Schedule-Dependent Interaction between Vinblastine and Cisplatin in Ehrlich Ascites Tumors in Mice

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Received January 25, 2002; accepted March 4, 2002 This article is available online at http://jpet.aspetjournals.org

ABSTRACT

Information on the in vivo antitumor efficiency of the combination of Vinca alkaloids in animal tumor models, especially vinblastine (VLB) with cisplatin [cis-diaminedichloroplatinum(II); CDDP] is very limited. Therefore, the aim of our study was to explore whether antitumor schedule dependence exists for the combination of CDDP and VLB on i.p. Ehrlich ascites tumors in mice. Animals were treated 3 days after tumor transplantation with VLB (0.006 mg/kg) or CDDP (0.05 mg/kg) alone, VLB followed by CDDP, and CDDP followed by VLB. The time interval between i.p. injections of the drugs was 24 h. Cell number was measured by counting viable cells using the trypan blue exclusion assay, cell platinum content by electrothermal atomic absorption spectrometry, DNA distribution pattern using flow cytometry, apoptosis by flow-cytometric terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, and cell morphology. Combination of CDDP and VLB resulted in additive interaction when VLB preceded CDDP as determined from cell survival data 24 h after completion of the therapy and in increased platinum content (two times) compared with the same combination in a reverse schedule (CDDP given before VLB), which resulted in antagonism. None of the treatment combinations induced apoptosis. We propose that the observed increase in antitumor effectiveness is mainly due to higher platinum accumulation in tumor cells, which we unambiguously demonstrated by measurement of platinum content in the tumor cells, leading to increased cytotoxicity as well as to cell cycle-dependent effects of VLB and CDDP.

Combined chemotherapy schedules including cisplatin [cis-diaminedichloroplatinum(II); CDDP] and various tubulin-binding agents are well established and are used for treatment of various malignancies, such as testicular cancer, lung cancer, and salivary gland, melanoma, and breast carcinomas (Auersperg et al., 1977; Mulder et al., 1982; Fuks et al., 1983; Newman et al., 1983; Huberman et al., 1986; Blumenreich et al., 1987; Creagan et al., 1987; Fraschini et al., 1988; Stefennelli et al., 1988; Haskell, 1990; Kosmidis et al., 1994; Eton et al., 1999; Airoldi et al., 2001; De Pas et al., 2001). Design of currently used combined chemotherapeutic schedules is based on data derived from preclinical studies, and Phase I and II clinical studies. However, little attention is paid to timing of drugs or possible interaction of drugs in a particular combined schedule. Both these factors could be crucial for the clinical effect of chemotherapy. The increasing knowledge and understanding of molecular mechanisms of drug-induced cytotoxicity forms the basis for rational planning of clinical chemotherapy. Several in vitro and some in vivo studies stressed the importance of the appropriate scheduling of the drugs to achieve pronounced antitumor effectiveness (Lee et al., 1989; Rowinsky et al., 1993; Milross et al., 1995; Vanhoefer et al., 1995; Barret et al., 2000). For example, it was shown in vitro that paclitaxel (Taxol) has to precede CDDP to achieve additive interaction, whereas CDDP given before paclitaxel leads to antagonism of the cytotoxic effects. In the case of vincristine combined with CDDP under in vitro conditions, the same interaction as for paclitaxel was demonstrated; CDDP has to follow vincristine administration to obtain additive cytotoxic effect (Lee et al., 1989; Rowinsky et al., 1993; Vanhoefer et al., 1995; Barret et al., 2000).

There were several hypotheses regarding the possible mechanisms for the observed effect, the most promising being cell cycle-dependent effect (Rowinsky et al., 1993; Milross et al., 1995). In addition, we have previously demonstrated that VLB affects cell membrane fluidity and, therefore, could increase cellular uptake of poorly or nonpermeant drugs, such as CDDP and bleomycin (Sentjurc et al., 1990; Sersa et al., 1994; Cemazar et al., 1997, 2000). Furthermore, we showed that combining VLB with bleomycin, a nonpermeant drug with very high intrinsic cytotoxicity, resulted in better antitumor effectiveness when VLB preceded bleomycin com-
pared with the combination bleomycin followed by VLB or simultaneous administration (Cemazar et al., 1997, 2000).

