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The combination of paclitaxel with cisplatin exhibits antagonism *in vitro* against human melanoma

Anil Mohith, Andrew Photiou and Spyros Retsas

Catherine Griffiths Cancer Research Laboratory, Melanoma Unit, Department of Medical Oncology, Charing Cross Hospital, London W6 8RF, UK. Tel: (+44) 181 746-8295; Fax: (+44) 181 746-8293.

The *in vitro* cytotoxicity of paclitaxel and cisplatin, alone and in combination, was evaluated against the established human melanoma cell line, G361, with either 1 or 24 h asynchronous paclitaxel exposure using the sulforhodamine B assay. As single agent, the mean cisplatin concentration which inhibited G361 cell growth by 50% (IC_{50}) was 10 000 nM for 1 h exposure. IC_{50} values obtained with 1 and 24 h paclitaxel exposure were, respectively, 63 and 3.8 nM, concentrations clinically achievable. The combination of paclitaxel with cisplatin was found to be antagonistic by the classical isobologram method, independent of drug sequence and of paclitaxel exposure time. The antagonism was significantly more pronounced for the sequence of paclitaxel followed by cisplatin compared with the reverse sequence for both 1 and 24 h paclitaxel exposure time ($p < 0.05$). Future clinical protocols employing paclitaxel and cisplatin, both active single agents for the treatment of metastatic malignant melanoma, should take into consideration that the combination of the two drugs may result in significant antagonism, irrespective of drug sequence, if administered within a short interval of each other.

Key words: Antagonism, cisplatin, cytotoxicity, isobologram, melanoma, paclitaxel.

Introduction

Cisplatin has measurable although limited clinical activity against metastatic malignant melanoma as single agent and has also been used in combination with other anti-neoplastic drugs in the treatment of this disease.[1–3]

More recently, paclitaxel (taxol), the first representative of the taxane group of drugs, has been shown in phase II studies to have clinical activity, albeit modest, in metastatic melanoma.[4,5] Combinations of paclitaxel and cisplatin in ovarian and lung cancer have shown encouraging preliminary results in pilot phase II studies in previously untreated patients with advanced disease, and are now being tested in prospective randomized clinical trials.[6,7] It is therefore conceivable that this drug combination could potentially be of therapeutic benefit to patients with metastatic melanoma and hence merits further evaluation.

In vitro, however, there is evidence of both antagonistic and synergistic interactions between paclitaxel and cisplatin, in leukemia, human gastric, ovarian and teratocarcinoma cell lines, depending upon the schedule used.[8–11] The synergistic results have been limited to the sequence of paclitaxel followed by cisplatin (P–C sequence) and, of interest, marked antagonistic interactions were observed either when cisplatin was given prior to paclitaxel (C–P sequence) or when both drugs were given concurrently.

To our knowledge, there are no studies which have explored the different sequences of paclitaxel combined with cisplatin on their optimal cytotoxic activity against human melanoma cell lines. The aim of this *in vitro* study was therefore to evaluate the interactive effect of this drug combination and to investigate the optimal sequence, if any, for drug administration with regard to the growth inhibition of a melanoma cell line.

Materials and methods

Cell line

The established melanotic human malignant melanoma cell line, G361, was used in this study. G361 was obtained from the European Collection of Animal Cell Cultures (PHLS, Porton Down, Salisbury, UK) and has previously been described.[12] Cells were cultured as monolayers in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L- glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Hyclone, Northumbria, UK). The

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Correspondence to A Mohith

cultures were incubated at 37°C in 5% CO_2 in a humidified atmosphere. Exponentially growing cells with a relative doubling time of approximately 30–36 h were used for all experiments.

Drugs

Paclitaxel (taxol) was generously provided by Bristol-Myers Squibb (Hounslow, UK) and stored at −20°C in 1% DMSO at 1 mM stock concentration. Cisplatin (David Bull Laboratory, Warwick, UK) was obtained as its clinical formulation with dilutions at 200 μM in growth medium made before each experiment and then serially diluted in the cell cultures.

