Dihydrotestosterone Sensitises LNCaP Cells to Death Induced by Epigallocatechin-3-Gallate (EGCG) or an IGF-I Receptor Inhibitor

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BACKGROUND. Compelling evidence has accumulated for chemopreventive effects for the active component of green tea Epigallocatechin-3-Gallate (EGCG) particularly for prostate cancer (CaP).
METHODS. We have assessed interactions between the effects of EGCG and two main regulators of prostate cell function, dihydrotestosterone (DHT) and insulin-like growth factor-1 (IGF-I). Using LNCaP (androgen-sensitive), PC3 and DU145 (androgen-resistant) CaP cell lines, we assessed the effect of EGCG alone on growth (0–200 μM) and on cell death (0–50 μM).
RESULTS. EGCG decreased the proliferation of all the CaP cancer cells in a dose-dependent manner with an increase in apoptosis from 30 to 50 μM. With DU145 cells, a sub-apoptotic dose of EGCG (10–20 μM) reduced IGF-induced growth. With LNCaP cells, a sub-apoptotic dose of EGCG (8 μM) switched DHT from a growth promoter to a growth inhibitor. A similar reversal of DHT effect was seen in the presence of an IGF-I receptor inhibitor, AG1024 (1 μM). These responses appeared to be due to DHT sensitizing the cells to apoptosis by EGCG and AG1024 ($P < 0.01$ and $P < 0.001$ respectively).
CONCLUSIONS. Our data suggests that both green tea and AG1024 are effective in inhibiting cell growth and inducing death in CaP cells but the effects of both are more effective in the presence of androgen. *Prostate 69: 219–224, 2009.*

KEY WORDS: **EGCG; CaP cells; androgen; IGF-IR**

INTRODUCTION

Prostate cancer (CaP) is the second leading cause of cancer-related deaths among men in Western countries, thus representing a major and growing health problem.

Prostate cancer represents an ideal disease for chemoprevention because it is typically a slowly developing cancer diagnosed in elderly men; therefore even a modest slowing in the neoplastic process achieved through pharmacological or nutritional intervention could result in a substantial reduction in the incidence of the clinically detectable disease. Life-style related factors, particularly diet, are considered to be the major contributors to CaP promotion. Of all dietary components the most compelling evidence for chemopreventive effects has accumulated for the active component of green tea Epigallocatechin-3-Gallate (EGCG) [1,2]. Several epidemiological studies have found a lower incidence of CaP in Asian countries (where green tea is consumed regularly) compared to

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Western populations [3]. In addition, Asian immigrants to the US who have abandoned original dietary habits soon develop the Western risk of CaP, which is associated with the adoption of a Western lifestyle [3]. A case-control study conducted in China showed that green tea consumption was protective against this disease [4]. Bettuzzi et al. [2] performed a double-blinded and placebo-controlled study with sixty male volunteers with high grade prostate intraepithelial neoplasia, (HGPIN), a premalignant condition of prostate growth [5] that is known to result in a substantial number of cancers within 1 year after repeated biopsy [6,7]. Half of the men consumed three 200 mg decaffeinated green tea catechin preparations orally, daily for 1 year, which resulted in a 90% reduction in the rate of HG-PIN positive men developing prostate cancer with no significant side effects [2].

The data from the epidemiological studies and clinical trial by Bettuzzi et al. [2] provide convincing evidence that EGCG has a protective role against the progression of CaP. However, the mechanisms of action of EGCG are not clearly understood and their delineation will be critical to understanding the effectiveness of EGCG and for optimizing future intervention studies for the prevention of CaP progression.

Androgens are an essential element in regulating prostate growth largely by driving proliferation [8] and so androgen withdrawal is often used as a treatment for prostate cancer [9]. However, the failure of anti-androgen therapy is common with progression to an androgen independent state occurring within 3 years of starting treatment in one third of patients [10]. This treatment failure illustrates the development of alternative growth pathways to drive CaP progression, of which insulin-like growth factor-I (IGF-I) is known to be a key player. Prospective studies have demonstrated an association between serum IGF levels with both the initiation and progression of CaP [11–13]. The IGF axis has a critical role in the establishment and maintenance of the transformed phenotype in numerous malignancies (reviewed in Ref. [14]). Their action is modulated by a complex network of molecules consisting of 2 ligands (IGF-I and IGF-II), 6 IGF binding proteins (IGFBP-1-6), and types I and II IGF receptors (IGF-IR and IGF-IIR). IGFs have potent mitogenic and anti-apoptotic effects on prostate tissue (reviewed in Ref. [15]). Expression and activity of components of this pathway are altered in many human malignancies, including CaP [16] and therefore it has been suggested that treatments (such as tyrosine kinase inhibitors and monoclonal antibodies) targeting the IGF-IR could enhance current therapeutics for CaP [17]. Many components of the IGF axis are regulated by androgens, such as IGF-I, the IGF-IR and IGFBPs [18–20].

