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Schedule-dependent Antagonism of Paclitaxel and Cisplatin in Human Gastric and Ovarian Carcinoma Cell Lines *In Vitro*

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Paclitaxel has demonstrated broad clinical activity in a variety of malignancies both alone and in combination with other chemotherapeutic agents. The *in vitro* cytotoxicity of paclitaxel and cisplatin alone, in combination and in sequence, were evaluated against established human gastric and ovarian carcinoma cell lines using 2-h drug exposure. The combination of cisplatin and paclitaxel was found to be additive or even synergistic when paclitaxel was given 24 h prior to cisplatin as demonstrated by isobologram analysis. However, when both drugs were given simultaneously or when cisplatin was given prior to paclitaxel, a strong antagonistic interaction was observed. This antagonism was evident for up to 72 h after a 2-h exposure to cisplatin. Pretreatment with cisplatin caused no alteration in [^{3}H]paclitaxel uptake in HM2 gastric carcinoma cells, but resulted in decreased intracellular retention of paclitaxel. Since cisplatin treatment led to a reduction in cellular glutathione content in these cells and reduced levels of glutathione have been associated with protection against cytotoxicity of paclitaxel, cells were pretreated with L-buthionine sulfoximine (L-BSO). However, depletion of glutathione had no influence on the activity of paclitaxel. A significant accumulation of cells in S-phase was observed 24 h after cisplatin, which resolved after 48 h and resulted in a pronounced increase of G_2M phase. These data demonstrate that the interactions of paclitaxel and cisplatin are highly schedule-dependent and applications of cisplatin simultaneously with or prior to paclitaxel may result in pronounced antagonism. These findings could have implications for the design of further clinical protocols.

Key words: paclitaxel, cisplatin, drug interaction, gastric cancer, ovarian cancer, pharmacokinetics, glutathione pathway

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INTRODUCTION

PACLITAXEL IS an anti-microtubule agent isolated from the western yew tree *Taxus brevifolia* with activity against a variety of malignancies. Paclitaxel has demonstrated clinical efficacy in ovarian cancer, breast cancer, non-small cell lung cancer and head and neck cancer [1–4]. Because of the high activity of paclitaxel in refractory ovarian carcinoma, phase II and III clinical studies of the combination paclitaxel and cisplatin are ongoing [5]. The exact mechanisms of the cytotoxic effects of paclitaxel are unknown, but there is evidence that paclitaxel inhibits cell division by binding to tubulin and stabilising the tubulin polymer formation [6–8]. Paclitaxel causes a G_2M-interphase blockade, which may be due to persistent interphase microtubule complexes [8, 9]. Cellular resistance to paclitaxel has been associated with the expression of p-glycoprotein, reduced cellular glutathione content, occurrence of abnormal tubulin subunits and alterations in karyotype [10–14]. There is evidence of antagonistic *in vitro* interactions between paclitaxel and cisplatin, depending on the schedule used [15, 16].

In order to clarify this issue, we investigated the interactions of paclitaxel and cisplatin given in different schedules, the role of paclitaxel updake and retention, the glutathione pathway and the cell cycle distribution.

MATERIALS AND METHODS

Drugs and chemicals

Paclitaxel, sulphorhodamin B reagent, 5,5 dithiobis (2-nitrobenzoic acid) (DTNB), L-buthionine sulphoximine (L-BSO), propidium iodide, RNase (Typ III-A, bovine pancreas), glutathione reductase (EC 1.6.4.2) and reduced nicotinamide adenine dinucleotide phosphate (NADP) were supplied by Sigma, (Deisenhofen, F.R.G.). L-15 medium was obtained from Boehringer Mannheim (Mannheim, F.R.G.), RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) from CIBO/Life Technologie (Eggenstein, F.R.G.). Cisplatin was supplied by Bristol (München, F.R.G.). The [^{3}H]paclitaxel (specific activity 19 Ci/mmol) was from Paesel-Lorei (Frankfurt, F.R.G.) and found to be 99.9% pure by high-pressure liquid chromatography. All drug solutions were prepared freshly before use.

