COMBINATIONS OF PACLITAXEL AND VINBLASTINE AND THEIR EFFECTS ON TUBULIN POLYMERIZATION AND CELLULAR CYTOTOXICITY: CHARACTERIZATION OF A SYNERGISTIC SCHEDULE

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Paclitaxel (PTX) and vinblastine (VBL) represent 2 classes of drugs that target tubulin but have separate binding properties and opposing mechanisms of action. To evaluate the potential use of these agents together in a chemotherapeutic regimen, we investigated their effects on the dynamics of tubulin polymerization and cellular cytotoxicity, when administered singly or in combination. In human epidermoid carcinoma KB cells and MCF-7 breast carcinoma cells, we observed a time- and dose-dependent effect on cytoskeletal dynamics for both PTX and VBL. Tubulin polymerization induced by PTX was stable for more than 24 hr. When PTX treatment was followed by VBL, a time- and dose-dependent reversal of tubulin polymerization was observed. In contrast, rapid tubulin polymerization occurred when VBL was followed by PTX. When both agents were added simultaneously, a diminution of PTX-induced tubulin polymerization was observed with increasing doses of VBL; a maximum reduction was achieved when equal concentrations were used. Examination of the tubulin pattern by immunofluorescence in MCF-7 breast cancer cells confirmed and extended our findings. Bundle formation followed treatment with PTX. Addition of increasing concentrations of VBL prevented bundling; however, the normal cytoskeletal architecture was not restored. Cytotoxicity studies carried out using the median dose effect principles and the combination index analysis showed synergism when VBL and PTX were administered sequentially and antagonism for simultaneous administration. Our results demonstrate changes in tubulin dynamics following drug treatment and provide a rationale for combined PTX/VBL therapy after careful evaluation of the schedule of administration. *Int. J. Cancer* 75:57–63, 1998.
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The cytoskeleton of eukaryotic cells participates in a number of cellular processes, including motility, secretion and proliferation (Hyams and Loyd, 1993). Microtubules (MTs) are an integral part of the cytoskeleton and are the molecular target of several currently utilized anti-cancer drugs (Hamel, 1996). MTs are composed of 2 major soluble proteins, α- and β-tubulin, which are similar polypeptides, each with a molecular weight of approximately 50 kDa (Hyams and Loyd, 1993). MTs are formed from α- and β-tubulin heterodimers that polymerize in the presence of GTP and several microtubule-associated proteins (MAPs) (Olmsted, 1995).

Paclitaxel (PTX), a complex diterpene originally isolated from the western yew *Taxus brevifolia* (Wani *et al.,* 1971), is an active agent in a wide range of malignancies, including breast and ovarian cancer (Rowinsky and Donehower, 1995). PTX binds reversibly to MTs, with a stoichiometry approaching 1:1, relative to the tubulin heterodimer (Diaz and Andrew, 1993). In the absence of exogenous GTP or MAPs, PTX binding stabilizes MTs and promotes their assembly by decreasing the critical concentration of microtubule protein required to initiate polymerization (Schiff and Horwitz, 1981). The effects of PTX are unlike those of other plant-derived anti-neoplastic agents, such as the podophyllotoxins and the Vinca alkaloids, which inhibit tubulin polymerization (Hamel, 1996; Sackett, 1995). The availability of 2 classes of compounds with a similar target, but distinct effects, prompted us to investigate the interactions between PTX and the Vinca alkaloids. The present study describes the effects of these drugs administered singly, or in combination, on the dynamics of tubulin polymerization and cellular cytotoxicity.

MATERIAL AND METHODS

Cell lines and tissue samples

KB 3-1 epidermoid carcinoma cells (a Hela subclone) and MCF-7 breast cancer cells were cultured in RPMI-1640 and IMEM (Biofluids, Rockville, MD) growth media, respectively. The growth media were supplemented with 10% FBS, 1% Pen-Strep and 2 mM glutamine (GIBCO, Grand Island, NY).

Materials

PTX was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute and kept as a stock solution of 6 mg/ml in Cremophor EL, stored at 4°C. Vinblastine (VBL) was purchased from Sigma (St. Louis, MO). Mouse IgG monoclonal antibodies (MAbs) against chick brain α-tubulin or rat brain β-tubulin were purchased from Sigma. Horseradish peroxidase (HRP)-conjugated sheep anti-mouse Ig antibody was purchased from Amersham (Arlington Heights, IL). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody was purchased from Boerhinger Mannheim (Indianapolis, IN). Enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham. All other chemicals were of reagent grade and were purchased from Sigma.

Tubulin polymerization assay

To quantitate tubulin polymerization we developed a simple assay by modifying a method originally described by Minotti *et al.* (1991). Cells grown to confluency in 24-well plates were treated with drug for a specified time. After washing each well twice with 1 ml of PBS in the absence of Ca^{2+} or Mg^{2+}, the cells were lysed at 37°C for 5 min in the dark with 100 µl of hypotonic buffer (20 mM Tris-HCl, pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% Nonidet P-40, 2 mM PMSF, 200 U/ml Aprotinin, 100 µg/ml soybean trypsin inhibitor, 5 mM ε-amino caproic acid and 1 mM benzamidine). The wells were scraped and the lysates transferred to 1.5 ml Eppendorf tubes. Each well was rinsed with an additional 100 µl of the hypotonic buffer, and this volume was pooled with the previous lysate.

