensitive LNCaP and androgen-insensitive DU-145 resulted in G_0/G_1 cell cycle arrest and induction of apoptosis (40-50% of cells) in a dose- and time-dependent manner (40-80 μg/ml and 48 h) (7).

An increasing number of studies have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) are effective in the prevention and treatment of many common cancers including prostate cancer (8-10). Ibuprofen, a non-selective NSAID, also effectively reduced cell proliferation and induced apoptosis in *in vitro* studies (LNCaP and DU-145 cells) (11) as well as enhancing the radiation response of prostate cancer cells in *in vitro* and *in vivo* xenograft models (12).

The Bcl-2 family proteins are key regulators of apoptosis, either inhibiting or promoting it (13). They include the anti-apoptotic family members Bcl-2, Bcl-X_L, Mcl-1_L and Bfl-1, and the pro-apoptotic members Bax, Bcl-Xs, Mcl-1s, Bak, Bik and Bid. By physical interaction with each other, the Bcl-2 family proteins are capable of forming a complex network of homo- and heterodimers *via* molecular regions named Bcl-2 homology (BH) domains (BH-1, BH2, BH3, BH4). These physical interactions sometimes play important roles in the net effects of pro- and antiapoptotic members of the family. Bfl-1/A1 possesses anti-apoptotic activity and localizes in the mitochondria, while its splicing variant, Bfl-1s, localizes to the nucleus *via* its distinct C-terminus created by alternative splicing. Both Bfl-1 and Bfl-1s act as anti-apoptotic proteins (14). It has become well established that many inducers of apoptosis activate caspases, and that the activation of these proteases is perhaps the point of irreversible commitment to the onset of apoptosis (15).

Studies linked the pro-oxidant property of EGCG to its induction of cell death in colon (16) and pancreatic cancer cells (17). Others showed that oxidative stress increased intracellular ceramide accumulation in lung epithelium (18, 19). Ceramide is a novel lipid second messenger, mediating

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the intracellular effects of various inducers to activate apoptosis program (20).

In this study, we determined the synergistic effect and the molecular mechanism of EGCG and ibuprofen on the apoptosis of cells of the DU-145 hormone-insensitive prostate cancer cell line. In addition, the expression of the antiapoptotic protein, Bfl-1/A1, was determined in these prostate cancer cells, and the effect of its inhibition by EGCG+ibuprofen.

Materials and Methods

Reagents. EGCG, ibuprofen and antibody to β-actin were purchased from Sigma (St. Louis, MO, USA). *N*-acetyl-L-cystein (NAC), fumonisin B1 (FB1), catalase, C_6-ceramide and caspase-9 specific antibody were purchased from EMD Biosciences (La Jolla, CA, USA). Phospho-specific and total antibodies against p42/44 MAPK and p53 were purchased from Cell Signaling (Danvers, MA, USA) and from Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

Cell culture. DU-145 cell line was obtained from ATCC (Manassas, VA, USA) and was maintained in culture medium (CM) containing Dulbecco's modified Eagle's medium (DMEM) (Invitrogen/Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin sulfate and 100 unit/ml penicillin G sodium. All cultures were maintained in a humidified 5% CO_2 incubator at 37˚C, and routinely passed when 80% to 90% confluent.

WST assay for cell proliferation. WST (Roche, Indianapolis, IN, USA) is a water-soluble tetrazolium salt that is cleaved to formazan only in viable cells. The formazan dye formed was quantified using an ELISA microplate reader ($Elx808_{IU}$ UltraMicroplate Reader, Bio-Tek Instruments, Inc, Winooski, VT, USA) and is directly related to the number of viable cells present in a well. DU-145 cells were seeded at 5,000 cells per well in 96-well plates and treated with none, EGCG (20 or 40 μg/ml), ibuprofen (1 mM), or EGCG (40 μg/ml)+ibuprofen (1 mM) for 1-2 days, followed by WST assay according to the manufacturer's protocol (Roche, Indianapolis, IN, USA). A standard curve was drawn from the WST value of known numbers of cells and the cell number of each WST value was calculated from the standard curve equation.

Nuclear staining assay. DU-145 cells were treated with none, ibuprofen 1 mM, EGCG 40 μg/ml, or EGCG (40 μg/ml) +ibuprofen (1 mM) for 24 h. Cells were then fixed in 4% formalin in PBS, permeabilized in methanol and mounted on slides with mounting medium containing 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI). Nuclear staining was seen under a fluorescence microscopy.

DNA histogram analysis. A quantitative measure of the percentage of apoptotic cells was determined by flow cytometric analysis of DNA histograms according to Darzynkiewicz *et al.* (21) with some modifications. Briefly, DU-145 cells were seeded in CM at 700,000 cells per 60 mm dish and allowed to adhere overnight. Cells were then treated with none, ibuprofen 1 mM, EGCG 40 μg/ml, or EGCG 40 μg/ml + ibuprofen 1 mM. To inhibit oxidation, MAPK, or p53, cells were pretreated with inhibitors (NAC 5 mM, PD98059 25 μM or pifithrin-α 2 or 10 μM, respectively) for 1 h prior to the addition of EGCG 40 μg/ml + Ibuprofen 1 mM. Cells were harvested on day 2 for DNA histogram analysis by flow cytometry. Cells were washed once with ice-cold PBS and fixed in 70% EtOH. After rehydration in PBS, cells were incubated in DNA extraction buffer (192 mM Na_2HPO_4, 4 mM citric acid, pH 7.8) for 5 min. After washing in PBS, cells were labeled in DNA staining solution (RNase A at 20 μg/ml and propidium iodide at 20 μg/ml in PBS). Cell fluorescence was measured in a Cytomics FC 500 Flow Cytometer (Beckman Coulter Corp., Miami, FL, USA). A minimum of 10^4 cells/sample were analyzed. Experiments were repeated twice.

