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Pattern analysis of microtubule-polymerizing and -depolymerizing agent combinations as cancer chemotherapies

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Abstract. Subcellular distribution of mass can be analyzed by a technique that involves culturing cells on interferometers and digitizing their interference contours. Contour sampling resulted in 102 variables per cell, which were predictors of oncogenic transformation. Cell phenotypes can be deconstructed by use of latent factors, which represent the covariance of the real variables. The reversal of the cancer-type phenotype by a combination of microtubule-stabilizing and -depolymerizing agents was described previously. The implications of these results have been explored by clinicians who treated patients with the combination of docetaxel and vinorelbine (Navelbine®). The current study was performed to determine the effects of different combinations on phenotype and in phases of the cell cycle other than mitosis. Combinations of paclitaxel with either colchicine, podophyllotoxin, nocodazole, or vinblastine caused phenotype reversal. Paclitaxel analogue, 7-deoxytaxol, by itself caused reversal. Factors #4, (filopodia), #5 (displacement and/or deep invaginations in the periphery), #8, and #12 took on values typical of normal cells, whereas the values of #7 (p21-activated kinase), and #13 (rounding up) shifted toward the cancer-type. All combinations altered microtubule arrangement at the cell edge. Delivery schedules and drug ratios used in clinical studies were subjected to analysis. Clinical response rates were better when the combination was not interspersed with a single agent ($P=0.004$). The results support the idea that efficacy depends upon simultaneous exposure to both agents, and suggest a novel mechanism for combination therapies. These therapies appear to restore in transformed cells some of the features of a contact-inhibited cell, and to impede progress through the cell cycle even when provided at nanomolar concentrations.

Introduction

Microtubule inhibitors are among the most commonly used chemotherapeutic drugs. Until recently, the mechanism of cytotoxicity has been assumed to rely upon interference with spindle formation. This may prevent the cells passing through a spindle checkpoint and cause mitotic arrest (1). It is thought that, subsequently, they may enter a cell death pathway. Cells from similar cell lines differed in their ability to arrest at M phase (2), however. When certain cell types are treated with high levels of anti-mitotic agents, they fail to sustain mitotic block and reconstitute a nucleus with a hyperdiploid chromosomal complement (3,4). As a result, such cells retain the chromosomal complement of a G2/M population (5,6). Both agents that enhance polymerization and those that promote depolymerization increase the number of multinucleated cells (7). Although both microtubule-polymerizing and -depolymerizing agents perturb progression through mitosis, there are occasional reports of paclitaxel affecting G1- and G2-phase cells (8). The current study was performed to explore microtubule reorganization occurring as a result of microtubule inhibitor combinations affecting cells in interphase of the cell cycle.

The microtubule is composed of protofilaments, each one made up of heterodimers assembled head-to-tail. The heterodimer is in turn made up of non-covalently bonded subunits, α - and β -tubulin. A GTP moiety is associated with β -tubulin. Its hydrolysis destabilizes the side-to-side binding between subunits, thereby limiting the lifetime of the microtubule. Microtubules show distinct kinetics at opposite ends, adding subunits faster at the plus end than the minus end. Some microtubules are anchored by their minus end in the centrosome or microtubule organizing center. Most microtubules are in a state of continuous polymerization or depolymerization (9). Moreover, a behavior called dynamic instability is characterized by periods of rapid disassembly (10,11). The sum of growth and shrinkage is called dynamicity. Dynamicity may be important for mitotic spindle assembly, as a 'search-and-capture' mechanism is thought to facilitate binding between the microtubule and the kinetochore (12). At low concentrations, paclitaxel inhibits dynamicity (13,14).

Paclitaxel decreases the magnitude of the dissociation constant for tubulin at both ends of the microtubule (15) and,

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at high concentrations, causes assembly of all the free tubulin and induces side-to-side aggregation of microtubules (16). It also arrests cell division and promotes apoptosis (17). At high concentrations, colchicine, vinblastine, podophyllotoxin, and nocodazole cause microtubule depolymerization but, like paclitaxel, they impair dynamicity at lower concentrations (3,5,13). All depolymerizing compounds used in this study had distinct binding sites on tubulin, with the exception of podophyllotoxin and colchicine (18). Even for these compounds, however, the mechanisms of action differ. Podophyllotoxin inhibits GTPase activity while colchicine enhances it (19). The reduction in microtubule dynamicity is generally considered important to the mechanism of cell killing by anti-mitotic drugs. Consistent with this hypothesis is some evidence showing that the level of circulating docetaxel in the first course of treatment is positively related to the time to progression in non-small cell lung cancer patients (20). Since the level was typically reduced to the low nanomolar range by one week after dose administration, docetaxel may have caused a persistent reduction of dynamicity (21). However, the evidence does not suggest that higher dosages of depolymerizing drugs, which affect microtubule integrity rather than dynamicity, are less effective therapeutically (see Discussion).

