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The combination of yondelis and cisplatin is synergistic against human tumor xenografts

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Abstract

Yondelis™ (trabectidin, ET-743) is a marine natural product that has shown activity both in preclinical systems and in human malignancies such as soft tissue sarcoma and ovarian cancers that are resistant to previous chemotherapies. Molecular pharmacological studies indicated that Yondelis interacts with DNA and DNA repair systems in a way that is different from Cisplatin (DDP). The current study was designed to investigate the effects of the combination of Yondelis and DDP in human cancer cell lines and in xenografts derived from different tumours. The *in vitro* studies performed in human TE-671 rhabdomyosarcoma, Igrov-1 and 1A9 human ovarian carcinoma cell lines showed additive effects or slight synergism. Several human tumour xenografts, such as TE-671 rhabdomyosarcoma, SK-N-DX neuroblastoma, FADU head and neck, LX-1 non-small cell lung cancer (NSCLC), H-187 melanoma and SKOV HOC 8 ovarian carcinoma, showed an antitumour effect for the combination that was greater than that of each drug when given as a single agent. No consistent changes in the activity were observed if Yondelis and DDP were given 1 h apart in sequence or simultaneously. An orthotopically transplanted human ovarian cancer HOC 8 growing in the peritoneal cavity of nude mice was used that is insensitive to Yondelis alone and only moderately sensitive to DDP alone. The combination of the two drugs produced a dramatic increase of survival lasting several months. In conclusion, the combination of Yondelis and DDP is synergistic *in vivo* (i.e. the antitumour effect is greater than that of each drug used as a single agent at the maximum tolerated dose (MTD)) in different human tumour xenografts. The two drugs can be combined at the MTD of each drug, thus indicating there are no overlapping toxicities. These results provide a rationale for testing the combination of Yondelis and DDP in the clinic.

Keywords: Marine natural products; Cisplatin; ET-743; Combination; Tumour xenografts

1. Introduction

Yondelis (trabectidin, ET-743) is a novel anticancer drug derived from the marine tunicate *Ecteinascidia turbinata* [1–3] with a potent antitumour activity in different *in vitro* and *in vivo* preclinical models [4–8]. In phase I and II clinical trials, Yondelis has shown significant antitumour activity against several human malignancies, including soft-tissue sarcomas [9–12] and ovarian carcinomas [13]. Because of these promising data, there is a strong clinical interest in evaluating the efficacy of Yondelis in combination with other anticancer drugs. We have focused our *in vitro–in vivo* studies on the effects of the combination of Yondelis and cisplatin (DDP) for the following reasons.

Molecular pharmacology studies have indicated that DDP and Yondelis have different mechanisms of interaction with DNA and involve different DNA repair mechanisms. DDP binds at the N7 position of guanine, in the major groove of DNA and forms intra- and

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interstrand crosslinks [14–16], whereas the monoadducts of Yondelis occur at the N2 position of guanine, in the minor groove [17]. Evidence exists that cell lines deficient in mismatch repair are partially resistant to DDP [18], whereas they are sensitive to Yondelis [19]. In addition, cell lines deficient in nucleotide excision repair (NER) are hypersensitive to DDP and are partially resistant to Yondelis [19–22]. The mechanism for this unusual mechanism of resistance is not yet understood, but the contrasting sensitivities of Yondelis and DDP in cell lines with different abilities to repair DNA damage might imply that the two drugs can be advantageously combined to effectively target different populations of cancer cells that may co-exist in the same tumour. Recently, following the observation that some Yondelis-induced DNA adducts are not efficiently repaired *in vitro* by the bacterial multisub-unit endonucleases uvrABC repair enzymes, it has been proposed that DNA repair enzymes might recognise the Yondelis unrepairable adducts and bind to them forming complexes with DNA, possibly playing a role in the drug's toxicity. If this hypothesis is correct one would anticipate that Yondelis would cause a temporary depletion of DNA repair proteins and thus the simultaneous treatment with other DNA damaging agents would produce a synergistic cytotoxicity and antitumour activity.

These considerations prompted us to investigate the effects of the combination of Yondelis and DDP both in human cancer cell lines growing *in vitro* and in xenografts derived from different human tumours.

