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Effects of Non-Cytotoxic Concentration of Anticancer Drugs on Doxorubicin Cytotoxicity in Human Breast Cancer Cell Lines

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Abstract: The effects of non-cytotoxic concentrations of tamoxifen, verapamil, and trifluoperazine on doxorubicin cytotoxicity in five human breast cancer cell lines were studied. A non-cytotoxic concentration of tamoxifen resulted in enhanced doxorubicin cytotoxicity in HTB-123, HTB-26, and MCF-7. In these three cell lines, a combination of tamoxifen with verapamil resulted in even more increased doxorubicin cytotoxicity. Addition of verapamil or trifluoperazine alone did not influence the doxorubicin cytotoxicity significantly. Only in HTB-19 did coincubation with verapamil increase the doxorubicin cytotoxicity. In HTB-123, combination of tamoxifen with trifluoperazine increased the doxorubicin cytotoxicity significantly. In the cell lines where co-incubation with tamoxifen increased doxorubicin sensitivity, high estrogen receptor expression was detected. However, HTB-20, where tamoxifen did not enhance doxorubicin action, was also estrogen receptor positive. None of the cell lines had multidrug resistance related drug efflux and drug retention was not increased by the treatment with tamoxifen and verapamil. Cell cycle traverses were not altered by incubation with tamoxifen, verapamil or combinations thereof. These observations suggest mechanism of non-cytotoxic concentrations of tamoxifen and verapamil on doxorubicin cytotoxicity may involve one or more other cellular processes besides those of interference of estrogen binding to its receptor, cell cycle perturbation, or drug efflux blocking.
Key words: doxorubicin cytotoxicity, tamoxifen, trifluoperazine, verapamil.

Doxorubicin is an important cancer chemotherapy agent (Crooke and Reich, 1980). Binding to DNA and production of free radicals has been suggested to be responsible for its cytotoxic action (Bachur *et al.*, 1978). Doxorubicin is highly fluorescent and has been used in flow cytometry studies relating doxorubicin cellular retention and its correlation with cytotoxicity (Ross *et al.*, 1988). Earlier studies have shown that cellular resistance to doxorubicin is due to rapid energy-dependent drug efflux (Skovsgaard, 1978). P-glycoprotein acts as a membrane-bound, ATP-dependent, drug-efflux pump and is believed to transport a number of functionally and structurally unrelated drugs, including many antibiotic agents (Roninson, 1991). Cellular expression of P-glycoprotein results in multidrug resistance in cancer. Several agents, such as calcium channel blockers (e.g., verapamil) and calmodulin inhibitors (e.g., trifluoperazine) by blocking active drug efflux can confer drug sensitivity on otherwise drug-resistant cells (Ganapathi and Grabowski, 1983).

Tamoxifen, a synthetic nonsteroidal antiestrogen, is one of the most studied and commonly used drugs in the endocrine therapy of breast cancer (Pearson *et al.*, 1982; Lerner and Jordan, 1990). Tamoxifen is best known for its ability to bind to the estrogen receptor and block the binding of estrogen to its receptor, thus preventing the proliferative effect of estrogen (Lippman *et al.*, 1976; Coezy *et al.*, 1982). However, non-estrogen receptor mediated tamoxifen activities which are involved in the inhibition of cell proliferation and in the alterations of membrane characteristics have been reported (Hanni and Wright, 1984; Biswas and Vonderhaar, 1987; Kellen *et al.*, 1992; Callaghan and Higgins, 1995).

In this study, human breast cancer cell lines treated with non-cytotoxic concentration of tamoxifen, verapamil, and trifluoperazine showed enhanced doxorubicin

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sensitivity and the possible mechanisms of these effects were investigated.

