

The Heat Shock Protein 90 Inhibitor, (–)-Epigallocatechin Gallate, Has Anticancer Activity in a Novel Human Prostate Cancer Progression Model

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Abstract

(–)-Epigallocatechin gallate (EGCG), a major tea polyphenol, elicits anticancer effects. However, the mechanism of action is not fully understood. Our laboratory previously showed that EGCG inhibits heat shock protein 90 (HSP90). We used nontumorigenic (NT), tumorigenic, and metastatic cancer cells from a novel human prostate cancer progression model to test the hypotheses that certain stages are more or less sensitive to EGCG and that sensitivity is related to HSP90 inhibition. Treatment of cells with EGCG, novobiocin, or 17-AAG resulted in more potent cytotoxic effects on tumorigenic and metastatic cells than NT cells. When tumorigenic or metastatic cells were grown *in vivo*, mice supplemented with 0.06% EGCG in drinking water developed significantly smaller tumors than untreated mice. Furthermore, EGCG prevented malignant transformation *in vivo* using the full prostate

cancer model. To elucidate the mechanism of EGCG action, we performed binding assays with EGCG-Sepharose, a C-terminal HSP90 antibody, and HSP90 mutants. These experiments revealed that EGCG-Sepharose bound more HSP90 from metastatic cells compared with NT cells and binding occurred through the HSP90 C-terminus. In addition, EGCG bound HSP90 mutants that mimic both complexed and uncomplexed HSP90. Consistent with HSP90 inhibitory activity, EGCG, novobiocin, and 17-AAG induced changes in HSP90-client proteins in NT cells and larger differences in metastatic cells. These data suggest that EGCG may be efficacious for the treatment of prostate cancer because it preferentially targets cancer cells and inhibits a molecular chaperone supportive of the malignant phenotype. *Cancer Prev Res*; 8(3); 249–57. ©2015 AACR.

Introduction

A risk factor for prostate cancer is consumption of a Western diet (1). Asian populations have a significantly lower incidence of prostate cancer. Among the many dietary differences, greater amounts of green tea are consumed in Eastern nations (2). Several studies have shown the health benefits of green tea consumption, including inhibiting prostate cancer progression (3). The major constituents of green tea are polyphenols (GTP), with (–)-epigallocatechin gallate (EGCG) making up about 60% (4). EGCG has shown efficacy in several animal models of cancer (5), including prostate cancer (6). Although EGCG has been shown to interact with several intracellular molecules and affect a number of signaling pathways (5), the exact mechanism of EGCG-elicited anticancer activity is not understood. Although the

anticancer effects of EGCG might be due to pleiotropic actions, it is also possible that many of these effects result from a common mechanism.

We (7, 8) and others (9, 10) have shown that EGCG inhibits heat shock protein 90 (HSP90) by binding to its C-terminal region and inhibiting dimerization (7, 8). As a molecular chaperone, HSP90 facilitates protein homeostasis by preventing misfolding and aggregation and enhancing protein stability. HSPs are upregulated in response to stressors including hypoxia, low nutrient availability, mutations, and overexpression of proteins/oncogenes (11, 12), characteristics important in cancer progression. HSP90 is also expressed at several-fold higher levels in many tumor cells (13), suggesting that tumor cell dependency on client proteins through mutations, aberrant expression, and improper cellular localization makes these cells critically reliant on HSP90 (14). HSP90 coordinates cancer-specific networks; its client proteins include signal effectors and transcription factors relevant to prostate cancer. These include AR, ErbB2, Akt, c-Src, VEGFR, and MMP2/9 (15, 16). Consistent with a role of HSP90 in supporting a malignant state through inappropriate stabilization of some client proteins, a significant fraction of HSP90 in cancer cells exists in multiprotein complexes, whereas in normal cells it remains predominantly free. HSP90 in tumor cells also appears to have a higher affinity for several HSP90 inhibitors (13). Taken together, these are likely reasons why normal cells are less sensitive to anti-HSP90 activity and why HSP90 is recognized as a critical factor in oncoprotein activity and support of the malignant phenotype. Thus, HSP90 may be a broad target for anticancer therapy (12).

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Studies indicating the ability of EGCG to affect diverse cellular targets are consistent with its ability to act as an HSP90 inhibitor, because most of the proteins reported to be affected by EGCG are HSP90 clients (16). These data provide a compelling basis for the hypothesis that the reported anticancer activity of EGCG is due to its effect on HSP90 function. That the levels/activity and biochemistry of HSP90 change with cancer progression also suggests the possibility for cancer stage-specific effects of HSP90 inhibitors. Here, we used a humanized mouse model of prostate cancer (17) to examine the relative sensitivity of different stages of prostate cancer to EGCG and to test the hypothesis that these effects are related to HSP90 inhibition.

Materials and Methods

Cell culture

BPH-1 [nontumorigenic (NT)] prostatic epithelial cells were from Dr. S. W. Hayward (Vanderbilt University, Nashville, TN). His laboratory authenticated these cells by full karyotyping, the presence of an active neomycin cassette (leading to G418 resistance), expression of the SV40 Large T-antigen, and PCR to ensure human specificity. BCaPT1 (early tumorigenic), BCaPT10 (late tumorigenic), and BCaPM-T10 (metastatic) human prostate cancer cells (see Supplementary Methods) were developed by Dr. W.A. Ricke (University of Wisconsin, Madison, WI) and derived from the BPH-1 line (17). His laboratory authenticated these cells by the same methods. These cells were cultured in RPMI-1640 (Cellgro) supplemented with 5% FBS, 25 mmol/L HEPES, and antibiotic/antimycotic (Cellgro). LNCaP cells (ATCC) were cultured in RPMI-1640 as above with 10% FBS. HEK293 (ATCC) were cultured in DMEM (Cellgro) supplemented with 10% FBS, 1 mmol/L sodium pyruvate, 2 mmol/L glutamine, and antibiotic/antimycotic. The ATCC authenticated these cell lines by short tandem repeat (STR) analysis. Cells were maintained in a 37°C and 5% CO₂ humidified incubator, used at low passage number, and cultured for no more than 3 months.

