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Advances in Brief

Dietary Genistein Negates the Inhibitory Effect of Tamoxifen on Growth of Estrogen-dependent Human Breast Cancer (MCF-7) Cells Implanted in Athymic Mice[1]

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Abstract

The use of dietary isoflavone supplements by postmenopausal women with breast cancer is increasing. We investigated interactions between the soy isoflavone, genistein, and an antiestrogen, tamoxifen (TAM), on the growth of estrogen (E)-dependent breast cancer (MCF-7) cells implanted in ovariectomized athymic mice. We hypothesized that weakly estrogenic genistein negate/overwhelm the inhibitory effect of TAM on the growth of E-dependent breast tumors. Six treatment groups were used: control (C); 0.25 mg estradiol (E_2) implant (E); E_2 implant + 2.5 mg TAM implant (2.5 TE); E_2 implant + 2.5 mg TAM implant + 1000 ppm genistein (2.5 TEG); E_2 implant + 5 mg TAM implant (5 TE), and E_2 implant +5 mg TAM implant +1000 ppm genistein (5 TEG). Treatment with TAM (2.5 TE and 5 TE) suppressed E_2-stimulated MCF-7 tumor growth in ovariectomized athymic mice. Dietary genistein negated/overwhelmed the inhibitory effect of TAM on MCF-7 tumor growth, lowered E_2 level in plasma, and increased expression of E-responsive genes (*e.g.*, pS2, PR, and cyclin D1). Therefore, caution is warranted for postmenopausal women consuming dietary genistein while on TAM therapy for E-responsive breast cancer.

Introduction

Antiestrogen administration of TAM[3] is a successful adjuvant therapy for patients with E-dependent breast cancer (1) based on significantly improved survival for those women (2). TAM and its metabolites act as E antagonists in breast tissue by binding to ER (3), and inhibiting ER-mediated gene transcription, DNA synthesis, and cancer cell growth (4). Studies in both tumor cell-implanted athymic mice and carcinogen-induced mammary cancer laboratory animal models have demonstrated that TAM inhibits E-dependent breast tumor growth (5). Increasing numbers of cancer patients are using complementary and alternative medicine to supplement their medical treatment or to enhance their overall health (6). After diagnosis of breast cancer, 28–91% of patients reported using one or more complementary dietary supplements (including soy isoflavones) not prescribed by physicians (7). The combined effects of soy isoflavones and TAM on menopausal symptoms after breast cancer are not clear (8). If a postmenopausal woman has E-dependent breast cancer, it is likely that she will be on TAM therapy and may also experience TAM-induced menopausal symptoms. Women may self-medicate with dietary isoflavone supplements to alleviate or reduce the TAM-associated menopausal-like symptoms without the knowledge of their physician.

We have demonstrated previously that genistein, the predominant soy isoflavone, is an E agonist and enhances human breast cancer (MCF-7) cell growth *in vitro* (10^{-8-6} M; Ref. 9) and *in vivo* (250–1000 ppm; Refs. 10, 11). Furthermore, the effective doses produced blood concentrations of total genistein that are relevant to human exposures. The estrogenic action of genistein may negate/overwhelm the beneficial effects of TAM on E-dependent breast tumor growth. This concern needs additional investigation to assess potential beneficial or adverse effects on women with an E-dependent breast cancer. To address this important concern, we used the same preclinical model that was used to characterize the antiestrogenic properties of TAM (12). In the present investigation we evaluated the interaction of dietary genistein and TAM to determine whether genistein could negate the beneficial effects of TAM.

Materials and Methods

Animals. Ovariectomized athymic BALB/c (nude) mice were purchased from Charles River Laboratories (Wilmington, MA) and handled as described in Ju *et al.* (11).

