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POTENTIATION OF TAMOXIFEN ACTIVITY BY VERAPAMIL IN A HUMAN BREAST CANCER CELL LINE

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Abstract—The tumor cell growth inhibitory activity of tamoxifen was enhanced significantly by verapamil treatment in an estrogen receptor positive human breast cancer cell line, MCF-7. Treatment of MCF-7 cells with 5 and 10 μg/mL verapamil produced a 1.8- and 2.8-fold increase, respectively, in tamoxifen activity. Unlike reversal of multi-drug resistance, the verapamil-mediated increase in tamoxifen activity was not associated with enhanced drug accumulation. Tamoxifen treatment alone or in combination with verapamil did not affect the activity of protein kinase C, an enzyme implicated in the anti-tumor activity of tamoxifen. Addition of 17β-estradiol in the cell survival assay system partially abrogated the modulatory effect of verapamil. These data suggest that potentiation of tamoxifen activity by verapamil may involve interaction of this agent with the estrogen receptor. In conclusion, potentiation of tamoxifen activity by calcium channel blockers represents a novel approach for improving the therapeutic results with tamoxifen in women with breast cancer.

Key words: tamoxifen; breast cancer; verapamil

Tamoxifen, a nonsteroidal antiestrogen, is the endocrine treatment of choice for metastatic breast cancer. Tamoxifen is effective in 50–60% of women with estrogen receptor positive metastatic breast cancer for a median duration of 1 year [1, 2]. This implies that 40–50% of the women do not respond to tamoxifen therapy, despite the presence of estrogen receptors. Most women who do respond eventually develop clinical resistance to this drug [3]. A small fraction of women with estrogen receptor negative metastatic breast cancer may also respond to tamoxifen [1]. Tamoxifen is useful in prolonging both disease-free and overall survival when administered as adjuvant therapy independent of the hormone receptor status [4, 5]. However, this benefit is observed in only one-third of the breast cancer patients [4]. A similar number of women have a decreased risk for the development of contralateral breast cancer with adjuvant tamoxifen therapy [4].

Although the primary mechanism of action of tamoxifen and other antiestrogens involves the inhibition of estrogen-stimulated tumor growth by blocking estrogen receptors [6, 7], several other mechanisms have also been proposed to account for the success or failure of tamoxifen therapy in both estrogen receptor positive and negative patients. These mechanisms include (i) effects of tamoxifen in modulating the levels of growth inhibitory/growth stimulatory factors for breast cancer [8–10], (ii) binding of tamoxifen with high-affinity microsomal binding proteins [11], (iii) inhibition of calcium influx and competition for calcium channel antagonist binding sites [12], and (iv) inhibition of protein kinase C activity [13]. However, the pharmacological and clinical significance of these observations remains to be established.

Therefore, studies that identify methods and mechanisms by which tamoxifen activity can be enhanced in breast cancer would have enormous clinical implications. In this paper, we report that verapamil, a calcium channel blocker and modifier of chemotherapeutic drug resistance [14], significantly increased the activity of tamoxifen in an estrogen receptor positive human breast cancer cell line, MCF-7. This cell line has been used extensively to elucidate the mechanism of action of tamoxifen and other hormones. The results of this study also suggest that the potentiation of tamoxifen activity by verapamil may involve interaction of the modulator with the estrogen receptor.

MATERIALS AND METHODS

Chemicals. Tamoxifen citrate and verapamil were purchased from the Sigma Chemical Co., St. Louis, MO. [^{3}H]Tamoxifen (sp. act. 85.7 Ci/mmol) was obtained from the Amersham Corp., Arlington Heights, IL. α-MEM and fetal bovine serum were purchased from Gibco Laboratories, Grand Island, NY, and Hyclone Laboratories Inc., Logan, UT, respectively.

Cell line. The MCF-7 human breast cancer cell line was provided by Dr. K. Cowan, National Cancer Institute, Bethesda, MD. Monolayer cultures of MCF-7 cells were maintained in phenol red containing α-MEM medium supplemented with 5% fetal bovine serum and penicillin/streptomycin.

