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# Simultaneous combination of microtubule depolymerizing and stabilizing agents acts at low doses

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**The combined activity of a stabilizing and a depolymerizing agent was studied on microtubule formation *in vitro* and on cellular parameters related to the cytoskeleton. New compounds in each class of microtubular drugs, docetaxel, as stabilizing agent, and CI 980, as colchicine analog, currently in clinical trials, were tested at high and low concentrations. Simultaneous combination of docetaxel and CI 980, both *in vitro* and in cell lines, induced microtubule structures resulting from the association of the effects of the two drugs: short and numerous microtubules *in vitro*, abnormal asters or short bundles in cells depolymerized in their periphery. Moreover, combining the two drugs at low concentrations inducing neither modification of the microtubular network nor variation in cell polymerized tubulin content showed synergistic effects in mitotic cell block and, at lower concentrations, in inhibition of proliferation in the KB 3-1 cell line. Similar qualitative results were obtained with paclitaxel and colcemid, used in the place of docetaxel and CI 980, respectively. At low doses, the taxoid/colchine analog combination seems to induce some degree of mitotic inhibition, resulting from a subtle effect on the inhibition of microtubule dynamics.**

*Key words:* CI 980, combination, docetaxel, microtubule, tubulin.

## Introduction

Paclitaxel (Taxol) and docetaxel (Taxotere) are new clinically active anticancer drugs, currently used in several classically refractory tumors, ovarian and breast carcinoma, lung tumors, leukemia, and malignant melanoma.[1,2] These two taxoids are antimitotic compounds, called stabilizing agents, whose mechanism of action is opposite to that of depolymerizing agents, *Vinca* alkaloids and analogs of colchicine. Indeed, unlike the latter which induce microtubule disassembly, taxoids promote assembly and stabilize the microtubules in the cold, in the presence of calcium or colchicine.[3] CI 980 is a new molecule which acts at the microtubule level. It is a very powerful deazapteridine compound[4,5] now undergoing clinical trials.[6,7] It inhibits tubulin assembly and binds at least partially on the colchicine site *in vitro*.[8] These antimitotic agents are potent inhibitors of cell proliferation and differently modify the microtubule network of cells. Taxoids predominantly induce bundles in interphase cells and pseudo-asters in mitotic cells.[9,10] The colchicine analogs (CLC-A) and *Vinca* alkaloids depolymerize cytoplasmic microtubules[11] and *Vinca* alkaloids at high concentration induce formation of tubulin paracrystals.[12] Recently, Jordan *et al.*[13,14] suggested that *Vinca* alkaloids and paclitaxel at low concentrations inducing no modification of the tubulin polymer mass similarly disturbed the organization of the mitotic spindle.

In the present study, we investigated the combined activity of a stabilizing agent and a depolymerizing agent on microtubule formation *in vitro* and on cellular parameters related to the cytoskeleton. New compounds in each class of microtubule drugs, docetaxel and CI 980, were chosen to perform this study. The effects of CI 980 were compared to those of colcemid (Cmd), a classical CLC-A, to avoid any specific effect due to this molecule. High (i.e. doses inducing classical modifications of microtubular network) and low concentrations were tested. Our main result, that the two agents at very low doses act synergistically in the inhibition of proliferation of KB 3-1 cells, suggests some degree of mitotic inhibition. Adenocarcinoma KB 3-1 cell line was chosen for low doses because taxoids induce both bundles and pseudo-asters in treated cells. This combination could be of clinical utility because it lowers the dose and hence the toxicity of each compound.

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## Materials and methods

### Chemicals

Docetaxel was a gift from Rhone Poulenc Rorer (Paris, France). CI 980 was given by Parke Davis (Ann Arbor, MI). Stock solutions (0.01 M) were made in dimethylsulfoxide (DMSO); the highest concentration of DMSO used was 0.2%. Colcemid was from Sigma (St Lous, MO); stock solutions (0.01 M) were made in water. All stock solutions were stored at −20°C. Mouse monoclonal anti-α-tubulin antibody, and antimouse Ig, fluorescein isothiocyanate (FITC)-linked whole antibody were obtained from Amersham (Buckinghamshire, UK).

### Cells

KB 3-1 human adenocarcinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 4.5 g glucose/l, 0.1 M sodium pyruvate (Sigma) plus 10% fetal bovine serum, L-glutamine, penicillin and streptomycin. J82 human bladder carcinoma cells were grown in RPMI medium supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics (Sigma). Doubling time was 18 ± 5 h for each cell line.

### Drug incubation for cellular studies

For the simultaneous combination experiments, exponentially growing cells were treated with culture medium containing the two drugs at various concentrations for the below-mentioned times. For pre-incubation study, log-phase cultures were incubated overnight with the first drug (docetaxel or colcemid) and washed once with drug-free medium before adding the second drug.

### Preparation of microtubular protein (MTP) and measurement of microtubule assembly

MTP was purified from pig brain by three cycles of temperature-dependent assembly/disassembly by using technique of Shelanksi[15] with some modifications.[16] For polymerization experiments, assembly buffer contained 0.1 M 2-[N-morpholino]ethanesulfonic acid, 1 mM ethyleneglycol-bis-(β-amino-ethyl ether)-*N,N'*-tetraacetic acid, 0.5 mM $MgCl_2 \cdot 6H_2O$ and 0.1 M GTP (Fluka, Buchs, Switzerland), pH 6.8. Stock solutions of docetaxel and CLC-A alone or in combination were diluted in the buffer and the reaction was started by adding MTP (2 mg/ml) in the 37°C thermostated 1-cm light path cell. For simultaneous incubation, assembly was monitored spectrophotometrically at 340 nm, 37°C, by following changes in turbidity with a Beckman DU 7400 during 20 min. For pre-incubation studies, the second drug was added at the end of polymerization as described above and turbidity was recorded during an additional 20 min. Depolymerization of preformed microtubles was started by a temperature shift from 37 to 4°C and turbidity was recorded during 20 min. Electron microscopy studies were performed using a Philips EM 400 T Instrument (Service Commun de Microscopie Electronique, Faculté des Sciences, Marseille, France). Samples were taken at the end of polymerization time or after 20 min at 4°C and then negatively stained with 2% uranyl acetate on carbon-coated grids.

