Antiangiogenic effect of combined treatment with curcumin and genistein on human prostate cancer cell line

N.P. Aditya a, Myeongkuk Shim b, Hanjoo Yang a, YoungJoo Lee b, Sanghoon Ko a, *

a Department of Food Science and Technology, Sejong University, 98 Gunja-dong, Guwangjin-gu, Seoul 143-747, Republic of Korea
b Department of Bioscience and Biotechnology, Sejong University, 98 Gunja-dong, Guwangjin-gu, Seoul 143-747, Republic of Korea

ABSTRACT

High cost and toxicity, low success rate and patient incompliance associated with existing anticancer drugs necessitated for finding new anticancer drugs which can overcome the aforementioned drawbacks. Toward this direction effort has been made to understand the anticancer activity of curcumin (cur) and genistein (gen) combination in human prostate cancer cell line (PC3) cells with respect to their antiangiogenic effect. Cur and gen has shown dose and time dependent decrease in cell viability, increase in apoptosis and cell cycle arrest at G0 phase. These effects were more noticeable when cur (20 μM) and gen (100 μM) were used in combination. To understand antiangiogenic effect of this combination, expression of ARNT and HIF-1α was studied. Significant decline in expression of ARNT and HIF-1α protein level was seen in comparison to control group and their respective monotherapy treated groups. Cur and gen are shown to be effective in abrogating the VEGF production by evading ARNT and HIF-1α complex formation as proved by immunoprecipitation assay. Thus this combination seems to be promising toward cancer treatment.

1. Introduction

Prostate cancer is more prominent in developed countries compared to developing countries (Baade, Youlden, & Krnjacki, 2009). Other than skin cancer, prostate cancer is the most common cancer in American men and account for about 29% of total cancer cases (Siegel, Ward, Brawley, & Jemal, 2011). Even though hormone therapy, radiation therapy, surgery and chemotherapy are effective in treating prostate cancer to some extent...

http://dx.doi.org/10.1016/j.jff.2014.03.014
1756-4646/© 2014 Elsevier Ltd. All rights reserved.
in early stages, outcome for advanced or recurrent cancer is not satisfactory. The mean survival period with these therapies is 12–18 months (Niu et al., 2008). In addition, most of the conventional antineoplastic drugs used today were designed to hit a single target. But, the physiological and mechanistic cause for deregulation accountable for prostate cancer initiation and promotion involves hundreds of genes or signaling cascades. Dose limiting toxicities and acquired resistance further limited their use. Thus, novel antineoplastic drugs should be able to act on multiple targets and nontoxic to normal cells.

Therefore, the ideal prototype of an antineoplastic drug should display anti-tumor activity by targeting multiple targets and damaging cancer cells without causing adverse effects or toxicity to healthy cells (Dhar, Kolishetti, Lippard, & Farokhzad, 2011). Since there are no such ideal drugs developed until now, intervention with compounds of natural origin such as phytotherapeutics agents is considered one such effective means to treat cancer and also to ward off cancer development. Since ages, phytotherapeutics have been in use to treat various ailments due to its pleiotropic effects and nontoxicity. Since role of inflammation in the progression of prostate cancer is apparent, anti-inflammatory, antioxidant phytotherapeutics play an important role in prostate cancer therapy (Trottier, Bostrom, Lawrentschuk, & Fleshner, 2010). From epidemiological studies it is evident that a prostate cancer case among Asian men is comparatively less than Caucasians who consumes more nutraceuticals in their daily diet (Ide et al., 2010). But, the major problems associated with these nutraceutical molecules are less stability and bioavailability. Hence it becomes very difficult to get the required therapeutic dosage at required site of action for required duration. Hence it becomes vital to find a suitable combination of molecules which can potentiate each other’s therapeutic efficiency and reduce the required therapeutic dosage.