2. Materials and methods

2.1. In vitro *studies*

To evaluate the effect of the combination Yondelis and DDP treatment, the dimethylthiazolyl-2,5-diphenyl tetrazolium bromide (MTT) test was used for Igrov-1 and 1A9 ovarian carcinoma cell lines, while a colony assay was used in TE671 rhabdomyosarcoma cells. The cells were exposed for 1 h to different concentrations of Yondelis or DDP, given alone or in combination.

2.2. *Data analysis for* in vitro *studies*

A factorial experimental design was adopted in each experiment, where single concentrations of Yondelis were coupled with several concentrations of DDP and *vice versa*, with three to six replicates in each experiment.

The data of each treatment were initially expressed as a percentage (or fraction 'unaffected' [fu]) of untreated samples, then best fit values of the concentration-effect relationships of Yondelis or DDP alone were used to calculate the Interaction Index according to Berembaum and the difference between the expected (based on the Lowe additivity criterion) and observed effects in each combination [23,24].

2.3. *The Interaction Index is defined as*

$$I = D_{YONDELIS}/IC_{X,YONDELIS} + D_{DDP}/IC_{X,DDP}$$

where $D_{YONDELIS}$ and D_{DDP} are the concentrations of ET-743 and DDP used in the combination, X is the observed effect in the combination, $IC_{X,YONDELIS}$ and $IC_{X,DDP}$ are the concentrations of the each individual drug that would produce the effect X if given alone.

Yondelis and DDP concentrations were grouped on the basis of the effect of the single drug in the Low (L, $fu > 0.8$), Intermediate (M, $fu \leqslant 0.8$ and > 0.5), High (H, $fu \leqslant 0.5$ and > 0.2) groups.

Eventually all combination data, referring to combinations of concentrations individually producing $fu > 0.2$ in any experiment with a given cell line, were entered in the analysis and the respective results were pooled into nine groups:

$L_{YONDELIS} + L_{DDP}$, $L_{YONDELIS} + M_{DDP}$, $L_{YONDELIS} + H_{DDP}$, $M_{YONDELIS} + L_{DDP}$, $M_{YONDELIS} + M_{DDP}$, $M_{YONDELIS} + H_{DDP}$, $H_{YONDELIS} + L_{DDP}$, $H_{YONDELIS} + M_{DDP}$, $H_{YONDELIS} + H_{DDP}$.

Means and the variance of the Interaction Indices in each group were calculated. The significance of the difference of the mean from $I = 1$ was evaluated using a two-tailed t-test: additivity when the mean value of I was not different from 1 at the 0.05 level of significance; synergism or antagonism when the mean I values were lower or higher than 1, respectively. Groups containing less than three data values were not considered.

2.4. *Animals*

Female NCr-nu/nu mice obtained from the animal production colony of the National Cancer Institute (NCI) Frederick Cancer Research and Development Center, Frederick, MD or eD1 nu/nu male mice from Charles River, Calco, Italy, were used for the study. Mice were used at eighteen weeks of age and with a mean body weight ± standard deviation (S.D.) of 22 ± 3 g. Throughout this study, nude mice were housed in filtered-air laminar flow cabinets and were manipulated following aseptic procedures.

Procedures involving animals and their care are conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 febbraio 1992, Circolare No. 8, G.U., luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

2.5. In vivo *studies*

Human ovarian carcinoma (HOC) xenograft, HOC8, was established and maintained intraperitoneally (i.p.) as a cell suspension in nude mice as previously described in Ref. [25]. HOC8 grows in the peritoneal cavity of all injected mice, producing ascites and solid lesions on abdominal organs: primarily the diaphragm, omentum and liver.

The human ovarian 1A9 cell line was kindly provided by Dr Tito Fojo (Medicine Branch-CCR-NCI-NIH Bethesda, MD, USA). 1A9 was derived from A2780 human ovarian carcinoma cells [26] and was established in nude mice from *in vitro* cell cultures. Briefly, the cell line was grown in Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal bovine serum (FBS) and 5 mM L-glutamine. A suspension of 1A9 cells (5×10^6) was injected subcutaneously (s.c.) into nude mice to obtain the corresponding xenograft. For all other xenografts used the number of cells transplanted s.c. ranged between 1.5×10^6 and 2.0×10^6. When the tumours were palpable (approximately 0.2 g) animals were divided randomly into test groups, consisting of 6–8 mice each and the drug treatment was started. In the case of ascitic HOC8, treatment was started 10 days after the i.p. injection of 10^7 cells (8–10 mice per experimental group).

