Influence of Tangeretin on Tamoxifen’s Therapeutic Benefit in Mammary Cancer

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Background: Tamoxifen and the citrus flavonoid tangeretin exhibit similar inhibitory effects on the growth and invasive properties of human mammary cancer cells in vitro; furthermore, the two agents have displayed additive effects in vitro. In this study, we examined whether tangeretin would enhance tamoxifen’s therapeutic benefit in vivo. Methods: Female nude mice (n = 80) were inoculated subcutaneously with human MCF-7/6 mammary adenocarcinoma cells. Groups of 20 mice were treated orally by adding the following substances to their drinking water: tamoxifen (3 x 10^−5 M), tangeretin (1 x 10^−4 M), tamoxifen plus tangeretin (3 x 10^−5 M plus 1 x 10^−4 M), or solvent. Results and Conclusions: Oral treatment of mice with tamoxifen resulted in a statistically significant inhibition of tumor growth compared with solvent treatment (two-sided \( P = .001 \)). Treatment with tangeretin did not inhibit tumor growth, and addition of this compound to drinking water with tamoxifen completely neutralized tamoxifen’s inhibitory effect. The median survival time of tumor-bearing mice treated with tamoxifen plus tangeretin was reduced in comparison with that of mice treated with tamoxifen alone (14 versus 56 weeks; two-sided \( P = .002 \)). Tangeretin (1 x 10^−6 M or higher) inhibited the cytolytic effect of murine natural killer cells on MCF-7/6 cells in vitro, which may explain why tamoxifen-induced inhibition of tumor growth in mice is abolished when tangeretin is present in drinking water. Implications: We describe an in vivo model to study potential interference of dietary compounds, such as flavonoids, with tamoxifen, which could lead to reduced efficacy of adjuvant therapy. In our study, the tumor growth-inhibiting effect of oral tamoxifen was reversed upon addition of tangeretin to the diet. Our data argue against excessive consumption of tangeretin-added products and supplements by patients with mammary cancer during tamoxifen treatment. [J Natl Cancer Inst 1999; 91:354–9]

The overall survival of patients with breast cancer is increased by adjuvant therapy with tamoxifen (1). Resistance of tumors to this drug, however, is one of the reasons for failure of treatment in patients with breast cancer. Many mechanisms of resistance to tamoxifen have been described (2). They vary from drug insensitivity of the cancer cells to altered drug metabolism. Dietary intake of some flavonoids has been invoked to explain resistance to tamoxifen because of direct estrogenic effects of flavonoids on the cancer cells (3) or their induction of tamoxifen metabolism in the liver (4).

Tangeretin, a flavonoid present in citrus fruits, affects certain activities of mammary cancer cells in a manner similar to that of tamoxifen, at least in vitro. Like tamoxifen (5), this flavonoid inhibits growth, activates cell–cell adhesion, and blocks invasion by human MCF-7/6 mammary adenocarcinoma cells (6). The effects on cell–cell adhesion and invasion of both tamoxifen and tangeretin are mediated by the E-cadherin/catenin complex, which serves as an invasion suppressor at the plasma membrane of epithelioid cells (7).

Inspired by these data, we wondered how a combined treatment with tamoxifen and tangeretin would affect mammary tumors in vivo, as compared with treatment with tamoxifen only. Our model consisted of MCF-7/6 tumors growing subcutaneously in nude mice, because, for ethical reasons, such a study could not be carried out with breast cancer patients.

Materials and Methods

MCF-7/6 cells. MCF-7/6 cells (a gift from Henri Rochefort, Unité d’Endocrinologie Cellulaire et Moléculaire, Montpellier, France) were derived...
from a pleural effusion of a mammary adenocarcinoma patient (8). In culture, these cells have retained a number of characteristics of their tumor of origin, including the expression of estrogen receptors (9) and the capacity to invade (10,11). They were maintained in a mixture of Dulbecco’s modification of Earle’s medium and Ham F12 (50:50; Gibco, Merelbeke, Belgium), supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B, and 10% fetal bovine serum.