Materials and Methods

Reagents and drugs

RPMI-1640, EMEM, fetal bovine serum, trypsin-EDTA, penicillin-streptomycin, and glutamine were purchased from GIBCO-BRL (Grand Island, USA). Doxorubicin was obtained from Adria Laboratories (Columbus, USA). Verapamil, tamoxifen, agar, ribonuclease, epidermal growth factor, transferrin, selenium, insulin, 3,3,5-triiodo-L-thyronine, progesterone, hydrocortisone, phosphorylethanolamine, and 2-aminoethanol were obtained from Sigma Chemical Co. (St, Louis, USA). MRK-16, a P-glycoprotein-specific monoclonal antibody, was purchased from Kamiya Biochemical Co. (Thousand Oaks, USA). Goat-anti-mouse IgG FITC and IgG2a were purchased from Sigma Chemical Co. A Vector Elite kit was purchased from Vector Laboratories (Burlingame, USA). Monoclonal antibody of estrogen receptor and isotype (IgG1) were purchased from DAKO Corporation (Caprinteria, USA). Biotinylated anti-Mouse IgG (γ chain specific, made from horse) was purchased from Vector Laboratories. Stock solutions of doxorubicin (1.5 mM) and verapamil (100 μM) were made in 0.85% sodium chloride and stored at 4℃. Stock solutions of tamoxifen (2.5 mM) in 95% ethanol were stored at −20℃.

Cell culture

Human breast tumor cell lines (MCF-7, HTB-19, HTB-20, HTB-26, and HTB-123) were obtained from the American Type Culture Collection (ATCC). All cell lines were grown as monolayers except for HTB-123, which grew in suspension. The MCF-7 cells were maintained in EMEM medium supplemented with 10% fetal bovine serum, insulin (0.1 U/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (292 μg/ml), sodium pyruvate (110 μg/ml), non-essential amino acids (0.1 mM), bombesin (10 nM) and growth factors (epidermal growth factor (7.5 ng/ml), transferrin (4.4 ng/ml), selenium (0.58 ng/ml), insulin (0.6 μg/ml), 3,3,5-triiodo-L-thyronine (1.5 ng/ml), progesterone (1.5 ng/ml), hydrocortisone (0.15 μg/ml), and phosphorylethanolamine (2.1 μg/ml). HTB-19, HTB-20, HTB-26, and HTB-123 cells were grown in RPMI-1640 medium, supplemented as above. In order to remove estrogenic components, the medium was changed to phenol red-free MEM plus 15% charcoal-treated fetal bovine serum for at least I week before using cells for estrogen receptor experiments. Cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2.

Flow cytometry

A FACScan flow cytometer (Becton and Dickinson Inc., San Jose, USA) equipped with a 15 mW air-cooled argon ion laser was used in the present study. Data were collected and analyzed on a Hewlett-Packard model 9,000-340 computer with Lysis II software.

Drug retention and cell cycle

Cells were harvested by treating with trypsin-EDTA, washed and resuspended at 10^6 cells/ml. Cells were pretreated with tamoxifen (10 μM) for 4 h at 37℃. These cells were exposed to verapamil (10 μM) and doxorubicin (2 μM) for 1 h and harvested. Intracellular doxorubicin fluorescence was analyzed by flow cytometry (Krishan *et al.*, 1986). The propidium iodide/hypotonic citrate method was used for the monitoring of cellular DNA content and cell cycle distribution (Krishan, 1975).

Flow cytometric analyses of P-glycoprotein and estrogen receptor expression

Exponentially growing cells (10^6) were washed with phosphate-buffered saline and incubated on ice for 30 min with MRK-16 (50 μg/ml). These cells were washed with 1 ml of phosphate buffered saline and pellets were mixed with Goat-anti-mouse IgG FITC (secondary Ab, 1:2000 diluted) and kept on ice for 30 min. Cells were washed with phosphate-buffered saline and resuspended in 1 ml of phosphate-buffered saline and fluorescences were determined with flow cytometric assays. Estrogen receptor expression was analyzed after incubating cells with estrogen monoclonal antibody followed by FITC-conjugated IgG.

Immunohistochemistry of estrogen receptor expression

Immunostaining was performed to determine the estrogen receptor expression (Paine *et al.*, 1992). For cytospin preparations, attached cells were removed from flasks with trypsinizations. The cells (2×10^5) were rinsed with phosphate-buffered saline (PBS) and deposited onto gelatin-coated slides at 750 rpm for 5 min and dried at room temperature for 10 min. Fixation was produced with 10% neutral buffered formalin, followed by rinses with phosphate-buffered saline for 10 min, two times, and incubated in cold (−20℃) methanol for 5 min and in cold (−20℃) acetone for 1 min, followed by rinses in phosphate-buffered saline for 5 min, twice, and air-dried for 10 min after fixation. The avidin-biotin-peroxidase complex (Vestastain ABC kit, Vector, Burlingame, USA) was used for immunoperoxidase staining as directed by the manufacturer. Nonspecific antibody binding was blocked by the applica-

