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Research paper

Sequence-dependent cytotoxicity of combination chemotherapy using paclitaxel, carboplatin and bleomycin in human lung and ovarian cancer

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Combination chemotherapy for non-small cell lung cancer (NSCLC) and ovarian cancer typically consists of a regimen of a taxane such as paclitaxel and a platinum-containing agent. Bleomycin, which halts cell cycle progression at G_2 phase, is an agent which might thereby increase taxane cytotoxicity. The goal of this study was to evaluate the effect of different paclitaxel–platinum or paclitaxel–bleomycin schedules on cytotoxicity in human NSCLC and ovarian cancer cells. The simultaneous combination of paclitaxel and carboplatin exhibited simple additivity *in vitro*, while sequential exposure studies indicated that carboplatin followed by paclitaxel produced greater than additive cytotoxicity using the isobologram analysis of combinatorial effects. In contrast, the simultaneous combination of paclitaxel and bleomycin consistently exhibited greater than additive effects indicating a potentially synergistic combination. Sequential exposure studies of bleomycin followed by paclitaxel produced similar synergistic findings. Experiments in SCID mice evaluating the combinations of paclitaxel and bleomycin supported the *in vitro* results, as significantly enhanced A549 lung tumor growth inhibition was observed when paclitaxel was administered 1 h after bleomycin. The synergistic activity shown by the combination of bleomycin and paclitaxel indicates a potentially beneficial novel combination for treatment of NSCLC and ovarian cancer. [© 2001 Lippincott Williams & Wilkins.]

Key words: Bleomycin, carboplatin, combination chemotherapy, isobologram, paclitaxel.

Introduction

The most common chemotherapeutic agents for lung and ovarian cancer include a combination of a taxane such as paclitaxel and a platinum-containing agent. Paclitaxel is one of the most broadly effective antineoplastic agents developed since the end of the 1980s. It has activity in many tumor types including advanced ovarian cancer,[1] breast cancer,[2] and both small-cell and non-small cell lung (NSCLC) cancers.[2] Paclitaxel is a cell-cycle phase specific drug with maximal effect throughout the G_2 and early M phases, and has demonstrated schedule dependence.[2]

Cisplatin, the first generation platinum-containing compound, also has activity in a wide variety of cancers, but exhibits dose-limiting nephrotoxicity.[3] Carboplatin, a second generation platinum-containing compound, is currently the most commonly used platinum-containing chemotherapeutic agent. Randomized trials in lung[4] and ovarian[5] cancer have shown that carboplatin-based chemotherapy produced equivalent survival when compared to a regimen containing cisplatin. Carboplatin has a lower potential for causing nephrotoxicity, neurotoxicity and emesis than cisplatin, but is significantly more myelosuppressive. It also has antitumor activity in head and neck cancer, and testicular cancer, but it is predominantly used in lung and ovarian cancers.[3] While carboplatin's cytotoxicity is not cell cycle phase specific, the effects can be maximized if the cells are exposed to carboplatin in S phase.[3]

Bleomycin was first isolated from culture broths of the fungus *Streptomyces verticillus* and contains at least 13 different fractions, with the major components being bleomycin A_2 and B_2.[6] It has significant activity in Hodgkin's and non-Hodgkin's lymphomas, testicular tumors, and lung cancer.[7] Bleomycin has also exhibited activity in previously untreated ovarian cancer patients,[8] but is not used in current combination regimens. Mechanistically, iron-bound bleomycin causes single- and double-stranded DNA damage which blocks cell cycle progression at G_2/M.[7] Toxic effects include cutaneous reactions, alopecia and pulmonary fibrosis. Myelosuppression is uncommon except in

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cases of patients with severely compromised bone marrow function resulting from previous chemotherapy.[9] This low toxicity in bone marrow allows bleomycin to be used at full strength in combination with myelosuppressive drugs[7] and was one rationale for evaluating it in combination with paclitaxel.