Trypan blue staining and cell counting. This protocol was modified from Chen *et al.* (16). After drug treatment with none, ibuprofen 1 mM, EGCG 40 μg/ml, or EGCG 40 μg/ml + ibuprofen 1 mM for 2 days, DU-145 cells were harvested by trypsinization, resuspended in PBS, and mixed with 0.4% trypan blue staining solution at a 1:1 ratio. Cells were counted on a hemocytometer. Unstained cells were counted as viable cells and stained blue cells as dead cells. The percentage of dead cells was calculated as the ratio between the number of stained cells and the total cell counts. To inhibit oxidation or ceramide generation, cells were pretreated with NAC 5 mM, catalase 5,000 units/ml, or FB1 100 μM for 1 h prior to the addition of EGCG 40 μg/ml + ibuprofen 1 mM. Experiments were repeated three times in triplicates per treatment per experiment.

Lysate preparation. For whole cell lysates, DU-145 cells were treated with none, ibuprofen 1 mM, EGCG 40 μg/ml, or EGCG 40 μg/ml + ibuprofen 1 mM and harvested at 4, 8, 16, and 24 h by scraping and washed in ice-cold PBS and lysed in M-PER reagent (100 μl/10^6 cells) (Pierce Chemical, Rockford, IL, USA) supplemented with 2 mM Na_3OV_4, 50 mM NaF and protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After 15 min incubation on ice, lysates were cleared by centrifugation at 10,000 rpm, at 4˚C. The resulting lysates were stored at –80˚C until needed. Protein concentration was measured by bicinchoninic acid (BCA) assay (Sigma, St. Louis, MO, USA). To inhibit oxidation or MAPK, cells were pretreated with NAC 5 mM or PD98059 25 μM, respectively, for 1h prior to the addition of EGCG 40 μg/ml + ibuprofen 1 mM. Cell lysates were prepared as described above.

Immunoblot analysis. Whole cell lysates (25-50 μg of protein) were separated on a 7.5% SDS polyacrylamide gel or 4-20% gradient gel (Pierce) under reducing conditions and then transferred to a PVDF membrane. The membrane was blocked for 1 h at room temperature (RT) in T-PBS, pH 7.5 (PBS with 0.1% Tween-20) with 5% non-fat dry milk. After washing, the blot was incubated with primary antibodies for 1 h at RT. The blot was washed 3 times in T-PBS and incubated with HRP-coupled secondary antibody (Pierce) for 1 h at RT. After extensive washing, the bands were detected using SuperSignal West Femto Maximum Sensitivity Substrate System (Pierce). The resulting chemiluminescence was visualized by a UVP BioImaging System (UVP, LLC, Upland, CA, USA) equipped with a CCD camera. Biotinylated protein standard (Bio-Rad, Hercules, CA, USA) and streptavidin-HRP conjugates were used to estimate protein MW on the blot. After stripping, the blot was reprobed with β-actin antibody to determine the equal loading of proteins. Experiments were repeated at least twice using lysates prepared at two different times with the same treatment.

RNA isolation and RT-PCR. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) isolated from cells was reverse transcribed to cDNA using oligo-dT and random primers. The cDNA was amplified by PCR using an ApoPrimer set for Bcl-2 family (Takara Bio Inc., Shiga, Japan). The set contains primer pairs for mcl-1, bfl-1, bax, bcl-2, bak, bik and bcl-x. The PCR was performed according to the manufacturer's instruction. The amplified products were visualized on 1% agarose gels. The RT-PCR was repeated at least three times using cDNAs prepared at three different times.

Caspase activity assay. DU-145 cells were seeded in CM (with 10% FBS) at 500,000 cells per 60 mm dish and allowed to grow for 2 days. Cells were treated with none, ibuprofen 1 mM, EGCG 40 μg/ml, or EGCG 40 μg/ml + ibuprofen 1 mM. Cell lysates were prepared in M-PER reagent (Pierce) on day 2 for caspase assay using Fluorometric Homogeneous Caspases Assay kit (Roche) with modification. Briefly, cell lysates were diluted 1:10 with the incubation buffer provided in the Caspase Assay kit and aliquoted 100 μl per well (triplicate wells per sample) in a 96-well plate. Freshly prepared substrate (DEVD-R110, Roche) solution was then added to each well. Fluorescence was measured at 60, 120 and 180 min with an excitation at 499 nm and emission at 521 nm in a SpectraMAX GeminiXS fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA). The protein concentration was measured by BCA assay. The measured relative fluorescence unit (RFU) was normalized to the protein amount and caspase activity was represented as RFU per μg of protein in each sample. Experiments were repeated twice in triplicates per treatment per experiment.

Statistical analysis. The percentage of apoptosis is expressed as mean ± standard deviation. The significance values between the control and treated groups were calculated by one-way analysis of variance (ANOVA) using InStat software (Graphpad, San Diege, CA, USA), and *p*-values less than 0.05 were taken as significant in all the experiments.

Results

Synergistic effect of EGCG+ibuprofen on the growth inhibition and apoptosis. In DU-145 cells (Figure 1a), treatment with ibuprofen 1 mM alone for 24 h and 48 h reduced cell numbers by 15% and 25%, respectively, compared to that of control cells. EGCG at 40 μg/ml alone also reduced cell numbers by 20% with 24 h and 48 h treatment. On the other hand, the combination of EGCG (40 μg/ml) + ibuprofen (1 mM) (EGCG+ibuprofen) augmented the growth inhibitory effect of ibuprofen or EGCG alone, resulting in 60% and 90% growth inhibition at 24 h and 48 h, respectively.

Next, we investigated if the growth inhibition of prostate cancer cells was due to cell death by examining the effect of EGCG and ibuprofen on cell morphology with DAPI nuclear dye staining (Figure 1b). Increased nuclear fragmentation and condensation implicating apoptosis was observed in cells treated with EGCG+ibuprofen (Figure 1b (iv)).