MATERIALS AND METHODS

Materials

All chemicals and inhibitors were purchased from Sigma-Aldrich (Dorset, UK) unless stated otherwise.

Cell Culture

DU145, LNCaP, and PC-3 cells were maintained in RPMI 1640 cell culture media (Lonza, Basel, Switzerland) supplemented with 10% foetal bovine serum (FBS, Gibco, Paisley, UK), 1% penicillin/streptomycin solution and 1% L-glutamine solution (200 mM). Cell growth and death were examined after the cells were grown in serum-free conditions for 24 hr. Serum free media for these cells was identical but with bovine serum albumin (BSA, 1 mg/ml) instead of FBS.

Tritiated Thymidine Incorporation (TTI)

DNA synthesis was measured using TTI as described previously [21,22]. In brief, the cells were incubated with 0.1 μCi [3H]thymidine per well for the final 4 hr of the dosing time period. After the removal of the supernatant, cells were then washed with 500 μl of 5% trichloroacetic acid (Merck Ltd., Middlesex, UK) at 4°C for 10 min followed by solubilization of DNA incorporated thymidine with 400 μl of 1 M NaOH (Fisher Scientific Ltd., Leicestershire, UK) for 1 hr at room temperature. The resulting suspension was placed into individual scintillation vials, and 3 ml of scintillation fluid was added. Samples were analyzed using a Beckman Scintillation Counter LS6500. Data were recorded as disintegrations per minute.

Cell Counting

Cell death was assessed using trypan blue cell counting as described previously [21].

Western Immunoblotting

Cells were lysed, loaded according to protein concentration of lysates and separated on an 8% SDS–PAGE gel and transferred to Hybond N+ nylon membranes (Amersham, Bucks, UK) as outlined previously [21]. Nonspecific binding sites on the nitrocellulose membranes were blocked overnight with 5% milk in Tris-buffered saline (TBS)/2% Tween for probing with anti-p85 cleaved subunit of PARP (Promega, Southampton, UK), anti-Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH; Chemicon, Hampshire, UK) anti-p-IGF-IR (Cell Signalling, Danvers, MA), anti-IGF-IR (Santa Cruz, CA) (all at 1:1,000) or blocked with 3% BSA for probing with phospho-MAPK (1:5,000). After the removal of excess unbound antibody appropriate secondary antibodies conjugated

to peroxidase were added for 1 hr. Binding of the peroxidase was visualized by enhanced chemiluminescence according to the manufacturer's instructions. Chemiluminescence was detected using the Chemi-Doc-It Imaging System (UVP) (Biorad, .Hertfordshire, UK) and analysed using Vision WorksTH ls Analysis Software (UVP, Inc., Upland, CA).

Statistics

The data were analyzed with the Microsoft Excel version 5.0a software package using ANOVA followed by least-significant difference *post hoc* test. A statistically significant difference was considered to be present at $P < 0.05$.

RESULTS AND DISCUSSION

In order to understand the mechanisms by which EGCG could protect against CaP, our goal was to assess its effects on the growth induced by the two main drivers of CaP progression, dihydrotestosterone (DHT) and IGF-I. To achieve this we initially characterized and compared the effects of EGCG on growth and the induction of cell death in three CaP cell lines: the androgen-responsive LNCaP cells and androgen-insensitive DU145 and PC3 cells. Figure 1A shows that EGCG decreased proliferation of all the prostate cancer cell lines in a dose-dependent manner. A significant decrease was first observed at 25 μM for DU145 cells ($P < 0.001$) and at 50 μM for both PC3 and LNCaP cells ($P < 0.001$ and $P < 0.05$ respectively). At the highest dose of 200 μM, there was a comparable reduction in tritiated thymidine incorporation for DU145 and PC3 cells (by 88.2% and 87.5% respectively; both $P < 0.001$), whereas the LNCaP cells seemed more resistant (decreased by 59.7%; $P < 0.001$). Figure 1B, shows that significant cell death was induced from 30 μM (DU145; $P < 0.01$, PC3; $P < 0.001$ and LNCaP, $P < 0.05$) and increased in a dose-dependent manner, with similar responses in all of the cells lines examined. The induction of cleaved PARP (Fig. 1B, insert) confirmed that this cell death was apoptotic. Doses of EGCG below 30 μM were anti-proliferative and sub-apoptotic, whereas doses above 30 μM decreased cell growth and were associated with the induction of apoptosis. These results are in keeping with previous studies [23,24].

