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ORIGINAL ARTICLE

Alexander D. Guminski · Paul R. Harnett
Anna deFazio

Carboplatin and paclitaxel interact antagonistically in a megakaryoblast cell line – a potential mechanism for paclitaxel-mediated sparing of carboplatin-induced thrombocytopenia

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Abstract *Purpose*: Clinical observation has shown that paclitaxel ameliorates the antiplatelet toxicity of carboplatin when the two drugs are combined, although antitumour activity and white cell toxicity are at least additive. We hypothesized that this is due to an interaction between the two drugs at the level of the platelet precursor. *Methods*: We measured inhibition of growth of the megakaryoblast cell line MEG-01 following exposure to paclitaxel and carboplatin singly or combined. Drug interaction was assessed by median effect analysis. *Results*: An antagonistic interaction was observed, and this was most marked at drug concentrations giving a low level of growth inhibition ($P < 0.002$, sign test). The interaction was not sequence-dependent. There was no significant difference in whole-cell accumulation of platinum or the amount of platinum adducts on DNA following combined treatment in comparison with carboplatin alone. *Conclusions*: These results provide the first evidence of an antagonistic interaction between paclitaxel and carboplatin in a platelet precursor and provide an explanation for the platelet-sparing effect of the combination of these chemotherapeutic agents. While the mechanisms underlying the interaction described in this report are yet to be fully elucidated, this study provides evidence that the antagonism between paclitaxel and carboplatin in MEG-01 cells is not due to reduced platination of DNA.

Keywords Chemotherapy · Platelets · Drug interaction · Platinum DNA adducts

A. D. Guminski (✉) · P. R. Harnett
Department of Medical Oncology, Westmead Hospital, Faculty of Medicine, University of Sydney at Westmead Hospital, Westmead, NSW 2145, Australia
E-mail: alexg@westgate.wh.usyd.edu.au
Tel.: +61-2-98456954
Fax: +61-2-98456391

A. deFazio
Department of Gynaecological Oncology, Westmead Hospital, Faculty of Medicine, University of Sydney at Westmead Hospital, Westmead, NSW 2145, Australia

A. D. Guminski · A. deFazio
Westmead Institute for Cancer Research, Faculty of Medicine, University of Sydney at Westmead Hospital, Westmead, NSW 2145, Australia

Introduction

Carboplatin is an effective chemotherapy agent in common malignancies including ovarian and non-small-cell lung carcinomas. Its dose-limiting toxicity is reduction in platelet count, or thrombocytopenia. This effect is dependent on total exposure to the drug which depends on drug clearance and body weight. As carboplatin clearance is primarily dependent on renal function, which in turn can be estimated by simple laboratory tests, an appropriate dose can be determined for each patient using area under the concentration-time curve (AUC) dosing [6, 10]. This ability to appropriately individualize dose prior to first treatment is unusual amongst chemotherapy drugs.

Paclitaxel is also effective against a number of tumour types and the combination of paclitaxel and carboplatin has been extensively investigated [4, 5]. An interesting observation from these studies is that the expected degree of thrombocytopenia is not seen despite the occurrence of significant neutropenia and antitumour effects. The basis of this interaction is unknown. A pharmacokinetic interaction between the two drugs appears unlikely from the data of Kearns et al. [13] and van Warmerdam et al. [21]. These authors compared the degree of thrombocytopenia for measured carboplatin AUC between patients receiving carboplatin combined with paclitaxel and historical values for carboplatin alone. Their analyses show an approximate 60% increase in the carboplatin AUC required to achieve a 50% fall in the platelet count when paclitaxel is given concurrently. We hypothesized that this platelet-sparing

effect of paclitaxel in combination with carboplatin might indicate an interaction at the level of the platelet precursor. We therefore sought to examine the antiproliferative effects of paclitaxel and carboplatin, singly and in combination, on a megakaryoblast cell line.

Materials and methods

Cell culture

The megakaryoblast cell line MEG-01 was obtained from the American Type Culture Collection, Rockville, Md. It is a Philadelphia chromosome-positive leukaemic cell line expressing megakaryocyte antigens GpIIb/IIIa and Factor VIII (but no lymphoid or myeloid surface markers) and with morphological features similar to megakaryocytes as described by Ogura et al. [17]. MEG-01 cells also show differentiation along a megakaryocytic pathway [16]. Cells were grown in RPMI-1640 medium buffered with sodium bicarbonate 5.6% (w/v) and HEPES 10 m*M* and supplemented with 20% (v/v) fetal calf serum, L-glutamine 2 m*M*, sodium pyruvate 1 m*M* and glucose 4.5 g/l (Astra, North Ryde, Australia). The final pH was approximately 7.2 and the medium was antibiotic-free. All supplements were from Trace Biosciences (Castle Hill, Australia) except where stated. Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were free from *Mycoplasma* contamination (Gen-Probe T.C. Rapid Detection System, BioMediq, Doncaster, Australia).