In this stare among the many nutraceuticals, curcumin (cur) which is a polyphenol derived from the root of curcuma longa Linn and the isoflavone genistin (gen) present in soya are among two widely studied phytotherapeutics for various diseases including cancer. Although, the anticancer potential of cur or gen has been adequately discovered, the information regarding synergistic anticancer activity especially the antiangiogenic effect of cur and gen combination is lacking. In this regard, Ide and coworkers have shown that cur and isoflavones (genistin, daidzein, and glycitein) combination activated the DNA damage response in LNCaP cells and thereby inhibited the cell proliferation (Ide et al., 2010). Since there are no such ideal drugs developed until now, intervention with compounds of natural origin such as phytotherapeutics agents is considered one such effective means to treat cancer and also to ward off cancer development. Since ages, phytotherapeutics have been in use to treat various ailments due to its pleiotropic effects and nontoxicity. Since role of inflammation in the progression of prostate cancer is apparent, anti-inflammatory, antioxidant phytotherapeutics play an important role in prostate cancer therapy (Trottier, Bostrom, Lawrentschuk, & Fleshner, 2010). From epidemiological studies it is evident that a prostate cancer case among Asian men is comparatively less than Caucasians who consumes more nutraceuticals in their daily diet (Ide et al., 2010). But, the major problems associated with these nutraceutical molecules are less stability and bioavailability. Hence it becomes very difficult to get the required therapeutic dosage at required site of action for required duration. Hence it becomes vital to find a suitable combination of molecules which can potentiate each other’s therapeutic efficiency and reduce the required therapeutic dosage.

In this paper among the many nutraceuticals, curcumin (cur) which is a polyphenol derived from the root of curcuma longa Linn and the isoflavone genistin (gen) present in soya are among two widely studied phytotherapeutics for various diseases including cancer. Although, the anticancer potential of cur or gen has been adequately discovered, the information regarding synergistic anticancer activity especially the antiangiogenic effect of cur and gen combination is lacking. In this regard, Ide and coworkers have shown that cur and isoflavones (genistin, daidzein, and glycitein) combination activated the DNA damage response in LNCaP cells and thereby inhibited the cell proliferation (Ide et al., 2010). Recently, we have shown that cur and gen combination therapy suppresses PC3 cell growth in vitro (Aditya et al., 2013). Earlier studies have clearly indicated that antiangiogenic therapies are hopeful treatments for the management of cancer. However, antiangiogenic effect of this combination is still unexplored. We hypothesize that by treating the prostate cancer cells by combination of cur and gen which are natural estrogen receptor (ER) modulators and are known to increase the expression of ERβ in PC3 cells, we can trim down the hypoxia stimulated HIF-1α-mediated VEGF expression (Mak et al., 2010; Piccolella, Crippa, Messi, Tetel, & Poletti, 2014). Thus, following research study was undertaken to evaluate in antiangiogenic effect of cur and gen combination. Our results clearly demonstrate that cur and gen combination treatment can effectively inhibit the angiogenesis in PC3 cells in vitro.

These results indicate that there is a huge potential for development of functional foods incorporating cur and gen as bioactive compounds. If functional foods are developed incorporating cur and gen, then that can be recommended for the consumption of elderly people (> 40 years) who are at the risk of getting ER associated cancer such as prostate cancer, and breast cancer, among others. Since both cur and gen are known to be non-toxic from several in vitro, in vivo and clinical studies, minimal effort will be needed to bring these functional foods to the market which have great demand and have become the choice of the consumers in recent years for health promotion and disease risk reduction (Im, Ravi, Kumar, Kuttan, & Maliakel, 2012; Vo & Kim, 2013).

2. Materials and methods

2.1. Materials

Curt with >95 purity (Sigma-Aldrich, St. Louis, MO, USA) and gen with >90 purity (Macro Care Ltd, Chungcheongbuk-do, Korea) were dissolved in dimethyl sulfoxide (DMSO). Required concentrations of cur and gen were obtained by diluting with culture media before treatment. Concentration of DMSO was <0.1%.

