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# The anti-cancer effects of carotenoids and other phytonutrients resides in their combined activity

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ABSTRACT

Epidemiological studies have consistently shown that regular consumption of fruits and vegetables is strongly associated with reduced risk of developing chronic diseases, such as cancer. It is now accepted that the actions of any specific phytonutrient alone do not explain the observed health benefits of diets rich in fruits and vegetables as nutrients that were taken alone in clinical trials did not show consistent preventive effects. The considerable cost and complexity of such clinical trials requires prudent selection of combinations of ingredients rather than single compounds. Indeed, synergistic inhibition of prostate and mammary cancer cell growth was evident when using combinations of low concentrations of various carotenoids or carotenoids with retinoic acid and the active metabolite of vitamin-D. In this study we aimed to develop simple and sensitive in vitro methods which provide information on potent combinations suitable for inclusion in clinical studies for cancer prevention. We, thus, used reporter gene assays of the transcriptional activity of the androgen receptor in hormone-dependent prostate cancer cells and of the electrophile/antioxidant response element (EpRE/ARE) transcription system. We found that combinations of several carotenoids (e.g., lycopene, phytoene and phytofluene), or carotenoids and polyphenols (e.g., carnosic acid and curcumin) and/or other compounds (e.g., vitamin E) synergistically inhibit the androgen receptor activity and activate the EpRE/ARE system. The activation of EpRE/ARE was up to four fold higher than the sum of the activities of the single ingredients, a robust hallmark of synergy. Such combinations can further be tested in the more complex in vivo models and human studies.



## Introduction

Diet provides desirable health benefits beyond basic nutrition. Life style and especially dietary habits have been closely linked to the risk of various chronic diseases. Considerable epidemiologic evidence indicates an association between the consumption of fruits and vegetables and reduced incidence of various types of cancer [1,2]. Carotenoids, an important group of dietary phytonutrients possess well-documented cancer-preventive activity [3,4]. For example, epidemiological studies have linked increased consumption of lycopene, the red pigment of tomato with decreased prostate [5,6] and breast [7] cancer risk. These findings are supported by in vitro and in vivo experiments showing reduced proliferation, induced apoptosis and a decrease in the metastatic capacity of prostate cancer cells as a result of lycopene treatment [8–10]. Another group of phytonutrients with well-documented cancer-preventive activity are the polyphenols [11,12]. The polyphenol curcumin, the major yellow pigment in turmeric, which is widely used as an Indian spice, attracts much attention due to its strong anti-inflammatory and anti-cancer effects [13], and the anti-proliferative effect of curcumin in prostate cancer cells has been established [14]. Other phytonutrients from human diet such as omega 3 fatty acids seem to provide a promising therapeutic approach [15].

Periodically, we are presented with a new "magic bullet", a dietary component with a therapeutic or preventive effect that should be consumed in high doses. Nonetheless, while most studies support a protective effect of the consumption of fruits and vegetables, significant associations with single compounds have shown conflicting results [16,17]. In addition, supplementation studies using synthetic or purified single agents did not show beneficial effects [18]. Of note, a substantial number of findings derive from cell based-studies using high concentrations of phytonutrients, which

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are often not physiological. The prevailing view is that "magic bullets" are not found, and the beneficial effect of fruits and vegetables is based on the synergistic effect of several components from whole foods, each of them present in low concentration and their combined activity is responsible for the antioxidant and anti-cancer effect of a diversified diet. Staple diet compositions are profoundly dependent on geographical or sociological factors, and have a marked impact on human health.

A tenable view is that the beneficial effects of phytochemical mixtures present in fruits, vegetables and other dietary components [19] reside, at least partly, on complementary and overlapping mechanisms of action of these nutrients on several cellular pathways. Regulation of transcription is a common mechanism for the chemopreventive activity of various phytonutrients and regulation of gene expression has been found to play a significant role in the effect of phytonutrients on many cellular processes including the antioxidant defense mechanism, cell proliferation and apoptosis and hormone signaling and metabolism [20–22].

Prostate cancer is the most common cancer in men [23]. Steroid hormones, particularly androgens, play a role in the initiation and progression of prostate cancer [24]. The pivotal role of androgens in the development of this malignancy prompted research regarding the potential use of phytonutrients, which could interfere with androgen metabolism or its signaling pathway. Another important mechanism in the prevention of cancer including the prevention of prostate cancer is activation of cellular antioxidant defense mechanisms. These include the activation of the Electrophile/Antioxidant Response Element (EpRE/ARE)[1] transcription system which is responsible for the induction of phase II detoxifying and antioxidant enzymes such as glutathione S-transferase, NAD(P)H: quinone oxidoreductase 1, superoxide dismutase, and heme oxygenase 1.