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Cell lines and culture techniques

The gastric carcinoma cell lines HM2 and HM51 were established from ascitic fluid and a lymph node of non-pretreated patients with gastric adenocarcinoma. The characteristics have been described previously [17]. The cell lines HM2 and HM51 were grown as monolayers in L-15 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), non-essential amino acids and L-glutamine. There was no evidence of expression of glycoprotein p-170 in either gastric cancer lines, using MRK16 fluorescence labelling flow cytometry analysis (data not shown). The ovarian cancer cell lines EOV1 and EOV2 were isolated from ascitic fluid of 2 patients with ovarian adenocarcinomas, pretreated with cisplatin and cyclophosphamide or carboplatin and cyclophosphamide, respectively, by our group at the West German Cancer Centre. Both ovarian cancer cell lines were grown as monolayers, and were used at approximately passage number 20. The ovarian cancer cell lines EOV1 and EOV2 were maintained in RPMI containing L-glutamine, 10% FCS and 25% DMEM. All cell lines were kept in an atmosphere of 5% CO_2 in air at 37°C.

Cytotoxicity assay

The cytotoxicity of cisplatin and paclitaxel was assessed by the sulphorhodamin B assay [18]. Cells in exponential growth were washed with phosphate-buffered saline (PBS), trypsinised with 0.25% trypsin/EDTA for 2 min at 37°, counted and seeded as a single-cell suspension at a density of 5000 cells/well in 96-well microtitre plates (Falcon, Heidelberg, F.R.G.), and allowed to attach overnight. After 24 or 48 h, 100 μl of medium containing different concentrations of either paclitaxel or cisplatin were added for 2 h. The drug-containing medium was aspirated from the plates and fresh medium was added. Control dishes without paclitaxel or cisplatin were treated identically. After a total incubation time of 120 h, cells were fixed with 50 μl of 50% trichloric acetic acid (TCA) for at least 1 h at 4°C, washed three times with PBS and stained as originally described. Eight wells were used for each drug concentration and all experiments were performed in triplicate. The drug concentration which inhibited cell growth by 50% (IC_{50}) was obtained from semi-logarithmic dose–response plots.

Two different protocols were used to determine the interactions of paclitaxel and cisplatin. In cell lines HM2 and HM51 the standard isobologram methodology (50% isodose) was employed. The schedule-dependent interactions of paclitaxel and cisplatin were classified as synergistic, additive or antagonistic [19]. In a second protocol, 10^6 cells of the gastric and ovarian cancer cell lines were seeded in 25-cm^2 plastic flasks, allowed to grow for 48 h and then incubated for 2 h either with 50% of the IC_{50} for cisplatin or drug-free medium. Then the cells were seeded in 96-well microtitre plates, exposed 24 h later to appropriate concentrations of paclitaxel for 2 h and measured as described above. The cell line HM2 was also pretreated with cisplatin 48 and 72 h before paclitaxel application as indicated in the results.

Glutathione assay

One million cells were seeded in plastic flasks and incubated either for 2 h with cisplatin (50% IC_{50}) or for 48 h with 5–40 μM L-BSO. The cells were trypsinised, washed twice with ice-cold PBS, lysed by sonication and centrifuged at 120000 *g* for 20 min at 2°C and vacuum. The intracellular concentration of glutathione was determined in the supernatant by the glutathione reductase recycling method of Griffith and associates [20]. L-BSO-pretreated cells were measured immediately after L-BSO incubation and cisplatin-pretreated cells were measured 24 and 48 h after cisplatin application, as described above. All experiments were performed in triplicate.

[^{3}H]Paclitaxel uptake and retention

Exponentially growing cells of the cell line HM2 were seeded in plastic flasks and incubated for 2 h either with 50% of the IC_{50} for cisplatin or drug-free medium. Twenty-four hours later cells were trypsinised, washed twice with PBS and counted. The [^{3}H]paclitaxel uptake was determined by exposing 10^6 cells for 2 h–50 nM (0.95 μCi) [^{3}H]paclitaxel and 150 nM unlabelled paclitaxel (200 nM final concentration) at 37°C. The [^{3}H]paclitaxel uptake was measured after 15, 30, 60 and 120 min. For the assessment of radioactivity, cells were centrifuged at 2000 U/min, washed three times with ice-cold PBS and lysed with 1 N NaOH for 24 h. The lysates were collected and counted in a liquid scintillation counter. Results are expressed as pmol paclitaxel (total concentration)/10^6 cells. For the measurement of [^{3}H]paclitaxel retention, the cells were centrifuged, washed with ice-cold PBS and resuspended in L-15 medium at 37°C. Samples were taken after 15, 30, 60 and 120 min as described above. All experiments were performed in triplicate.