Following a brief but vigorous vortex the samples were centrifuged at 16,000 *g* for 10 min at room temperature. The 200 µl supernatants containing soluble or unpolymerized (cytosolic) tubulin were carefully separated from pellets containing insoluble or polymerized (cytoskeletal) tubulin and transferred to separate tubes. The pellets were resuspended in 200 µl of hypotonic buffer containing 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% Nonidet P-40 and the protease inhibitors described above. Each tube containing either the soluble or the polymerized fraction was mixed with 70 µl of 4× SDS-PAGE sample buffer (0.3 M Tris-HCL, pH 6.8, 45% glycerol, 20% β-mercaptoethanol, 9.2% SDS and 0.04 g/100 ml bromophenol blue) and heated at 95°C for 5–10 min; 20 µl aliquots of each sample were analyzed by

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SDS-PAGE, performed as described by Laemmli (1970), on a 10% resolving gel with a 3% stacking gel.

Following immunoblotting using a primary anti-tubulin MAb, the signal was quantitated by densitometry. In some experiments we observed 2 bands instead of one. This most likely represents resolution of α- and β-tubulin, both of which were detected by the antibodies used in the current study. Where 2 bands were detected, both of them were included in the quantitative analysis. The percent of polymerized tubulin (%P) was determined by dividing the value of polymerized tubulin by the total tubulin content (soluble and polymerized). Although most tubulin exists in the soluble form in untreated cells (Minotti *et al.,* 1991), the distribution of tubulin between the soluble and polymerized fractions could be altered to some degree by the experimental conditions. We determined that the presence of PTX in the lysis buffer or an increase in the temperature during harvesting increased the amount of tubulin in the polymerized fraction. Care was taken to maintain consistency from one experiment to the next, and each experiment was performed with the appropriate set of controls. In each experiment the percent of polymerized tubulin in the untreated cells was measured and used as a control value for comparison with values obtained after drug treatment. Identical results were observed whether a MAb against α- or β-tubulin was used for immunodetection.

Indirect immunofluorescence

Cells were plated on glass slides 1 day prior to treatment with drug for 8 hr. Cells were then fixed in 1:1 methanol/acetone for 5 min at room temperature and washed with PBS. Incubation with the primary anti-α-tubulin MAb for 1 hr was followed by a 30 min incubation with the secondary fluorescein-conjugated goat anti-mouse IgG antibody. All antibody incubations and washes were performed at room temperature.

Cytotoxicity assay and synergism analysis

Cytotoxicity assays were performed by seeding 500 cells/well in 100 μl of growth medium in 96-well microtiter plates. After 24 hr, cytotoxic agents diluted in 100 μl of the medium were added to each well, and the cells were incubated for an additional 48 hr, after which time the first drug was removed. Cells receiving sequential drug treatment had the second drug added for an additional 48 hr, while the others were grown in drug-free medium for an additional 48 hr.

Fixation and sulforhodamine B (SRB) staining were performed as previously described (Monks *et al.,* 1991). Survival curves were obtained with PTX and VBL alone and with combinations of both drugs at a constant 1:1 ratio. To determine synergism, additivity or antagonism for the combination of PTX and VBL, we used the median dose effect principles and the combination index method developed by Chou *et al.* (1994). This method takes into account both the potency of each drug alone or each drug combination and the shapes of the dose-effect curves. The dose ranges studied were chosen based on the IC$_{50}$ values for each drug individually, and the mathematical analysis was performed using a computer software program for automated analysis and simulation (Chou *et al.,* 1994).

RESULTS

Effect of paclitaxel and vinblastine on tubulin polymerization

To compare the effect of single, sequential or simultaneous administration of PTX and VBL on tubulin polymerization, we chose experimental conditions that resulted in a minimal basal level of polymerization. In this way, the sensitivity for detecting polymerization was maximized. The 2 cell lines used in the present study, KB3-1 and MCF-7, had similar total tubulin levels, as assessed by quantitative immunoblot (data not shown). Each experiment was performed at least 3–4 times, and a representative result is shown in each case. The drug doses used to treat the cells were estimated based on IC$_{50}$ values of the 2 drugs determined in a

4-day growth inhibition assay, and data in other cell lines indicate that the 1–24 hr IC$_{50}$ values are on average 10- to 50-fold greater (data not shown). These values were 0.7 and 0.5 ng/ml for PTX and 0.3 and 0.1 ng/ml for VBL, in KB3-1 and MCF-7 cells, respectively.

