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INVITED REVIEW

Synergic effect of α-tocopherol and naringenin in transglutaminase-induced differentiation of human prostate cancer cells

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Abstract Prostate cancer is the second most common cancer in men worldwide. Its prevention and treatment remain a challenge to clinicians. Thus, there is an urgent need to discover novel, less toxic, and more effective therapies for patients. Many vitamins and related chemicals, including vitamin E, (tocopherols) have shown their anti-cancer activities as anti-oxidants, activators of transcription factors or factors influencing epigenetic events. Although laboratory tests including the use of animal models showed that this vitamin may have anticancer properties, whether it can effectively prevent the development and/or progression of prostate cancer in humans remains to be intensively studied. This review provides up-to-date information regarding the recent outcomes of laboratory, epidemiology and/or clinical trials on the effects of tocopherols on prostate cancer development, along with our last observations on a combined treatment of a prostate cancer cell line (PC-3) with two natural antineoplastic compounds, naringenin (NG) and α-tocopherol (α-TOC). We report the synergic effect of α-TOC and NG in transglutaminase-induced differentiation of human PC-3 prostate cancer cells. While our results are based on one histological class of tumor, the most significant implication of this observation is that establishes a new way in the screening for detecting new differentiative antineoplastic agents.

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Introduction

Prostate cancer is the most common noncutaneous malignancy in man and the second leading cause of cancer death. Rates of detection of prostate cancers vary widely across the world, with South and East Asia detecting less frequently than in Europe, and especially the United States. Despite the fact that widespread screening by prostate-specific antigen (PSA) has led to a substantial rise of prostate cancer-incidence in younger men, this tumor entity still remains a disease of the elderly. Sixty-five percent of new cases are diagnosed in men older than 65 years and 25% in men older than 75 years (Fitzpatrick 2008). Prostate cancer in senior adults, as well as in younger men, ranges in severity from a slow-growing tumor to a highly aggressive and potentially fatal disease. Hence, optimal management of prostate cancer in senior adults is of considerable clinical relevance yet associated with many uncertainties (Fitzpatrick 2008). The risk of being diagnosed with prostate cancer rises almost linearly with age (Stangelberger et al. 2008). In an autopsy study, it has been shown that 46% of men between 70 and 80 years of age have histological evidence of cancer (Yin et al. 2008). The incidence and mortality of prostate cancer shows strong worldwide variations (Breslow et al. 1977). Epidemiological investigations identify lifestyle, above all diet, as the major leads. Genetic predisposition is likely. A high rate of concurrence of prostate cancer in identical twins has been revealed (Ahlbom et al. 1997). Lifestyle, and specifically what has been termed as the 'Western diet', is likely to

induce prostate cancer (Adlercreutz 2002). Nutritional factors, such as meat, fat and dairy intake, which are common in Western diets, have been linked to a greater risk of the disease (Chan et al. 1998). It is possible if not likely, however, that foodstuffs used in countries with a very low prostate cancer mortality are protective. These include plant-derived anti-oxidants and soy products, which contains isoflavones such as daidzeine and genisteine, which have weak estrogenic properties and may function as selective androgen receptor modulators. For many of the epidemiological findings, mechanisms have not been established. It is possible, therefore, that the Western diet does not initiate or promote prostate cancer, but that the lower incidence and mortality rates in Far Eastern countries are due to the protective mechanism available in foods that are not commonly used in the West. This would allow the application of dietary supplements with a preventive action to Western populations without having to change their dietary habits. A vegetarian diet, as is used in Eastern countries, was also shown to influence the levels of circulating androgens in men (Hamalainen et al. 1983). This provides a link to the endocrine dependence of prostate cancer, which may play an important role in the phase of promotion to clinically relevant disease.

Anti-oxidants are one of the major candidates for chemoprevention of prostate cancer. Their potential effectiveness is based on the working hypothesis that residual oxygen species (ROS), mainly super-oxide that accumulates in the aging body, may play an important role in prostate cancer pathogenesis. Anti-oxidants catalyze the process of transforming super-oxide to O_2 and to water at several levels. Selenium, one of the anti-oxidants under study, catalyses glutathione peroxidase, an enzyme important in the removal of hydrogen peroxide. Food products with anti-oxidant activity include green tea-containing flavonoids, red wine (quercetin), vitamin E, selenium and lycopene, the red color of the tomato and other substances from vegetables. Epidemiological studies have shown marked variations in prostate cancer incidence and mortality across different geographic regions, leading to the rising interest in the role of nutrition in prostate cancer risk (Chan et al. 2009). Although the impact on prostate cancer risk differs among various vegetables and their constituent nutrients, the overall benefits of plant based diet on cancer prevention and other diet-related diseases should be promoted.