DNA flow cytometry

To assess changes in the cell cycle distribution, flow cytometry analysis was performed 24 and 48 h after cisplatin and paclitaxel application. Fixed cells were incubated in a DNA staining solution containing propidium iodide (50 μg/ml) and RNase (Type III-A, bovine pancreas, 4 KU/ml) and kept cold and dark for at least 30 min until flow cytometry analysis was carried out [21]. Cells were analysed in a Coulter flow cytometer equipped with an argon laser (488 nm) (Coulter Electronics, Hialeah, Florida, U.S.A.), and data were registered and stored in list mode. Debris and damaged cells were excluded by gating on a forward and side scatter dot plot or on a DNA histogram. DNA was recorded in the FL3 channel (635 nm) using linear amplification. Data obtained were evaluated with the Multicycle software (Phoenix Flow Systems, San Diego, California, U.S.A.).

Statistical analysis

The differences between the mean values were analysed for significance using the unpaired two-tailed Student's *t*-test for independent samples; P values <0.05 were considered to be statistically significant.

RESULTS

The IC_{50} values of cisplatin and paclitaxel for the cell lines HM2, HM51, EOV1 and EOV2 are shown in Table 1.

Table 1. IC_{50} values for cisplatin and paclitaxel (2-h exposure)

Cell line	IC_{50} (μM) Cisplatin	Paclitaxel
HM2	133	0.22
HM51	27	0.45
EOV1	13	0.06
EOV2	10	0.14

The results are presented as the mean values from three independent experiments.

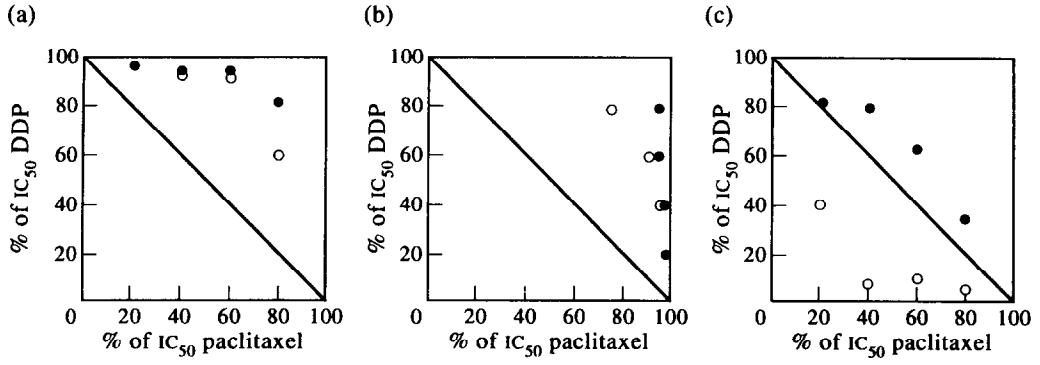

Figure 1. Isobologram analysis (50% isodose) of paclitaxel and cisplatin given in different schedules in cell lines HM2 (closed circles) and HM51 (open circles). (a) Cisplatin and paclitaxel simultaneously for 2 h. (b) Cisplatin (2 h) 24 h prior to paclitaxel. (c) Paclitaxel (2 h) 24 h prior to cisplatin.

The isobologram analysis of the schedule-dependent interactions between paclitaxel and cisplatin in the gastric carcinoma cell lines HM2 and HM51 are displayed in Figure 1. The simultaneous application of paclitaxel and cisplatin resulted in a marked antagonism in both cell lines (Figure 1a). This antagonism was also seen when cisplatin was given first followed 24 h later by paclitaxel (Figure 1b). In contrast, additive or synergistic interactions were observed in the cell lines HM2 and HM51 when paclitaxel was applied 24 h prior to cisplatin (Figure 1c). To further assess the magnitude and duration of the cisplatin-induced protection against paclitaxel, we incubated the cells with a fixed concentration of cisplatin (50% of the IC_{50} for each cell line) at various times before the paclitaxel application. The results are shown in Table 2. For all cell lines, a pronounced increase in the concentration of paclitaxel needed to inhibit cell growth was seen 24 h after cisplatin exposure. Furthermore, this protection against paclitaxel cytotoxicity was demonstrated for up to 72 h after cisplatin exposure in the cell line HM2 (Figure 2).

