

Tamoxifen and Genistein Synergistically Down-Regulate Signal Transduction and Proliferation in Estrogen Receptor-Negative Human Breast Carcinoma MDA-MB-435 Cells

FEI SHEN^{1,2}, XINJIAN XUE¹ and GEORGE WEBER¹

¹Laboratory for Experimental Oncology and the ²Department of Obstetrics and Gynecology, Indiana University School of Medicine, 699 West Drive, Indianapolis, IN 46202-5119, U.S.A.

Abstract. *Purpose.* Tamoxifen and genistein were tested for synergism in estrogen receptor-negative human breast carcinoma MDA-MB-435 cells because the two compounds decrease signal transduction activity through different biochemical mechanisms and arrest the cell cycle at different phases. *Materials and Methods.* The combination effect of tamoxifen and genistein on signal transduction was determined by measuring IP₃ concentrations and on cell proliferation and colony formation by growth inhibition assay and clonogenic assay. *Results.* In growth inhibition assays, for tamoxifen and genistein in the carcinoma cells the IC₅₀s were (mean ± SE) 17 ± 0.9 and 27 ± 1.6 μM; in clonogenic assays the LC₅₀s were 0.9 ± 0.4 and 12.5 ± 1.1 μM, respectively. When tamoxifen and genistein were simultaneously added to the cells, synergism was observed in growth inhibition, in cytotoxicity and in the reduction of inositol 1,4,5-trisphosphate concentration. *Conclusion.* The synergistic down-regulation of signal transduction by tamoxifen and genistein may explain, in part at least, the synergistic antiproliferative and cytotoxic actions of the two compounds. The synergism of tamoxifen and genistein may be of interest in the clinical treatment of breast carcinoma.

Recently, tamoxifen was shown in this Laboratory to down-regulate signal transduction activity in a time- and dose-dependent fashion in ER⁻ human breast carcinoma MDA-

MB-435 cells (1-3). This new observation might be helpful in throwing light on the mechanism of action of tamoxifen in cancer cells.

Tamoxifen, a nonsteroidal antiestrogen used to prevent and treat breast carcinomas, was thought to act primarily through occupying the estrogen receptor sites in ER⁺ human breast carcinoma cells. However, the clinical outcome of the treatment showed that not all ER⁺ cancer patients responded to it and unexpectedly some ER⁻ patients responded well. Studies in vitro demonstrated that tamoxifen was cytotoxic not only to the ER⁺ breast carcinoma cells, but also to the ER⁻ breast carcinoma cells (4,5). This suggested that not only its antiestrogen property but also other possible mechanism(s) are involved in the anticancer action. The novel observation that tamoxifen down-regulates signal transduction provides a new understanding and possible novel targets in the treatment of human breast carcinoma. On the basis of earlier results in this Laboratory that tamoxifen is synergistic with tiazofurin, where the two drugs down-regulate signal transduction at different biochemical sites (3), we tested the interaction of tamoxifen with genistein, a natural isoflavone, also known to reduce signal transduction. Genistein possesses chemopreventive and chemotherapeutic properties (6,7) and its anticancer mechanisms are still under investigation. The hypothesis was tested that tamoxifen and genistein which act at different biochemical sites (8-10), in different phases of the cell cycle (7,11-13) (Figure 1), and both induce cytotoxicity (3,14), apoptosis and differentiation (5,12,13,15,16) (Figure 2) should be synergistic.

MDA-MB-435 human breast carcinoma cells were selected for study because they have elevated signal transduction activity (1) and, therefore, should be sensitive to tamoxifen and genistein. In this paper we report that tamoxifen and genistein exert synergism in these cells in reducing signal transduction activity and in acting as antiproliferative and cytotoxic agents.