Based on the cell cycle phase specificity of the two drugs, we hypothesized that bleomycin could enhance paclitaxel activity by blocking dividing cells in G_2/M phase, the most sensitive portion of the cell cycle for paclitaxel-induced cytotoxicity. Therefore, we evaluated the efficacy of this combination and contrasted it with the established combination of paclitaxel and carboplatin. To accomplish this we examined the effects of the individual drugs against human NSCLC cells and human ovarian cancer cells *in vitro*. We then analyzed simultaneous and sequential combinations of paclitaxel with either bleomycin or carboplatin to determine the most effective sequence and combination *in vitro* using the isobologram method. The isobologram method was chosen to avoid interpretation problems that can arise with other combination methods, such as median-effect analysis.[10] Finally, we confirmed the effects of the combination of paclitaxel and bleomycin on severe combined immune deficient (SCID) mice implanted with human lung tumor xenografts.

Materials and methods

Chemicals

Paclitaxel (Taxol®), carboplatin (Paraplatin®) and bleomycin (Blenoxane®) were purchased from Bristol-Myers Squibb Oncology Products (Syracuse, NY) and stored in rubber-stopped glass vials. Paclitaxel was supplied as a 6 mg/ml concentrate for injection and was stored at 4°C prior to dilution. Carboplatin was supplied as 50 mg of lyophilized powder and was stored at 4°C prior to reconstitution in sterile water for injection, USP. After reconstitution, carboplatin was stored at −80°C. Bleomycin was obtained as 15 U of lyophilized powder, stored at 4°C prior to reconstitution and was manufactured by Nippon Kayaku Company (Tokyo, Japan) for Bristol-Myers Squibb. Bleomycin was reconstituted in sterile water for injection, USP, and then stored at −20°C before use.

Cell culture

Human NSCLC cells (A549, CCL 185), originally derived from a human alveolar cell carcinoma, were obtained from ATCC (Rockville, MD). Ovarian cancer cells (HEY), derived from a patient with differentiated papillary cystadenocarcinoma of the ovary,[11] were obtained from the laboratory of Dr Evan Hersh (Arizona Cancer Center, Tucson, AZ). Cells were maintained in PDRG basal media (Hyclone, Logan, UT) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco/BRL Products, Grand Island, NY) and 5% iron-enriched calf serum (Hyclone, Logan, UT). Cells were incubated at 37°C in air with 100% relative humidity.

Flow cytometry

Flow cytometry was used to assess the effect of bleomycin on the percent of cells accumulated in G_2 phase. A549 cells were plated at a density of 500 000 in 25 cm^2 flasks and allowed to attach for 24 h. The following day the cells were exposed to concentrations of bleomycin ranging from 0 to 25 μg/ml for 24 h. On day 2, the drug and media was removed, and the cells were washed with phosphate-buffered saline (PBS), removed from the flasks and resuspended in 1 ml of Krishan's buffer (0.1% sodium citrate, 0.02 mg/ml RNase, 0.3% NP-40 and 50 μg/ml propidium iodide) and stored at 4°C overnight. The samples were analyzed using a Becton Dickinson FACScan flow cytometer (San Jose, CA) and ModFit LT (version 2.0, Verity Software House, Topsham, ME) data analysis program.

Sulforhodamine B (SRB) assay

The sulforhodamine B (SRB) assay was used to assess the growth inhibitory effects in the A549 and HEY cell lines.[12] Tumor cells were plated at densities of 2500/well in 96-well microtiter plates. On day 2, drugs diluted in sterile water were added at concentrations ranging from 1 ng/ml to 100 μg/ml. For short-term drug exposures, the plates were rinsed with PBS and incubated with drug-free media at 37°C in air with 100% relative humidity for a total of 5 days. After the 5-day incubation period, the plates were washed with PBS and fixed with trichloroacetic acid. Total protein in the cells was then stained with 0.4% (w/v) SRB stain for 10 min. Unbound dye was rinsed off using 1% acetic acid and the bound dye was solubilized by 0.01 M Tris base buffer. The protein was quantified by visible absorbance at 557 nm on an automated microculture plate reader (CeresUV900HDi; Bio-Tek Instruments, Winooski, VT). Results were calculated as percent of absorbance from untreated cells. Each drug concentration was tested in three independent experiments, and the IC_{50} values (inhibitory concentration at 50%) were determined using the dose–response analysis function in the data graphing software

package Microcal Origin (version 5.0; Microcal Software, Northampton, MA).