**Growth of MCF-7/6 tumors in nude mice.** At the age of 4 weeks (1 week before cell inoculation), 80 female Swiss nu/nu mice were primed with a 1-mg estradiol pellet (Organon Laboratories, Cambridge, U.K.) implanted subcutaneously. MCF-7/6 cells were injected into the mammary fat pad as a 100-μL suspension of 1 × 10^6 cells in Matrigel® (Becton Dickinson, Erembodegem-Aalst, Belgium). The larger (a) and smaller (b) diameters of the subcutaneous tumors were measured every 2 weeks, and the tumor volume (V = 0.4 × a × b^2) was estimated after caliper measurements (12). The animals were fed “RO4 Aliment Composé Complet” (UAR, Épinay-sur-Orge, France). The pellets have the following ingredients: barley grains, wheat and corn bran, soy cake, fish protein concentrate with minerals (composition: 17.5% protein, 3% lipid, 4% carbohydrate, 12.5% water, 5.2% inorganic material, and 25 mg/kg copper), 7000 IU/kg vitamin A, 2000 IU/kg vitamin D₃, and 15 mg/kg vitamin E. Animal care was in accord with the guidelines of the local ethics committee of our University Hospital.

**Treatment of mice.** From the day of tumor cell inoculation on, groups of 20 mice were treated orally with tamoxifen (Nolvadex®; Zeneca, Destelbergen, Belgium), tangeretin (a gift from John Attaway, Department of Citrus, Lakeland, FL), or a combination of both products. The animals were allowed to drink 3 × 10⁻⁹ M tamoxifen and/or 1 × 10⁻⁴ M tangeretin in acidified (pH 6.2) tap water ad libitum. A solvent control group of 20 mice received 1% ethanol (referring to ad libitum.

**Assay for tamoxifen and its principal metabolites.** When moribund, the mice were killed by chloroform inhalation. Blood samples of 1 mL were obtained by heart puncture, and serum was stored frozen. Tumors were excised in toto; after formalin fixation of part of the tissue for histology, the remainder was extracted with phosphate-buffered salt solution (pH 7.4), after which extracts were frozen down at −70°C. Fractions of approximately 1 g from liver, fat tissue, and kidney were extracted and frozen as well. Both serum and tissue extracts were subjected to high-pressure liquid chromatography (HPLC) analysis of tamoxifen and its principal metabolites (N-desmethyl tamoxifen and 4-hydroxyltamoxifen) (14). Concentrations were expressed per milliliter (in serum) or per milligram wet tissue (in tumor fragments).

**Assay for growth of MCF-7/6 cells in vitro.** MCF-7/6 cells were seeded in microtiter plates at an initial density of 1.5 × 10⁴ cells in 200-μL culture medium. After an incubation period of 5 days, the amount of cell protein in each well was estimated with the sulfhydradamine B assay (15).

**Assay for P-glycoprotein.** The expression of P-glycoprotein as an antigen (16) and function (17) were measured by flow cytometry. The antibodies used to detect the antigen were 4E3 (Dako, Glostrup, Denmark) and MRK16 (Research Diagnostics, Inc., Flanders, NY). The function of P-glycoprotein was measured as the accumulation and retention of rhodamine 123 in the presence and the absence of verapamil. Two variants of a human erythroleukemia cell line (K562 and K562-VLB) were used as negative and positive control cells, respectively.

**Circulating lymphocyte counts.** Peripheral blood samples (0.5 mL), obtained by heart puncture of nude mice, were collected in EDTA-containing tubes. Total leukocyte counts and differentiation were performed with an H2D Hematology Analyzer (Technicon, Tarrytown, NY).

**Assay for natural killer (NK) cell activity in vitro.** Spleen leukocytes from 8-week-old nude mice were activated with 1000 U/mL recombinant interleukin 2 (a gift from R. Devos, Roche Research, Gent, Belgium) for 5 days to trigger NK cell activity (18). In accordance with the method of Leclercq et al. (19), MCF-7/6 cells were loaded with 5¹⁴Cr and co-incubated with lymphokine-activated killer (LAK) cells at different effector-to-target ratios (from 64:1 to 1:1). After incubation periods of 4 and 22 hours (20), the amount of ⁵¹Cr released into the supernatant culture medium was measured with a 1450 MicroBeta PLUS liquid scintillation counter (Wallac Oy, Turku, Finland). This release of ⁵¹Cr from the target cells is considered to be a measure of the sensitivity of the cells to LAK-mediated cytolysis. K562 cells were used as a positive control cell line for killing by the NK cells. Cytolysis was calculated as 100 × (R₂ - R₁)/(R₀ - R₁), where R₀ is the release of ⁵¹Cr in the presence of LAK cells, R₁ the spontaneous release in the absence of LAK cells, and R₂ the total release after treatment with Triton X-100. Viability of LAK, MCF-7/6, and K562 cells after 22 hours of treatment with tamoxifen and/or tangeretin was assessed by trypan blue dye exclusion. In the case of MCF-7/6 cells, aggregation during these treatments was prevented by adding a mouse monoclonal antibody against human E-cadherin (0.5 μg/mL MB2) (21).