Analysis of drug effects on proliferation

Carboplatin (Delta West, Perth, Australia) and paclitaxel (Taxol, Bristol Myers Squibb, Noble Park, Australia) were obtained commercially. This paclitaxel formulation is dissolved in Cremophor EL/ethanol. A vehicle control was prepared using Cremophor EL (Sigma, Steinheim, Germany) plus absolute ethanol, equivalent to the concentration of vehicle in the highest concentration of drug to which cells were exposed. Drugs and vehicle control were diluted in complete medium to obtain final dilutions. Concentration response curves for each agent alone were obtained to define an appropriate concentration range and IC_{50} (concentration inhibiting proliferation by 50% compared with control). Experiments were conducted by plating logarithmically growing cells at 4×10^3 cells in 50 µl medium in each well of a 96-well plate and allowing them to grow for 24 h before drug addition. Drug exposure was continuous from the time of addition. Cell proliferation was assessed every 24 h for 144 h using a colorimetric assay (Cell Titre 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, Wis.) which relies on the conversion by mitochondrial dehydrogenases of a tetrazolium salt to a formazan product [15]. Absorbance was measured on a plate reader (Biorad Microplate Reader Model 550; Biorad, Hercules, Calif.) at 570 nm minus background at 630 nm. Proliferation for each concentration was performed in quadruplicate on each plate and is expressed as the mean of four wells relative to vehicle control.

For the combined-agent experiments, cells were exposed to drug 24 h after seeding and read 48 or 72 h later. Cells on each plate were exposed to five different concentrations of paclitaxel, five of carboplatin and to the same concentrations of each drug combined (see below) in addition to appropriate vehicle controls. The value for each concentration of drug alone or in combination is the mean of four wells. Paclitaxel was added either 3 or 19 h prior to, or 1 or 3 h following, carboplatin.

Analysis of drug interaction

Potential drug interactions were analysed using the median-effect method of Chou and Talalay [7]. This model is based on administering two drugs in the ratio of their individual IC_{50} values (or "median effect" dose) and at severalfold higher or lower concentrations of each drug. At all drug concentrations, the ratio remains fixed. This analysis is valid if the median-effect plot [log(fraction of cells affected/fraction of cells unaffected) versus log(dose)] is linear, as was found in our experiments for each drug alone and in combination (r-values ranged between 0.948 and 0.998, data not shown). A further consideration in the model is whether there is any observed interaction between drugs that share a common receptor or pathway (termed mutually exclusive) or whether they interact via distinctive mechanisms (mutually nonexclusive), bearing in mind that a common pathway may occur some way downstream.

From these plots the doses required for a certain level of cell kill for each drug alone and for the drugs in combination can be determined. A combination index (CI) can then be determined at that level of cell kill according to the following equations. For mutually exclusive drugs:

$$\mathrm{CI} = \frac{[\text{drug 1 in combination}]}{\text{drug 1 alone}} + \frac{[\text{drug 2 in combination}]}{\text{drug 2 alone}}$$

For mutually nonexclusive drugs:

$$\mathrm{CI} = \frac{[\text{drug 1 in combination}]}{\text{drug 1 alone}} + \frac{[\text{drug 2 in combination}]}{\text{drug 2 alone}} + \frac{[\text{drug 1 in comb}]\times[\text{drug 2 in comb}]}{[\text{drug 1 alone}] \times [\text{drug 2 alone}]}$$

As demonstrated by Chou and Talalay, $\mathrm{CI} > 1$ indicates antagonism, $\mathrm{CI} = 1$ indicates additivity and $\mathrm{CI} < 1$ indicates synergy.

In the situation where the median-effect plots of each individual drug reveal a different slope, as occurred in our experiments (data not shown), then it is not possible to say whether the interaction is mutually exclusive or nonexclusive. We applied the analysis assuming mutual exclusivity as this is the most conservative for identifying antagonism, as can be seen from the two equations above. It is intuitively implicit that two drugs in combination would exert a greater cell kill if they worked via separate pathways than if they utilized the same pathway.