Previous studies from this laboratory suggested that, in samples obtained from lines that underwent transformation over a long period of *in vitro* culture, time could be used as an independent variable to define differences between the transformed and the normal phenotypes. The resulting standard curve of morphogenetic changes could be used to analyze the phenotypes of cells after experimental treatment, as described previously (22-27). If the phenotypes were altered to resemble long-term changes, the effect was considered promoter-like. Such an effect was found after colchicine treatment (26). Cells exposed to colchicine with a molar excess of paclitaxel showed the opposite effect, and this treatment was therefore considered an anti-promoter (24). Reversal of the shape phenotype was reported using this combination on unsynchronized cell populations. Thus, the greatest fraction of the population was necessarily composed of cells in the G1, S, and G2 phases (24,28). These and other studies (7) gave clinicians a rationale to conduct tests of such inhibitor combinations. A vinblastine analogue, 5'-noranhydrovinblastine, was used as the depolymerizing agent in clinical studies (29-51; Culine S, *et al.*, *Am Soc Clin Oncol*: abs. 515, 1998). Since phenotype reversal implies that interphase cells show an unusual response to microtubule inhibitor combinations, the current study investigates the question of whether microtubule function is integrally bound up with key structural and cytoskeletal characteristics of the cells. To investigate this hypothesis, the studies explore the phenotype and shape features of cells and draw comparisons where possible with the efficacy of inhibitor combination chemotherapy.

Materials and methods

Cell culture. The IAR20 PC1 line was derived from the liver of inbred BD-VI rats. It was grown in Williams E medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum, as previously described (22,52,53).

Microtubule inhibitors. All chemicals were from Sigma-Aldrich Company (St. Louis, MO) unless otherwise noted. Colchicine, nocodazole, podophyllotoxin, and vinblastine sulphate were made up and stored at -20°C , as previously described (28). Paclitaxel (Taxol[®]) was obtained from the Development Therapeutics Program, Division of Cancer Treatment, National Cancer Institute and from Dabur, Inc., India. In preliminary experiments, we had used paclitaxel analogue, 7-deoxytaxol, which reversed the phenotype to a similar extent as did the combination of paclitaxel and colchicine. It was purchased from the LC Laboratories (Woburn, MA) and made up like paclitaxel. It was reported previously that substituting taxanes, baccatin III or cephalomannine, for paclitaxel did not lead to phenotype reversal (28). Therefore, paclitaxel was the only microtubule-stabilizing compound used in combination treatments.

Computerized morphometry. Cells were plated on Tolansky substrates in replicate dishes and left overnight. They were exposed to various combinations of agents for 2 h and then fixed, dried, and viewed as previously described (22,27). Interference contours were derived from each cell and primary shape variables were extracted as previously described (53). To determine whether inhibitor combinations affected cell shape, latent factors were computed by a principal components procedure, Proc Factor, in SAS software using a database that included representatives of both highly malignant and precancerous cells (27). The 'Score' procedure was used to compute factor scores for each cell in an experiment based on the primary variable values. We determined the value of the overall cancer-type phenotype for each cell, in units of days in culture, using an equation based on the factor scores. Differences among group means were evaluated for statistical significance by procedures in the Statistical Applications Software (54).

Microtubule arrangement. To determine whether the reversal effect of inhibitor combinations was related to the arrangement of microtubules, we imaged the microtubule arrays. Cells were subcultured onto glass coverslips, left for 18-48 h to attach, and either treated with various agents for 2 h or with solvent alone. Concentrations of $6\ \mu\text{M}$ paclitaxel and $2\ \mu\text{M}$ depolymerizing agent were used, unless otherwise noted. In separate experiments, lower concentrations of compounds were supplied for 20 h. The coverslips were collected by methanol immersion at -70°C . N357 monoclonal antibody against β -tubulin from Balb/C57 mouse (Amersham Biosciences, Piscataway, NJ) was diluted 1:600 for use. Secondary antibodies, FITC-conjugated goat anti-mouse (U.S. Biochemical Corp., Cleveland, OH) or Alexa 488-conjugated goat anti-mouse (Molecular Probes, Eugene, OR), were used as previously described (28). Representative cells were photographed on a Zeiss Axiophot microscope equipped with x63 and x100 Neofluar lenses, using Kodak Ektachrome film or else recorded with a Princeton Instruments RTE/CCD camera and IBM-PC running MetaMorph 4.6r5 software (Universal Imaging Corp., Buckinghamshire, UK).

Analysis of clinical reports. Clinical reports issued by 23 research groups, collectively including 909 patients who

underwent a course of combination therapy, were compiled. Combinations of microtubule-polymerizing and -depolymerizing agents consisted of docetaxel or paclitaxel with vinorelbine (Navelbine®). Commonly reported variables such as dosage levels and dosing schedules, as well as the duration of treatment, the length of treatment cycle, and the drug ratio were tabulated. The sum of partial and complete response was designated as the dependent variable. Generalized linear model regression was used to evaluate the ability to predict the dependent variable's values on the basis of the other variables. Interactions among the various variables were also tested, and the results graphed using Minitab software (State College, PA).