**Statistical methods.** Tumor volumes (and their logarithms) for the different groups of mice first underwent analysis of variance. Then the probabilities of statistically significant differences between the logarithms of the tumor volumes were adjusted by use of the multiple-comparison techniques of Fisher, Scheffé, or Bonferroni/Dunn. Survival curves were calculated by the method of Kaplan-Meier and analyzed with the Mantel–Cox logrank test. In all other instances, Student’s t test was used. All P values are two-sided and considered statistically significant for P < 0.05. Statistical calculations were done with the Statview 4.1 software (Abacus Concepts, Inc., Berkeley, CA).

**RESULTS**

Oral treatment of nude mice with tamoxifen inhibited the growth of subcutaneous MCF-7/6 tumors as compared with solvent-treated controls. At 16 weeks after tumor cell inoculation, analysis of variance indicated differences between the group means, and this result was valid for both the tumor volumes as such and the logarithms of the volumes (P = .001). Multiple-comparison techniques applied to the logarithms of these volumes showed differences only between the tamoxifen group and the other three groups (solvent control, tangeretin alone, and tamoxifen plus tangeretin) at the 1% significance level (data not shown). In tangeretin-treated mice, the mean tumor volumes were higher than those in the solvent-treated group, but the difference was not statistically significant. When added to the drinking water together with tamoxifen, tangeretin completely neutralized the favorable effect of tamoxifen (Fig. 1).

[Fig. 1. Growth of subcutaneous implants of human MCF-7/6 breast cancer cells in female nude mice. Tumor volumes were calculated as 0.4 × a × b², where a and b are the larger and the smaller diameters, respectively. The groups of mice (n = 10 in each of two groups) were treated orally with tamoxifen (tam, 3 × 10⁻⁴ M), tangeretin (tang, 1 × 10⁻⁴ M), the combination of both drugs (tam + tang, 3 × 10⁻⁴ M and 1 × 10⁻⁴ M, respectively), or with a mixture of the respective solvents (solvent). Symbols indicate mean values; error bars indicate the 95% confidence intervals.]
Furthermore, treatment with tamoxifen plus tangeretin reduced the median survival time of the tumor-bearing mice compared with that of the tamoxifen-treated group (14 weeks versus 56 weeks; $P = .002$, Mantel–Cox logrank test) (Fig. 2). Remarkably, the growth-inhibiting effect of tamoxifen during the 36-week period was reversed upon addition of tangeretin to the tamoxifen-containing drinking water: Tumor growth resumed after a median lag period of 14 weeks (Fig. 3). No regression, however, could be obtained with tumors previously grown in mice treated with tamoxifen plus tangeretin, for which the treatment was switched to tamoxifen only (data not shown).

In contrast to its effect on tumors in vivo, tangeretin was unable to neutralize the growth inhibition by tamoxifen in MCF-7/6 cell cultures (Fig. 4). Both drugs inhibited cell growth at $1 \times 10^{-6} M$ (tamoxifen) and $1 \times 10^{-4} M$ (tangeretin), and combined treatment amplified the growth inhibition as compared with separate treatments ($P < .001$, Student’s $t$ test). MCF-7/6 cells derived from tumors grown in mice treated with tamoxifen plus tangeretin behaved ex vivo in the growth assay as did their parent cells. In the parent and the tumor-derived cells, P-glycoprotein—the product of the multidrug resistance gene—was undetectable with both immunologic and functional assays. Also, in MCF-7/6 cells grown in vitro under tangeretin selection pressure ($10^{-5} M$ for 30 days), no P-glycoprotein could be measured (data not shown).

Induction of tamoxifen’s liver metabolism by tangeretin was ruled out by HPLC determinations of tamoxifen and its principal metabolites in tumors, different normal tissues (liver, fat tissue, and kidney), and serum. Tamoxifen concentrations were not lower in tumors and tissues from mice treated with tamoxifen plus tangeretin than in those from mice treated with tamoxifen alone. In serum, these concentrations were even higher in the first group (mean = 2.7 pg/mL) than in the second one (mean = 0.7 pg/mL) ($P = .0008$), and the same holds true for the concentrations of N-desmethyl tamoxifen ($P = .0025$) (Fig. 5). The active metabolite 4-hydroxytamoxifen was undetectable in both groups. No additional information could be obtained from tumor pathology, because histology did not distinguish between the four treatment groups.