2.6. Drug treatment

Yondelis, provided by Pharma Mar, S.A. (Tres Cantos, Madrid, Spain), was prepared as a 1 mg/ml stock solution in absolute ethanol (stored at −20 °C) and further diluted with phosphate buffer pH 4.0 immediately before administration. Yondelis (0.05 and 0.15 mg/kg) was given intravenously (i.v.) to mice either a single 0.2 mg/kg injection or one 0.1–0.2 mg/kg injection three times every 4 days (Q4×3). DDP (Sigma) was dissolved in saline and given either by a single i.v. injection or Q4×3. Appropriate vehicles were injected, using the same schedule and route of injection as the drug therapies. Yondelis and DDP were given 1 h apart in sequence or simultaneously.

2.7. Treatment evaluation

In xenografts transplanted s.c., tumour growth was monitored and tumour weight (TW) was determined by measuring the tumour diameters with a Vernier caliper every 2–4 days and using the formula $TW = d^2\times D/2$ (where d and D represent the shortest and the longest diameter, respectively).

Tumour Weight Inhibition (TWI) in treated versus control mice was calculated according to the formula: $100-(T/C\times100)$.

In i.p. HOC, antitumour activity studies were performed as previously described in Ref. [27]. Mice were monitored twice a week for body weight loss and tumour formation (abdominal distension) in the peritoneal cavity. Animals were sacrificed when they became moribund and the day of sacrifice was considered as a survival time. The presence of the tumour in the peritoneal cavity was ascertained by microscopic examination at autopsy.

Surviving animals that did not present gross evidence of tumours in the peritoneal cavity were euthanised and autopsied no earlier than 90 days after the death of the last control animal. The absence of tumour in 'cured' mice was confirmed by cyto-histological examination, as previously described in Ref. [28].

Briefly, lavages of the peritoneal cavity were spun in a cytocentrifuge and the cells were fixed and stained with haematoxylin/eosin (H&E). The ovary/uterus, pancreas, omentum, liver, diaphragm and lung were collected, fixed in 10% phosphate-buffered formalin and processed for standard histological analysis.

3. Results

Tables 1, 2 and 3 show the mean interaction index of the combination of Yondelis and DDP, found in human TE-671 rhabdomyosarcoma, Igrov-1 and 1A9 human ovarian carcinoma cell lines growing *in vitro*, respectively. No evidence of antagonism was seen in any of all these three cell lines. In TE-671, we found synergism when the two drugs were combined at a relatively high concentration (i.e. a concentration producing a cytotoxic effect between 50 and 80% when given alone) or for intermediate concentrations of one drug (i.e. a concentration producing a cytotoxic effect between 20 and 50% when the drug was given alone) combined with concentration of the other. In Igrov-1 cells, the synergism was evident again at the high concentrations of the two drugs, although it was also observed at a low con-

Table 1
Mean interaction index of the combination of ET-743 and DDP in TE-671 rhabdomyosarcoma cell line

	L_{DDP}	M_{DDP}	H_{DDP}
L_{ET}	(3) 0.88±0.1 ADD	(9) 1.1±0.12 ADD	(3) 1.09±0.05 ADD
M_{ET}	ND[a]	(14) 0.96±0.22 ADD	(18) 0.82±0.18 SYN
H_{ET}	(6) 0.94±0.33 ADD	(9) 0.79±0.21 SYN	(21) 0.85±0.21 SYN

The figure represents the mean and standard deviation (in parentheses: number of combination data in the specified effect ranges). ET-743 and DDP concentrations were grouped on the basis of the effect of the single drug in the Low (L, % survival >80%), Intermediate (M, % survival between 50 and 80%), High (H, % survival between 20 and 50%) groups. ET, ET-743; DDP, cisplatin.

[a] ND, not determined because less than three data values were available.

Table 2
Mean interaction index of the combination of ET-743 and DDP in Igrov-1 ovarian cancer cell line

	L_{DDP}	M_{DDP}	H_{DDP}
L_{ET}	(3) 0.88±0.08 ADD	(4) 0.99±0.29 ADD	(9) 0.93±0.14 ADD
M_{ET}	ND[a]	(6) 0.88±0.18 ADD	(16) 0.83±0.14 SYN
H_{ET}	(3) 0.80±0.02 SYN	(13) 0.87±0.22 ADD	(30) 0.77±0.20 SYN

The figure represents the mean and standard deviation (in parentheses: number of combination data in the specified effect ranges). ET-743 and DDP concentrations were grouped on the basis of the effect of the single drug in the Low (L, % survival >80%), Intermediate (M, % survival between 50 and 80%), High (H, % survival between 20 and 50%) groups.