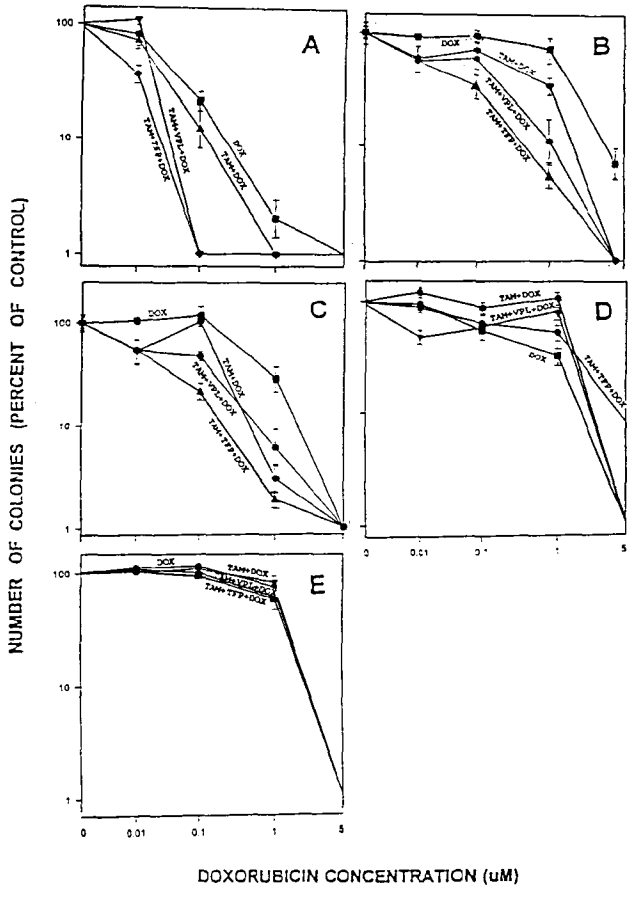

Fig. 1. Modulating effect of tamoxifen (TAM), verapamil (VPL), and trifluoperazine (TFP) on doxorubicin (DOX) cytotoxicity of human breast cancer cell lines. (A) HTB-123 (B) HTB-26 (C) MCF-7 (D) HTB-19 (E) HTB-20. Cells pretreated with or without 10 μM TAM for 4 h were exposed to various DOX concentrations (0, 0.01, 0.1, 1, 5 μM) for 1 h with or without VPL (10 μM) or TFP (15 μM).

tion of 10% normal serum for 20 min. Cells were covered with primary antibody, followed by washings in PBS for 5 min, twice, and incubated with secondary ab for 30 min, followed by washings in PBS for 5 min, twice. Slides were incubated in ABComplex (Avidin and Biotinylated horseradish peroxidase macromolecular Complex) for 30 min and washed in water for 5 min twice. Slides were incubated in diaminobenzidine (DAB) for 3 min and washed in PBS for 5 min, twice. Cells were counterstained with hematoxylin. Cytospin preparations were dehydrated and mounted in permount.

Clonogenic assay

Drug sensitivity of breast cancer cells was determined by soft-agar clonogenic assay (Gupta and Krishan, 1982). Log phase cells were incubated with tamoxifen and without tamoxifen (10 μM) for 4 h at 37℃. Cells were centrifuged at 80×*g*, washed with PBS and resuspended in medium containing doxorubicin (0.01, 0.1, 1, and 5 μM) for 1 h at 37℃ with verapamil and without verapamil (10 μM) or with trifluoperazine and without trifluoperazine (15 μM). Cells (2×10^4) were washed with PBS and plated in 0.3% agar over an underlayer of 0.5% agar. Both agar layers were prepared in EMEM medium containing 15% fetal bovine serum, 10 μM mercaptoethanol, insulin (0.1 U/ml), penicillin (100 μ/ml), streptomycin (100 μg/ml) and glutamine (2 mM), sodium pyruvate (110 μg/ml), non-essential amino acids (0.1 mM), bombesin (10 nM), and growth factors. Colonies larger than 50 μm in size were counted after 11-21 days incubation at 37℃ in a humidified atmosphere of 5% CO_2. Data plotted in Fig. 1 is based on mean of colonies from a minimum of three different experiments.

Results and Discussion

Determination of the drug sensitivity by clonogenic assays

To investigate the effects of noncytotoxic concentration of tamoxifen, verapamil, and trifluoperazine, log phase cells preincubated with/without tamoxifen for 4 h, washed with PBS, and then incubated with verapamil (trifluoperazine) and various concentrations of doxorubicin for 1 h were used in soft-agar clonogenic assays.