### Immunofluorescence microscopy

For immunofluorescence microscopy, cells were treated as previously described (10). They were stained with monoclonal anti-α-tubulin, with antimouse Ig, fluorescein-linked whole antibody and then with 4,6-diamino-2-phenylindole (DAPI) (Sigma). Plurinuclear cells were counted among the DAPI stained cells (200 cells). Experiments were performed in triplicate.

### Determination of polymerized tubulin in cells

Tubulin was quantified using enzyme-linked immunoessay (ELISA) technique. Cells were plated at 50 000 cells/ml (0.2 ml) in 96-well plates and cultured for 1 day. All assays were conducted in triplicate with the drugs for the desired time. They were then lysed and fixed as described.[17] Each measurement was performed three times.

### Inhibition of cell proliferation

Cell monolayers ($2.5 \times 10^5$) were incubated with various amounts of drugs for 72 h, harvested at different times and then the cells without Trypan blue (Sigma) were counted. Inhibition of cell proliferation by combinations of agents was considered synergistic if the surviving fraction of cells was smaller

than the product of the surviving fractions obtained with the individual agents at the designated concentration.[18]

### Flow cytometry analysis

DNA content was measured by flow cytometry after 24 h contact time. Cells were harvested with trypsin–EDTA, fixed in cold methanol, incubated with RNAse and then stained with propidium iodide (Sigma). Measurements were performed in triplicate on 5000 cells by using an ATC 3000 flow cytometers as previously described.[10]

## Results

### Microtubule assembly studies *in vitro*

Figure 1 shows the effect of docetaxel (1 μM) and CI 980 (1 μM) alone or in combination on microtubule assembly. Docetaxel (1 μM) decreased the nucleation time and induced an increase in turbidity similar to that of MTP; the microtubules obtained were comparable with control (Figure 2) except that they were resistant to cold. In contrast, CI 980 inhibited the polymerization of MTP in a dose-dependent manner. CI 980 was a 10-fold more potent microtuble inhibitor than Cmd [half-inhibitory concentration ($I_{50}$) was 1 and 10 μM with 2 mg/ml MTP for CI 980 and Cmd, respectively]. As reported for other CLC-A,[19] microtubules polymerized with CI 980 were shorter and less numerous than control (Figure 2). Like Cmd but contrary to colchicine, binding of CI 980 to tubulin does not take longer than 20 min. The polymerization of MTP in presence of the docetaxel (1 μM)/CI 980 (1 μM) combination was similar to that obtained with docetaxel alone (lack of lag-time, same rate of assembly) except for the lower absorbance due to shorter microtubules. Indeed, the microtubules formed were shorter than those obtained with docetaxel but more numerous than those obtained with CI 980 and they were partially stable at 4°C.

These effects were dose-dependent. Indeed, the docetaxel (1 μM)/CI 980 (10 μM) combination decreased the plateau absorbance value at 37°C and increased the percentage of depolymerization at 4°C, whereas docetaxel (1 μM)/CI 980 (0.1 μM) showed the opposite effects (Table 1). Docetaxel (0.1 μM) and CI 980 (0.1 μM) alone or in combination did not modify microtubule assembly. Similar results were obtained with Cmd but at 10-fold higher doses than for CI 980, according to the $I_{50}$ measured for each drug alone.

Table 2 reports the results of the pre-incubation studies. Docetaxel (1 μM) added to microtubules preformed with CI 980 induced an increase in turbidity (e.g. increase in turbidity of 26% with 1 μM

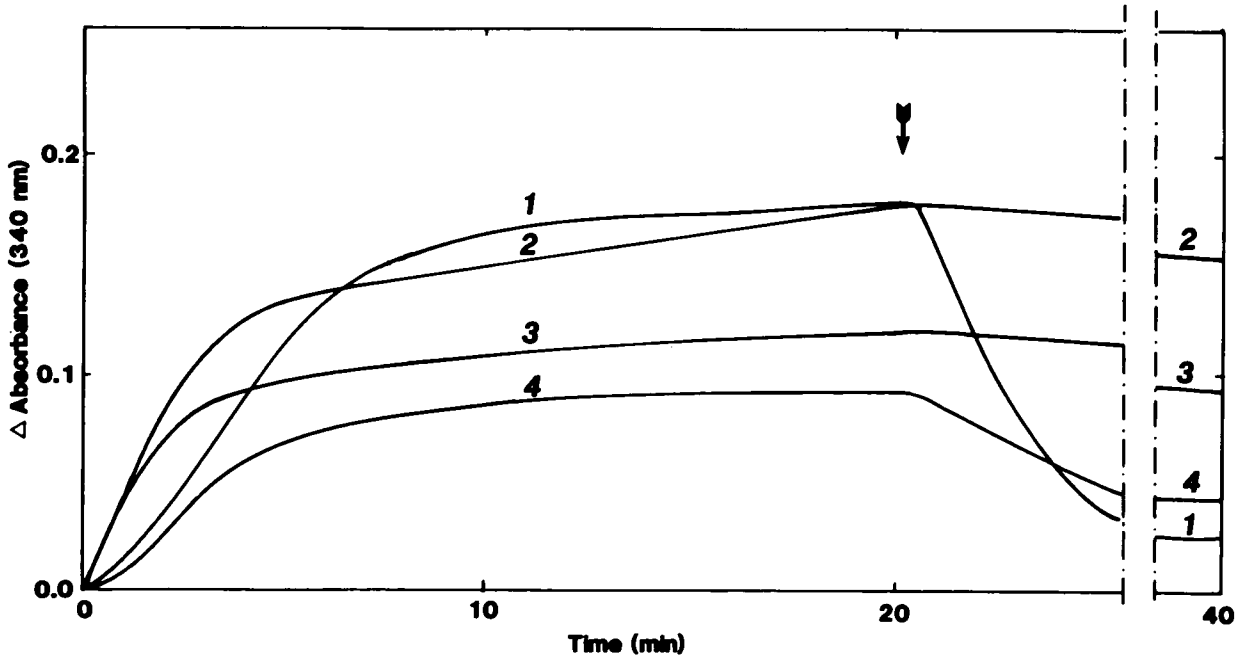


**Figure 1.** Turbidity time course of *in vitro* microtubule assembly: MTP 2 mg/ml (1), with docetaxel 1 μM (2), with docetaxel (1 μM)/CI 980 (1 μM) (3) and with CI 980 1 μM (4). At the time indicated by the arrow, the sample was cooled to 4°C during 20 min.