Cell survival assay. Exponentially growing MCF-7 cells (4×10^3) were seeded in 5 mL of the complete medium and exposed to different concentrations of tamoxifen dissolved in ethanol. The final concentration of ethanol, <0.3%, did not affect

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colony formation by MCF-7 cells. After gassing with 5% CO_2 and air, the flasks were incubated at 37° for 9–10 days. Subsequently, the colonies were fixed with 10% formalin and counted under an inverted microscope. To determine the effect of verapamil on tamoxifen cytotoxicity, cells were incubated with both agents during the colony formation assay. To determine the effect of 17β-estradiol on the modulatory activity of verapamil, all three agents were added simultaneously. The same batch of fetal bovine serum was used in all experiments, and the concentration of endogenous 17β-estradiol in the cell culture medium was 0.33 pmol/mL.

Cellular accumulation of tamoxifen. Log phase cultures of MCF-7 cells were treated with 1 μg/mL [^{3}H]tamoxifen in the absence or presence of 10 μg/mL verapamil at 37°. After specified time intervals, the cells were washed twice with ice-cold saline. The cell pellet was digested with 0.5 M Solvable solution (NEN Research Products, Boston, MA), and the radioactivity was determined by liquid scintillation counting.

Efflux of tamoxifen. MCF-7 cells were incubated with 1 μg/mL [^{3}H]tamoxifen in the absence or presence of 10 μg/mL verapamil for 1 hr at 37°. Cells were washed twice with ice-cold medium. The cell pellet was resuspended in fresh complete medium and incubated further at 37° for 30, 60 or 90 min. Subsequently, the cells were washed twice with ice-cold saline and processed for radioactivity determination as described above.

Protein kinase C assay. Log phase MCF-7 cells were treated with 3 μg/mL tamoxifen in the absence or presence of 10 μg/mL verapamil for 24, 48 and 72 hr at 37°. After specified time intervals, the cells were trypsinized and washed twice with phosphate-buffered saline. The cells were then suspended in lysis buffer [20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.33 M sucrose]. The cells were sonicated, and the cell extract was centrifuged at 100,000 *g* for 1 hr. Calcium-dependent protein kinase C activity was measured by the procedure described by Kitano *et al.* [15] using histone III as a substrate. Protein content was determined by the method of Bradford [16]. Enzyme activity is expressed as picomoles [^{32}P]ATP hydrolyzed per minute per microgram of protein.

RESULTS AND DISCUSSION

Figure 1 shows the effect of verapamil on growth inhibition of MCF-7 cells by tamoxifen. The IC_{50} value for tamoxifen in the MCF-7 cell line was 2.11 ± 0.30 μg/mL. The IC_{50} of tamoxifen in serum-containing medium observed in the present study was comparable with those values reported previously [17]. Treatment of MCF-7 cells with 5 or 10 μg/mL verapamil alone did not affect the colony-forming ability of these cells. Verapamil treatment produced a significant increase in tamoxifen activity in a concentration-dependent manner (Fig. 1). Treatment of MCF-7 cells with 5 and 10 μg/mL verapamil caused a 1.8- and 2.8-fold increase, respectively, in the tumor cell growth inhibitory activity of tamoxifen ($P < 0.005$). The IC_{50} values for tamoxifen in the presence of 5 and 10 μg/mL verapamil were 1.18 ± 0.15 and 0.76 ± 0.27 μg/mL, respectively. Lower concentrations of verapamil (0.1, 0.5, or 1.0 μg/mL) did not affect the activity of tamoxifen (data not shown). The potentiation of tamoxifen activity by the relatively less toxic analog *R*-verapamil [18] was similar to that of racemic verapamil (data not shown).

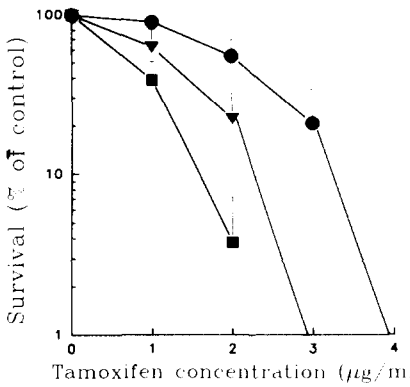

Fig. 1. Effect of verapamil on MCF-7 cell growth inhibition by tamoxifen. Key: survival of MCF-7 cells exposed to tamoxifen alone (●); tamoxifen + 5 μg/mL verapamil (▼); and tamoxifen + 10 μg/mL verapamil (■). Values are means ± SD of at least three independent experiments. The plating efficiency of the MCF-7 cell line was about 6%.