Table I. Effect of EGCG and ibuprofen, alone or in combination, on DU-145 cells. EGCG+ibuprofen synergistically induce apoptosis in DU-145 cells. % of cells present in sub G_0 population of DNA histogram analysis were considered apoptotic and shown here. The data are from two independent experiments.

DU-145	% Apoptosis on day 2	
Experiment	1	2
Control	5.3	5.2
Ibu 1 mM	13.2	11.6
EGCG 20 μg/ml	5.7	5.4
EGCG 20+Ibu 1	23.4	24.5
EGCG 40 μg/ml	5.3	5.4
EGCG 40+Ibu 1	27.9	29.9

We also analyzed cell death by DNA histogram assays in which apoptotic cells appeared in the subG_0 population (Table I). As shown in Table I, synergistic effect of ibuprofen on EGCG-mediated cell death was observed. While control cells showed 5.3% death, EGCG+ibuprofen treatment increased cell death to 27.9% on day 2 of treatment, compared to 13.2% with ibuprofen and 5.3% with EGCG 40 μg/ml alone (experiment 1). Experiment 2 showed comparable results.

Taken together, these results clearly demonstrated that ibuprofen exerted synergistic growth inhibitory effect with EGCG on prostate cancer cells, which was in part due to cell death induced by the EGCG+ibuprofen treatment.

Regulation of Bcl-2 family proteins by EGCG+ibuprofen treatment. We investigated the expression of Bcl-2 family of proteins by RT-PCR analysis in DU-145 cells (Figure 2). DU-145 cells do not express Bcl-2, Bax, or Mcl-1 proteins as determined by our RT-PCR analysis and by other studies (22, 23). RT-PCR analysis showed no change in the mRNA expression of Bcl-X (long and short forms) or Bak in EGCG+ibuprofen-treated cells (Figure 2b).

However interestingly, the EGCG+ibuprofen treatment inhibited the mRNA expression of anti-apoptotic protein Bfl-1 as determined by RT-PCR analysis (Figure 2b). Neither EGCG nor ibuprofen alone had a significant effect on the mRNA expression of Bfl-1 (Figure 2a).

MAPK activation by EGCG+ibuprofen treatment. We investigated the effect of EGCG+ibuprofen treatment on the p42/44 MAP kinase (MAPK) signaling pathways and p53 protein in DU-145 prostate cancer cells by immunoblotting analysis (Figure 3).

Most interestingly, we detected two stages of MAPK regulation with the EGCG+ibuprofen treatment (Figure

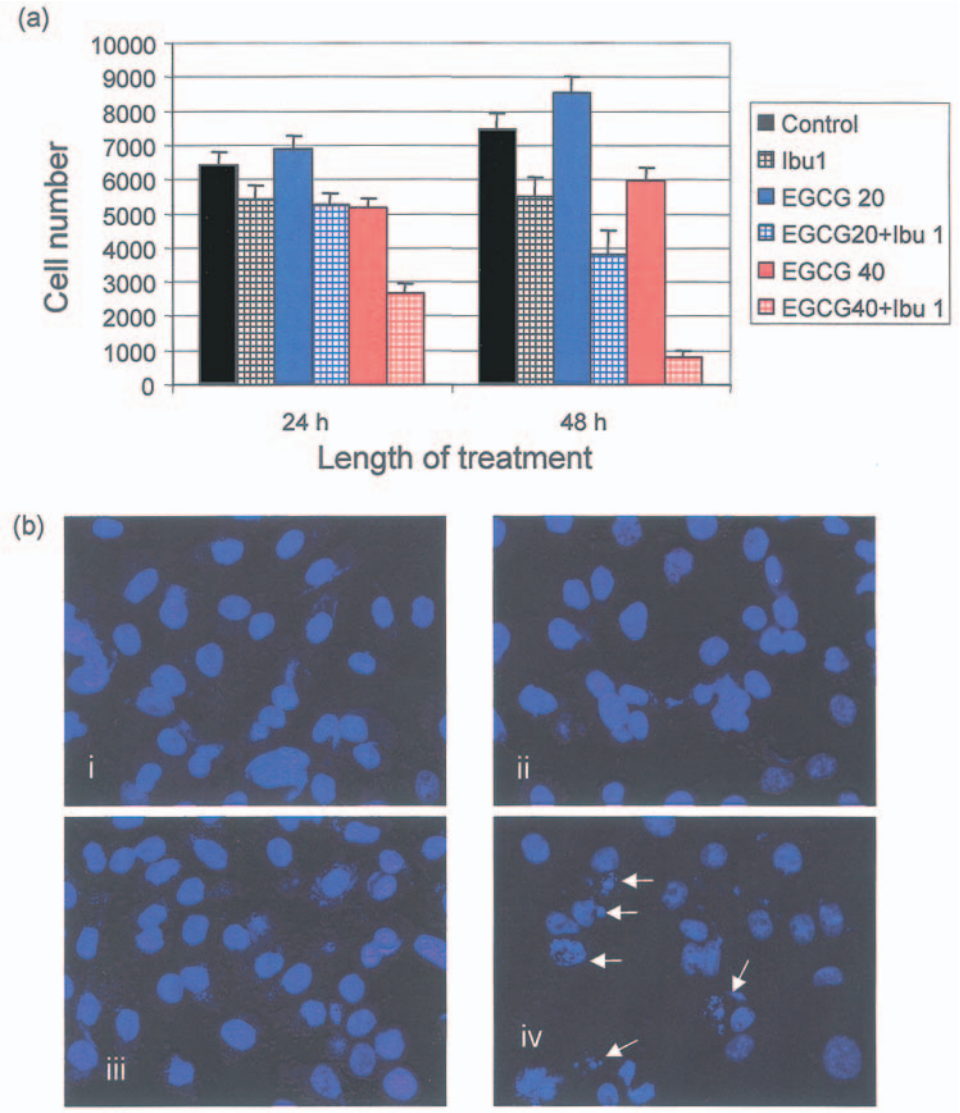
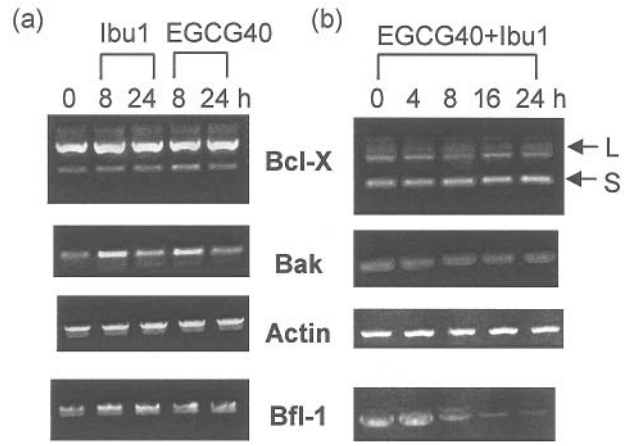