Materials and Methods

Cell culture. ER⁻ MDA-MB-435 human breast carcinoma cells (4) were maintained in MEM (GIBCO, Grand, NY), cultured and incubated in

Abbreviations: ER⁻, estrogen receptor-negative; ER⁺, estrogen receptor-positive; IP₃, inositol 1,4,5-trisphosphate; IC₅₀, the drug concentration which inhibits cell proliferation by 50%; LC₅₀, the drug concentration which inhibits colony formation by 50%; MEM, minimum essential medium; PI, 1-phosphatidylinositol; PIP kinase, PI 4-phosphate 5-kinase (EC 2.7.1.68); PIP₂, PI 4,5-bisphosphate; C.I., combination index.

Correspondence to: George Weber, M.D., Laboratory for Experimental Oncology, Indiana University School of Medicine, 699 West Drive, Indianapolis, IN 46202-5119, U.S.A.

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5% CO₂:95% humidified air at 37°C as reported (3). Plateau phase cultures were seeded at a density of 2×10^5 cells/ml and allowed to proliferate for 48 to 72 h.

Drugs. Tamoxifen was purchased from the Sigma Chemical Co. (St. Louis, MO) and genistein from Indofinc Chemical Company, Inc. (Somerville, NJ). Stock solutions of tamoxifen and genistein were prepared, filtered, diluted with the MEM culture media and added to the cell culture to produce the desired drug concentrations (3,17).

Growth inhibition and clonogenic assays. Exponentially growing MDA-MB-435 cells were seeded for growth inhibition assays and clonogenic assays as reported (3). After 24 h of seeding, various concentrations of tamoxifen and genistein were added to the cells except in the control groups where only the corresponding cell culture media were added. The cells were also treated with tamoxifen and genistein simultaneously. For the growth inhibition assay cells were harvested after 3 days of drug exposure and counted in a Coulter Counter Model-ZM (Coulter Electronics Ltd., Luton, UK). Cytotoxicity was measured by clonogenic assays. After 7 days of drug treatment colonies were stained with 1% crystal violet and counted.

Radioassay of IP₃. Exponentially growing cells were harvested and seeded in tissue culture flasks. After 24 h of seeding, cells were treated with tamoxifen or genistein. Other groups of cells were treated with tamoxifen and genistein simultaneously. Controls received only the solvents for the drugs. After 3 days of drug exposure, cells were extracted and prepared for IP₃ assay as reported (18). The IP₃ concentration was determined with assay kits (DuPont NEN Kit, NEK064). The determination is based on the competition between unlabeled IP₃ and a fixed quantity of tritium-labeled IP₃ for a limited number of binding sites in a bovine adrenal binding protein preparation.

Evaluation of drug action. For evaluation of the interaction between tamoxifen and genistein on cell proliferation and colony formation the Chou-Talalay method was applied (19). Synergism in drug interaction is indicated by the C.I. of < 1. The Webb method was used for evaluation of changes in IP₃ concentration (20). Synergism is indicated by reduction of IP₃ to concentration <70% of the predicted reduction of IP₃ concentration. IP₃ concentration is expressed in pmol/mg protein. Data obtained from three or more experiments are also expressed as percentages of control. The interaction of drugs was also determined by constructing isobolograms based on dose responses (21). Results are statistically evaluated by the *t* test for small samples. Differences between means yielding a probability of < 5% are considered significant.

Results and Discussion

Antiproliferative action of tamoxifen and genistein in human breast carcinoma MDA-MB-435 cells. In the growth inhibition assay, dose-dependent antiproliferative effect was observed with IC₅₀s for tamoxifen and genistein of 17 ± 0.9 and 27 ± 1.6 μM, respectively (Figure 3). In clonogenic assay, dose-dependent cytotoxicity was seen with LC₅₀s of 0.9 ± 0.4 and 12.5 ± 1.1 μM, respectively (Figure 4). Therefore, in MDA-MB-435 cells tamoxifen is 1.6 and 13.9 times more effective than genistein in growth inhibition and clonogenic assays, respectively. Our results are in line with studies which reported that tamoxifen or genistein had antiproliferative effects in MDA-MB-435 cells (4,22,23). Our study has clinical relevance because the IC₅₀s reported here are achievable in the plasma of patients treated with tamoxifen (24) or in the