Effect of microtubule inhibitors on the cell cycle. The effect of microtubule inhibitors on the cell cycle was studied in synchronized cell populations. Cells were subcultured at 2×10^6 per 100-mm dish and allowed to remain for 15-30 h to enter log phase. To arrest the population in S phase, aphidicolin was added to a final concentration of $1 \mu\text{g/ml}$. After 12-14 h, cultures were washed twice with incubation for 20 min between washes, and then treated with microtubule inhibitors. Beginning 4 h later, the cells were harvested by replacing the medium with a trypsin solution. Soybean trypsin inhibitor was used at a final concentration of $25 \mu\text{g/ml}$ to terminate the trypsin digestion. For cell counts, the suspension was diluted 1:9 in saline and counted in a Coulter counter. For flow cytometry, the cultures were prepared according to the method of Vindelov and coworkers (55). Samples were analyzed for DNA content with a Beckman Coulter EPICS XL-MCL flow cytometer (Miami, FL), and data were analyzed using Multicycle AV (Phoenix Flow Systems, San Diego, CA).

The frequency of aberrant metaphase plates and multinucleated cells was determined 48 h after treatment with the combination of colchicine and paclitaxel or with these single agents. Cells were subcultured as above into 60-mm plastic tissue culture dishes and then treated with the agents. The cells were fixed in ethanol:glacial acetic acid (7:1), stained with 10% Giemsa solution, mounted in ImmunoFluore (ICN, Costa Mesa, CA) and viewed on a Zeiss Axiophot microscope (Thornwood, NY) equipped with a x40 Planapo lens. At least 200 mitotic cells were counted in each sample.

Results

Cancer-type reversal by inhibitor combinations. Previous results showed that inhibitor combinations composed of paclitaxel and colchicine caused reversal of the transformed cell phenotype (28). The combination of paclitaxel with different depolymerizing agents was studied in the current experiments. The two inhibitors were also supplied with a molar excess of paclitaxel or on an equimolar basis. All combinations caused reversal that was significant at the 98% level of confidence. The greatest effect was observed after the equimolar combination of paclitaxel and colchicine, although the 3:1 combination also reversed the phenotype. Paclitaxel analogue, 7-deoxytaxol, had an equally great effect. The samples treated with equimolar paclitaxel and colchicine, or a high concentration of 7-deoxytaxol alone, separated from the combinations composed from paclitaxel

Table I. Phenotype classification with significance testing by Duncan's multiple range test.^a

Duncan grouping	Predicted time (days)	Combination of agents (sample)
A	146.32	Control (solvent only)
B	124.38	Podophyllotoxin + paclitaxel (1:3)
B	120.33	Podophyllotoxin + paclitaxel (1:1)
B C	117.82	7-deoxytaxol ($6 \mu\text{m}$)
B C	116.62	Colchicine + paclitaxel (1:3)
B C	111.13	Nocodazole + paclitaxel (1:3)
B C	108.88	Nocodazole + paclitaxel (1:1)
B C	107.96	Vinblastine + paclitaxel (1:3)
B C	101.89	Vinblastine + paclitaxel (1:1)
C	94.70	7-deoxytaxol ($18 \mu\text{m}$)
C	94.29	Colchicine + paclitaxel (1:1)

^aMeans with the same letter are not significantly different at the level $P < 0.02$.

and podophyllotoxin on a statistically significant basis (Table I).

Deconstruction of phenotype reversal effects. As shown in previous studies (27,28,56), the phenotype of cells could be broken down by analysis of latent factors. Factors show a one-to-one correspondence to cell features. Thus, features undergoing promotion or reversion in an experiment, relative to the control, could be identified by this method. Of all the factors correlated with cancer-type phenotype, #4 accounted for the greatest proportion of transformation-related variance. It measured the prevalence of flat, tapering extensions called filopodia. The samples treated with equimolar paclitaxel and colchicine, or a high concentration of 7-deoxytaxol alone, significantly affected #4 values at the 98% level. Their values were reverted to values like those of normal cells. In addition, one of the treatments with paclitaxel and podophyllotoxin appeared to affect factor #4 values. In the case of factor #5 values, six out of the ten samples were also reverted toward the normal phenotype (Table II). Most of these changes were also significant at the 98% level. With respect to a third edge feature, #7, the phenotype came to resemble that of transformed cells. Equimolar combinations of paclitaxel with colchicine, nocodazole, and vinblastine, as well as certain 3:1 combinations with various agents, significantly elevated its values. Although none of the samples' values were shifted toward the normal type, 3:1 molar combinations of paclitaxel with colchicine or nocodazole were indistinguishable from the sample treated with solvent alone (Table II).

The above data indicated subtle differences between treated cells and control cells with respect to edge features. Some of the additional changes identified by factor analysis, namely #1, #8, and #12, reflected differences in the processing of endocytosed fluids (26,27). Analysis of the treated populations showed that the agents generally had little effect on factor

Table II. Factors indexing cell edge features that are altered significantly by treatment with microtubule inhibitor combinations.