2.2. Cell culture and hypoxic condition

PC3 cells were obtained from American Type Cell Culture Collection and maintained in Dulbecco’s Modified Eagle Medium (DMEM- Phenol red free) supplemented with 10% fetal bovine serum (FBS) (WelGENE, Daegu, Korea). Cells were grown in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. The test medium in cytotoxicity and other assays was DMEM supplemented with 5% charcoal–dextran stripped FBS (CD-FBS). For the hypoxic condition, cells were incubated at a CO2 level of 5% with 1% O2 balanced with N2 using a hypoxic chamber (Forma).

2.3. Cell viability assay

The cytotoxicity of cur and gen alone or in combination was determined using the cell proliferation reagent WST-1 (4-[3-(4-iiodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio])-1,3-benzene disulfonate) (Roche Applied Science, Indianapolis, IN, USA). Briefly, PC3 cells were seeded in triplicate at 5 × 104 cells/well in 96 well plates. After 24 h, cells were treated with cur (20 μM), gen (100 μM) or cur + gen (20 μM + 100 μM) and cells were cultured under aforesaid conditions for the indicated periods of time. At the end of the treatment, the cells were washed twice with PBS to remove the yellow color which was due to the presence of cur which may interfere with the final product and 200 μL of fresh medium was added. For the WST-1 assay, 20 μL of tetrazolium salt WST-1 solution was added to each well and incubated for 2 h at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Cleavage of WST-1 to yellow formazan was quantified by using microplate reader in accordance with the
manufacturer’s instructions. Minimum three independent sets of experiments were performed.

2.4. Cell cycle analysis

Fate of cell cycle progression in PC3 cells after treatment with cur and gen was studied using flow cytometry following staining with propidium iodide as described previously (Herman-Antosiewicz & Singh, 2005). Briefly PC3 cells were treated with cur and/or gen and after 48 h both floating and attached cells were collected and fixed with 70% ethanol at 4 °C. Cells stained with propidium iodide were used to study the cell cycle using FACS (BD Biosciences, San Jose, CA, USA).

2.5. Caspase-3 and -7 activity assays

For quantitative apoptotic assay, PC3 cells were seeded at a density of 5 × 10^4 cells/well in 96 well plates in triplicate. Cells were incubated with cur (20 μM), gen (100 μM) or cur + gen (20 + 100 μM). After 24 or 48 h incubation, caspase-3 and caspase-7 activities were measured in accordance with the manufacturer’s directions (Promega, Madison, WI, USA) with slight modification. Briefly, treated cells were washed three times with PBS to remove cur which may interfere with flurometric measurement due to its autofluorescence and fresh medium was added. 100 μL of caspase-Glo 3/7 substrate was added to each well and incubated for 2 h in ambient condition. Fluorescence was measured at an excitation wavelength of 490 nm and emission wavelength of 510 nm using Varioskan Flash microplate reader. Caspase-3 and caspase-7 activity was expressed as fold increase in comparison to control. Minimum of three independent sets of experiments were performed.

2.6. Enzyme-linked immunosorbent assay (ELISA)

To examine the antiangiogenic effect of cur and gen combination, PC3 cells were treated with either cur or gen or in combination and incubated both in hypoxia and normoxia condition for 48 h. After hypoxic or normoxia exposure, culture medium was collected and stored at −70 °C until assayed. These conditioned media from cultured cells treated with cur (20 μM), gen (100 μM) or cur + gen (20 + 100 μM) were subjected to VEGF-ELISA according to the manufacturer’s instruction (R&D System, Minneapolis, MN, USA). In brief, 50 μL of assay diluents and 200 μL of samples were added to a 96 well plate coated with human VEGF monoclonal antibody. After 2 h of incubation, wells were washed and once again incubated for 2 h with VEGF conjugate which is then washed using wash buffer and color was developed using substrate solution. After stopping the reaction by using stopping buffer, optical density was measured at 450 nm using microplate reader with correction at 560 nm. Standard graph was constructed for each experiment. Minimum of three independent sets of experiments were performed.