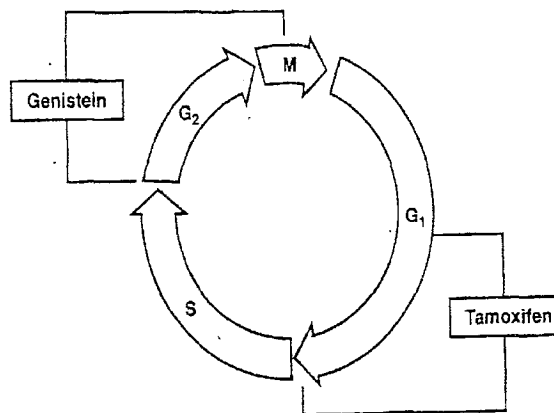


Figure 1. Attacking points of tamoxifen and genistein in the cell cycle.

range of human physiological plasma concentration of genistein (25).

Effect of combination of tamoxifen and genistein in MDA-MB-435 cells. Synergism in growth inhibition was obtained when tamoxifen (5 to 15 μM) was added simultaneously with genistein (5 to 15 μM) (Table I). The best results were obtained with 5 μM tamoxifen and 5 μM genistein at which concentrations the drug combination decreased cell proliferation to 44% and yielded a synergistic C.I. of 0.53. Synergistic action was also observed in clonogenic assays when tamoxifen (0.25 to 0.75 μM) was given simultaneously with genistein (2 to 10 μM) (Table II). Treatment for 7 days with individual drugs, tamoxifen (0.25 μM) and genistein (2 μM), reduced colony counts to 94% and 80%, respectively. The combination of the two drugs, at the above concentrations, synergistically depressed colony formation to 49% yielding a C.I. of 0.24. The drugs exerted a more pronounced synergistic interaction if either tamoxifen or genistein concentrations were increased. Synergistic interaction was also shown in the isobologram constructed from the clonogenic assay (Figure 5).

Action of tamoxifen and genistein on IP₃ concentration in MDA-MB-435 cells. Treatment for 3 days with either tamoxifen (5 μM) or genistein (5 μM) decreased IP₃ concentration in the cells only to 99%. However, treatment with the two drugs added simultaneously reduced IP₃ concentration to 61% indicating synergistic action (Table III). The synergism in the reduction of IP₃ concentration might explain, in part at least, the synergistic antiproliferative and cytotoxic interactions of the two drugs.

Tamoxifen and genistein attack different targets in signal transduction and different phases in the cell cycle. The signal transduction activity of the conversion of PI through PIP

