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ORIGINAL ARTICLE

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Sequence-dependent cytotoxicity of etoposide and paclitaxel in human breast and lung cancer cell lines

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Abstract *Purpose*: To evaluate the effect of schedule on the interaction of etoposide with paclitaxel in vitro against the A549 human lung cancer cell line and the MDA-231 and MCF-7 human breast cancer cell lines. *Methods*: Exposure schedules that were 24-h concurrent, 24-h sequential, and sequential 24-h with a 24-h intervening drug-free period were quantitatively evaluated by the use of the median-effect principle and the combination index. The clonogenic assay was used to assess cytotoxicity, and calculations were done with computer software. *Results*: Concurrent exposures were less than additive in two of the three cell lines tested. Sequential 24-hour and sequential 24-h with an intervening 24-h drug-free period showed synergism at high effect levels in all three cell lines. Similar synergistic interactions were found when either agent was administered first. *Conclusions*: These results show a schedule-dependent cytotoxic interaction between etoposide and paclitaxel in the human lung and breast cancer cell lines evaluated, with optimal synergism occurring with sequential, but not with concurrent, treatment.

Key words Cell culture · Breast · Lung · Etoposide · Paclitaxel

Introduction

Both paclitaxel and etoposide have a wide range of antitumor activity as well as different potential mechanisms of action and resistance [1, 6–8, 10–14]. Paclitaxel is the prototype of a novel class of antineoplastic agents that inhibit growth of cells in the G2 and M phases of the cell cycle by promoting microtubule assembly [14]. As a microtubule promoter, paclitaxel shifts the equilibrium in favor of the microtubule and thus decreases the concentration of tubulin necessary for subsequent assembly [1, 8]. This mechanism of inhibition is unique from that of other mitotic inhibitors affecting the microtubules, such as vinca alkaloids, which inhibit assembly. Human studies have shown activity against a variety of solid tumors, including ovarian and breast carcinomas, small-cell and non-small-cell lung cancers, head and neck tumors, and malignant melanoma [7].

Etoposide is a semisynthetic podophyllotoxin derivative from the plant *Podophyllum peltatum*. This agent appears to exert its cytotoxic activity as a phase-specific, schedule-dependent, spindle-damaging agent, causing metaphase arrest [4, 5, 10]. Etoposide also inhibits cells from entering mitosis through the production of DNA strand breaks by inhibiting topoisomerase II [9].

Studies evaluating the cytotoxic interaction of different schedules of paclitaxel in combination with other chemotherapy drugs have yielded results consistent with sequence-dependent activity [4, 5]. A previous in vitro study has shown that paclitaxel and etoposide interact in an antagonistic manner when used concurrently in human cancer cell lines; however, the potential effect of scheduling was not evaluated [9]. As the individual efficacy of each of these two agents is significant, we decided to evaluate the combination of paclitaxel and etoposide in relation to a variety of schedules in an attempt to determine an optimal treatment schedule and to find out whether sequencing contributes to the in vitro interaction of these agents. The cytotoxicity of these agents alone and in combination was assessed with a clonogenic assay, and their interaction was quantitatively analyzed using the median-effect principle and the combination index (CI) [2, 3].

Materials and methods

Cell lines and culture media

The human breast cancer cell lines MDA-231 and MCF-7 and the human lung cancer cell line A549 were obtained from the American Type Culture Collection, Rockville, Md., and

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maintained in RPMI-1640 and 5% (v/v) fetal calf serum supplemented with 50 U/ml penicillin, 50 U/ml streptomycin, and 2 m*M* of L-glutamine (A549, MDA-231) or in minimal essential medium with Earle's salts, nonessential amino acids, 10% (v/v) fetal calf serum, 1 m*M* sodium pyruvate, and 10 μg/ml bovine insulin (MCF-7). RPMI-1640, minimal essential medium with Earle's salts, bovine insulin, and penicillin/streptomycin/L-glutamine solution and powders were purchased from Gibco/BRL, Gaithersburg, Md.

Antineoplastic agents

Etoposide and paclitaxel were provided by Bristol-Myers Squibb, Princeton, N.J. Both agents were prepared as 10-m*M* stock solutions in dimethylsulfoxide and stored at −20 °C.