Figure 1. *EGCG+ibuprofen synergistically inhibited cell proliferation of DU-145 cells. (a) Cell proliferation assay. Cells were seeded at 5,000 cells per well in 96-well plates and treated with the indicated doses of ibuprofen, EGCG, or both. The data represent the means from three independent experiments, each performed in triplicate; bars, Standard Deviation. (b) Induction of apoptosis. DU-145 cells were treated with none (i), ibuprofen 1 mM (ii), EGCG 40 μg/ml (iii), or EGCG+ibuprofen (iv) for 24 h. Arrows indicate apoptotic cells with condensed and fragmented nuclei.*

3a). Compared to that of control samples at 0 h, the phosphorylation of MAPK decreased at 4-8 h, but increased at 16-24 h after treatment with EGCG+ibuprofen. On the other hand, EGCG alone or ibuprofen alone reduced the phosphorylation of MAPK, but was unable to reactivate these kinases at later times (Figure 3b).

We also determined the increase in the Ser15 phosphorylation of p53 protein with the concomitant increase of total p53 protein by EGCG+ibuprofen treatment (Figure 3c), while no significant increase in Ser15 phosphorylation or total protein of p53 was found with EGCG or ibuprofen alone (Figure 3d).

Oxidative stress and ceramide on EGCG+ibuprofen induced cell death. As shown in Figure 4, anti-oxidant NAC pre-treatment completely abolished EGCG-induced cell death, while no effect was seen in ibuprofen-treated cells. Moreover, NAC pre-treatment significantly reduced cell death in EGCG+ibuprofen treated cells (11.7% cell death in NAC pretreated *vs.* 24.7% in non-NAC pretreated cells). This result indicated that oxidative stress was involved in EGCG- and EGCG+ibuprofen-induced cell death in DU-145 cells. To further investigate the oxidative stress caused by EGCG, we added catalase to the DU-145 cell culture prior to EGCG- and EGCG+ibuprofen treatment. Catalase



Figrure 2. *RT-PCR analysis of Bcl-2 family of protein expression in DU-145 cells. Cells were treated with (a) ibuprofen 1 mM (Ibu1), EGCG 40 μg/ml (EGCG40), or (b) both (EGCG40+Ibu1) for the indicated times and harvested for total RNA preparation and cDNA synthesis. RT-PCR analysis of Bcl-2 family of proteins was performed as described in Materials and Methods. The RT-PCR results are representative of at least three separate experiments.*

neutralizes the oxidative effect of H_2O_2. The results showed that the addition of catalase completely blocked EGCG- and H_2O_2-induced cell death (Table II). It significantly reduced DU-145 cell death by EGCG+ibuprofen treatment, but was unable to block cell death completely. Neither catalase nor NAC had any significant effect on ibuprofen-induced cell death. Interestingly, the pretreatment of fumonisin B1 (FB1), a fungal toxin and ceramide synthase inhibitor (24), significantly reduced EGCG+ibuprofen-induced cell death, suggesting a role of ceramide generation in cell death.

Role of oxidative stress in the MAPK signaling pathway and p53 protein. We investigated whether oxidative stress after EGCG+ibuprofen treatment led to MAPK activation and p53 phosphorylation. NAC pretreatment prevented EGCG+ibuprofen-induced MAPK activation at 16 h (Figure 5a, p-p42/44), suggesting a role of oxidative stress in MAPK activation. However, NAC pretreatment did not prevent the Ser15 phosphorylation of p53 protein (Figure 5a, p-p53-ser15, marked by arrow) or the increase of total p53 protein (Figure 5a, t-p53). DNA histogram analysis showed that NAC pretreatment reduced the number of the apoptotic cells in the EGCG+ibuprofen treated group (Figure 5b). Thus this result suggested that cell death prevention by NAC pretreatment was associated with the inhibition of MAPK activation, but not with Ser15 phosphorylation of p53 protein.

Role of MAPK activation in EGCG+ibuprofen-induced apoptosis. Immunoblot analysis (Figure 6a, p-p42/44) showed that pretreatment with PD98059, a pharmacological

Table II. *Effect of catalase pretreatment on DU-145 cell death induced by EGCG, ibuprofen or their combination.*

Experiment	1		2	
Pretreatment	None	Catalase	None	Catalase
Treatment				
Control	4.96	4	3	4.8
Ibu 1 mM	12	7.57	7.5	6.7
EGCG 40 μg/ml	6.9	4.6	4.9	3.3
EGCG 40+Ibu 1	28.3	13	32	15.6
H2O2- 250 μM	34.8	2.9	32.5	4.8

Pretreatment with catalase reduced DU-145 cell death in response to EGCG+ibuprofen. Cells treated with 250 μM H_2O_2 acted as control. The percentage cell death was determined by traypan blue staining as described in Materials and Methods.

inhibitor of MAPK, 1 h prior to EGCG+ibuprofen treatment abolished MAPK activation by EGCG+ibuprofen treatment and reduced cell death by 50% compared to EGCG+ibuprofen treatment (Figure 6b). However, the addition of PD98059 alone increased Ser15 phosphorylation of p53 but did not prevent the Ser15 phosphorylation of p53 protein at 16 h in EGCG+ibuprofen-treated cells (Figure 6a, p-p53-ser15).

