PRECIPITOUS DOSE-RESPONSE CURVES FOR THE ANTICANCER ACTIONS OF MICROTUBULE-DISRUPTING AGENTS IN HUMAN BREAST CANCER CELLS: IMPLICATIONS FOR HIGH-DOSE REGIMEN IN ANTICANCER CHEMOTHERAPY

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RESEARCH ARTICLE

ABSTRACT. DISRUPTION OF THE NORMAL FUNCTIONS of mitotic microtubules is a common target of attack for many anticancer drugs. We report here our observations that several well-known microtubule-disrupting agents (paclitaxel, vinblastine, vincristine, vinorelbine, colchicine, and 2-methoxyestradiol) each had almost identical and unusually precipitous dose-response curves for their inhibition of the growth of five human breast cancer cell lines in culture. The cancer cell lines examined in the present study included three estrogen receptor-positive cell lines (MCF-7, T-47D, and ZR-75-1) and two estrogen receptor-negative cell lines (MDA-MB-231 and MBA-MB-435s). These observations led us to suggest that for this class of anticancer agents, the use of adequately high doses, rather than opting for lower and safer doses, should be considered for breast cancer patients in order to assure effective anticancer activity. Otherwise, a cancer patient may face the possibility of reaping far less therapeutic benefits as they would have if these agents are given at relatively lower doses that may produce drug concentrations even slightly below the maximally-effective levels. Further studies are needed to determine whether such a precipitous dose-response relationship observed in vitro for paclitaxel, vinca alkaloids, and 2-methoxyestradiol is also seen in breast cancer patients.

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1. INTRODUCTION

Disruption of the formation and normal functioning of mitotic microtubules has been a common target of action for many anticancer drugs. The vinca alkaloids, such as vinblastine, vincristine, and vinorelbine, are in common with colchicine and paclitaxel (a taxane), which are cell cycle-specific agents, blocking cells in mitosis. Mechanistically, vinca alkaloids induce destabilization of the polymerized tubulins and ultimately block the polymerization of tubulins to form microtubules during mitosis, resulting in cell division arrest in metaphase [1-3]. In comparison, paclitaxel inhibits the disassembly of microtubules by binding to the tubulin subunit, subsequently resulting in the formation of aberrant microtubule-like structures [4-6]. Notably, 2-methoxyestradiol (2-MeO-E_2), a well-known nonpolar endogenous metabolite of 17β-estradiol (E_2) in humans [7,8], has also been found to have antimicrotubule activity [9,10]. This estrogen metabolite has strong antiangiogenic and antiproliferative activity when present at pharmacological concentrations [11,12], and it is currently under extensive study for its potential use as an effective chemotherapeutic agent for certain types of human cancer [13].

In this report, we describe our interesting observations that several microtubule-disrupting agents (paclitaxel, vinblastine, vincristine, vinorelbine, and 2-MeO-E_2) all have highly similar and unusually precipitous dose-response curves for their growth-inhibitory actions in five human breast cancer cell lines tested, including three estrogen receptor (ER)-positive human breast cancer cell lines (MCF-7, T-47D, and ZR-75-1) and two ER-negative human breast cancer cell lines (MDA-MB-435s and MDA-MB-231). In addition, colchicine, a prototypic microtubule-disrupting agent, was also found to have a similarly precipitous dose-response curve pattern in these five cell lines. Although the precise mechanism(s) for the observed precipitous dose-response curves of these anticancer agents is not understood at present, our observations, nevertheless, led to the suggestion that the use of a high-dose regimen may be necessary for this class of agents in order to assure effective anticancer activity in breast cancer patients.