Cell viability assay

Cells were seeded in 96-well plates at 3,000 cells per well. The following day, cells were treated with vehicle (DPBS), EGCG (Sigma) ± 30 U/mL catalase, and 30 U/mL superoxide dismutase (SOD; Sigma), novobiocin (Sigma), or 17-AAG (LC Laboratories) for 48 hours. After incubation, 10 µL Alamar Blue (AB; Life Technologies) was added to each well, plates were incubated at 37°C for 3 hours, and percentage of AB reduced was quantified.

Cell proliferation assay

Cells were seeded and treated with EGCG as indicated above (no enzymes). After 96 hours, medium was removed and the plates were frozen at -80°C. DNA content was measured using the FluoroReporter Blue Fluorometric dsDNA Quantitation Kit (Molecular Probes).

Apoptosis assay

Cells were seeded in 4-well glass chamber slides. Once 60% to 70% confluent, EGCG (no enzymes) was added for 48 hours. Apoptotic cells were detected using the Fluorescein *In Situ* Cell Death Detection Kit (Roche Applied Biosciences).

Wound-healing assay for motility

BCaPT10 cells were seeded at 300,000 cells per well in a 6-well plate. Once confluent, a plastic pipette tip was used to create a "wound" and cells were treated ± EGCG (± enzymes). Micrographs were taken at the time of wounding and 6 hours later at fixed locations. At these locations, distance traveled was quantified using a 500 µm reference ruler and ImageJ analysis.

SDS-PAGE and Western blotting

Cells were seeded at 500,000/100 mm dish. Twenty-four hours later, cells were treated with vehicle, EGCG (+30 U/mL catalase and SOD), novobiocin, or 17-AAG for 24 hours. Some cells were dosed a second time with vehicle or 50 µmol/L EGCG for 24 more hours. Cell lysate was prepared in lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% TritonX-100, and protease/phosphatase inhibitors], centrifuged at 4°C, 20,000 × g for 30 minutes, and protein concentration determined by Bradford assay. Protein (50 µg) was separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked in TBS-T [50 mmol/L Tris (pH 7.5), 300 mmol/L NaCl, and 0.5% Tween 20] containing 5% nonfat milk, cut horizontally to allow for probing of multiple proteins, and incubated with primary antibody in blocking buffer overnight at 4°C. Membranes were then washed in TBS-T and incubated with species-specific secondary antibody in blocking buffer at room temperature. Protein bands were visualized using the Li-Cor Odyssey or Bio-Rad ChemiDocMP Imaging system. Densitometry was performed using ImageJ.

Binding assay

EGCG was conjugated to cyanogen bromide-activated Sepharose (Sigma) as described previously (8). Where applicable, cell lysate (50 µg) was incubated with vehicle or excess EGCG for 1 hour, followed by incubation with EGCG-Sepharose (30 µL), or unconjugated Sepharose for 1 hour with continuous rotation at 4°C. For immunoprecipitation, cell lysate (300 µg) was incubated with vehicle or excess EGCG or novobiocin for 1 hour before addition of C-terminal HSP90 primary antibody (sc-7947) for 3 hours and protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) overnight at 4°C. Beads were pelleted by microcentrifugation and washed with binding buffer [0.05 mol/L Tris (pH 7.5), 0.15 mol/L NaCl]. Bound proteins were analyzed by Western blotting.

Transfection

HEK293 cells were seeded at 250,000 cells per well in a 6-well plate. After 24 hours, cells were transfected with 2 µg pcDNA3.1-FLAG-tagged HSP90 constructs (WT HSP90α, HSP90α-E47A, or HSP90α-D93A), kindly provided by Dr. Len Neckers (National Cancer Institute, Bethesda, MD), using TransFast (Promega) according to the manufacturer's instructions. Cell lysate was collected 36 hours after transfection for binding assays and Western blotting.

Chaperone function assay

See Supplementary Methods.

Tumor xenograft assay

Animals were maintained and treated in accordance with the guidelines set by the University of Rochester Committee on Animal Resources and the American Association for Laboratory

Animal Science. Six-week-old male athymic mice (Charles River) were allowed to acclimate for 1 week. Mice were then given sterile deionized water ($n = 8$) or 0.06% EGCG in water ($n = 8$) *ad libitum* using amber-colored bottles for 1 week prior to surgery and throughout the experiment, with water changed every Monday, Wednesday, and Friday. BCaPT10 or BCaPM-T10 cells (100,000) were resuspended in 15 μ L rat tail collagen (BD) titrated to pH 7.4, and after polymerization, collagen grafts were placed subcutaneously into mice. Animals with BCaPT10 cells were euthanized after 2 months, while animals with BCaPM-T10 cells were euthanized after 1 month, and tumor mass was determined. Tumors were formalin-fixed, paraffin-embedded, cut in 8- μ m sections, and stained with H&E.

