Anti-Cancer Drugs 1999, **10**, pp. 213–218

Research paper

In vitro antagonistic cytotoxic interactions between platinum drugs and taxanes on bone marrow progenitor cell CFU-GM

Michael de Graaff, Marc Mallepaard, Dick Pluim, Ben JG Floot, Ineke CM Slaper-Cortenbach[1] and Jan HM Schellens

Department of Experimental Therapy and Medical Oncology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. [1]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands.

We have designed and used an *in vitro* bone marrow cell culturing system for investigating pharmacodynamic interactions between platinum anti-cancer drugs and taxanes. With this system, in which the bone marrow progenitor cell CFU-GM is proliferating and differentiating into granulocytes and monocytes, we could show a strong antagonistic cytotoxicity of the combination carboplatin and Taxotere, in three different schedules, and of the combination cisplatin and Taxol, in two out of the three schedules tested. Modulation of intracellular platinum drug accumulation in granulocytes and monocytes does not seem to be a plausible explanation for the observed antagonism. *In vitro* co-incubation of granulocytes/monocytes with the combination carboplatin and Taxotere did not reveal an effect of Taxotere on intracellular platinum accumulation. Although Taxol reduced intracellular cisplatin levels by 12%, this effect was not significantly different from the co-incubation of cisplatin with Cremophor EL, the solvent for paclitaxel in Taxol. The toxicity data obtained in this study seem to be in accordance with recent clinical trials where combination therapies with platinum drugs and taxanes resulted in marked reductions in myelosuppression in patients. Therefore, these types of assays could be useful as to the assessment of bone marrow toxicities of clinically important drug combinations. [© 1999 Lippincott Williams & Wilkins.]

Key words: Cellular uptake, CFU-GM, cytotoxic interactions, platinum drugs, taxanes.

Introduction

Taxane drugs, such as paclitaxel and docetaxel, are suitable candidates for combination therapies with platinum drugs, since they also exhibit high activities against solid tumors,[1,2] their mechanism of action differs from platinum drugs and their toxicity profiles only partly overlap with those of platinum drugs. The cytotoxic activity of taxanes is based on their preferential binding to tubulin, thereby stabilizing microtubuli,[3] arresting cells in mitosis[4] which eventually leads to cell death. For their clinical use paclitaxel is dissolved in dehydrated ethanol/Cremophor EL 50/50% (v/v): Taxol; and docetaxol in 100% polysorbate 80 (Tween 80): Taxotere.

Various clinical trials are ongoing using a combination of platinum drugs and taxanes. The combination cisplatin and Taxol is now standard therapy in first-line advanced epithelial ovarian cancer. An important observation was that significant and schedule-dependent myelotoxicity is observed, whereby the sequence Taxol→cisplatin results in much less myelosuppression than the reverse sequence.[5–8] Furthermore, the combination carboplatin/Taxol resulted in much less thrombocytopenia than was anticipated.[9,10] Thus far no pharmacokinetic interaction can explain these observations.[8,11]

It seems possible that pharmacodynamic interactions take place between platinum drugs and taxanes at the level of the bone marrow progenitor cells. To test this hypothesis we have used an *in vitro* bone marrow cell culturing system and determined the cytotoxic effects of platinum drugs and taxanes, and assessed their interaction using the median effect analysis principle.[12,13] As a model system we used cultures of the progenitor cell CFU-GM (colony forming unit granulocytes and monocytes).

In this paper we show that strong antagonistic cytotoxic interactions take place at the level of the

Correspondence to JHM Schellens, Department of Experimental Therapy and Medical Oncology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.
Tel: (+31) 20 5122564; Fax: (+31) 20 5122572;
E-mail: jhm@nki.nl

CFU-GM *in vitro*. Moreover, we have attempted to elucidate the mechanism of the observed interactions.

Material and methods

Chemicals

Taxol [stock of 6 mg/ml paclitaxel in 50%/50% (v/v) Cremophor EL/ethanol] was a generous gift from Bristol Meyers Squibb (Woerden, the Netherlands). Taxotere (6 mg/ml docetaxel in 100% Tween 80) was obtained from Rhone-Poulenc Rorer (Antony, France). Cisplatin (1 mg/ml platinol) was obtained from TEVA Pharma (Mijdrecht, The Netherlands) and carboplatin (10 mg/ml paraplatin) was obtained from Bristol Meyers Squibb.