2. MATERIALS AND METHODS

2.1. CHEMICALS

Vinblastine, vincristine, paclitaxel, doxorubicin, mitomycine C, 5-fluorouracil, mathotrexate, E_2, crystal violet, Triton-100, 50% glutaradehyde, dithiothreitol, dextran-coated charcoal, and fetal bovine serum (FBS) were all obtained from the Sigma Chemical Co. (St. Louis, MO). Vinorelbine tartrate (vinorelbine) was provided by GlaxoSmith-Kline (Research Triangle Park, NC). 2-MeO-E_2 was purchased from Steraloids (Newport, RI). Our HPLC analysis showed that 2-MeO-E_2 from Steraloids only had ~94% purity, but no E_2 or estrone was detected. We re-purified the 2-MeO-E_2 with HPLC when it was used in all of the cell culture experiments described in this paper. The antibiotics solution (containing 10,000 U/mL penicillin and 10 mg/mL streptomycin), Trypsin-Versene (containing 0.25% trypsin and 0.02% EDTA), RPMI-1640 medium (phenol red-free), Eagle's modified minimum essential medium (EMEM; phenol red-free), Iscove's modified minimum essential medium (IMEM), and calf bovine serum were purchased from Life Technology (Rockville, MD).

2.2. CULTURE OF HUMAN BREAST CANCER CELLS

The ER-positive MCF-7, T-47D, and ZR-75-1 human breast cancer cells and the ER-negative MDA-MB-231 and MDA-MB-435s human breast cancer cell lines were all obtained from the American Type Culture Collection (ATCC, Manassas, VA). The methods for the in vitro culture of the ER-positive MCF-7 and T-47D cells as well as the ER-negative MDA-MB-231 and MDA-MB-435s cells were described in our recent study [14]. For culturing the ER-positive ZR-75-1 cells, we used RPMI-1640 medium supplemented with 10% FBS and the same amount of the antibiotics.

The human breast cancer cells were first propagated in the 75-cm^2 flasks under 37°C air with 5% CO_2 and 95% humidity to ~80% confluence. They were then detached from the flask by treatment with 3 mL of the trypsin-EDTA solution for ~5 min. Cell suspensions were centrifuged and the cell sediments were re-suspended in the culture medium at the desired 10^5 cells/mL density. A 0.1-mL aliquot of the cell suspension was then added to

each well of the 96-well microplate at a final density of 10^4 cells per well. After the cells were allowed to attach and grow for 24 hrs, the cell culture medium was changed and different treatments were also given at that time. In most experiments (unless otherwise indicated), the drug treatment lasted for 3 days with one medium change on the third day following the initial drug treatment.

2.3. PREPARATION OF THE ANTICANCER DRUG SOLUTIONS

Due to their high lipophilicity, the stock solutions of paclitaxel (0.2 mM) and 2-MeO-E$_2$ (10 mM) were prepared in pure ethanol (200 proof). The stock solutions of doxorubincin (5 mM), mitomycin C (0.1 mg/mL), and vinorelbine (1 mM) were prepared in phosphate buffer (pH 7.4). The stock solutions containing 10 mM of 5-fluorouracil or methoxetrate were prepared by first making a 50-mM drug concentration in 1.0 N potassium hydroxide and followed by dilution with phosphate buffer (pH 7.4) to a 10-mM drug concentration. All these stock solutions were filtered with a Millex syringe filter (0.22 μm acetatecellulose membrane), and the filtrates were stored at –20°C in tightly-sealed sterile tubes. Shortly before introducing the anticancer agents to the cultured cancer cells, each chemical was freshly diluted with a buffer to the desired concentrations and an aliquot (usually 10 μL) of the drug-containing solution was added to each well. Usually <0.1% of the original solvent of the stock solution was present in the final cell culture medium.

2.4. MEASUREMENT OF CELL GROWTH

The cell density in the 96-well microplates was determined by using the crystal violet staining method [14-16]. Briefly, the culture medium in the microplates was first removed by aspiration, and then the cells in each well were fixed with 1% glutaradehyde for 20 min. After removing the fixation solution, each well was rinsed with PBS buffer and allowed to dry at room temperature. The cells in each well were then stained with 50 μl of 5% crystal violet (dissolved in 20% methanol and 80% deionized water) for 15 min at room temperature, and the plates were rinsed carefully with tap water to remove residual crystal violet. The stained dye was then dissolved in 100 μL of 0.5%

Triton-100 for overnight. After addition of 50 μL of 200-proof ethanol, the optical density values of each well were measured at 405 nm and 560 nm with a UVmax microplate reader (Molecular Device, Palo Alto, CA), and the difference in the optical density values at these two wavelengths were used to represent the cell density.

