Palm oil tocotrienols and plant flavonoids act synergistically with each other and with Tamoxifen in inhibiting proliferation and growth of estrogen receptor-negative MDA-MB-435 and -positive MCF-7 human breast cancer cells in culture

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Palm oil, unlike many other dietary oils, does not increase the yield of chemically-induced mammary tumors in rats when fed at high levels in the diet. This difference appears to be due to the vitamin E fraction of palm oil, which is rich in tocotrienols, since palm oil stripped of this fraction does increase tumor yields. Experiments in our laboratory have shown that tocotrienols inhibit proliferation and growth of both MDA-MB-435 and MCF-7 cells in culture much more effectively than α-tocopherol. In addition, it was found that combinations of tocotrienols with Tamoxifen, a drug widely used for treatment of breast cancer, inhibit these cells more effectively than either tocotrienols or Tamoxifen alone. The present studies have now shown synergistic effects between tocotrienols and a number of other flavonoids from various plant sources, including citrus fruits, in the inhibition of both MDA-MB-435 and MCF-7 cells (IC_{50}s 0.05-25 and 0.02-5 μg/mL respectively). In the MCF-7 cells, 1:1:1 combinations of tocotrienols, flavonoids and Tamoxifen were even more effective, with the best combination being δ-tocotrienol, hesperetin and Tamoxifen (IC_{50} 0.0005 μg/mL). These results suggest that diets containing palm oil may reduce the risk of breast cancer, particularly when eaten with other plant foods containing flavonoids, and may also enhance the effectiveness of Tamoxifen for treatment of breast cancer.

Introduction

Previous studies have shown that diets containing a high level of palm oil do not promote chemically-induced mammary carcinogenesis in rats[1,2]. Evidence that this inhibition is related to the vitamin E fraction of palm oil, consisting mainly of tocotrienols, was provided by Nesaretnam *et al.*[3] They showed that rats treated with the mammary carcinogen, 7,12-dimethylbenz(a) anthracene (DMBA), and fed vitamin E free palm oil developed more tumors than those fed palm oil containing vitamin E. Tocotrienols also caused a delay in the onset of subcutaneous lymphoma in HRS/J hairless mice by 2-4 weeks[4] and the life span of mice inoculated with transplanted tumor cells was increased by tocotrienols[5-7].

Flavonoids are polyphenolic compounds that occur ubiquitously in plant foods and are important constituents of the human diet[8-10]. They have also been investigated for their anticancer properties[11]. Genistein, an isoflavone found in soybeans, has been extensively studied as a possible anti-cancer agent[12-15]. Quercetin, another flavonoid found in many fruits and vegetables, has also been investigated for anticancer activity. It has been shown to have growth inhibitory activity in vitro in human breast cancer cells[16] and to reduce the incidence of chemically-induced mammary tumors in rats[17].

Previous studies in our laboratory have shown that both tocotrienols[18,19] and citrus flavonoids[20] are effective inhibitors of human breast cancer cells in culture. We have also shown that rats treated with the mammary carcinogen DMBA and given orange juice, developed fewer tumors than controls[20] which may be due to the flavonoid, hesperetin, in orange juice.

A number of epidemiological studies have been concerned with relationships between diet and cancer and have provided evidence that consumption of fruits and vegetables protects against various types of cancer[21]. Although this protective effect has been generally attributed to the antioxidant capacities of vitamin C and β-carotene present in these foods, it may also be related to other constituents of vegetables and fruits, such as the flavonoids[22].

Tamoxifen, a non-steroidal estrogen antagonist, has been extensively used in the treatment of hormone-responsive breast cancer[23]. It acts mainly by blocking the stimulatory action of estrogens in hormone-responsive breast cancer cells[24]. Most breast cancers consist of hormone-independent as well as dependent cells[25] and tumors invariably develop resistance to tamoxifen[26].