Malignant transformation

See Supplementary Methods.

Urogenital mesenchyme isolation. Timed pregnant (E13) Sprague-Dawley female rats (Charles River) were allowed to acclimate until E18. Urogenital mesenchyme (UGM) was then isolated from rat embryos as described previously (18).

Preparation/implantation of grafts. Tissue recombinants/grfts were prepared by mixing 250,000 UGM cells with 100,000 BPH-1 cells in rat tail collagen as described above. Grafts were placed under the renal capsule of preacclimated 6- to 8-week-old male athymic mice (1 graft/mouse) along with subcutaneous pellets containing testosterone (25 mg) and 17 β -estradiol (2.5 mg). Mice were given water ($n = 5$) or 0.06% EGCG ($n = 5$) as above. After 3 months, grafts were dissected and processed for cell culture.

Isolation/purification of BPH-1-derived cells and regrafting. A piece of graft from each animal was cut into small fragments, cultured, and the neomycin-resistant/large T-antigen/cytokeratin-positive

(BPH-1 derived) cells were purified as described previously (17). Cells were expanded in culture and engrafted (1×10^6 cells of each line, prepared as above) subcutaneously in new untreated 6-week-old male athymic mice ($n = 10$) for 2 months. The number of isolated cell lines from previously untreated/treated animals that formed tumors was determined.

Results

Metastatic cells are more sensitive than NT counterparts to EGCG and other HSP90 inhibitors

To test potential differences in sensitivity to HSP90 inhibitors, human NT (BPH-1), tumorigenic (BCaPT1 and BCaPT10), and metastatic (BCaPM-T10) prostatic epithelial cells were treated with EGCG and cell viability assays were performed. NT cells were less sensitive to EGCG relative to tumorigenic and metastatic cells from this model. Concentrations up to 100 μ mol/L EGCG had a relatively minor effect on the viability of NT cells, while the same concentrations led to significant decreases in viability of tumorigenic and metastatic cells (Fig. 1A). It has been suggested that hydrogen peroxide and superoxide are produced when EGCG is auto-oxidized in cell culture medium (19–21), which could affect the observed differences in sensitivity to EGCG. Therefore, using the least (BPH-1) and most (BCaPM-T10) sensitive cell lines from Fig. 1A, viability assays were repeated in the presence of catalase and SOD. In the presence of these enzymes, the difference in sensitivity between NT and metastatic cells to EGCG was still observed, albeit the effects were less potent than in the no-enzyme controls (Fig. 1B), indicating that free radicals do contribute to some toxicity under these culture conditions, but not to the stage sensitivity. To investigate whether exposure to other HSP90 inhibitors results in similar stage sensitivity, viability assays were performed following treatment with another C-terminal inhibitor, novobiocin, or an N-terminal inhibitor, 17-AAG. Both elicited

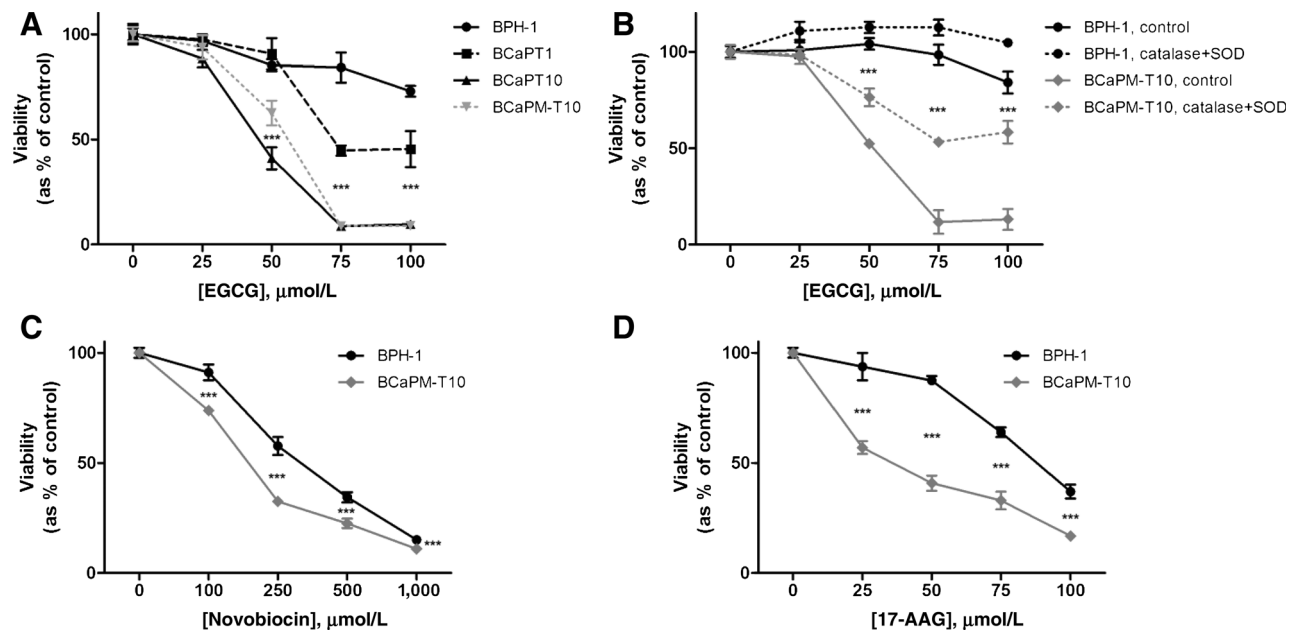


Figure 1.