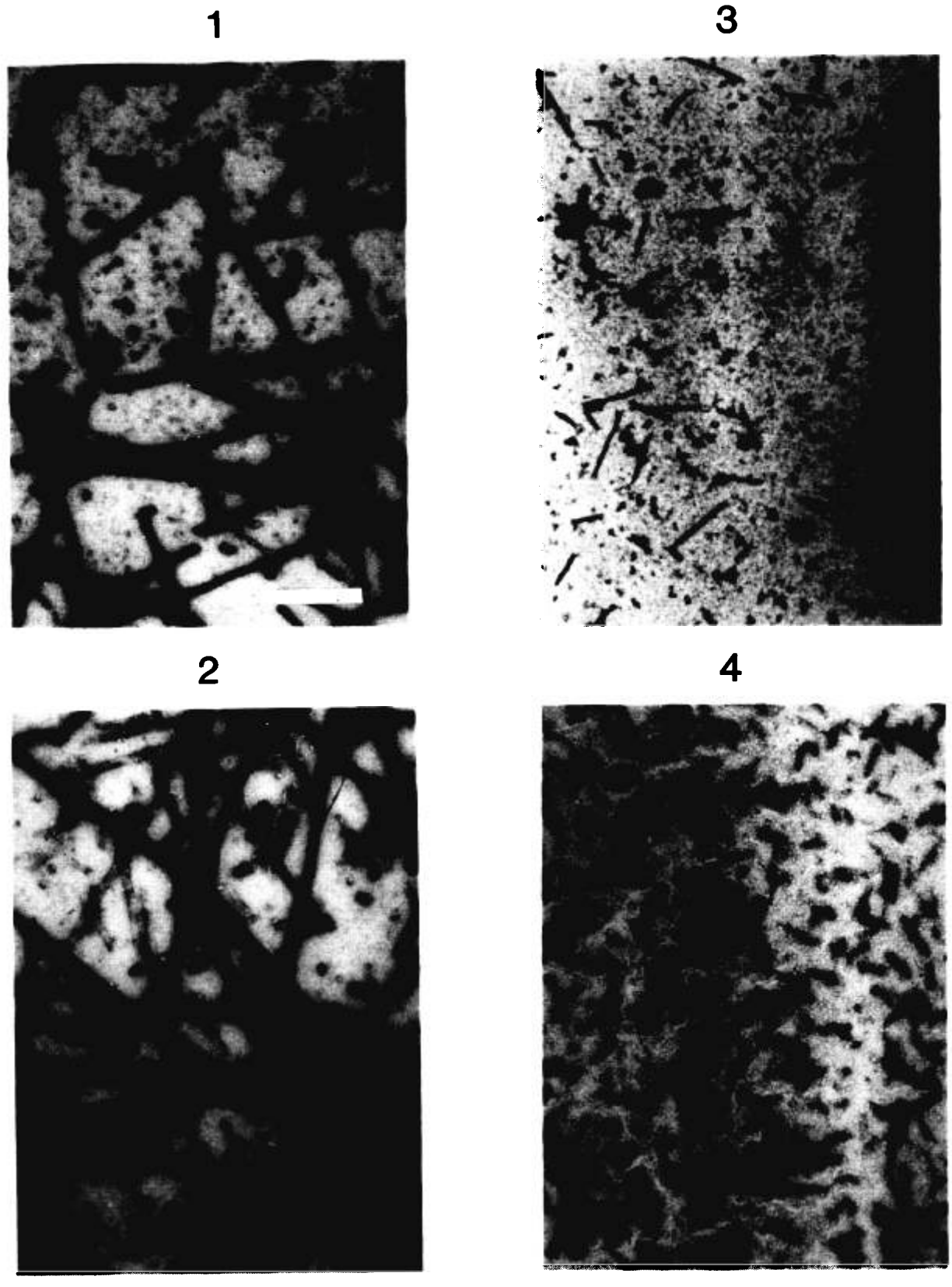


**Figure 2.** Electron micrograph of microtubule formation: MTP 2 mg/ml (1), with docetaxel 1 μM (2), with CI 980 1 μM (3) and with the combination docetaxel (1 μM)/CI 980 (1 μM) (4). Scale bar, 1 μM.

**Table 1.** Dose effect of the simultaneous combination of docetaxel/CLC-A on microtubule assembly

| [Docetaxel] (μM) | [CI 980] (μM) | [Cmd] (μM) | Polymerization (%) |
|---|---|---|---|
| 0.1 | — | — | 98 |
| 1.0 | — | — | 95 |
| 10.0 | — | — | 94 |
| — | 0.1 | — | 100 |
| — | 1.0 | — | 52 |
| — | 10.0 | — | 40 |
| 0.1 | 0.1 | — | 98 |
| 1.0 | 0.1 | — | 85 |
| 1.0 | 1.0 | — | 66 |
| 1.0 | 10.0 | — | 47 |
| 10.0 | 0.1 | — | 91 |
| 10.0 | 1.0 | — | 80 |
| 10.0 | 10.0 | — | 45 |
| — | — | 1.0 | 96 |
| — | — | 10.0 | 52 |
| 0.1 | — | 1.0 | 94 |
| 0.1 | — | 10.0 | 51 |
| 1.0 | — | 1.0 | 90 |
| 1.0 | — | 10.0 | 58 |
| 10.0 | — | 10.0 | 81 |

The polymerization was expressed as the percentage of the control value obtained from MTP (2 mg/ml) alone after 20 min of assembly at 37°C.

docetaxel on microtubules polymerized with 1 μM CI 980). On the contrary, as described for the other CLC-A,[20] CI 980 (1 μM) induced no significant depolymerization of microtubules preformed with docetaxel (1 μM). Whatever the order of compound addition, the microtubules obtained at the end of incubation were stable at 4°C.