Factor number, definition	Variance explained	Change in transformation	Value	Combination of agents (ratio)
#4, filopodia/microspikes	24%	↓	-1.2 ^{a,h}	7-deoxytaxol (18 μ M)
			-1.2 ^{a,h}	Colchicine + paclitaxel (1:1)
			-1.2 ^a	Podophyllotoxin + paclitaxel (1:3)
			-1.6 ^b	Control (solvent treated)
#5, blunt or long projections or large invaginations	5%	↑	-0.6 ^c	Control (solvent treated)
			-1.1 ^d	Colchicine + paclitaxel (1:1)
			-1.1 ^{d,h}	Podophyllotoxin + paclitaxel (1:1)
			-1.1 ^{d,h}	Vinblastine + paclitaxel (1:3)
			-1.2 ^{d,h}	Nocodazole + paclitaxel (1:1)
			-1.3 ^{d,h}	Nocodazole + paclitaxel (1:3)
			-1.5 ^{d,h}	7-deoxytaxol (18 μ M)
#7, p21-activated kinase-dependent protrusions	9%	↑	0.64 ^{e,h}	Colchicine + paclitaxel (1:1)
			0.30 ^{e,f,h}	Podophyllotoxin + paclitaxel (1:3)
			0.21 ^{e,f,h}	Nocodazole + paclitaxel (1:1)
			0.14 ^{f,h}	Vinblastine + paclitaxel (1:1)
			0.11 ^{f,h}	7-deoxytaxol (18 μ M)
			0.065 ^{f,h}	7-deoxytaxol (6 μ M)
			0.056 ^{f,h}	Vinblastine + paclitaxel (1:3)
			-0.46 ^g	Control (solvent treated)

^{a-g}Samples designated by the same letter are indistinguishable at the level of significance $P < 0.05$. Samples that were indistinguishable from control are not shown. ^hSample means differ from that of control at the level of significance $P < 0.02$.

Table III. Factors for internal and global features that are altered significantly by treatment with microtubule inhibitor combinations.

Factor number, definition	Variance explained	Change in transformation	Value	Combination used (ratio)
#8, spiky structures in 2nd contour	4%	↓	2.76 ^{a,k}	7-deoxytaxol (18 μ M)
			2.71 ^{a,k}	Nocodazole + paclitaxel (1:1)
			1.47 ^b	Control (solvent treated)
#12, hollowed-out structures in 2nd contour	4%	↓	10.4 ^{c,k}	7-deoxytaxol (18 μ M)
			10.3 ^{c,k}	Nocodazole + paclitaxel (1:1)
			10.2 ^{c,d,k}	Vinblastine + paclitaxel (1:3)
			10.1 ^{c,d,k}	Colchicine + paclitaxel (1:1)
			9.6 ^{c,d,e,k}	Nocodazole + paclitaxel (1:3)
			9.4 ^{d,e,f,k}	Colchicine + paclitaxel (1:3)
			9.1 ^{e,f,k}	Vinblastine + paclitaxel (1:1)
			8.9 ^{e,f,k}	Podophyllotoxin + paclitaxel (1:3)
#13, overall rounding up	7%	↑	7.9 ^g	Control (solvent treated)
			2.82 ^{h,k}	7-deoxytaxol (18 μ M)
			2.73 ^{h,i,k}	Vinblastine + paclitaxel (1:3)
			2.52 ^{h,i,k}	Nocodazole + paclitaxel (1:1)
			2.51 ^{h,i,k}	Colchicine + paclitaxel (1:1)
			2.48 ^{h,i,k}	Nocodazole + paclitaxel (1:3)
			2.07 ^{i,k}	Colchicine + paclitaxel (1:3)
1.86 ^j	Control (solvent treated)			

^{a-j}Samples designated by the same letter are indistinguishable at the level of significance $P < 0.05$. Samples that are indistinguishable from control are not shown. ^kSample means differ from that of control at the level of significance $P < 0.02$.