The effects of a tomato extract containing lycopene and other phytonutrients and fish oil containing omega-3 fatty acids on metabolic pathways in prostate cancer patients were recently examined [25]. In this double-blind randomized, placebo-controlled study performed on non-aggressive prostate cancer patients, cDNA microarray analysis revealed that supplementation of these dietary extracts modulate at least two regulatory pathways which are important for the progression of this malignancy: the androgen and estrogen metabolism and the EpRE/ARE transcription system which regulate the oxidative stress response pathway. In view of the importance and relevance of these two pathways in the prostate, we examined in the current study the effect of phytonutrient combinations (including lycopene and omega-3 fatty acids) on the differential modulation of the Androgen Response Element (AnRE) and the EpRE/ARE transcription system in LNCaP prostate cancer cells. To this end, we utilized the sensitive reporter gene assay system in order to explore the effect of different phytonutrient combinations as a selection tool for assessing the best combinations that could be used for prostate cancer prevention in the clinical setting.

## Materials and methods

### Materials

Crystalline lycopene preparations, purified from tomato extract (>97%), phytoene + phytofluene – a 1:1 mixture (the indicated concentrations are the sum of both carotenoids), tomato extract LycoMato®, vitamin E (Tocopherol mix) and a 1.5:1 mixture of eicosapentaenoic acid and docosahexaenoic acid (EPA/DHA) omega-3 fatty acids (Quimica Industrial Spes S.A, Chile) were a gift of Lycored Ltd., Beer Sheva, Israel. Astaxanthin, silibinin and all-trans retinoic acid were purchased from Sigma Chemicals (Rehovot, Israel). β-Carotene was a gift of DSM (Basel Switzerland). Crystalline curcumin (>95%) was purchased from Cayman Chemicals (Ann Arbor, MI). Carnosic acid (93–97%) was purchased from Alexis Biochemicals (Switzerland) and tetrahydrofuran (THF), containing 0.025% butylated hydroxytoluene as an antioxidant, was purchased from Aldrich (Milwaukee, WI, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 and phenol red free RPMI medium, fetal calf serum (FCS), dextran-coated charcoal-stripped FCS (DCC-FCS), and sodium pyruvate were purchased from Biological Industries (Beth Haemek, Israel). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemicals. Acrodisc syringe filter 0.8/0.2 μm (PALL Corporation, MI, USA). Dihydrotestosterone (DHT) was purchased from Ikapharm, Ramat Gan, Israel. 1α,25-Dihydroxyvitamin $D_3$ (1,25vit$D_3$) was a gift from Dr. Andrzej Kutner (Pharmaceutical Research Institute, Warsaw, Poland).

### Cell culture and treatment

LNCaP – androgen-responsive, PC-3 and DU-145 – androgen-nonresponsive, human prostate cancer cells and MCF-7 – hormone-dependent human mammary cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA).

LNCaP, PC-3 and DU-145 cells were grown in RPMI containing sodium pyruvate (0.11 mg/ml) and DHT ($10^{-9}$ M). MCF-7 cells were grown in DMEM medium containing insulin (0.6 μg/ml). Human foreskin fibroblasts, provided by Soroka University Medical Center skin bank, Beer Sheva, Israel, were grown in DMEM medium. All culture media were supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), nystatin (12.5 μg/ml), and 10% FCS.

Carotenoids were dissolved in THF, solubilized in cell culture medium and the absorption spectra of the compounds were determined as described previously [26,27]. The absorption spectra were measured in each experiment to evaluate the stability and to calculate the actual concentrations of the carotenoids. Curcumin and silibinin were dissolved in DMSO and added to the culture media at the final concentrations specified in the figure legends. Carnosic acid, vitamin E and 1,25vit$D_3$ were dissolved in ethanol. EPA/DHA mixture was first diluted 1:1 in ethanol and then 1:500 in culture media to receive the final concentrations specified in the figure legends. The final concentrations of the solvents in both the treated and control cells were 0.5% THF, 0.1% ethanol, and 0.05–0.1% DMSO. The compound vehicles did not affect the measurements performed. All procedures were performed under reduced lighting.

Since LNCaP cells detach easily from tissue culture plate surface, the medium was never completely removed from the wells. Medium containing double concentration of all compounds was prepared and diluted 1:2 in the medium present in each well. Final concentrations in wells are specified in figure legends.

### Reporter constructs and expression vectors

4xARE reporter construct was kindly provided by Dr. M. Hannink (University of Missouri-Columbia, Columbia, MO). PSA E4-LUC containing the wild type enhancer of the prostate specific antigen (PSA) human gene (496 bp) includes 6xAnRE and was kindly provided by Dr. H.P. Koeffler (Cedars-Sinai Medical Center, Los Angeles). Renilla luciferase (P-RL-null) expression vectors which served as internal transfection standards were purchased from Promega (Madison, WI, USA).

[1] *Abbreviations used:* AnRE, Androgen Response Element; CI, Combination Index; DHT, dihydrotestosterone; DMSO, dimethyl-sulfoxide; DMEM, Dulbecco's modified Eagle's medium; DCC-FCS, dextran-coated charcoal-stripped FCS; EpRE/ARE, Electrophile/Antioxidant Response Element; EPA/DHA, eicosapentaenoic acid and docosahexaenoic acid; FCS, fetal calf serum; NFkB, nuclear factor-kappa B; PSA, prostate specific antigen; THF, tetrahydrofuran; 1,25vit$D_3$, 1α,25-dihydroxyvitamin $D_3$.

