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Preclinical report

In vitro evaluation of synergism or antagonism with combinations of new cytotoxic agents

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New cytotoxics with significant activity both in preclinical and clinical situations continued to be applied in the clinic by empiric means. The use of defined cell lines allows unanticipated antagonism between agents to be identified and to suggest synergistic combinations which need to be tested. By means of a semi-automated MTT assay and median effect analysis, we have identified antagonism in two couplets being evaluated in the clinic: etoposide with paclitaxel and vinorelbine with gemcitabine. Optimal use of these agents in man may require spacing these agents in time to prevent an adverse drug interaction between the agents which may diminish the potential response rate. [© 1998 Lippincott Williams & Wilkins.]

Key words: Etoposide, gemcitabine, median effect analysis, paclitaxel, vinorelbine.

Introduction

The past several years has seen the development of a plethora of new cytotoxic agents for the treatment of malignant disease and an even greater number of compounds expected to reach clinical trials shortly.[1] However, the use of combinations of agents remains largely empiric with the only rationale being single-agent activity in man. The use of this approach has been very successful in Hodgkin's disease.[2] Such combinations may actually result in synergism as has been claimed for paclitaxel-doxurubicin[3] or in turn offer potential antagonism in the control of human malignancy.

In an attempt to make combination therapy in man more rational, we and others[4–7] have been using median effect analysis for several years to preclinically evaluate drugs in combination and also evaluate the sequence of administration. The advantage of this approach is the use of a semi-automated technique, and a long history of this approach in both non-malignant and malignant disease.[8] A similar approach has been taken by European investigators using classical isobologram analysis.[9] In a previous report, we have validated median effect analysis against isobologram analysis in our cell lines and culture conditions.[10]

Combinations such as paclitaxel with doxorubicin may result in enhanced cardiotoxicity in man,[11] which may then limit the number of effective treatments.[12] We therefore have concentrated our effort on non-anthracycline-containing combinations which might be of value, particularly in patients with either breast or prostate cancer, as these diseases reflect the interest of our clinical group.

As new drug combinations have been developed combining paclitaxel with etoposide and are touted to be active in small phase I–II trials in man,[13–18] but have conflicting results *in vitro*,[9,19–21] we have re-examined this combination for synergy using six cell lines. In a similar manner, vinorelbine and gemcitabine are being empirically combined in human trials because of their broad antitumor activity in man. Therefore, the question of sequencing and synergy of the latter two agents was examined in breast cancer cell lines. We herein report our findings. Preliminary findings were previously presented in abstract form.[22]

Materials and methods

Cell lines and culture methods

For the evaluation of paclitaxel combinations with etoposide, six cell lines were used: DU145, PC3 and LnCaP, which are prostate cancer cell lines obtained from the ATCC (Rockville, MD); MCF_7-wt (wild-type)

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and MCF-/adr (doxorubicin resistant) as a gift from Dr Kenneth Cowan (National Cancer Institute, Bethesda, MD). LL 86 is a human sarcoma line obtained from the ATCC. The tissue culture methodology has previously been described.[10] In brief, the cell lines were grown to confluence in T 150 tissue culture flasks (Corning Glass Works, Corning, NY) using RPMI 1640 (Gibco, Grand Island, NY) with 5% CO_2 and 10% fetal bovine serum. LnCap cells were grown in McCoy's 5A media (Gibco). All cultures contained penicillin (100 μg/ml), streptomycin (0.25 μg/ml) and glutamine (Gibco) to a final concentration of 2 mM. To maintain drug resistance in the MCF-/adr cell line, cells were grown in media containing 10 μM of doxorubicin. Doxorubicin was deleted in the MCF-/adr cell line culture 3 days prior to harvesting cells for studies. All cell lines were repetitively tested for mycoplasma and had viability by Trypan blue exclusion greater than 95%. Cells were harvested at confluence, washed and aliquoted into 96-well dishes (Falcon 3072; Baxter Scientific, McGraw Park, IL) at concentrations of 5000–8000 cells/well in a total volume of 200 μl/well.