Oral treatment of nude mice ($n = 10$ animals in each of two groups) with tangeretin $\left(1 \times 10^{-4} M\right)$ alone for 4 months led to a significant decrease in the number of circulating lymphocytes ($P = .0075$) (Fig. 6). Because in nude mice an important fraction of lymphocytes consists of NK cells (22), we expanded the NK cell population by interleukin 2 stimulation to obtain LAK cells and further studied the effect of tangeretin on these cells. In contrast with K562 cells, MCF-7/6 cells appeared to be insen-
the ED50 (i.e., dose that is effective in half the cultures) for the flavonoid cytotoxicity to LAK cells was between $1 \times 10^{-6}$ and $1 \times 10^{-4} \text{M}$ respectively; open bars for 5 weeks. Values are means with upper bounds of 95% confidence intervals shown.

Fig. 6. Effect of tangeretin on peripheral blood concentrations of different classes of murine leukocytes. Animals (n = 10 in each of two groups) were treated orally with either tamoxifen (1 × 10^-5 M; dashed bars) or solvent (open bars) for 4 months. Concentrations of circulating monocytes (mono), neutrophils (neutro), and lymphocytes (lympho) are compared. Values are means with upper bounds of 95% confidence intervals shown.

Oral tangeretin (3 × 10^-5 M) and 1 × 10^-4 M, respectively) or with tamoxifen plus tangeretin (3 × 10^{-5} M and 1 × 10^{-4} M respectively; open bars) for 5 weeks. Values are means with upper bounds of 95% confidence intervals shown.

sitive to LAK cells after 4 hours of incubation. After 22 hours, however, cytosis of the Mcf-7/6 cells was observed (mean = 68%; 95% confidence interval = 31%–100%, at an effector-to-target ratio of 16:1). Preculturing the Mcf-7/6 cells in the presence of tamoxifen (1 × 10^{-6} M) for 7 days and performing the cytosis assay during 22 hours in the presence of the drug resulted in a slight, statistically nonsignificant decrease in killing by the Mcf-7/6 cells. The same treatment with tangeretin (1 × 10^{-4} M) or with tamoxifen plus tangeretin (1 × 10^{-6} M and 1 × 10^{-4} M, respectively), however, completely inhibited the Mcf-7/6 cell-mediated cytosis (P = .0009 and .0001 respectively). This inhibition was seemingly due to a selective toxicity of tangeretin for LAK cells (Fig. 7). While trypan blue dye exclusion was not affected by 1 × 10^{-6} M tangeretin with Mcf-7/6 and KS62 cells, the ED50 (i.e., dose that is effective in half the cultures) for the flavonoid cytotoxicity to LAK cells was between 1 × 10^{-6} M and 1 × 10^{-5} M.

DISCUSSION

Our main finding is that tangeretin has a neutralizing effect on the growth inhibition of Mcf-7/6 tumors by tamoxifen in vivo. This effect was observed not only when tangeretin was added to tamoxifen-containing drinking water immediately after inoculation of nude mice with tumor cells, but also after a delay of 36 weeks. Furthermore, tangeretin addition shortened the survival time of the tumor-bearing mice.

Looking for the mechanism of this tangeretin effect, we could exclude a number of possible targets for the flavonoid. We found evidence against a target in the Mcf-7/6 cells themselves. In combined treatments of the parent cells in vitro, tangeretin did not neutralize but rather amplified the growth-inhibitory effect of tamoxifen. This finding and the observations in vivo and in vitro that treatment with tangeretin did not stimulate growth make it difficult to consider this flavonoid as a molecule with an estrogen-like effect on Mcf-7/6 cell growth. Therefore, although the estrogenic activity of a number of dietary flavonoids has been shown to compete with the antiestrogenic effect of tamoxifen on Mcf-7/6 cells (23), this mechanism cannot be invoked to explain our results. Furthermore, selection of a subpopulation of tamoxifen-resistant Mcf-7/6 cells due to oral tangeretin treatment is also unlikely. Selection pressure by tangeretin in vivo and in vitro yielded Mcf-7/6 cell populations that behaved as their parent cells did in the presence of tamoxifen and tangeretin. Because we could not exclude a priori induction of P-glycoprotein by tangeretin to explain our results (24), we studied its expression in tumor-derived Mcf-7/6 cells and in Mcf-7/6 cells grown under tangeretin selection pressure in vitro; however, we found no indication that tangeretin induced this drug resistance molecule.