Isobologram analysis

The cytotoxic relationship of paclitaxel in combination with either carboplatin or bleomycin was determined using isobologram analysis.[13] In this analysis, the maximal concentration of each drug in combination is limited to the 50% inhibitory (IC_{50}) value when used as a single agent. If a specific combination of two drugs resulted in an IC_{50} value, that number was plotted on the graph. This method requires testing multiple concentrations of each of the agents used in the combinations, such that each combination would be expected to yield 50% growth inhibition if there is simple additivity. In brief, the IC_{50} value of each individual drug is normalized to 1.0. Fractions of these values are then tested in combination and if the subsequent cytotoxic effect results in IC_{50} values of the combination, these points are plotted on the graph. If the data points lie along the 45° line connecting the two individual IC_{50} points on each axis, the relationship is termed additive. If the points lie above or below this line, the relationship is deemed to be less than additive (antagonistic) or greater than additive (synergistic), respectively.

Animal studies

SCID male mice (C.B-17/IcrACCscid) were obtained from the colony developed at the University of Arizona and maintained under specific pathogen-free conditions. These animals are deficient in mature B and T lymphocytes,[14] and thereafter support the growth of various human tumor cells.[15] The mice were housed in microisolator cages, and were provided NIH-31 modified mouse sterilizable diet pellets and ultraviolet-irradiated water, *ad libitum*.

Subcutaneous tumor cell injections of 10^6 A549 cells were given on the mouse's lower right flank on day 0. On days 1, 5 and 9, injections of paclitaxel (20 mg/kg) and bleomycin (10 mg/kg) were given i.p. Following development of the tumors at roughly 2 weeks, the tumor weight was estimated using the formula (tumor width^2 × tumor length/2) twice weekly.

Statistical analysis

Linear regression and exponential decay analyses for the isobolograms were performed using the data graphing software package Microcal Origin (version 5.0; Microcal Software). Statistical analysis of the flow cytometry results was performed using one-way analysis of variance (ANOVA). Tumor growth rates were compared using ANOVA and Tukey's multiple comparison.[16]

Results

Cytotoxicity studies

Antitumor effects of paclitaxel, carboplatin and bleomycin were evaluated *in vitro* using the SRB assay in both a non-small cell lung cancer cell line (A549) and a human ovarian cancer cell line (HEY). Individual IC_{50} values were obtained for paclitaxel (0.006 and 0.02 μg/ml), carboplatin (10 and 15 μg/ml) and bleomycin (1.0 and 0.07 μg/ml) in A549 and HEY cells, respectively, treated for 120 h. One hour IC_{50} values were also obtained for the three drugs in each cell line (Table 1). The 1-h timecourse was chosen to best approximate clinical situations, but this also could reduce cytotoxic potential for those drugs that are schedule-dependent. The results show that human A549 lung cells were more sensitive than the ovarian HEY cells to the cytotoxic effects of paclitaxel and carboplatin. Conversely, the lung cancer cells were slightly less sensitive to the effects of bleomycin. As expected, the IC_{50} values for the 1-h exposures were considerably higher than those for 120-h exposures, which may be due to both the schedule-dependent nature of the drugs as well as the short duration of incubation time for the slowly activated drugs such as carboplatin.

Flow cytometry was used to evaluate the cell cycle effects of bleomycin on A549 cells for 24 h (Figure 1). Concentrations of bleomycin ranged from 0 to 25 μg/ml. The greatest amount of increase in the G_2/M cell population was seen at 25 μg/ml. However, at all bleomycin concentrations of 5 μg/ml or above, statistically significant decreases in the population of cells in S phase were observed. Significant increases in the G_2/M populations were also observed at both the 10 and 25 μg/ml concentrations.