2.5. STATISTICAL ANALYSIS

In the in vitro growth inhibition experiments, the growth rates of the control and drug-treated cells were expressed as MEAN ± S.E. of the values obtained from 6 to 8 replicate wells. The IC$_{50}$ values were calculated according to the equation for sigmoidal dose-response curves (with variable slopes) using the non-linear regression curve-fitting model of the Prism software. A P value <0.05 was considered to be statistically significant, and a P value <0.01 was considered statistically very significant.

3. RESULTS

When each of the five human breast cancer cell lines were treated with increasing concentrations (0.01-100 nM) of paclitaxel, a very precipitous dose-response curves were observed (FIG. 1). Notably, paclitaxel had little or no growth-inhibitory effect in these cell lines at its concentrations ≤1 nM, but a near complete inhibition of cell growth was observed in several of the cell lines when its concentration was increased to 10 nM. The IC$_{50}$ values of paclitaxel in these five breast cancer cell lines were quite comparable, regardless of the ER status. Such dose-response experiments were repeated more than once in three of the five cell lines (MCF-7, MDA-MB-231, and MDA-MB-435s), and highly consistent results were obtained.

Similarly, when each of the five human breast cancer cell lines were treated with increasing concentrations (0.01–100 nM) of vinblastine, vin-cristine or vinorelbine, highly-similar patterns of the dose-response curves were also observed (FIG. 1). The IC$_{50}$ values for each of the vinca alkaloids in all these cell lines appeared to be not markedly different from each other, regardless of the ER status. Typically, the anticancer activity of each of the vinca alkaloids started quite abruptly when the concentrations of the drug reached certain threshold
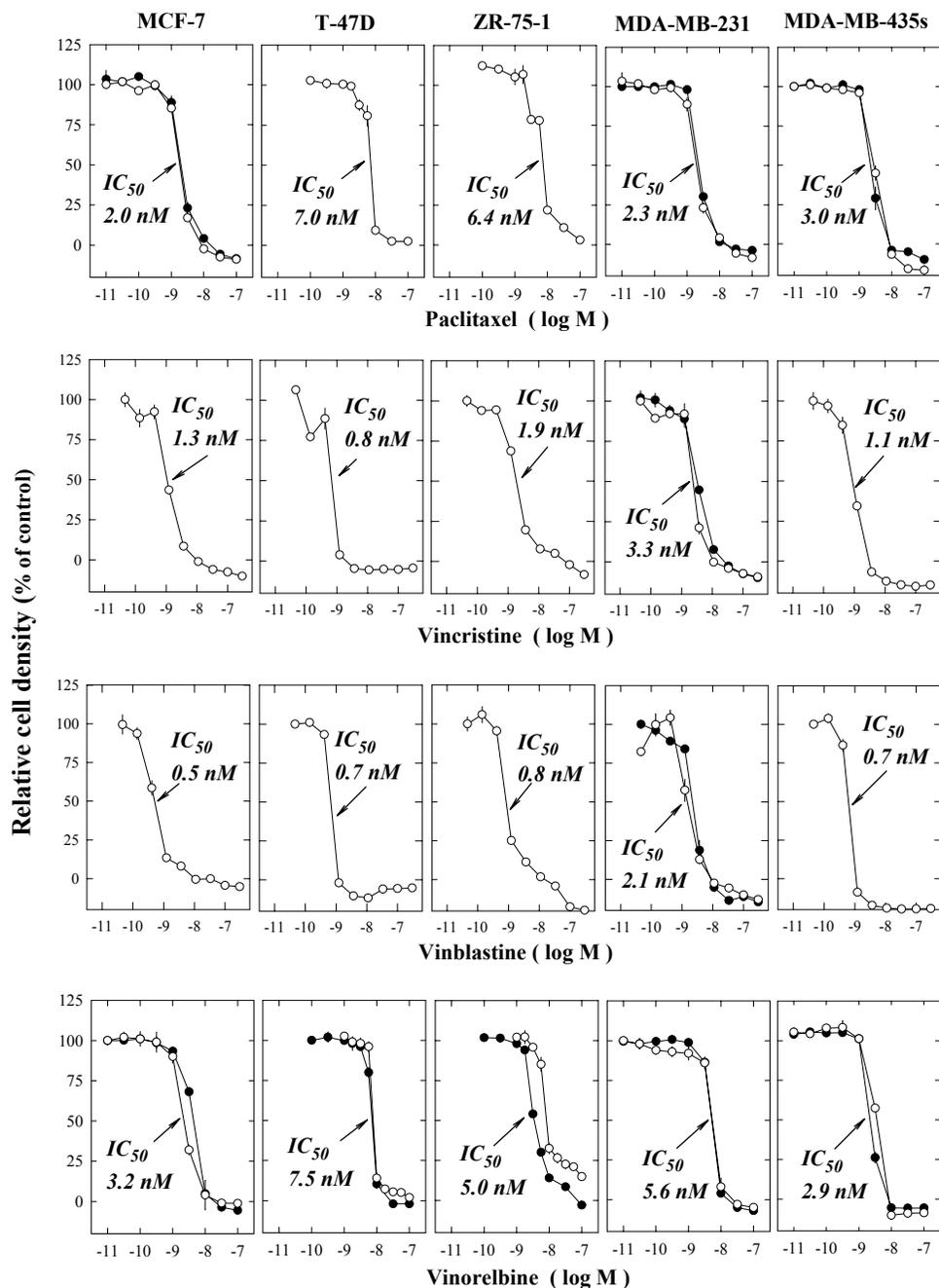
FIGURE 1. EFFECTS OF PACLITAXEL, VINBLASTINE, VINCRISTINE, AND VINORELBINE ON THE GROWTH OF THE ER-POSITIVE MCF-7, T-47D AND ZR-75-1 CELLS AND THE ER-NEGATIVE MAD-MD-231 AND MDA-MB-435S CELLS. The methods for culturing these cancer cells were described in the MATERIALS AND METHODS section. Twenty-four hrs after an aliquot of the cell suspension (containing 10^4 cells) was placed into the 96-well microplate, the culture medium was