E_2 and TAM Implants. E_2 implants contained 0.25 mg of E_2 mixed in 1.75 mg of cholesterol in a silastic tube (0.04 inner diameter × 0.023 wall). TAM implants contained 2.5 mg TAM (in 17.5 mg of cholesterol) or 5 mg TAM (in 1.5 mg of cholesterol) in a silastic tube (0.062 inner diameter × 0.032 wall). The level of E_2 in these implants allows the tumors to grow at submaximal rate and permits the inhibition of E_2-stimulated MCF-7 tumor growth at these levels of TAM (12). Silastic tubing was used to release E_2 or TAM slowly. The E_2 and TAM implants were then placed in the i.p. region of athymic mice.

Analysis of Tumor Growth Induced by Genistein in Athymic Nude Mice. Four days after the E_2 and/or TAM implantation, E-dependent human breast cancer (MCF-7) cells (1×10^5 cells/40 μl/site) were injected into two sites on the flank of the mouse. MCF-7 cells were maintained and prepared as described in Ju *et al.* (11). Mice were divided into six treatment groups; C, E, 2.5 TE, 5 TE, 2.5 TEG, and 5 TEG (11–12 mice/group). Isoflavone-free American Institute of Nutrition 93 growth semipurified diet was used as a base diet for C, E, 2.5 TE, and 5 TE groups. Mice in 2.5 TEG and 5 TEG groups were fed American Institute of Nutrition 93 growth diet plus genistein (1000 ppm). During the study, tumor growth and body weight were monitored weekly, and feed intake was measured.

RNA Preparation and Analysis of Changes in Gene Expression Using RT-PCR. E responsive genes *pS2* and *PR*, and cell cycle-regulated gene *cyclin D1* mRNA expressions were analyzed using RT-PCR. RNA from frozen tumor (≤200 mg) was prepared as described in Ju *et al.* (11). cDNAs were generated using 10 ng of RNA and TaqMan Reverse Transcription Reagents (PE Applied Biosystem, Foster City, CA). The pS2, PR, and cyclin D1 primers and fluorescence (6-FAM)-labeled probes were designed using Primer and Probe Design Express (Applied Biosystems). The human GAPDH primers and

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[3] The abbreviations used are: TAM, tamoxifen; E, estrogen; C, control; 2.5 TE, E_2 implant + 2.5 mg TAM implant; 2.5 TEG, E_2 implant + 2.5 mg TAM implant + 1000 ppm genistein; 5 TE, E_2 implant + 5 mg TAM implant; 5 TEG, E_2 implant + 5 mg TAM implant + 1000 ppm genistein; LC-ES/MS/MS, liquid chromatography Electrospray/mass-spectrometry/mass-spectrometry; LOQ, limit of quantitation; ER, estrogen receptor; E_2, 17β-estradiol; PR, progesterone receptor; RT-PCR, reverse transcriptase-PCR; 4OH-TAM, 4-hydroxytamoxifen; HRT, hormone replacement therapy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ct, comparative threshold; COV, coefficient variation.

a fluorescent (6-FAM)-labeled probe were used as a control. PCR and analysis of PCR products were performed using the ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). Data were analyzed using a Ct cycle method (User Bulletin; PE Applied Biosystems). The parameter Ct was defined as the point at which the amplification plot, representing the fractional cycle number of fluorescence generated by cleavage of the probe (ΔRn), passed a fixed threshold above baseline. Ct was reported as the cycle number at this point. A comparative Ct method detected relative gene expression. Amplicons were run as triplicates in separate tubes to permit quantification of target genes normalized to a control, GAPDH.

E_2 Level in Plasma. E_2 level in plasma was measured using an Ultrasensitive Estradiol RIA kit and a company protocol (Dynamics System Laboratory, Webster, TX). E_2 in the plasma samples (50 μl) was extracted using toluene. The primary antibodies and [^{125}I]E_2 were used in 1:4 dilution of company protocol. Controls included plasma containing a low and a high concentration of E_2, 0.1% gel-PBS control, and a plasma blank plus a known amount of E_2 (for recovery). The sensitivity of this RIA is 1.4 pg/ml ($\sim 5 \times 10^{-12}$ M), and the interassay COV was 2–5%.