In order to explain the marked schedule-dependent antagonism between cisplatin and paclitaxel, the effect of cisplatin on the cellular accumulation of [^{3}H]-labelled paclitaxel was measured. Figure 3a depicts the paclitaxel uptake 24 h after a 2-h incubation with 50% of the IC_{50} of cisplatin in the gastric carcinoma cell line HM2. The results demonstrated that an exposure to cisplatin had no significant effect on the initial

Table 2. Cytotoxicity of paclitaxel after exposure to cisplatin or buthionine-sulphoximine (L-BSO)

	IC_{50} (μM) of paclitaxel				
	No exposure	Cisplatin*	L-BSO 5 μM	L-BSO 10 μM	L-BSO 20 μM
HM2	0.22	>100	0.18	0.19	0.25
HM51	0.45	5.5	0.23	0.25	0.38
EOV1	0.06	>33.3		n.d.	
EOV2	0.14	10.0		n.d.	

* Cisplatin was given at a concentration corresponding to 50% of the individual IC_{50} for each cell line. Cells were preincubated with cisplatin or L-BSO as described in Materials and Methods. The results are presented as the mean values from three independent experiments. n.d., not determined.

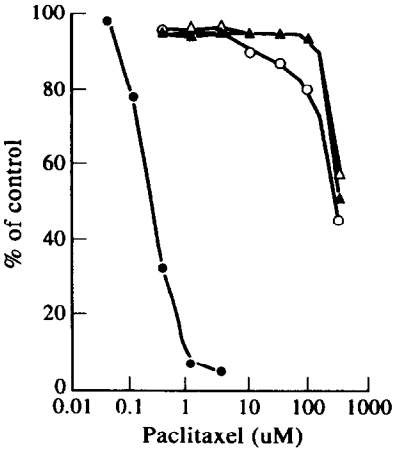

Figure 2. Cytotoxicity of paclitaxel in cell line HM2. Cells were pretreated with cisplatin (50% of IC_{50}) for 2 h (open circles), 48 h (closed triangles) or 72 h (open triangles) prior to paclitaxel or not pretreated (closed circles).

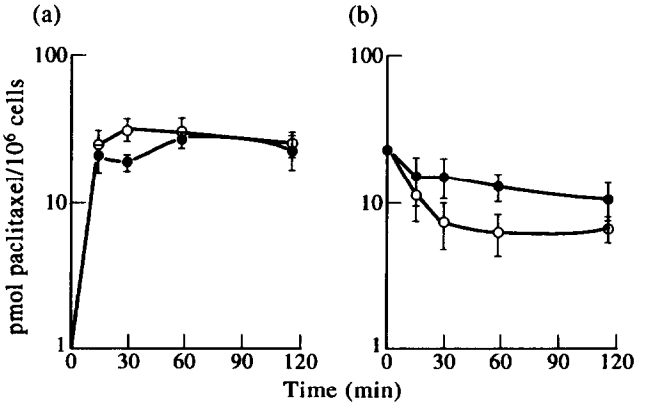

Figure 3. (a) Uptake of [^{3}H]paclitaxel in cell line HM2. Cells were pre-incubated for 2 h with 50% of IC_{50} of cisplatin and exposed 24 h later to 50 nM [^{3}H]paclitaxel/150 nM paclitaxel (open circles: pretreated with cisplatin; closed circles: not pretreated). The difference was significant at 30 min. (b) Retention of [^{3}H]paclitaxel in HM2. Cells were washed and resuspended in drug-free medium (open circles: pretreated with cisplatin; closed circles: not pretreated). The differences were significant at 30 and 60 min.