2.7. Quantitative real-time-polymerase chain reaction (qPCR)

Trizol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) was used to isolate total RNA according to the manufacturer’s instruction. Diethylpyrocarbonate-treated water was used to dissolve the RNA before its concentration was checked using nanodrop at 260 nm (Malcom Co., Ltd, Tokyo, Japan). Samples were aliquoted and stored at −70 °C until further processing. Three microgram total RNA was incubated at 70 °C for 5 min with 0.5 μg of random hexamer and deionized water to synthesize cDNA (up to 11 μL). The reverse transcription reaction was performed using 40 units of M-ML reverse transcriptase (Promega, Madison, WI, USA) in 5× reaction buffer (250 mmol/L Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 50 mM DTT), RNase inhibitor at 1 unit/μL, and 2.5 mM dNTP mixtures at 37 °C for 60 min. Quantitative real-time PCR (qPCR) was used to detect VEGF mRNA using IQ™ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers used were β-actin sense primer, 5′-CAA ATG CTT CTA GCC GGA CTA TG-3’; anti-sense primer, 5′-TGC GCA AGT TAG GTT TTG TCA-3’; VEGF sense primer, 5′-CGT CAT GCA GCT CAT AGC TC-3’; anti-sense primer, 5′-ATG AAC TTT CTG CTC TCT GG-3’. A final volume was 20 μL, and an iCycler iQ™ Real Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for qPCR. The amplification data were analyzed by IQ™5 optical system software version 2.1 and calculated using the ΔΔCT method. The ΔΔCT method was used to calculate relative mRNA expression. The relative target gene expression was calculated using 2-ΔΔCT, where ΔCT = target CT – control CT, ΔΔCT = ΔCT target – ΔCT calibrator.

2.8. Transient transfection and luciferase assay

PC3 cells were seeded in 24-well plates at a density of 5 × 10^4 cells/well. After 24 h, plasmids were transiently transfected with hypoxia-responsive element (HRE)-luciferase reporter plasmids into the cell by using the polyethylenimine (PEI, Polysciences) method. Further cells were incubated for 12 h and later treated with either cur and/or gen and incubated under normoxic or hypoxic conditions for 24 h. After 24 h of treatment, plates were frozen at −70 °C at least for 2 h to lyse the cells. Luciferase activity was determined with an AutoLumat LB953 luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany) using the luciferase assay system (Promega, Madison, WI, USA) and expressed as relative light units. All transfection experiments were repeated at least three times.

2.9. Western analysis

Radioimmune precipitation buffer (containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% NP-40 and 0.5% deoxycholic acid with a protease inhibitor cocktail) was used to extract protein from PC3 cells. Proteins were separated by centrifuging the cell lysate for 20 min at 13,000 × g. Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to determine the protein concentration. Before transferring the proteins on the polyvinylidene difluoride membrane for immunoblotting they were separated by SDS-PAGE. Five percent nonfat dry milk in Tris-buffered saline/0.05% Tween-20 (TBST) was used to block the membranes. This membrane was incubated with antimouse polyclonal antibody to alyl hydrocarbon receptor nuclear translocator (ARNT) (BD Transduction Laboratories, Lexington, KY, USA), anti-rabbit monoclonal antibody to hypoxia inducible factor-1α (HIF-1α) (BD Transduction Laboratories,
Lexington, KY, USA), or anti-mouse polyclonal antibody to b-actin (Sigma Life-Science, St. Louis, MO, USA). After overnight incubation at 4 °C, blots were washed at least thrice with TBST and again incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA), and washed again three times with TBST. The transferred proteins were visualized with enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.10. Immunoprecipitation

Immunoprecipitation assay was conducted as described earlier with suitable modification (Lim et al., 2011). Briefly, 200 μg of PC3 cell lysate was mixed with 1 μg of antibody and at constant rotation it was incubated for overnight at 4 °C. One hundred fifty microgram of protein A- sepharose, diluted 1:1 in PBS was added to the sample and kept on ice and rotated for additional 2 h to obtain immunoprecipitated complex. Beads were pelleted down using centrifugation. The bound proteins were eluted by using 2× SDS loading buffer by boiling beads for 5 min. The eluted proteins were analyzed by immunoblot analysis.