changed and different concentrations of each anticancer drug were introduced at that time. Each of the drug treatments lasted for 4 days with one medium change on the third day following the initial drug treatment. Cell density in each well was determined by using the crystal violet staining method followed by spectrometric measurement with a UVmax microplate reader, and the starting cell density immediately prior to estrogen treatment was subtracted. For comparison, the rate of cell growth in the absence of the anticancer drug was arbitrarily assigned to be 100%, and the rate of cell growth in the presence of the drug was expressed as "% of control". Each point is the MEAN ± S.E. of 5-7 replicate measurements.
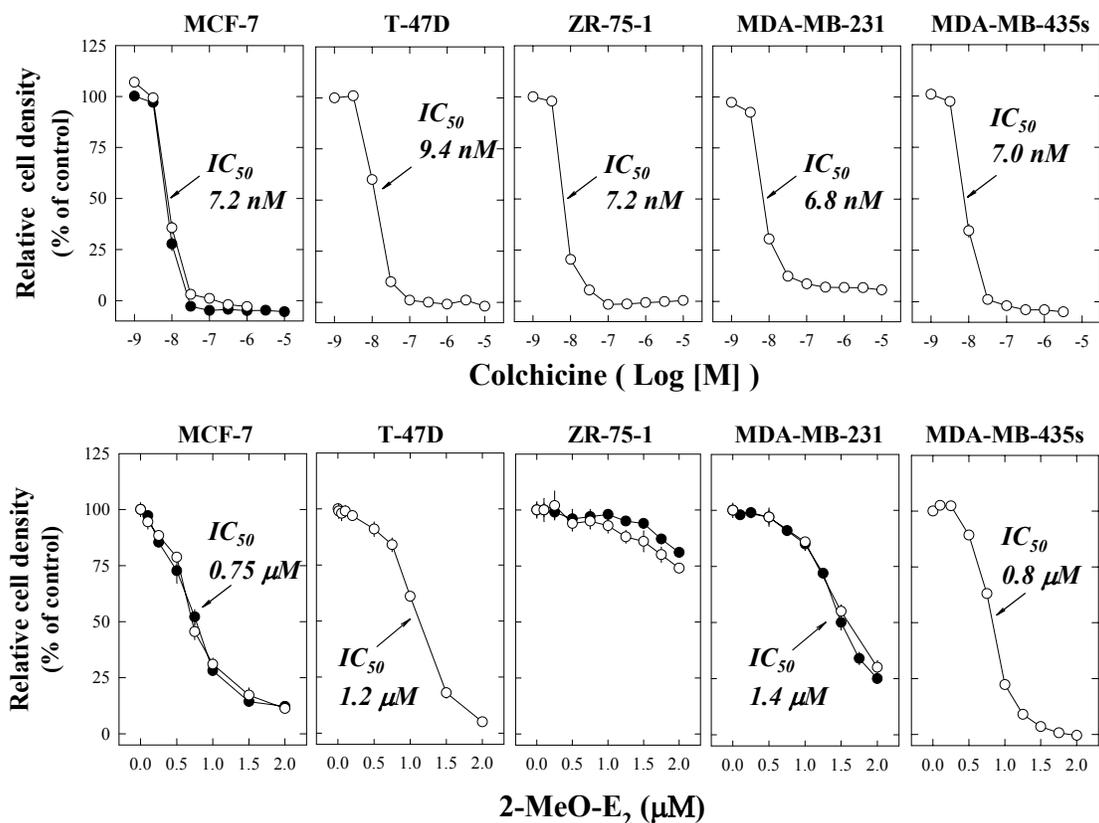
FIGURE 2.   EFFECTS OF COLCHICINE AND 2-MeO-E$_2$ ON THE GROWTH OF THE ER-POSITIVE MCF-7, T-47D AND ZR-75-1 CELLS AND THE ER-NEGATIVE MAD-MD-231 AND MDA-MB-435S CELLS. The methods for culturing these cancer cells were described in the MATERIALS AND METHODS section. The experimental conditions were described under the legend to FIGURE 1. Each point is the MEAN ± S.E. of 5-7 replicate measurements.