[a] ND, not determined because less than three data values were available.

Table 3
Mean interaction index of the combination of ET-743 and DDP in 1A9 ovarian cancer cell line

	L_{DDP}	M_{DDP}	H_{DDP}
L_{ET}	(9) 0.86±0.15 SYN	(7) 0.93±0.26 ADD	(7) 0.88±0.2 ADD
M_{ET}	(6) 0.95±0.16 ADD	(6) 1.05±0.16 ADD	(16) 1.06±0.2 ADD
H_{ET}	(5) 0.97±0.19 ADD	(4) 1.06±0.19 ADD	(4) 1.2±0.32 ADD

The figure represents the mean and standard deviation (in parentheses: number of combination data in the specified effect ranges). ET-743 and DDP concentrations were grouped on the basis of the effect of the single drug in the Low (L, % survival >80%), Intermediate (M, % survival between 50 and 80%), High (H, % survival between 20 and 50%) groups.

centration of DDP (i.e. a concentration producing a cytotoxic effect less than or equal to 20%), whereas in 1A9, the effect was always additive at all the concentrations investigated.

In order to evaluate the effects of the combination of the two drugs *in vivo*, we selected some xenografts that were relatively resistant to a single dose of DDP and moderately sensitive to a single dose of Yondelis. The maximal single i.v. doses of DDP and Yondelis that caused no toxic deaths were 12 and 0.2 mg/kg, respectively. The same dose of each drug could be given when the two drugs were administered in combination with a tolerable toxicity, with a maximal weight loss ranging from 10 to 26% in different experiments ($n=14$) and a median value of 15%. Treatment with the combination caused only a slightly higher weight loss than treatment with each drug alone. The toxicity did not appear to be different when the two drugs were given simultaneously or given one after the other with an interval of 1 h in either of the two sequences.

Table 4
Tumour weight inhibition (TWI) of ET-743 combined with DDP compared with each drug alone[a]

Tumour	Treatment	TWI (%)	BW loss (%)
TE-671 Rhabdomyosarcoma	ET (0.2 mg/kg)	62	10
	DDP (12 mg/kg)	55	10
	ET→DDP	81	15
	DDP→ET	75	15
	ET+DDP	78	20
SK-N-DZ Neuroblastoma	ET (0.2 mg/kg)	52	11
	DDP (12 mg/kg)	16	12
	ET→DDP	68	14
	DDP→ET	91	15
	ET+DDP	59	14
FADU Head and neck carcinoma	ET (0.2 mg/kg)	41	5
	DDP (12 mg/kg)	57	13
	ET→DDP	70	26
	DDP→ET	71	22
LX-1 NSCLC	ET (0.2 mg/kg)	39	0
	DDP (12 mg/kg)	51	0
	ET→DDP	82	14
	DDP→ET	87	19
H-187 Melanoma	ET (0.2 mg/kg)	52	0
	DDP (12 mg/kg)	52	0
	ET→DDP	57	5
	DDP→ET	69	5
SKOV Ovarian carcinoma	ET (0.2 mg/kg)	30	5
	DDP (12 mg/kg)	10	5
	ET→DDP	58	10
	DDP→ET	33	15
1A9 Ovarian carcinoma	ET (0.1 mg/kg×3)	28	14
	DDP (4 mg/kg×3)	37	12
	ET+DDP	73	16

NSCLC, non-small cell lung cancer; BW, body weight; TWI, tumour weight inhibition.

[a] The drugs were given either simultaneously (ET+DDP) or in sequence with a 1-h interval between the two drugs (ET→DDP or DDP→ET).

As can be seen in Table 4, the antitumour activity of the combination was greater than that of each drug alone in all models. In TE-671 rhabdomyosarcoma and SK-N-DZ neuroblastoma, all three combinations (i.e. Yondelis given 1 h before DDP or at the same time or 1 h after DDP) were compared and no significant differences in the antitumour activity were observed. In addition, in head and neck (H&N) FADU, in non-small cell lung carcinoma (NSCLC) LX-1, in melanoma H-187 and in the ovarian SKOV, where the two sequences were compared, no consistent differences related to the sequence was found. Collectively, all the data indicate that the antitumour activity of the combination was greater than that of each drug alone and the sequence does not influence the treatment efficacy and toxicity in a consistent fashion.