*Transient transfection and reporter gene assay*

Cells were transfected using jetPEI reagent (Polyplus Transfection, Illkrich, France) in 24 well plates. LNCaP cells (70,000 cells per well) were transfected with 0.2 μg reporter construct and 0.05 μg normalizing plasmid. The ratio of DNA to jetPEI was 1:10. Cells were seeded in culture media containing 3% FCS. On the next day, 50 μl of DNA mixed with jetPEI was added to 0.5 ml medium in the wells. Cells were then incubated for 4–6 h at 37 °C. Medium was replaced with fresh medium containing the test compounds, supplemented with 3% FCS and cells were incubated for additional 16–20 h.

EpRE/ARE and AnRE reporter activity were measured in cell extracts and normalized to renilla luciferase using the Dual Luciferase Reporter Assay System, (Promega, Madison, WI) according to the manufacturer's instructions. All luminescence measurements were performed in 20/20$^n$ Luminometer (Turner Biosystems, Sunnyvale, CA). Of note, in different experiments transfection level as well as other parameters like cell batch and passage were variable. In combination experiments, the comparison between the different compounds in the same experiment were reproducible, however, basal reporter activity as well as the fold induction varied between experiments.

*Cell proliferation*

The androgen-responsive LNCaP cells were depleted of bound steroid hormones by incubation for 3 days in phenol red-free RPMI 1640 medium supplemented with 3% DCC-FCS and then seeded in a 96-well plate (7000 cells/well in 100 μl medium). Two days later medium was changed to 1% DCC-FCS. The next day, cells were treated with $10^{-9}$ M DHT alone or in combination with the different compounds at the indicated concentrations or with THF as control. These media were replaced daily for 3 days. The other types of the cells (30,000 cells/well) were treated as specified for LNCaP, with the exception of inducing proliferation with 3% serum and without DHT.

Cell proliferation was measured by [$^3$H]thymidine incorporation: 5 μCi/well of [$^3$H]thymidine (specific radioactivity 5 mCi/mmol) containing unlabeled thymidine (100 μM) was added (for LNCaP) or medium was replaced to one containing 2.5 μCi/well thymidine for all other types of cells (in fibroblast cells 300 μM cold thymidine was used). Plates were incubated at 37 °C for 3 h (LNCaP), 2 h (DU-145, PC-3), 1 h (MCF-7) or for 3 h (fibroblasts). Nucleotide incorporation was stopped by adding unlabeled thymidine (0.5 μmol). The cells were then trypsinized and collected on a glass–fiber filter using a cell harvester (Inotech, Switzerland). Radioactivity was determined by a radioactive image analyzer (BAS 1000, Fuji, Japan).

*PSA secretion*

LNCaP cells were depleted of steroid hormones by incubation for 3 days in phenol red-free RPMI 1640 medium supplemented with 3% DCC-FCS and then seeded in 24 well plates (70,000 cells per well). Two days later cells were treated with $10^{-8}$ M DHT alone or in combination with the different compounds at the indicated concentrations or with THF as control. Medium containing the secreted PSA was collected at 48 h after addition of compounds. PSA protein level was measured using the ADVIA Centaur® system. Results were normalized to cell number as measured by Coulter Counter Z1 (Coulter Electronics Luton, England).

*Combination Index*

The inhibitory effect of single agents and their combination on cancer cell proliferation was analyzed according to the classical isobologram equation [28]:

$$\text{Combination Index (CI)} = (D1)/(DX1) + (D2)/(DX2)$$

In this equation DX1 or DX2 are the doses of one compound alone required to produce an effect, and D1 and D2 are the doses of both compounds (present together) that produce the same effect. From this analysis, the combined effects of two compounds can be assessed as either additive (or zero) interaction indicated by CI = 1, synergism as indicated by CI < 1, or antagonism indicated as CI > 1.

*Statistical analysis*

All experiments were repeated at least twice. The significance of the differences between the means of the various subgroups was assessed by two-tailed Student's *t*-test using the Microsoft Excel program. $p \leqslant 0.05$ was considered statistically significant.