Initial studies with LNCaP cells revealed that increasing doses of DHT stimulated DNA synthesis up to a dose of 0.01 μM ($P < 0.001$), but at higher doses the responses were reduced (Fig. 2A). In addition, we found that addition of exogenous IGF-I (0–200 ng/ml) to these cells had no effect on basal DNA synthesis (data not shown). However, we did observe in Figure 2B, that proliferation of these cells was dose dependently inhibited in the presence of an IGF-IR tyrosine kinase

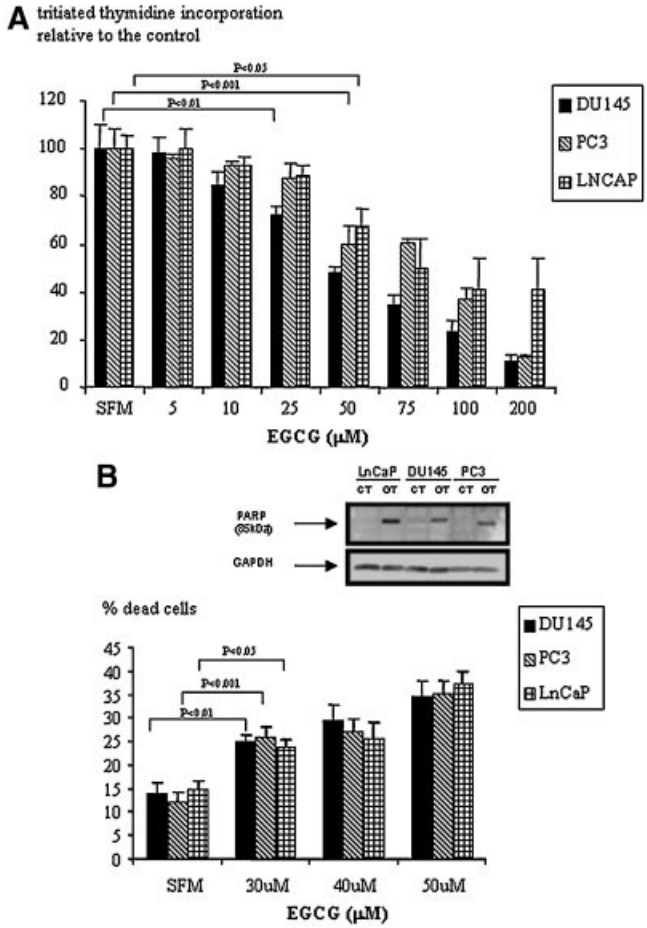

Fig. 1. Effects of EGCG on the growth and survival of DU145, PC3, and LNCaP prostate cancer (CaP) cells. **A**: CaP cells were seeded in growth media (GM; 1 ml) in 24-well plates ($0.025 - 0.2 \times 10^6$ cells/well) for 24 hr. Following 24 hr in serum-free media (SFM) they were treated with EGCG (0–200 μM) for 48 hr and DNA synthesis was measured using TTI (**B**) CaP cells were seeded in GM (2 ml) in 6-well plates ($0.1 - 0.2 \times 10^6$ cells/well) for 24 hr. Following 24 hr in SFM, cells were dosed with EGCG (0–50 μM) for 48 hr and cell death was assessed using trypan blue cell counting. For confirmation of apoptosis we monitored PARP cleavage (insert). CaP cells (0.5×10^6) were grown to 60% confluence in T25 cm^2 flasks then incubated in SFM for a further 24 hr prior to dosing as in (B). Cells were then processed as described in materials and methods. The graphs representing the TTI and cell counting experiments (A,B) are the mean of three experiments each repeated in triplicate. The Western blot (insert B) is representative of an experiment repeated three times.