The role of p53 phosphorylation in EGCG+ibuprofen-induced cell death. As shown in Figure 7, pifithrin-α, a relatively specific p53 inhibitor (25), was not able to prevent cell death by EGCG+ibuprofen treatment as determined by DNA histogram analysis.

Role of oxidative stress and MAPK in Bfl-1 expression and caspase activation. To further determine the mechanism of oxidative stress and MAPK in cell death, we investigated their roles in the expression of anti-apoptotic protein, Bfl-1 in DU-145 cells treated with EGCG+ibuprofen.

As shown in Figure 8a, EGCG+ibuprofen treatment down-regulated Bfl-1 mRNA (lane 2). On the other hand, pretreatment with NAC or PD98059 prevented the down-regulation of Bfl-1 mRNA (lanes 3 and 4, respectively).

Figure 8b showed that EGCG+ibuprofen treatment activated pro-caspase-9 (46 kDa, closed arrow) in 24 h lysates of DU-145 cells resulting in the appearance of the cleaved form (36 kDa, open arrow). We also determined caspase activity in EGCG+ibuprofen treated cells. As shown in Figure 8c, caspase activity increased in EGCG+ibuprofen-treated cells by approximately 2-fold as compared to that of control cells. On the other hand, NAC pretreatment prevented caspase activation and abrogated caspase activity induced by EGCG+ibuprofen treatment.

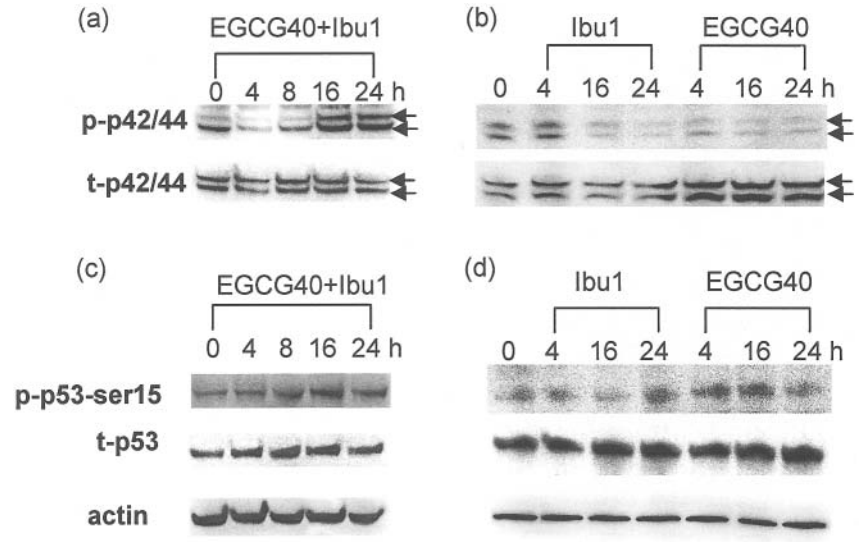


Figure 3. *MAPK and p53 activation by EGCG+ibuprofen treatment. Cell lysates were prepared from EGCG+ibuprofen treated cells (a) and (c) or from ibuprofen 1 mM (Ibu 1) or EGCG 40 μg/ml (EGCG40) treated cells (b) and (d) at the indicated times. Immunoblotting was performed using phosphorylation specific antibodies to p42/44 MAP kinase (p-p42/44) (a) and (b), or to Ser15-p53 (p-p53-ser15) (c) and (d) in DU-145 cells. The same blots were stripped and re-probed with antibodies recognizing total kinases (t-p42/44), total p53 (t-p53) or with actin to confirm equal loading of proteins. Each blot is a representative from 3 independent blots of 3 independents experiments with similar results.*

Role of ceramide in EGCG+ibuprofen-induced cell death. Our results suggested that EGCG+ibuprofen treatment might generate ceramide which could play a role in apoptosis (Figure 4). Our results also showed that EGCG+ibuprofen caused oxidative stress in DU-145 cells (Figures 4 and 5). As shown in Figure 9a, EGCG+ibuprofen treatment activated MAPK signaling at 16 h treatment which was abrogated by NAC pretreatment. Interestingly, addition of ceramide was able to rescue the MAPK activation even in the presence of NAC. Moreover, ceramide treatment inhibited the expression of Bfl-1 in DU-145 cells (Figure 9b).