## Cellular studies

*Immunofluorescence anlaysis at drug doses inducing modifications of microtubule network.* As previously described,[10] docetaxel induced bundles on J82 cell line (250 nM docetaxel induced bundles in 80% of J82 cells) and pseudo-asters on KB 3-1 cells (50 nM docetaxel induced pseudo-asters in 45% of KB 3-1 cells). CI 980 was a more potent depolymerizing agent of the microtubular network than was Cmd (1 and 50 nM for CI 980 and Cmd, respectively, whatever the cell line tested) (data not shown).

Overnight cell treatment with the docetaxel/CLC-A combination induced abnormal microtubule structures: short bundles surrounded by depolymerized network, pseudo-asters of short microtubules and multiple foci scattered in the cytoplasm (Figure 3). The abnormal structures were observed whatever the cell line tested and whatever the drug combined with docetaxel: CI 980 (1 nM) or Cmd (50 nM). Abnormal condensation of chromatin, as observed by DAPI staining, was associated with pseudo-asters of short microtubules and multiple foci (data not shown). KB cells treated with docetaxel (50 nM)/CI 980 (0.25 nM) showed the effects of docetaxel alone, whereas only the depolymerized network was observed with docetaxel (50 nM)/CI 980 (5 nM), indicating that the formation of these abnormal structures was clearly dose-dependent.

The formation of abnormal structures was also time-dependent, e.g. short bundles and pseudo-asters of short microtubules were observed at 3 and 6 h contact time, respectively. Overnight pre-incubation of KB 3-1 cells with Cmd (50–100 nM) followed by a wash allowed formation of pseudo-asters by docetaxel (50 nM) after shorter contact time (1 h) than without pre-incubation (6 h); the abnormal structures induced by the combination were not detected in this experiment. On the contrary, pretreatment of J82 cells with Cmd 250 nM, followed by a wash and incubation with docetaxel

**Table 2.** Pre-incubation study on microtuble assembly

| First drug (μM) | | Polymerization (%) | Second drug (μM) | | Variation of polymerization induced by the second drug (%) |
|---|---|---|---|---|---|
| CI 980 | 1 | 52 | docetaxel | 1 | 26 |
| CI 980 | 1 | 52 | docetaxel | 10 | 70 |
| CI 980 | 10 | 40 | docetaxel | 10 | 30 |
| Docetaxel | 1 | 95 | CI 980 | 1 | NS[a] |
| Docetaxel | 10 | 94 | CI 980 | 1 | NS |
| Docetaxel | 1 | 95 | CI 980 | 10 | NS |

MTP was polymerized in the presence of the first drug during 20 min, then the second drug was added. Variation in optical density after 20 min of polymerization in the presence of the two drugs was expressed as the percentage of the value of polymerization (second column) before adding the second drug (corrected, if necessary, from the absorbance of CI 980 alone).
[a] Not significant.