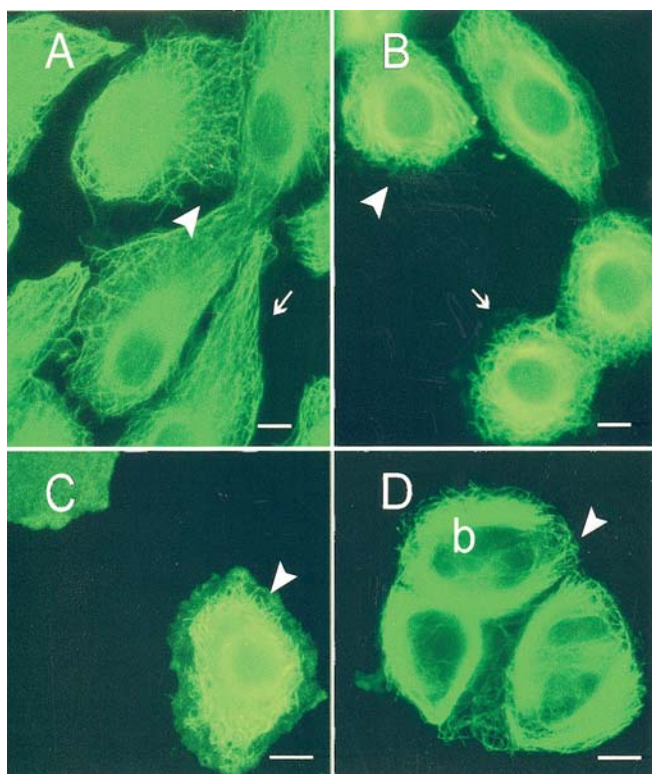


Figure 1. Combinations of paclitaxel and a depolymerizing agent caused microtubule rearrangement. (A) Control cells showed peripheral microtubules curving around in the direction of the cell margin (arrow). In areas where cells were in contact, microtubules went straight out toward the cell edge (arrowhead). (B) After treatment with 2 μ M paclitaxel and 2 μ M vinblastine, cells had peripheral microtubules extending perpendicular to the edge (arrowhead). In some cells, the microtubule ends were seen 1-3 μ m internal to the edge of the cytoplasm (arrow). (C) After treatment with 2 μ M paclitaxel and 2 μ M podophyllotoxin, some cells showed complete microtubule depolymerization (upper left). In others, microtubules were concentrated in the center, and the cell edge was outlined by free tubulin. Microtubules pointed out toward the cell edge, but were well behind the edge (arrowhead). (D) After treatment with 0.6 μ M paclitaxel and 0.2 μ M nocodazole, the microtubules assembled into bundles (b) in the central portion of the cytoplasm. They still terminated in individual microtubules that extended to the cell edge. Bar = 10 μ m.

#1 values. Only a sample exposed to equimolar colchicine and paclitaxel differed significantly from control, suggesting promotion of a transformed property (data not shown). The opposite was true of factor #8. Here, only a 7-deoxytaxol and a paclitaxel- and nocodazole-treated sample exceeded the values for control. With respect to #12, all samples except those exposed to podophyllotoxin and paclitaxel differed significantly from control, indicating reversal relative of the phenotype. Factor #13 (overall rounding up) was affected by the unequal vinblastine and paclitaxel combination and both treatments with colchicine and nocodazole. Elevated values reflected changes in the direction taken by cancer-type cells (Table III).

Microtubule rearrangement. Cell features, evaluated above by factor values, were compared with patterns of rearrangement of microtubules in cells treated with the inhibitor combinations. In control cells, microtubules were typically arranged parallel to the cell edge. In areas of cell contact, however, they extended out toward the neighboring cell. Cells exposed to all

microtubule inhibitor combinations showed zones of marginal cytoplasm that were clear of microtubules (Fig. 1A and B). This effect was particularly obvious after podophyllotoxin exposure. Cells treated with the paclitaxel and podophyllotoxin combinations showed evidence of microtubule destabilization, and they were occasionally filled with depolymerized tubulin. As noted previously (28), combinations containing a molar excess of paclitaxel over a depolymerizing agent caused side-to-side aggregation (bundling) of microtubules. However, combinations of podophyllotoxin and paclitaxel did not show bundling at either ratio (data not shown). Samples treated with all combinations showed a tendency to have microtubules extending straight out toward the cell edge (Fig. 1B-D). In addition to short-term treatment with high concentrations of inhibitors, we treated cells with 200 nM of each depolymerizing agent in combination with 600 nM paclitaxel over a longer period. Although 3:1 combinations of inhibitors permitted more bundling after long-term than short-term exposures, effects were otherwise similar. Again, podophyllotoxin was an exception, and complete depolymerization of the microtubules occurred in some cells.

Analysis of chemotherapy clinical trials. The microtubule-polymerizing and -depolymerizing drug combinations showed greater efficacy than the same agents delivered singly (57,58). To determine what, if any, relationship existed between therapeutic efficacy and the reversal of cancer-type features, we tabulated the objective response rate reported in clinical trials employing a combination of vinorelbine and either docetaxel or paclitaxel. The variables, dosage level, ratio of drugs, length of treatment cycle, complexity of delivery schedule, and duration of treatment, were compared with objective response rate for 23 phase I, II, and III trials. There were several variables that showed no relationship to the average response rate (tests 1-4, Table IV). Neither the dose level of either drug alone nor the ratio of drugs showed a relationship to the average response rate. Response rate was significantly related to the duration of treatment, however ($P=0.012$). Since some protocols allowed treatment termination in the case of patients who did not respond to treatment, or an extended course of treatment for patients who showed an objective response, the duration may have been affected by the rate of early responses.

When we tested the relationship of other variables to the response rate, breast cancer trials characteristically showed a greater response rate than trials on pooled cancers for other sites (test 6, $P<0.015$). Moreover, inspection of the data suggested that protocols consisting only of the two-drug combination, delivered as simultaneously as possible, were more efficacious than other protocols (test 7, Table IV). Since the depression of response rate in the protocols employing a single drug may have been complicated by the fact that all cancer sites were pooled for analysis, the interaction between site and response rate was explored. The variables, site and schedule complexity, interacted to affect response rate at a level of significance, $P<0.001$ (test 9, Table IV). Schedules that only employed docetaxel or vinorelbine together gave a higher response than any other schedules for cancers of the lung, ovary, prostate, and head and neck. In breast cancer trials, there was little or no dependence of response rate on

Table IV. Meta-analysis of variables related to the efficacy of inhibitor combinations in clinical trials.^a

