Pergamon

Biochemical Pharmacology, Vol. 47, No. 8, pp. 1449–1452, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(94)E0052-M

Antagonism between tamoxifen and doxorubicin in the MCF-7 human breast tumor cell line

(*Received* 27 *September* 1993; *accepted* 21 *December* 1993)

Abstract—Tamoxifen, an antiestrogen, and doxorubicin, an anthracycline antibiotic, are each utilized alone and in combination in the treatment of breast carcinoma. In view of conflicting reports relating to the interaction between these drugs, studies were undertaken to characterize the influence of tamoxifen on growth inhibition by doxorubicin in the MCF-7 breast tumor cell line *in vitro*. Studies combining 5 μM tamoxifen, a clinically relevant concentration, with various concentrations of doxorubicin, indicated that this drug combination produces antiproliferative effects that appear to be less than additive. Concentration-dependent growth inhibition was analyzed further using various concentrations of tamoxifen and doxorubicin by the combination index–isobologram method; this quantitative approach provided clear evidence of antagonism between these agents, a finding with potential relevance to the treatment of breast cancer.

Key words: doxorubicin; tamoxifen; MCF-7; breast cancer; antagonism

Tamoxifen is the drug of choice in the treatment of estrogen-receptor positive breast cancer [1, 2], while doxorubicin may be utilized after relapse in patients treated with tamoxifen or in estrogen-receptor negative tumors [3]. Although these drugs have been utilized in combination, the advantage of this combination, in terms of therapeutic efficacy, remains controversial [4].

In vitro studies using doxorubicin-sensitive, estrogen-receptor positive breast tumor cell lines have been reported to show: (i) a slightly additive interaction between tamoxifen and doxorubicin [5]; (ii) a lack of interaction [6]; or (iii) protection by tamoxifen from doxorubicin toxicity [7]. Consequently, it appeared to be of importance to further evaluate this interaction, which could be of clinical relevance in the treatment of breast carcinoma.

Materials and Methods

Doxorubicin was purchased from the Sigma Chemical Co., St. Louis, MO, maintained as a frozen stock solution in distilled water, and protected from light [8]. Tamoxifen citrate (Sigma) was dissolved as a stock solution in 95% ethanol. The final concentration of ethanol for experimental conditions using 5 μM tamoxifen was 0.05%. MTT*, used in the cellular proliferation assay, was obtained from the Sigma Chemical Co.

MCF-7 human breast cancer cells were provided by Dr. Kenneth H. Cowan of the National Cancer Institute, National Institutes of Health, Bethesda, MD. Cells were maintained in high glucose Dulbecco's minimal essential medium (Hazelton Biologics, Lenexa, KS) supplemented with 5% fetal bovine serum (Life Technologies, Grand Island, NY) and 5% defined bovine serum (Hyclone Laboratories, Logan, UT). The medium also contained glutamine (29.2 mg/100 mL) and penicillin/streptomycin (0.5 mg/100 mL).

While some laboratories utilize charcoal-stripped medium to eliminate the estrogenic effects of the pH indicator, phenol red [9, 10], previous studies have demonstrated that the responsiveness of MCF-7 breast tumor cells to tamoxifen is reduced in charcoal-stripped medium [11]. Consequently, the studies in this manuscript utilize "regular" medium in order to simulate a physiological milieu, where breast tumor cells are exposed to estradiol.

Cellular proliferation studies. The effects of tamoxifen and doxorubicin on cellular proliferation were monitored using a modification of the MTT tetrazolium dye assay [12–14], as described previously [15]. All growth studies were performed utilizing a continuous (72 hr) exposure to drug. The IC_{50} values generated using this type of analysis are comparable with those using cytotoxicity (clonogenic) assays [13, 14].

Intracellular accumulation of doxorubicin. Cellular doxorubicin levels were assessed using high performance liquid chromatography, as previously described [16, 17]. This methodological approach also serves to monitor drug metabolism [16, 18].

Ribonuclease protection assay. Expression of mRNA for the estrogen receptor was determined by the RNase protection assay [19] where 0.001 mg of pGEM-4Z-HEO plasmid DNA linearized with *Eco*RI was utilized in standard *in vitro* transcription reactions with [α-^{32}P]GTP, RNasin, and T7 RNA polymerase, respectively. RNase digestion of the non-hybridized sequences followed, and protected RNA was recovered by phenol/choroform extraction and ethanol precipitation.