Genistein, TAM, and 4OH-TAM Levels in Plasma. Concentrations in plasma of total genistein, TAM, and its metabolite 4OH-TAM concentration was determined using validated isotope dilution LC-ES/MS/MS methods. Briefly, liquid-liquid extraction and LC with ES/MS/MS with multiple reaction monitoring detection were used to quantify TAM and 4OH-TAM.[4] The methods were highly sensitive with an LOQ <0.1 ng/ml from 10 μl of serum with acceptable accuracy/precision for TAM (COV 2–7%) and 4OH-TAM (COV 6–20%). For genistein, the LOQ was $<1 \times 10^{-9}$ M with a COV of 3–8% (13).

Statistics. Data from tumor area, RT-PCR, serum concentrations of E_2, TAM, and genistein, and feed intake were analyzed accordingly using one-way or repeated-measures ANOVA according to the characteristics of the data set using the SAS program. If the overall treatment *F*-ratio was significant ($P < 0.05$), the differences between treatment means were tested with Fisher's Least Significant Differences test.

Results

Effect of the Interaction of Genistein and TAM on MCF-7 Tumor Growth. The average cross-sectional tumor area of E group (E_2 silastic implant) reached 116.5 mm^2 16 weeks after E_2 implantation. Animals in the remaining treatment groups were terminated at 32 weeks. Average cross-sectional area of 2.5 TEG and 5 TEG groups were 75.1 mm^2 and 50.9 mm^2, respectively (Fig. 1*A*). Tumor sizes in 2.5 TE (14.4 mm^2) and 5 TE (13.7 mm^2) groups were not statistically different from that in the C (5.9 mm^2) group (Fig. 1*B*). Tumor areas from the 2.5 TEG (75.1 mm^2) and 5 TEG (50.9 mm^2) groups were significantly different from the C group ($P < 0.05$). Feed intake was measured during the study, and no significant difference was observed among any of the treatment groups (data not shown). Final body weights in 2.5 TE, 5 TE, 2.5 TEG, and 5TEG were significantly lower than those in C and E groups as has been observed by others in animals (14).

Effect of the Interaction of Genistein and TAM on E-responsive and Cell Cycle-regulated Gene Markers. Tumors with areas similar to the average tumor surface area of the 2.5 TEG, 5 TEG, or E group were used for mRNA analysis. The following E-responsive gene markers, pS2 and PR, and cell cycle-regulated gene marker, cyclin D1, were evaluated by quantitative RT-PCR using Taqman 7700 Sequence Detector System.

pS2. We observed a significant increase in pS2 expression from E_2 treatment (4.4× over the C) and that TAM treatment inhibited E_2-stimulated pS2 expression in 2.5 TE (1.5× over the C) and 5 TE (1.4×) groups (Fig. 2*A*). The addition of dietary genistein treatment increased pS2 expression in 2.5 TEG (3.3×) and 5 TEG (2.8×) groups ($P < 0.05$).