Verapamil has been shown to increase the cytotoxicity of certain cancer chemotherapy drugs in cells with a multi-drug resistance phenotype by enhancing drug retention through inhibition of P-glycoprotein-mediated rapid drug efflux [14]. To determine whether verapamil-mediated potentiation of tamoxifen activity was associated with increased drug retention, tamoxifen accumulation was determined in the absence and presence of 10 μg/mL verapamil (Fig. 2). The cellular accumulation of tamoxifen following a 10-, 20- or 60-min incubation

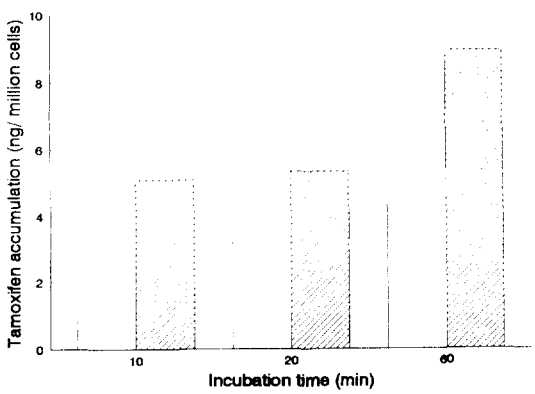

Fig. 2. Effect of verapamil on cellular accumulation of tamoxifen in MCF-7 cells. Key: tamoxifen retention in the absence of verapamil (□) and in the presence of 10 μg/mL verapamil (▨). Values represent averages of two independent experiments.

Table 1. Effect of 17β-estradiol on verapamil-mediated increase in tamoxifen activity

17β-Estradiol (μM)	Cell survival* (% of control) + 5 μg/mL Verapamil	+ 10 μg/mL Verapamil
None	50.6 ± 8.0†	17.6 ± 4.6
0.01	63.7 ± 12.8	35.6 ± 7.4
0.1	74.5 ± 8.2	54.8 ± 5.1
1.0	83.4 ± 8.0	64.0 ± 6.4

* Cell survival in the presence of 1 μg/mL tamoxifen alone was 79.3 ± 3.1% of the control. 17β-Estradiol treatment, as low as 0.01 μM, produced complete reversal (100%) of the cell-killing effect of tamoxifen alone.

† Values are means ± SD of three independent experiments.

in the presence and absence of verapamil was comparable. Similarly, accumulation of tamoxifen following longer periods of incubation (3, 6, 12 and 24 hr) was not affected by verapamil treatment (data not shown). Furthermore, verapamil treatment did not affect the rate of tamoxifen efflux (data not shown). Thus, our results indicate that the potentiation of tamoxifen activity by verapamil is independent of alterations in drug retention/efflux.

The primary mechanism of tumor cell growth inhibition by tamoxifen is believed to be due to its interaction with the estrogen receptor. We proposed that potentiation of tamoxifen activity by verapamil may involve interaction of this agent with the estrogen receptor. If so, the potentiation of tamoxifen activity by verapamil should be reversed by inclusion of 17β-estradiol in the cell survival assay system. 17β-Estradiol treatment partially reversed the potentiation of tamoxifen activity by verapamil in a concentration-dependent manner (Table 1). At 1 μM 17β-estradiol, a 100-fold excess of estradiol than that required to reverse the effects of tamoxifen alone, the reversal of verapamil modulatory activity should have been complete. Since this concentration of 17β-estradiol produced only partial reversal, it appears that non-estrogen receptor interactions may also contribute to the potentiation of tamoxifen activity by verapamil.