We became interested in tocotrienols as a result of the observation that palm oil stripped of its vitamin E fraction promotes chemically-induced carcinogenesis in rats as effectively as other fats[3] and in flavonoids because of our observation that naringenin, a flavonoid in grapefruit, is a more effective inhibitor of proliferation and growth of human breast cancer cells *in vitro* than genistein[27]. Since combinations of drugs are often more effective than single drugs in chemotherapy[28], we tested 1:1 combinations of tocotrienols and flavonoids as well as 1:1:1 combinations of tocotrienols, flavonoids and Tamoxifen on proliferation, growth and viability of both estrogen receptor-negative and -positive human breast cancer cells.

Material and methods

Materials. The tocotrienol-rich fraction of palm oil (TRF), as well as α-tocopherol and the individual tocotrienols, were obtained from the Palm Oil Research Institute of Malaysia (PORIM), Kuala Lumpur. Nobiletin and tangeretin were obtained from the State of Florida Department of Citrus, Lake Alfred, FL. Apigenin, 17-β estradiol, genistein, hesperetin, naringenin, quercetin and Tamoxifen were purchased from the Sigma Chemical Co, St

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Louis, MO. MDA-MB-435 cells[29] were obtained from Dr. Janet Price (MD Anderson Cancer Center, Houston, TX) and MCF-7 cells[30] were obtained from the American Tissue Culture Collection, Rockville, MD. Tissue culture medium, fetal calf serum and fungizone (antibiotic/ antimycotic) were purchased from Gibco Chemical Co, Burlington, ON. Fetal calf serum treated with dextran-coated charcoal (FCS/DCC) was obtained from Cocalico Biologicals Inc, Reamstown, PA. Trypsin was purchased from Difco Laboratories, Detroit, MI and [^{3}H] thymidine (6.7 Ci/mmol) from ICN, Irvine, CA. All other chemicals were from Sigma.

Cell culture. MDA-MB-435 estrogen receptor-negative human breast cancer cells were maintained at 37°C in minimum essential medium (alpha modification) containing 3.7 g of sodium bicarbonate per liter, supplemented with 10% v/v fetal calf serum, in a humidified atmosphere of 5% carbon dioxide. Stock cultures were seeded at a density of 2 x 10^5 cells and allowed to multiply for 48-72 hours.

MCF-7 estrogen receptor-positive human breast cancer cells were maintained in minimum essential medium (alpha modification) containing 3.7 g of sodium bicarbonate supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 10 μg/ mL insulin and 1% v/v fungizone (antibiotic/antimycotic, 10 000 units/mL penicillin G sodium, 10 000 μg/mL streptomycin sulphate and 25 μg/mL amphotericin B in 0.85% saline). Cells were grown to confluence at 37°C in a humidified atmosphere containing 5% carbon dioxide and were passaged weekly using 0.25% trypsin.

Experimental media. Stock solutions of TRF, α-tocopherol, α-, γ- and δ-tocotrienols, and the various flavonoids were dissolved in DMSO at a concentration of 50 mg/mL and then diluted into the culture medium and filter sterilized with an 0.2 μM syringe filter. The final concentration of DMSO was 0.1% and a similar amount was added to control cells. Tamoxifen was dissolved in ethanol at a concentration of 50 mg/mL and ethanol was likewise added to control cells.

Incorporation of [^{3}H] Thymidine into DNA. MDA-MB-435 cells were plated at a density of 2 x 10^4 cells/well in 96-well, flat-bottomed tissue culture plates in a total volume of 200 mL of medium and incubated at 37°C for 48 hours, with or without the test compounds. [^{3}H] thymidine (0.5 μCi/well) was then added to determine the number of dividing cells at each concentration and after 4 hours the medium and excess radiolabel were removed and the cells were trypsinized and harvested onto a glass fiber filter paper, using a semi-automatic 12-well harvester (Skatron Inc, Sterling, VA). Radioactivity on the filter paper was counted, using BCS scintillant in a liquid scintillation counter[31]. For the MCF-7 cells, the growth medium was exchanged for phenol red-free medium containing 10% fetal calf serum that had been treated with dextran-coated charcoal (FCS/DCC) five days prior to use. The cells were then trypsinized and 2 x 10^4 cells/well were plated as described above. Two days later, the medium was replaced with an experimental one containing 2.5% FCS/DCC and the test compounds for 5 days[32]. Untreated cells were used as a control. [^{3}H] thymidine was then added and the cells harvested as described above. The concentration at which 50% inhibition occurred (IC_{50}) was determined by comparing the number of disintegrations per minute for the treated cells with that obtained for the control cells. The IC_{50}s reported for the 1:1 combinations represent the total concentration of both compounds present.