Cell viability was analyzed by AB assay after incubation with EGCG (A), EGCG+30 U/mL catalase+30 U/mL SOD (B), novobiocin (NB; C), or 17-AAG (D). Experiments were repeated three times (representative results shown) and data represent a mean of four to six replicates \pm SD. ***, $P < 0.001$ by one-way ANOVA or unpaired t test comparing individual cell lines to BPH-1 at the same concentration. B, ***, $P < 0.001$ for BPH-1 (catalase+SOD) versus BCaPM-T10 (catalase+SOD).

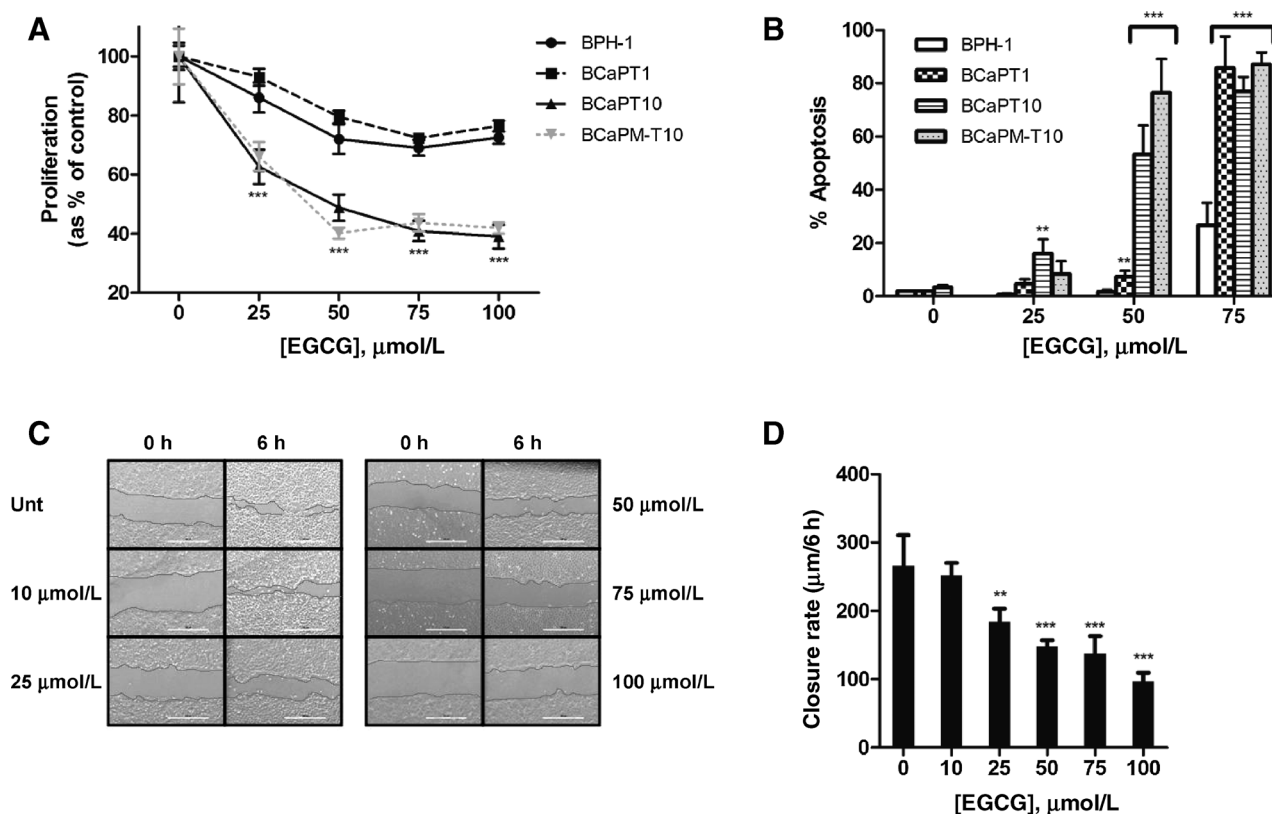


Figure 2.

A, cells were analyzed for DNA content after incubation with EGCG. Data represent a mean of four to six replicates \pm SD. B, apoptotic cells were quantified by TUNEL staining after incubation with EGCG. Data represent mean percentage of positive TUNEL cells in three random fields of view \pm SD. C and D, motility of BCaPT10 cells was determined \pm EGCG exposure by scratch assay. Data represent mean distance traveled from three random fields of view \pm SD. Experiments were repeated three times; representative results shown. **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA or unpaired t test by comparing to (A and B) BPH-1 at the same concentration or (D) untreated cells.

dose-dependent effects on viability with greater potency in metastatic cells compared with NT cells (Fig. 1C and D). Collectively, these data suggest that EGCG mimics the anticancer effects of other HSP90 inhibitors on cell viability, with cancer cells being more sensitive than NT counterparts.

The effect of EGCG on the viability of these cells could be the result of a decrease in proliferation and/or an induction of apoptosis. We found that EGCG (25–100 $\mu\text{mol/L}$) led to a significant decrease in the total DNA content of tumorigenic (BCaPT10) and metastatic cells compared with NT cells (Fig. 2A). A dose-dependent increase in terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells was also observed in tumorigenic (BCaPT10) and metastatic cells treated with 25 to 75 $\mu\text{mol/L}$ EGCG, whereas negligible effects were observed in NT cells treated with 25 to 50 $\mu\text{mol/L}$ (Fig. 2B). Both of these assays indicated that cancer cells are more sensitive to EGCG than are the NT.