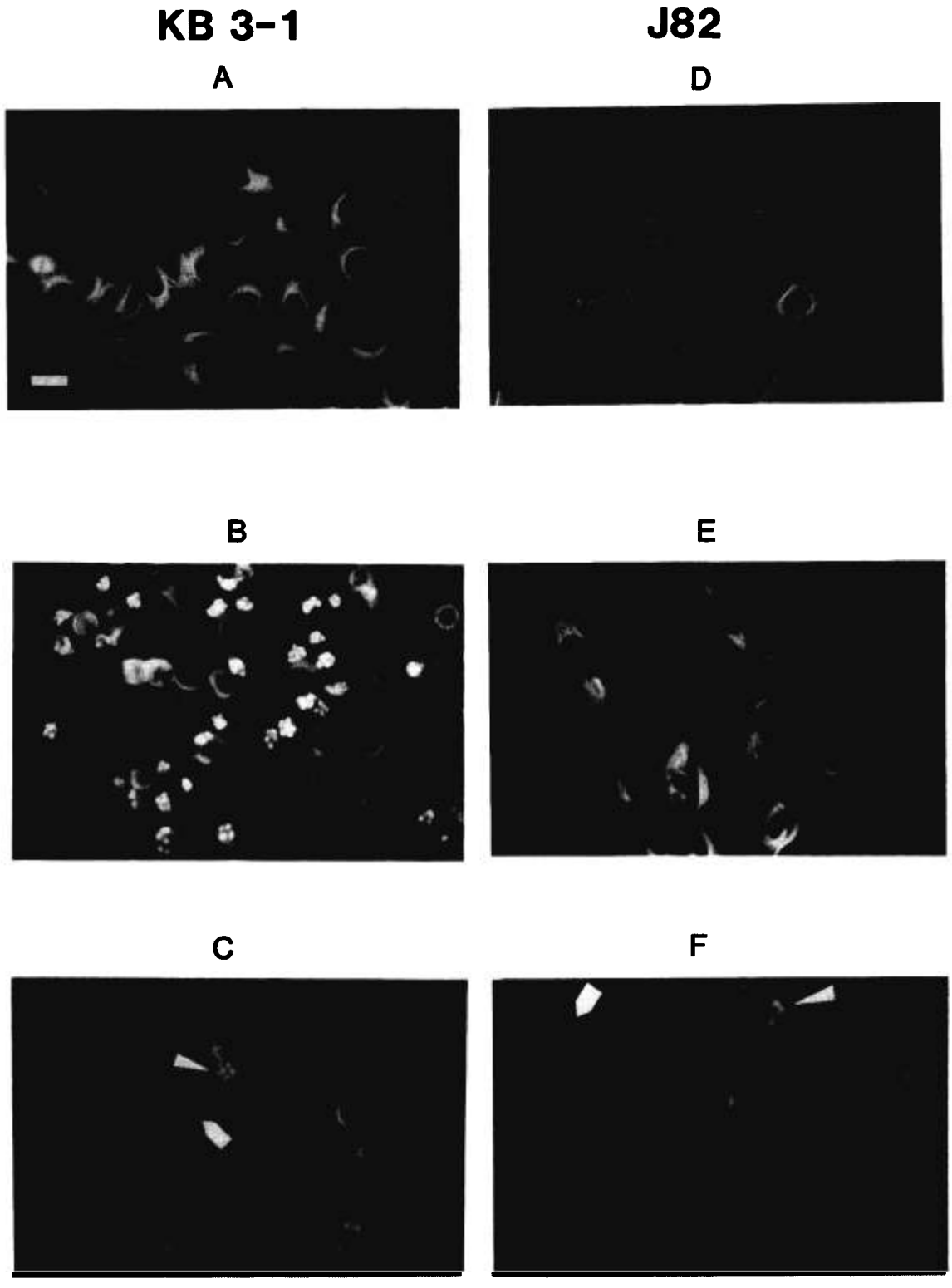


**Figure 3.** Indirect immunofluorescence staining of tubulin in the KB 3-1 (left) and J82 (right) cell lines. Cells were incubated overnight at 37°C without drug (A and D), docetaxel alone (50 and 250 nM for B and E, respectively), and combination docetaxel/CI 980 (docetaxel 50 nM/CI 980 1 nM and docetaxel 250 nM/CI 980 1 nM for C and F, respectively). Abnormal structures (white triangle indicates pseudo-asters of short microtubules, white arrow indicates shorter bundles surrounded by depolymerized microtubule network) were present only in cells treated with the combination (C and F), and took the place of pseudo-asters and microtubule bundles (B and E) usually induced by docetaxel alone. Scale bar, 20 $\mu$m.

250 nM transitorily showed the formation of short bundles. Overnight pre-treatment with docetaxel, followed by a wash and incubation with Cmd, also temporarily showed the formation of abnormal structures (short bundles, short pseudo-asters), whatever the cell line tested.

Since the combination of docetaxel and CI 980 at these high doses induced abnormal microtubular structures different from those obtained with the drugs alone, it was of interest to test these compounds alone and in combination at lower doses on some cellular parameters. This study was performed on KB 3-1 cells because taxoids induced both bundles and pseudo-asters in treated cells.

*Immunofluorescence analysis and DAPI staining at low concentrations.* The lowest concentrations of docetaxel and CI 980 alone which induced a slight modification of microtubule network of KB 3-1 cells were 5 and 1 nM, respectively. The combination of the two drugs at these doses induced the formation of the abnormal structures described above in only some of the cells.

No modification of the microtubule network was observed in cells treated with lower doses of docetaxel (1 nM), CI 980 (0.05–0.25 nM) or Cmd (2.5–10 nM), alone or in combination. Moreover at these very low doses, docetaxel (1 nM) induced formation of multinucleated cells (53% at 24 h contact time) (Table 3), the combination of docetaxel (1 nM)/CI 980 (0.05 nM) slightly increased this value (63%) but only a few multinucleated cells were counted with CI 980 alone (5%). Cells were heterogeneous in number and size of nuclei, as observed by DAPI staining (Figure 4). The percentage of multitinucleated cells increased over 72 h contact time, but none of the pseudo-asters, bundles or abnormal structures described above were observed.

*Cell DNA content.* After 24 h incubation, at doses inducing subtle modifications of the microtubular network (combination of 5 nM docetaxel and 0.5 nM CI 980), 52% of cells were blocked in $G_2M$, whereas less than 15% were in $G_2M$ in control cells or cells treated with each drug alone (Figure 5). Flow cytometry analysis of KB 3-1 cells treated with the combination at lower concentration [docetaxel (1 nM)/CI 980(0.05 nM)] was not possible because of the formation of multinucleated cells.

*Inhibition of cell proliferation.* The inhibition of KB 3-1 cell proliferation was clearly evidenced at 48 h contact time with very low doses of docetaxel (1 nM) and CI 980 (0.05–0.25 nM) alone, or in combination (Figure 6). The inhibition of proliferation of KB 3-1 cells treated by the combination was significantly higher ($p < 0.01$) than that predicted from the sum of each drug effect separately (Table 4).

*Quantification of polymerized tubulin.* No modification of polymerized tubulin was evidenced by ELISA technique in KB 3-1 cells treated with low concentrations of drugs: docetaxel (1–5 nM), CI 980 (0.05–1 nM), Cmd (5–20 nM), alone or in combination. For example, a similar quantity of polymerized tubulin was observed either after 4 h incubation with docetaxel (5 nM)/CI 980 (0.5 nM), or after overnight incubation with docetaxel (1 nM)/CI 980 (0.05 nM), or in untreated cells.

## Discussion

### *In vitro* polymerization studies

Results of *in vitro* MTP polymerization studies indicated that microtubules obtained in presence of the docetaxel/CI 980 combination resulted from

**Table 3.** Time effect of the docetaxel/CI 980 combination on the multinucleation of the KB 3-1 cells

| Drugs | Percentage of multinucleated cells | | |
|---|---|---|---|
| | after 24 h contact | after 48 h contact | after 72 h contact |
| Docetaxel (1 nM) | 52.7 ± 2.3 | 63.0 ± 2.1 | 61.3 ± 3.2 |
| Docetaxel (1 nM)/CI 980 (0.05 nM) | 63.3 ± 1.8 | 69.8 ± 1.6 | 72.0 ± 4.5 |
| Docetaxel (1 nM)/CI 980 (0.10 nM) | 65.0 ± 0.8 | 73.7 ± 1.8 | 71.0 ± 2.4 |
| CI980 (0.05 nM) | 4.7 ± 1.4 | 6.2 ± 0.9 | 9.3 ± 0.9 |
| CI980 (0.10 nM) | 5.4 ± 1.9 | 6.8 ± 1.1 | 10.1 ± 1.6 |

Multinucleated cells are those with more than one nucleus. To quantify the percentage of multinucleated cells, a minimum of 200 consecutive cells was scored on each coverslip. Experiments were performed in triplicate; these data are the mean values ± SEM. Whatever the contact time, the percentage of multinucleated control KB 3-1 cells was not significant.