Experiments on cell growth. The effects of tocotrienols, flavonoids and Tamoxifen, alone and in combination, on the growth of both types of cells were also studied. MDA-MB-435 and MCF-7 cells were plated at 1 x 10^4 cells/dish in 60 mm dishes, with or without the test compounds at their IC_{50} concentration in a total volume of 7 mL. The cells were removed by trypsinization at the specified times and counted using a hemocytometer. Results are presented as the average of 3 determinations ± SD.

Viability of cells. The viability of the cells was measured using the MTT assay[33]. In this assay, a tetrazolium salt, 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT), is reduced to a blue formazan product by mitochondrial dehydrogenases that are active in viable living cells. The intensity of the blue color that develops is a measure of cell viability.

MDA-MB-435 and MCF-7 cells (8 x 10^4 cells/well) were seeded in 96-well, flat-bottomed tissue culture plates with various concentrations of tocotrienols, flavonoids and Tamoxifen, alone or in combination, in a total volume of 200 μL/well of medium. Forty-eight hours later, MTT (25 μL of 5 mg/mL) was added to each well. After three hours, 100 μL of extraction buffer, consisting of 20% SDS, dissolved in a 1:1 dimethylformamide : water solution at pH 4.0, was added. The blue color formed was measured at 570 nm in a Dynatech MRX Micoplate Reader. For the flavonoids and combinations containing flavonoids, a control was prepared to account for side reactions observed between them and MTT. This contained all compounds, medium and MTT without cells. The percentage of cells surviving was determined by comparing the absorbance of the treated cells with that of the control. Results are the average of three experiments ± SEM.

Results

MDA-MB-435 cells. The ability of different tocotrienols and flavonoids to inhibit proliferation of MDA-MB-435 human breast cancer cells was investigated by measuring the incorporation of [^{3}H] thymidine into DNA of the cells in the presence of varying concentrations of the compounds. The IC_{50}s for the compounds, alone and 1:1 combinations are presented in Table 1. Synergistic effects between the tocotrienols and flavonoids were observed in most cases, with γ-tocotrienol and tangeretin being the most effective combination (IC_{50}-0.05 μg/mL).

Table 1. Inhibition of proliferation of MDA-MB-435 cells by 1:1 combinations of flavonoids and tocotrienols.

	IC_{50}(μg/mL)				
		Tocotrienols			
Flavonoids	None	TRF	α	γ	δ
None		180	90	30	90
Genistein (soybeans)	140	20	13	4	16
Naringenin (grapefruit)	18	16	8	1	4
Hesperetin(oranges)	18	6	2	19	19
Tangeretin (tangerines)	0.5	0.25	0.1	0.05	0.1
Nobiletin (tangerines)	0.5	0.5	2	0.5	0.25
Quercetin (various plants)	10	1	0.4	25	19
Apigenin (various plants)	3	8	2	2	4

Estrogen receptor-negative MDA-MB-435 human breast cancer cells were cultured with or without various concentrations of the test compounds. The concentration required to inhibit cell proliferation by 50% was determined, as measured by the incorporation of [^{3}H] thymidine into DNA. The experiments were done in triplicate, and the results are averages of three experiments.