Table 3. Intracellular glutathione (GSH) content after cisplatin and L-BSO treatment

	Control	Cisplatin* 24 h	Cisplatin* 48 h	L-BSO 5 μM	L-BSO 10 μM	L-BSO 20 μM
HM2	35.0	31.5	25.3†	27.0	18.5†	16.0†
	(5.0)	(2.6)	(4.6)	(1.4)	(3.5)	(1.4)
HM51	30.2	34.5	23.0‡	25.0	22.5‡	14.0‡
	(2.8)	(2.1)	(2.6)	(4.2)	(0.7)	(5.6)

* Cisplatin was given at a concentration corresponding to 50% of the individual IC_{50} for each cell line. † $P < 0.05$. ‡ $P < 0.01$. Glutathione content expressed as nmol GSH/mg soluble protein. Cells were pre-incubated with cisplatin or L-BSO as described in Materials and Methods. The results are presented as the mean values from three independent experiments, standard deviations are shown in parentheses.

paclitaxel uptake. However, a significantly reduced retention of paclitaxel was shown for pretreated cells compared to those not exposed to cisplatin (Figure 3b).

Since it has been demonstrated that a depletion of the cellular glutathione concentration can result in a marked reduction of the activity of paclitaxel, the effects of a cisplatin-mediated depletion of cellular glutathione content on the cytotoxicity of paclitaxel were measured. A significant time-dependent depletion of cellular glutathione content was observed in both gastric cancer cell lines 24 and 48 h after a cisplatin exposure. However, when a comparable depletion of cellular glutathione was produced by BSO (Table 3), there was no effect on the cytotoxicity of paclitaxel, indicating that the changes in glutathione content were not responsible for the observed antagonism (Table 2).

The influence of both drugs on the cell cycle distribution is shown in Figure 4. As expected, paclitaxel produced an accumulation of cells in G_2M phase for up to 48 h. Twenty-four hours after treatment with cisplatin, the majority of cells exhibited an S phase DNA content, at 48 h most of the cells were found in G_2M phase.

DISCUSSION

The recently demonstrated clinical activity of paclitaxel against a variety of malignancies, especially ovarian and breast cancer, is encouraging [2, 4]. Clinical trials of the combination of paclitaxel and cisplatin have been initiated in ovarian cancer using empirically derived schedules [5]. Since *in vitro* data are suggesting a schedule-dependent antagonism between cisplatin and paclitaxel [15, 16], we investigated different sequences of paclitaxel and cisplatin in human gastric and ovarian cancer cell lines in order to develop guidelines for an optimal clinical scheduling of both drugs. The sequence of paclitaxel followed 24 h later by cisplatin resulted in additive or even synergistic interactions in the gastric carcinoma cell lines HM2 and HM51. This is in accordance with reported data showing that paclitaxel and cisplatin act synergistically when a 24-h exposure to paclitaxel is followed 24 h later by a 1-h exposure to cisplatin in the ovarian cancer cell lines A2780 and CP70 [22]. This synergism has been associated with a paclitaxel-induced inhibition of the platinum–DNA adduct repair, whereas the cellular accumulation of cisplatin was not effected by paclitaxel [22, 23]. Jekunen and colleagues recently reported synergistic interactions when paclitaxel preceded cisplatin in the ovarian cancer cell line 2008 and the 11-fold DDP-resistant subline 2008/C13*. There was no evidence for paclitaxel-induced alterations of the platinum uptake, the cellular concentration of glutathione or metallothionein content and the permeability of the plasma membrane, which could explain the observed synergism for this schedule [16].

However, when paclitaxel and cisplatin were administered concurrently or when cisplatin was given prior to paclitaxel, we found a strong antagonistic interaction in both gastric carcinoma cell lines. This cisplatin-induced protection against the cytotoxicity of paclitaxel was also seen for the ovarian carcinoma cell lines EOV1 and EOV2, and was evident for up to 72 h after cisplatin exposure in the gastric cancer cell line HM2. In the above-mentioned paper by Jekunen and associates, antagonistic interactions were observed when cisplatin was administered prior to a 20-h exposure to paclitaxel or when both drugs were given concurrently for 1 h [16]. Similar data for the sequence in which cisplatin is followed by paclitaxel were also demonstrated in the leukaemic cell line L1210 [15].