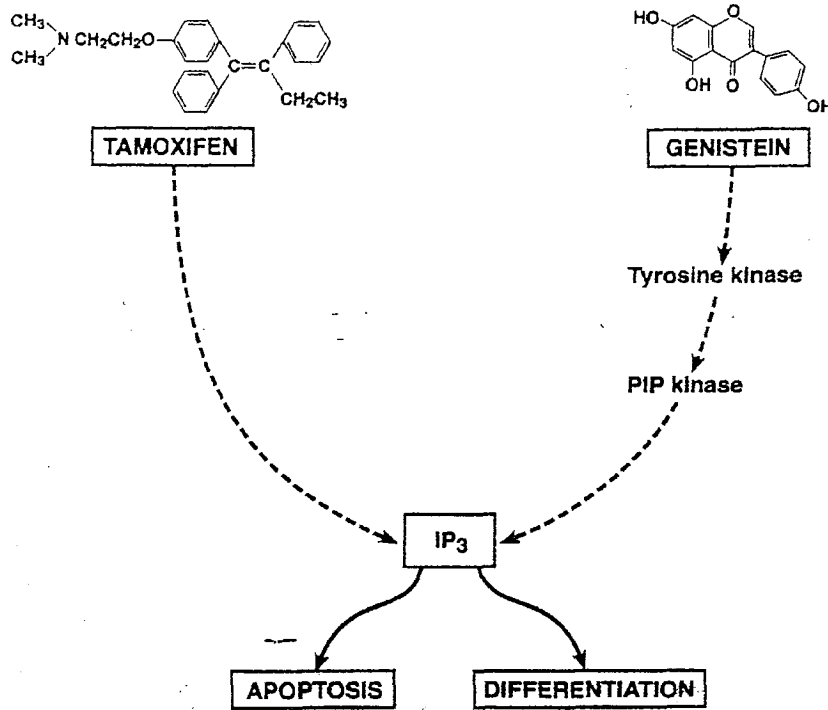


Figure 2. Hypothesis for the synergistic action of tamoxifen and genistein. The inhibition of tyrosine kinase and PIP kinase activities, the depression of IP₃ concentration by genistein, the reduction of IP₃ concentration by tamoxifen and the induction of apoptosis and differentiation by each of the drugs are reported observations. The hypothesis indicated in Figure 2 that the reduction of IP₃ levels is a required final common pathway needs further evidence.

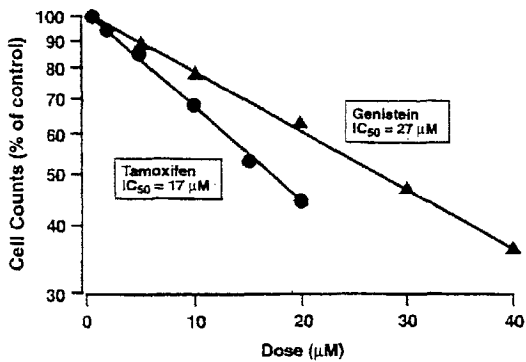


Figure 3. Antiproliferative effect of tamoxifen and genistein in MDA-MB-435 cells. Values are means of three or more experiments in triplicate. Standard errors were less than 10%.

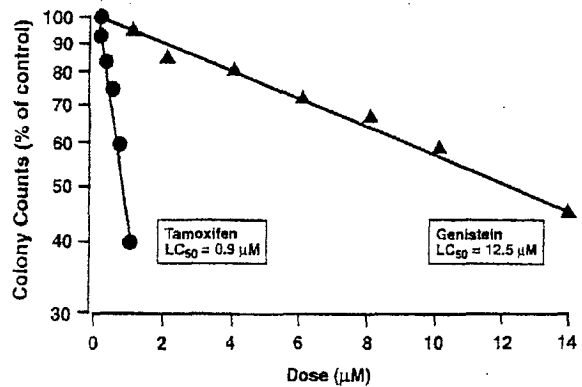


Figure 4. Cytotoxic effect of tamoxifen and genistein in MDA-MB-435 cells. Values are means of three or more experiments in triplicate.

and PIP₂ to second messengers, IP₃ and diacylglycerol, is linked with neoplastic transformation and progression (1,18,26,27). Higher IP₃ concentration was observed in all examined experimental and clinical samples of transformed cells (1,18,26-28). Our previous results indicate that breast

carcinoma MDA-MB-435 cells have higher PI kinase, PIP kinase activities and IP₃ concentration than normal breast HMEC cells (1,27). Therefore, the cells should be sensitive to drugs which lower signal transduction activity. Tamoxifen, an antiestrogen compound, has chemo-

Table III. Effect of combination of tamoxifen and genistein on IP₃ concentration in MDA-MB-435 cells.

Tamoxifen (μM)	Genistein (μM)	IP ₃ concentration		Predicted value	% observed of predicted
		Mean ± SE (pmol/mg protein)	% of control		
Control	Control	14.5 ± 0.51	100		
5		14.2 ± 0.19	99		
	5	14.2 ± 0.43	99		
5	5	8.9 ± 0.37	61 *	98	62

IP₃ assay was performed as described in Materials and Methods. Evaluation was done by Webb method (20). Observed reduction of IP₃ concentration < 70%, 70-100% and > 100% of the predicted reduction of IP₃ concentration indicates synergism, additive effect and antagonism, respectively.