inhibitor (TKI; AG1024, 0–1 μM), suggesting that the lack of response to exogenously added IGF-I was due to the cells own endogenous production stimulating autocrine growth. We also found that the DHT-induced increase in DNA synthesis in LNCaP cells was completely blocked in the presence of the IGF-IR TKI at doses of 0.5–0.75 μM (Fig. 2B) confirming that the ability of DHT to increase cell proliferation was at least in part dependent upon the IGF1-R. This is in keeping

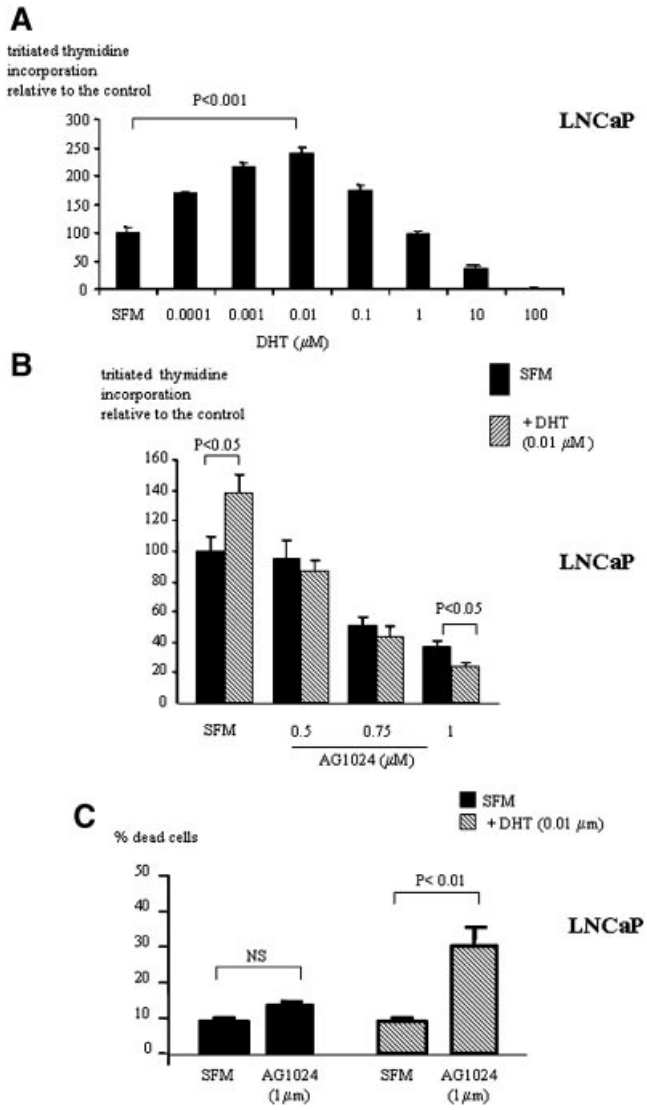

Fig. 2. Effects of an IGF-IR inhibitor, AGI024 on DHT-induced growth of LNCaP cells LNCaP cells were seeded in growth media (GM; 1 ml) in 24-well plates (0.2×10^6 cells/well) for 24 hr. Following 24 hr in serum-free media (SFM) they were treated with (**A**) DHT (0–100 μM) either alone or +/− (**B**) AGI024 (0–1 μM). TTI was performed as described in Figure 1B. **C**: Cells were seeded as in Figure 1B and following 24 hr in SFM were dosed with DHT (10 nM), AGI024 (1 μM) or the combination for 48 hr and then cell death was assessed as in Figure 1B. The graphs each show the mean of three experiments each repeated in triplicate.

with previous reports in LNCaP cells showing that androgens increase IGF-1R expression and IGF-1 levels [18,19]. At the highest dose of 1 μM AG1024, we noticed that the effect of DHT was reversed ($P < 0.05$). On investigating this further using trypan blue cell counting, we found that the DHT was sensitizing the cells to induction of cell death by the IGF-IR TKI. Figure 2C shows that AG1024 (1 μM) and DHT (10 nM) each alone had no effect but in the presence of DHT, the IGF-IR TKI then caused a significant increase in cell death ($P < 0.001$).