Platinum accumulation

MEG-01 cells in logarithmic growth phase were incubated for 3 h in serum-free medium containing either paclitaxel (final concentration 0.105 µ*M*) or paclitaxel vehicle control, or in serum-free medium alone. Carboplatin was then added to a final concentration of 1 m*M* and the cells, including an untreated control, were incubated at 37°C for a further 4 h. Cells were then harvested, washed twice in ice-cold PBS and finally resuspended in 2 ml PBS. A 0.5-ml aliquot was taken and stored at –20°C for whole-cell platinum and protein determination. The remaining volume was incubated for 1 h at 37°C with 0.1 *M* ammonium bicarbonate to inactivate non-DNA-bound platinum. DNA was then extracted using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.). The samples taken for whole-cell determination were subsequently sonicated and aliquots taken for spectroscopic protein determination (Bio-Rad Protein Assay; Bio-Rad Laboratories) and whole-cell platinum measurement. Platinum concentrations were determined using an inductively coupled plasma mass spectrometer (ICPMS; Elan 5000; Perkin Elmer, Sydney; Department of Biochemistry, Royal Prince Alfred Hospital, Sydney, Australia). Experiments were performed in triplicate on two separate occasions. After drug exposure aliquots from drug-treated cells and controls were taken, washed, then resuspended in complete medium and used to seed a colorimetric cytotoxicity assay as above with cell survival measured every 24 h for 72 h following removal from drug exposure.

Statistical analysis

All data were statistically analysed using either Microsoft Excel 97 for Windows or SPSS ver. 8. A sign test was used to assess the significance of the differences between the observed CIs. Analysis of

variance with repeated measures with Bonferroni correction was used to test for differences in whole-cell platinum accumulation and DNA platination.

Results

As shown in Fig. 1, both carboplatin and paclitaxel exposure resulted in a concentration-dependent decrease in survival of MEG-01 cells. The IC_{50} values after 48 h were found to be 85 ± 7 μM for carboplatin alone and 9 ± 1 nM for paclitaxel alone. The ratio between the IC_{50} values was 9500:1 (carboplatin:paclitaxel). The growth-inhibitory effects of the drugs in combination were determined by calculation of the percentage survival of MEG-01 cells with increasing concentrations of carboplatin plus paclitaxel at this fixed ratio of 9,500:1. As shown in Fig. 1, the combination of carboplatin and paclitaxel was more effective at inhibiting growth than either drug alone. The sequence of drug addition (paclitaxel 3 h before carboplatin or carboplatin 3 h before paclitaxel) did not significantly alter the growth inhibitory effects of the drug combination.

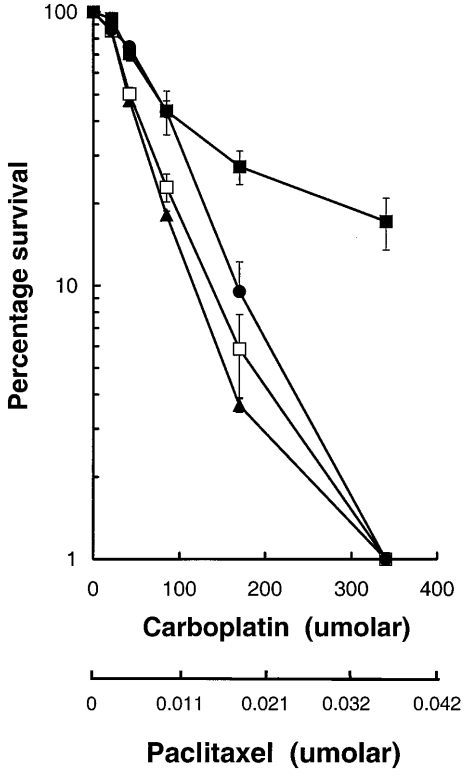

Fig. 1 Percentage of surviving MEG-01 cells following paclitaxel alone (■), carboplatin alone (●) or both drugs combined – paclitaxel 3 h before carboplatin (▲) and carboplatin 3 h before paclitaxel (□) – after 48 h of continuous drug exposure, measured by a colorimetric assay. The carboplatin alone survival points are the mean of six separate experiments performed in quadruplicate and the paclitaxel alone and both drugs combined are the means of at least two separate experiments performed in quadruplicate. Each point represents the mean ± SE

In view of the known antiproliferative effects of the paclitaxel vehicle (Cremophor/ethanol) [9] the equivalent vehicle control for each of five paclitaxel concentrations encompassing the range used in the combined drug experiments (36 to 2 nM) was coadministered with carboplatin. In addition paclitaxel vehicle control up to a concentration equal to that in 300 nM paclitaxel solution was tested as a single agent for its inhibitory effect on proliferation. No significant difference in cytotoxicity was seen between any of the vehicle controls and carboplatin alone treated cells or untreated cells, respectively (data not shown).