2.11. Statistical analysis

Values shown represent the mean ± S.D. Statistical analysis was performed by Student’s t-test, and a P-value < 0.05 was considered significant.

3. Results and discussion

3.1. Cell viability-mitochondrial activity assay

PC3 cells were treated with different concentrations of cur and/or gen for different time intervals and cell viability was determined by WST-1 assay. As shown in Fig. 1A, PC3 cells were treated with three different concentrations of cur (20, 40 and 60 μM) and three different concentrations of gen (60, 80 and 100 μM) for 24 h. Cur treatment reduced the cell viability in dose dependent manner and cell viability was reduced to 83% at 60 μM concentration. When treated with gen, more than 90% cell viability was observed in all tested doses. Even though 60 μM concentration of cur was able to effectively inhibit the PC3 alone, this concentration is physiologically unachievable through dietary consumption (Beevers, Li, Liu, & Huang, 2006). However, cell viability in PC3 cells were more efficiently inhibited when treated with cur and gen combination in all tested doses. Next, to check the time dependent effect of cur and gen treatment PC3 cells, cells were treated with either 20 μM cur, 100 μM gen or 20 μM cur + 100 μM gen combination for 24, 48 and 72 h. Compared to 24 and 48 h treatments, 72 h treatment had shown minimum cell viability when treated individually or in combination. Cur and gen combination treatment reduced the cell viability up to 57% after 72 h treatment compared to control (Fig. 1B). This clearly indicated that gen alone is not potent molecule to inhibit the cell viability but it can potentiate the activity of cur when used in combination. Similarly, earlier Verma and co-workers have shown lessened cell viability when MCF-7 cells were treated with cur and gen combination compared to individual molecules (Verma, Salamone, & Goldin, 1997).

3.2. Cell cycle analysis

Since cell cycle plays a vital role in cell proliferation and growth, we determined the effects of cur and/or gen on the cell cycle development by propidium iodide staining using FACS. As shown in Fig. 2 cur and gen combination treatment for 48 h effectively arrested cell cycle progression in PC3 cells in G0 phase. Total cells in G0 phase were 15.4% in cur and 16.4% in gen treated groups. It rose up to 23.6% when PC3 cells were treated with combination of cur and gen. This cell cycle arrest in G0 phase either will provide an opportunity for cell to repair the damage before proceeding further or unrepaired cells will undergo apoptosis. These results are in corroborations with earlier results which also showed that treatment of LNCaP cells with isoflavones and cur results in activation of DNA damage response which inhibit the tumor progression in early stages of cell cycle (Iđe et al., 2011). Further decrease in G1 phase clearly indicates that cur and gen combination had stopped the start of new cell cycle.

3.3. Curcumin and genistein combination induces cell death by apoptosis

Since more cells were found in the G0 phase we tested the caspase -3 and -7 activity. These executioner caspase-3 and caspase-7 are among the family of cysteine proteases required to induce apoptosis in the cell (Gururaj, Belakavadi, Venkatesh, Marme, & Salimath, 2002). To validate that decreased cell viability in combination treatment is due to enhanced cellular apoptosis compared to monotherapy, quantitative apoptotic analysis was performed. Cur and gen combination showed significantly (P < 0.05) enhanced caspase-3 and caspase-7 activities compared to cur or gen monotherapy after 48 h treatment (Fig. 3). Earlier it has been shown that cur induce apoptosis in PC3 cells via activation of caspase independent pathway (caspase-8) whereas gen is known to induce apoptosis in PC3 cells via caspase dependent pathway (caspase-9) (Hilchie et al., 2010; Yu et al., 2012). Our result indicates that increased apoptosis in cur and gen combination therapy compared to monotherapy may be due to activation of both caspase dependent and independent pathways.