The reasons for this schedule-dependent antagonism have not yet been elucidated. It has been shown recently that a depletion of the cellular glutathione content by L-BSO, a specific inhibitor of the enzyme gamma-glutamyl cysteine synthetase [24], which is required for glutathione synthesis, produced a marked *in vitro*

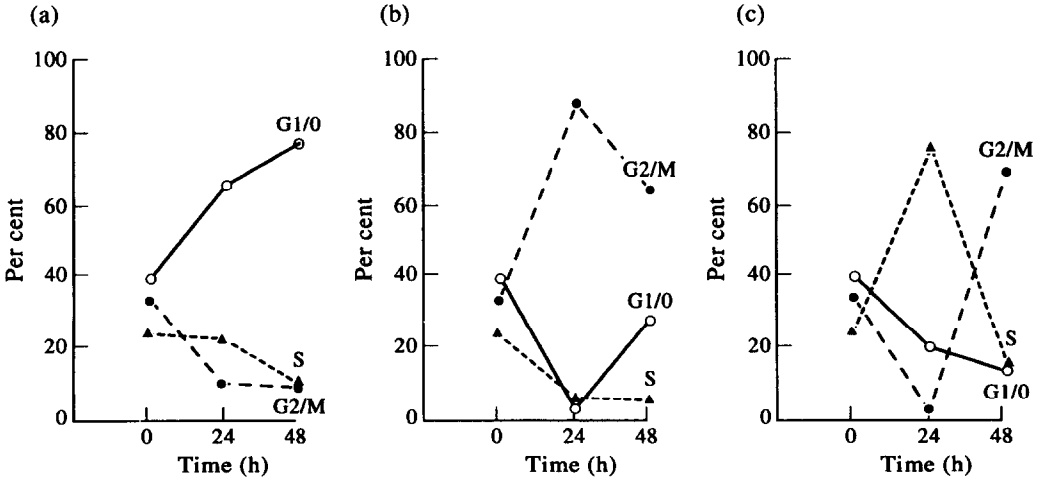

Figure 4. Cell cycle distribution in cell line HM2 24 and 48 h after a 2-h exposure to (a) drug-free medium, (b) paclitaxel (50% of IC_{50}) or (c) cisplatin (50% of IC_{50}).

resistance to paclitaxel. This protection against the cytotoxicity of paclitaxel might be due to the oxidation of tubulin sulphydryl groups, resulting in a disaggregation of the tubulin polymers. There was no evidence that the changes of cellular glutathione content by L-BSO did effect the cellular binding of ^{3}H-labelled paclitaxel [10]. In the present study, both gastric carcinoma cell lines showed a time-dependent significant decrease of cellular glutathione after an exposure to cisplatin. However, when a comparable reduction of glutathione content was produced by L-BSO, no difference in the cytotoxicity to paclitaxel emerged, suggesting that the observed schedule-dependent antagonism was not caused by a depletion of cellular glutathione content.

Both drugs, cisplatin and paclitaxel, exhibited a pronounced influence on the cell cycle distribution which might be associated with the observed interactions. Paclitaxel produced a significant increase of the proportion of cells in the G_2M-phase, consistent with its major action as a mitosis inhibitor [9]. Twenty-four hours after cisplatin exposure, we saw an accumulation of cells with an S-phase DNA content followed at 48 h by a synchronised traverse to G_2M-phase, indicating a delay of the transition through the S-phase as has also been reported by others [25–27]. It may be speculated that this could contribute to the observed antagonism for the sequence of cisplatin followed by paclitaxel since such a blockade at the S–G_2M boundary would prevent cells from entering the paclitaxel-sensitive M-phase [9]. However, these changes in the cell cycle transition are not sufficient to explain the antagonism which was seen with concurrent short-term (2 h) exposure to both drugs.

In the present study, cisplatin had no effect on the cellular uptake of ^{3}H-labelled paclitaxel, but we found a significantly increased retention of paclitaxel in non-pretreated cells compared to those exposed prior to cisplatin. This decreased retention of paclitaxel may be related to cisplatin-induced alterations in the cellular specific and non-specific binding sites for paclitaxel. Currently, there are no data which indicate that cisplatin will induce perturbations of the cellular membrane leading to an increased efflux of paclitaxel. Whether these changes in the cellular pharmacokinetics are solely responsible for the altered activity of paclitaxel after cisplatin exposure remains to be determined. Interestingly, clinical data have shown that *in vivo* prior exposure to cisplatin decreases the total body clearance of paclitaxel, which was associated with more profound myelosuppression for this schedule [28]. The biochemical basis of this interaction between both drugs is, as yet, unclear. It has been speculated that cisplatin induces an inhibition of the paclitaxel metabolising enzymes, the cytochrome P_{450} pathway, which might be responsible for the observed changes in paclitaxel pharmacokinetics. However, we found no differences in the enzyme activities of the NADPH-dependent cytochrome-C reductases in the presence or absence of a prior exposure to cisplatin in either gastric carcinoma cell lines (data not shown).