When PTX (20 or 200 ng/ml) was added alone to either KB 3-1 or MCF-7 cells (Fig. 1), a time- and dose-dependent increase of tubulin polymerization was observed in both cell lines. A maximum effect of PTX-driven tubulin polymerization was observed within 4 hr of treatment in both cell lines, whether 20 or 200 ng/ml PTX were used. Thus, the above conditions for PTX treatment were chosen to examine the effect of sequential administration of PTX and VBL on tubulin polymerization. In the experiments shown in Figure 2, KB3-1 cells were initially treated with 200 ng/ml PTX for 4 hr. After the 4 hr PTX incubation, fresh drug-free medium or medium containing 2–200 ng/ml VBL was added. Cells were harvested at different time points and the percent of tubulin in the polymerized form was determined. PTX treatment alone, represented by the 0 time point, which designates the end of the 4 hr PTX incubation, resulted in tubulin polymerization. This effect was stable during the 25 hr after PTX removal, in the absence of VBL or in the presence of 2 ng/ml VBL. A time- and dose-dependent depolymerization of tubulin was observed when PTX treatment was followed by treatment with higher (20 and 200 ng/ml) VBL concentrations. Cell death after 25 hr precluded an accurate determination of the degree of polymerization at this time point. Thus, PTX-induced polymerization persists after removal of PTX but can be reversed by the addition of VBL.
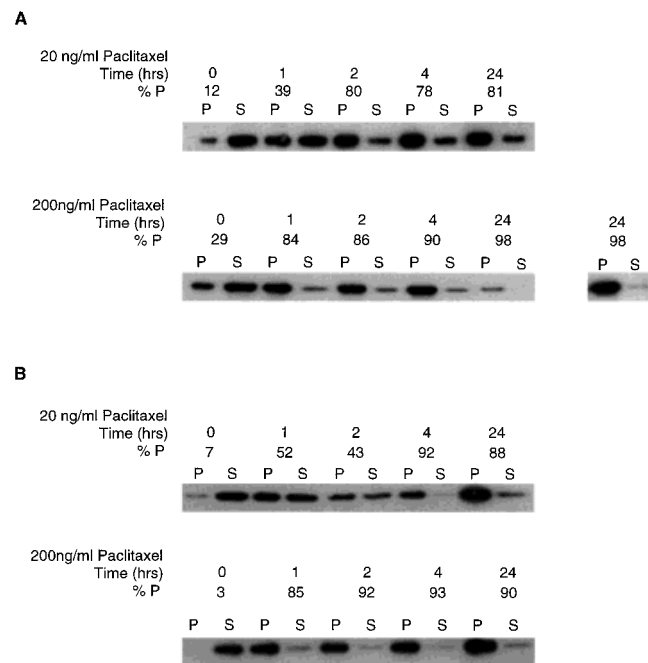

FIGURE 1 – PTX effect on tubulin polymerization in KB3-1 and MCF-7 cells. KB3-1 or MCF-7 cells (*a* and *b,* respectively) were treated with either 20 ng/ml PTX (upper row) or 200 ng/ml PTX (lower row), over a 24 hr period. Cells were harvested at different time points, and tubulin polymerization was assessed. For each experiment the different time points are: 0, untreated control cells, harvested at the same time as cells treated with PTX for 1 hr; 1, 2, 4 or 24, cells treated with PTX for 1, 2, 4 or 24 hr, respectively. In the second row of *a,* a darker exposure of the 24 hr time point is shown, enabling the acquisition of an accurate densitometric measurement. For each time point, the percent of polymerized tubulin (%P) was determined by dividing the value of polymerized tubulin by the total tubulin content (the sum of P and S).
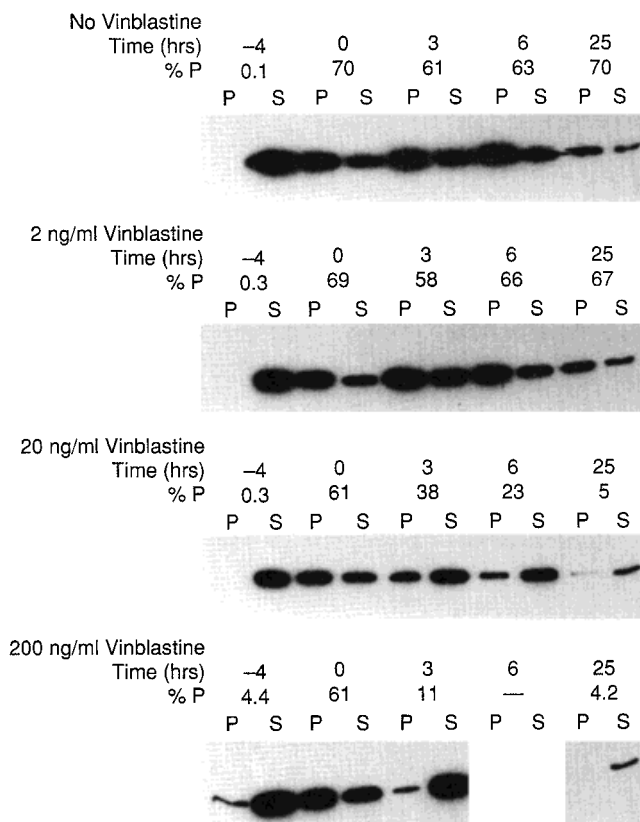
FIGURE 2 – Sequential treatment: VBL effect on tubulin polymerization in PTX-treated KB3-1 cells. Cells were treated with 200 ng/ml PTX for 4 hr, followed by incubation in drug-free medium or in 2–200 ng/ml VBL over a 25 hr period. Cells were harvested at different time points, and tubulin polymerization was assessed. For each experiment the different time points are: −4, untreated control cells, harvested at the same time as cells treated with PTX for 4 hr (0); 3, 6 or 25, 3, 6 or 25 hr chase with drug-free medium or VBL after PTX removal, respectively. The percent of polymerized tubulin (%P) was calculated as described in Figure 1.
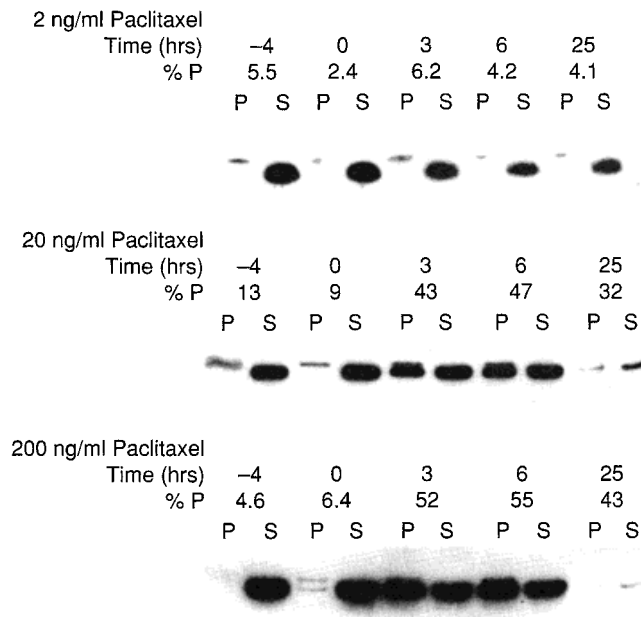