The observation that the toxicity of the combination appeared very modest prompted us to test the effect of the combination of Yondelis and DDP splitting the

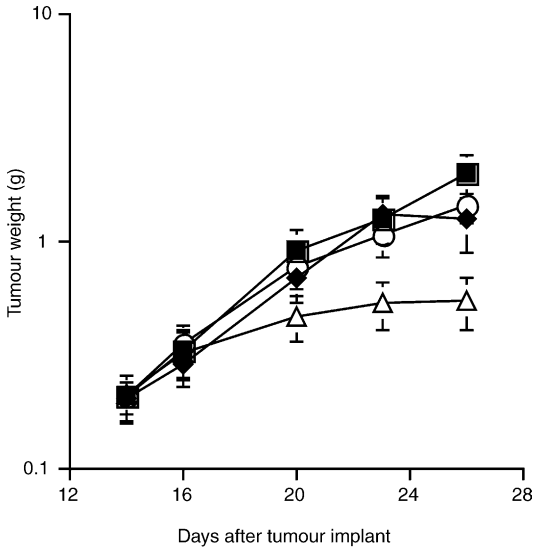

Fig. 1. *In vivo* antitumour activity of the combination of Yondelis and DDP on the human ovarian 1A9 xenograft. Nude mice were transplanted subcutaneously (s.c.) with 1A9 and treatment was started when the tumour reached the size of approximately 0.2 g. ■ = vehicle; ◆ = DDP 4 mg/kg intravenously (i.v.), days 15, 19, 23 after the tumour implant. ○ = Yondelis 0.1 mg/kg i.v., days 15, 19, 23 after the tumour implant; △ = Yondelis 0.1 mg/kg i.v. + DDP 4 mg/kg i.v., days 15, 19, 23 after the tumour implant. Values are mean ± standard error of the mean (SEM) $N = 6$ mice per group.

dose of both drugs into three administrations with an interval of 4 days.

As shown in Fig. 1, 1A9 ovarian carcinoma xenografts were relatively resistant to the two drugs used as monotherapy. In contrast, DDP at 4 mg/kg (Q4×3) (for a total dose of 12 mg/kg) given simultaneously with Yondelis at 0.1 mg/kg (Q4×3) (for a total dose of 0.3 mg/kg) induced a significant TWI of 73%.

Again, we observed no toxic deaths or severe toxicity with the combination (mean body weight loss 16%) compared with the single drugs (14 and 12% with Yondelis and DDP, respectively).

In ovarian carcinoma patients, the tumour spreads into the peritoneal cavity. Therefore, to mimic the clinical disease we selected a human ovarian xenograft, HOC 8, which was transplanted intraperitoneally from ascites and disseminated in the peritoneal cavity. This tumour is partially sensitive to DDP (increase in life span ILS = 139%) and insensitive to Yondelis (ILS = 21 and 23% with 0.05% and 0.15 mg/kg doses given Q4×3).

When the two drugs were combined the effect was much greater than that of each drug given as a single agent with a dramatic increase in survival (Fig. 2). Both the low (ILS = 258% versus vehicle) and the high (ILS = 322% versus vehicle) dose of Yondelis combined with DDP increased the survival time of mice bearing HOC8. This time was significantly improved compared with that when DDP was given as monotherapy (ILS = 49 and 76% versus DDP with low and high doses of Yondelis, respectively). Three animals were still alive after 12 months, two of them belonging to the group receiving the high Yondelis dose. They were sacrificed and a detailed macroscopic and microscopic pathological evaluation was performed. The mouse belonging to the group receiving the low Yondelis dose was apparently cured as a microscopic analysis of the liver, spleen, pancreas, bone marrow, diaphragm, ovary, uterus, omentum and several lymph nodes were negative. However, both of the other long-term surviving mice