Growth inhibition assays

The growth inhibitory effect of the drugs was determined using the sulfofodamine B (SRB) assay.[13] Briefly, exponentially growing cell lines were washed with phosphate-buffered saline (PBS), harvested in 0.01% trypsin and 0.004% EDTA solution, resuspended in growth medium, and were seeded as a single-cell suspension (3000 viable cells/well/100 μl growth medium—a cell density previously shown to allow exponential cell growth for the duration of the assay) in 96-well microplates in triplicate. After allowing cells to attach overnight, cultures were exposed, in succession, first to cisplatin (serially diluted 2- fold from an initial concentration of 100 μM to produce nine decreasing concentrations) for 1 h, washed sequentially with PBS and growth medium at 37°C, and then finally exposed for another hour to fixed concentrations of paclitaxel, ranging between 0.1 and 100 nM for separate experiments. The reverse P–C sequence was used in separate but otherwise identical experiments.

Alternatively, cells were exposed first to taxol for 24 h (at concentrations ranging between 0.01 and 2.5 nM for successive experiments), washed and then exposed to cisplatin for 1 h (serially diluted from an initial concentration of 100 μM), and the reverse C–P sequence repeated with the same drug exposure time for separate but otherwise identical experiments. (These drug concentrations are well within clinically achievable doses, with reported end-of-infusion plama levels of paclitaxel administered over 24 h averaging between 0.21 and 0.83 μM for doses between 110 and 200 mg/m^2, respectively, in phase I studies.[14]) The maximum concentration of DMSO solvent in the cell cultures, after dilutions, did not exceed 0.02%, a dose previously shown not to have any significant cytotoxic effect on G361 cell growth.[15]

After 6 days incubation (equivalent to at least three cell doubling times) following removal of the second drug from the cell cultures by washing, cell number was assessed indirectly by measuring basic amino acid content using SRB. Briefly, cultures were fixed with 40% trichloroacetic acid for 1 h at 4°C, washed and stained for 1 h with 0.4% SRB dissolved in 1% acetic acid. Unbound dye was washed out with 1% acetic acid and the protein-bound dye was extracted with Tris base [tris(hydroxymethyl)aminomethane] for determination of optical density at 492 nm in a 96-well microplate reader. Control plates without paclitaxel or cisplatin were otherwise treated identically. The drug concentration which inhibited cell growth by 50% relative to controls (IC_{50}) was obtained from semi-logarithmic dose–response computer plots.

Two different methods were used to determine the interaction of paclitaxel and cisplatin, first by the standard isobologram analysis (50% isodose) using variable dose ratios of both drugs in combination, and the drug interaction classified as either synergistic, additive or antagonistic, with respect to an 'additivity envelope', as previously described.[16] The median effect analysis was used as a complementary analytical method to quantify the degree of antagonism or synergy and dose–response curves were determined with constant dose ratio of both drugs in combination.[11] Computer-generated combination indices (CI) were obtained at the level of 50% growth inhibition (IC_{50}). CI values of < 1 indicate synergy, a value of 1 additivity and values > 1 indicate antagonism; and, the further away the CI values are from the line of additivity ($CI = 1$), the greater the degree of antagonism or synergy.

Statistical analysis

The differences between the mean values were analysed for significance using the paired two-tailed Student's *t*-test. *p* values below 0.05 were considered to be statistically significant.

Results

The individual dose–response plots for cisplatin and paclitaxel are shown in Figure 1. The isobologram