Drug treatment and *in vitro* culturing of CFU-GMs

Bone marrow aspirates from patients with various malignancies were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and stored under liquid nitrogen. For every experiment a sample was thawed in Iscove's modified Dulbecco medium (IMDM) supplemented with 20% (v/v) fetal calf serum (FCS), 5 mM $MgCl_2$ and 1.5 U/ml DNase (Sigma, St Louis, MO), and was subsequently left for 30 min at room temperature. The cells were centrifuged at 266 *g* for 10 min, resuspended in IMDM+20% (v/v) FCS and left for another 30 min at 37°C. The cells were again centrifuged and resuspended in IMDM+20% (v/v) FCS at a final concentration of 2500 CFU-GM/ml (as was determined by previous growing assays). In total 200 CFU-GMs were added to 2.5 ml methylcellulose medium (Methocult GF; StemCell Technologies, Vancouver, BC, Canada) after which platinum drugs and taxanes were added either as single agents or in combination. Three different schedules for the combination experiments were used: (i) simultaneous administration, (ii) taxane drug followed by platinum drug after 24 h and (iii) platinum drug followed by taxane drug after 24 h. Platinum drugs were added in concentrations ranging from 0 to 13 μM; taxanes ranging from 0 to 52.6 nM. As controls, equivalent amounts of vehicle (Cremophor EL in the case of Taxol; Tween 80 in the case of Taxotere) were added to the cells. When all the drugs were added (in case of the simultaneous administration at day 1, in case of the schedules at day 2), the 2.5 ml samples were plated in two 3.5 cm Petri dishes (100 CFU-GMs per dish). The CFU-GMs were grown during 14 days at 37°C in a humidified atmosphere with 5% CO_2 in air in the presence of the drugs, after which the colonies of granulocytes, monocytes or both were scored. A group of cells consisting of 40 or more cells was defined as a colony.

In the experiments where the CFU-GMs were exposed to drugs during a shorter period than 24 h, a large batch of bone marrow cells ($50–100 \times 10^6$ cells) was thawed as described above. After the second centrifugation step the cells were resuspended in Long Term Medium [LTM: IMDM with Glutamax 1/300 (Gibco), 10% (v/v) FCS, 5% horse serum, 10^{-5} M hydrocortisone, 100 U/ml penicillin, 100 U/ml streptomycin, 0.1 mM β-mercaptoethanol] at a concentration of 10^6 cells/ml. The cells were left at 37°C for 24 h in T25 tissue culture flasks (5 ml/flask) before adding the drugs. After adding the drugs, the cells were incubated for another 24 h at 37°C. After this incubation step the cells were transferred to a fresh tube, after which the flask was washed with 5 ml cold PBS. Only a small sample (usually 100–150 μl, corresponding to 200 CFU-GMs) was added to 2.5 ml methylcellulose medium, plated and cultured for 14 days as described above. Using this method drug concentrations during the 14 day cell culture period were reduced 20- to 25-fold compared to the above-described method.

As positive controls in all the culturing experiments, CFU-GMs were grown in the absence of drugs. These controls should yield approximately 100 colonies per Petri dish after 14 days of culture. All experiments were performed at least 3 times using bone marrow samples of three different patients.

Cytotoxic interactions

Cytotoxic interactions between platinum drugs and taxanes on CFU-GMs were determined by calculation of the combination indices (CIs) for each fractional effect using the median-effect analysis.[12,13] This was done by using the following equation:

$$CI = d_1/D_1 + d_2/D_2.$$

in which D_1 and D_2 stand for the concentrations of the drugs to produce a given effect as single agent, d_1 and d_2 for the concentrations of the drugs in combination. The results were interpreted as follows:

CI = 1	additive cytotoxicity or no drug interaction
CI < 1	synergistic cytotoxicity
CI > 1	antagonist cytotoxicity

Measuring intracellular platinum drug accumulation

To test the effect of taxanes or their solvents on intracellular platinum accumulation, a CFU-GM derived mixture of granulocytes and monocytes was harvested from Petri dishes after a 14 day culturing period, and resuspended in IMDM+10% FCS. To each sample of 2×10^6 cells drugs were added to final concentrations of 10 μg/ml platinum drug in the absence or presence of 1 μg/ml taxane. As controls, platinum drugs were incubated in the presence of 0.0083%/0.0083% Cremophor EL/ethanol (solvent control equivalent to 1 μg/ml for Taxol) or in the presence of 0.017% Tween 80 (solvent control for 1 μg/ml Taxotere). Cells were incubated for 2 h at 37°C in the case of cisplatin accumulation and 4 h in the case of carboplatin accumulation. After incubation, cells were centrifuged at 2000 *g* for 5 min at 4°C, washed twice with PBS, and resuspended and lysed in 1 ml distilled water. Protein content was determined using the Bradford method.[14] Subsequently, samples were dried under vacuum, proteins were digested with nitric acid for 2 h at 70°C and intracellular platinum content was determined by flameless atomic spectrometry (AAS) as described earlier.[15] Platinum concentration was calculated as pg Pt/mg protein. The samples incubated with platinum drugs in the absence of taxanes or vehicles were used as positive controls and were set at 100%. Other samples were calculated relative to this control. All experiments were carried out using at least three different patient's samples.