EGCG inhibits cancer cell motility

We examined whether EGCG affects cancer cell motility using tumorigenic (BCaPT10) cells because these originated from the site of a primary tumor and had yet to metastasize. Representative micrographs (Fig. 2C) and quantified data (Fig. 2D) show that untreated tumorigenic cells almost completely closed a pipette

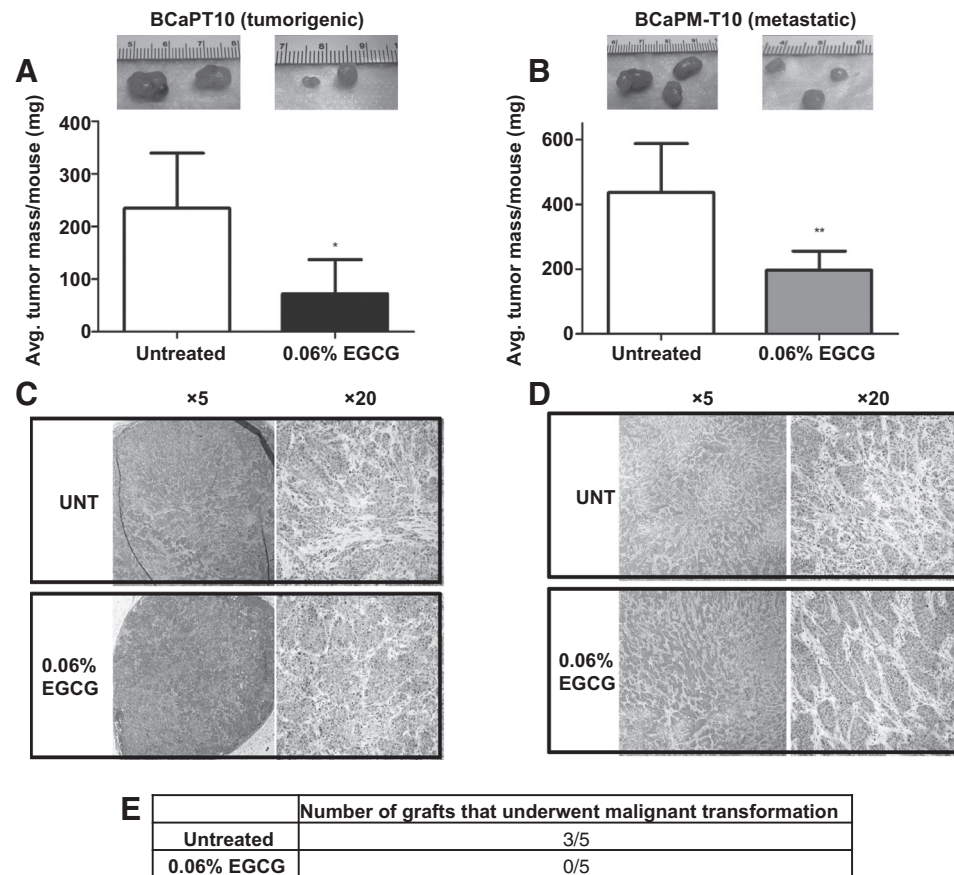
tip-created "wound" by 6 hours, whereas a significant inhibition of this effect was observed at concentrations of 25 to 100 $\mu\text{mol/L}$ EGCG (Fig. 2D), with no significant effects on cell viability in response to EGCG over this time (data not shown). Furthermore, the addition of catalase and SOD did not affect the ability of EGCG to inhibit the motility of BCaPT10 cells (data not shown).

EGCG elicits anti-prostate cancer activity *in vivo*

To test whether EGCG consumed in drinking water affects the *in vivo* growth of human prostatic epithelial cells at different stages, mice were engrafted with cancer cells and given sterile deionized water with or without 0.06% EGCG. This concentration has shown efficacy in the TRAMP model of prostate cancer (22), and is based on other studies that used GTP concentrations of 0.1% (23). We observed an approximate 50% decrease in tumor size in mice engrafted with tumorigenic (Fig. 3A) or metastatic (Fig. 3B) cells after 2- and 1-month EGCG treatment, respectively, relative to controls. No change in body weight was observed (data not shown), indicating this regimen was well tolerated. These results are the first to demonstrate that oral consumption of EGCG elicits anticancer activity in two different stages (primary tumor growth and metastatic tumor growth) of human prostate cancer from this model system. Microscopic examination revealed EGCG-treated and untreated tumors from both cell types to be high-grade with

Figure 3.

Tumor mass was determined after engrafting mice with BCaPT10 (A) or BCaPM-T10 (B) cells and continuing a regimen of sterile deionized water \pm 0.06% EGCG for (A) 2 or (B) 1 month(s). Data represent average tumor mass/mouse \pm SD with $n = 8$ mice/group. *, $P < 0.05$; **, $P < 0.01$ by unpaired *t* test. C and D, H&E staining of late tumorigenic (A) and metastatic tumors (B) from untreated and EGCG-treated mice. E, mice ($n = 5$ /group) were engrafted with one of the individually isolated/purified NT BPH-1-derived prostate epithelial populations from previously untreated or EGCG-treated mice for 2 months. The extent of malignant transformation was then examined (# of tumors formed). Data represent number of mice in which tumors were observed for that group.



equally identifiable mitotic figures and morphology (Fig. 3C and D). As such, it does not appear (at least visibly) that EGCG is selecting for a more aggressive cancer type.