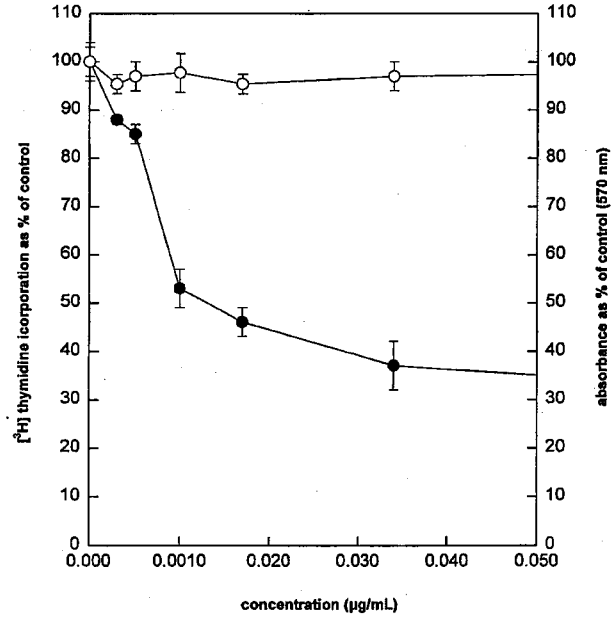
The compounds also showed synergism with Tamoxifen when tested in 1:1 or 1:1:1 combinations (Table 2). Tamoxifen alone had an IC_{50} of 90 μg/mL for these cells. The lowest IC_{50} (0.01 μg/mL) was again obtained with γ-tocotrienol and tangeretin, in combination with Tamoxifen. The inhibition of proliferation and the cytotoxic effect of this combination are illustrated in Figure 1. Most cells were viable at the IC_{50}, indicating that the antiproliferative effect was not due to nonspecific cytotoxicity. The ability of a 1:1 combination of γ-tocotrienol and tangeretin to suppress growth of the cells over a ten-day period is illustrated in Figure 2.

Table 2. Inhibition of proliferation of MDA-MB-435 cells by 1:1 and 1:1:1 combinations of flavonoids, tocotrienols and tamoxifen.

	IC_{50} (μg/mL)				
		Tocotrienols			
Flavonoids	None	TRF	α	γ	δ
None		4	1.5	2	6
Genistein (soybeans)	21	10	3	2	6
Naringenin (grapefruit)	10	6	6	0.5	2
Hesperetin (oranges)	13	6	2	9	6
Tangeretin (tangerines)	0.5	0.25	0.1	0.01	0.1
Nobiletin (tangerines)	0.5	0.5	2	0.5	0.25
Quercetin (various plants)	6	1	0.4	5	3
Apigenin (various plants)	3	5	2	1	2

Estrogen receptor-negative MDA-MB-435 human breast cancer cells were cultured with or without various concentrations of the test compounds. Tamoxifen was present in each case. The concentration of each combination required to inhibit cell proliferation by 50% was determined, as measured by the incorporation of [^{3}H] thymidine into DNA. The experiments were done in triplicate, and the results are averages of three experiments.

Figure 1. Effect of a 1:1:1 combination of γ-tocotrienol, tangeretin and Tamoxifen on the proliferation (•) and viability (o) of MDA-MB-435 cells. The cells were incubated with various concentrations of these compounds for 48 hours, [^{3}H] thymidine (0.5 μCi/well) was then added and the cells were harvested after four hours to evaluate the incorporation of thymidine into DNA. For viability, cells were incubated with various concentrations of the test compounds for 48 hours, MTT was then added (25 μL) and after three hours, extraction buffer was added (100 μL) and OD measurements made at 570 nm. Points are the average of mean values from three experiments ± SEM.



MCF-7 cells. We have also tested the ability of the above combinations to inhibit proliferation of MCF-7 estrogen receptor-positive human breast cancer cells. Table 3 shows the IC_{50}s for the compounds alone and in 1:1 combinations. Synergistic effects were observed in these cells as well. The most effective combinations were tangeretin with γ- and δ-tocotrienols (IC_{50}s of 0.02 and 0.04 μg/mL respectively). Tamoxifen had an IC_{50} of 0.04 μg/mL in these cells. Combinations (1:1:1) of tocotrienols, flavonoids and Tamoxifen were more effective than the 1:1 combinations of tocotrienols and flavonoids. The best results were obtained with a combination of δ-tocotrienol, hesperetin and Tamoxifen (IC_{50}-0.0005 μg/mL) (Table 4). Viability studies showed that most of the cells were viable at the IC_{50}s of the triple combinations.