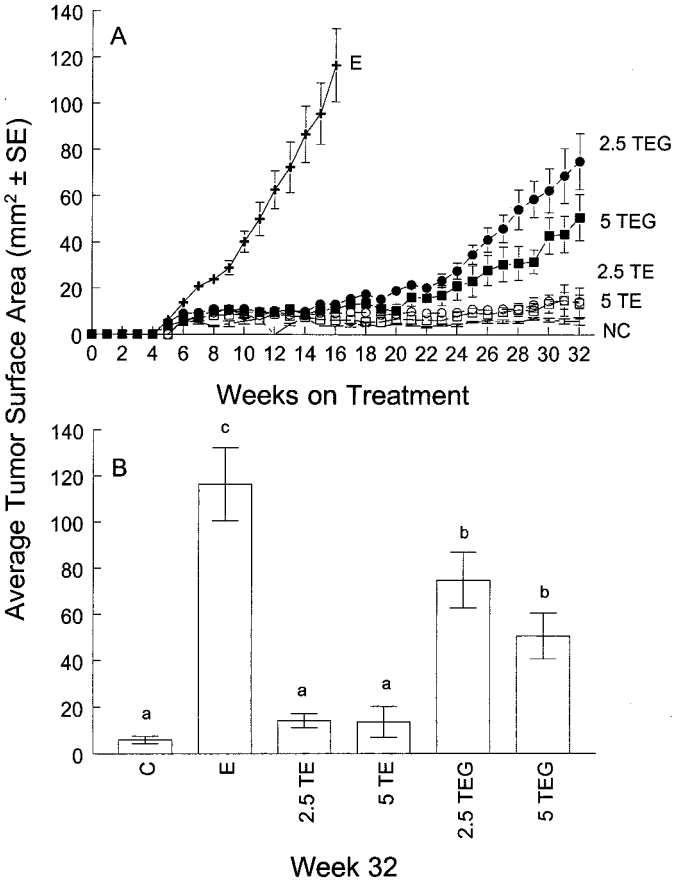

Fig. 1. *A*, the interaction of genistein and TAM on growth of E-dependent human breast cancer cells (MCF-7) implanted in ovariectomized athymic mice. At the time when E_2 and/or TAM implants and MCF-7 cells were placed into mice, animals were randomly assigned into six treatment groups: C (11 mice; $n = 9$ tumors), E (12 mice; $n = 24$ tumors), 2.5 TE (11 mice; $n = 20$ tumors), 5 TE (10 mice; $n = 12$ tumors), 2.5 TEG (11 mice; $n = 20$ tumors), and 5 TEG (11 mice; $n = 21$ tumors). Data are expressed as average cross sectional area (mm^2) for all tumors in each treatment; *bars*, ± SE. *B*, effect of the interaction of genistein and TAM on growth of MCF-7 tumors. Mice in the E group were terminated when tumors reached an average cross-sectional area of 116.5 (± 15.8) mm^2 16 weeks after E_2 implants were placed because of their high tumor burden. Mice in other treatment groups were terminated at 32 weeks. The average cross-sectional area of the 2.5 TEG treatment group was 75.1 (± 12.1) mm^2 and 50.9 (± 10.0) for the 5 TEG treatment groups 32 weeks after dietary genistein treatment was started. At the termination of the study, the average cross-sectional area of the C group reached 5.9 (± 1.6) mm^2, 2.5 TE treatment group reached 14.4 (± 3.0) mm^2, and 5 TE group reached 13.7 (± 6.7) mm^2. *Bars with different letters* are significantly different, $P < 0.05$; *bars*, ± SE.

PR. E_2 implantation increased expression of another E-responsive marker, PR (3.2× over C). TAM treatment also increased PR expression by 1.7× for 2.5 TE and by 1.8× for 5 TE group even although the increase observed was lower than that in E group ($P < 0.05$). Dietary genistein plus TAM treatment increased PR expression to a level similar to the E group (2.9× and 2.7× for 2.5 TEG and 5 TEG, respectively; Fig. 2*B; P* < 0.05).

Cyclin D1. E_2 implantation induced cyclin D1 expression (3.7× over the C), and TAM treatment inhibited E_2 stimulation by 1.24× (for 2.5 TE) and 1.28× (for 5 TE) to a level similar to C. Dietary genistein treatment increased cyclin D1 expression by 2.6× (for 2.5 TEG) and 2.2× (for 5 TEG; Fig. 2*C; P* < 0.05).

E_2, Total Genistein, and TAM/4OH-TAM Concentrations in Plasma. Table 1 shows the levels of E_2, TAM/4OH-TAM, and total genistein in the plasma. The average E_2 concentration for C group was 3.45×10^{-11} M. E_2 implantation elevated E_2 levels to 1.73×10^{-10} M in the E group. E_2 levels in 2.5 TE (1.72×10^{-10} M) and 5 TE (1.49×10^{-10} M) groups were not statistically different from that in the E group. Dietary genistein treatment significantly lowered E_2 level

[4] Twaddle, unpublished observations.