Results

Cytotoxicity of platinum drugs and taxanes on *in vitro* cultured CFU-GMs

CFU-GMs were cultured in methylcellulose medium during 14 days in the presence or absence of taxanes or platinum drugs. When cisplatin, carboplatin, Taxol or Taxotere were added to this system as single agents, clear cytotoxic effects could be observed. For cisplatin the IC_{50} value was 4.5 μM. The IC_{50} values found for the other drugs used in this system, carboplatin, Taxol and Taxotere, are shown in Table 1 (left column, drugs present during 14 days). No significant difference in cytotoxic effect of the drugs was observed on the formation of either granulocytes or monocytes (not shown). Apparently, neither the taxanes nor the platinum drugs preferentially inhibit colony formation of either cell type, but affect both cell types with approximately the same efficacy. The solvents for paclitaxel in Taxol (Cremophor EL/ethanol) and for docetaxol in Taxotere (Tween 80) did not significantly inhibit or stimulate colony formation compared to the controls (not shown).

To test the cytotoxic interaction between platinum drugs and taxanes, the combinations cisplatin/Taxol or carboplatin/Taxotere were added to the CFU-GMs using their respective IC_{50} ratio, and cells were grown in the presence of the drugs. Three different administration schedules were tested: (i) simultaneous administration, (ii) taxane drug followed by platinum drug after 24 h and (iii) the reverse sequence. We then determined combination indices (CIs) of these schedules.

The results of these experiments are shown in Figure 1, where the CIs are represented as a function of the fractional effect. Strikingly, in the case of cisplatin/Taxol two out of the three schedules resulted in a marked antagonism (Figure 1A). The strongest antagonism was found when administering cisplatin and Taxol simultaneously (a CI was found of 1.76 at the IC_{50} of the combination). Only the schedule in which cisplatin preceded Taxol by 24 h resulted in an almost additive cytotoxicity (a CI was found of 1.1 at the IC_{50} value), suggesting that in this case no interaction, or only a weak antagonism, between the drugs seemed to take place. Antagonistic interactions are not confined to the combination cisplatin/Taxol, since also the combination carboplatin and Taxotere resulted in strong antagonistic cytotoxicities in all the schedules tested (Figure 1B). The CIs found for the three schedules of this combination were not significantly different (ranging from 1.41 to 1.57 at the IC_{50}). Possibly, on the level of the CFU-GM, antagonistic cytotoxicities could be a general feature of this type of combination of anti-cancer drugs.

A disadvantage of the above-described method is that the CFU-GMs proliferate and differentiate into granulocytes and monocytes during 14 days in the presence of the drugs. The cytotoxic effects of the drugs will therefore not be limited to the progenitor

Table 1. IC_{50} values (±SD) of platinum drugs taxanes in the CFU-GM colony-forming assay using two different methods (left column: 14 day drug exposure; right column: drug diluted 20- to 25-fold after 24 h of incubation)

Drug	Drugs present during 14 days	Drugs diluted after 24 h
Cisplatin	4.5±2.3 μM (*n*=6)	1.4±0.6 μM (*n*=3)
Carboplatin	6.0±1.1 μM (*n*=8)	7.6±1.1 μM (*n*=3)
Taxol	12.7±1.3 nM (*n*=6)	19.1±4.3 nM (*n*=3)
Taxotere	12.4±1.8 nM (*n*=8)	18.5±2.8 nM (*n*=3)

cells but will also affect their daughter cells during the culturing period. We therefore modified our *in vitro* system in such a way that after 24 h of drug exposure the cells were diluted into the methylcellulose medium, therewith reducing drug concentrations 20- to 25-fold during the 14 day culturing period. Using this modified method the drugs showed similar cytotoxicities as in the above described experiments, albeit that the IC_{50} values for the taxanes were somewhat higher. The IC_{50} values obtained using this method are shown in Table 1 (right column, drugs diluted after 24 h). Strikingly, to obtain a cytotoxic effect using taxane drugs, bone marrow samples needed to be incubated in LTM at 37°C for at least 24 h prior to addition of the drug (not shown). Possibly, this incubation period is needed for the cells to recover from the thawing procedures and to re-start cell division. In contrast, platinum drugs exerted a cytotoxic effect when added immediately after thawing of the bone marrow sample (not shown). The cytotoxic effect obtained in this way was not significantly different from the experiments in which the drugs were incubated during 14 days.