Vitamin E and cancer prevention

Vitamin E is a natural, highly tolerable and cost effective molecule. This generic term is used for tocopherol and tocotrienols consisting of two rings with a hydrocarbon

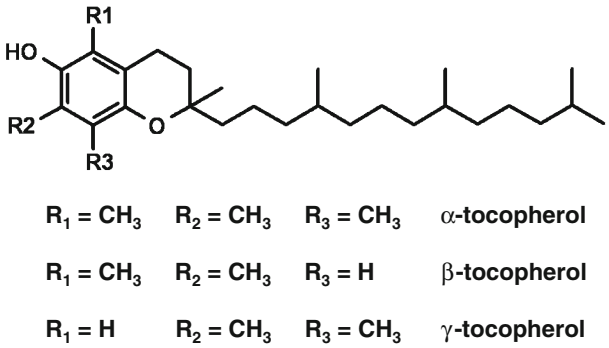

Fig. 1 Chemical structure of natural vitamin E

chain. Both structures are similar, although the tocotrienol structure has double bonds on the isoprenoid units. Natural tocopherols are known as α, β, γ, and δ according to the methyl or proton groups that are bound to their benzene rings, and the most common and biologically active form is α-TOC; (Fig. 1) (Brigelius-Flohe and Traber 1999). When produced synthetically, it is composed of eight stereoisomers in which RRR-α-tocopherol is the most biologically active form (Sies and Murphy 1991). The principal reserve of natural tocopherols is vegetable oil where its function is to protect tissue from oxidative damage. It is a liposoluble molecule, and, therefore, after dietary intake, vitamin E is not only absorbed easily from the intestinal lumen but is also dispersed between lipids and proteins in cell membranes. Vitamin E molecules can interrupt free radical chain reactions by capturing the free radical. This imparts to them their antioxidant properties. The free hydroxyl group on the aromatic ring is responsible for the antioxidant properties. The hydrogen from this group is donated to the free radical, resulting in a relatively stable free radical form of vitamin E. Regarding the pharmacodynamics of tocopherols, it has been reported in a study conducted in human eyes that the retinal levels of vitamin E are higher than those of the choroid or vitreous and is correlated with serum levels of vitamin E (Bhat 1986). Some animal experiments and human studies suggest that vitamin E may protect against cancer and serum α-TOC concentration was studied for its prediction (Knekt et al. 1991). It has been suggested that vitamin E has anticancer effects as a lipid antioxidant and free radical scavenger. Animal experiments and in vitro studies have shown that vitamin E can block the formation of carcinogenic nitrosamines. Studies on the effect of vitamin E on other carcinogens have, however, yielded somewhat conflicting results (Knekt 1988; Chen et al. 1988). Most of the studies have concentrated on mammary gland, colon, oral, and skin carcinogenesis. Vitamin E has been shown to inhibit oral carcinogenesis and to inhibit or to have no effect on mammary gland carcinogenesis. Vitamin E has also been shown to inhibit or

to enhance skin carcinogenesis and to inhibit, to have no effect on, or to enhance colon carcinogenesis (Birt 1986). The effect of vitamin E possibly depends on several factors, including the amount of vitamin E administered, the level of modifying factors (e.g., dietary selenium and fat), the dosage and the type of carcinogens used. Although the animal studies provide some support for the hypothesis that vitamin E has an anticancer effect, the role of this micronutrient in cancer prevention in humans is equivocal. In several human epidemiologic studies on diet and cancer, consumption of foodstuffs rich in vitamin E such as vegetables has been found to be inversely related to the risk of different cancers. However, the possibility that the associations in these studies, are mainly due to carotene or certain other substances in the foodstuff, cannot be excluded. Possibly, for methodological reasons, the results of the few studies on the association between vitamin E intake and cancer risk do not generally show any evidence that vitamin E has a protective effect (Knekt et al. 1991). It was originally thought that vitamin E chemopreventive properties could be attributed to its ability to neutralize the free radicals generated by the fecal bacteria in the gut and thereby prevent DNA damage. Nevertheless, accumulating evidences in the literature suggest that signal transduction activities independent of the antioxidant functions of vitamin E may also be responsible for the observed cancer chemopreventive effects (Packer 1991; Campbell et al. 2006).