We then wished to assess the effects of EGCG on the actions of both IGF-I and DHT on prostate cancer cells. Using DU145 cells, which unlike LNCaP cells respond to addition of exogenous IGF-I, we show in Figure 3, that IGF-I induced a significant increase in tritiated thymidine incorporation over 48 hr ($P < 0.001$), which was dose-dependently abrogated in the presence of EGCG (10–30 μM). This effect of EGCG was associated with a reduction on the phosphorylation of the IGF-IR and p-MAPK (insert Fig. 3). We also found that EGCG was able to decrease the basal phosphorylation of the IGF-IR in LNCaP cells (insert Fig. 3). Such an effect of EGCG on the phosphorylation of the IGF-IR has been shown previously in colon cancer cells [25] and in mouse 3T3 fibroblasts, in which the IGF-IR was found to be a novel binding partner of EGCG [26].

However, previous data in the prostate have demonstrated that oral infusion of green tea polyphenols over 24 weeks to the mouse model (TRAMP; transgenic adenocarcinoma of the mouse prostate)

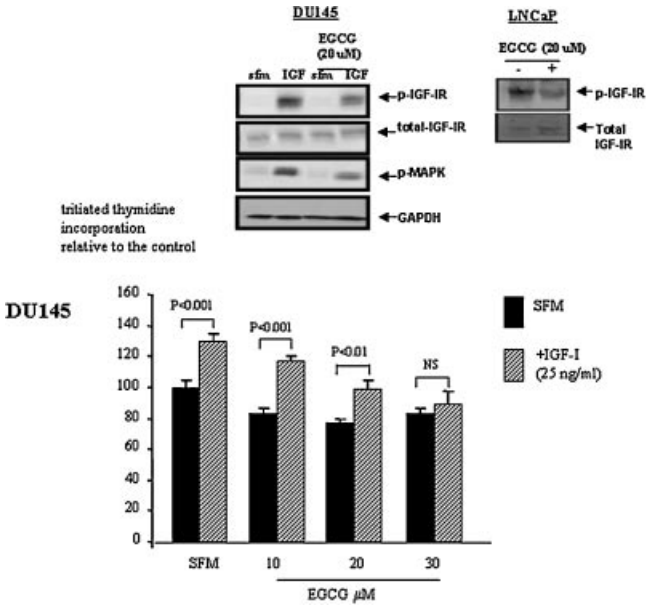

Fig. 3. Effects of EGCG on IGF-induced growth of DU145 cells. DU145 cells were seeded in growth media (GM; 1 ml) in 24-well plates (0.025×10^6 cells/well) for 24 hr. Following 24 hr in serum-free media (SFM) they were treated with IGF-I (25 ng/ml) +/− EGCG (10–30 μM) for 48 hr. DNA synthesis was measured as described in Figure 1A. To assess activation of the IGF-I receptor (DU145 and LNCaP) and p-MAPK (DU145) cells were prepared as in Figure 1B, dosed with IGF-I (25 ng/ml) (DU145) +/− EGCG (20 μM) (DU145 and LNCaP) for 10 min and then lysed and run on a gel as in Figure 1B. The TTI graph shown in (A) is the mean of three experiments each repeated in triplicate. The Western blots (insert) are representative of experiments repeated three times.

caused a reduction in the levels of IGF-I and increased concentrations of IGFBP-3 in the dorso-lateral prostate and that this was associated with a decrease in p-Akt and p-MAPK [27]. In Du145 and LNCaP cells, EGCG decreased levels of p-Akt but increased levels of p-MAPK over a period of 12–24 hr [28].