We then applied this modified method to test both the combinations cisplatin/Taxol and carboplatin/Taxotere, using a simultaneous administration schedule, allowing for a 24 h 'recovery' incubation prior to adding the drugs. The results of the calculated CIs as a function of the fractional effects are shown in Figure 2. Despite the fact that the IC values for the taxanes appeared to be somewhat elevated using this modified method, the CIs were strikingly similar to the ones obtained using the method where drugs were present during 14 days (compare Figure 2 with Figure 1, simultaneous schedule, open circles). Interestingly, no significant difference in CIs was observed between the two combinations tested (1.31 for Taxol+cisplatin at the IC_{50}; 1.41 for Taxotere+carboplatin at the IC_{50}).

Effect of taxanes on intracellular platinum drug accumulation in CFU-GM-derived granulocytes and monocytes

In order to determine the mechanism of the observed antagonisms *in vitro* on the level of the CFU-GMs, we investigated the effect of taxane incubation on intracellular platinum accumulation in CFU-GM-de-

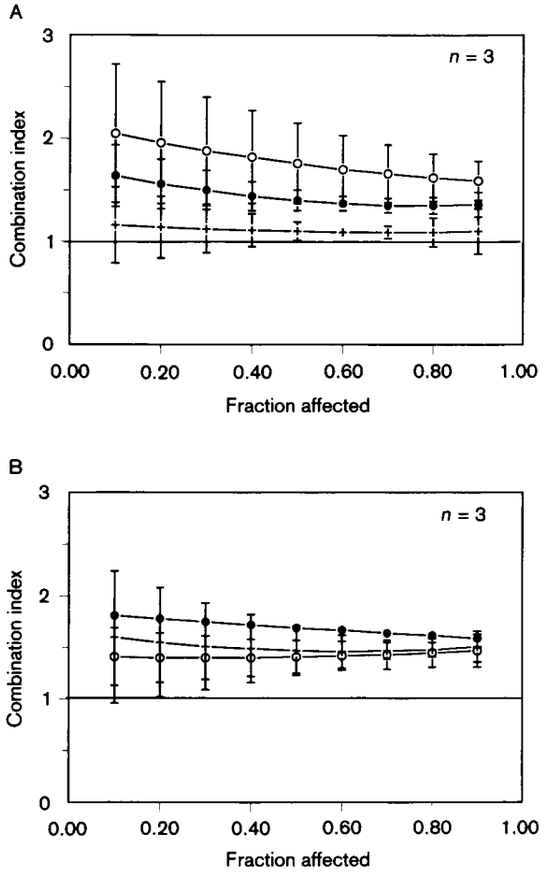

Figure 1. Combination indices of the drug combinations Taxol/cisplatin (A) or Taxotere/carboplatin (B) as a function of the fractional effect on *in vitro* cultured CFU-GMs. Drugs were present during the 14 day culturing period. Three different drug administration schedules were used: simultaneous administration (○), taxane drug followed by platinum drug after 24 h (●) and platinum drug followed by taxane after 24 h (+).

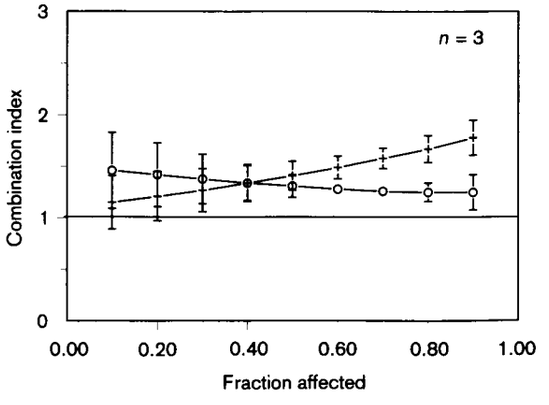

Figure 2. Combination indices of the drug combinations Taxol/cisplatin (○) or Taxotere/carboplatin (+) as a function of the fractional effect on *in vitro* cultured CFU-GMs. Drugs were added to the bone marrow samples simultaneously. Just prior to plating, after 24 h of incubation, drugs were diluted 20- to 25-fold and cells were cultured for 14 days.