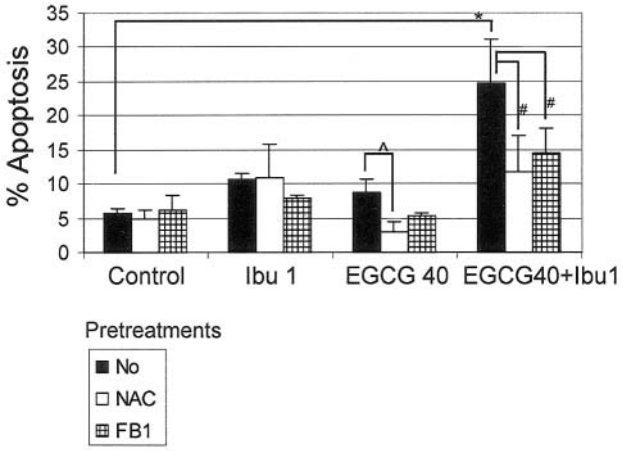


Figure 4. *Pretreatment with antioxidant NAC or ceramide synthase inhibitor, FB1 reduced DU-145 cell death in response to EGCG+ibuprofen. DU-145 cells were pretreated with 5 mM NAC or 100 μM FB1 for 1 h prior to treatment with none, ibuprofen 1 mM, EGCG 40 μg/ml, or both for 48 h. The percentage of cell death was calculated using trypan blue staining as the ratio between dead cells (stained blue) and the total cell counts. Closed bar, no pretreatment; open bar, NAC at 5 mM pretreatment; hatched bar, FB1 at 100 μM pretreatment. The error bars indicate the standard deviation. *p<0.01 vs. none (closed bar); ^p<0.01 vs. EGCG 40 (closed bar); #p<0.05 vs. EGCG40+Ibu1 (closed bar).*

Discussion

Although progress is being made, no chemotherapeutic agent or regimen has been able to demonstrate an improvement in survival of patients with hormone-refractory prostate cancer (26). Numerous *in vitro* and *in vivo* studies have demonstrated the anticarcinogenic and antiangiogenic properties of green tea, most noticeably regarding its main component EGCG (5, 27). In addition, ibuprofen, a non-selective NSAID, was shown to be effective in the inhibition of prostate cancer cell growth *via* the inhibition of NF-κB activity (28). In this study, we determined that the combination of EGCG and ibuprofen (EGCG+ibuprofen) exerted synergistic effect on growth inhibition and apoptosis of prostate cancer cell, DU-145. We also determined for the first time the expression of anti-apoptotic protein Bfl-1/A1 in prostate cancer cells, DU-145. Our study determined that the apoptotic effects of EGCG+ibuprofen were attributed to the inhibition of Bfl-1 expression, activation of MAPK and caspase activation. These pro-apoptotic events were

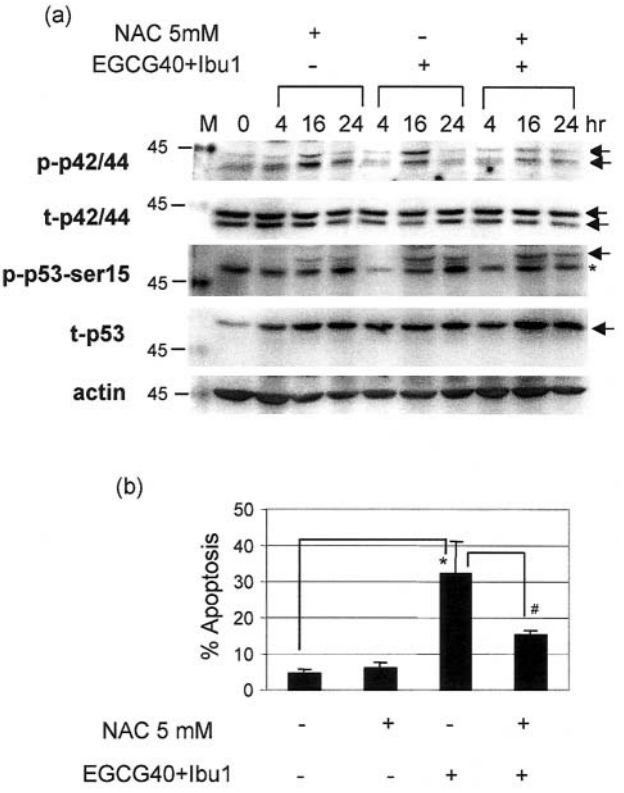


Figure 5. *Effect of antioxidant NAC on EGCG+ibuprofen-activated MAPK and p53 signals and cell death. (a) NAC (5 mM) was added into the media 1 h before EGCG (40 μg/ml) + ibuprofen (1 mM) (EGCG40+Ibu1) treatment. Immunoblotting was performed as described in Materials and Methods. Arrows indicate specific bands on each blot. *indicates non-specific band on phospho-p53 blot. (b) Cell death was determined by DNA histogram analysis as described in Materials and Methods. The error bars indicate the standard deviation. *p<0.001 vs. none; # p<0.01 vs. EGCG40+Ibu1.*

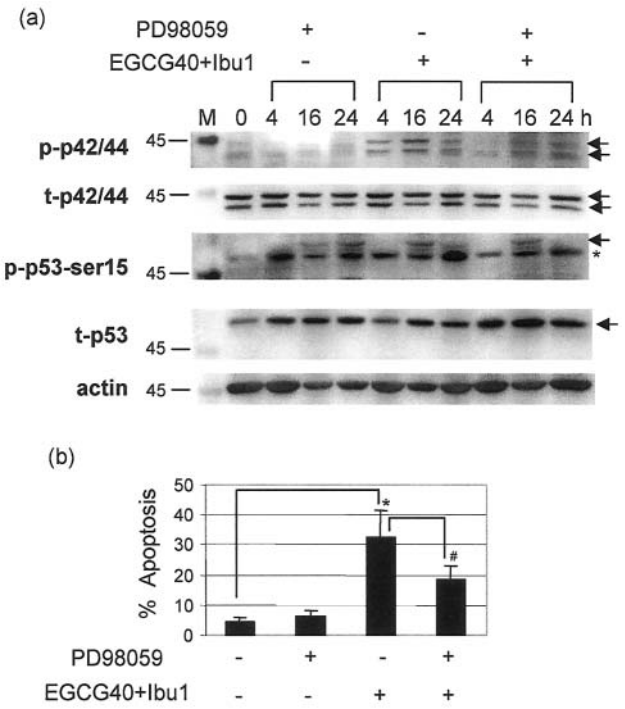


Figure 6. *Effect of MAPK inhibition on EGCG+ibuprofen-activated MAPK and p53 signals and cell death. PD98059 (25 μM) was added into the media either 1 h before EGCG (40 μg/ml) + ibuprofen (1 mM) (EGCG40+Ibu1) treatment. (a) Immunoblotting was performed as described in Materials and Methods. Arrows indicate specific bands on each blot. *indicates non-specific band on phospho-p53 blot. (b) Cell death was determined by DNA histogram analysis as described in the Materials and Methods. The error bars indicate the standard deviation. *p<0.001 vs. none; #p<0.01 vs. EGCG40+Ibu1.*