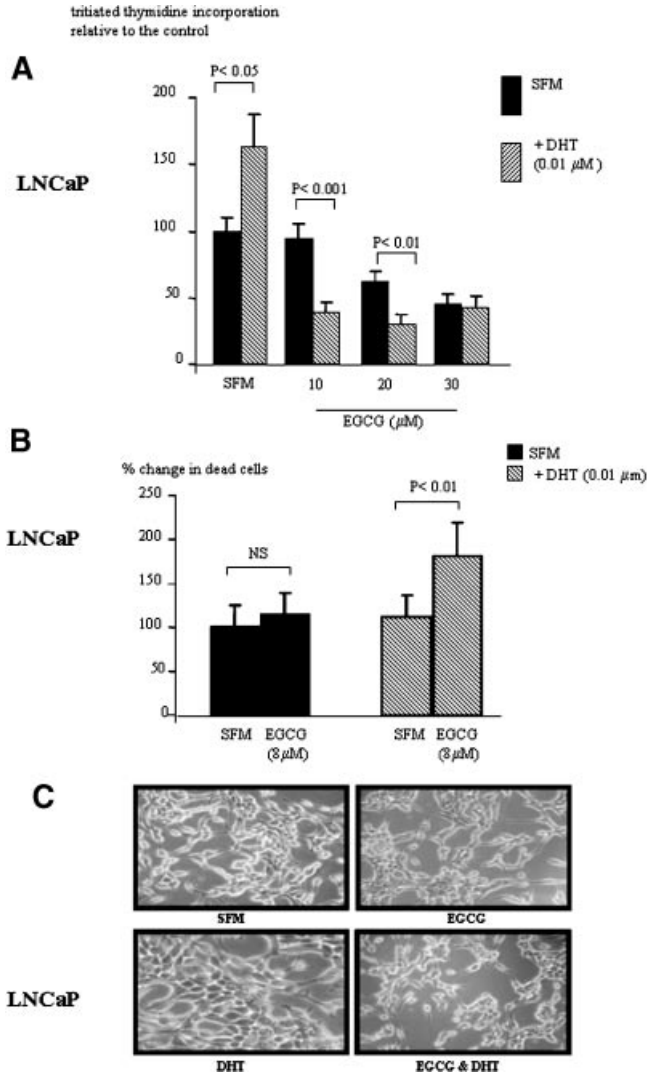

Fig. 4. Effects of EGCG on DHT-induced growth of LNCaP cells. LNCaP cells were seeded in growth media (GM; 1 ml) (**A**) in 24-well plates (0.2×10^6 cells/well) for 24 hr. Following 24 hr in serum-free media (SFM) they were treated with (A) DHT (0.01 μM) +/− EGCG (0–30 μM). TTI was performed as described in 1B and the graph shows the mean of three experiments each repeated in triplicate. Subpart **B** in 6-well plates (0.3×10^6 cells/well) for 24 hr. Following 24 hr in serum-free media (SFM) they were treated with DHT (0.01 μM) +/− EGCG (10 μM). Cell death was assessed as in Figure 1B and the graph shows the mean of three experiments each repeated in triplicate. **C**: LNCaP cells were treated as in (B) and are represented pictorially (D). Cells were viewed using a Leica DMI RB microscope and photographs were taken using a Nikon E450 digital camera (magnification 100×).

As we had found that the IGF-IR played a role in the ability of DHT to increase DNA synthesis and that the growth response to IGF-I was blocked by EGCG, we next investigated the effect of EGCG on DHT-induced proliferation. It has been shown that treatment with EGCG reduces circulating testosterone in murine models of CaP [29,30]. In addition, with in vivo models of CaP, inhibitors of 5 α-reductase slowed the growth of established tumors [31] and EGCG is capable of blocking 5 α-reductase activity [32]. In vitro, EGCG has also been shown to down-regulate expression of androgen receptor mRNA in LNCaP cells [33]. Figure 4A shows that in the presence of sub-apoptotic doses of EGCG (10–20 μM), the growth response to DHT was in fact significantly reversed (10 μM, $P < 0.001$ and 20 μM, $P < 0.01$) and blocked at the higher dose of 30 μM. In Figure 4B, we determined that in the presence of androgen the cells became sensitized to EGCG such that a sub-apoptotic dose then induced death. In the presence of EGCG or DHT alone there was no effect, but together there was a significant induction of cell death ($P < 0.01$). We show photomicrographs in Figure 4C to illustrate that coincident with the induction of apoptosis, there were fewer cells attached to the plate with the combination of DHT and EGCG in relation to either on their own. We also established that in contrast to DHT, exogenous IGF-I was unable to sensitize these cells to EGCG (data not shown), despite the growth response to DHT being dependent on activation of the IGF-IR.

Our data suggests that both green tea and the IGF-IR TKI are both effective in inhibiting cell growth and inducing death in CaP cells and they both become more effective in the presence of androgen. Our data further suggests that these agents might therefore be compromised if used in combination with anti-androgen therapy. This study highlights that potential combination therapy has to be considered carefully in order to develop effective chemoprevention and therapy of prostate cancer.

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