Table 3. Inhibition of proliferation of MCF-7 cells by 1:1 combinations of flavonoids and tocotrienols.

	IC_{50} (μg/mL)				
		Tocotrienols			
Flavonoids	None	TRF	α	γ	δ
None		4	6	2	2
Genistein (soybeans)	3.9	3.3	3.1	2.6	3.1
Naringenin (grapefruit)	18	2	1	0.4	0.7
Hesperetin (oranges)	12	2	2	3	0.1
Tangeretin (tangerines)	0.4	0.6	0.4	0.02	0.04
Nobiletin (tangerines)	0.8	0.8	1.6	0.8	0.8
Quercetin (various plants)	5.1	3.3	2.6	2.1	1.6
Apigenin (various plants)	2.4	3.1	3.1	3.1	2.4

Estrogen receptor-positive MCF-7 human breast cancer cells were cultured with or without various concentrations of the test compounds. The concentration required to inhibit cell proliferation by 50% was determined, as measured by the incorporation of [^{3}H] thymidine into DNA. The experiments were done in triplicate, and the results are averages of three experiments.

Figure 2. Growth of MDA-MB-435 cells in the presence (Δ) or the absence (▲) of 0.01 μg/mL of γ-tocotrienol, tangeretin and Tamoxifen. Cells were plated in triplicate in 60 mm culture dishes. Cells were removed by trypsinization at the specified times and were counted using a hemocytometer.

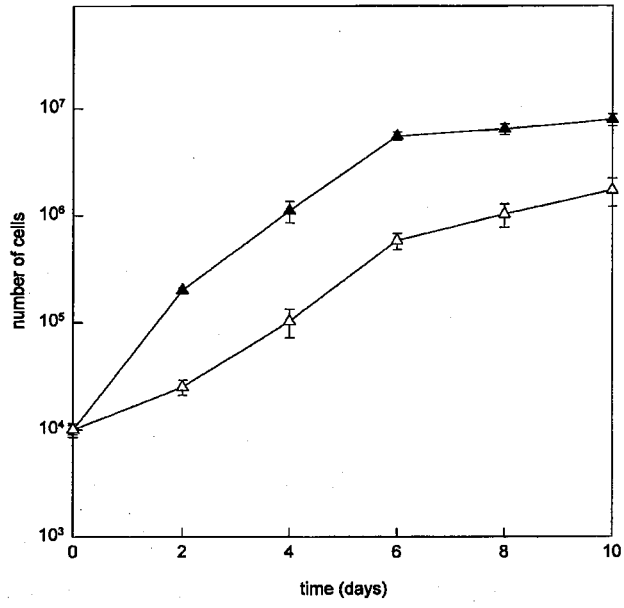


Table 4. Inhibition of proliferation of MCF-7 cells by 1:1 and 1:1:1 combinations of flavonoids, tocotrienols and tamoxifen.

	IC_{50} (μg/mL)				
		Tocotrienols			
Flavonoids	None	TRF	α	γ	δ
None		0.5	0.1	0.01	0.003
Genistein (soybeans)	2.1	1.1	1.6	0.8	0.05
Naringenin (grapefruit)	1.2	0.4	0.1	0.008	0.4
Hesperetin (oranges)	0.3	0.4	0.4	0.8	0.0005
Tangeretin (tangerines)	0.08	0.04	0.4	0.02	0.02
Nobiletin (tangerines)	0.004	0.4	0.07	0.09	0.001
Quercetin (various plants)	1.1	1.2	3.3	0.08	0.02
Apigenin (various plants)	1.9	1.6	1.6	2.4	0.8

Estrogen receptor-positive MCF-7 human breast cancer cells were cultured with or without various concentrations of the test compounds. Tamoxifen was present in each case. The concentration of each combination required to inhibit cell proliferation by 50% was determined, as measured by the incorporation of [^{3}H] thymidine into DNA. The experiments were done in triplicate, and the results are averages of three experiments.

Discussion

Earlier studies in our laboratory have shown that tocotrienols and flavonoids inhibit proliferation and growth of both MDA-MB-435 and MCF-7 cells[18-20,34,35]. The present studies provide evidence that combinations of these compounds act synergistically in the inhibition of these cells. This synergistic effect was enhanced further by the addition of Tamoxifen.