Table 1. IC_{50} values for effects of paclitaxel, carboplatin and bleomycin on human NSCLC (A549) and human ovarian cancer calle (HEY) (results are the mean of three independent experiments ± SEM)

Cells	Agent (μg/ml)		
	Paclitaxel	Carboplatin	Bleomycin
A549 (120 h)	0.006 ± 2.79	10 ± 3.76	1.0 ± 0.51
A549 (1 h)	1.0 ± 7.13	250 ± 2.10	40 ± 2.25
HEY (120 h)	0.02 ± 7.08	15 ± 4.01	0.7 ± 1.99
HEY (1 h)	0.2 ± 9.27	500 ± 7.20	25 ± 1.96

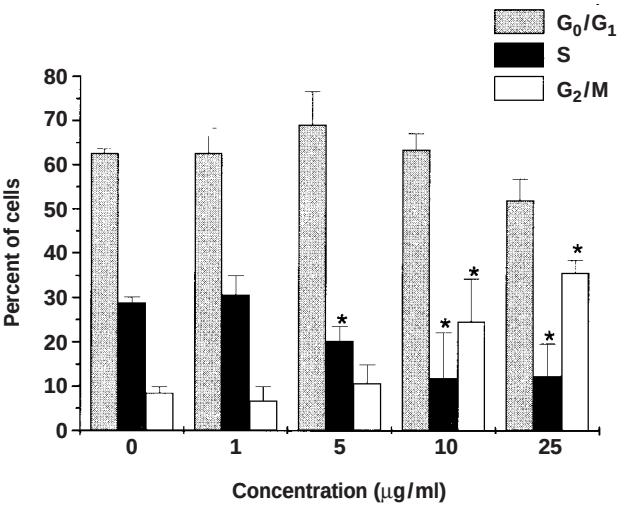

Figure 1. Effect of bleomycin on the cell cycle of A549 cells for 24 h. Results are the mean of three independent experiments ± SEM and $*p < 0.05$ versus control as analyzed by one-way analysis of variance (ANOVA).

Simultaneous combination studies

Fractions of the individual IC_{50} values for paclitaxel and carboplatin or paclitaxel and bleomycin were combined and administered to the cells for 120 h. The combination of paclitaxel and carboplatin exhibited additivity in both the A549 and HEY cells (Figure 2). However, the simultaneous combination of paclitaxel and bleomycin exhibited greater than additive effects in both the A549 and HEY cells (Figure 3).

Sequential combination studies

After determining the effects of simultaneous drug exposure on the cell lines, we examined the effects of different sequences on growth inhibition. First, we evaluated the sequence-dependent effects of paclitaxel and bleomycin since this combination was the most active in the simultaneous exposure studies. The first drug was placed on the cells for 1 h, the cells were

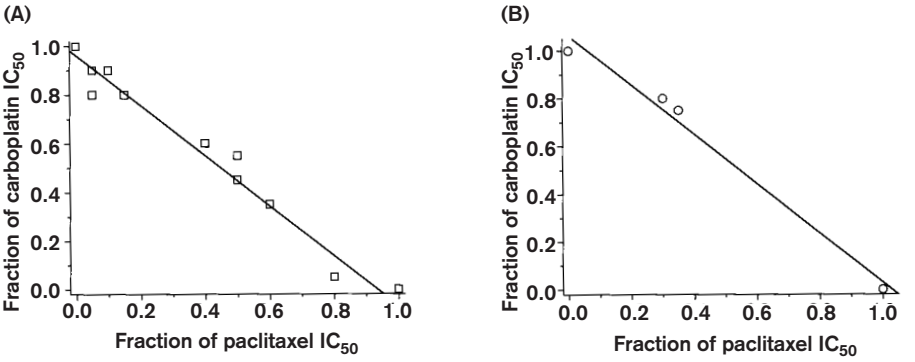

Figure 2. Isobologram analysis of simultaneous combination of paclitaxel and carboplatin for 120 h in A549 cells (A) and HEY cells (B).