Some clinical data have suggested chemopreventive effects of GTPs given to patients with high-grade intraepithelial neoplasia (HG PIN), an early prostate cancer lesion (24). The NT BPH-1 cells described here are large T-antigen immortalized (representing an "initiated state"), comparable with early lesions in humans. To determine whether EGCG would prevent malignant transformation of the NT BPH-1 cell line, mice were engrafted with UGM+BPH-1 tissue recombinants and T+E2 implants, and given sterile deionized water with or without 0.06% EGCG for 3 months (a time determined to produce small, localized tumors in untreated animals; ref. 17). At this time, BPH-1-derived epithelial cells from these grafts were purified and grafted subcutaneously into new hosts without EGCG, T+E2, or stroma. After 2 months, the presence of tumors was determined. In all 5 treated mice, EGCG had prevented malignant transformation, because no tumors grew in secondary hosts. In contrast, tumors grew in 3 of 5 new hosts engrafted with cells from animals not treated with EGCG (Fig. 3E). These data suggest that EGCG prevents malignant transformation of a human cell type that mimics HG PIN.

EGCG-Sepharose binds more HSP90 from metastatic cells compared with NT cells

Most of the putative "targets" of EGCG are HSP90-client proteins, leading to a unifying hypothesis that the mechanism of EGCG's anticancer activity is related to HSP90 inhibition.

Because the above data suggest differences in sensitivity to EGCG, we sought to determine whether there were also stage differences in HSP90 characteristics that may mediate this. We found that EGCG-Sepharose bound more HSP90 from metastatic cells than from NT cells, even though total amounts of HSP90 were similar (Fig. 4A). Excess EGCG was able to specifically compete with EGCG-Sepharose for binding to HSP90 in metastatic and LNCaP (another metastatic line) cell lysate. This was not reproduced in NT cells, suggesting the possibility that a specific subpopulation of HSP90 present in cancer cells may have a greater ability to bind EGCG-Sepharose, and/or the "affinity" of NT HSP90 binding to EGCG-Sepharose is low and cannot be further perturbed at the EGCG concentration used.

To verify, as suggested previously (7), that the C-terminal region of HSP90 is the target of EGCG, immunoprecipitation experiments were performed. In the presence of excess EGCG, the amount of HSP90 pulled down with a C-terminal antibody from NT and metastatic cell lysates was diminished (Fig. 4B). EGCG also closely mimicked the response generated by novobiocin, another C-terminal HSP90 inhibitor (25), in a chaperone-mediated luciferase refolding assay (Supplementary Fig. S1). In contrast, excess novobiocin had little effect on the ability to bind the C-terminal antibody (Fig. 4B), indicating that it has lower affinity for HSP90, it does not share the same binding site as EGCG or the sc-7947 HSP90 antibody, and/or novobiocin binding elicits different conformational changes in HSP90 compared with EGCG.

HSP90 subpopulations exist that display different affinities for N-terminal HSP90 inhibitors and differentially interact with

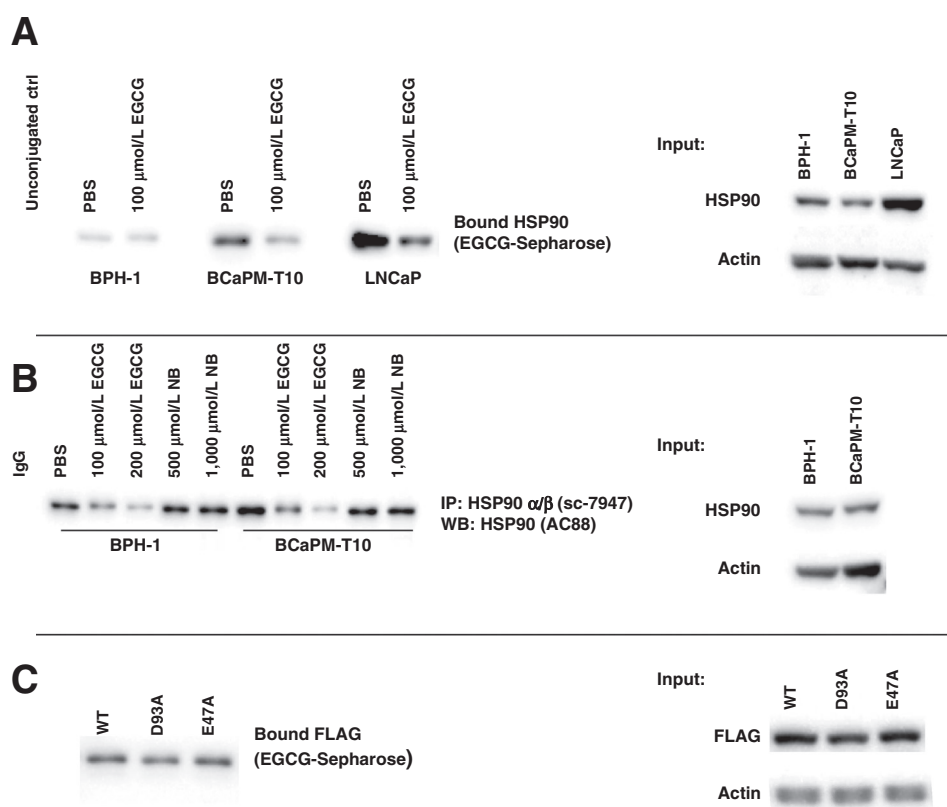


Figure 4.