Determination of cytotoxicity and synergy

Harvested cells were cultured for 24 h to allow adherence to the well. The culture medium was then replaced (with or without active drug) every 24 h. For cytotoxic experiments, cells were exposed for 24, 48, 72 or 96 h. Cytotoxic agents were studied in combination concurrently or sequentially with the first agent washed out prior to the introduction of the second drug. Controls contained cells, media and vehicle. Media changes were every 24 h. The following drugs were obtained in powder form from commercial sources: gemcitabine base (Eli Lilly, Indianapolis, IN), etoposide (Sigma-Aldrich, St Louis, MO), vinorelbine (Glaxo Wellcome, Research Triangle, NC) and paclitaxel (Sigma-Aldrich).

Cell growth was evaluated by a previously described MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) assay with absorption measured at 595 nm using a Biorad 3550 Microplate Reader (Biorad, Hercules, CA).[23] ID_{50} concentrations were determined by the EZ-ED50 program (Perrella Scientific, Conyers, CA).[24,25] All reported values are the means of two to four experiments with each study having four wells per dose level.

After the ID_{50} values for 24, 48 and 72 h incubations were established for each cytotoxic agent in each of the cell lines, the following combinations were evaluated for synergy, additivity or antagonism using median effect analysis[8,26] and the computer program of Chou and Chou.[27] Drug combinations studied to reflect clinical use were (i) etoposide with paclitaxel and (ii) vinorelbine with gemcitabine. Fixed drug ratios were examined above and below the ID_{50} with a range from $0.03125N$ to $8N$, where N is a value close to the ID_{50} of an individual drug as previously described.[27] This model is a variant of the Hill equation where $f_a/f_u=[D/D_m]^m$ is defined as the median effect equation. f_a is the fraction of cells affected. f_u is the fraction not affected $(1-f_a)$. D is the dose of drug. D_m is the dose of drug needed to cause a median effect and m is the slope of the median effect curve.[8] The combination index (CI) to define synergism, additive effect or antagonism is further defined as

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

where $\alpha = 0$ for drugs with mutually exclusive mechanisms of action (no drug or biologic interaction), $\alpha = 1$ for drugs with mutually non-exclusive mechanism, $(D)_1$ is the dose of drug 1, $(D)_2$ is the dose of drug 2 and (D_x) is the dose required to cause a median effect. Median effect plots were done [log dose versus log $(f_a/1-f_a)$] as the slopes (m) of the curves are parallel if there is no biologic interaction between drugs (mutually exclusive) thus suggesting the appro-

Table 1. ID_{50} for the cell lines for varying times.

Cell line	Drug	Incubation time (h)	
		24	48
DU 145	paclitaxel	403.12 ± 58.72	28.34 ± 6.70
	etoposide	1151.33 ± 116.58	425.83 ± 55.83
	vinorelbine	23.82 ± 11.29	14.55 ± 1.26
	gemcitabine		245.66 ± 31.97
PC 3	paclitaxel	263.43 ± 26.08	126.56 ± 20.21
	etoposide	439.96 ± 75.34	243.10 ± 58.81
	vinorelbine	12.13 ± 1.21	4.77 ± 0.80
	gemcitabine		686.35 ± 1.06
LnCaP	paclitaxel	112.82 ± 30.83	51.68 ± 28.76
	etoposide	335.33 ± 56.88	245.45 ± 19.07
	vinorelbine	12.22 ± 3.23	9.07 ± 3.05
	gemcitabine		28.69 ± 15.94
MCF_7/wt	paclitaxel	83.67 ± 21.96	43.67 ± 19.66
	etopside	570.54 ± 107.80	454.26 ± 74.98
	vinorelbine	9.04 ± 1.93	7.04 ± 1.24
	gemcitabine	567.43 ± 79.17	545.95 ± 18.88
MCF_7/adr	paclitaxel	678.42 ± 106.95	93.37 ± 17.51
	etoposide	453.03 ± 34.41	312.16 ± 83.32
	vinorelbine	26.67 ± 2.79	11.71 ± 2.81
	gemcitabine	216.23 ± 57.39	116.53 ± 44.51