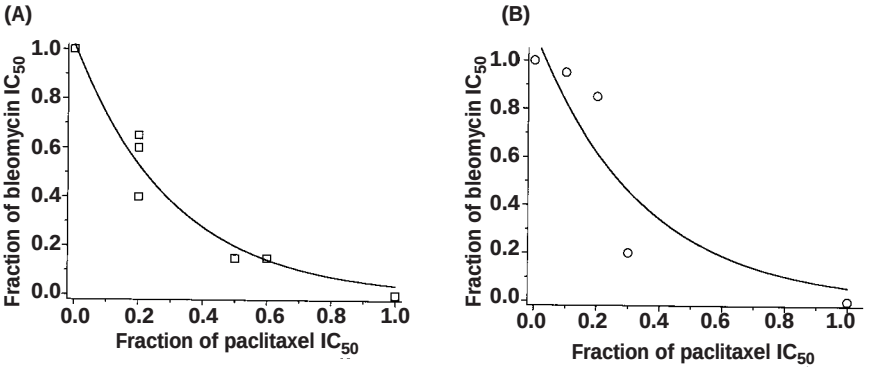

Figure 3. Isobologram analysis of simultaneous exposure of paclitaxel and bleomycin for 120 h in A549 cells (A) and HEY cells (B).

washed and new media plus the second drug was placed on the cells for 1 h. This was followed by an additional washing and then incubation in drug-free media for a total of 120 h. Several sequence-dependent effects were observed—the combination of paclitaxel followed by bleomycin (Figure 4a) exhibited additivity, while the reverse schedule (Figure 4b) showed synergism using the isobologram analysis. The dose–response curves for the sequence of paclitaxel first indicated that this schedule is not very effective (data not shown); however, the lack of a large number of data points in this isobologram means less than additive and greater than additive effects could not be ruled out.

Next, the combination of paclitaxel and carboplatin was examined to determine the effect of the sequence of exposure. Paclitaxel followed by carboplatin (Figure 5a) exhibited additivity, while the reverse schedule, carboplatin first (Figure 5b), exhibited greater than additive effects as analyzed by the isobologram method. However, less than additive effects cannot be ruled out for the first schedule since only three points were obtained for analysis. Isobolograms could not be obtained for the sequential combinations in the HEY cell line due to marked sensitivity to the combined drugs, which precluded achieving reliable IC_{50} values. However, dose–response curves of the combinations tested suggested that the schedule of carboplatin followed by paclitaxel was more effective than the reverse schedule. Similarly, the sequence of bleomycin followed by paclitaxel appeared more effective than the opposite sequence in this cell line (data not shown).

Overall, these *in vitro* data show that there is marked potential synergy for combinations of paclitaxel and bleomycin, particularly when bleomycin is administered first. The flow cytometry studies confirm that bleomycin was able to block the cell cycle progression at the paclitaxel-sensitive G_2/M phase. In contrast, combinations of paclitaxel and carboplatin appeared to be largely additive, no matter which