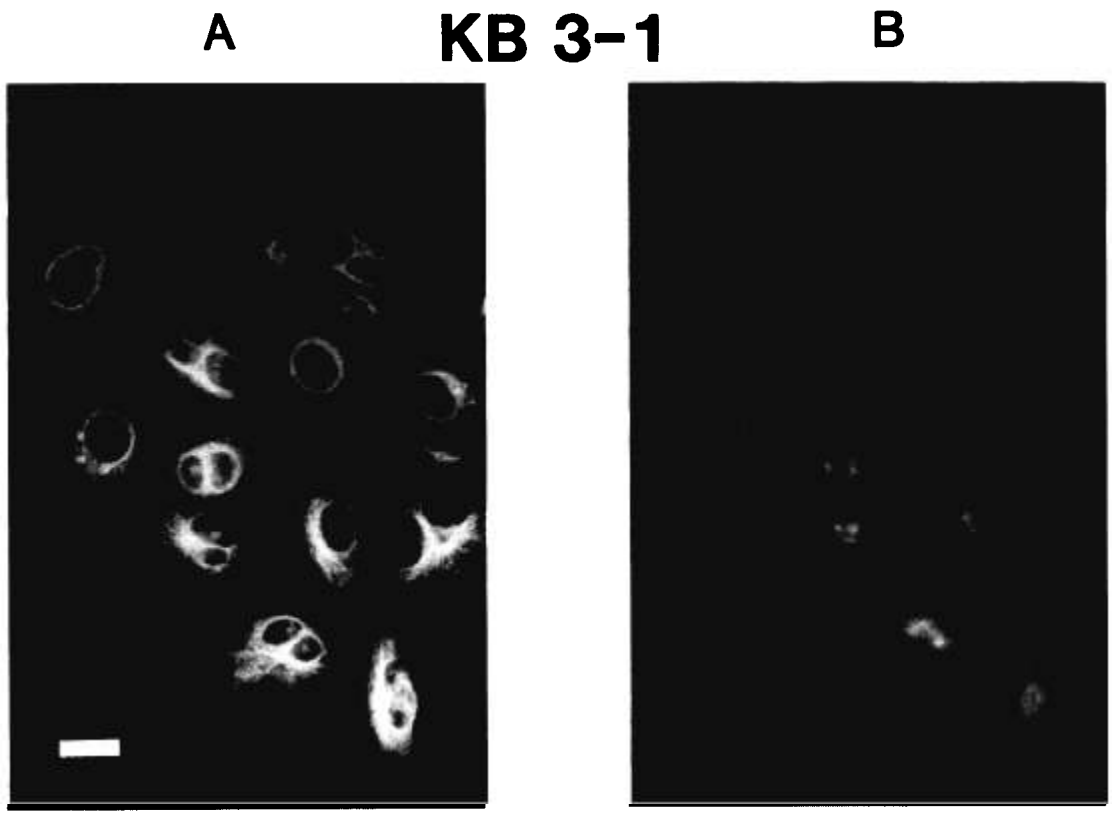


**Figure 4.** KB 3-1 cells were treated overnight with docetaxel (1 nM)/CI 980 (0.05 nM). Staining of tubulin and chromatin showed no modification of their microtubular network (A) and appearance of multinucleated cells (B). Scale bar, 20 $\mu$M.

the concomitant action of the two drugs. After 20 min of polymerization, microtubules were the only structure observed, but they differed in length, quantity and in resistance to cold according to the dose of each compound. The most obvious action of the combination was obtained with half inhibitory concentrations of CLC-A (1 $\mu$M for CI 980 and 10 $\mu$M for Cmd) and with 1 $\mu$M of docetaxel; at higher doses of one compound, its own effect was predominant. Combining each compound at low doses did not modify the polymerization of microtubules relative to that of MTP alone.

## Cellular studies at high doses of drugs

Overnight simultaneous incubation of cells with docetaxel/CLC-A induced abnormal microtubule structures different from the pseudo-asters and bundles observed with docetaxel or the network depolymerization due to CLC-A alone. Among these structures, foci were previously observed in mitotic cells with high concentrations of depolymerizing agent,[19] whereas short bundles surrounded by a depolymerized network and pseudo-asters of short microtubules were specific to the combination. The presence of partially depolymerized bundles and asters indicate that CLC-A did not prevent the specific organization of cellular microtubules which is due to taxoids; it can be suggested that the shorter microtubules making up the pseudo-asters or bundles observed with the combination were due to the depolymerizing effect of CLC-A. These structures appeared at doses at which each compound alone induced its classical effect on the microtubular network; an excess of one drug relative to the other prevented their formation. These structures were spontaneously developed in the case of simultaneous incubation, but they may result from transformation of bundles or pseudo-asters in the case of pre-incubation of docetaxel. The presence of classical pseudo-asters, instead of pseudo-asters partially depolymerized, after 1 h contact with docetaxel preceded by overnight incubation of CLC-A may be explained by the fast reversibility of CLC-A effect on the microtubule network in KB 3-1 cells.[20]