Table 1. IC_{50} DOX and dose modification factor after coincubation with TAM, VPL, TFP and DOX.

	DOX	DOX/VPL	DOX/TFP	TAM/DOX	TAM/DOX/VPL	TAM/DOX/TFP
HTB-123	0.056	0.094(0.6)	0.072(0.8)	0.041(1.4)	0.020(2.8)	0.008(7.0)
HTB-26	1.35	1.05 (1.3)	ND	0.37 (3.7)	0.29 (4.7)	ND
MCF-7	0.6	0.5 (1.2)	ND	0.40 (1.5)	0.12 (4.8)	ND
HTB-19	0.83	0.38 (2.2)	0.85 (1.0)	3.16 (0.3)	2.74 (0.3)	1.42 (0.6)
HTB-20	1.10	0.9 (1.2)	1.12 (1.0)	1.15 (1.0)	1.25 (0.9)	1.22 (0.9)

TAM-pretreated and control cells were exposed to different concentrations of DOX (0, 0.01, 0.1, 1, 5 μM), VPL (10 μM), or TFP (15 μM), washed, and plated in soft agar. Numbers in parenthesis indicate the dose modification factor of the combination derived by IC_{50} DOX÷IC_{50} of DOX with other drug(s). DOX : doxorubicin; VPL : verapamil; TFP : trifluoperazine; TAM : tamoxifen. The abbreviations used are: DMF, dose modification factor; DOX : doxorubicin; TAM : tamoxifen; TFP : trifluoperazine; VPL : verapamil; ND : not determined.

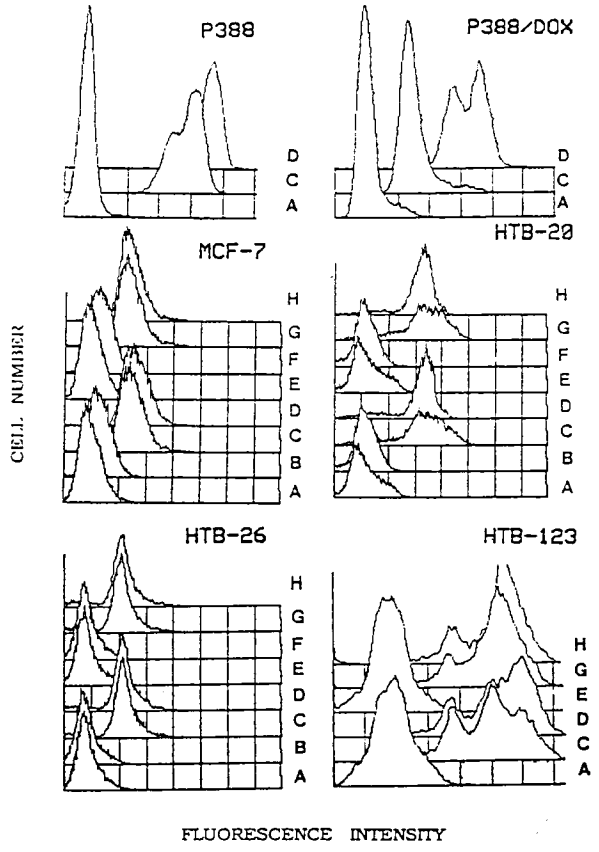

Fig. 2. Doxorubicin (DOX) fluorescence profiles of human breast cancer cells. Cells pretreated with or without 10 μM tamoxifen (TAM) for 4 h were exposed to 10 μM verapamil (VPL) and/or 2 μM DOX for 1 h. A) Control, B) VPL, C) DOX, D) DOX+VPL, E) TAM, F) TAM+VPL, G) TAM+DOX, H) TAM+DOX+VPL. DOX is one of the fluorescent anticancer drug, it is used in the monitoring cellular drug transport and retention by laser flow cytometry. TAM and/or VPL did not result in enhancement of drug retention in these cell lines. Murine leukemic cell lines, P388, and P388/DOX, were used as a negative and a positive control of drug retention change.