FIGURE 3 – Sequential treatment: PTX effect on tubulin polymerization in VBL-treated KB3-1 cells. Cells were treated with 20 ng/ml VBL for 4 hr, followed by incubation with 2–200 ng/ml PTX, over a 25 hr period. Cells were harvested at different time points, and tubulin polymerization was assessed. The different time points represent: −4, untreated control cells, harvested at the same time as cells treated with VBL for 4 hr (0); 3, 6 or 25, 3, 6 or 25 hr chase with PTX after VBL removal, respectively. The percent of polymerized tubulin (%P) was calculated as described in Figure 1.
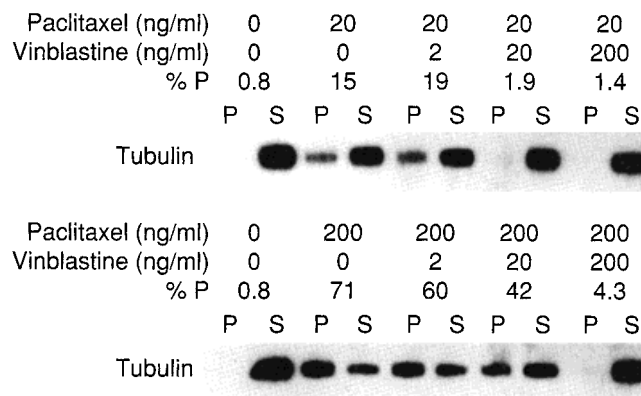

FIGURE 4 – Simultaneous treatment: effect of PTX and VBL on tubulin polymerization in KB3-1 cells. Cells were treated for 4 hr with a fixed PTX concentration of 20 ng/ml or 200 ng/ml PTX (upper and lower panel, respectively) and without or with increasing VBL concentrations (2–200 ng/ml). 0, 0, untreated control cells; 20, 0, 20 ng/ml PTX alone; 200, 0, 200 ng/ml PTX alone. Other combinations as indicated.

The effect of reversing the order of drug administration is shown in Figure 3. Treatment of KB3-1 cells with 20 ng/ml VBL for 4 hr followed by incubation with PTX also demonstrated time- and dose-dependent effects. The effect of 20 ng/ml VBL treatment alone is demonstrated at the 0 hr time point, which designates the end of the 4 hr VBL incubation. Little or no polymerization was observed during the ensuing 25 hr in the absence of PTX (data not shown) or in the presence of 2 ng/ml PTX. In contrast to PTX concentrations of 20 and 200 ng/ml, polymerization was observed within 3 hr.

Simultaneous administration of PTX and VBL is shown in Figure 4. KB3-1 cells were incubated with a fixed PTX concentration of 20 or 200 ng/ml, without or with increasing concentrations of VBL (2–200 ng/ml), for 4 hr prior to harvesting. The 4 hr treatment of KB3-1 cells with 20 ng/ml PTX in the absence of VBL resulted in polymerization of 15% of the total tubulin, compared with 71% when 200 ng/ml PTX was used. An apparent dose-dependent depolymerization was observed with increasing VBL concentrations. The percent of polymerized tubulin in cells incubated with equitoxic concentrations of PTX and VBL (20 or 200 ng/ml of each drug) is comparable to the levels observed in untreated cells.