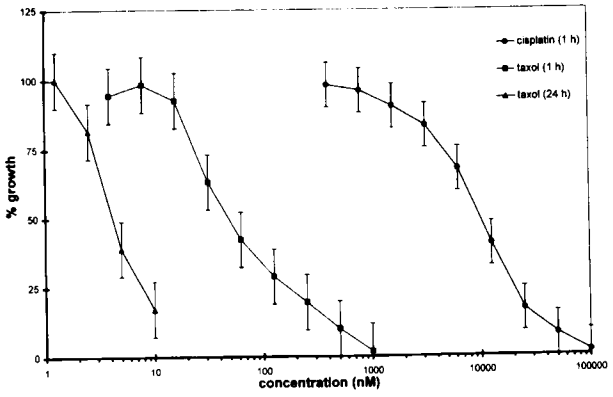
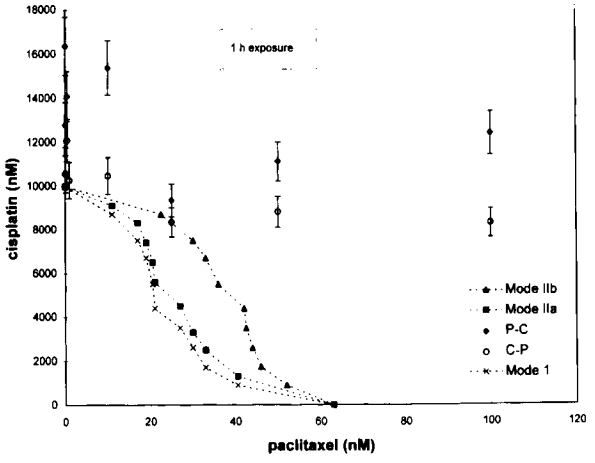


Figure 1. Dose–response curves of single-agent cisplatin and paclitaxel after 1 and 24 h drug exposure against the G361 melanoma cell line. Each point represents the mean percentage of cell growth relative to controls as determined by SRB assay derived from three separate experiments, each in triplicate cultures. Vertical error bars indicate the standard error of the mean.



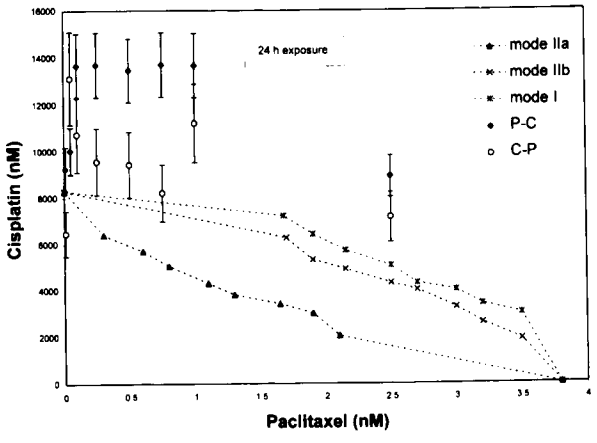


Figure 2. Isobologram analysis at 50% iso-effect level of combinations of paclitaxel and cisplatin given in different sequences using G361 melanoma cell line. (a) Cisplatin (1 h) followed by paclitaxel (1 h) and the reverse P–C sequence paclitaxel (1 h) followed by cisplatin (1 h). (b) Cisplatin (1 h) followed by paclitaxel (24 h) and the reverse P–C sequence paclitaxel (24 h) followed by cisplatin (1 h). Each point represents the mean of three separate experiments each using triplicate cell cultures. The envelope of additivity was constructed from data obtained from the respective dose–response curves (Figure 1) of the individual drugs as described by Okano *et al.*[18]

analysis of the interaction between paclitaxel and cisplatin in the G361 melanoma cell line following 1 and 24 h exposure to paclitaxel is displayed in Figure 2(a and b), respectively. IC_{50} values of the drug combinations have been plotted as isobolograms according to the methods described by Berenbaum[16] and by Steel and Peckham.[17] An envelope of additivity was individualized for the combination by plotting three lines (mode I, mode IIa, mode IIb) which were constructed using data calculated from the dose–response curves for each drug as described by Okano *et al.*[18] Antagonism is seen irrespective of the sequence and duration of paclitaxel exposure, with all the experimental plots lying to the right of the envelope of additivity.

As shown in Table 1, for the 1 h experiments, data from three separate experiments demonstrate more pronounced antagonism ($p < 0.001$, two-tailed paired *t*-test) for the P–C sequence than the reverse C–P sequence over a three-log range of paclitaxel concentrations (range from 0.1 to 100 nM). Similar results were obtained for experiments using the 24 h paclitaxel exposure, irrespective of drug sequence ($p = 0.018$, two-tailed paired *t*-test).