Analysis of data. Analysis of statistical data was performed using the unpaired Student's *t*-test. Effects resulting in P values of 0.05 or below were considered to be statistically significant. For growth analysis and intracellular doxorubicin accumulation, values are expressed as means ± SEM, or means ± range for experiments with two replicates. The IC_{50} values were calculated by graphic extrapolation.

Computer software [20, 21] based on the median-effect principle and the combination index-isobologram equations [22, 23] was used for analysis of the interaction(s) between tamoxifen and doxorubicin at a 50:1 ratio.

Results and Discussion

Competitive radioligand binding studies [24] have established the presence of the estrogen receptor in MCF-7 breast tumor cells. Prior to evaluating the interaction of tamoxifen with doxorubicin in MCF-7 cells, expression of message for the estrogen receptor was confirmed by RNase protection (not shown).

MCF-7 breast tumor cells were exposed to 5 μM tamoxifen for 24 hr prior to doxorubicin as well as during the subsequent 48-hr incubation with doxorubicin. Exposure

* Abbreviations: IC_{50} drug concentration inhibiting 50% of cellular growth; and MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

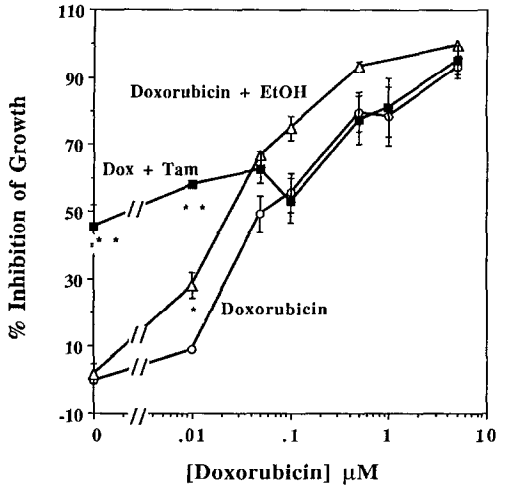

Fig. 1. Interaction between the antiproliferative activities of tamoxifen and doxorubicin in MCF-7 breast tumor cells. Cells in logarithmic growth were pretreated with 5 μM tamoxifen or 0.05% ethanol for 24 hr. At this time, cells were treated with various concentrations of doxorubicin (0.01 to 5.0 μM) with or without a simultaneous incubation with 5 μM tamoxifen or 0.05% ethanol, respectively. The number of viable cells was determined after 72 hr utilizing the MTT tetrazolium dye assay as described in Materials and Methods. The data presented are mean values for percent inhibition of growth ± SEM (as compared with untreated control) or as mean ± range (when only two replicate experiments were performed) for 2–15 individual experiments. Key: (*) significantly different from doxorubicin alone at $P \leq 0.01$, as determined by Student's *t*-test; (**) different from doxorubicin alone at $P \leq 0.001$, as determined by Student's *t*-test; (○) doxorubicin alone; (△) doxorubicin + ethanol; and (■) doxorubicin + tamoxifen.

of MCF-7 cells to 5 μM tamoxifen alone for 72 hr resulted in a 45.6 ± 6.3% inhibition of growth (Fig. 1 at 0 concentration of doxorubicin). Figure 1 further suggests that the combination of tamoxifen and doxorubicin produced less than additive effects on MCF-7 cell growth. At doxorubicin concentrations of 0.05 and 0.1 μM, growth inhibition never significantly exceeded that produced by tamoxifen alone, despite the fact that doxorubicin at these concentrations inhibited growth by 49.1 ± 5.5 and 55.6 ± 5.8%, respectively. At concentrations of 0.05 μM doxorubicin and above, growth inhibition by the combination of tamoxifen and doxorubicin was essentially identical to that produced by doxorubicin alone. (Ethanol, the vehicle in which the tamoxifen was dissolved, at a final concentration of 0.05%, demonstrated minimal effects on the growth of MCF-7 cells, although a small but significant increase in growth inhibition was observed at 0.01 μM doxorubicin.)