Information on the in vivo antitumor efficiency of the combination of Vinca alkaloids in animal tumor models, especially VLB with CDDP, is very limited. Therefore, the aim of our study was to explore whether schedule dependence exists for the combination of CDDP and VLB in vivo. For this purpose, we determined platinum content, cell survival, changes in cell morphology, apoptosis, and cell cycle distribution of i.p. Ehrlich ascites tumors (EATs) in mice 24 h after completion of treatment with VLB, CDDP, and the combination of these drugs.

Materials and Methods

Drug Formulation. VLB (vinblastine sulfate; Lilly France S.A., Fegersheim, France) was dissolved in 0.9% NaCl solution at a concentration of 2.5 μg/ml. CDDP (Platamine; Pharmacia & Upjohn S.p.a., Milan, Italy) was dissolved in distilled water at a concentration of 500 μg/ml. Each animal was injected i.p. with an adjusted volume of drug solution to achieve a VLB dose of 62.5 μg/kg and different CDDP doses ranging from 0.01 to 4 mg/kg. This low VLB dose was selected according to our previous studies, in which we demonstrated that this dose significantly affected cell membrane fluidity with minimal effect on cell survival (Sersa et al., 1994; Cemazar et al., 1997, 2000). According to Freireich et al. (1966), the corresponding doses for VLB in humans would be 0.2 mg/m² (0.005 mg/kg) and for CDDP from 0.03 to 12.3 mg/m² (0.0008 to 0.33 mg/kg). All doses used are far below the maximal tolerated dose and also below doses used in clinical chemotherapy schedules.

Animals. Inbred CBA mice were purchased from the Institute of Pathology, Medical Faculty Ljubljana (Slovenia). Mice were maintained at a constant room temperature (22°C) and a natural day/night light cycle in a conventional animal colony. Before experiments, mice were subjected to an adaptation period of at least 10 days. Mice of both sexes, in good condition, weighing 22 to 30 g, were included in the experiments. Animal studies were carried out according to the guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia, and in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD).

Tumor Model. Intraperitoneal Ehrlich ascites carcinoma syngeneic to CBA mice was used in the study. The tumor was maintained i.p. as ascites by serial transplantation once a week. For transplantation of i.p. tumors, tumor cells from the donor mouse were harvested by peritoneal lavage with 4 ml of 0.9% NaCl solution, washed, and resuspended at a concentration of 3 × 10⁶ cells/ml. Tumors were transplanted by i.p. injection of 1.5 × 10⁶ viable EAT cells in 0.5 ml of 0.9% NaCl solution. Cell viability, determined by trypsin dye exclusion test, was ≥95%.

Treatment Protocol. Three days after tumor transplantation, animals were randomly allocated into the following groups: control (i.p. treated with 0.9% NaCl solution), VLB alone, CDDP alone, VLB followed by CDDP, and CDDP followed by VLB. Time interval between i.p. injection of the first and second drug was 24 h. In the case of monochemotherapy, 0.9% NaCl was injected 24 h after the first drug (Table 1). Mice were killed 24 h after the completion of therapy by cervical dislocation. Each experimental group consisted of at least six mice, and the data were pooled from two to three independent experiments.

| TABLE 1 |
| Treatment schedule |

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Injection of t.c.</td>
<td>Saline</td>
<td>Saline</td>
<td>Harvesting</td>
</tr>
<tr>
<td>VLB (24 h)</td>
<td>Injection of t.c.</td>
<td>VLB</td>
<td>Harvesting</td>
<td></td>
</tr>
<tr>
<td>VLB (48 h)</td>
<td>Injection of t.c.</td>
<td>VLB</td>
<td>Harvesting</td>
<td></td>
</tr>
<tr>
<td>CDDP (24 h)</td>
<td>Injection of t.c.</td>
<td>CDDP</td>
<td>Harvesting</td>
<td></td>
</tr>
<tr>
<td>CDDP (48 h)</td>
<td>Injection of t.c.</td>
<td>CDDP</td>
<td>Saline</td>
<td>Harvesting</td>
</tr>
<tr>
<td>VLB-24 h-CDDP</td>
<td>Injection of t.c.</td>
<td>VLB</td>
<td>CDDP</td>
<td>Harvesting</td>
</tr>
<tr>
<td>CDDP-24 h-VLB</td>
<td>Injection of t.c.</td>
<td>CDDP</td>
<td>VLB</td>
<td>Harvesting</td>
</tr>
</tbody>
</table>

t.c., tumor cells.
eter (Hitachi, Tokyo, Japan), adjusted to a wavelength of 265.9 nm. In our previous studies, it was demonstrated that total platinum content in the washed cells isolated from solid tumors correlated well with the amount of platinum bound to DNA, which is responsible for CDDP cytotoxicity. In addition, the reproducibility of measurements, linearity of calibration curve, and low limit of detection demonstrated in previous studies proved that this method is sensitive and accurate and therefore suitable for determination of platinum content in the tumor cells (Milacic et al., 1997; Cemazar et al., 1999; Scancar et al., 2000).