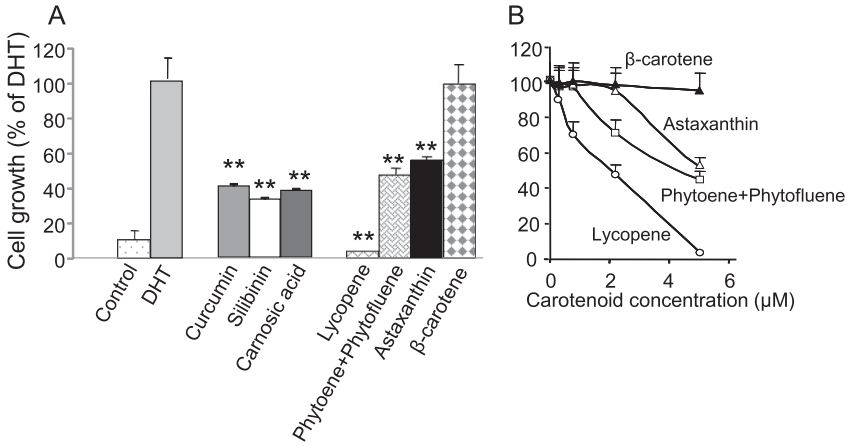


**Fig. 1.** Phytonutrients inhibit DHT-induced cell proliferation. (A) LNCaP cell proliferation was measured by incorporation of [$^3$H]thymidine into DNA as described under Materials and methods. Cell number measured 3 days after incubation in the presence of $10^{-9}$ M DHT was defined as 100%. Concentrations used: curcumin (5 μM), silibinin (30 μM), carnosic acid (10 μM), various carotenoids (5 μM). $^*p \leqslant 0.05$, $^{**}p \leqslant 0.01$. (B) LNCaP cells were treated with $10^{-8}$ M DHT and with varying carotenoid concentrations. The results are presented as the mean ± SE of 5 experiments each done with 8–10 replicates.

**Table 1**
$IC_{50}$ values (μM) of carotenoids in growth inhibition of prostate cancer and fibroblast cells.

| Cell line | Prostate cancer | | | Fibroblasts | | |
|---|---|---|---|---|---|---|
| | LNCaP | DU-145 | PC-3 | Fb 1 | Fb 2 | Fb 3 |
| *Carotenoid* | | | | | | |
| Lycopene | 2.0 ± 0.2 | 3.0 ± 0.3 | 4.0 ± 0.4 | >15 | >15 | >15 |
| Astaxanthin | 5.5 ± 0.5 | 11.0 ± 2.2 | 14.0 ± 2.8 | >15 | >15 | >15 |
| β-Carotene | >15 | >15 | 13.0 ± 2.6 | >15 | n.d. | n.d. |
| Phytoene + phytofluene | 5.2 ± 0.5 | n.d. | 9.0 ± 1.8 | >15 | n.d. | n.d. |

Cell growth was measured as described in the legend to Fig. 1. Following 24 h incubation with 0.5% serum, cell growth was induced in LNCaP cells by administration of $10^{-9}$ M DHT and in DU-145, PC-3 and normal fibroblasts by 3% serum. The human primary foreskin fibroblasts (Fb 1, Fb 2 and Fb 3) were obtained from three individuals. The results are the mean ± SE of 5 experiments, each performed with 8–10 replicates. n.d.: not done.

## Results

### *Phytonutrients inhibit prostate cancer cell proliferation*

The interference with androgen cancer promoting activity by various phytonutrients was assessed firstly by examining their effect on cancer cell proliferation. To this end we used the human LNCaP prostate cancer cell line which expresses androgen receptors and respond to DHT, the active androgen in the prostate, with enhanced proliferation as can be seen in Fig. 1A. Most of the carotenoids tested as well as polyphenols such as curcumin, silibinin and carnosic acid significantly inhibited DHT-induced LNCaP cell proliferation (Fig. 1). The inhibition by carotenoids was dose-dependent (Fig. 1B). The $IC_{50}$ values, calculated from these results, are shown in Table 1. LNCaP cell growth inhibition was more pronounced in lycopene-treated cells than in cells treated by phytoene plus phytofluene or by astaxanthin, whereas β-carotene was the least effective.

The inhibition of LNCaP cell proliferation by carotenoids was compared to that obtained in two human androgen-independent prostate cancer cell lines and to three human primary non-malignant fibroblast cell lines (Table 1). The carotenoid inhibition of proliferation of DU-145 and PC-3 cells was somewhat lower than that found for the LNCaP cells. Interestingly, carotenoids inhibited only marginally proliferation of fibroblast cell lines and the $IC_{50}$ was higher than 15 μM. This concentration is at least 10 times higher than the carotenoid concentrations in blood and tissues, suggesting that the growth of normal cells such as fibroblasts is not significantly affected by these dietary compounds.

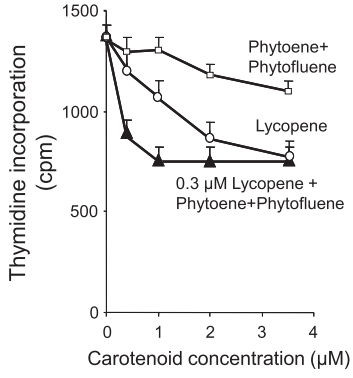


**Fig. 2.** Carotenoid combinations induce synergistic inhibition of LNCaP prostate cancer cell proliferation. LNCaP cell growth was measured as described in Fig. 1. The results are shown as cpm of incorporated [$^3$H]thymidine into DNA after 3 days in culture in the presence of DHT and the indicated carotenoid concentrations. Mean ± SE of 3 experiments each done with 8–10 replicates. The solid triangles show the data obtained with a combination of 0.3 μM lycopene with the indicated concentrations of the phytoene plus phytofluene preparations.