Cloning assay

Aliquots of exponentially growing cells, 500 for A549, 1000 for MDA-231, and 1500 for MCF-7, were plated on 35-mm tissue culture dishes in a total volume of 2 ml of the media described above. Plated cells were incubated overnight in an atmosphere containing 5% CO_2 at 90% humidity and 37 °C. Drug and vehicle in 1000× concentrates were dispensed into the appropriate triplicate culture dishes as 2-μl aliquots. After a 24-h incubation, the media were aspirated and the plates washed twice with the appropriate serum-free media and then replenished with 2 ml of the appropriate complete media. Immediately thereafter, the plates were returned to the incubator for the remainder of 8 to 14 days or treated with 2 μl of the second 1000× drug and returned to the incubator for 24 h or returned to the incubator for 24 h and subsequently treated with 2 μl of the second 1000× drug. After a 24-h exposure to the second drug, the media were aspirated and the plates washed twice with serum-free media, replenished with 2 ml complete media, and returned to the incubator for the remainder of 8 to 14 days. When the colonies became visible macroscopically (generally after 8 days of total incubation for A549, 12 days for MDA-231, and 14 days for MCF-7), the plates were washed twice with phosphate-buffered saline or Tris-buffered saline and immediately fixed and stained with Coomassie Brilliant Blue R (2.5 mg/ml) in 15% acetic acid and 50% methanol. The plates were rinsed with distilled water, and colonies containing more than 30 cells were counted.

Data analysis

Raw colony counts were analyzed by the median effect and CI method, as described previously by Chou and Talalay [2, 3]. The calculations were performed on a digital computer, model DEC-pc466ST, with a Quattro Pro spreadsheet software package from Novell, Orem, Utah. Only data from experiments in which the median effect curves had an r^2 value greater than 0.80 were considered to be reliable, and they were incorporated into the mean values displayed here. A minimum of four replicates were used to calculate mean values.

The median-effect principle involves plotting dose-effect curves for each agent and for multiply diluted fixed-ratio combinations of agents by the application of the median-effect equation:

$$f_a/f_u = (D/D_m)^m$$

in which D is dose, D_m is the dose required for 50% effect (e.g. 50% inhibition of cell growth), f_a is the fraction affected by dose D (e.g. 0.95 if cell growth is inhibited by 95%), f_u is the unaffected fraction, and m is a coefficient of sigmoidicity of the dose-effect curve; $m = 1$, >1, and <1 indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curves, respectively. The dose-effect curve is plotted by a logarithmic conversion of this equation to log $(f_a/f_u) = m \log (D) - m \log (D_m)$ for the median-effect plot: $x = \log (D)$ vs $y = \log (f_a/f_u)$, which determines the m (slope) and D_m (x intercept) values. On the basis of the slope of the dose-effect curves, it may be decided whether the agents have mutually exclusive effects (e.g. similar mode of action) or mutually nonexclusive effects (e.g. independent mode of action). A CI is then determined with the following equation:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{a(D)_1(D)_2}{(D_x)_1(D_x)_2}$$

in which $(D_x)_1$ is the dose of agent 1 required to produce x percent effect alone and $(D)_1$ is the dose of agent 1 required to produce the same x percent effect in combination with $(D)_2$. Similarly, $(D_x)_2$ is the dose of agent 2 required to produce x percent effect alone, and $(D)_2$ is the dose required to produce the same effect in combination with $(D)_1$. If the agents are mutually exclusive, a is 0 (i.e. CI is the sum of two terms); if the agents are mutually nonexclusive, a is 1 (i.e. CI is the sum of three terms). If it is uncertain whether the agents act in a similar or an independent manner, the formula may be solved both ways. Different values of CI may be obtained by solving the equation for different values of f_a (e.g. different degrees of inhibition of cell growth). CI values of <1 indicate synergy, values >1 indicate antagonism, and values equal to 1 indicate additive effects.

Graphs illustrating these principles are shown in Fig. 1.