3.4. Curcumin and genistein combination inhibits VEGF

Since, previous study had shown that both cur and gen had antiangiogenic effect in various cancer models both in vitro and in vivo, here effort had been made to assess the antiangiogenic effect of cur and gen combination over the individual compounds (Gururaj et al., 2002; Yu et al., 2012) by using VEGF, a very well-studied HIF-1α target gene (Park, Kim, Shim, & Lee, 2012). As shown earlier, increase in VEGF mRNA level was observed when PC3 cells were exposed to hypoxic condition (Darrington, Zhong, Vo, & Khan, 2012). There was no much variation was observed when cells were treated with either cur or gen compared to hypoxia group cells. This is in corroborations with earlier results which have shown that treatment with either cur or gen regulates the VEGF expression only.
post-transcriptionally (Levy, Levy, & Goldberg, 1996; Subramaniam et al., 2011). Surprisingly, when PC3 cells were treated with combination of cur and gen VEGF mRNA level reduced significantly (Fig. 4A) compared to hypoxia group and even slight reduction was seen compared to control group. This result of the present study clearly indicates that combination of cur with gen will be more potent in inhibiting the angiogenesis compared to monotherapy.

Fig. 1 – Dose- and time-dependent inhibitory effects on proliferation of PC3 cells by cur and/or gen. (A) PC3 cells were treated with cur, gen or cur + gen combination for 24 h. (B) PC3 cells were treated with 20 μM curcumin, 100 μM genistein or 20 μM cur + 100 μM gen for 24, 48 and 72 h. Curcumin and genistein combination therapy after 72 h exposure inhibited PC3 cell proliferation more efficiently compared to monotherapy and 24 and 48 h time intervals. Each bar represents the mean ± SD of triplicate data points from three separate experiments.
Fig. 2 – Cell cycle analysis of PC3 cells after treatment with the cur and/or gen examined by flow cytometry following propidium iodide staining for DNA content. PC3 cells were treated with (A) control, (B) 20 μM cur, (C) 100 μM gen, and (D) 20 μM cur + 100 μM gen combination for 48 h. (B) Effects of cur and/or gen on the percentages of cells at sub G0, G1, S and G2/M phase. Cur and gen combination resulted in more cells accumulation in G0 phase indicating increased apoptosis or activation DNA damage response. Graphs are representative of data collected from three experiments.

<table>
<thead>
<tr>
<th></th>
<th>sub G0</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>con</td>
<td>16.26</td>
<td>41.30</td>
<td>7.82</td>
<td>34.62</td>
</tr>
<tr>
<td>cur</td>
<td>15.54</td>
<td>45.01</td>
<td>9.01</td>
<td>30.44</td>
</tr>
<tr>
<td>gen</td>
<td>16.52</td>
<td>38.83</td>
<td>9.66</td>
<td>34.99</td>
</tr>
<tr>
<td>cur+gen</td>
<td>23.75</td>
<td>26.74</td>
<td>11.16</td>
<td>38.35</td>
</tr>
</tbody>
</table>

Fig. 3 – Cur and/or gen activate caspase-3 and caspase-7, an apoptosis mediator. PC3 cells incubated with 20 μM cur, 100 μM gen or 20 μM cur + 100 μM gen were analyzed for apoptosis by caspase-3 and caspase-7 activation after 24 and 48 h. Cur and gen combination treatment after 48 h treatment increased the caspase-3 and caspase-7 activation compared monotherapy group. Fold increase was calculated by taking control as reference. Each bar represents the mean ± SD of triplicate data points from three separate experiments.
To check the VEGF protein level induced by cur and/or gen level of VEGF protein present in the media was studied. After treatment with either cur or gen, VEGF concentration decreased by 2.2-fold in cur treated cells and 1.6-fold in gen treated cells compared to conditioned media. But always VEGF protein level was higher compared to control media. The decrease in VEGF protein level in cur treated cells may be due to increase in the expression of RNA binding protein CUGBP2, which inhibits the translation of VEGF mRNA (Subramaniam et al., 2011). The decrease in VEGF mRNA in gen treated cells may be due to reduced hypoxia-induced stabilization of VEGF 3′-UTR transcripts (Levy et al., 1996). On the other hand, cur and gen combination treatment resulted in 6.8-fold decrease in VEGF protein level compared to conditioned media and even it was decreased 2.6-fold compared to control media (Fig. 4B). This significant decrease in VEGF protein in combination therapy may be attributed by the dual action of cur and gen (i.e. decreased VEGF mRNA translation by cur and reduced VEGF protein stability by gen). This clearly indicated that cur and gen combination therapy is more potent than monotherapy for post-transcriptional inhibition of angiogenesis.