### *Carotenoid combinations produce synergistic inhibition of LNCaP prostate cancer cell proliferation*

The carotenoid concentrations which are required for significant inhibition of prostate cancer cell proliferation when added alone (Fig. 1 and Table 1) are higher than those present in human plasma and tissues. To explain the efficacy of carotenoids in vivo, we surmised that low concentrations of different active nutritional ingredients act in combination to produce additive or synergistic effects. Accordingly, we tested whether a more pronounced inhibition could be achieved at low concentrations of carotenoids which are present in tissues combined with one another or with other dietary derived compounds such as 1,25vit$D_3$ or retinoic acid (Fig. 2 and Table 2). Fig. 2 shows the inhibition of LNCaP cell growth by a combination of lycopene with phytoene plus phytofluene. Significant inhibition of cell growth by lycopene was achieved at 1 μM, a concentration found in the blood of individuals who consume large amounts of tomato products. At 0.3 μM, lycopene, when administered alone, did not have any effect. Similar results were obtained when using a mixture of two other tomato carotenoids, phytoene and phytofluene, which are active only at high concentration. However, when the low (0.3 μM), non-active concentration of lycopene is combined with a low concentration of phytoene and phytofluene (solid triangles), synergistic inhibition of cancer cell growth was clearly evident.

To examine the statistical significance of the synergy described in Fig. 2 and in similar experiments, a Combination Index (CI) was calculated according to Cutts et al. [28] (Table 2). For example, the calculated CI value for the combination of lycopene with phytoene + phytofluene in LNCaP cells is much smaller than 1, clearly

**Table 2**
Combination Index values for the inhibition of prostate and mammary cancer cells growth by combinations of carotenoids or carotenoids with retinoic acid and 1,25vit$D_3$.

| Cell line | Micronutrient (1) + (2) | Dx1 | Dx2 | D1 | D2 | CI |
|---|---|---|---|---|---|---|
| LNCaP | Lycopene + phytoene phytofluene | 5 | 12 | 0.3 | 0.8 | 0.13 |
| LNCaP | Lycopene + β-carotene | 2 | 4 | 0.3 | 2 | 0.65 |
| MCF-7 | Lycopene + phytoene phytofluene | 2 | 4 | 0.3 | 2 | 0.65 |
| MCF-7 | Lycopene + β-carotene | 2 | 13 | 0.3 | 1 | 0.23 |
| MCF-7 | Lycopene + astaxanthin | 3 | 4.5 | 0.3 | 3.5 | 0.88 |
| MCF-7 | Astaxanthin + 1,25vit$D_3$ (nM) | 5 | 100 | 0.3 | 1 | 0.07 |
| MCF-7 | Lycopene + retinoic acid (nM) | 3 | 8 | 0.3 | 1 | 0.23 |

Cell growth was induced in LNCaP cells by DHT as described in the legend to Fig. 1 and in MCF-7 mammary cancer cells by 3% serum, after 1 day partial starvation with 0.5% serum. The values of carotenoid concentrations are expressed in μM, and those of 1,25vitD and retinoic acid in nM.
The Combination Index (CI) was calculated according to the following equation: CI = (D1)/(DX1) + (D2)/(DX2) as shown under Materials and methods.
In this equation Dx1 and Dx2 are the doses of each compound alone required to produce an effect, and D1 and D2 are the doses of both compounds (present together) that produce the same effect. From this analysis, the combined effects of two compounds can be assessed as either additive (no interaction) indicated by CI = 1; synergism, as indicated by CI < 1; or antagonism indicated by CI > 1.

indicating a strong synergistic interaction of these tomato carotenoids in the inhibition of prostate cancer cell growth. Moreover, a similar synergistic interaction was evident not only in the prostate cancer cells, but also in MCF-7 mammary cancer cells (Table 2) supporting the generality of this phenomena. Of note, such a synergy is shown for all the tested combinations described in Table 2, albeit to a different extent. These results support the view that tomato carotenoids in combination with other phytonutrients at concentrations that can be achieved in blood are effective in the inhibition of prostate cancer cell proliferation.

*Phytonutrients synergistically inhibit androgen signaling*

Inhibition of androgen-induced prostate cancer cell proliferation implies the effect of phytonutrients on androgen signaling. Therefore, we next examined whether these compounds inhibit androgen-induced transcription. We found that both carotenoids and polyphenols exerted a marked inhibition of DHT-induced AnRE reporter gene activity (Fig. 3A).

An important marker for androgenic activity (linked to prostate cancer progression) is the secretion of PSA. Indeed, PSA secretion by LNCaP cells in response to DHT treatment was inhibited by carotenoids and polyphenols (Fig. 3B).

Combination of carotenoids and polyphenols exhibited a synergistic inhibition of DHT-induced AnRE reporter activity, as well as PSA protein secretion (Fig. 4). At the concentrations used, when administered alone, curcumin, vitamin E, the omega-3 fatty acids EPA and DHA and tomato extract containing lycopene, each exhibited only a marginal effect on AnRE reporter activity. However, different combinations of two compounds showed a synergistic inhibition of the transcriptional activity. Moreover, combination of three compounds such as in the case of curcumin, vitamin E and the tomato extract showed a stronger synergistic effect than each pair of compounds. The inhibition of PSA secretion by such combinations was at least additive or even more than additive. The inhibition of DHT-induced androgen dependent transcription and PSA secretion by combination of phytonutrients is an important mechanism supporting the effect of a diverse diet on prostate cancer prevention.