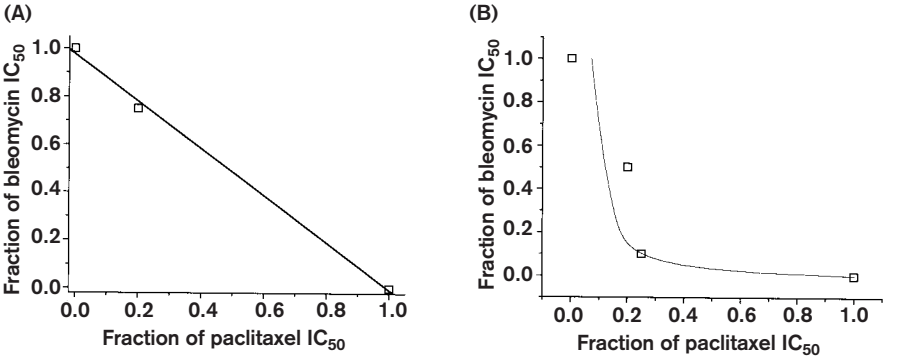

Figure 4. Isobologram analysis of a 1-h sequential exposure of paclitaxel (IC_{50} = 1 μg/ml) followed by bleomycin (IC_{50} = 40 μg/ml) in A549 cells (A) and the reverse schedule (B).

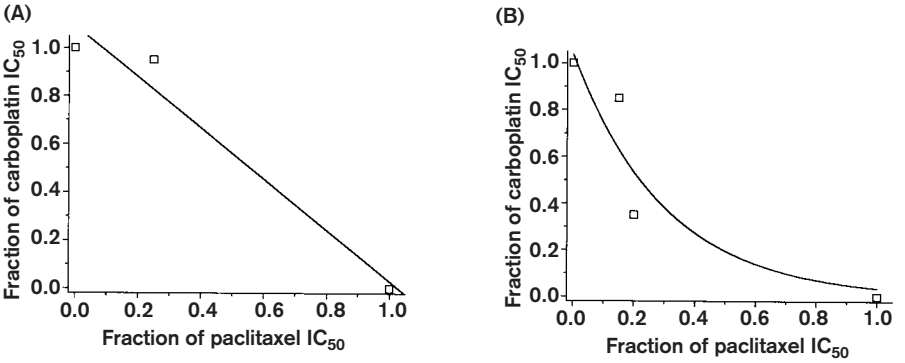

Figure 5. Isobologram analysis of a 1-h sequential exposure of paclitaxel (IC_{50} = 1 μg/ml) followed by carboplatin (IC_{50} = 250 μg/ml) in A549 cells (A) and the reverse schedule (B).

sequence of exposure was tested. Because of the apparent synergy, bleomycin/paclitaxel combinations were evaluated in SCID mice.

Animal studies

Six groups of eight SCID mice were injected s.c. with 10^6 A549 cells on day 0. They then received various combinations of paclitaxel (20 mg/kg) and bleomycin (10 mg/kg) on days 1, 5 and 9. The sequential combinations consisted of paclitaxel administered 1-h prior to the administration of bleomycin and vice versa. A previous dose-finding experiment with paclitaxel and bleomycin on A549 cells in SCID mice determined these doses were tolerated in combination by the animals (data not shown). Animals were monitored for total body weight (Figure 6a) and tumor weight (Figure 6b) for 48 days. The control mice were sacrificed at 44 days after the tumors averaged 2 g in weight, and the group of mice receiving bleomycin 1 h prior to paclitaxel was sacrificed at 54 days. Animals that received bleomycin singly and in combination lost approximately 15% of their total body weight following administration of the drug, but regained the weight once dosing was finished.

Tumor growth in each treatment group was significantly inhibited as compared to the control group. Consistent with the *in vitro* experiments, the most effective combination in this study was the administration of bleomycin 1 h prior to paclitaxel. This produced a profound inhibition of tumor growth which was not matched by the single agent or the opposite sequence of the two-drug combination. Indeed in the bleomycin first group, the tumors never grew beyond 200 mg, even up to 54 days post-implantation. According to NCI standards,[17] the tumor growth inhibition value (T/C value) was calculated on day 44 using the median tumor weights of all the groups (Table 2). T/C $\leqslant$42% is considered to be the minimum level for activity and T/C $<$10% indicates a high antitumor activity level which warrants further investigation.[17] All of the treatment groups exhibited levels of activity according to the NCI standards and the bleomycin first group clearly indicated a highly active combination.