A and B, binding assays were performed by incubating BPH-1, BCaPM-T10, or LNCaP lysate with (A) EGCG-Sepharose or (B) a C-terminal HSP90 antibody and Agarose beads after competition with excess ligand. C, lysate from HEK293 cells transfected with FLAG-tagged HSP90 constructs was used for EGCG-Sepharose binding assays. Membranes were cut horizontally to probe for different proteins and bound/total proteins were determined by Western blotting. Experiments were repeated three times; representative results shown.

HSP90 clients (15, 26). Furthermore, a significant fraction of HSP90 in cancer cells exists in multiprotein complexes, whereas in normal cells it remains predominantly uncomplexed (13). To determine whether EGCG prefers different conformations of HSP90 that might exist in NT versus metastatic cells, EGCG-Sepharose assays were performed using lysate from HEK cells transfected with FLAG-tagged WT HSP90 α , HSP90 α -E47A, or HSP90 α -D93A. HSP90 α -E47A binds but cannot hydrolyze ATP, and thus in cells, gets trapped in an N-domain dimerized, p23-binding (complexed) conformation not preferred by N-domain drugs. In contrast, HSP90 α -D93A is thought to exist primarily in an apo, N-domain open (uncomplexed) conformation that does not bind ATP or N-terminal inhibitors (L. Neckers; personal communication, Oct 21, 2013). As shown in Fig. 4C, EGCG-Sepharose bound FLAG-WT HSP90 α , HSP90 α -E47A, and -D93A, suggesting that EGCG (such as novobiocin; ref. 27) binding is not affected by either ATP-dependent alterations in N-domain conformation or by nucleotide occupancy of the N-domain binding pocket. Moreover, these data indicate that EGCG binds both complexed and uncomplexed HSP90 conformations.

EGCG elicits HSP90 client protein changes in NT and metastatic cells

Because EGCG, novobiocin, and 17-AAG demonstrate stage-specific cytotoxicity (Fig. 1), and EGCG binds more strongly to HSP90 in metastatic cells (Fig. 4A), we sought to determine how HSP90 function/activity differs between these two cell lines by examining changes in HSP90 client protein levels in response to HSP90 inhibition. Within 24 hours, EGCG treatment led to a

dose-dependent loss of ErbB2, Raf-1, and Akt in both NT and metastatic cells. No significant or consistent changes were observed for total HSP90 levels in either cell line. Repeated treatment with 50 $\mu\text{mol/L}$ EGCG ("2 \times 50"; Fig. 5) led to a more pronounced decrease in HSP90 clients. However, certain clients appear to be more affected than others. Densitometric analyses suggested that p-Akt was more sensitive to EGCG in metastatic relative to NT cells (Fig. 5 and Supplementary Table S1A). Moreover, metastatic cells have higher total basal levels of Akt, p-Akt, and Raf-1 (Supplementary Table S1B). Therefore, the total decrease in the levels of these client proteins with increasing dose of EGCG is much greater in metastatic than NT cells (Fig. 5), which could be associated with increased sensitivity.

When comparing EGCG to novobiocin and 17-AAG, loss of the same clients was observed, although the relative potencies of these inhibitors were different. 17-AAG at 25 $\mu\text{mol/L}$ led to an almost complete loss of the clients tested (Fig. 5). In contrast to EGCG and novobiocin, it also induced HSP70, consistent with its effects on the pro-survival heat shock response (HSR; Fig. 5; ref. 28). Novobiocin also led to HSP90-client protein changes, but at higher concentrations. Raf-1 levels also increased (with a band shift) in response to novobiocin (Fig. 5), whereas this effect was not elicited by EGCG, suggesting that although these compounds both bind to the HSP90 C-terminus, different binding sites within this region may lead to distinct changes in HSP90-regulated networks. Finally, the parental BPH-1 and its derived cell lines contain large T-antigen. Although this protein is an HSP90-client, its levels were largely unaffected by EGCG, novobiocin, or 17-AAG (Fig. 5) at all concentrations tested, ensuring that the

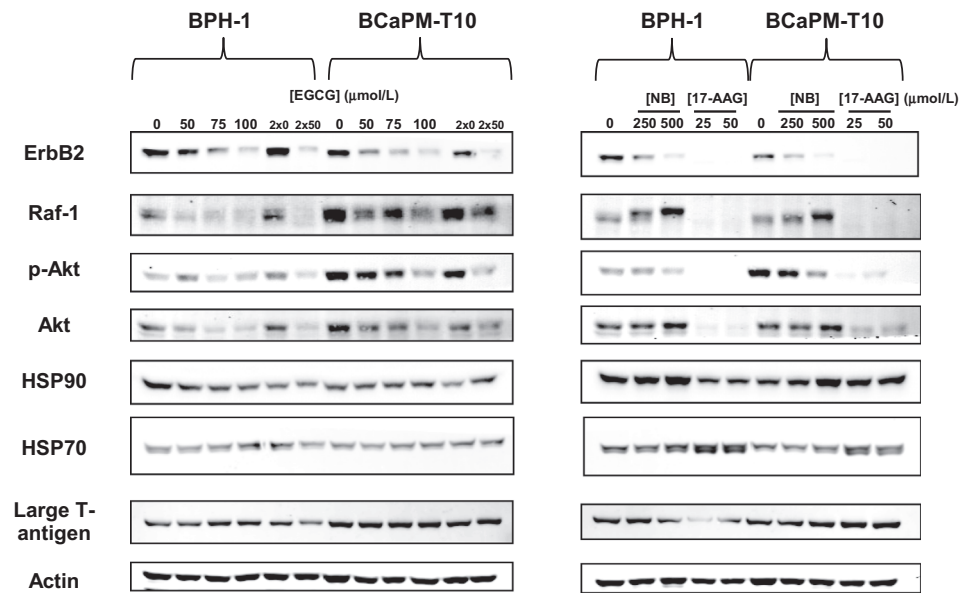


Figure 5.