Immunofluorescence of tubulin in MCF-7 cells

While these biochemical results suggested that the simultaneous addition of PTX and VBL prevented PTX-induced polymerization,

we wanted to assess the cellular morphology and the tubulin organization of the drug-treated cells by immunofluorescence. The results with MCF-7 breast cancer cells are shown rather than those with KB3-1 cells, since similar biochemical observations were made for each cell type, and the greater cytoplasmic to nuclear ratio and larger size of the MCF-7 cells provided a better display of cytoskeletal architecture. Untreated MCF-7 cells (control) demonstrate a well-organized tubulin network (Fig. 5a). Treatment with 200 ng/ml PTX for 8 hr leads to a collapse of the network with the
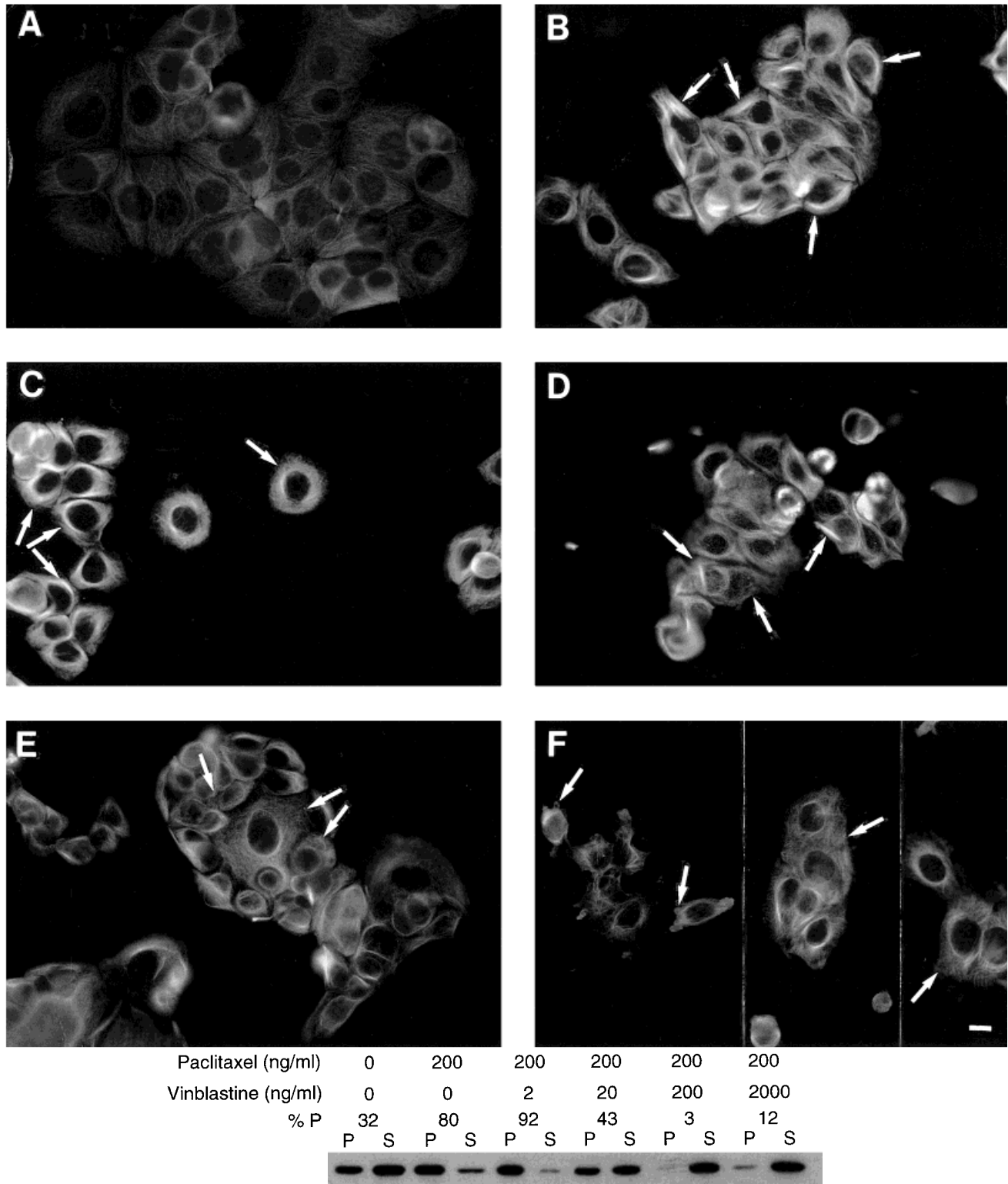
FIGURE 5 – Indirect immunofluorescence of microtubules in MCF-7 cells. Cells treated with 200 ng/ml PTX without or with increasing VBL concentrations for 8 hr were stained with anti α-tubulin after fixation. *(a)* No drug treatment (control). *(b)* 200 ng/ml PTX (arrows point to tubulin bundles). *(c)* 200 ng/ml PTX and 2 ng/ml VBL (arrows point to tubulin bundles). *(d)* 200 ng/ml PTX and 20 ng/ml VBL (the 2 upper arrows point to bundles, and the lower one points to a disorganized tubulin network). *(e)* 200 ng/ml PTX and 200 ng/ml VBL, (arrows point to a disorganized tubulin network). *(f)* 200 ng/ml PTX and 2,000 ng/ml VBL (the 2 left arrows point to blebbing cells, and the 2 right arrows point to a disorganized tubulin network). Below the immunofluorescence figure, an identical experiment performed using the tubulin polymerization assay is displayed. MCF-7 cells were treated with 200 ng/ml PTX for 8 hr, without or with increasing VBL concentrations (2–2,000 ng/ml). 0, untreated control cells; 200, 0, 200 ng/ml PTX alone; 200, 2, 200 ng/ml PTX with 2 ng/ml VBL; 200, 20, 200 ng/ml PTX with 20 ng/ml VBL; 200, 200, 200 ng/ml PTX with 200 ng/ml VBL; 200, 2,000, 200 ng/ml PTX with 2,000 ng/ml VBL. Scale bar = 10 μm.