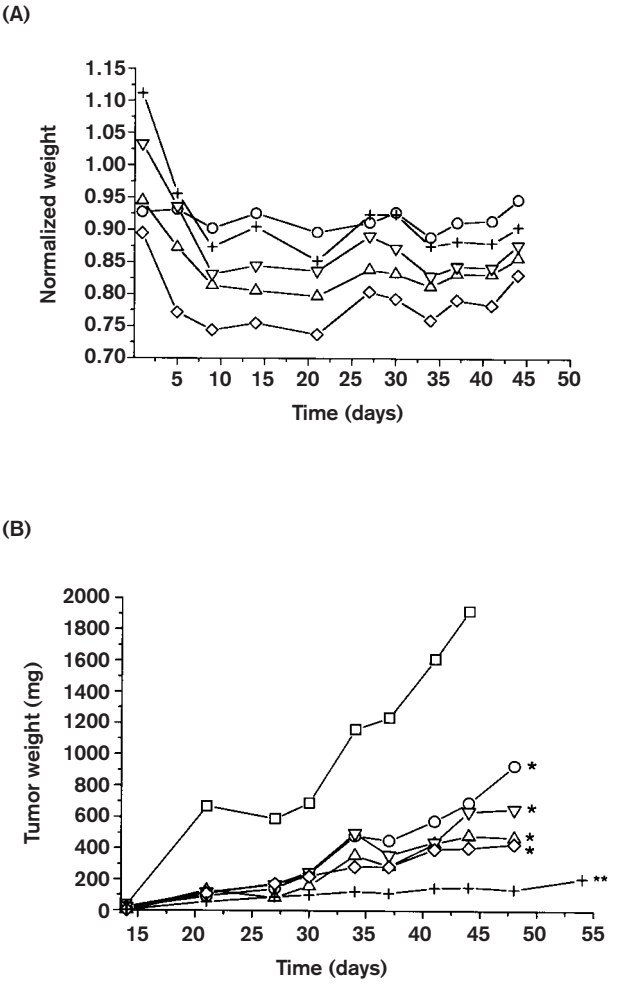

Figure 6. Effect of paclitaxel (20 mg/kg) and bleomycin (10 mg/kg) on total body weight normalized to control group (A) and tumor weight (B) in SCID mice with control (□), paclitaxel (○), bleomycin (△), paclitaxel and bleomycin administered simultaneously (▽), paclitaxel administered 1 h before bleomycin (◇), and bleomycin administered 1 h before paclitaxel (+). *$p<0.05$ versus control using ANOVA and **$p<0.05$ versus paclitaxel alone using Tukey's multiple comparison.[16]

Table 2. Tumor growth inhibition (T/C value), the NCI standard for determination of antitumor activity for early stage disease,[17] was calculated using median measurements of tumor weights from day 44

Group	T/C (%)
Paclitaxel	36.1
Bleomycin	25.2
Paclitaxel and bleomycin	33.2
Paclitaxel before bleomycin	21.1
Bleomycin before paclitaxel	7.9

Discussion

The combination of paclitaxel and carboplatin has been used successfully in the treatment of both NSCLC and ovarian cancer, but to our knowledge the combination of paclitaxel and bleomycin has never