The observed synergism suggests that these compounds may be acting by different mechanisms. Tamoxifen is widely used in the treatment of hormone-responsive tumors[36], and acts mainly by competing with estrogen for its receptor. Our data indicate that tocotrienols and flavonoids act via an estrogen receptor-independent pathway, since they inhibit both receptor-positive and -negative cell lines. We have previously shown that tocotrienols do not compete with estrogen for the receptor on the MCF-7 cells (unpublished observation). Isoflavones such as genistein have been shown to act as weak estrogens and to inhibit MCF-7 cells by occupying the estrogen receptor[37], but experiments in our laboratory have indicated that other flavonoids tested do not act by this mechanism[38].

The precise mechanism for the observed inhibition of cell proliferation of these compounds is at present unknown, but may be related to their antioxidant properties[21,39] or to inhibition of key enzymes involved in the regulation of cellular proliferation, such as protein tyrosine kinases and protein kinase C. Both genistein and quercetin have been shown to inhibit the activities of tyrosine-specific protein kinases[40,41]. Quercetin also inhibits protein kinase C[42]. Preliminary results in our laboratory have shown that tocotrienols abolish protein kinase C activity at their IC_{50} concentration in MDA-MB-435 human breast cancer cells in culture[43]. Thus, tocotrienols and flavonoids may exert their anti-proliferative properties by interfering with signal transduction events involving protein kinases.

Previous studies have shown that increased phosphorylation of the estrogen and progesterone receptors can alter their activity[44] and tocotrienols and/or flavonoids may be interfering with this phosphorylation state. Other mechanistic possibilities include the potential involvement of a second set of estrogen-binding sites, referred to as type II estrogen binding sites[45]. These sites are occupied by an endogenous ligand with growth-inhibitory activity and evidence has suggested that it may be a flavonoid-like molecule[46].

These results have important clinical implications since most breast cancers are heterogeneous and consist of estrogen receptor-positive as well as -negative cells[25]. An agent that inhibits the growth of both estrogen receptor-positive and -negative tumors would be of great interest. Also, a treatment regimen coupled with an anti-hormonal drug, such as Tamoxifen, could effectively target both types of tumor cells. Our results indicate that tocotrienols and flavonoids effectively inhibit both cell types and that 1:1 combinations are synergistic. The addition of Tamoxifen enhanced this inhibition in the estrogen receptor-negative cells and in some cases in the estrogen receptor-positive cells as well.

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在培養基中，棕櫚油生育三烯酚，植物黃酮類化合物和Tamoxifen相互協同，可最近抑制MDA－MB－435和MCF－7人類乳癌細胞的作用

摘要

棕櫚油不象其他食用油類，以高棕櫚油膳喂養大鼠不會增加乳瘤．這種差异是由于棕櫚油富含生育三烯酚的緣故．因爲除去生育三烯酚的棕櫚油，可再引起乳瘤增加．我們的實驗顯示生育三烯酚在培養基中抑制MDA－MB－435和MCF－7細胞的增長與增殖較α－生育酚有效得多．再者，我們發現同時應用生育三烯酚和Tamoxifen（一種廣泛用以治療乳癌的藥物）抑制這些細胞的作用較單獨用生育三烯酚或Tamoxifen的效果好得多．目前研究顯示，生育三烯酚和一系列來自不同植物（包括柑橘類）的黃酮類化合物可協同增加抑制MDA－MB－435和MCF－7細胞（分別爲IC_{50} S 0．05－25和0．02－5微克/毫升）．對MCF－7細胞，用生育三烯酚，黃酮類化合物和Tamoxifen 1：1：1甚至可獲得更好的效果．δ－生育三烯酚，橙皮素和Tamoxifen（IC_{50} 0．0005微克/毫升）聯合使用的效果是最好的．這些結果指出了膳食含棕櫚油，特別是與含黃酮類植物同時進食，也許可增加Tamoxifen對乳癌的治療效果．

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