As shown in Figure 1 and Table 1, the IC_{50} of doxorubicin in soft agar assay of the five breast cencer cell lines was between 0.056 μM (HTB-123) and 1.35 μM (HTB-26). Co-incubation with verapamil (10 μM) or trifluoperazine (15 μM) for 1 h did not alter (less than 2 fold) the IC_{50} of doxorubicin in soft agar assays. Only in HTB-19 cells did coincubation with verapamil change the doxorubicin IC_{50} by 2.2 fold. Preincubation of cells with tamoxifen (10 μM) for 4 h before exposure to doxorubicin changed the IC_{50} in HTB-123 and HTB-26 cells by a factor of 1.4 to 3.7. Addition of verapamil (10 μM) to cells preincubated with tamoxifen for 4 h reduced the IC_{50} of doxorubicin further with a dose modification factor of 4.7 and 4.8 in HTB-26 and MCF-7 cell lines, respectively. A similar effect was seen in HTB-123 cells coincubated with trifluoperazine (verapamil) and doxorubicin after preincubation with tamoxifen. In HTB-20 cells, no major effect of preincubation with tamoxifen or coincubation with verapamil, or trifluoperazine on doxorubicin IC_{50} was evident.

No doxorubicin retention change with the treatment of tamoxifen and verapamil

Doxorubicin is fluorescent and can be used to monitor cellular uptake, retention, and efflux. To investigate whether or not the observed dose modifying effects of tamoxifen, verapamil, or trifluoperazine were due to changes in cellular doxorubicin retention, we analyzed cells incubated under various conditions by laser flow cytometry. Histograms of cellular doxorubicin retention (Fig. 2) indicate that the observed effects were not due to the enhancement of cellular doxorubicin retention.

Drugs effects on cell cycle distributions

Doxorubicin is effective against the cancer cells, causing a decrease in cell growth. The effect is dose dependent. High concentrations of doxorubicin cause a block in the cell cycle at the G2/M phase which may lead to cell death. Incubation with low concentrations of verapamil, tamoxifen, or combinations did not alter the cell cycle traverses of HTB-26 (Fig. 3) and other four breast cancer cell lines (data not shown).

Determination of P-glycoprotein expression

The multidrug resistance phenotype is of importance since it leads to resistance to a group of chemically dissimilar but highly active drugs such as doxorubicin, etoposide, and the Vinca alkaloids. The multidrug resistance is associated with high efflux of the cytotoxic drugs. Membrane alterations, including overexpression of the drug transport P-glycoprotein, appear to be related to this multidrug resistance. With the flow cytometric assays, expression of p-glycoprotein in the five cell lines was investigated (Fig. 4). All five cell lines were expected to be p-glycoprotein negative because there was no drug retention change with the calcium channel blocker (Fig. 2), however, HTB-123 produced p-glycoprotein. This P-glycoprotein in HTB-123 is either insensitive to efflux blocker or non-functional. HTB-123 can possibly be used as an *in vitro* model system in which the changes that destroy the functionality of p-gp can be investigated.

Status of the estrogen receptor expression

Tamoxifen appears to inhibit cell proliferation predominantly by blocking the action of estrogen by binding to the estrogen receptor. In the present study, a flow cytometric method was used to determine the estrogen receptor expression (Fig. 5a) and compared with the

traditional immunostaining method (Fig. 5b). MCF-7 and HTB-20 cell lines showed high estrogen receptor-positivity compared to isotype controls, while HTB-26 and HTB-123 cell lines were 75% and 48% positive,