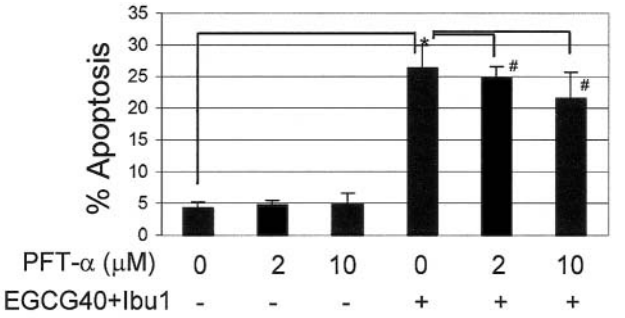


Figure 7. *Effect of p53 inhibition on cell death. Pifithrin-α (PFT-α) at 2 μM or 10 μM was added to the media 1 h before EGCG (40 μg/ml) + ibuprofen (1 mM) (EGCG40+Ibu1) treatment. Cell death was determined by DNA histogram analysis as described in Materials and Methods. The error bars indicate the standard deviation. *p<0.001 vs. none; #p>0.05 vs. EGCG40+Ibu1.*

most likely mediated by oxidative stress, either directly or indirectly *via* ceramide generation.

Our results argue for the pro-apoptotic role of MAPK activation in EGCG+ibuprofen-induced cell death. We speculated that the activation of MAPK played an important role in synergistic induction of apoptosis by EGCG+ibuprofen treatment. First, separate treatment with EGCG or ibuprofen alone inhibited the phosphorylation of p42/44 MAPK, but did not re-activate its phosphorylation at a late stage. Secondly, pre-treatment with PD98059, a MAPK inhibitor, reduced cell death and prevented Bfl-1 down-regulation by EGCG+ibuprofen, and this was related to the inhibition of MAPK activation in DU-145 cells. Thus these data strongly argue for the role of MAPK activation in the apoptosis induction of DU-145 cells treated with EGCG+ibuprofen. In a previous study, resveratrol treatment induced MAPK activation which mediated pro-apoptotic signaling *via* p53 protein phosphorylation at Ser15 (29). Inhibition of MAPK and p53 protein reduced resveratrol-induced cell death.

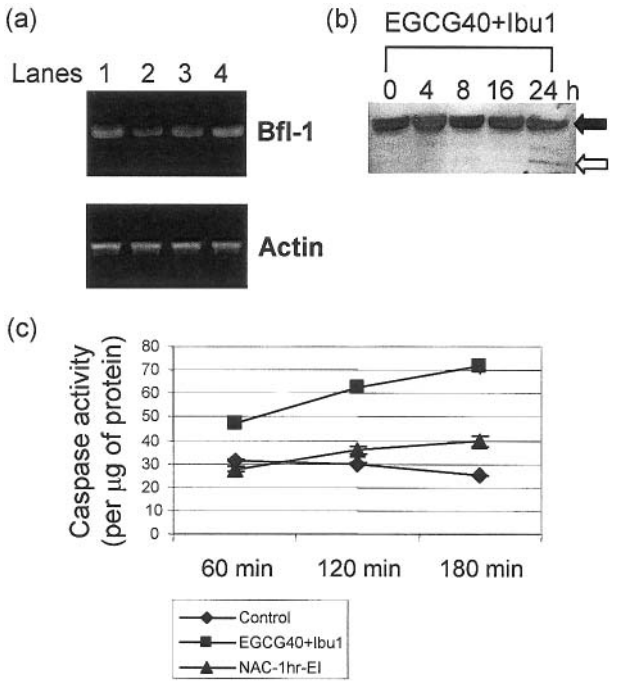


Figure 8. *Regulation of Bfl-1 expression and caspase activation. (a) Bfl-1 expression. DU-145 cells were pretreated with none (lane 2), 5 mM NAC (lane 3) or 25 μM PD98059 (lane 4) for 1 h prior to treatment with EGCG+ibuprofen for 48 h (lanes 2-4). Vehicle-treated cells served as control (lane 1). Bfl-1 expression was determined by RT-PCR analysis as described in Materials and Methods. (b) Immunoblot analysis of caspase-9. Cell lysates were prepared from EGCG+ibuprofen treated cells at the indicated times and immunoblotted using anti-caspase-9 antibody which recognizes pro-caspase-9 at 47 kDa (closed arrow) and cleaved caspase-9 at 35 kDa (open arrow). (c) Inhibition of caspase activation by NAC pretreatment. Caspase activity was determined by incubation of lysate (10 μl) with fluorogenic substrate, DEVD-R110 at 37˚C. The release of fluorescence was measured at 60, 120, and 180 min in a fluorescence microplate reader (excitation=499 nm; emission=521 nm). The fluorescence intensity was normalized to the protein amount present in 10 μl of lysate. The caspase activity was represented as RFU per μg of protein per sample.*