**Statistical Analysis.** Data are presented as arithmetic means ± S.E.M. The significance of the effect was determined using a post hoc Tukey's t test after one-way analysis of variance was performed; levels of less than 0.05 were taken as indicative of significant differences. Spector’s formula was used to assess the combined effects (additivity, synergism, and antagonism) of two chemotherapeutic drugs with independent mechanisms (Spector et al., 1982).

**Results**

**Effect of CDDP on Number of i.p. EAT Cells.** Cytotoxicity of CDDP to EAT cells in vivo was demonstrated in a dose-dependent manner for all of the doses tested (Fig. 1). For up to a 0.05-mg/kg dose there was no significant decrease in cell number (Fig. 1). The highest dose tested (4 mg/kg) reduced cell number to approximately 20% of the control. A relatively noncytotoxic CDDP dose (0.05 mg/kg), which reduced cell number to 85% of control, was chosen for subsequent experiments combining VLB and CDDP, to detect possible potentiation of CDDP cytotoxicity in combination with VLB; thus, an evident cell kill caused by the CDDP action alone was not desirable.

**Effect of VLB or CDDP and Their Combinations on Number of i.p. EAT Cells.** Cytotoxic effects of VLB or CDDP as single treatments and in the combined treatment schedules on i.p. EAT cells were determined 24 h after completion of therapy. VLB as a single treatment, in contrast to CDDP as a single treatment, significantly reduced the number of cells in peritoneal lavage compared with control, saline-treated animals (Fig. 2). The interaction between the drugs was antagonistic in the combination in which CDDP preceded VLB. Moreover, in this particular combination group, the number of cells in ascites was higher compared with treatments with single drugs. In contrast, VLB followed by CDDP resulted in a greatly reduced number of cells in ascites and resulted in additive interaction between the two drugs (Fig. 2). The effects of VLB and CDDP on the number of cells were not sex-related, and no drug-related toxicity was observed regardless of the drug sequence used in the time span of the experiment.

**Effect of CDDP and Combination of VLB and CDDP on Platinum Content in i.p. EAT Cells.** To obtain adequate control values for total platinum content in the cells after combined VLB and CDDP treatment, platinum content was determined in cells 24 h and 48 h after the treatment of animals with CDDP. Platinum content in the cells 24 h after the CDDP treatment served as a control for the combination of VLB followed by CDDP, and after 48 h for the combination of CDDP followed by VLB. There was no significant difference in total platinum content in CDDP-treated animals compared with animals receiving the combination in which CDDP preceded VLB (Fig. 3). In contrast, an approximately 2 times higher total platinum content was determined in the cells treated with the reverse combination, where VLB was followed by CDDP.
Effect of CDDP or VLB and Their Combinations on Cell Cycle Phase Distribution of i.p. EAT Cells. Both CDDP and VLB caused marked changes in cell cycle distribution 24 h after the treatment (at the time of the second drug administration in the combination groups) compared with controls (Fig. 4). VLB increased the number of cells with DNA values greater than in the G2M compartment of cell cycle. Furthermore, no distinct tumor peaks were observed in the DNA histogram and a lot of signals with DNA values lower than in G1 phase, which was most probably due to the formation of micronuclei as a consequence of impaired mitoses. CDDP reduced number of cells in G1 phase of cell cycle, slowed down the passage of cells through S phase with a block in late S phase. An increased number of cells in G2M compartment was observed compared with control (Fig. 4). The combination of CDDP followed by VLB had an even greater effect on cell cycle distribution compared with treatment with single drugs. The DNA histogram showed an increased number of cells in the G2M compartment, whereas no distinct G1 peak was observed (Fig. 4). In the DNA histogram of cells treated with the combination VLB followed by CDDP, no distinct peaks were observed; cells were distributed almost evenly through different DNA values (Fig. 4).