The median effect analysis provides a method of determining the degree of antagonism at various levels of cell kill. The greater the value of CI above 1, the greater the antagonism. Figure 3 shows the CI versus F_a (fraction affected) plot for two independent experiments where combinations of cisplatin 50 μM and paclitaxel 0.5 μM were asynchronously exposed to G361 cell lines for 1 h each, in a fixed molar ratio of 100:1, respectively (approximately equal to the ratio of their respective individual IC_{50}). The plot shows marked antagonism between cisplatin and paclitaxel in the growth inhibition of G361 cells when the cells were treated with either the P–C or C–P sequences. At the levels of 25, 50, 75 and 90% growth inhibition, CI was, respectively, 5.8, 4.1, 3.1 and 2.4 for the P–C sequence, and 4.1, 2.8, 2.0 and 1.5 for the reverse sequence, indicating consistent and marked antagonism. The P–C sequence is also shown to be significantly more antagonistic than the

Table 1. IC_{50} values (nM) obtained for standard isobologram analysis using combinations of varying concentrations of paclitaxel with a fixed concentration of cisplatin (100 μM) in G361 melanoma cell line

Paclitaxel		Experiment 1		Experiment 2		Experiment 3		Mean of 1–3	
exposure (h)	[PTXL]	P–C	C–P	P–C	C–P	P–C	C–P	P–C	C–P
1	0.1	19400	14100	9400	7700	9600	9900	12800	10567
1	0.5	20500	15500	10900	9900	10900	10900	14100	12100
1	1	20900	12000	17700	6200	10500	12600	16367	10266
1	10	18000	12900	17000	7800	11100	10700	15367	10466
1	25	5300	10300	8300	6900	14400	7800	9333	8333
1	50	7100	10100	9800	6200	16400	10100	11100	8800
1	100	11500	11800	10700		15000	4800	12400	8300
t-test			($p = 0.1791$)		($p = 0.0496$)		($p = 0.0620$)		($p = 0.00098$)
		Experiment 4		Experiment 5		Experiment 6		Mean of 4–6	
		P–C	C–P	P–C	C–P	P–C	C–P	P–C	C–P
24	0.01	13100	6400	9200	6900	5500	6100	9267	6467
24	0.05	13400	7100	11500	15000	5200	17300	10033	13133
24	0.1	9400	10700	28100	8600	3500	12900	13667	10733
24	0.25	11300	12200	16100	7800			13700	10000
24	0.5	12400	12700	25100	5800	2900	9800	13467	9433
24	0.75	11300	9600	16100	8900			13700	9250
24	1	13100	9600	20500	12200	7400	11800	13667	11200
24	2.5	2300	4600	15500	6700			8900	5650
t-test			($p = 0.216$)		($p = 0.015$)		($p = 0.0939$)		($p = 0.0186$)

P–C = paclitaxel followed by cisplatin sequence, C–P = cisplatin followed by paclitaxel sequence, [PTXL] = paclitaxel concentrations (nM). For each of six separate experiments, the data for each pair of drug sequences were compared using the two-tailed, paired *t*-test. *p* values < 0.05 are significant.