In conclusion, the present study demonstrated a schedule-dependent marked *in vitro* antagonism between paclitaxel and cisplatin in ovarian and gastric carcinoma cell lines. This antagonism was seen for the sequence of cisplatin followed by paclitaxel or when both drugs were given simultaneously. In contrast, the schedule of paclitaxel followed by cisplatin resulted in additive or synergistic interactions. The observed antagonism might be related to cisplatin-induced cell cycle effects or alterations in the specific or non-specific binding sites of paclitaxel.

Our *in vitro* data suggest that clinical protocols using the sequence of cisplatin followed by paclitaxel could have reduced therapeutic efficacy and should therefore be avoided. Further studies will have to clarify the exact biological and biochemical mechanisms which are responsible for these significant schedule-dependent interactions.

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Insulin Sensitivity, Hormonal Levels and Skeletal Muscle Protein Metabolism in Tumour-bearing Exercising Rats*

P. Daneryd, L. Hafström, E. Svanberg and I. Karlberg

We have previously shown that spontaneous physical exercise can delay onset of experimental anorexia and cachexia, and retard tumour growth; we now report the effects on insulin sensitivity, hormonal levels and skeletal muscle protein metabolism. Insulin sensitivity determined with a euglycaemic hyperinsulinaemic clamp revealed a normalised glucose disposal rate in tumour-bearing exercising (TBE) versus sedentary (TBS) animals (TBE 15.55 ± 2.71 versus TBS 2.47 ± 2.12 mg/kg/min; $P < 0.05$). Both TBE and TBS animals had decreased levels of corticosterone during the clamp. Serum levels of insulin during tumour progression were unaffected by exercise, but the insulin : glucagon ratio increased and the progressive decrease in rT_3 was attenuated. The concentration of glucagon decreased in both tumour-bearing groups during the experiment, while TBE animals showed a relative reduction in corticosterone. Capacity for skeletal muscle protein synthesis, expressed as RNA : protein ratio, was normalised in TBE animals in two tumour protocols (TBE 5.9 ± 0.6 versus TBS 4.7 ± 0.3; TBE 2.9 ± 0.4 versus TBS 1.8 ± 0.2; $P < 0.05$, respectively). Incorporation rate of ^{14}C-phenylalanine into skeletal muscle protein was increased in the TBE group *in vitro* and *in vivo*. In the postexercise period, protein degradation evaluated by tyrosine release *in vitro* was increased, but decreased over time. This study has confirmed a positive skeletal muscle protein balance in exercising tumour-bearing animals, partly explained by the increased insulin sensitivity. This conclusion was further supported by the less catabolic pattern indicated by hormonal levels.

Key words: cancer, exercise, insulin resistance, insulin, glucagon, thyroid hormones, corticosterone, proteins
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INTRODUCTION

WASTING OF skeletal muscle tissue is a predominant feature of progressive malignant disease [1, 2]. This is the combined effects of inanition, altered protein metabolism and inactivity [1]. As to protein metabolism in skeletal muscle, there is evidence indicating a decreased synthesis rate [3, 4], and the degradation rate has been shown to be either unaffected [5] or increased [6]. This altered metabolic state contrasts with reports on increased whole body protein turnover, previously described in viscera and the immune system [1, 7, 8].

As physical exercise is a powerful stimulus for an anabolic state in skeletal muscle, successful attempts to counteract the catabolic state in cancer have been made with forced physical exercise in an experimental model [9]. In an experimental model with spontaneous physical exercise, we have recently reported a better preserved body composition in the tumour-bearing exercising host and an increased capacity for protein synthesis in skeletal muscle [10]. We have also found a decrease in tumour weight, despite an early increase in food intake in the tumour-bearing exercising animals. These tumour-bearing animals in