*Combination of dietary compounds synergistically induce EpRE/ARE activity*

Another transcription system important for cancer prevention is the Electrophile/Antioxidant Response Element transcription system. This system has been shown by us and by others to be induced by various phytonutrients such as carotenoids and polyphenols (see Introduction). Similar to their ability to cooperatively inhibit androgen signaling, various plant derived compounds markedly synergized to induce EpRE/ARE reporter gene activity in prostate cancer cells (Fig. 5).

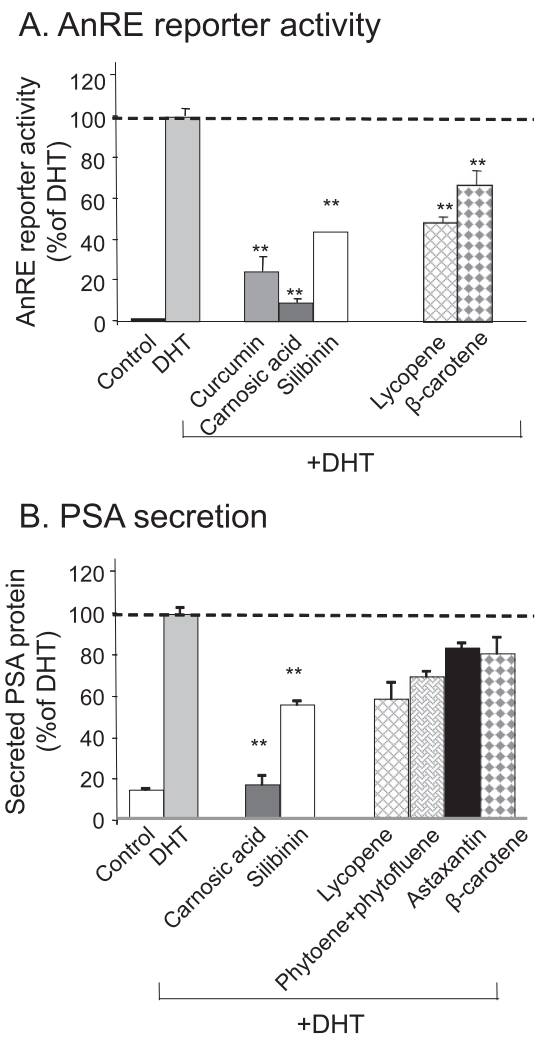


**Fig. 3.** Phytonutrients inhibit the DHT-induced AnRE reporter activity and PSA secretion. (A) AnRE reporter activity. LNCaP cells were transiently transfected with the AnRE-LUC reporter construct and renilla luciferase for normalization. The reporter gene assay was performed as described under Materials and methods. Five hour after transfection, cells were incubated with $10^{-8}$ M DHT and with polyphenols at the concentrations indicated in Fig. 1 or with lycopene (8 μm), or β-carotene (6.5 μm). Values in the presence of DHT alone were defined as 100%. Data are the mean ± SE of 3 experiments, performed in triplicates. $^{**}p \leqslant 0.01$. (B) PSA secretion. LNCaP cells were depleted of steroid hormones as described under Materials and methods, and stimulated with DHT for 48 h in the presence of phytonutrients. The level of PSA protein in the medium was measured in the ADVIA Centaur® system, and normalized to cell number measured by Coulter counter. Concentrations of polyphenols were as indicated in Fig. 1. Concentrations of carotenoids were: lycopene 2.5 μm, phytoene plus phytofluene 4 μm, astaxanthin 9.6 μm and β-carotene 6.5 μM. Values in the presence of DHT alone were defined as 100%. Data are the mean ± SE of 3 experiments, performed in triplicates. $^{**}p \leqslant 0.01$.

## Discussion

The results of this study support the hypothesis that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities, and that the benefit of a diet rich in fruits and vegetables is attributable to the complex mixtures of phytonutrients. In the current study, we examined the effects of different phytonutrients alone and in combination on proliferation of breast and prostate cancer cells and on two signaling pathways which play a determining role in the initiation and progression of prostate cancer: (i) the androgen signaling measured by the androgen receptor transcriptional activity as well as by PSA secretion, a marker of androgenic activity which is of major clinical significance in the identification and monitoring of prostate cancer progression, and (ii) the antioxidant defense mechanism measured by activation of the EpRE/ARE transcription system. It should be emphasized that the latter system is important also for breast cancer as well as for several other types of cancer. Both androgen signaling and the EpRE/ARE activity were measured by very sensitive reporter gene assays concurrently with the relevant, but less sensitive, determination of cell proliferation. Clearly, the in vitro reporter gene experiments serve as an early step which later should be verified and validated in carcinogenesis models in vivo. Such in vivo studies will be necessary to provide firm and definitive answers to the question of synergy among phytochemicals.