Median effect analysis

The median effect method of analysing drug interaction requires combining two drugs at simultaneously varying concentrations with the ratio between them fixed to equal the ratio of their individual IC_{50} values. The results of the median effect analysis are expressed as a CI at each level of drug effect, in this case cell cytotoxicity. Figure 2A shows the CIs for the sequence of paclitaxel preceding carboplatin from five separate experiments plotted against the fraction of cells affected. To mimic clinical schedules of combined paclitaxel and carboplatin administration, paclitaxel was added 3 or 19 h prior to carboplatin. Small differences were found in the median effect dose used to calculate the CIs (7–9 nM) reflecting interexperimental variation in the IC_{50} values. The shapes of the curves were similar and values close to 1 (additive or no interaction) were seen at high levels of growth inhibition, whereas the CI became increasingly positive (indicating an antagonistic interaction) as the level of growth inhibition decreased. Despite differences in the interval between drug administration, the shapes of the curves were similar and the majority of CI values were positive.

In previous studies, sequence-dependent effects on in vitro cytotoxicity between paclitaxel and cisplatin in ovarian cancer cell lines have been observed [12, 14]. Therefore alternate sequences were included in these experiments. Figure 2B shows the CIs for the sequence of carboplatin added prior to paclitaxel. The results are similar in quality and magnitude to those obtained when paclitaxel preceded carboplatin, suggesting that the antagonistic interaction was independent of the order of drug administration. Given the similarity in the distributions of the CIs between the two sequences of administration, all values were combined to estimate significance of the CIs calculated. Applying a sign test, the probabilities of the observed positive values occurring by chance at each fraction of cells affected is shown in Table 1. Significance was also assessed by constructing a 95% confidence envelope around the mean, and this resulted in a mean CI greater than 1 with $P < 0.05$ when the percentage of cells unaffected was greater than 55%.

Paclitaxel has very limited water solubility and is reconstituted in a mixture of Cremophor and ethanol prior

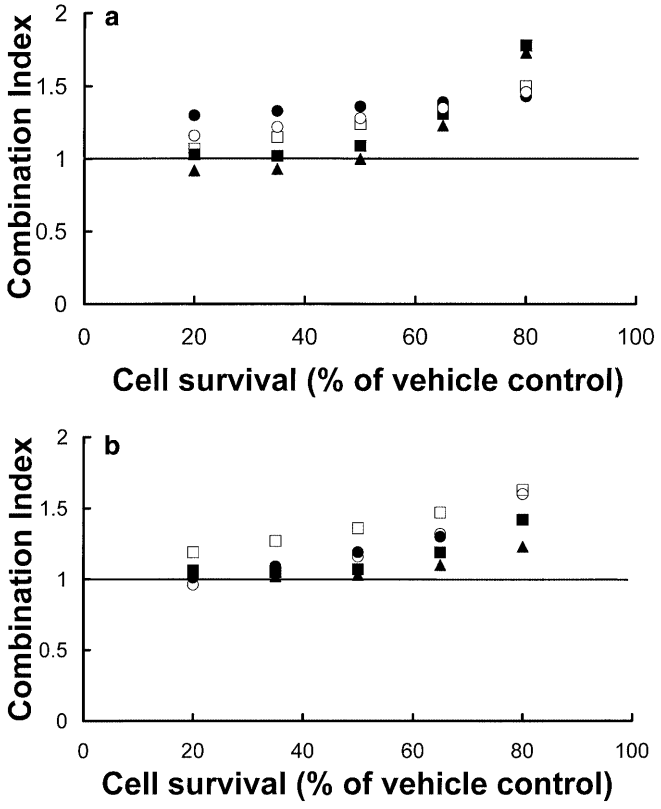

Fig. 2a,b Drug interaction assessed in terms of CIs (where CI > 1 indicates antagonism, CI < 1 indicates synergy and CI = 1 indicates additivity or no interaction). **a**) MEG-01 cells exposed to paclitaxel prior to carboplatin plotted against cell survival for five separate experiments, represented by individual symbols: ● paclitaxel 19 h before; ▲, ■ 3 h before; □, ❍ 3 h before. **b**) MEG-01 cells exposed to carboplatin prior to paclitaxel plotted against cell survival for five separate experiments, represented by individual symbols: ● carboplatin 1 h before; ▲, ■ 3 h before; □, ❍ 3 h before. Median paclitaxel/carboplatin doses calculated for individual experiments: 0.007/85 μ*M* (●), 0.008/65.5 μ*M* (▲, ■), 0.009/85 μ*M* (□, ❍)