Clinical trials have supported that prostate cancer can be suppressed by α-TOC. In the post intervention follow-up study, they found that the benefits of α-TOC on prostate cancer prevention rapidly disappeared after discontinuation of the supplement (Virtamo et al. 2003). Moreover, their results show that low levels of α-TOC in serum increased prostate cancer mortality (Eichholzer et al. 1996). Daily oral supplementation of α-TOC and its analogs have been applied in preclinical animal studies. For example, Nakamura et al. (1991) reported that a diet containing an antioxidant mix, including α-TOC, could reduce the incidence and lesions of experimental rat prostate carcinogenesis. Others showed that administration of a mix of three compounds (vitamin E, selenium and lycopene) in the diet dramatically inhibits prostate cancer development in Lady transgenic mice, which spontaneously develop prostate tumor (Venkateswaran et al. 2004). Collectively, those studies indicated that α-TOC could be combined with other nutrients (antioxidants) to prevent prostate cancer. α-TOC could alter cell cycle progression in prostate cancer cells. It was reported that α-TOC inhibited DNA synthesis in prostate cancer LNCaP, PC-3, and DU145 cells (Israel et al. 1995). In agreement with this notion, it has been shown that α-TOC could block cell cycle progression in prostate cancer cells (Ni et al. 2003). Venkateswaran and colleagues (2002) showed that vitamin E could reduce the cells in S phase in both LNCaP and PC-3 cells. This S phase reduction was accompanied by G1/S phase arrest in LNCaP cells and G2/M phase arrest in PC-3 cells. Additional analysis revealed that α-TOC significantly up regulates p27 expression, leading to the inactivation of cyclin E/cdk2, which contributes to the G1 arrest in prostate cancer cells (Venkateswaran et al. 2002). Extensive examinations revealed that α-TOC could regulate the expression of cyclin D1, D3, cdk2, cdk4, but not cdk6, resulting in disrupting Rb-E2F pathway in prostate cancer LNCaP cells. Interestingly, those results indicated that reduced cyclin D and cdk4 expression are earlier responsiveness for VES treatment (Ni et al. 2003). Gunawardena and collaborators (2000) showed that 3 days of α-TOC treatment can stimulate apoptosis in actively dividing prostate cancer LNCaP cells, but not in confluent quiescent cells. They assessed nucleosome fragmentation by cell death detection ELISA as apoptosis index. In general, the apoptosis can be induced through mitochondria, lysosomes, endoplasmic reticulum stress/cytokine signaling pathway, and extracellular death pathway-mediated manners (Zu and Ip 2003).

Transglutaminase and prostate cancer

The term transglutaminase (TG) was first introduced by Clarke et al. (1957) to describe the transamidating activity observed in guinea pig liver. Since this finding, proteins showing TG activity have now been described following in microorganisms, plants, invertebrates, amphibians, fish and birds. TGs are a widely distributed and peculiar group of enzymes that catalyze the post-translational modification of proteins by the formation of isopeptide bonds. This may occur either through protein cross-linking via ε-(γ-glutamyl)lysine bonds (Fig. 2) or through incorporation of many primary amines at the level peptide-bound glutamine residues (Folk and Finlayson 1977). In mammals, nine distinct TGs isoenzymes have been identified at the genomic level; however, only six have so far been isolated and