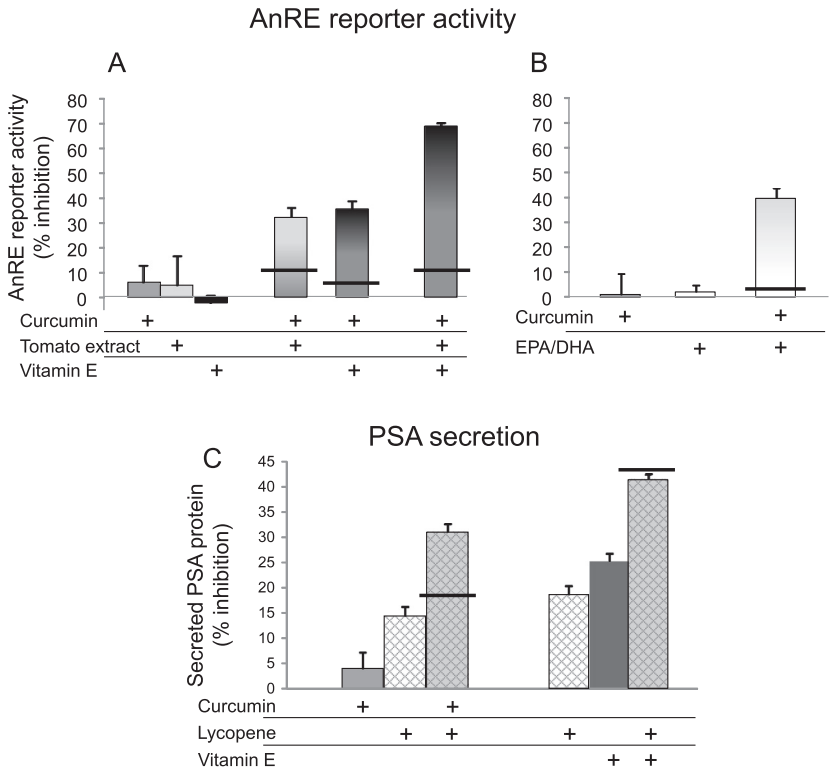


**Fig. 4.** Synergistic inhibition of androgen signaling by combinations of phytonutrients. (A and B) LNCaP cells were transiently transfected with the AnRE-LUC reporter and renilla luciferase as in Fig. 3A, stimulated with DHT and treated with the compounds alone or in the indicated combinations. Results are presented as % inhibition relative to the effect of DHT alone. The horizontal lines in the various combinations represent the sum of the inhibitory effects of individual compounds. A representative experiment out of 3 experiments performed in triplicates is presented. Concentrations of the different compounds were: curcumin (2.5 μm in (A) or 1.25 μm in (B)), vitamin E (200 μm), tomato extract (4 μm lycopene), EPA/DHA (500 μm). (C) LNCaP cells were treated as shown in Fig. 3B and supplemented with the different compounds alone or in combinations. PSA protein level was measured in the medium as in Fig. 3B and the results are presented as % inhibition relative to the effect of DHT alone. A representative experiment out of 3 experiments performed in triplicates is presented. Concentrations of the different compounds were: curcumin, 5 μM, vitamin E, 100 μM, lycopene 4 μM.

While carotenoids can influence various signaling pathways [22], in our previous studies we have shown that at least some of these activities are in fact mediated by carotenoid derivatives. For example, we have recently observed that carotenoid derivatives are the active mediators in nuclear factor-kappa B (NFkB) inhibition [29] as well as EpRE/ARE induction [27]. In these studies, we used a partially oxidized lycopene preparations that contained a mixture of spontaneously oxidized carotenoid derivatives. We separated these derivatives from the intact carotenoid by ethanolic extraction and found that the activity in both transcription systems resided in the oxidized derivatives. Many other publications support this view by demonstrating the importance of carotenoid derivatives in various cellular signaling pathways [30–32]. Notably, we found that the effect of lycopene in inhibition of AnRE activity in prostate cancer cells is mediated by carotenoid derivatives as well (Linnewiel-Hermoni, unpublished results). In the current study we used several lycopene preparations, each containing different proportions of oxidized derivatives and a tomato extract preparation. This tomato oleoresin is less prone to spontaneous oxidation during its storage. Furthermore, the tomato extract contains other compounds in addition to lycopene such as phytoene phytofluene and natural tocopherols. Therefore, it is difficult to compare the activity of the tomato extract to that of the crystalline lycopene preparations.

Some in vitro experiments showing a beneficial effect of a single phytonutrient and specifically of carotenoids [33] were performed at high carotenoid concentrations. In the current study, 1 μM lycopene, a concentration found in the blood of individuals who consume large amounts of tomato products significantly inhibited prostate cancer cell growth (Figs. 1B, and 2). However, low concentration of lycopene (0.3 μM), such as that found in the blood of Japanese and North Europeans who consume small amounts of such products, did not have any effect. Importantly, when this low non-effective concentration of lycopene was combined with similar non-effective concentration of phytoene plus phytofluene, a synergistic inhibition of cancer cell proliferation was clearly evident.

Our results are in line with previous publications showing synergistic effect of different phytonutrients in various assay systems. For example the synergistic effect of lycopene and vitamin E found in the current study both in EpRE/ARE activation and in PSA secretion is supported by studies showing the combined effect of these phytochemicals on prostate cell growth in vitro [34] and in vivo [35]. In their study, Pastori et al. found that while lycopene alone was not a potent inhibitor of prostate carcinoma cell proliferation, the combination of lycopene with vitamin E resulted in a strong inhibition of prostate cell proliferation. Similarly, the growth of PC-346C human prostate cancer cells in nude mice was not inhibited by each compound alone but the combination of lycopene and vitamin E significantly suppressed the growth of PC-346C prostate tumors and increased median survival time of the mice [35].