to administration. We investigated the possibility that the antagonistic interaction may occur between carboplatin and the paclitaxel vehicle (Cremophor/ethanol) rather than with the paclitaxel itself, as reduced cellular uptake of cisplatin by peripheral blood leucocytes in the presence of Cremophor has been reported [9]. This was investigated by replacing paclitaxel with vehicle over the same range of concentrations. No difference was observed between growth inhibition by carboplatin alone and inhibition by carboplatin plus paclitaxel vehicle (data not shown).

Table 1 Significance of the calculated CIs for the interaction between paclitaxel and carboplatin. Both sequences of drug administration were combined. Significance was calculated from the probability of the observed number of positive values for ten independent observations occurring by chance

Cell survival (%)	*P*-value by sign test (two-sided) for observed CI
20	> 0.1
35	> 0.1
50	0.008*
65	< 0.002*
80	< 0.002*

*$P < 0.05$

Platinum accumulation

The mechanism of cisplatin cytotoxicity is believed to depend upon the formation of platinum adducts with DNA. Carboplatin has been found to produce similar adducts in vivo [2], although at a slower rate, and in vitro cell cytotoxicity and adduct formation have been demonstrated to correlate with carboplatin dose [19]. Table 2 shows the whole-cell accumulation of platinum and DNA adduct formation present after 4 h exposure of MEG-01 cells to 1 m*M* carboplatin. No significant differences were observed in either whole-cell accumulation or platinum on DNA between cells pretreated for 3 h with either paclitaxel or serum-free medium alone. In keeping with a published protocol [11], accumulation studies were performed using a higher concentration of carboplatin (1 m*M*) and a shorter exposure than that

Table 2 Whole-cell platinum accumulation and DNA platination of MEG-01 cells treated with carboplatin and carboplatin plus paclitaxel. MEG-01 cells were pretreated for 3 h with either 0.105 μ*M* paclitaxel, paclitaxel vehicle control or serum-free medium prior to the addition of 1 m*M* carboplatin for a further 4 h. Whole-cell platinum accumulation and adducts on DNA were measured using an inductively coupled plasma mass spectrometer. Aliquots for cell survival were washed twice in medium and 4×10^3 cells per well plated in quadruplicate on a 96-well plate. Cell survival was measured by a colorimetric assay (MTT) at 24 and 48 h after completion of drug exposure. Whole-cell and DNA platinum accumulation experiments were performed twice in triplicate and the cell survival experiment was performed once. Significance for the comparison between carboplatin alone and carboplatin plus paclitaxel was calculated using two-way ANOVA with repeated measures with Bonferroni correction

	Whole-cell platinum (pg/μg protein, ± SD)	Platinum on DNA (pg/μg DNA, ± SD)	Proliferation (% of control)		Equivalent CI	
			24 h	48 h	24 h	48 h
Carboplatin	532 ± 179	10.6 ± 3.0	97	57		
Carboplatin + paclitaxel vehicle control	353 ± 55	8.7 ± 2.6	91	55		
Carboplatin + paclitaxel	520 ± 141	13.0 ± 4.2	89	55	1.52 ± 0.28	1.29 ± 0.28
P-value	> 0.8	0.13				

used to measure cytotoxicity. In order to confirm that cytotoxicity was occurring in the range of an antagonistic interaction, cytotoxicity of the drug exposure under these conditions was assessed by a colorimetric assay at 24 and 48 h. The cytotoxicity of combined treatment showed equivalent CIs of 1.52 ± 0.28 at 24 h and 1.29 ± 0.28 at 48 h, indicating an antagonistic interaction for the drug exposures used in the platinum accumulation experiments.