Using partially purified enzyme preparation from rat brain, O'Brian *et al.* [13] have shown inhibition of protein kinase C activity by tamoxifen *in vitro*. These investigators postulated that the tumor cell growth inhibition by tamoxifen may be attributed, at least in part, to the inhibition of calcium-dependent protein kinase C activity [13]. To evaluate the role of non-estrogen receptor-mediated mechanisms, we determined if part of the potentiation of tamoxifen activity by verapamil is manifested through inhibition of protein kinase C activity in human breast cancer MCF-7 cells (Table 2). Interestingly, up to 3 μg/mL tamoxifen treatment did not inhibit protein kinase C activity. Similarly, verapamil treatment alone (10 μg/mL) or in combination with tamoxifen had no effect on protein kinase C activity (Table 2). It is important to emphasize that we used the same substrate (histone III) for protein kinase C activity determination as used by O'Brian *et al.* [13]. The inhibition of protein kinase C activity in the study by O'Brian *et al.* [13] was observed at tamoxifen concentrations that were several-fold higher than those used in the present study. The possibility that the high concentrations of tamoxifen needed to demonstrate inhibition of protein kinase C activity may be due to non-specific lipophilic effects cannot be ruled out. Furthermore, these investigators used partially purified enzyme preparations rather than intact cells, which may account for this discrepancy. Our results not only question the role of protein kinase C in tamoxifen activity in human breast cancer cells but also suggest that verapamil-mediated potentiation of tamoxifen activity is independent of protein kinase C inhibition at the cellular level.

Verapamil has been shown to increase the activity of the natural product class of anti-tumor drugs, such as doxorubicin, by increasing their cellular retention [14]. However, cellular accumulation of tamoxifen did not seem to be affected by verapamil treatment. These results suggested that the potentiation of tamoxifen activity by verapamil may involve a mechanism(s) other than altered drug transport. Our results are in agreement with those of Clarke *et al.* [19], who reported that transfection of the P-glycoprotein gene into MCF-7 cells does not change their sensitivity to 4-hydroxytamoxifen. Similarly, the verapamil-mediated increase in tamoxifen activity appeared to be independent of protein kinase C activity.

The results of this study suggest that potentiation of tamoxifen activity by verapamil may involve, at least in part, interaction of a calcium channel blocker with the estrogen receptor, as the modulatory effect of this agent was partially reversed by 17β-estradiol. Potential estrogen receptor-mediated mechanisms for the observed enhancement of tamoxifen activity by verapamil include: (i) up-regulation of the estrogen receptor or change in binding affinity, (ii) increased expression of growth inhibitory transforming growth factor β and decreased expression of growth stimulatory transforming growth factor α and insulin-like growth factor, (iii) phosphorylation of the estrogen receptor, (iv) increased receptor dimerization, (v) increased binding of tamoxifen–estrogen receptor complex to the estrogen response element, and (vi) alterations in tamoxifen metabolism. However, the precise nature of the interaction of verapamil with the estrogen receptor and the role of non-estrogen receptor mechanisms in the enhancement of tamoxifen activity by this agent remains to be elucidated.

In summary, although tamoxifen represents the endocrine treatment of choice in the clinical management of metastatic breast cancer [1, 4], most patients respond for only a short time or do not show any response to this agent. Therefore, identification of agents that can enhance the activity of tamoxifen would have enormous clinical implications. Results of the present study indicate that non-cytotoxic concentrations of the calcium channel blocker verapamil significantly enhances the

Table 2. Effects of verapamil and/or tamoxifen treatments on protein kinase C activity

Treatment	Protein kinase C activity (pmol/min/μg protein)		
	24 hr	48 hr	72 hr
Control	0.11 ± 0.03*	0.12 ± 0.00	0.13 ± 0.05
Tamoxifen (3 μg/mL)	0.10 ± 0.03	0.12 ± 0.02	0.13 ± 0.05
Verapamil (10 μg/mL)	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.04
Combined treatment	0.09	0.13	0.13 ± 0.04

* Values are means ± SD of three independent experiments, except for the 24- and 48-hr combined treatment where N = 2.

activity of tamoxifen in the human breast cancer cell line MCF-7. Since potentiation of tamoxifen activity by verapamil occurred at concentrations that cannot be achieved clinically, we are currently screening other calcium channel blockers that may have modulatory activity at clinically achievable concentrations. Nonetheless, our results represent a potentially novel approach to enhance the activity of tamoxifen in metastatic breast cancer.

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