Drug accumulation studies failed to show significant differences in intracellular doxorubicin levels in cells exposed to doxorubicin alone or in the presence of tamoxifen (Table 1). High pressure liquid chromatographic analysis of drug extracted from MCF-7 cells after 2 hr of incubation indicated the absence of any doxorubicin metabolites (data not shown).

To perform a more quantitative evaluation of the interaction between tamoxifen and doxorubicin, MCF-7 cells were exposed to various concentrations of tamoxifen alone, doxorubicin alone, or to tamoxifen + doxorubicin in a 50:1 ratio, and growth inhibition was analyzed using the dose–effect analysis program developed by Chou and Talalay [22, 23]. Figure 2 presents a computer extrapolation of the combination index–dose–effect relationship (based on data

Table 1. Lack of effect of 5 μM tamoxifen on the intracellular accumulation of doxorubicin in MCF-7 breast tumor cells

	Intracellular doxorubicin concentration (pmol/10^6 cells)	
Doxorubicin [μM]	Doxorubicin alone	Doxorubicin and tamoxifen
5	1222 ± 167 (4)	1194 ± 154 (4)
10	2098 ± 751 (4)	2807 ± 721 (3)

Cells in logarithmic growth were grown in medium alone or in the presence of 5 μM tamoxifen for 24 hr. At this time, cells were incubated for 2 hr with doxorubicin at concentrations of 5 and 10 μM with or without a simultaneous incubation with 5 μM tamoxifen. Following drug treatments, cells were harvested and intracellular doxorubicin concentrations were determined as described under Materials and Methods. Results were normalized for cellular protein content. The data presented are means ± SEM for the number of replicate experiments indicated in parentheses. No significant differences in doxorubicin accumulation were noted between cells exposed to doxorubicin alone or those exposed to the combination of doxorubicin and tamoxifen.

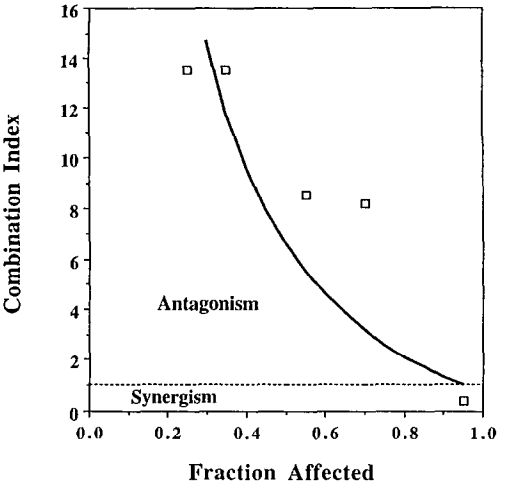

Fig. 2. Fractional inhibition of growth–combination index plot for the combination of tamoxifen + doxorubicin at a 50:1 ratio. Symbols indicate the actual combination data points obtained when MCF-7 human breast tumor cells were exposed to tamoxifen (for 24 hr) followed by doxorubicin for an additional 48 hr. The total incubation period was 72 hr. The interrupted line is derived from a computer simulation of this drug combination assuming that the actions of the two drugs are mutually non-exclusive. A more extensive discussion of this approach to analysis of drug interaction is found in Ref. 25.

derived from a representative experiment), which indicates that the combination of tamoxifen + doxorubicin was antagonistic over virtually the entire range of growth inhibition (up to approximately 95% growth inhibition). This conclusion is based on the fact that the combination index is greater than one over the entire range [20, 21]. Also shown are calculated combination index values for the five experimental conditions where tamoxifen + doxorubicin were combined at a ratio of 50:1 (from which the extrapolated curve was derived).

The basis for antagonism at the lower range of concentrations of doxorubicin may be related to the capacity of tamoxifen to decrease growth of estrogen-receptor positive MCF-7 cells, and to stabilize these cells in the G_0–G_1 phase of the cell cycle [26, 27], resulting in decreased sensitivity to cytotoxic drugs [28]. In this context, DNA synthesis inhibition has been shown to protect against cytotoxicity mediated by topoisomerase II inhibitors such as doxorubicin [29]. Antagonism at elevated concentrations of doxorubicin may be related to the findings of Clarke *et al.* [30], indicating a reduction in estrogen binding capacity in MCF-7 cells following a 24-hr exposure to 0.018 to 1.8 μM doxorubicin, presumably due to a decrease in the rate of estrogen-receptor cycling or its synthesis. In this case, the presence of doxorubicin would serve to abrogate any antiproliferative effects of the antiestrogen tamoxifen. Consequently, both cell cycle arrest and alterations in estrogen-receptor content may contribute to antagonism between doxorubicin and tamoxifen over a relatively wide range of doxorubicin concentrations.