All values are means ± SD of two to eight experiments expressed in μM.

priate model for this study.[8] Based upon this model, CI values less than 1.0 indicate synergism, 1.0 indicates additive effects and values greater than 1.0 indicate antagonistic effects.

Results

The drug concentrations needed for 50% inhibition of cell growth (ID_{50}) for the various agents in our cell lines over varying periods of exposure are shown in Table 1. A time-dependent effect on all cell lines is apparent for all study drugs but most apparent for paclitaxel when a 24 h period of incubation is compared with a 48 h incubation. The ID_{50} values differ dramatically between cell lines (Table 1).

A plot of log dose versus log $(f_a/1-f_a)$ suggested that the cytotoxic effects were non-exclusive (data not shown) as the slopes were not parallel. Hence, the results of drug interaction by median effect analysis are shown for the non-exclusive criteria which has an assumption in the model that the biologic effects of the two drugs interact. This criteria is also more conservative in assigning an effect to be synergistic.[8] Table 2 describes the interaction of etoposide with paclitaxel in the six cell lines. Using a f_a of 0.5 as the Chou-Tallay model has been criticized as not being accurate at extreme values due to a linear estimation of a non-linear equation,[28,29] five of the cell lines failed to demonstrate a synergistic effect when etoposide was combined concurrently with paclitaxel over a 24 h incubation period (Table 2). Similar findings occurred when a simultaneous 48 h exposure of both agents was applied to the cell lines. When the exposure to cytotoxic agents was extended to 48 h, etoposide and paclitaxel were synergistic only in the MCF_7/adr cell line. This synergy was independent of schedule of administration of the agents (Table 2). The LL 86 sarcoma line demonstrated borderline synergy under differing drug exposure times. Prolonged exposure in a sequential fashion for 24 h of the first agent, the drug washed out and a subsequent 24 h exposure to the second drug (48 h sequential results, Table 2) indicated that the combination of etoposide and paclitaxel remained antagonistic. When the incubation times were increased further to 48 h per drug (96 h sequential results), only the MCF_7/adr line, known to express MDR-1 resistance protein, demonstrated a synergistic effect.

Similar studies were done with gemcitabine and vinorelbine in the breast cancer cell lines MCF_7 and MCF_7/adr. As demonstrated in Table 3, all permuta-

Table 3. Median effect analysis of vinorelbine (V) and gemcitabine (G) combinations both concurrently and sequentially

Cell line	Drug sequence	Total exposure time (h)	CI at $f_{a(50)}$
MCF_7/wt	simultaneously	24	1.68±0.17
	V→G	48	1.24±0.15
	G→V	48	1.35±0.15
	simultaneously	48	1.48±0.17
	V→G	96	2.26±0.25
	G→V	96	3.31±0.33
MCF_7/adr	simultaneously	24	1.77±0.18
	V→G	48	1.58±0.15
	G→V	48	1.86±0.18
	simultaneously	48	2.72±0.27
	V→G	96	2.35±0.26
	G→V	96	1.45±0.17