3.5. Curcumin and genistein down regulates hypoxia induced HRE complex

Even though cur and gen monotherapy failed to control the VEGF level, cur and gen combination was successful (Fig. 4B). Thus to study the effect of this combination on formation of HRE complex, HIF-1α and ARNT were studied. For this PC3 cells transformed with a HRE-Luc reporter construct was used to assess whether HIF-1α activity was compromised in the presence of cur and/or gen. We found that cur treatment alone was incapable to either reduce or increase the HRE activation. But gen treatments significantly increase the HRE activity. Whereas cur and gen combination treatment almost abrogated HRE activity in the PC3 cells at the tested concentration (Fig. 5A). This clearly indicates that cur and gen combination affect the transcriptional activity of HIF.

Next, we checked the effects of cur and/or gen on its sub-units HIF-1α and ARNT levels. Hypoxia increased HIF-1α level compared to control which corroborate with earlier results (Park et al., 2012). Treatment with either cur or gen has a very little effect on HIF-1α and ARNT level in PC3 cells, but both HIF-1α and ARNT levels were totally reduced when PC3 cells were treated with cur and gen combination (Fig. 5B). This effect may be due to the increase in ERβ expression in PC3 cells by the cur and gen combination treatment more efficiently than monotherapy which are well known natural ER modulators (Piccolella et al., 2014). Earlier results from our laboratory, using MCF-7 and Hep3B cells, have proven that sufficient level of ERβ expression down regulates the ARNT protein levels and down regulation of ARNT leads to the HIF-1α repression (Lim et al., 2011).

3.6. Effect of curcumin and genistein combination on ARNT binding with HIF-1α

Our data strongly suggest that cur and gen combination therapy decreases HIF-1α mediated gene transcription through HIF-1α and ARNT down-regulation. To study the functional consequence which might arise from down regulation of ARNT after cur and gen combination in PC3 cells, formation of ARNT/HIF-
1α complex was evaluated. As shown in Fig. 5C, combination therapy resulted in decreased HIF-1α/ARNT complex levels under hypoxic condition determined by immunoprecipitation more efficiently than the monotherapy. In line with this study earlier studies have shown that inactivation of ARNT is sufficient to suppress HIF target gene induction (Maltepe, Schmidt, Baunoch, Bradfield, & Simon, 1997). A recent study from our laboratory has shown decrease in HIF-1α and ARNT complexation in the presence of ERβ (Lim et al., 2011). Since cur and gen are known to enhance the ERβ expression in cancer cells, particularly in prostate cancer cell lines (PC3), increase in ERβ concentration might result in reduced formation of ARNT/HIF-1α complex (Lim et al., 2011; Piccolella et al., 2014).
4. Conclusion

Due to the presence of molecular heterogeneity and complexity of the signaling pathways in cancer cell growth, phytotherapeutics capable of targeting cancer cell in multiple ways are seem to be valuable tools for control, prevention, and cure of cancer. Cur and gen combination therapy which has been known to inhibit the cancer cells by multiple ways in earlier studies (Ide et al., 2010, 2011) has also been shown to inhibit angiogenesis by acting on VEGF protein expression by down regulating HIF-1α and ARNT levels and inhibiting the formation of HIF-1α and ARNT complex formation (Fig. 6). The current study provides valuable input regarding suitability of cur and gen combination for the management of prostate cancer in vitro. However, additional studies, using suitable in vivo models and clinical trials, are required before recommending functional foods enriched with cur and gen for cancer prevention and treatment in humans.

Acknowledgments

This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science, ICT & Future Planning (No. 2013078159).

REFERENCES