HSP90 client protein levels were examined in BPH-1 and BCaPM-T10 cells after treatment with EGCG (+30 U/mL catalase, 30 U/mL SOD), novobiocin (NB), or 17-AAG. Membranes were cut horizontally to probe for different proteins by Western blotting. Experiments were repeated three times; representative results shown.

stage-specific sensitivity is unrelated to expression of this protein in these cell lines.

Discussion

The efficacy of targeting HSP90 in human cancer has been demonstrated with various N-terminal HSP90 inhibitors using preclinical animal models of cancer where HSP90 client proteins have known roles in tumor progression and survival (12, 29, 30). Our data suggest a mechanism by which EGCG elicits anti-prostate cancer activity specifically in cancer cell subtypes by binding to HSP90 and affecting a number of client proteins critical to the malignant phenotype.

Previous studies have indicated PC-3 prostate cancer cells are more sensitive than primary and NT prostatic epithelial cells (RWPE-1) to the anticancer effects of EGCG (31–33). However, these cell models all have different genetic origins. An advantage of our model is that the cancer cells are derived from the clonal NT BPH-1 cell line, thus offering a means to identify and characterize changes in HSP90 that may be associated with differential sensitivity. Using this model, we have shown that NT cells are less sensitive than metastatic cells to EGCG and other HSP90 inhibitors. This may be a result of oncogenic mutation and/or over-expression of HSP90 client proteins in cancer cells, necessitating a greater reliance on HSP90 to stabilize client load and conformations of mutant proteins. In metastatic cells, compared with NT cells, Raf-1, p-Akt, and Akt are all present at higher levels, thus providing support to this hypothesis. Although the HSP90 clients examined appeared to change in both cell types, quantitative differences in the effects of EGCG on total levels of particular clients may be associated with differential sensitivity, given that metastatic cells may be uniquely dependent on these proteins. It is also possible that there are other clients whose expression may be more dramatically affected by EGCG or other C-terminal inhibitors in metastatic cells compared with NT. Taken together, it is not unreasonable to suspect that normal/NT cells are less sensitive to HSP90 inhibitors because they are less dependent than cancer cells on one or more HSP90 client, and/or client proteins other than those we examined are more sensitive to HSP90 inhibition in

metastatic cells. Furthermore, given the evidence that structurally distinct N-terminal inhibitors trap different subpopulations of HSP90 (15, 26), it is possible that in metastatic cells, a critical stage-specific, procancer subpopulation of HSP90 exists, leading to the observed binding by EGCG-Sepharose, and whose inhibition leads to anticancer effects.

Consistent with our *in vitro* data, these human tumorigenic/metastatic cells were also sensitive to EGCG *in vivo* (Figs. 3A and B). Published epidemiologic and clinical studies of GTPs and prostate cancer chemoprevention/treatment have been inconclusive regarding the ability of GTPs to affect any particular stage of prostate cancer (34). It is possible that all stages of prostate cancer are affected by EGCG, but with varying degrees of sensitivity. Furthermore, in some cases, differences in stage sensitivity may be small or not observed because of other genetic and/or metabolic differences that influence HSP90 biochemistry or EGCG kinetics. Clearly, a variety of factors that influence the sensitivity of prostate cancer, or other tumor types, within a given individual need to be considered in any therapeutic approach.

Nevertheless, together, our results offer a unifying hypothesis to account for the numerous reported putative "targets" of EGCG in that most of these "targets" are HSP90 client proteins (7, 16). An advantage of the use of C-terminal HSP90 inhibitors, such as EGCG, is the lack of or low induction of the prosurvival HSR (35). This response, characterized by upregulation of HSP70 (28), is induced by N-terminal inhibitors and is likely counterproductive for anticancer efficacy. Furthermore, the ability of EGCG-Sepharose to bind more HSP90 from metastatic cells, and bind several HSP90 conformations, as well as the decreased activity of EGCG in normal/NT cells suggest that C-terminal inhibitors should be further pursued for treating prostate cancer. In addition, EGCG may have additive/synergistic effects when combined with specific and traditional anticancer agents (36), as has been observed with N-terminal HSP90 inhibitors (30). The use of EGCG in combination may maximize clinical benefit by sensitizing cancer cells to other anticancer therapies, potentially allowing for the use of lower drug doses. These clinical benefits may limit serious side effects and oncogene switching (12), which can result in cancer recurrence.

Overall, determining how HSP90 characteristics change with cancer progression and whether this affects binding to EGCG/ other HSP90 inhibitors using this prostate cancer model system will provide critical details about specific and distinct changes in HSP90 networks and allow for more directed therapies to target HSP90 in human prostate cancer. The studies described here may lay the foundation to evaluate structure–activity relationships for analogs of EGCG that have increased bioavailability, while inhibiting HSP90 function to elicit stronger anticancer properties with limited side effects (37).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.A. Moses, E.C. Henry, W.A. Ricke
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.A. Moses, W.A. Ricke, T.A. Gasiewicz

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