Table 2. Median effect analysis for drug combinations

Cell line	Paclitaxel+ etoposide 24 h simultaneously [$f_{a(50)}$]	Paclitaxel+ etoposide 48 h simultaneously [$f_{a(50)}$]	Paclitaxel to etoposide 48 h sequentially [$f_{a(50)}$]	Etoposide to paclitaxel 48 h sequentially [$f_{a(50)}$]	Paclitaxel to etoposide 96 h sequentially [$f_{a(50)}$]	Etoposide to paclitaxel 96 h sequentially [$f_{a(50)}$]
DU 145	5.92 (0.56)	2.10 (0.77)	3.07 (0.45)	3.21 (0.39)	3.48 (0.43)	0.99 (0.13)
PC 3	6.32 (0.60)	3.21 (0.88)	1.18 (0.14)	1.04 (0.13)	2.48 (0.36)	1.33 (0.19)
LnCaP	1.59 (0.17)	1.02 (0.30)	4.73 (0.50)	2.38 (0.23)	1.10 (0.20)	0.52 (0.10)
MCF_7/wt	3.49 (0.44)	3.78 (0.54)	1.44 (0.19)	2.21 (0.23)	1.10 (0.22)	1.18 (0.15)
MCF_7/adr+	3.88 (0.45)	0.90 (0.03)	3.65 (0.41)	6.23 (0.44)	0.22 (0.04)	0.54 (0.09)
LL 86	0.90 (0.17)	2.90 (0.32)	0.89 (0.15)	1.08 (0.17)	2.63 (0.44)	5.13 (0.88)

The number given is the CI. CI < 1.0 indicates synergy, CI > 1.0 indicates antagonism. $f_{a(50)}$ is 50% of the cells are affected (killed) by the drug combination. All values are means of two to three experiments ± (SE).

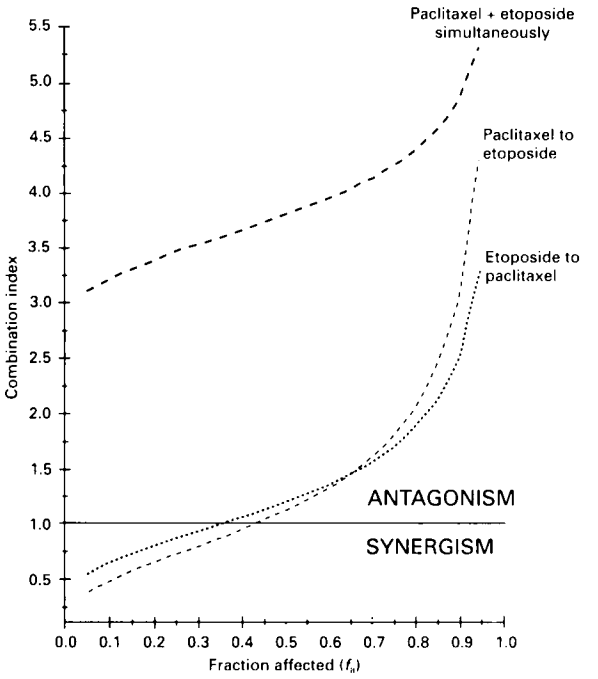

Figure 1. Prolonged incubation of concurrent etoposide and paclitaxel (48 h) or sequential (96 h) as measured by median effect analysis in MCF_7 wild-type cells.

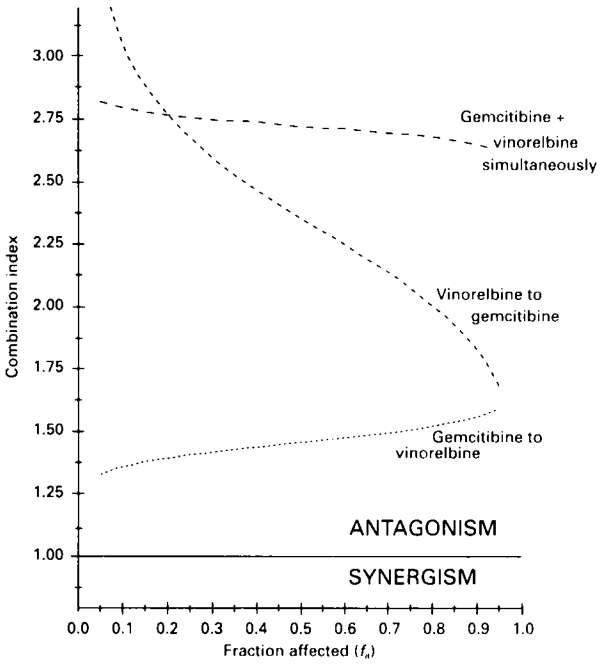

Figure 2. Concurrent vinorelbine with gemcitabine (48 h) or sequential exposure (total 96 h) as evaluated by median effect analysis.