appearance of microtubule arrays and tubulin bundles (Fig. 5*b*). The addition of increasing VBL concentrations results in a dose-dependent prevention of the PTX-induced bundle formation (Fig. 5*c–f*). However, although bundle formation occurs only infrequently, the addition of VBL results in a disrupted tubulin network (Fig. 5*e*) instead of restoration of the well-organized structure seen in untreated cells (compare Fig. 5*d–f* with Fig. 5*a*). When occasional bundle-like structures were seen, they were accompanied by blebbing of the plasma membrane (Fig. 5*f*), probably indicating compromised cell viability. Thus, while VBL can prevent PTX-induced bundle formation, the 2 drugs together lead to cytoskeletal disorganization.

To confirm the data obtained by immunofluorescence, we performed exactly the same experiment in MCF-7 cells using the tubulin polymerization assay. Eight hour treatment of MCF-7 cells with 200 ng/ml PTX resulted in polymerization of 80% of the total tubulin, whereas an apparent dose-dependent depolymerization was observed with increasing VBL concentration. When PTX was combined with the same or a higher concentration of VBL (200 or 2,000 ng/ml, respectively), the percent of polymerized tubulin was lower than that observed in the untreated cells, which confirms the observation that the normally well-organized cell architecture was not restored (Fig. 5*e,f*). Similar results were observed with both cell lines (data not shown).

Combination cytotoxic effects of PTX and VBL

Having demonstrated an interaction between PTX and VBL on tubulin polymerization and cytoskeletal organization, we investigated their effects on cellular cytotoxicity in KB3-1 and MCF-7 cells. The cytotoxic interaction between PTX and VBL administered sequentially or simultaneously over a period of 4 days was assessed. Since PTX and VBL bind to different sites on the same macromolecular target, their combination could lead to synergism, additivity or antagonism, depending on the nature of the interactions. For this type of analysis and for each drug separately (*i.e.,* PTX or VBL alone), we measured how the fraction affected (FA; *i.e.,* fractional cell growth inhibition) varied with differing doses. For 2 drugs in combination (*i.e.,* PTX and VBL), we varied the doses of the 2 drugs while monitoring the FA; however, the doses were varied such that a constant ratio of drug 1 to drug 2 was maintained. The dose ratio of the 2 drugs was fixed at 1:1, based on median dose concentrations determined in preliminary experiments. The combination index (CI) equation evaluates the interaction of the 2 cytotoxic drugs. A plot of FA on the horizontal axis and of CI on the vertical axis yields a complex curve describing the extent of synergism, antagonism or additivity at each effect level. A CI value greater than 1 indicates antagonism and a value less than 1 indicates synergism; a value of 1 indicates additivity between the 2 drugs used in the combination.

From previous unpublished observations in which the same drug was used in the combination as drug 1 and drug 2, we have determined that the range of additivity lies between CI values of 0.75 and 1.25 (Fig. 6, dotted lines). The data were calculated using both the mutually exclusive assumption (*i.e.,* similar mechanism of action and/or resistance) and the mutually non-exclusive assumption (*i.e.,* dissimilar mechanism of action and/or resistance). When exclusivity is unknown, the CI value is routinely calculated in both ways. The combination index results are summarized in Figure 6. Sequential drug administration (Fig. 6*a,b*) is compared with a schedule whereby each drug is administered alone for 2 days (PTX/0, 0/VBL or VBL/0, 0/PTX), while co-administration of the 2 drugs is compared with a schedule whereby each drug is given alone for 4 days (PTX/PTX and VBL/VBL). Sequential administration of PTX and VBL resulted in synergism, independent of the order of administration (Fig. 6*a,b*). In contrast, when PTX and VBL were given together, antagonism was obtained. This was true for both cell lines, in agreement with the similar biochemical data obtained for the 2 cell lines. In addition, when simultaneous

administration of PTX and VBL was compared with sequential administration (Fig. 6*d*), antagonism was also obtained for KB3-1 cells with at best additivity for MCF-7 cells, indicating that co-administration of PTX and VBL is the least preferential combination.