Results

Each single-agent 24-h exposure IC_{50} (inhibitory concentration for 50% of the cells) was determined for each cell line (A549, 3.10 n*M* paclitaxel, 0.66 μ*M* etoposide; MDA-231, 3.75 n*M*, 0.77 μ*M*; MCF-7, 2.38 n*M*, 0.59 μ*M*, respectively). These IC_{50} values were then used in the design of the combination experiments. The concentrations used and their relationship to IC_{50} are shown in Table 1. The mean values for the multiply repeated combination experiments for the human lung and breast cancer cell lines are listed in Table 2. For the A549 lung cancer cell line, the concurrent 24-h combination of etoposide and paclitaxel was antagonistic or less than additive at all levels of cytotoxicity. The CI generated by averaging the CI values of multiply repeated concurrent exposure assays generated values greater than 1 over the entire range of cell kill. All other schedules tested (paclitaxel for 24 h followed by etoposide for 24 h, etoposide for 24 h followed by paclitaxel for 24 h, and both sequences with an intervening 24-h drug-free incubation) showed synergism. The CI value fell below 1 (greater than additive or synergistic) at all cell kill levels above 75% for each schedule tested.

For the MDA-231 breast cancer cell line, like the A549 cell line, the concurrent 24-h combination of etoposide and paclitaxel was less than additive or antagonistic at all levels of cytotoxicity. The average CI values were greater than 1 over the entire range of cell kill. Also, like the A549 cell line, the MDA-231 cell line showed some degree of synergy with all other schedules tested. The average CI value for the 24-h paclitaxel schedule followed by the 24-h etoposide schedule dropped below 1 between 50% and 55% cell kill. All other sequential exposures generated CI curves that dropped below 1 at 40–50% cell kill, indicating synergy.

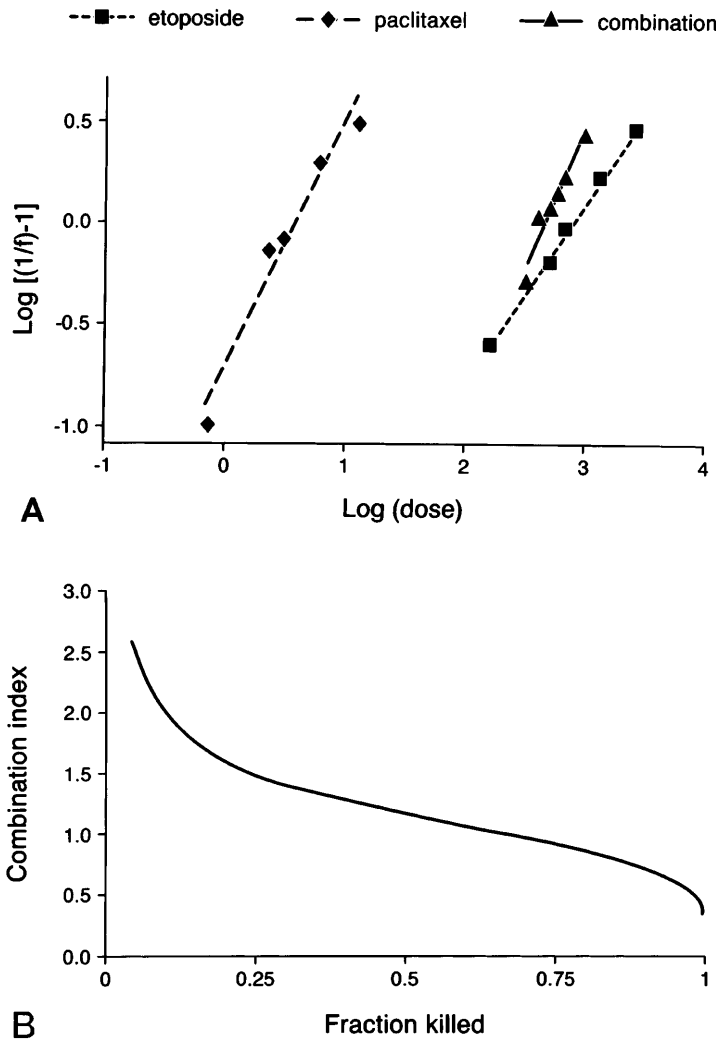

Fig. 1A,B Sample median effect and combination index graphs generated utilizing the median effect and combination index principles developed by Chou and Talalay [3]. **A** The median-effect plots for the concurrent 24-h combination of paclitaxel and etoposide in the MCF-7 breast cancer cell line, (*symbols*, actual data points; *lines*, calculated regressions with r^2 values of 0.99, 0.96, and 0.93 for etoposide alone, paclitaxel alone, and their combination, respectively; *f*, fraction affected. **B** The corresponding combination index curve