been investigated. Previous clinical studies have evaluated paclitaxel combined in the PEB regimen (with etoposide and bleomycin) in advanced solid tumors[18] or in patients with germ cell cancer.[19] While these combinations were active, neither of these studies evaluated sequential drug administration, and bleomycin always followed paclitaxel administration. The effect of sequence on paclitaxel combinations with platinum-containing agents has been rigorously studied with cisplatin but not with carboplatin.[20] Based on cell cycle theory, the impact of different schedules of carboplatin and paclitaxel should be negligible since carboplatin is active in all phases of the cell cycle.[3] This is consistent with our data for simultaneous exposure and the sequence of paclitaxel before carboplatin in the treatment of NSCLC.[21] Our *in vitro* data for the paclitaxel/carboplatin combination showed additive cytotoxic effects when both drugs were added simultaneously, as well as with the sequence of paclitaxel followed by carboplatin. Other studies in both ovarian and lung cancer have indicated that varied schedules of paclitaxel and carboplatin produced no difference in clinical outcome.[22] In contrast, the reverse sequence of carboplatin before paclitaxel produced some evidence for greater than additive cell killing. One caveat for this finding is that a relatively short carboplatin exposure time of 1 h was used and this might not be sufficient to maximize the drug's activity because of the slow dissociation of carboplatin to its active form.[23] Conversely, the effects of synergism observed with the sequence of carboplatin before paclitaxel could also be partly due to the effect of a G_1/S arresting agent, such as carboplatin. This could potentially block the apoptotic effects of paclitaxel which are believed to be maximized in G_2 phase.[24] For example, the *in vitro* studies of Johnson *et al*. clearly showed that the antitumor effect of G_1/S arresting anticancer agents was not greater in combination with an antimitotic agent (like the taxanes) than when the antimitotic agent was used alone.[24]

The combination of paclitaxel and bleomycin has not been reported previously, but because of their unique mechanisms of action and non-overlapping toxicities, their theoretical combination shows promise. Bleomycin inhibits progression of cells out of G_2 phase and if administered prior to paclitaxel, should allow the maximal effect of paclitaxel to be expressed at the G_2/M portion of the cell cycle. We used flow cytometry to analyze lung cancer cells treated with bleomycin for 24 h to confirm this G_2 block. Statistically significant increases in the population of cells in G_2/M were observed at 10 and 25 μg/ml ($p < 0.05$). This finding confirmed bleomycin's blockade in the cell cycle at the critical G_2/M juncture.

Other sequence-dependent effects with paclitaxel and other anticancer drugs have been reported both *in vitro* and in clinical trials. In cells treated with paclitaxel and etoposide, several researchers determined that the simultaneous combination exhibited additivity while greater than additive effects were observed when either agent was administered first.[25,26] Similar results were seen with the combination of paclitaxel and SN-38, the active metabolite of irinotecan.[27] In these *in vitro* studies several sequences of paclitaxel and either etoposide or SN-38 resulted in greater cell killing than simultaneous exposure. Phase I results also suggest that the sequence of paclitaxel administration in a combination regimen is important in reducing toxicity. For example, a phase I study with doxorubicin indicated that paclitaxel given after doxorubicin facilitated reduced stomatitis over that seen with the reverse sequence.[28] Conversely, more severe toxicity was seen clinically when paclitaxel was administered before cyclophosphamide[29] and when cisplatin was administered prior to paclitaxel.[20] These studies suggest that the sequence of drug administration is an important determinant of efficacy for paclitaxel-containing combinations.

While the isobologram method of analysis that we used is useful in differentiating synergy, additivity and antagonism, it has limited utility since only a portion of the data obtained can be plotted unless the specific inhibitory concentration value (in this case, an IC_{50} value) is obtained for the combination. Analysis of combination data using the median-effect principle of Chou and Talalay,[10] which includes a wider spectrum of data, is possible, but the ratio of the drugs used in combination is typically fixed to avoid analysis difficulties. A further problem with median-effect analyses is that multiple effects (synergism, additivity or inhibition) can be observed at different parts of the concentration–effect curve.[30] For these reasons, we chose to use the less equivocal but more labor-intensive isobologram method for the current studies.

Importantly, we have identified an active new drug combination of paclitaxel and bleomycin. We also confirmed the synergistic effect of bleomycin followed by paclitaxel in A549 human lung tumors in SCID mice. According to our hypothesis, bleomycin followed by paclitaxel should be more effective based on cell cycle theory. The sequential study results support this theory as paclitaxel administered before bleomycin exhibited simple additivity, while the reverse sequence showed marked cytotoxic synergy. Our results suggest that bleomycin followed by paclitaxel may be an effective combination in the treatment of NSCLC and ovarian cancer, and clinical trials should be

feasible since these two drugs produce non-overlapping clinical toxicities, thereby enhancing potential clinical utility.

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