*Significantly different from control ($p < 0.05$).

observed synergistic antiproliferative action and cytotoxicity of the two compounds. The synergism may also be attributed to their actions in different phases of the cell cycle. Tamoxifen attacked cells at early G₁ phase in ER⁺ MCF-7 human breast carcinoma cells (11) and ER⁻ A2780 human ovarian carcinoma cells (12). Genistein arrested cells at G₂ or early M phase in both ER⁻ and ER⁺ breast carcinoma cells (13).

The synergism of tamoxifen and genistein in ER⁻ human breast carcinoma MDA-MB-435 cells should be of interest for clinical treatment of breast carcinoma because the combination should allow the use of lower concentrations of tamoxifen, thus decreasing its possible adverse effects.

Novel aspects of this investigation include the following. 1. This is the first report on the combination of tamoxifen and genistein in the treatment of human breast carcinoma cells. These drugs were tested because of their different mechanisms of action on signal transduction and on the cell cycle. 2. IC₅₀s of the two compounds were in the range achievable in human plasma. 3. Synergistic growth inhibition and cytotoxicity were observed when tamoxifen and genistein were given simultaneously. This should make it possible to use lower concentrations of tamoxifen, thus decreasing its side effects. 4. Tamoxifen or genistein, as single agents, decreased IP₃ concentrations. The combination of tamoxifen and genistein yielded synergistic reduction of IP₃ concentration which may explain, in part at least, the synergistic antiproliferative and cytotoxic action of the two drugs. 5. This drug combination should be of interest in clinical treatment of breast carcinoma since it provides a new drug combination protocol for tamoxifen.

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References

- Weber G, Shen F, Yang H, Prajda N and Li W: Amplification of signal transduction capacity and down-regulation by drugs. *Advan Enzyme Regul* 39: Accepted, June, 1999.
- Weber G, Shen F, Yang H, Li W and Prajda N: Regulation of signal transduction in normal and cancer cells. *Anticancer Res*: Accepted, 1999.
- Shen F and Weber G: Tamoxifen down-regulates signal transduction and is synergistic with tiazofurin in human breast carcinoma MDA-MB-435 cells. *Oncol Res* 10: 325-331, 1998.
- Charlier C, Chariot A, Antoine N, Merville M, Gielen J and Castronovo V: Tamoxifen and its active metabolites inhibit growth of estrogen receptor-negative MDA-MB-435 cells. *Biochem Pharmacol* 49: 351-358; 1995.
- Perry RR, Kang Y and Greaves B: Effect of tamoxifen on growth and apoptosis of estrogen-dependent and -independent human breast cancer cells. *Ann Surgical Oncol* 2: 238-245, 1995.
- Kelloff GJ, Boone CW, Crowell JA, Steele VE, Lubet RA, Doody LA, Malone WF, Hawk ET and Sigman CC: New agents for chemoprevention. *J Cellular Biochem* 26: 1-28, 1996.
- Zhou JR, Mukherjee P, Gugger ET, Tanaka T, Blackburn GL and Clinton SK: Inhibition of murine bladder tumorigenesis by soy isoflavones via alterations in the cell cycle, apoptosis, and angiogenesis. *Cancer Res* 58: 5231-5238, 1998.
- Friedman ZY: The antitumor agent tamoxifen inhibits breakdown of polyphosphoinositides in GH4C₁ cells. *J Pharmacol Exp Ther* 271: 238-245, 1994.
- Cheng AL, Chuang SE, Fine RL, Yeh KH, Liao CM, Lay JD and Chen DS: Inhibition of the membrane translocation and activation of protein kinase C, and potentiation of doxorubicin-induced apoptosis of hepatocellular carcinoma cells by tamoxifen. *Biochem Pharmacol* 55: 523-531, 1998.
- Ozaki Y, Yatomi Y, Jinnai Y and Kume S: Effects of genistein, a tyrosine kinase inhibitor, on platelet function. Genistein attenuates thrombin-induced Ca²⁺ mobilization in human platelets by affecting polyphosphoinositide turnover. *Biochem Pharmacol* 46: 395-403, 1993.
- Osborne CK, Boldt DH, Clark GM and Trent JM: Effects of tamoxifen on human breast cancer cell cycle kinetics: Accumulation of cells in early G₁ phase. *Cancer Res* 43: 3583-3585, 1983.
- Ercoli M, Scambia G, Fattotossi A, Raspaglio G, Battaglia A, Cicchillitti L, Malorni W, Rainaldi G, Panici PB and Mancuso S: Comparative study on the induction of cytostasis and apoptosis by ICI 182,780 and tamoxifen in an estrogen receptor-negative ovarian cancer cell line. *Int J Cancer* 76: 47-54, 1998.
- Shao ZM, Alpaugh MI, Fontana JA and Barsky SH: Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by p21 WAF1/CIP1 induction, G₂/M arrest, and apoptosis. *J Cellular Biochem* 69: 44-54, 1998.
- Peterson G and Barnes S: Genistein inhibition of the growth of human breast cancer cells: Independence from estrogen receptors and the multi-drug resistance gene. *Biochem Biophys Res Commun* 179: 661-667, 1991.
- Macinga D, Jain V, Sizemore N, Gorodeski GI, Eckert RL and Rorke EA: Tamoxifen regulation of ectocervical cell differentiation. *J Soc Gynecol Invest* 2(6): 754-761, 1995.
- Constantinou A and Huberman E: Genistein as an inducer of tumor