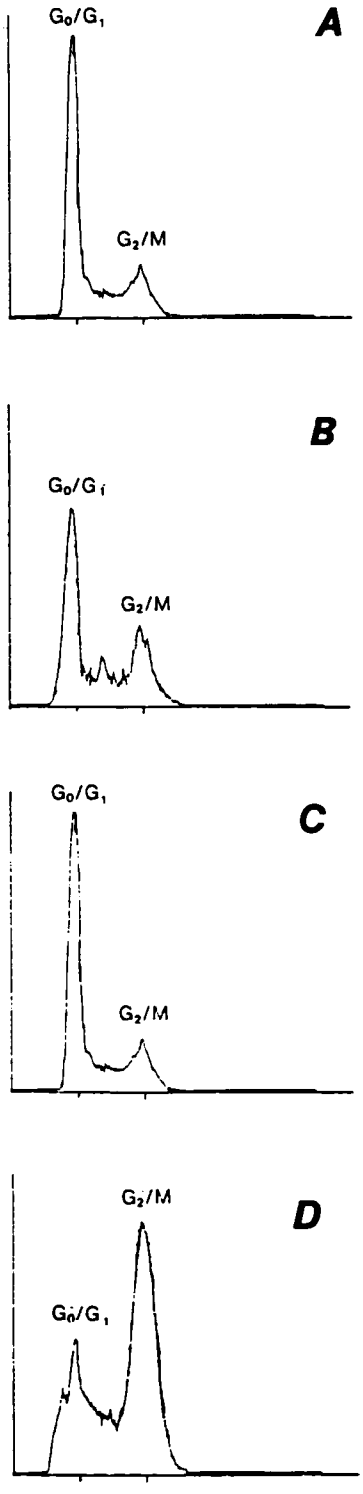


**Figure 5.** Variation of KB 3-1 cell DNA content in KB 3-1 cells: control (A) or treated overnight with docetaxel 5 nM (B) and CI 980 0.5 nM (C) alone or in combination (D). DNA was stained with propidium iodide. Measurement was performed on 5000 cells by flow cytometry.

## Comparison *in vitro–in vivo* studies

Both studies *in vitro* and *in vivo* demonstrated that the microtubular structures obtained with the docetaxel/CLC-A combination result from the association of the effects of the two drugs: short and numerous microtubules *in vitro*, abnormal asters or short bundles in cells depolymerized in their periphery. The appearance of these structures in cells with lower extracellular drug concentrations than those used *in vitro* may be explained by the mechanism of intracellular concentration of the drugs.[13] CI 980, however, was 50 times more active than Cmd at the cellular level but only 10 times more so *in vitro* on MTP polymerization. This discrepancy may be explained by the following hypotheses:[8] (i) high affinity of CI 980 for some cellular tubulin isoforms not present in the brain preparations used or (ii) transformation *in vivo* of CI 980 into more potent metabolites. The hypothesis of an additional target of CI 980 is unlikely because the qualitative data for Cmd and CI 980 were identical.

## Cellular studies at low concentrations of drugs

Our study demonstrates that the docetaxel/CLC-A combination modifies cellular parameters at low doses. These doses induced neither modification of microtubule network of cells nor polymerization of MTP *in vitro*.

To avoid a specific effect due to docetaxel, we tested a paclitaxel/CI 980 combination at low doses. Similar results were obtained with paclitaxel at doses two-fold higher than those of docetaxel, as previously demonstrated on KB 3-1 cell line.[10]

The blockage in $G_2M$ cycle phases after overnight contact was evident only with the docetaxel (5 nM)/CI 980 (0.5 nM) combination but not with each drug alone, demonstrating a greater than additive effect of the combination. The mitotic blockage was accompanied by a subtle modification of the microtubule network, as observed by immunofluorescence analysis, without variation of the intracellular content of polymerized tubulin. Mitotic blockage may involve kinetic stabilization of mitotic spindle microtubule dynamics, as previously described by Jordan *et al.*[21] These authors demonstrated that this mechanism was shared by depolymerizing agents and Taxol. The cumulative effect of docetaxel and CI 980 on the inhibition of spindle microtubule dynamics is not sufficient to explain the supra-additivity observed.