Test	Variable analyzed	Number of observations	Mean	F	P
1	Length of cycle	25	21.3 days	0.03	0.860
2	Docetaxel dose	25	77.8 mg/m ²	0.24	0.631
3	Ratio D:V	25	3.3	0.27	0.606
4	Vinorelbine dose	25	23.6 mg/m ²	0.34	0.569
5	Treatment duration	23	105.5 days	7.72	0.012
6	Breast vs. organ site	25	-	7.01	0.015
7	Simple vs. complex	25	-	10.31	0.004
8	Interaction 3 and 7	25	-	6.34	0.020
9	Interaction 6 and 7	25	-	14.05	0.000

^aRegression between various variables and response rate was tested by analysis of variance. Response rates for 23 clinical trials were averaged and tested for statistical significance against conditions varied in the trials. The number of observations exceeds the number of trials, because one trial employed three different schedules. Also reported are the mean, where known, the F statistic (F), and the probability of finding the F-value as a result of random chance alone (P). Interactions among the variables were also tested as described in Materials and methods.

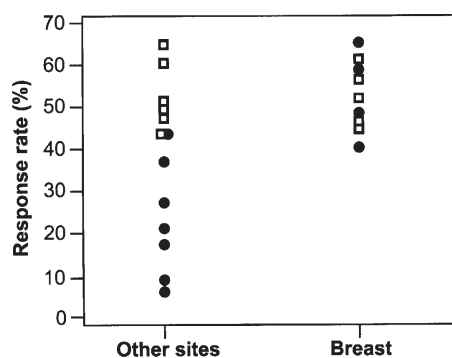


Figure 2. Response rate as a function of cancer site and dosage schedule. □, Schedules which only delivered microtubule-polymerizing and -depolymerizing agents on the same day. ●, More complex schedules, with interspersed doses of vinorelbine or docetaxel singly. Simple and complex schedules showed equal efficacy against breast cancer. When the therapy was directed against cancer of other sites, simple schedules were more effective. The interaction between site and dosage schedule was significant at $P < 0.001$ (Table IV, test 9).

dosing schedule (Fig. 2). The schedule variable also interacted with the ratio of docetaxel and vinorelbine, suggesting that the ratio was only important if the clinicians chose to intersperse single drug doses between treatments with the combination of drugs (test 8, Table IV). The results of the tests were similar when additional trials, mainly with paclitaxel substituted for docetaxel, were included in the dataset (57,59-77).

Cell cycle. The above results suggested that the combination of microtubule inhibitors had an effect that was qualitatively different from those of either drug alone. To determine whether the effect was specific to any one phase of the cell cycle, we analyzed cultures that had been synchronized by aphidicolin blockade. Control cultures showed an elevation in cell number beginning 5 h after washout. This increase, corresponding

to cell division by 60% of the population, was inhibited by low nanomolar concentrations of paclitaxel and colchicine (Fig. 3A). These samples still showed a small increase in numbers, which represented 15% or less of the amount represented in the control cultures. Since it was possible that the missing cells, representing three-fourths of the 60% that were expected to divide, were due to arrest in G2 or M phase, the amount of DNA present was determined by flow cytometry in replicate experiments. DNA determinations showed no enhancement in the fraction of G2/M cells. Moreover, if the cells had failed to exit S phase, the proportion of S-phase cells in the treated samples would have exceeded that in the control samples. However, evidence indicated that the shortfall in dividing cells was not due to a failure to exit S phase. The percentage of the control population in S phase dropped from 66% to 29% over 8 h (data not shown). The S-phase fraction was slightly larger than 29% in the treated sample but was within the range of sample-to-sample variability (Fig. 3B).

An increase in cell numbers in controls occurred at 24 h, which was also suppressed in treated cells (Fig. 3A). There was no actual decline in cell number with the 3:1 nM combination of paclitaxel and colchicine, however. These data showed that these concentrations inhibited progress through the cell cycle, and that the cells did not pause at any single cell cycle phase. Frank cell killing was observed in samples treated with 30 nM paclitaxel and 10 nM colchicine (Fig. 3A). In addition, DNA content determinations on replicate samples showed that approximately 50% of the cells that should have exited S phase remained in that phase. Thus, many cells failed to resume cycling. The fraction of cells in G2/M phase was also elevated (Fig. 3B).

Treatment with either paclitaxel or colchicine singly caused an increase in mitotic aberrations and multinucleated cells over the levels found in control samples. For both endpoints, aberrant mitoses and multinucleated cells, the effective dose (ED50) of paclitaxel was ~50 nM. In the case of colchicine,