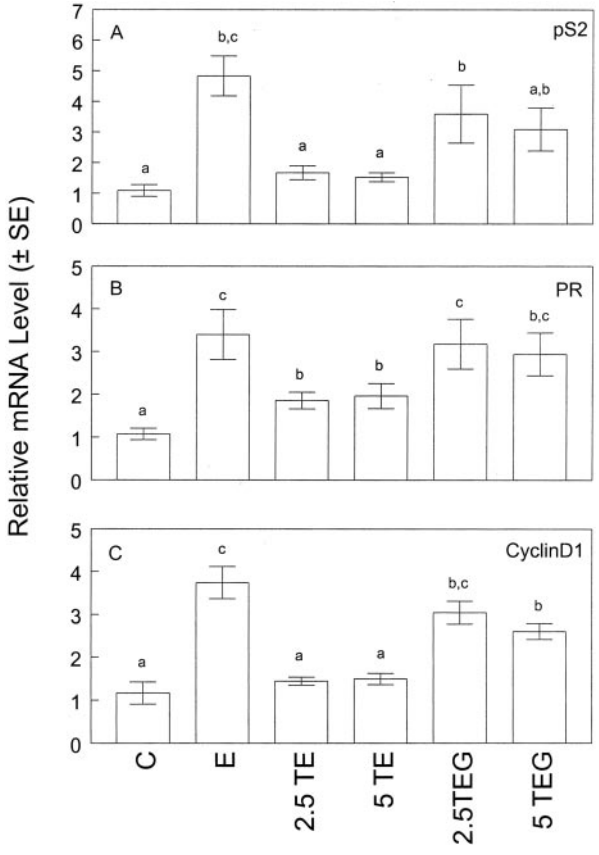

Fig. 2. Relative pS2, PR, and cyclin D1 mRNA levels. Six or seven tumors per treatment group were analyzed. mRNA expression levels were evaluated using RT-PCR, and numbers on the *Y* axis represent the relative mRNA level; *bars*, ± SE. GAPDH was used as a standard. *A*, pS2 expression; *B*, PR expression; and *C*, Cyclin D1 expression. *Bars with different letters* are significantly different, $P < 0.05$.

in 2.5 TEG (6.41×10^{-11} M) and 5 TEG (9.32×10^{-11} M) groups when compared with the level in the E or TE groups. Plasma TAM level of animals in 2.5 TE group was 2.06×10^{-9} M, 1.61×10^{-9} M for 5 TE group, 1.21×10^{-9} M for 2.5 TEG group, and 2.94×10^{-9} M for 5 TEG group, respectively. 4OH-TAM levels in the plasma were 14–18% of TAM.

The level of total genistein (aglycone + conjugated) was 5×10^{-6} M and 3.9×10^{-6} M for 2.5 TEG and 5 TEG, respectively, and was not statistically different. Less than 5% of total genistein was present as free genistein.

Discussion

Breast cancer is the most commonly diagnosed cancer among women in the United States. Breast cancer rates increase with age, and ~75% of breast cancer patients are over the age of 50. The majority of these cancer cases are E dependent, and it is likely that many of these women are being treated with TAM. During the period of perimenopause and postmenopause, many women experience one or more symptoms such as hot flashes, depression, mood swings, sleeping disorders, vaginal dryness, and joint pain, largely because of decreases of E and progesterone levels (15). TAM therapy is known to worsen these symptoms. HRT may relieve some of these symptoms, and HRT has also been shown to reduce the risk of osteoporosis and certain types of cancer. However, because of a concern for increased breast cancer risk, most oncologists do not recommend HRT to E-dependent breast cancer patients. As an alternative to HRT, some postmenopausal women may self-medicate with dietary supplements containing phytoestrogens without the knowledge of their physician.