tions of these two agents demonstrated antagonism both at 24 and 48 h incubations. The results of these findings are shown graphically for a 96 h exposure in Figure 1 (etoposide and paclitaxel) and Figure 2 (vinorelbine and gemcitabine) over the entire f_a range.

Discussion

The use of an *in vitro* model and median effect analysis allows the cytotoxic interaction of several agents to be defined. This model has been criticized as it uses a non-weighted linear approximation and thus is inaccurate at extremes of f_a.[28] We have previously validated this model at $f_{a(50)}$ comparing the results to classical isobologram analysis[10] and thus report our results using this point of drug effect. As the amount of cell kill in any human tumor can vary from virtually nil (low f_a) to several logs (high f_a), the *in vitro* results thus serve as a crude measure of expected drug effect in man. In addition, this model does not address therapeutic index or perturbation of metabolism of drugs and therefore remains an imperfect preclinical tool. Another concern is that the effect(s) of combinations of the agents on the tumor microenvironment or host remain outside of the scope of this model system.[6] With the above caveats, we have demonstrated that the combination of etoposide with paclitaxel or vinorelbine with gemcitabine are antagonistic in several cell lines. This data suggests that these active agents in human malignancy may be better used if they are spaced in time sufficiently distant from each other to preclude a drug interaction leading to antagonism of cytotoxic effect rather than combined drugs for convenience. These findings are of further concern as there are ongoing trials of these combinations of agents which may actually reduce the maximal response rate because of scheduling interactions. We have applied this sequence spacing in our current exploratory trial in inoperable non-small cell lung cancer with vinorelbine on day 1 and paclitaxel with gemcitabine on day 3.

Previous investigators have examined the interaction of etoposide with paclitaxel. Hahn *et al.* evaluated three cell lines (MCF-, A549 and OVG1) by a clonagenic assay and demonstrated reduced cell kill when 24 h paclitaxel exposure was combined with 1 h etoposide exposure.[20] As etoposide has a significantly longer plasma exposure in man,[30] we exposed our cells to a minimum of 24 h. However, our results stand in contrast to Perez *et al.*, who evaluated three cell lines (MCF-, A549 and MDA231) with median effect analysis. The latter group of investigators, using a 24 h incubation time for etopo-

side and/or paclitaxel, noted antagonism when paclitaxel preceded etoposide and synergism with the inverse schedule.[19] These results contrast with our findings of antagonism between the two agents on all schedules. The only exception to our general finding is the results of a 48 h exposure of the two drugs in the MCF_7/adr which is known to express MDR-1. Hence, the mechanism of synergy in this cell line may result from interference with efflux as has been previously demonstrated with other agents in another cell line.[5] The reasons why there is a discrepancy between the two laboratories may also relate to differences in cell lines or culture conditions.

Gemcitabine has broad antitumor properties in man with recently demonstrated activity in non-small cell lung cancer.[31,32] This agent has also shown antitumor effects in metastatic breast cancer.[33] We are therefore interested in combining it with vinorelbine, an established agent used in the treatment of these diseases.[34] Phase I trials are combining these two agents in the hope of creating a non-cardiotoxic combination with broad antitumor activity. However, our *in vitro* results suggest that these two agents may exhibit better cytotoxicity if distantly spaced in time separately from each other, as all sequences and combinations examined were antagonistic.

The results of anticancer treatment for inoperable disease in man remain modest. A re-evaluation of the sequence and timing of the currently available agents may lead to enhanced results based upon *in vitro* findings.

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