rived granulocytes and moncoytes. Co-incubation of Taxol and cisplatin resulted in a slightly, but significantly, reduced intracellular platinum accumulation (88.1 ± 2.2; $n=4$), compared to cisplatin alone (which was set at 100%). We also investigated the effect of the solvent Cremophor EL/ethanol in Taxol. In this case we found no significant reduction in cisplatin accumulation [$101.0 \pm 11.6\%$ ($n=4$) compared to control levels]. The difference in effect of Taxol and Cremophor EL on platinum accumulation was not statistically significant ($p=0.11$, paired t-test). Moreover, co-incubation of Taxotere and carboplatin on CFU-GM-derived granulocytes and monocytes did not result in a significantly reduced intracellular platinum accumulation ($102.9 \pm 15.5\%$; $n=4$) compared to controls where carboplatin was incubated alone. As expected, the solvent for docetaxel in Taxotere (Tween 80) did not affect intracellular carboplatin accumulation either ($105.6 \pm 16.0\%$ of control levels; $n=4$).

Discussion

In our *in vitro* culturing assay the combination of taxanes and platinum drugs showed marked antagonistic cytotoxic effects on the level of the CFU-GMs. In the assays where drugs were present during the 14 day culturing period, antagonistic interactions occurred in five out of six of the tested schedules using two different drug combinations (Figure 1). The cytotoxic effects of the drugs most likely occurred within the first 24 h of drug incubation, since the experiments in which drug concentrations were reduced after 24 h resulted in similar cytotoxic interactions (Figure 2). This implies that indeed the progenitor cells are the target of the cytotoxic effect of the drugs and that a possible cytotoxic effect on the daughter cells is less important. Possibly, the drugs are largely inactivated after 24 h of incubation by binding to proteins in the culturing medium or by other mechanisms. The observation that the bone marrow cells had to be left at 37°C for 24 h before adding the taxanes in order to obtain a cytotoxic effect suggests that actively cycling cells are absent during the first 24 h after thawing. Taxanes are known to be antimitotic agents, interfering with the cell cycle by inducing a G_2/M block.[16,17]

Strikingly, the combination carboplatin/Taxotere, which is not yet used in the clinic, showed a schedule-independent antagonism. Possibly, this combination might need further attention in future clinical trials. With the combination Taxol/cisplatin a schedule-dependent interaction was found, where the schedule cisplatin→Taxol resulted in an additive cytotoxicity and the reverse showed an antagonistic interaction. Possibly, due to a lack of a cytotoxic effect of the taxane during the first 24 h of incubation, no significant difference was found between the schedules where the drugs were added simultaneously and where Taxol preceded cisplatin.

These results also seem to be in accordance with data obtained in clinical trials where the sequence Taxol→cisplatin was shown to be less toxic to bone marrow than the reverse sequence.[5,6,8,18] Possibly, the technique presented here might be a useful diagnostic tool to study cytotoxic interactions at the level of bone marrow cells, with other drug combinations as well.

The cytotoxic interactions between platinum drugs and taxanes described here were not due to interactions with the solvents in Taxol and Taxotere (Cremophor EL and Tween 80, respectively). It has been shown earlier that taxane drugs reduced intracellular platinum accumulation in peripheral white blood cells *in vivo* and *in vitro*.[15] We and others showed that this effect was entirely caused by the solvent for the taxane drugs (M de Graaff *et al.*, in preparation). De Vos *et al.*[19] speculated in their paper that possibly the reduced accumulation of platinum drugs by Cremophor EL in blood cells could play a role in the bone marrow sparing effect of the drug combination. In the present paper we show that both Cremophor EL and Tween 80 have no cytotoxic effect on CFU-GMs cultured *in vitro* nor do they significantly affect intracellular platinum accumulation in CFU-GM-derived granulocytes and monocytes, making the proposed explanation by De Vos less likely.

Future improvements of our system would be to use a better defined cell fraction in the experiments. One could think of performing a $CD34^+$ selection prior to drug administration and cell culture. This would exclude drug interactions and possible effects on stroma cells, which are still present in our system at the time that the cytotoxic effects take place (the first 24 h of drug incubation). It would also be of interest to extend the investigations to other types of bone marrow progenitor cells (e.g. CFU-Meg: megakaryocytes), since it was shown that the combination carboplatin/Taxol resulted in less thrombocytopenia than was anticipated.[9,10]

Acknowledgments

We are indebted to Professor Jos H Beijnen for his active support. We wish to thank Dr Olaf van Tellingen and Heidi van der Woude for their expertise and kind assistance using the flameless atomic spectrometer.