Table 1 *E_2 genistein, TAM, and 4OH-TAM concentrations in plasma*

	E_2 ($\times 10^{-12}$ M ± SE)	TAM ($\times 10^{-9}$ M ± SE)	4OH-TAM ($\times 10^{-9}$ M ± SE)	Total genistein ($\times 10^{-6}$ M ± SE)
C	34.49 ± 1.9^{a}	ND	ND	ND
E	173.0 ± 36.5^{c}	ND	ND	ND
2.5 TE	171.8 ± 31.4^{c}	$2.1 \pm 0.5^{a,b}$	$0.40 \pm 0.09^{a,b}$	ND
5 TE	$148.8 \pm 44.2^{b,c}$	1.6 ± 0.2^{a}	0.30 ± 0.07^{a}	ND
2.5 TEG	64.1 ± 7.4^{a}	1.2 ± 0.2^{a}	0.29 ± 0.06^{a}	5.0 ± 0.85^{a}
5 TEG	$93.2 \pm 16.2^{a,b}$	2.9 ± 0.5^{b}	0.59 ± 0.09^{b}	3.9 ± 0.76^{a}

Numbers with different letters are significantly different, $P < 0.05$; ND, not detected.

Phytoestrogens are a diverse group of nonsteroidal plant compounds, such as isoflavones, lignans, and coumestans. Because they are structural mimics to E_2, they can bind to both ERα and β, albeit with weaker affinity than E_2, and act as E agonists (16).

Genistein is a well-documented E agonist, and in cultured cells physiologically relevant concentrations are sufficient to mediate ER agonism and reverse the inhibitory effects of 4OH-TAM on ER-responsive reporter genes (17). ERα is predominantly expressed in MCF-7 cells and in human breast tumors. Genistein binds to ERα and ERβ with the affinity of 4 and 87% that of E_2, respectively (18). Its induction of ER-dependent transcriptional expression characterizes genistein as an agonist for both ERα and ERβ (19, 20). A relevant plasma level of dietary genistein can transactivate both ERα and ERβ (18).

The studies reported here were designed to determine whether dietary genistein could negate/overwhelm the inhibitory effects of TAM on E-dependent tumor growth *in vivo*. To address this important issue we evaluated the interaction between genistein and TAM on MCF-7 tumor growth. The E_2 implants produced plasma levels in these mice that are in the range observed in postmenopausal women (21). Furthermore, these levels were sufficient to stimulate MCF-7 tumor growth (Fig. 1, *A and B*). TAM implants produced enough TAM and its metabolites in plasma (Table 1) to antagonize the stimulatory effect of E_2 on MCF-7 tumor growth without altering blood E_2 concentration (Fig. 1, *A* and *B*). In postmenopausal women, TAM treatment did not affect serum concentrations of E_2 or progesterone (22). Serum E_2 concentrations observed in postmenopausal women are ~1–2 $\times 10^{-10}$ M (21), and <5% of E_2 is free (*i.e.*, not bound to serum proteins). Concentrations of E_2 observed in breast tumors from postmenopausal women are ~10-fold higher than those seen in serum (23). Oral intake of 30 mg TAM/day produces up to 1.1×10^{-6} M of TAM and its metabolites in plasma (24), and <2% of TAM in women is free (not bound to serum proteins). In humans, concentrations of TAM and its metabolites in tissues were 10–60-fold higher than in serum (in rats, 8–70-fold higher than in serum; Ref. 25). In the breast tumors of women taking 40 mg TAM/day, concentrations of TAM and its metabolites were 0.67–14 $\times 10^{-9}$ M (21, 26).