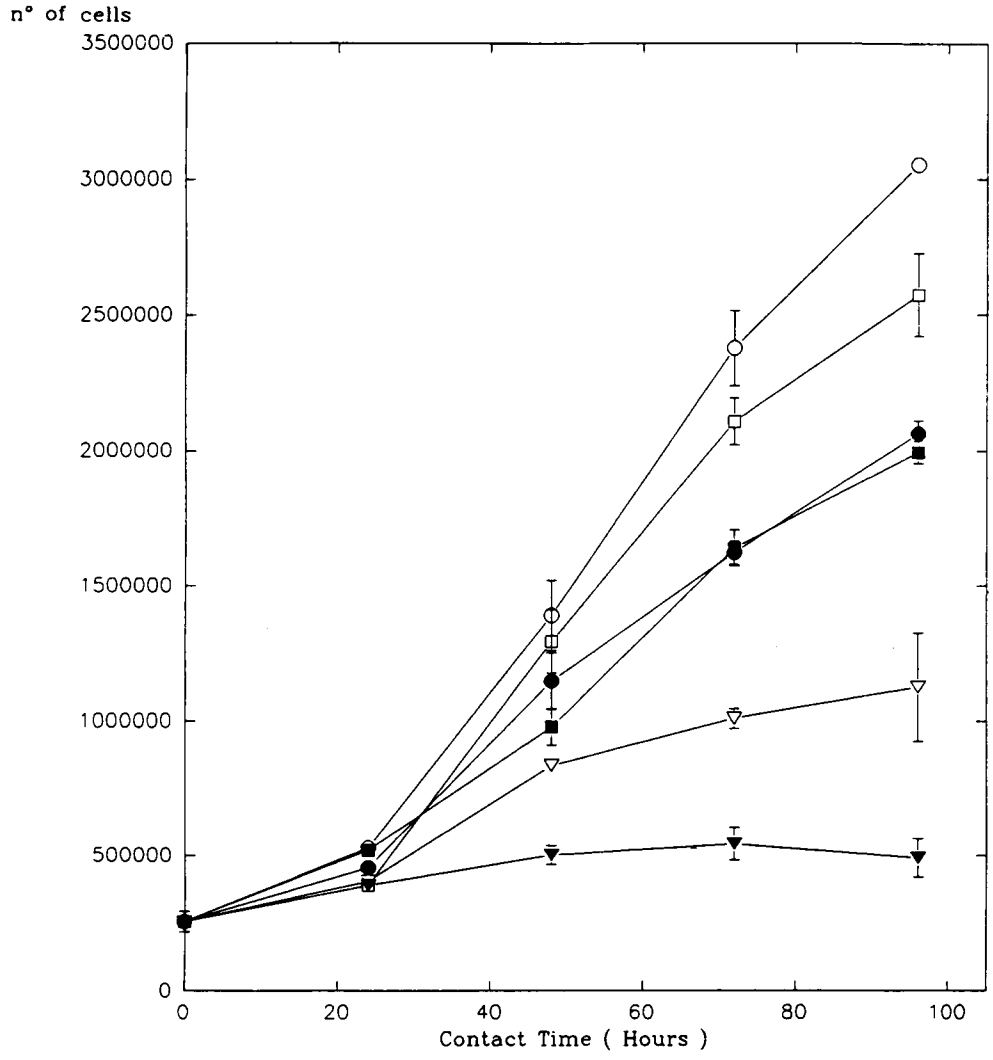


**Figure 6.** Time effect of docetaxel and CI 980 alone or in combination on KB 3-1 cell proliferation. Cells were treated with drug free medium (○), docetaxel 1 nM (●), CI 980 0.05 nM (□), CI 980 0.25 nM (■), docetaxel (1 nM)/CI 980 (0.05 nM) (▽) and docetaxel (1 nM)/CI 980 (0.25 nM) (▼). At the designated times, viable cells were numerated as described in Materials and methods. Data are the mean ± SEM of three experiments.

**Table 4.** Synergy of the docetaxel/CLC-A combination of the inhibition of KB 3-1 cell proliferation ($p < 0.01$)

| | Observed surviving fraction/predicted surviving fraction | |
|---|---|---|
| Combination of drugs | 48 h | 72 h |
| Docetaxel 1 nM/CI 980 0.05 nM | 0.82 ± 0.14 | 0.70 ± 0.04 |
| Docetaxel 1 nM/CI 980 0.25 nM | 0.64 ± 0.02 | 0.48 ± 0.02 |
| Docetaxel 1 nM/Cmd 2.50 nM | 0.82 ± 0.09 | 0.65 ± 0.01 |
| Docetaxel 1 nM/Cmd 10.00 nM | 0.75 ± 0.14 | 0.54 ± 0.09 |

The predicted surviving fractions are the products of multiplying the surviving fraction obtained with one agent at the designated time and concentration by the surviving fraction obtained with the other agent at the same time and concentration. Each point is the mean value of three experiments. See Material and methods for definition of synergy.

**Table 5.** Effect of the combination docetaxel/CLC-A on the intracellular polymerized tubulin content

| Drugs | Contact time (h) | Polymerized tubulin (%) |
|---|---|---|
| Docetaxel 5 nM | 4 | 103 |
| Docetaxel 5 nM/Cl 980 0.5 nM | 4 | 104 |
| Docetaxel 5 nM/Cl 980 1.0 nM | 4 | 101 |
| Cl980 0.5 nM | 4 | 97 |
| Cl980 1.0 nM | 4 | 92 |
| Docetaxel 5 nM/Cmd 5.0 nM | 4 | 98 |
| Docetaxel 5 nM/Cmd 20.0 nM | 4 | 94 |
| Cmd 5.0 nM | 4 | 97 |
| Cmd 20.0 nM | 4 | 92 |
| Docetaxel 1 nM | 20 | 98 |
| Docetaxel 1 nM/Cl 980 0.05 nM | 20 | 101 |
| Docetaxel 1nM/Cl 980 0.25 nM | 20 | 98 |
| Cl 980 0.05 nM | 20 | 98 |
| Cl 980 0.25 nM | 20 | 98 |
| Docetaxel 1nM/Cmd 5 nM | 20 | 92 |
| Docetaxel InM/Cmd 10 nM | 20 | 97 |
| Docetaxel InM/Cmd 20 nM | 20 | 96 |
| Cmd 5.0 nM | 20 | 99 |
| Cmd 10.0 nM | 20 | 86 |
| Cmd 20.0 nM | 20 | 82 |

Polymerized tubulin content of treated KB 3-1 cells was expressed as the percentage of control cells. SEMs are less than 5% of the mean values.

This combination was then studied at lower does. Measurements of cell cycle by flow cytometry were impossible to perform because multinucleated KB 3-1 cells formed when the concentrations of docetaxel were decreased. We therefore tested the docetaxel (1 nM)/CI 980 (0.05 nM) combination on the inhibition of cell proliferation. The supra-additive effect observed cannot be explained by a modification of the microtubule network and by a variation in polymerized tubulin content, or by $G_2M$ blockage, since most cells were multinucleated with interphasis nuclei.

Multinucleated cells may result from an abnormal formation of the mitotic spindle, which is unable to segregate the sister chromatids properly, resulting in the reformation of the nuclear membrane around groups of polyploid chromosomes.[22] The abnormal cell division associated with multinucleated cells may be all the more implicated in the inhibition of cell proliferation as the inhibition was not evident before 48 h contact time.

Finally, the multinucleation, the absence of data excluding block or delay in $G_2M$ at very low concentrations and the synergistic $G_2M$ block at higher concentrations argue for some degree of mitotic inhibition with the combinations of drugs.

Recently, it has been suggested that the causes of cytotoxicity of depolymerizing agents and stabilizing agents differ. The former interfere with spindle function whereas the latter disturb spindle formation.[22] Moreover, the suppression of microtubule dynamics by taxol is dose dependent. At the lowest effective binding stochiometries, taxol inhibits only the rate and extent of shortening, suggesting that progression through metaphase may be slowed but progression to anaphase is not blocked.[23] This difference in mechanisms of cytotoxicity may explain the synergistic effect observed in the inhibition of proliferation and in $G_2M$ block with the Taxoid/CLC-A combination at low doses.

A similar study is in progress concerning *Vinca* alkaloids; preliminary results on high doses of vinblastine showed abnormal structures similar to those described above, but it was reported that the combination paclitaxel/vinblastine was not synergistic on survival of some cell lines.[24,25]

The doses of docetaxel used in the combination are much lower than those administered in clinical trials.[2] It may be of interest to combine these drugs in cancer therapy, thereby lowering the toxicity of each drug without decreasing the therapeutic efficacy.

## Conclusion

Our study demonstrated firstly that the combination of a taxoid and a colchicine analog induces mod-

ifications of the microtubular network different from those described with each drug alone. Secondly, our main result indicates that the simultaneous combination of these two agents at very low doses induces a synergistic inhibition of proliferation of KB-3-1 cells.

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