Table 1 Actual concentrations of etoposide and paclitaxel and their relationship to IC_{50} fractions

No.	Concentration (n*M*)		Approximate fraction of IC_{50}
	Paclitaxel	Etoposide	
1	1.500	320	4/8
2	1.875	400	5/8
3	2.250	480	6/8
4	2.625	560	7/8
5	3.000	640	8/8
6	4.500	960	12/8

Unlike the A549 and MDA-231 cell lines, the MCF-7 breast cancer cell line showed a greater-than-additive, or synergistic, sensitivity to the 24-h concurrent combination exposure to paclitaxel and etoposide. The CI curve generated by averaging CI values of multiply repeated assays dropped below the entire range of cell kill. All other schedules tested showed synergy to an even greater degree. The sequential exposures generated CI curves that dropped below 1 at 40–50% cell kill.

Discussion

Paclitaxel and etoposide are two chemotherapy agents with broad cytotoxic activity but different mechanisms of action and resistance. Because previous studies of their combined cytotoxicity had yielded conflicting results, we thought that evaluation of the potential impact of sequential and concurrent administrations warranted investigation. We therefore evaluated the effects of drug scheduling on cell growth inhibition in lung and human breast cancer cell lines. The clonogenic assay and median-effect analysis were used to assess drug interactions. The results of our experiments are consistent with a schedule-dependent synergism between paclitaxel and etoposide in these in vitro models.

Previous in vitro studies of paclitaxel and etoposide have been reported by Hahn et al. [9]. These investigators concluded that when etoposide and paclitaxel are administered in combination, a possible antagonism takes place. This was consistent with some but not all of our findings. Concurrent 24-h exposures in two of the three cell lines used displayed antagonism. One cell

Table 2 Combination index mean values ± standard deviation at IC_{50} to IC_{99} cytotoxicity levels for each drug exposure schedule for each of the three human cell lines tested (*Tax* paclitaxel, *Eto* etoposide, *ND*, 24-h drug-free period)

Drug schedule	Combination index value at					No.
	IC_{50}	IC_{75}	IC_{90}	IC_{95}	IC_{99}	
A549 cancer cell line						
Concurrent	1.36 ± 0.29	1.28 ± 0.24	1.21 ± 0.21	1.16 ± 0.19	1.09 ± 0.19	4
Tax then Eto	1.39 ± 0.52	0.92 ± 0.36	0.61 ± 0.25	0.46 ± 0.20	0.26 ± 0.12	5
Eto then Tax	1.39 ± 0.57	0.98 ± 0.41	0.69 ± 0.30	0.55 ± 0.24	0.34 ± 0.15	5
Tax, ND, Eto	1.61 ± 0.21	0.97 ± 0.15	0.61 ± 0.12	0.45 ± 0.11	0.24 ± 0.08	4
Eto, ND, Tax	1.52 ± 0.19	0.94 ± 0.17	0.60 ± 0.15	0.45 ± 0.14	0.25 ± 0.11	4
MDA-231 cancer cell line						
Concurrent	1.49 ± 0.27	1.30 ± 0.57	1.32 ± 0.83	1.42 ± 1.04	1.91 ± 1.65	4
Tax then Eto	1.08 ± 0.58	0.50 ± 0.20	0.29 ± 0.11	0.22 ± 0.10	0.15 ± 0.09	4
Eto then Tax	0.85 ± 0.29	0.39 ± 0.15	0.22 ± 0.15	0.17 ± 0.12	0.11 ± 0.13	4
Tax, ND, Eto	0.89 ± 0.19	0.57 ± 0.16	0.39 ± 0.13	0.30 ± 0.12	0.19 ± 0.09	4
Eto, ND, Tax	0.93 ± 0.04	0.54 ± 0.09	0.34 ± 0.12	0.26 ± 0.11	0.15 ± 0.09	4
MCF-7 cancer cell line						
Concurrent	1.20 ± 0.16	0.92 ± 0.07	0.73 ± 0.08	0.63 ± 0.11	0.49 ± 0.19	4
Tax then Eto	0.90 ± 0.22	0.58 ± 0.14	0.40 ± 0.13	0.32 ± 0.13	0.22 ± 0.15	4
Eto then Tax	1.27 ± 0.51	0.70 ± 0.08	0.42 ± 0.06	0.31 ± 0.09	0.18 ± 0.09	4
Tax, ND, Eto	0.99 ± 0.23	0.61 ± 0.15	0.39 ± 0.11	0.29 ± 0.09	0.16 ± 0.07	4
Eto, ND, Tax	0.89 ± 0.27	0.50 ± 0.16	0.30 ± 0.12	0.21 ± 0.09	0.11 ± 0.06	4