We selected a dietary genistein concentration based on our previous study that produced estrogenic responses in mice (11). This level of dietary genistein (1000 ppm) produced ~4 $\times 10^{-6}$ M of total genistein in the plasma, a value well within the range of reported human exposures (27, 28). It is important to note that only aglycone genistein is estrogenically active, and the level in genistein-treated mice is ~2.4 $\times 10^{-7}$ M (≈6% of total) in this study. A recent study reported that in rats (given the 100 and 500 ppm genistein) the total genistein concentrations in endocrine-responsive tissues were higher than that observed in serum, and aglycone genistein in tissues was 10–100% of total genistein (1–5% of total in blood; Ref. 13). Of particular significance in this study is the observation that dietary genistein treatment produces sufficient aglycone genistein concentrations in the tumor tissue to overwhelm the inhibitory effects of TAM on tumor growth.

In addition, dietary genistein lowered blood E_2 levels. In humans,

modulation of E_2 circulating level by isoflavones has been reported but the mechanism responsible for these effects is unclear. It is important to note that even though genistein is weakly estrogenic, the estrogenic effects of genistein was still sufficient to negate/overwhelm the inhibitory effect of TAM and its metabolites on E-dependent MCF-7 tumor cell growth.

The interaction of the weak E agonist, genistein, with E_2 and TAM is complex. We have demonstrated that genistein can enhance pS2 expression in MCF-7 cells both *in vitro* (9) and *in vivo* (10, 11). Genistein can also reverse the inhibitory effects of 4OH-TAM on E_2-stimulated ER-mediated reporter gene activity *in vitro* (17). In the present study, pS2 and cyclin D1 mRNA expressions were enhanced by E_2 implantation (Fig. 2, *A* and *C*). TAM treatment inhibited E_2-induced pS2 and cyclin D1 expression. Dietary genistein was able to reverse the inhibitory effect of TAM on E-responsive pS2 (Fig. 2*A*) and the G_1 phase-regulated marker, cyclin D1 expressions. These data suggest that the increase in tumor growth is ER-mediated and that dietary genistein can reverse the blockade in cell cycle progression caused by TAM (Fig. 2*C*). PR expression was up-regulated by E_2 implants (Fig. 2*B*). TAM treatment (2.5 and 5 TE groups) reduced PR expression. However, dietary genistein in combination with TAM treatment increased PR expression. In summary dietary genistein can overwhelm the inhibitory effects of TAM on markers of E-dependent gene expression in E-dependent MCF-7 tumors *in vivo*.

Whereas 4OH-TAM has higher binding affinity to ERα than E_2, TAM, N-desmethylTAM, and genistein are ~20-fold lower (18). Under the conditions used in this study, the concentrations of TAM and its metabolite(s) are clearly sufficient to compete with E_2 binding to ERα. However, a weak E like genistein can also compete with TAM and its metabolites for binding to ERα. In this case, activation of ER-mediated processes occurs resulting in up-regulation of E-responsive and cell cycle progression-regulated gene expressions, and negation of the TAM inhibitory effect on MCF-7 tumor growth. It is also possible that other cellular mechanisms are involved (*e.g.*, ER-dependent actions on host cells). For example, dietary genistein may act through nontranscriptional pathways such as growth factors, protein tyrosine phosphorylation, and activation of the mitogen-activated protein kinase pathway. Although the primary mechanism of action of TAM is believed to be through the antagonism of ER-mediated processes, research over the years has indicated that other mechanisms exist (*e.g.*, modulation of signaling proteins such as protein kinase C, calmodulin, transforming growth factor β, and the proto-oncogene c-myc).

In summary, TAM suppressed E_2-stimulated MCF-7 tumor growth, and dietary intake of genistein negated the protective effect of TAM. Results from this study raise concerns about the consumption of dietary isoflavone supplements in conjunction with TAM therapy in postmenopausal women with E-dependent breast cancer. Additional studies will be needed to evaluate the specific concentration range of dietary genistein sufficient to overcome the inhibitory effect of TAM on tumor growth and to understand the molecular mechanisms of the interaction between genistein and TAM. Furthermore, understanding the mechanisms by which dietary genistein alters growth of E-dependent breast tumors will be important so that scientifically based recommendations can be made to women with E-dependent breast cancer regarding genistein consumption.

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