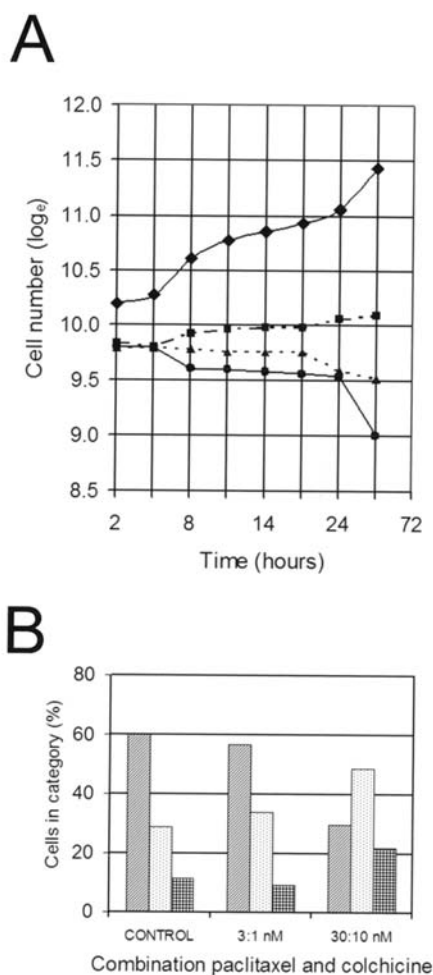


Figure 3. Resumption of cell cycling after blockade in S phase, in control and treated samples. (A) Samples treated with the solvent vehicle only showed increases in cell number at 6 and 24-48 h, corresponding to passage through M phase (●). In cultures treated with 3:1 nM paclitaxel and colchicine (■), replication was less, and little increase in cell number was observed. A decline in number, indicating cell killing, occurred after treatment with concentrations of 30:10 nM (▲) and 300:100 nM (●) paclitaxel and colchicine. (B) DNA content in samples analyzed at 8 h after exposure to solvent vehicle or experimental agents. A majority of cells were in G1 phase (dashed columns) for both control samples and those treated with the lowest concentration of paclitaxel and colchicine (3 and 1 nM respectively). For the treated sample, the number of cells in S phase (dotted columns) was elevated by 5%, which was within the range of sampling error. A majority of cells in samples treated with a higher concentration of agents (30 nM and 10 nM respectively) were in S and G2 phases, suggesting that both emergence from aphidicolin blockade and progress through the cell cycle were retarded. G2 cells are designated by the hatched column.

ED50 values were again similar for both endpoints but were approximately 15-fold lower than those observed for paclitaxel (Table V). For paclitaxel alone, these ED50s exceeded by at least 10-fold those found for the combination of paclitaxel with colchicine. In cells exposed to varied concentrations of paclitaxel in 3:1 molar proportion with colchicine, there was a slight divergence in the endpoints compared to cells treated with either compound alone. The combination was slightly more synergistic when the ED50 for multinucleated cells was determined (Table V), suggesting that the cells underwent only a brief mitotic arrest before reconstituting their nuclei. The results indicated that paclitaxel and colchicine synergized in both mitotic arrest and mitotic slippage endpoints.

Table V. ED50 values on various endpoints for aberrations related to spindle formation.^a

Compound tested	ED50 (nM) for biological effect	
	Multinucleated cells	Abnormal mitoses
Paclitaxel	58	53
Colchicine	3.8	3.6
Paclitaxel + colchicine	1.8+0.9	3.6+1.2

^aThe number of cells with multiple nuclei was counted for cultures after 48 h of treatment with agents specified. Similarly, abnormal versus normal mitotic figures were counted. Determinations were made as described in Materials and methods.

Discussion

Anti-mitotic agents. The results of clinical trials suggest that the therapeutic effect of other drug combinations, e.g. platinum compounds with anti-mitotic agents, is superior to that of either drug alone (78). In these cases, the agents' mechanisms of toxicity were clearly different, so that it was reasonable to expect them to be synergistic. Since docetaxel and vinorelbine were both thought to work by arresting cells in mitosis, synergy was more difficult to explain. One explanation for the unexpected efficacy of the combination was an additive effect, as suggested by *in vivo* tests with P388 leukemia cells. The agents individually had little anti-tumor activity but, in combination, each one could be employed at a level close to its maximum tolerated dosage (79). If their efficacy in clinical trials depended on additivity, however, the results obtained by pooling and comparing different trials would be expected to indicate a positive relationship between response and dosage. On the contrary, analysis of 23 published reports suggested no relationship between increased vinorelbine or docetaxel concentration and the response rate. Nevertheless, the expected relationship may not have been seen because clinicians often lowered or deferred the scheduled doses on account of toxicity. Secondly, the drugs are transported and the ultimate concentrations attained in the cells may have been a greater source of inter-patient variability than the dose delivered by the investigators.

Although clinical researchers often relied upon *in vitro* cytotoxicity tests to predict the therapeutic effects of drugs, these tests gave inconsistent results with combinations of microtubule inhibitors. Synergy occurred when vinorelbine was applied before paclitaxel, or when the drugs were applied concurrently and left for 96 h (8). Antagonism was observed when the order was reversed (80) or with alternating applications starting with either agent (81). The opposite was observed in studies of human lung cancer lines, where synergy was observed with alternating application (82). The results of clinical trials suggested that alternating dosages were relatively ineffective (31,42,83). The contradictory results obtained on the agents' additivity, antagonism, or synergy indicates that

new approaches are needed to improve the power of *in vitro* tests. Because unsynchronized cell populations were used in previous studies identifying the reversal of the phenotype by microtubule inhibitor combinations (24,28), these effects could not be attributed to mitotic arrest. Thus, this study was performed to gain additional information about the effects of the experimental agents on the functions of interphase cells, such as microtubule organization and shape features, and to compare these effects to the therapeutic efficacy of combination treatments.