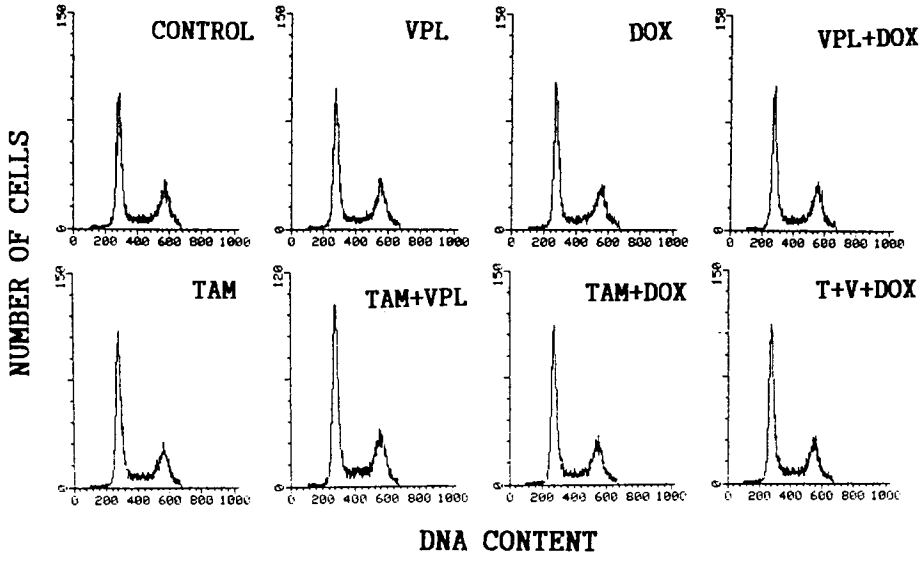

Fig. 3. Effect of tamoxifen (TAM) and/or efflux blocker (VPL, verapamil) on cell cycle distribution of breast cell line (HTB-26). Histograms were obtained in a flow cytometer after 4 h preincubating cells with a non-cytotoxic concentration of tamoxifen (10 μM) or verapamil (10 μM) for 1 h. No cell cycle traverses were observed by incubation with tamoxifen and verapamil. No significant effect on cell cycles was noted in other cell lines also (data not shown).

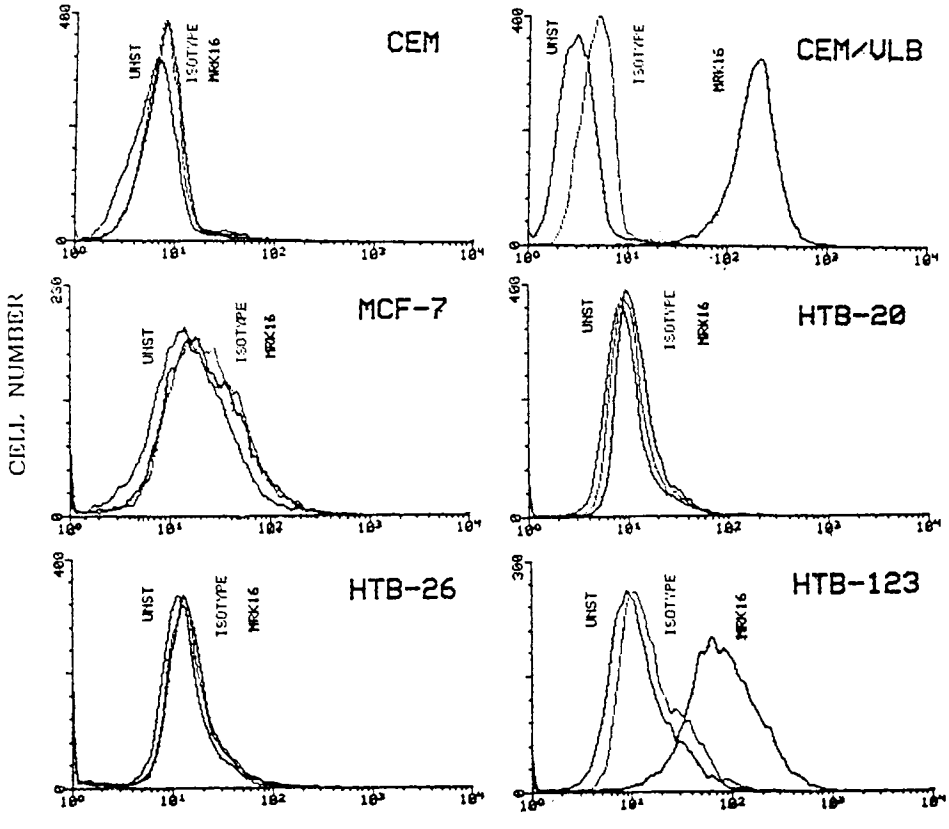

Fig. 4. P-glycoprotein expression in human breast cancer cell lines. Cells were reacted with P-glycoprotein specific MRK-16 antibody and FITC-conjugated secondary antibody. Fluorescences were determined with flow cytometric assays. Among the cell lines investigated, only HTB-123 cells produced large amount of P-glycoprotein. Human leukemic cell lines CEM and CEM/VLB were used as a negative and a positive control of P-glycoprotein expression.