Although tamoxifen has been shown to increase the effectiveness of doxorubicin against multidrug-resistant breast tumor cells *in vitro* [6], the fact that the combination of tamoxifen and doxorubicin in MCF-7 cells is antagonistic may contribute to the observation that the utilization of conventional doses of these agents in combination has not demonstrated markedly greater clinical utility than either drug administered alone [4]. However, it should be noted that a number of different drugs are used in these combination regimens, and that other factors such as the extent of hormone dependency, innate drug resistance, and the clinical pharmacokinetics of the administered drugs may also influence the ultimate response to antitumor drugs in combination regimens.

Departments of Pharmacology/Toxicology and Medicine
Medical College of Virginia
Richmond, VA 23298, U.S.A.

KAREN E. WOODS
JOYCE K. RANDOLPH
DAVID A. GEWIRTZ*

REFERENCES

1. Jordan VC and Murphy CS, Endocrine pharmacology of antiestrogens as antitumor agents. *Endocr Rev* **11**: 578–610, 1990.
2. Jordan VC, Long-term tamoxifen: Balancing benefits and risks. *Contemp Oncol* **3**: 26–33, 1991.
3. Henderson IC and Canellos GP, Cancer of the breast, the past decade. *New Engl J Med* **302**: 78–90, 1980.
4. Early Breast Cancer Trialists' Collaborative Group, Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy: 133 randomized trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet* **339**: 71–85, 1992.
5. Osborne CK, Kitten L and Artega CL, Antagonism of chemotherapy-induced cytotoxicity for human breast cancer cells by antiestrogens. *J Clin Oncol* **7**: 710–717, 1989.
6. Foster BJ, Grotzinger KR, McKoy WM, Rubinstein LV and Hamilton TC, Modulation of induced resistance to Adriamycin in two human breast cancer cell lines with tamoxifen or perhexiline maleate. *Cancer Chemother Pharmacol* **22**: 147–152, 1988.
7. Hug V, Hortobagyi GN, Drewinko B and Finders M, Tamoxifen-citrate counteracts the antitumor effects of cytotoxic drugs *in vitro*. *J Clin Oncol* **3**: 1672–1677, 1985.
8. Bosanquet AG, Stability of solutions of antineoplastic agents during preparation and storage for *in vitro* assays. *Cancer Chemother Pharmacol* **17**: 1–10, 1986.
9. Sapino A, Pietribiasi F, Bussolati G and Pier CM, Estrogen- and tamoxifen-induced rearrangement of cytoskeletal and adhesion structures in breast cancer MCF-7 cells. *Cancer Res* **46**: 2526–2531, 1986.
10. Wilding G, Lippman ME and Gelmann EP, Effects of steroid hormones and peptide growth factors on protooncogene c-*fos* expression in human breast cancer cells. *Cancer Res* **48**: 802–805, 1988.
11. Katzenellenbogen BS, Norman MJ, Eckert RL, Peltz SW and Mangel WF, Bioactivities, estrogen receptor interactions, and plasminogen activator-inducing activities of tamoxifen and hydroxytamoxifen isomers in MCF-7 human breast cancer cells. *Cancer Res* **44**: 112–119, 1984.
12. Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**: 55–63, 1983.
13. Carmichael J, DeGraff WG, Gazdar AF, Minna JD and Mitchell JB, Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemo-sensitivity testing. *Cancer Res* **47**: 936–942, 1987.
14. Vistica DT, Skehan P, Scudiero D, Monks A, Pittman A and Boyd MR, Tetrazolium-based assays for cellular viability: A critical examination of selected parameters affecting formazan production. *Cancer Res* **51**: 2515–2520, 1991.
15. Munger C, Ellis A, Woods K, Randolph J, Yanovich S and Gewirtz D, Evidence for inhibition of growth related to compromised DNA synthesis in the interaction of daunorubicin with H-35 rat hepatoma. *Cancer Res* **48**: 2404–2411, 1988.
16. Gewirtz DA and Yanovich S, Metabolism of the anthracycline antibiotic daunorubicin to daunorubicinol and deoxydaunorubicinol aglycone in hepatocytes isolated from the rat and the rabbit. *Biochem Pharmacol* **35**: 4059–4064, 1986.
17. Woods KE, Ellis AL, Randolph JK and Gewirtz DA, Enhanced sensitivity of the rat hepatoma cell to the daunorubicin analogue 4-demethoxydaunorubicin associated with induction of DNA damage. *Cancer Res* **49**: 4846–4851, 1989.
18. Gewirtz DA and Yanovich S, Metabolism of Adriamycin in hepatocytes isolated from the rat and the rabbit. *Biochem Pharmacol* **36**: 1793–1798, 1987.
19. Gilman M, Ribonuclease protection assay. In: *Current Protocols in Molecular Biology* (Eds. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K), pp. 4.7.1–4.7.8. John Wiley, New York, 1987.
20. Chou J and Chou T-C, *Dose–effect analysis with microcomputers: Quantitation of ED_{50}, LD_{50}, synergism, antagonism, low dose risk, receptor-ligand binding and enzyme kinetics*. Manual and Software. Biosoft, Cambridge, U.K. 1987.
21. Chou J, Quantitation of synergism and antagonism of two or more drugs by computerized analysis. In: *Synergism and Antagonism in Chemotherapy* (Eds.