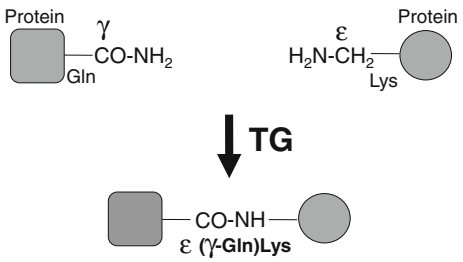

Fig. 2 TG-catalyzed protein cross-linking via ε-(γ-glutamyl)lysine bond formation

characterized at the protein level, after purification either from natural sources or as recombinant proteins. The best characterized enzymes include (a) the circulating zymogen Factor XIII, which is converted, by a thrombin-dependent proteolysis, into the active TG Factor XIIIa, (plasma TG) involved in stabilization of fibrin clots and in wound healing; (b) the keratinocyte TG (type 1 TG), which exists in membrane-bound and soluble forms, is activated by proteolysis and is involved in the terminal differentiation of keratinocytes; (c) the ubiquitous type 2 tissue TG (tTG) (Fesus and Piacentini 2002); (d) the epidermal/hair follicle TG (type 3 TG), which also requires proteolysis to become active and, like type 1, is involved in the terminal differentiation of the keratinocyte; (e) the prostatic secretory TG (type 4 TG, TG-4), essential for fertility in rodents, and (f) the recently characterized types 5–7 TGs. All mammalian forms have a good structural homology, are members of the papain-like super family of cysteine proteases and are the products of different genes arising from duplication and rearrangement (Lentini et al. 2009). In the prostate, the expression of TG-4 is restricted to luminal epithelial cells of the gland. Expression of the TG-4 protein could occasionally be observed in high-grade prostatic intraepithelial neoplasia, but was either at a lower level in prostate cancer compared with normal tissues or absence in certain prostate carcinoma cells. The expression pattern observed for TG-4 in the prostate has not been found thus far for any other prostate-specific marker (Dubbink et al. 1999). Metastatic prostate tumors also showed loss of expression of TG-4 (An et al. 1999). TG-4 has a relatively wide profile of expression in human cancer cell lines and is strongly expressed in the low invasive CA-HPV-10 prostate cancer cell line. This enzyme is associated with the invasive potential of prostate cancer cells (Davies et al. 2007). The function of the prostate TG is not clear. It has been reported that a 30- and a 100-kDa GTPase are linked to the prostatic secretion of TG-4 (Spina et al. 1999). The rat homolog, rat prostate TG (dorsal prostate TG or Dorsal protein 1) has been suggested to be responsible for the cross-linking during the copulatory plug formation and may be involved to some degree in sperm cell motility and immunogenicity (Williams-Ashman 1984; Ablin and Whyard 1991) and immunoregulation (Ablin et al. 1987). Together with the report that TG-4 can be up-regulated by androgen in PC-346C, but not in LNCaP cells (despite that both are androgen responsive cell lines) (Dubbink et al. 1996), this suggests that the enzyme may also play a role in the control of invasiveness of prostate cancer cells. Cancer prevention by dietary factors or other agents is likely to represent one of the strategies to reduce the risk of development of malignancy in humans. The identification of new molecules able to increase TG activity in cancer cell lines, with the aim of reducing their proliferative and metastatic potential is the goal of the differentiation therapy (Jimenez and Yunis 1987; Thiele et al. 2000; Leszczyniecka et al. 2001). Therefore, it was suggested that one of the intriguing strategies to control cell proliferation, apoptosis and metastatic ability of different tumors by cell differentiation is linked to the post-translational modification of cell proteins catalyzed by tissue TG (Beninati et al. 1993). In last couple of decades, the use of natural compounds like flavonoids as chemopreventive agents has gained much attention. NG is a flavonoid considered to have a bioactive effect on human health as antioxidant, free radical scavenger, anti-inflammatory, carbohydrate metabolism promoter, and immune system modulator. It is the predominant flavanone in grapefruit (Gábor 1986). Although the antioxidant effects of NG due to their capability to scavenge free radicals have attracted a great deal of attention, there are effects beyond antioxidation that may be important in determining the anticancer activity of phytochemicals, such as inhibitory effects on tumor cell proliferation, decrease of polyamines production and TG activation (Lentini et al. 2007). Moreover, the chemopreventive action of NG has been demonstrated to be exerted through the induction of apoptosis in prostate cancer cells, as well as through the stimulation of DNA repair mechanisms (Gopalakrishnan et al. 2006; Gao et al. 2006).