concentrations, and the maximal anticancer activity was rapidly reached when the drug concentrations were further increased. These experiments were also repeated more than once with most of the agents in some of the cell lines tested, and highly consistent results were observed.

For the purpose of comparison, we also determined the growth-inhibitory effect of the prototypic antitubulin agent colchicine in these human breast cancer cell lines (FIG. 2, upper panel). Similarly precipitous dose-response curves were obtained with colchicine, and the IC$_{50}$ values of cholchicine in all these cell lines were very close.

2-MeO-E$_2$ has previously been shown to have strong antiangiogenic and antiproliferative activity when present at pharmacological concentrations [9-12]. Several earlier studies have suggested that its aniangiogenic and antiproliferative actions are largely attributable to its antitubulin activity [9,10].

Therefore, we have also compared the dose-response curve pattern of 2-MeO-E$_2$ in these five human breast cancer cell lines. 2-MeO-E$_2$ inhibited the growth of the ER-positive MCF-7 and T-47D cells in a concentration-dependent manner (IC$_{50}$ of 0.75 and 1.2 μM, respectively), and very precipitous dose-response curves were observed in these two cell lines (FIG. 2, lower panel). Although 2-MeO-E$_2$ had little anticancer activity at <0.3 μM in these two cell lines, a >90% inhibition of their growth was achieved when 2 μM 2-MeO-E$_2$ was present. However, the ER-positive ZR-75-1 cells were selectively insensitive to the antiproliferative actions of 2-MeO-E$_2$. We recently have shown that this insensitivity is due to a very rapid metabolic conversion of 2-MeO-E$_2$ to the inactive 2-methoxy-estrone in this cell line [17,18]. In the two ER-negative human breast cancer cell lines (MDA-MB-231 and MDA-MB-435s), similarly precipitous
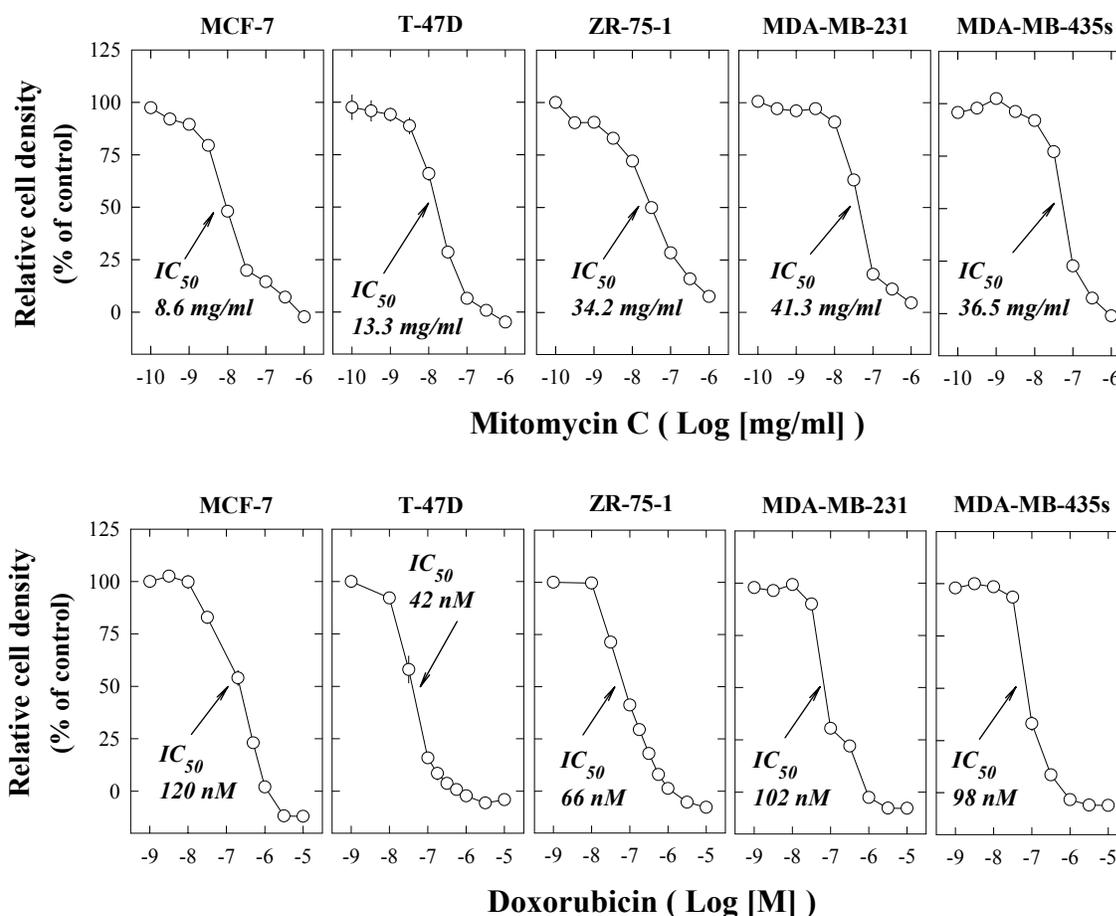
FIGURE 3.   EFFECTS OF MITOMYCIN C AND DOXORUBICIN ON THE GROWTH OF FIVE HUMAN BREAST CANCER CELL LINES IN CULTURE. The methods for culturing the cancer cells were described in the MATERIALS AND METHODS section. The experimental conditions were described under the legend to FIG. 1. Each point is the MEAN ± S.E. of 5-7 replicate measurements.

dose-response curves were observed. It is apparent that the growth-inhibitory effect of 2-MeO-E$_2$ in sensitive human breast cancer cells had a similarly precipitous dose-response curve pattern, and its growth-inhibitory action is also independent of the ER status.