There are several aspects of anti-mitotic drug effects that are difficult to reconcile with the conventional hypothesis given to explain their effects, namely the synergistic inhibition of dynamicity. Several anti-mitotic agents are more potent depolymerizing agents than vinblastine but do not show greater efficacy against tumors (84-87). Secondly, when microtubule inhibitor combinations were used, heavily pretreated patients were nearly as likely to respond as patients who had not received previous treatment. This suggested that resistance mechanisms developed against other drugs were not valid against the combination of inhibitors. In schedules in which the combination was followed by one or more doses of vinorelbine singly, there was a significant relationship between taxane dosage and response rate. This suggested that taxane pharmacokinetics may be important to the success of these complex schedules. In summary, these facts support the concept that simultaneous exposure to the microtubule inhibitor combination has a unique effect on cells.

Cancer-type reversal. These experiments showed that the microtubule inhibitor combinations' effects on interphase cells are qualitatively different from the effect of either inhibitor as a single agent. The unique effect of such combinations was supported by a meta-analysis of clinical trials on docetaxel and vinorelbine, which showed that dosage schedules were more efficacious when the combination was not complicated by interspersing vinorelbine or docetaxel doses singly. We determined whether various depolymerizing agents could be substituted for colchicine in the combination and found that all combinations reverted the phenotype. Thus, a combination of paclitaxel with any depolymerizing agent is likely to cause phenotype reversal. One compound, 7-deoxytaxol, showed the same effect as the inhibitor combinations. When cell features contributing to the reversal were investigated, all of the samples showed alterations in at least one feature, and each treatment affected a different assemblage of features. Features represented by #4, #5, #8, and #12 were reverted towards the normal phenotype. Factors #7 and #13 were changed to resemble the cancer-type. Thus, reversal did not apply uniformly to all of the properties recognized by factor analysis.

Deconstruction of phenotype reversal effects. It is possible that changes in the factor describing the edge feature, #4, may have reflected the rearrangement of microtubules in cells treated with inhibitor combinations. Observations of the microtubule array suggested that the treatments prevented the full extension of microtubules into the cell periphery. There is a well-known antagonism between microtubule- and actin-based cytoskeletal components, which may affect formation and turnover of focal contacts at the cell edge (88,89). If

focal contacts were stabilized by microtubule withdrawal, this could explain the increased prevalence of filopodia in treated cells. Another difference between treated and control cells was that, in the former, microtubules impinging on the cell edge tended to project out at right angles. That this arrangement was typical of cell-cell contact regions in untreated cells suggested that the pattern adopted at the cell edge resembled that of contact-inhibited cells. Our preliminary results suggest that this arrangement is associated with changes in vesicle trafficking (data not shown).

The inference that interphase cells were affected by microtubule inhibitor combinations was supported by observations of the microtubule arrangement in cells exposed to combinations with different ratios of paclitaxel and depolymerizing agent or different depolymerizing agents. Sidewise aggregation of microtubules occurred with unequal ratios of the inhibitors, except for those containing podophyllotoxin. The latter cleared microtubules from the cytoplasm to a greater extent than the other agents studied. Likewise, the combination of paclitaxel and colchicine tended to suppress the microtubule organizing center (28). Since all ratios and depolymerizing agents were effective in reversing the overall phenotype, the current results suggested that neither partial dissolution of microtubules nor their aggregation were crucial mechanisms of phenotype reversal. This conclusion coincided with results from clinical studies. If the investigation were restricted to dosing schedules that only delivered the two drugs on the same day, there was no significant relationship between drug ratio and response rate. When levels of the agents were adjusted to approximate those attained during treatment of clinical patients, the rearrangements observed were similar to those found with short-term treatment.

Cell cycle specificity. To determine whether the agents had a greater impact on cells in any one cell cycle phase, we examined cycle kinetics in treated and control samples. When treated with low nanomolar concentrations of agents, most cells failed to divide indicating that their progress through the cell cycle was abnormal. Since the data showed little accumulation of these cells in either S or G2 phase of the cell cycle, it must be assumed that the missing cells left the culture plates in all phases. This conclusion is supported by data from other laboratories, which reported significant cell killing by 8 h after the combination treatment (90) and DNA damage within a few hours of exposure to paclitaxel alone (91). Thus, nanomolar concentrations caused killing of a low percentage of interphase cells. Cells treated with higher concentrations, e.g. 30 nM paclitaxel and 10 nM colchicine, paused in S and G2/M phase. Their accumulation in the pre-mitotic phases of the cell cycle caused the reduced number of cells in G1 phase at 5 h and longer after washout. Taken together, the results suggest that the combination of paclitaxel with a depolymerizing agent has a dual mode of action. High concentrations caused more cell killing, but they also slowed passage through the cell cycle and caused the population to pile up in G2/M. A possible result, in this case, would be mitotic slippage leading to cell death. However, killing also took place at low nanomolar concentrations of the combined agents. The latter mechanism would be especially important in the clinical setting, where some cells already transit the cell cycle at a slow pace.

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