DISCUSSION

The present studies were undertaken to investigate the potential use of both PTX and a Vinca alkaloid in a chemotherapeutic regimen. Although PTX and VBL share the same cellular target, the cytoskeletal protein tubulin, they have distinct non-overlapping binding sites and different modes of action (Hamel, 1996; Sackett, 1995). PTX has been shown to bind to the microtubule polymer stabilizing MTs and promoting their assembly (Rao *et al.,* 1994), whereas VBL binds to the tubulin dimer and causes depolymerization of MTs into free tubulin subunits (Sackett, 1995; Toso *et al.,* 1993). Since each drug shifts the dynamic equilibrium that normally exists in cells between polymerized and soluble tubulin in an opposite direction, we examined their effect on tubulin polymerization.

PTX caused a dose- and time-dependent polymerization, and a similar pattern was observed for the KB3-1 and MCF-7 cell lines. A concentration of 20 ng/ml was able to cause maximal polymerization by 4 hr, while 200 ng/ml of PTX exhibited a maximal effect within 1 hr of exposure. In contrast, when 2 ng/ml PTX was used, no increase of polymerization was observed, even after 25 hr of exposure. These observations correlate with a lack of significant cytotoxicity of the 2 ng/ml dose and indicate that a critical concentration is required to initiate polymerization. To determine the optimal schedule for the administration of 2 tubulin active agents in combination we examined the effect of sequential drug administration on drug activity. A schedule whereby PTX precedes VBL was examined by treating KB3-1 cells with 200 ng/ml PTX for 4 hr followed by VBL. PTX alone caused significant tubulin polymerization, which was stable over a 25 hr observation period, in the absence of drug or in the presence of 2 ng/ml VBL. In contrast, although cytotoxicity studies had shown that PTX and VBL were equitoxic, 20 ng/ml VBL was able to reverse the effect of 200 ng/ml PTX almost completely by 25 hr, while 200 ng/ml of VBL reversed the effect of PTX within 3 hr. A similar pattern was observed when VBL treatment preceded PTX administration. Rapid tubulin polymerization occurred with 20 ng/ml PTX following 20 ng/ml of VBL, and this effect was maximal since a PTX concentration of 200 ng/ml did not increase polymerization further. These observations suggest that with sequential administration, each drug retains activity so that pretreatment with one class of antimitotic drugs does not preclude the other drug's effect on tubulin.

We also determined the effect of co-administration of PTX and VBL and found that tubulin polymerization induced by PTX was prevented when both PTX and VBL were administered simultaneously. The immunofluorescent studies demonstrated that a disorganized pattern was evident rather than a normal cytoskeletal architecture, suggesting that both drugs had some effect but that neither had achieved its maximum activity. These results, together with those discussed in the previous paragraph with sequential drug administration, support the cytotoxicity data demonstrating synergism when PTX and VBL were administered sequentially, but not when the 2 drugs were given simultaneously. In our models, synergism with sequential administration was observed with both cell lines and with either sequence of administration, when comparably toxic doses were utilized. Our data clearly demonstrate that co-administration is not indicated, as shown by inhibition of PTX induced polymerization and the antagonism demonstrated by the combination index analysis.