References

1. Rowinsky EK, Donehouwer RC. Paclitaxel. *N Engl J Med* 1995; **332**: 1004-14.
2. Schellens JHM, Ma J, Planting ASTh, *et al.* Relationship between the exposure to cisplatin, DNA-adduct formation in leukocytes and tumor response in patients with solid tumors. *Br J Cancer* 1996; **73**: 1569-75.
3. Horwitz SB. Mechanism of action of taxol. *Trends Pharm Sci* 1992; **13**: 134-6.
4. Schiff PB, Horwitz SB. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc Natl Acad Sci USA* 1980; **77**: 1561-5.
5. Bookman MA, McGuire III WP, Kilpatrick D, *et al.* Carboplatin and paclitaxel in ovarian carcinoma: a phase I study of the Gynaecologic Onc Group. *J Clin Oncol* 1996; **14**: 1895-902.
6. Huizing MT, van Warmerdam LJ, Rosing H, *et al.* Phase I and pharmacologic study of the combination paclitaxel and carboplatin as first-line chemotherapy in stage III and IV ovarian cancer. *J Clin Oncol* 1997; **15**: 1953-64.
7. Rowinsky EK, Gilbert MR, McGuire WP, *et al.* Sequences of Taxol and cisplatin: a phase I pharmacologic study. *J Clin Oncol* 1991; **9**: 1692-703.
8. Obasaju CK, Johnson SW, Rogatko A, *et al.* Evaluation of carboplatin pharmacokinetics in the presence and absence of paclitaxel. *Clin Cancer Res* 1996; **2**: 549-54.
9. Calvert AH. A review of the pharmacokinetics and pharmacodynamics of combination carboplatin/paclitaxel. *Semin Oncol* 1997; **24** (suppl 2): 85-90.
10. Van Warmerdam LJ, Huizing MT, Giaccone G, *et al.* Clinical pharmacology of carboplatin administered in combination with paclitaxel. *Semin Oncol* 1997; **24** (suppl 2): 97-104.
11. Huizing MT, Giaccone G, van Warmerdam LJC, *et al.* Pharmacokinetics of paclitaxel and carboplatin in a dose-escalating and dose-sequencing study in patients with non-small-cell lung cancer. *J Clin Oncol* 1997; **15**: 317-29.
12. Chou T-C, Talalay P. Quantitative analysis of dose-effect relationships: the combined effect of multiple drugs or enzyme inhibitors. *Adv Enz Reg* 1984; **22**: 27-55.
13. Chou T-C, Motzer RJ, Tong Y, Bosl GJ. Computerized quantitation of synergism and antagonism of taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design. *J Natl Cancer Inst* 1994; **20**: 1517-24.
14. Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein using the principles of protein-dye binding. *Anal Biochem* 1976; **72**: 248-54.
15. Ma J, Verweij J, Planting AST, *et al.* Docetaxel en paclitaxel inhibit DNA-adduct formation and intracellular accumulation of cisplatin in human leukocytes. *Cancer Chemother Pharmacol* 1996; **37**: 382-4.
16. Liebmann JE, Cook JA, Lipschultz C, *et al.* The influence of Cremophor EL on cell cycle effects of paclitaxel (Taxol^{R}) in human tumor cell lines. *Cancer Chemother Pharmacol* 1994; **33**: 331-9.
17. Lopes NM, Adams EG, Pitts TW, Bhuyan BK. Cell kill kinetics and cell cycle effects of Taxol on human and hamster ovarian cell lines. *Cancer Chemother Pharmacol* 1993; **32**: 235-42.
18. Planting ASTh, van der Burg MEL, De Boer-Dennert M, Stoter G, Verweij J. Phase I/II study of a short course of weekly cisplatin in patients with advanced solid tumors. *Br J Cancer* 1993; **68**: 789-92.
19. De Vos AI, Nooter K, Verweij J, *et al.* Differential modulation of cisplatin accumulation in leukocytes and tumor cell lines by the paclitaxel vehicle Cremophor EL. *Ann Oncol* 1997; **8**: 1145-50.

(Received 21 July 1998; revised form accepted 27 October 1998)