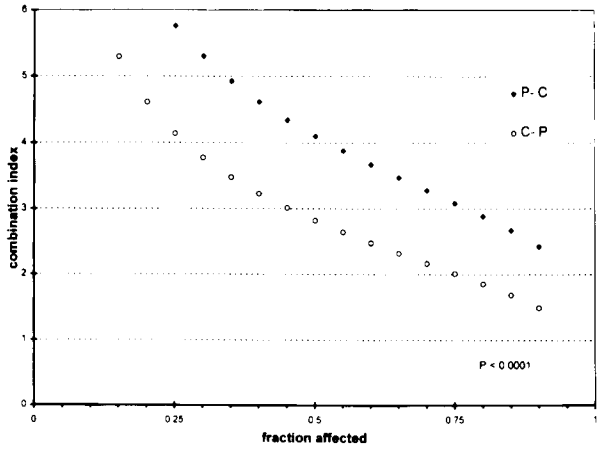


Figure 3. Combination index (CI) expressing the type of drug interaction at various levels of growth inhibition of G361 cells, using the median-effect principle. CI values were generated from computer software using data from growth inhibition assays using SRB. Cells were exposed to the combination of paclitaxel (1 h) followed by cisplatin (1 h) [P–C, closed triangles] and to the reverse sequence of cisplatin (1 h) followed by paclitaxel (1 h) [C–P, open circles], the drugs being used in 2-fold serial dilutions in cultures with fixed molar ratio equivalent to the ratio of their individual IC_{50}. Each point in either curve represents the mean of two independent experiments each using triplicate cell cultures. CI < 1 indicates synergy, CI > 1 antagonism and CI ~ additivity.

reverse sequence, in line with the isobologram results ($p < 0.0001$, *t*-test).

Discussion

In this study, we have explored in our own *in vitro* system different sequences and duration of exposure of cisplatin and paclitaxel against human melanoma cells in culture. There are reports of schedule-dependent *in vitro* synergy between these two drugs in ovarian and gastric cancer, teratocarcinoma and leukemia cell lines.[8–11] This observation has, however, not been reproduced in our experiments using the G361 human melanoma cell line. Furthermore, in our study, which involved the use of formal drug combination analysis by both the isobologram and median effect/combination index methods, the P–C sequence was found to be more antagonistic than the reverse C–P sequence against the G361 melanoma cell line, in contrast to the synergism reported in other tumors.

Jekunen *et al.*[9] reported that the interaction between taxol and cisplain was highly synergistic

in human ovarian carcinoma when 19 h taxol exposure was followed by 1 h concurrent exposure to taxol and cisplatin. However, the same study demonstrated antagonism when 1 h exposure to cisplatin was followed by 20 h exposure to taxol or when the cells were exposed to cisplatin and taxol for 1 h concurrently.

Vanhoefer *et al.*[10] reported similar results with synergism or additivity observed when paclitaxel was given 24 h prior to cisplatin (2 h drug exposure) to established human gastric and ovarian carcinoma cell lines, and antagonism when the drugs were given simultaneously or when cisplatin was given before paclitaxel.

Although the exact mechanism of the *in vitro* antagonistic effect of cisplatin with paclitaxel is not fully understood, both drugs have been reported to interact with tubulin. Paclitaxel is known to induce microtubule assembly by increasing tubulin polymerization, leading to stable and dysfunctional microtubules. Cisplatin has been reported by Rixe *et al.*[19] to cause polymerization of tubulin, but by Peyrot *et al.*[20] to cause depolymerization, in addition to its known DNA adduct formation.

Jekunen *et al.*[9] also suggested that the synergistic interaction for the P–C sequence in the ovarian carcinoma cell line 2008 is not a taxol-induced alteration in cell cycle kinetics, as mitotic arrest was observed only at taxol concentrations well above those required for synergy with cisplatin. It has therefore been speculated that the interaction between paclitaxel and cisplatin may be occurring at the tubulin level rather than at the DNA level.

It is noteworthy that the P–C sequence had been selected as the treatment sequence for the phase III Gynaecologic Oncology Group (GOG) study of taxol (24 h schedule) plus cisplatin versus cyclophosphamide plus cisplatin in patients with suboptimally debulked ovarian epithelial neoplasms on the basis that this particular sequence was more cytotoxic *in vitro* and was associated with less profound neutropenia in phase I studies than the reverse C–P sequence. Our study would suggest that future clinical trials using combinations of paclitaxel and cisplatin in metastatic melanoma should address the issue of sequence dependence futher and take into account the possibility of antagonism between these two antitumor agents if administered within a short interval of each other. Whether an interval of 24 h as suggested from our *in vitro* data can be translated directly to clinical protocols will only be determined from clinical studies which take into consideration possible pharmacological interactions between these two agents.

Although each drug individually exhibits significant *in vitro* cytotoxicity against the G361 melanoma cell line, their combination in various sequences using different drug exposure times has shown no evidence of synergistic interaction.

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