Discussion

This study showed an antagonistic interaction between paclitaxel and carboplatin in a cell line with features of a platelet precursor. Several clinical studies have shown that paclitaxel given in combination with carboplatin reduces the degree of thrombocytopenia (but not neutropenia) expected following carboplatin administration. It has been estimated that the required patient exposure to carboplatin to achieve a 50% fall in platelets is increased by approximately 60% when paclitaxel is coadministered [13, 21]. We hypothesized that a pharmacodynamic interaction occurs between the drugs at the level of the platelet precursor. Our results show that there was an antagonistic interaction as defined by the median effect analysis [7] between carboplatin and paclitaxel in the inhibition of in vitro proliferation of platelet precursor MEG-01 cells.

Although the precise mechanisms of the cytotoxic action of platinum compounds are not completely understood there are several factors influencing their effect (reviewed in reference 18). DNA is the eventual platinum drug target but there must be initial accumulation of the platinum compound within the cell. This provides an opportunity for decreasing reactive platinum via quenching by intracellular thiols and drug export. The amount of platinum adduct formation on DNA, balanced against removal by DNA repair mechanisms, is believed to be a major contributing factor to the cytotoxic effect of platinum compounds. Also of relevance may be the type of adduct, interactions between DNA containing adducts and other proteins, and hence the recognition of unrepairable damage and the cell response of effective apoptosis. Measurement of whole-cell and DNA platinum accumulation reveals net cellular transport and indicates the sum of the effects of DNA adduct formation and early repair. In our study there was no difference in platinum accumulation or DNA adduct formation in MEG-01 cells treated with paclitaxel prior to carboplatin as compared with those treated with carboplatin alone. This suggests that the mechanism of the antagonistic interaction observed between the two drugs is unlikely to involve altered platinum transport or accelerated early repair.

De Vos et al. [9] have shown a sequence-dependent reduction in whole-cell cisplatin accumulation in peripheral leucocytes in vitro due to the taxane-solubilizing agents Cremophor EL or Tween 80, which was not seen in ovarian or other tumour cell lines. They did not see a similar reduction in platinum accumulation in peripheral leucocytes when carboplatin was used in place of cisplatin, however. These results suggest that a component of the paclitaxel preparation inhibits the DNA adduct formation of one platinum compound, but not another, and that the effect depends on cell origin. This is consistent with our observation of no reduction in platinum accumulation in MEG-01 cells treated with paclitaxel and carboplatin, compared with carboplatin alone. Similarly, de Graaff et al. [8] have reported that combinations of cisplatin and paclitaxel or carboplatin and the paclitaxel analogue Taxotere have an antagonistic cytotoxic interaction when administered to CFU-GM (bone marrow granulocyte-monocyte progenitor) cells in vitro. They also observed a 12% reduction in cisplatin adduct formation attributable to Cremophor EL in the paclitaxel formulation but saw no difference in carboplatin-derived adducts when given with Taxotere, despite demonstrable antagonism. It is thus likely that different mechanisms account for tissue-specific interactions between taxanes and either cisplatin or carboplatin.

Paclitaxel cytotoxicity is believed to depend upon tubulin binding, activation of protein kinases, including Raf 1, protein kinase A and MAP kinases, and hyperphosphorylation of proapoptotic molecules including bcl-2 [1, 22]. Induction of p53 and $p21^{WAF1/CIP1}$ genes also occurs but their relevance to paclitaxel-induced apoptosis is controversial, as reviewed by Blagosklonny and Fojo [1]. Both paclitaxel and cisplatin have demonstrated synergy in vitro against OVCAR-3 ovarian cancer cells when administered with an antisense oligonucleotide targeting protein kinase A [20]. The in vitro cytotoxicity of cisplatin can also be enhanced by coadministration with the agent UCN-01 (7-hydroxystaurosporine) which is a potent inhibitor of protein kinase C and to a lesser extent protein kinase A [3]. It is thus possible that the antagonistic interaction of paclitaxel with carboplatin we saw in MEG-01 cells, occurring despite no change in DNA platination, arises downstream in their respective effector pathways.

Antagonistic interactions between carboplatin and paclitaxel in other tumour cell lines, or in clinical trials, have not been reported, suggesting that the effect we observed in the megakaryoblast cell line MEG-01 may have been due to a lineage-specific effect of the drug combination. Although consistent with the clinical observations, absolute proof of a lineage-specific interaction will require demonstration of a similar effect in normal megakaryocyte precursors and comparison with their neutrophil counterparts, notwithstanding the limitations of progenitor cell assays. Further investigation of this interaction may enhance our knowledge of the actions of these chemotherapeutic agents and suggest ways in which tumours may be made more susceptible to, or normal tissues protected from, cytotoxicity.

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