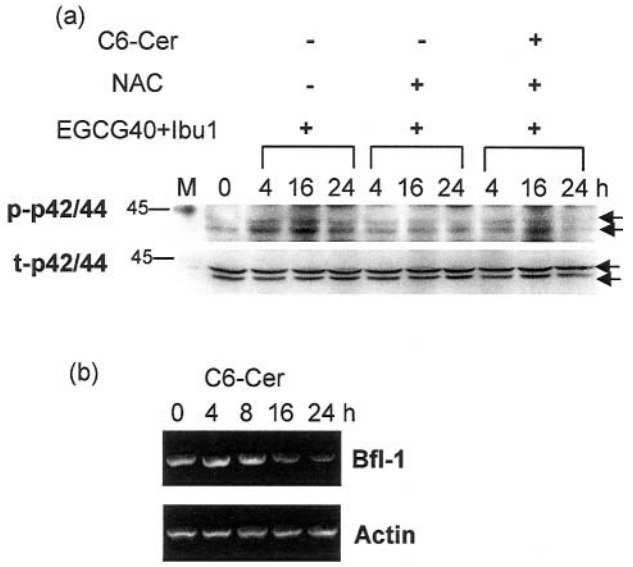


Figure 9. *Ceramide activated MAPK and inhibited Bfl-1 expression. (a) Immunoblot analysis of MAPK. NAC (5 mM) or vehicle was added to DU-145 cells 1 h before EGCG+ibuprofen treatment. Ceramide (C6-Cer, 25 μM) was then added 1 h after EGCG+ibuprofen. Cell lysates were prepared at the indicated times after EGCG+ibuprofen addition and analyzed for MAPK as described in Materials and Methods. (b) RT-PCR analysis of Bfl-1 expression. Cells were treated with ceramide (C6-Cer) at 25 μM for the indicated times and harvested for total RNA preparation and cDNA synthesis. RT-PCR analysis of Bfl-1 was performed as described in Materials and Methods. The RT-PCR results are representative of at least three separate experiments.*

We also determined an increase of Ser15 phosphorylation of p53 protein in EGCG+ibuprofen-treated cells along with an increase in total p53 protein. However, antioxidant NAC or PD98059 pretreatment did not prevent p53 phosphorylation at Ser15 by EGCG+ibuprofen treatment. Moreover, a p53-specific inhibitor, pifithrin-α, pretreatment had no significant effect on reducing the cell death induced by EGCG+ibuprofen. Taken together, these data suggest that p53 protein might not be an important contributor in EGCG+ibuprofen-induced cell death, although we could not completely exclude the possibility of its apoptotic role.

Bfl-1 is an anti-apoptotic Bcl-2 family member and preferentially expressed in hematopoietic cells and endothelium (30). It is known that Bfl-1 is not expressed constitutively, but induced by stimuli that activate NF-κB (31). We speculate that constitutive activation of NF-κB (28) may be responsible for Bfl-1 expression in DU-145 cells. Nevertheless, EGCG+ibuprofen treatment down-regulated the expression of Bfl-1, likely mediated by NF-κB inhibition (28). Pretreatment with NAC and PD98059 prevented Bfl-1 down-regulation by EGCG+ibuprofen, suggesting that oxidative stress and MAPK activation played a role in down-regulation of Bfl-1.

Caspases function as initiators and effectors in the apoptosis process. After proteolytic activation from procaspases, activated caspases specifically cleave the target proteins after aspartic acid, which then leads to inhibition of protein activities, dismantling of the cytoskeletal and cellular structure, and eventually cell death (15). Our study determined that EGCG+ibuprofen treatment resulted in the cleavage of caspase-9 to its activated form and hence the induction of caspase activity in DU-145 cells. Pretreatment with antioxidant NAC significantly reduced the caspase activity observed in EGCG+ibuprofen-treated cells. Thus, these data suggested that oxidative stress played a role in caspase activation which in turn contributed to the cell death of DU-145 cells.

Oxidative stress played an important role mediating apoptotic signaling from EGCG+ibuprofen treatment in

prostate cancer cells. Our data showed that pretreatment with antioxidant, NAC, prevented activation of MAPK, and caused down-regulation of Bfl-1 expression and caspase activation by EGCG+ibuprofen. Previous studies showed that EGCG as a potential pro-oxidant caused cell death in colon (16) and pancreatic cancer cells (17), which was blocked by anti-oxidant NAC pretreatment. It has been shown that EGCG may induce the production of hydrogen peroxide (H_2O_2) in the culture media (32). Indeed, pretreatment with catalase in our study reduced EGCG-induced apoptosis in DU-145 cells, suggesting that oxidative stress caused by EGCG+ibuprofen might be related to the generation of H_2O_2. Thus these result strongly suggested that oxidative stress involving H_2O_2 production contributed to EGCG+ibuprofen induced cell death, but we cannot rule out that other intermediates of oxidative stress may also contribute to EGCG+ibuprofen-induced cell death.

Others showed that H_2O_2-caused oxidative stress increased ceramide accumulation, leading to apoptosis of lung epithelium (18, 19). Our results also argued for the role of ceramide in EGCG+ibuprofen-induced cell death. First, pretreatment with fumonisin B1 (FB1), a fungal toxin and ceramide synthase inhibitor (24), reduced cell death, suggesting that EGCG+ibuprofen may increase ceramide generation by a *de novo* synthesis pathway. Secondly, addition of ceramide rescued the inhibition of EGCG-ibuprofen-induced activation of MAPK by NAC pretreatment. Lastly, ceramide treatment inhibited the expression of the anti-apoptotic protein, Bfl-1, in DU-145 cells. Thus, we speculate that ceramide may be a second messenger in pro-apoptotic signaling initiated by EGCG+ibuprofen-induced oxidative stress.

Taken together, our results from this study showed that EGCG+ibuprofen treatment elicits a synergistic effect on the apoptosis of hormone-refractory prostate cancer DU-145 cells. In addition, we report, for the first time, the expression of anti-apoptotic protein, Bfl-1/A1, in prostate cancer cells. The pro-apoptotic signal was mediated by oxidative stress which either directly or indirectly *via* ceramide generation, activated MAPK, leading to the down-regulation of Bfl-1 and activation of caspases.

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