Effect of α-tocopherol and naringenin on human PC-3 prostate cancer cells

We have evidenced that both treatments with 10 μM NG and 100 μM α-TOC exerted antineoplastic properties on the human PC-3 prostate cancer cell line. Interestingly, the simultaneous treatment of tumor cells with both molecules potentiates their proliferation-inhibitory effect (Fig. 3a), as well as their pro-differentiative action, as shown by the strong increase of TG activity (Fig. 3b). Furthermore, cytofluorimetric cell cycle and apoptosis studies demonstrated that NG and α-TOC were able to induce cell death, and that the combination between the two molecules induced an increase of the apoptotic rate (Table 1). This data were confirmed by Western blot analysis of p21 and p16 (Fig. 4). In fact, the tumor suppressor protein p16, a specific inhibitor of the cdk4/cyclin D kinase, plays an important suppressive role in the neoplastic process. Alteration in p16 protein expression level, mutation and homozygous deletion of the p16 gene are frequently detected in human cancers (Iemelynova et al. 2009). Under normal conditions, cyclin-dependent kinases (cdks) exist predominantly in multiple quaternary complexes, each containing a cdk, cyclin, proliferating cell nuclear antigen and the p21. p21 is one of several kinase inhibitors that are known to control cell proliferation (Geng et al. 2010). With

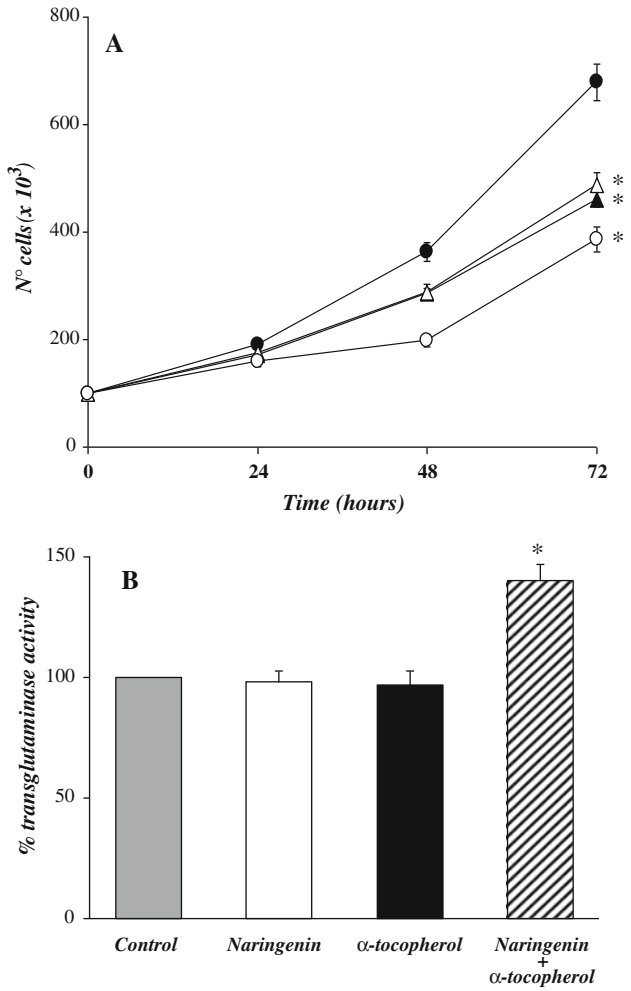

Fig. 3 **a** Proliferation curve of human PC-3 prostate cancer cells treated with 10 μM naringenin (NG) (*filled triangle*), or 100 μM α-tocopherol (α-TOC) (*open triangle*), or 10 μM NG + 100 μM α-TOC (*open circle*) after 24, 48 and 72 h. Control cells (*filled circle*) were incubated with 0.1% ethanol only. **b** Determination of TG activity in PC-3 cells treated with 10 μM NG and 100 μM α-TOC for 48 h. Cells were incubated with ^{14}C-methylamine and specific incorporation of labeling into proteins was detected. Data represent the mean of three different determinations ± SD (*$p < 0.001$; Student's t test)

these preliminary results, we have provided evidence about the antineoplastic role of α-TOC and its synergism with NG on prostate cancer cells. In addition, a possible correlation between inhibition of cell growth, differentiation and apoptosis induction and TG activation could be hypothesized.