Recent studies have used MT depolymerizing agents in combination with PTX. In agreement with the present results, the sequential
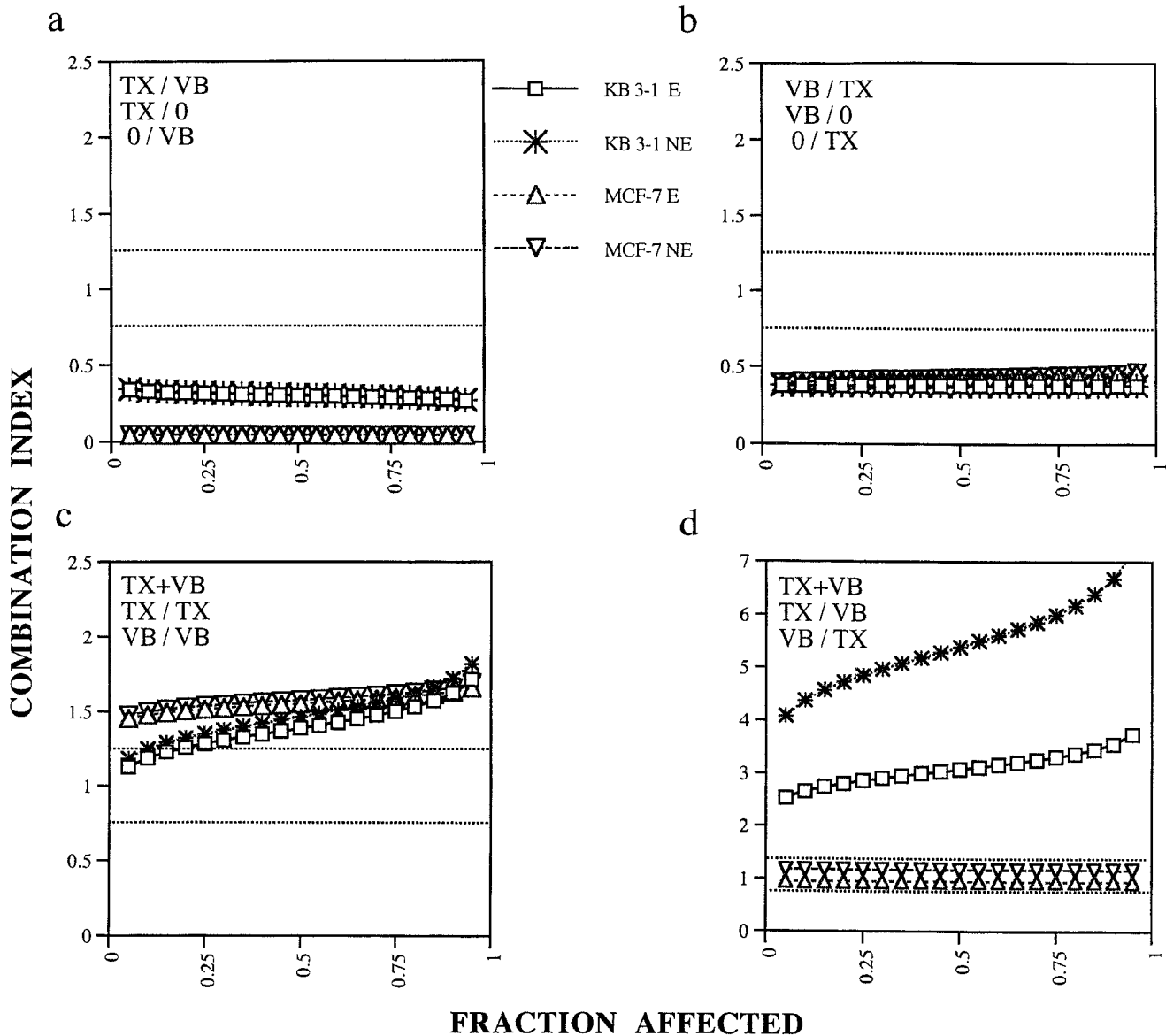
FIGURE 6 – Combination index plots for the combination of PTX and VBL in KB3-1 and MCF-7 cells. Computer-simulated combination index (CI) at various effect levels (FA or fraction affected) for the combination of PTX or VBL, in KB3-1 and MCF-7 cell lines. For each cell line the CI is calculated using assumptions for the drug action both mutually exclusive (E) and mutually non-exclusive (NE). KB3-1 and MCF-7 cells were treated with drug for 4 days. *(a)* Sequential drug exposure with PTX treatment prior to that of VBL. *(b)* Sequential drug exposure with VBL treatment prior to that of PTX. *(c)* Simultaneous drug exposure. *(d)* Simultaneous drug exposure compared with sequential drug exposure, both when PTX is followed by VBL and when VBL is followed by PTX. The symbols used in the graphs (open squares, asterisks and open triangles) represent the actual combination data points, and the curve that connects them was obtained by computer simulation. Dotted lines in each graph across CI values 0.75 and 1.25 represent the range of drug additivity. In *a* and *b,* the data points for each cell line and for each set of calculations (E *vs.* NE) are so close together that the graphs almost overlap.

administration of vinorelbine tartrate and PTX was additive *in vivo* against P388 murine leukemia cells, independent of the order of administration of the 2 drugs (Knick *et al.,* 1995). Also, in agreement with our findings, the simultaneous administration of *vincristine* plus PTX in the human teratocarcinoma cell line 833K was antagonistic (Chou *et al.,* 1994). However, unlike the results described herein, the co-administration of VBL and PTX to human non-small cell lung cancer cell lines did not produce antagonism (Viallet *et al.,* 1996), while the co-administration of vinorelbine and PTX for 1 hr to human melanoma cell lines resulted in synergism (Photiou *et al.,* 1997). The difference in the experimental models used and in the latter case the short time of exposure

might account for the apparent discrepancies. Taken together, the published data and the current study provide evidence that these 2 classes of compounds retain activity when administered together. Preliminary clinical studies suggest that the preclinical data may be predictive of clinical results. Studies combining a taxane (PTX or docetaxel) with a Vinca alkaloid (vinorelbine) are currently under way in numerous centers; the preliminary data indicate that some schedules may be more effective (Bissery *et al.,* 1996; Fumoleau *et al.,* 1996; Kourousis *et al.,* 1996; Weiselberg *et al.,* 1996). However, a final answer will require a randomized trial. Caution is warranted, and a combination of an MT stabilizing and destabilizing agent should be pursued only after careful evaluation of

efficacy, especially in diseases in which both are active as single agents.

The availability of 2 classes of tubulin acting agents (taxanes and vincas) with a similar target but separate effects remains to be exploited efficiently. *In vitro* evidence suggests that when the resistance is mediated by tubulin, collateral sensitivity can be expected for the alternate class of tubulin targeting agents (Minotti *et al.,* 1991). While this offers the possibility of exploiting any emerging resistance by means of an agent to which such cells may be collaterally sensitive, the present results suggest that the schedule of administration must be carefully considered. It is hoped that these studies will aid in the design of such trials.

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