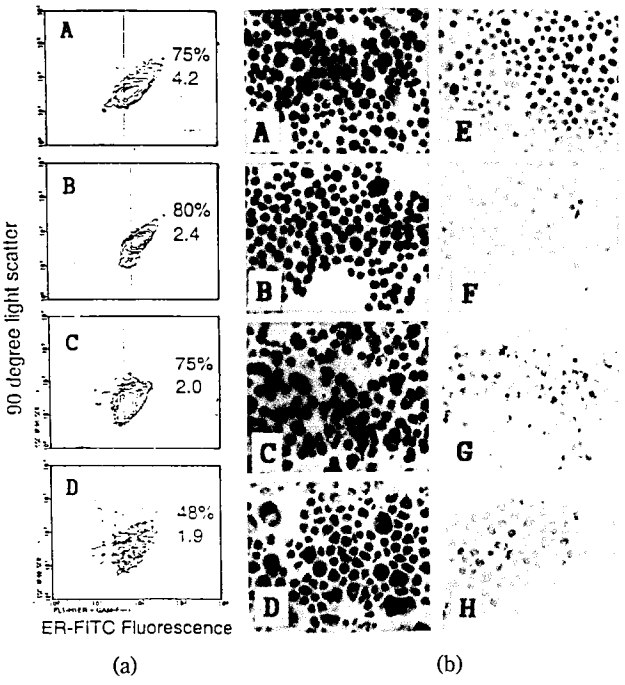

Fig. 5. Flow cytometric assays (a) and Immunoperoxidase staining (b) of estrogen receptor in human breast cancer cell lines. A, B, C, and D show the degrees of estrogen receptor expression in MCF-7, HTB-20, HTB-26, and HTB-123, respectively. E, F, G, and H were the negative controls of above cells. MCF-7 and HTB-20 were highly estrogen receptor positive. HTB-26 and HTB-123 produced less estrogen receptors.

respectively. Comparison of mean of log fluorescence values revealed that MCF-7 cells had the highest expression with a relative fluorescence values index (RFI) of 4.2. HTB-20 had medium expression (RFI: 2.4), while HTB-26 and HTB-123 had low expression (RFI: 1.9 and 2.0, respectively). Parallel analysis of immunoperoxidase stained slides showed good concordance with the flow cytometric data. MCF-7 and HTB-20 cell lines had dense expression of estrogen receptor by immunoperoxidase (+4 and +3.5, respectively), while HTB-26 and HTB-123 were less dense (+2 and +1, respectively), and had more heterogeneous expression.

In the present study, the effects of low concentrations of tamoxifen, verapamil, and trifluoperazine on doxorubicin cytotoxicity were investigated. A noncytotoxic concentration of tamoxifen increased doxorubicin cytotoxicity in three cell lines but had no effect in the other two cell lines. In the cell lines where tamoxifen increased doxorubicin sensitivity, a combination of tamoxifen with verapamil and/or trifluoperazine significantly increased doxorubicin cytotoxicity. In cancer chemotherapy, single drugs such as doxorubicin are rarely used alone; and in most protocols, several drugs with different mechanisms of action are combined. Several studies have shown that calcium channel blockers (e.g., verapamil) and calmodulin inhibitors (e.g., trifluoperazine) will reduce doxorubicin efflux and increase the cytotoxicity in drug-resistant cells (Ganapathi and Grabowski, 1983). However, according to our flow cytometric data analyses, low concentrations of tamoxifen and verapamil that increased doxorubicin cytotoxicity did not enhance the drug retention. Tamoxifen competes with estrogen for binding to the estrogen receptor, therefore the status of estrogen receptor is important in responsiveness of the breast cancer cells to tamoxifen treatment. In this study, the modulatory effects on doxorubicin cytotoxicity by low concentrations of tamoxifen did not depend on the status of estrogen receptor in the breast cancer cell lines. The mechanism by which non-cytotoxic concentration of tamoxifen, verapamil, and trifluoperazine increases doxorubicin sensitivity is not understood. However, we could exclude the possible involvements of increased cytotoxicity with estrogen-receptor mediated response, alteration of the drug retention, and perturbation of the cell cycles.

Doxorubicin has dose-limiting cardiotoxicity as one of the major limitations to its continued clinical use (Crooke and Reich, 1980). To avoid the side effects that can be caused by the combination of high concentration of anticancer drugs with doxorubicin and to increase the doxorubicin cytotoxicity, combinations of low concentrations of anticancer drugs with doxorubicin may be possible alternative protocols. To support the use of the low concentration of drugs that enhances the doxorubicin cytotoxicity, we need more information on the activities of non-cytotoxic concentrations of anticancer drugs.

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