Metastasis represents the final step in the progression of malignancy, and continues to be the primary cause of mortality among cancer patients. While there is still debate as to the exact nature of the pathogenesis of metastasis, the process is generally considered to follow a stochastic, sequential cascade involving tumor cell intravasation, dissemination via the circulatory and/or lymphatic compartments, extravasation into a remote location, angiogenesis, and finally overt growth (Chambers et al. 2002). Every step in the metastatic pathway must be successfully negotiated for a tumor cell to establish itself as an overt metastasis, and it is usually an extremely inefficient process (Luzzi et al. 1998). Mounting evidence supports the view that extracellular proteinases, such as the matrix metalloproteinases (MMPs), mediate many of the changes in the microenvironment during tumor progression. The antimetastatic effects of NG and α-TOC were evaluated by means of a series of in vitro metastatic assays, including wound healing migration and the Boyden chamber invasion assays. Results were shown in Table 2. Cell migration ability was investigated by a wound healing in vitro assay, in which cells migrate bidirectionally from the edges of a scratch wound. Migration ability was reduced by about 58%, with respect to control, in 100 μM α-TOC and NG-treated human PC-3 prostate cancer cell line, and by about 65% after combined treatment. The antimetastatic activity of the two molecules was further studied, evaluating the adhesion and invasion pattern of 100 μM α-TOC or 10 μM NG-treated human PC-3 prostate cancer cells. Invasion capability, estimated by a Boyden Chamber assay, was impaired. In fact, the computerized analysis performed on matrigel (MG)-coated porous filters showed a decrease of the invasive power of α-TOC and NG-treated human PC-3 prostate cancer cell line, with respect to the control, by 26% and by 53%. Combined treatment reduced invasion ability by about 78%, with respect to the control. In order to quantify the activity of secretory MMPs in human PC-3 cells treated with α-TOC and NG, we performed a gelatin-zymographic analysis. The evaluation of the integrated optical density (IOD) of the 72 kDa matrix MMP-2 and the 92 kDa MMP-9 shows that MMP-9 activity, in PC-3 cell after treatment with α-TOC in combination with NG, was reduced, with respect to the control, by about 33% (Table 2). On the contrary, MMP-2 activity was unaffected (data not shown).

Conclusive remarks

Despite the many therapeutic regimens introduced recently, human prostate cancer is still an incurable disease. Thus, there is an urgent need to discover novel, less toxic, and more effective therapies for these patients. Among several options, NG, a biologically active flavonoid, commonly found in fruits and vegetables, exhibit antitumor effects, against breast cancer, hepatoma and melanoma cell lines, through the induction of cell differentiation (Lentini et al. 2007). Differentiation therapy of cancer was conceived from the observation that tumor cells, can regain control of growth and differentiation in response to a number of natural and synthetic compounds. Although, several

Table 1 Effect of naringenin and α-tocopherol on apoptosis in human PC-3 prostate cells

	% apoptotic cells(APOAC detection kit)	% subG1 cells (cytofluorimetry)
Control	3.5 ± 0.2*	4.4 ± 0.2*
10 μM NG	14.0 ± 0.6*	33.2 ± 1.7*
100 μM α-TOC	10.2 ± 0.5*	25.8 ± 1.3*
NG + α-TOC	18.6 ± 0.9*	53.7 ± 2.5*

For cytofluorimetric studies, PC-3 cells were stained with propidium iodide and analyzed with a FACScan Flow Cytometer (Becton-Dickinson, Bedford, MA). Apoptosis rate was determined by the APOAC Annexin V-Cy3 apoptosis detection kit (Sigma, Milan, Italy), according to the manufacturer instructions

Each point represents the mean ± SD of three different determinations (*$p < 0.001$; Student's t test)

NG naringenin, *α-TOC* α-tocopherol

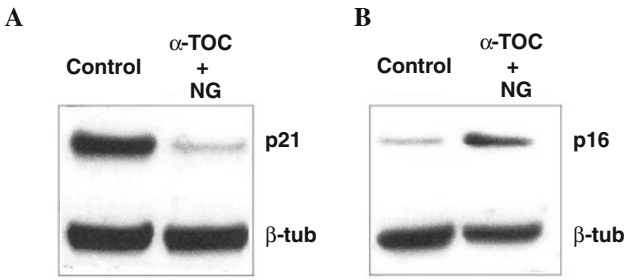

Fig. 4 Differential expression of p21 (**a**) and p16 (**b**) protein: western blot data of PC-3 cells treated with a combined 10 μM naringenin (NG) and 100 μM α-tocopherol (α-TOC). Total cell lysates were subjected to western blot analysis with p16-, p21-, and tubulin-specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