Like many other flavonoids, tangeretin can interfere with the gastrointestinal absorption of xenobiotics and can induce cytochrome P450 activity and other drug-metabolizing enzymes in hepatocytes (25). Such mechanisms can lead to lower blood concentrations of the xenobiotic and to relatively higher concentrations of its metabolites. However, this was not the case in our experimental model: Tamoxifen concentrations in the blood were higher in mice that received tamoxifen plus tangeretin in vivo.
the drinking water than in those that were treated with tamoxifen only. Furthermore, tangeretin did not alter the ratio between tamoxifen and its principal (inactive) metabolite, N-desmethyl tamoxifen, in blood. Thus, tangeretin does not seem to interfere with the metabolism of tamoxifen in our mouse model.

The differences between our results in vivo and in vitro point toward the presence of a tangeretin-sensitive host activity in the mouse that can counteract growth inhibition by tamoxifen. The elimination of cancer cells by NK cells is such a candidate activity, because it has been reported to be sensitive to both tamoxifen and certain flavonoids. Although we were unable to confirm the data reported by Baral et al. (26), who demonstrated an increased sensitivity of tamoxifen-treated MCF-7 cells to NK cells, we did find an inhibition of MCF-7/6 cell lysis in the presence of tangeretin, as a result of selective toxicity of the flavonoid for NK cells. The ED$_{50}$ of this effect was between $1 \times 10^{-6}$ M and $1 \times 10^{-5}$ M in vitro, a concentration of tangeretin metabolites also found in liver and kidney tissues of mice drinking $1 \times 10^{-4}$ M tangeretin ad libitum (27). This phenomenon may override the tamoxifen effect in vivo and may explain the lack of inhibition of tumor growth in mice treated with tamoxifen plus tangeretin. We can only speculate why the tumor growth-stimulating effect of tangeretin in vivo is less dramatic in the absence of tamoxifen than in its presence, by reasoning that tumor growth is a balance between the effects of multiple growth promoters and suppressors. Tamoxifen acts as a tumor growth suppressor, which—on top of other growth suppressors—is able to bring the balance toward “prevention” of tumor growth, while the addition of tangeretin brings the balance back toward “permission” for tumor growth. In the absence of tamoxifen, however, tangeretin may be unable to change the primary position of this balance and provokes only a minor effect on tumor growth.

It is unlikely that the effect of tangeretin is related to the higher concentrations of tamoxifen and its metabolites in the serum of mice also receiving tamoxifen. Data from the literature indicate that a 100-fold higher concentration of tamoxifen than that which we measured in serum does not inhibit NK cell activity, at least not that which we measured in serum does not inhibit NK cell activity in vitro (26). Furthermore, we have shown in this study that $1 \times 10^{-6}$ M tamoxifen has only a very small and statistically nonsignificant effect on nude mouse NK cell viability and function. Again, this concentration is two orders of magnitude higher than the serum concentration found in mice. We must admit that no in vitro data are available for the tamoxifen metabolites.

We believe that our mouse model is a relevant tool for study of the influence of dietary xenobiotics in vivo on the effect of tamoxifen treatment of mammary cancer. Although oral administration of tamoxifen is associated with lower blood levels in mice than in humans (13), we were able to control the growth of human mammary cancer cells in our animals as occurs in human patients. The human equivalent of the amount of tangeretin used in our experiments would be 280 mg per day. Since tangeretin is poorly water soluble, this dose cannot be ingested by drinking reasonable amounts of citrus juices; nor do we underestimate the numerous health benefits that accrue from eating or drinking citrus products, even for cancer patients. (Our data certainly do not indicate that citrus flavonoids would be carcinogenic!). Methoxylated flavones, however, are components of citrus peel oil, and the equivalent human dose of tangeretin (280 mg) corresponds to about 20 mL of tangerine oil. The addition of citrus peel oils to beverages and foods, baked goods, candy, and confections—both in the United States (28) and in Europe—may lead to a higher tangeretin intake than with juices. We are even more concerned about the promotion and sale of dietary supplements containing citrus flavonoids (i.e., tablets that are currently marketed with a recommended daily dose of about 1 g of citrus flavonoids). Furthermore, we recently found that another methoxylated flavonoid, named nobiletin, is 10 times more potent than tangeretin in inhibiting NK cell activity in vitro. In addition, nobiletin is the major methoxylated flavone in orange oil (0.2%–0.4%). Taken together, our results plead against excessive consumption of tangeretin-added products or dietary supplements by breast cancer patients during tamoxifen treatment.

**References**


NOTES

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