In order to determine whether the precipitous dose-response curve pattern is only characteristic for the cell lines or for the anticancer agents used in the present study, we also compared the dose-response curves for the growth-inhibitory actions of several other anticancer agents (such as mitomycin C, doxorubicine, 5-fluorouracil, and methotrexate) in these five human breast cell lines under exactly the same experimental conditions.

Mitomycin C and doxorubincin are two commonly-used multi-functional chemotherapeutic antibiotics that inhibit DNA synthesis and also cause DNA alkylation and strand breaks. Mitomycin C inhibited the growth of five human breast cancer cells in a concentration-dependent manner (FIG. 3, upper panel). The effective drug concentrations covering the 5-95% inhibition of the cancer cell growth span ~2 orders of magnitude. Similarly, the overall dose-response curve patterns of doxorubicin observed in these cell lines were similar to those of mitomycin C (FIG. 3, lower panel).

5-Fluorouracil and methotrexate are two classical antimetabolism agents, inhibiting cancer growth by blocking thymidylate synthesis and ultimately DNA synthesis. In the three ER-positive human breast cancer cell lines tested for comparison, these two anticancer agents also exhibited a rather wide dose-response curve pattern.

4. DISCUSSION

Paclitaxel and various vinca alkaloids are among the most commonly-used anticancer agents for the treatment of a variety of solid tumors, including late-stage human breast cancer [1-6,19-22]. Mechanistically, these agents exert their anticancer actions primarily through disturbing the polymerization of tubulins (in the case with vinca alkaloids [1-3]) or the disassembly of microtubules (in the case with paclitaxel [4-6]), consequently resulting in mitotic arrest and cell death. The results of our present study showed that paclitaxel, vinblastine, vincristine, vinorelbine, and colchicine each had almost identical and very precipitous dose-response curves for their growth-inhibitory actions in five human breast cancer cell lines, i.e., their anticancer activity started abruptly when the concentrations of the anticancer agents reached certain threshold concentrations, and also their maximal anticancer activity was rapidly reached when the drug concentrations were further increased. Our observations provide an experimental basis for the notion that adequately high doses of these microtubule-disrupting agents should be used in cancer patients in order to achieve effective anticancer activity. Otherwise, a cancer patient might face the possibility of reaping far less therapeutic benefits as they should have when these agents are given at doses that would yield drug concentrations even slightly below the maximally-effective levels. More studies are needed to determine whether such precipitous dose-response curves observed in vitro for paclitaxel, various vinca alkaloids, and 2-MeO-E_2 are also seen in breast cancer patients. In this context, it is of note that some clinical studies have suggested that there appeared to be a threshold dose or concentration for paclitaxel to exert its anticancer effect in cancer patients, and only negligible anticancer activity was observed when the drug was used below the threshold dose [23,24]. In addition, there also appeared to be a plateau dose or concentration for paclitaxel, and further increase of the drug dose beyond the plateau dose did not produce any further anticancer activity in cancer patents [23,24]. These in vivo observations are in line with the in vitro observations described in the present study using cultured human breast cancer cell lines.

Notably, the precipitous dose-response curve pattern appears to be quite characteristic for various microtubule-disrupting agents, because several other anticancer agents that were also tested in the present study did not have a similarly precipitous dose-response curve pattern in these cell lines. It is likely that the unique dose-response curve pattern is reflective of the antitubulin mechanism of their anticancer actions. In this context, it is also of note that the high degree of similarity of the dose-response curves of 2-MeO-E_2's anticancer actions to those of other antitubulin agents may offer support for the earlier mechanistic suggestion [9,10] that the anticancer actions of 2-MeO-E_2 is attributable to its antitubulin activity.

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