markers have been identified on tumor cells, their casual relationship to neoplastic competence has not been characterized in sufficient detail, to warrant their evaluation as novel pharmacological targets for the design of new differentiative agents. In recognition of this information, and in search of a novel approach for cancer chemoprevention, we have focused our attention on the investigation of the possible involvement of TG in the antineoplastic role of α-TOC. Several lines of evidence show a key role of TG in the antineoplastic activity of phytochemicals, through the induction of terminal cell differentiation followed by apoptosis (Lentini et al. 2010). The apoptotic functions of TG are linked to the ability of this enzyme to irreversibly cross-link proteins in the presence of Ca^{2+}. It is likely that phytochemicals are able to induce a stressful condition, followed by a massive release of Ca^{2+} from intracellular stores or influx of Ca^{2+} from outside the cell, leading to activation of TG resulting in post-translational modification of key proteins and onset of apoptosis (Fig. 5). In our study, we observed an increase in p16 and a reduction of

Table 2 Effect of naringenin and α-tocopherol on the metastatic potential of human PC-3 prostate cells

	% migration	% invasion	% MMP-9 activity
Control	100	100	100
10 μM NG	44.8 ± 4.4*	74.2 ± 7.1*	–
100 μM α-TOC	40.9 ± 4.1*	47.8 ± 4.3*	–
NG + α-TOC	34.6 ± 3.5*	21.7 ± 2.5*	76.9 ± 5.5*

Wound healing migration was performed by growing cells to confluency in 12-well plates and wounds were made with a sterile plastic tip. Cells were further incubated for 24 h with or without compounds in D-MEM (without FCS) and photographed under microscope at the time 0 point and after 24 h. The number of treated migrating cells was quantified with Image J software (NIH; http://rsb.info.nih.gov/ij/)

Invasion assay was carried out in a Boyden chamber using polycarbonate polyvinylpyrrolidone-free membranes (8-μm pore size; Neuroprobe, Cabin John, MD, USA). The number of invasive cells was evaluated by means of Image J software

MMP-9 activity was measured in PC-3 cells conditioned media using a gelatin zymography technique. Samples were loaded under nonreducing conditions onto 7% polyacrylamide gel polymerized with 1% gelatin. Finally, the gel was washed in 2.5% Triton X-100 for 30 min under gentle shaking, incubated overnight at 37°C in the substrate buffer (0.5 mol/l Tris–HCl, pH 7.5, 50 mmol/l $CaCl_2$, 2 mol/l NaCl and 0.2% Brij35), stained in Coomassie blue (G-250) and then destained. Digested regions corresponding to MMP-9 (and their zymogens) activity was quantified using the Gel-Pro Analyzer (Media Cybernetics Inc., Bethesda, Maryland, USA) optical densitometric software

Each point represents the mean ± SD of three different determinations (*$p < 0.001$; Student's t test)

NG naringenin, *α-TOC* α-tocopherol

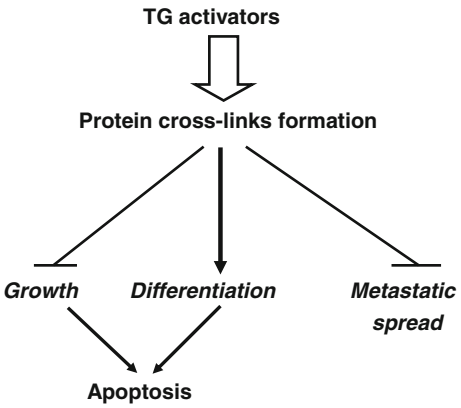

Fig. 5 Induction of TG activity is involved in cancer cell growth, differentiation, apoptosis and metastasis

p21 expression together with an increase in the activity of TG in the PC-3 cultures treated with α-tocopherol and NG. Consequently, a higher rate of apoptosis caused by the combined treatment was observed. Therefore, the use of NG in combination with α-TOC allows a better reduction of the PC-3 proliferation and invasion, because of the synergic effect of the combination of these drugs. The

synergism with NG makes α-TOC a potential drug for further study on the differentiation therapy in prostate cancer patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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