It is noteworthy that some of our results were obtained using the LNCaP hormone-dependent cancer cell line. The inhibition of androgen-induced transcriptional activity and PSA secretion is one mechanism for prostate cancer prevention. However, the inhibition observed in this study of the proliferation of androgen-independent cell lines, such as DU-145, by lycopene and other carotenoids (Table 1) suggests that other mechanisms in addition to inhibition of androgen signaling may be involved in carotenoid anti-cancer activity in prostate cancer. Such mechanisms may

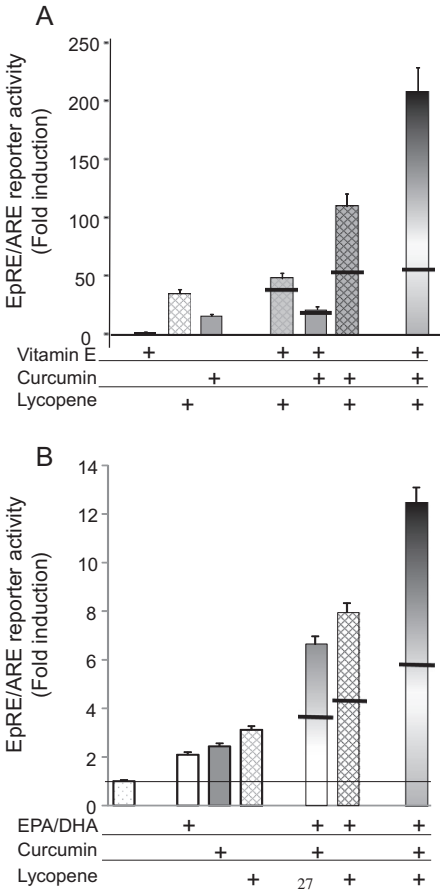


**Fig. 5.** Synergistic induction of EpRE/ARE reporter activity by combination of lycopene and curcumin with vitamin E (A) or omega-3 fatty acids (B). LNCaP cells were transiently transfected with the ARE-LUC reporter construct and with renilla luciferase for normalization. Five hour after transfection cells were treated with the compounds alone or in the indicated combinations. Results are presented as fold induction. The horizontal lines in the various combinations represent the sum of fold induction by the individual compounds. A representative experiment out of 3 experiments performed in triplicates is presented. Concentrations of the different compounds were: vitamin E 25 μM, curcumin 2.5 μM, EPA/DHA 500 μM and lycopene 5 μM (A) or 2 μM (B).

include inhibition of growth factor activity and interference with cell cycle progression as previously shown by us [36–38] and by others [39–41] in mammary and prostate cancer cells.

Other synergistic combinations were previously described, for example, the two polyphenols curcumin and resveratrol exhibited a synergistic antioxidant effect in vitro which was greater than that of curcumin in combination with the flavonol quercetin [42]. Interestingly, the combination of curcumin and resveratrol showed synergistic activation of the EpRE/ARE transcription system in mammary cancer cells (Hagar Salman et al. unpublished results). Moreover, a synergistic inhibition of NFkB activity and the anti-inflammatory effects of several carotenoids in combination as well as the combination of carotenoids with the polyphenol carnosic acid were recently shown in mouse peritoneal macrophages as well as in an in vivo mouse model of peritonitis [43]. The synergy found between the omega-3 fatty acids and other phytonutrients is of special importance since tomatoes and fish are a hallmark of Mediterranean diet which was found to be inversely associated with overall cancer mortality and also with individual cancers among which is prostate cancer [44]. For example, in the Health Professionals Follow-up Study, greater adherence to the Mediterranean diet after diagnosis of non-metastatic prostate cancer was associated with lower overall mortality [45]. Similarly, in north Sweden men, increased Mediterranean diet score was inversely associated with total cancer mortality [46].

The synergistic effects of distinct phytonutrients on a specific transcription system imply that the phytonutrients may modulate this transcription system via different molecular mechanisms, for example, direct interaction with different proteins in the same cellular pathway. We have previously suggested that carotenoid derivatives activate the EpRE/ARE transcription system by interaction with the inhibitory protein Keap1 via its reactive thiol groups, thereby releasing nuclear factor E2-related factor 2 (Nrf2), the key transcription factor of this transcription system and allowing its nuclear translocation. Other phytonutrients used in the current study, such as vitamin E and the omega 3 fatty acids EPA and DHA, may affect EpRE/ARE activity via interaction with additional proteins in the cascade, or by other mechanisms yet to be determined. Another valid option is the possibility of interaction between the different phytonutrients which could enhance their stability or their ability to enter the cells.

*Conclusions:* the beneficial effects of a diet rich in fruits and vegetables in general, and particularly in carotenoids has long been appreciated. In the current study we focused on the combined effect of phytonutrients on the prevention of prostate cancer. Our study may assist in providing optimized combinations of foods that could be efficient in cancer prevention in the clinical setting.

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