line, MCF-7 breast cancer, exhibited a greater-than-additive, or synergistic, reaction to concurrent 24-h exposure. However, we found that the apparent antagonistic relationship of etoposide and paclitaxel was restricted to concurrent exposures. With the three cell lines used in this study, a synergistic relationship was revealed through manipulation of the administration schedule. Although the molecular details of this interaction in a particular sequence remain to be elucidated, it can be suggested that the antagonistic interaction observed with concurrent but not sequential drug exposure in two of the three cell lines tested could be related to etoposide's interference with the religation of DNA cleaved by topoisomerase II and paclitaxel's interference with cell proliferation (by inducing a sustained block at the metaphase/anaphase boundary of the cell cycle).

Therefore, concurrent use of the agents could be associated with a proliferation block (by paclitaxel) that would not allow for etoposide's activity in interfering with DNA repair mechanisms. However, a drug-free period between the two agents could allow for partial cell repair of the cytotoxic activity of each agent, making it possible for the cell kill to be additive or synergistic when the second agent is added. Another potential explanation for the observed antagonism with the sequence-dependent synergism observed is as yet unexplained differential effects on the apoptotic mechanisms [which may include the proteolytic cleavage of poly(ADP-ribose)polymerase (PARP) associated with etoposide] and their relevance to paclitaxel-induced apoptosis, or effects of paclitaxel-induced Bcl-2 phosphorylation during the G2/M phase of the cell cycle and their relevance to etoposide's cytotoxicity.

Perhaps the greater-than-additive effect seen with concurrent exposure to MCF-7, which is in contrast to the findings of Hahn et al. [9], can be explained by the differences in our treatment exposure lengths. We exposed the cells to etoposide for 24 h, whereas Hahn et al. tested a 1-h exposure. They cited possible mechanistic explanations, as evidenced through cell cycle analysis. For example, part of their hypothesis was that paclitaxel stalls the cells in G2/M long enough for the repair of any etoposide-induced damage. By exposing cells to etoposide for only 1 h, Hahn et al. may have allowed transient DNA damage to be repaired during the paclitaxel 24-h exposure. In contrast, our etoposide exposures were probably long enough to cause irreversible damage to etoposide-sensitive cells. Hahn et al. also suggested that the G1/S arrest caused by etoposide could protect the cells from subsequent paclitaxel damage, since only mitotic cells are sensitive to paclitaxel. However, experiments to evaluate potential differences in cell cycle effect by the different exposure times for these two agents have not been performed.

When the drugs were administered sequentially, or sequentially with an intervening 24-h drug-free incubation period, synergism occurred at higher (clinically relevant) levels of cell kill and less than additive and antagonistic effects at lower (less clinically relevant) levels of cell kill. It is not uncommon for drug combinations to show antagonism at low doses and synergism at higher doses [13]. Since clinical doses of these agents are generally given to produce a high level of cell kill, the synergism at higher doses is more compelling and pertinent than the apparent antagonism at lower doses.

In conclusion, our experiments are consistent with a schedule-dependent synergism between etoposide and paclitaxel in the tested human lung and breast cancer cell lines. Enhanced cytotoxic interaction was noted with sequential rather than concurrent exposures. This basic laboratory observation should be taken into

consideration for the design of clinical trials evaluating etoposide and paclitaxel in combination.

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