- cell differentiation: Possible mechanisms of action (43841). Proc Soc Exptl Biol Med 208: 109-115, 1995.
- 17 Shen F and Weber G: Synergistic action of quercetin and genistein in human ovarian carcinoma cells. Oncol Res 9: 597-602, 1997.
 - 18 Singhal RL, Prajda N, Yeh Y and Weber G: 1-phosphatidylinositol 4-phosphate 5-kinase (EC 2.7.1.68): a proliferation- and malignancy-linked signal transduction enzyme. Cancer Res 54: 5574-5578, 1994.
 - 19 Chou TC and Talalay P: Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. Advan Enzyme Regul 22: 27-55, 1984.
 - 20 Webb JL: Enzyme and metabolic inhibitors. vol. 1. New York: Academic Press: 107-510, 1963.
 - 21 Berenbaum MC: Criteria for analyzing interaction between biological active agents. Advan Cancer Res 35: 269-335, 1981.
 - 22 Guthrie N, Gapor A, Chambers AF and Carroll KK: Inhibition of proliferation of estrogen receptor-negative MDA-MB-435 and positive MCF-7 human breast cancer cells by palm oil tocotrienols and tamoxifen, alone and in combination. J Nutr 127: 554s-548s, 1997.
 - 23 So FV, Guthrie N, Chambers AF, Moussa M and Carroll K: Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. Nutr Cancer 26: 167-181, 1996.
 - 24 Johnston SRD, Haynes BP, Sacks NPM, McKinna JA, Griggs LJ, Jarman M, Baum M, Smith IE and Dowsett M: Effect of estrogen receptor status and time on the intra-tumoral accumulation of tamoxifen and N-dimethyltamoxifen following short-term therapy in human primary breast cancer. Breast Cancer Res Treat 28: 241-250, 1993.
 - 25 Zava DT and Duwe G: Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. Nutr Cancer 27(1): 31-40, 1997.
 - 26 Weber G, Shen F, Prajda N, Li W, Csokay B, Olah E and Look KY: Regulation of signal transduction program by drugs. Advan Enzyme Regul 37: 35-55, 1997.
 - 27 Singhal RL, Yeh Y, Look KY, Sledge GW Jr and Weber G: Coordinated increase in activities of the signal transduction enzymes PI kinase and PIP kinase in human cancer cells. Life Sci 55: 1487-1492, 1994.
 - 28 Junghahn I, Bergmann J, Langen P, Thun I, Vollgraf C and Brachwitz H: Effect of ALP analogs on inositol trisphosphate formation in H184 mammary epithelial cells before and after transfection with v-erb B oncogene. Anticancer Res 15: 449-454, 1995.
 - 29 Peterson G: Evaluation of the biochemical targets of genistein in tumor cells. J Nutr 125: 784s-789s, 1995.

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