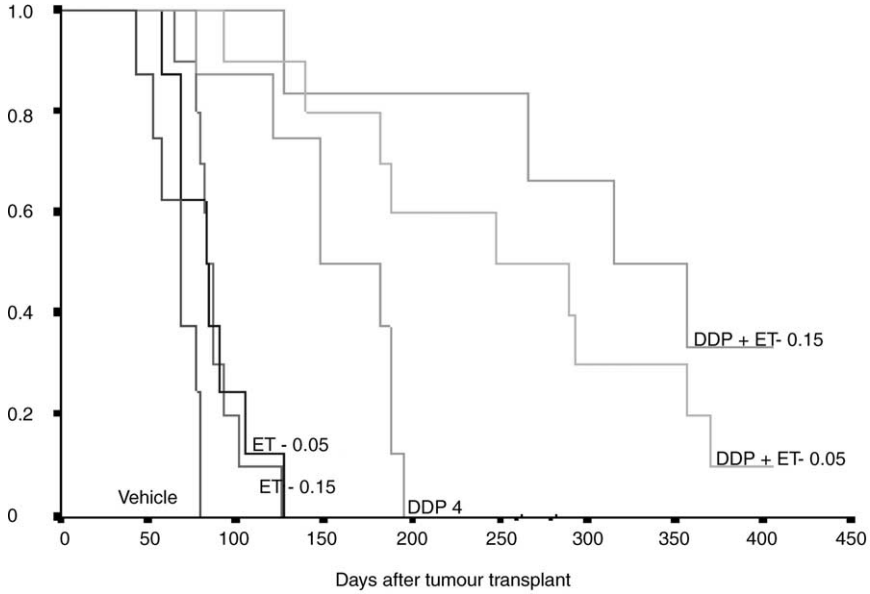

Fig. 2. Survival of HOC8-bearing mice treated with the combination of Yondelis and DDP. Nude mice were inoculated intraperitoneally (i.p.) with HOC8 cell suspension and treatment started 10 days later. Yondelis (0.05 and 0.015 mg/kg) and DDP (4 mg/kg) were administered i.v. alone or in combination on days 10, 14, 18 after tumour transplant ($N = 8$–10 mice per group).

showed a residual tumour of the omentum and in one of them a single metastasis in the uterus was found, although no metastases were detected in the other organs.

4. Discussion

The different pattern of DNA damage induced by Yondelis and DDP and the different mechanisms involved in the repair of the DNA damage induced by the two drugs [14,19] provided a strong rationale to investigate the biological activity of the combination of the drugs. The present study shows that the combination of Yondelis and DDP has an additive or synergistic effect *in vitro* and a synergistic activity *in vivo* against human xenografts, i.e. an antitumour activity greater than that of each drug used as a single agent at the maximum tolerated dose (MTD). The best results were obtained *in vivo*, in xenografts, suggesting that the synergism cannot be entirely explained on the basis of an enhanced cytotoxicity against cancer cells, but may involve other mechanisms mediated by the host. While further studies should be performed to investigate the mechanisms responsible for the strong synergism observed *in vivo*, the results obtained provide a strong rationale to undertake investigations on this combination at the clinical level. The fact that full doses of each of the two drugs can be given with only a marginal increase in the toxicity should encourage a phase I study in which full doses of DDP can be associated with escalating doses of Yondelis. It should be noted that the two drugs have a different clearance mechanism; DDP is cleared by renal elimination [29], whereas the renal clearance of Yondelis seems to be negligible, hepatic clearance being the more important [28]. In addition, the pattern of toxicity of the two drugs is different, the target of toxicity for Yondelis is the bone marrow and liver, whereas the main toxicities of DDP treatment are certainly nephro- and peripheral neurotoxicity [29]. These pharmacokinetic and toxicological considerations help explain why the two drugs can be combined without requiring a dose reduction from the doses used for treatments with each drug alone.

The finding that giving the two drugs simultaneously or one after the other in either sequence, with an interval of 1 h, produced a similar antitumour activity could be important information when designing future clinical protocols. It should be emphasised that even in tumours in which the two drugs produced no significant activity given alone, there was evidence of activity for the combination. Therefore, the combination of Yondelis and DDP should be tested not only in tumours in which the two drugs alone are effective, but also in tumours that are considered resistant to these drugs given alone.

Recently a phase II study of Yondelis in ovarian cancer has shown an high percentage of partial responses (approximately 40%), in patients resistant to carboplatin or DDP and paclitaxel [30].

In view of the present study, which clearly indicates a strong synergism for the combination of Yondelis and DDP, it seems attractive to test the combination of the two drugs in patients who are relapsing or refractory to first-line chemotherapy.

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