* Coresponding author: Dr. David A. Gewirtz, Department of Medicine, Medical College of Virginia, Box 230, MCV Station, Richmond, VA 23298. Tel. (804) 786-9523; FAX (804) 371-8079.

Chou T-C and Rideout DC), pp. 223–244. Academic Press, New York, 1991.
22. Chou T-C and Talalay P, Generalized equations for the analysis of inhibitors of Michaelis–Menten and higher order kinetic systems with two or more mutually exclusive or nonexclusive inhibitors. *Eur J Biochem* **115**: 207–216, 1981.
23. Chou T-C, The median effect principle and the combination index for quantitation of synergism and antagonism. In: *Synergism and Antagonism in Chemotherapy* (Eds. Chou T-C and Rideout DC), pp. 61–102. Academic Press, New York, 1991.
24. Vickers PJ, Dufresne MJ and Cowan KH, Relation between cytochrome P450-Ia1 expression and estrogen receptor content in human breast cancer cells. *Mol Endocrinol* **1**: 157–164, 1989.
25. Chou T-C, Tan Q-H and Sirotnak FM, Quantitation of the synergistic interaction of ederate and cisplatin *in vitro*. *Cancer Chemother Pharmacol* **31**: 259–264, 1993.
26. Taylor IW, Hodson PJ, Green MD and Sutherland RL, Effects of tamoxifen on cell cycle progression of synchronous MCF-7 human mammary carcinoma cells. *Cancer Res* **43**: 4007–4010, 1983.
27. Sutherland RL, Green MD, Hall RE, Reddie RG and Taylor IW, Tamoxifen induces accumulation of MCF7 human mammary carcinoma cells in the G_0/G_1 phase of the cell cycle. *Eur J Cancer Clin Oncol* **19**: 615–621, 1983.
28. Koa WYJ and Collins JL, A rapid *in vitro* screening system for the identification and evaluation of anticancer drugs.*Cancer Invest* **7**: 303–311, 1989.
29. Holm C, Covey JM, Kerrigan D, Kohn KW and Pommier Y, Protection by DNA synthesis inhibition against cell killing by topoisomerase blocking drugs. In: *DNA Topoisomerases in Cancer* (Eds. Potmesil and Kohn K), pp. 161–171. Oxford University Press, New York, 1991.
30. Clarke R, Morwood J, van den Berg HW, Nelson J and Murphy RF, Effect of cytotoxic drugs on estrogen receptor expression and response to tamoxifen in MCF-7 cells. *Cancer Res* **46**: 6116–6119, 1986.