Effect of CDDP or VLB on Cell Morphology and Apoptosis of i.p. EAT Cells. Treatment with VLB resulted in about 50% of multinucleated cells and cells with micronuclei as a consequence of the impaired mitoses (Fig. 5), whereas after treatment with CDDP, enlarged cells with enlarged nuclei compared with the untreated cells were found (Fig. 5). However, in the cytological smears after CDDP, the number of mitoses was comparable with that of the controls. After treatment with CDDP followed by VLB, more than 50% of cells were in mitoses. Besides, several bi- and multinucleated cells and fragments of cytoplasm were observed (Fig. 5), whereas after treatment with the reverse combination, VLB followed by CDDP, various degenerative changes, such as vacuolization of the cytoplasm, potocytosis, and micronuclei, in the cells were observed (Fig. 5). Neither the results from the cell morphology and DNA histograms, nor the APO-BRDU method showed detectable apoptosis after treatment with VLB or CDDP alone or as combinations. The APO-BRDU method showed that the number of cells accepted as positively labeled varied from 0.1% in the control group to 0.5% in the group CDDP followed by VLB. However, this difference was not statistically significant (Table 2).

Discussion

Our study shows that the combination of CDDP and VLB results in additive interaction when VLB precedes CDDP and that the same combination in a reverse schedule (CDDP given before VLB) results in antagonism, as determined from cell number data 24 h after therapy. The additive effect of the combination in which VLB preceded CDDP could be explained by increased CDDP concentration (2-fold) within the tumor cells, which consequently leads to increased cytotoxicity, in comparison with the schedule of CDDP alone or CDDP followed by VLB. The antagonism in the combination CDDP followed by VLB was determined 24 h after completion of the therapy from cell number data. Although, the number of cells in ascites was in the range of the control, untreated animals, these cells had degenerative changes, and therefore, it can be

Fig. 4. Representative DNA histograms of EAT cells after treatment with CDDP or VLB alone and a combination of both drugs.
speculated that they would die afterward in the following cell cycles.

CDDP combined with several tubulin-binding agents is used in chemotherapeutic schedules for treatment of many different types of cancer, such as non-small-cell-lung cancer (Fuks et al., 1983; Blumenreich et al., 1987; DePas et al., 2001), urothelial cancer (Dodd et al., 1999), melanoma (Mulder et al., 1982), breast carcinoma (Fraschini et al., 1988), and small cell carcinoma of the lung (Newman et al., 1983). In most of the clinical reports on combined chemotherapeutic schedules in solid tumors, the exact data on sequence of particular drugs are not given. Usually, there are only data on the type of administration; i.e., continuous infusion or i.v. push, and the day of administration. Most of these clinical trials showed that the combination of Vinca alkaloids and CDDP did not produce a significant response rate or prolonged survival, and in many cases, increased toxicity was observed (Mulder et al., 1982; Fuks et al., 1983; Newman et al., 1983; Blumenreich et al., 1987; Fraschini et al., 1988). Two recent reports on salivary gland malignancies (Airoldi et al., 2001) and non-small-cell lung cancer (DePas et al., 2001) showed positive results of this schedule.

The exact mechanism responsible for the observed schedule-dependent effects of CDDP and VLB is still unknown. Our results support several recent in vitro studies showing the schedule-dependent effect of CDDP with other tubulin-binding agents (Lee et al., 1989; Rowinsky et al., 1993; Vanhoever et al., 1995; Barret et al., 2000). Rowinsky et al. (1993) showed on leukemia L1210 cells that cytotoxicity resulting from the combination of paclitaxel and CDDP was significantly increased over that obtained with CDDP alone, only when CDDP was administered after paclitaxel. They demonstrated that paclitaxel or vincristine did not modulate intracellular thiol content, inhibit repair of critical CDDP-induced DNA lesions, or increase the intracellular accumulation of CDDP. Therefore, they suggested that the interaction of paclitaxel or vincristine with CDDP in vitro was due to cell cycle-dependent effects (Rowinsky et al., 1993). In contrast, Christen et al. (1993) found that in 2008 human ovarian carcinoma cells, pretreatment with paclitaxel increased accumulation of CDDP, which was ascribed to paclitaxel-induced polymerization of tubulin and, consequently, cell membrane alteration (Christen et al., 1993).

Already, in 1990, Sentjurc et al. demonstrated changes of cell membrane fluidity of soft tissue sarcoma tumors in patients. In our early studies, we demonstrated an increased accumulation of labeled bleomycin after pretreatment with low doses of VLB (Auersperg et al., 1975); an effect of VLB on cell kinetics and cell membrane permeability was the possible explanation. Subsequent work by our group proved increased cell membrane fluidity after VLB in human and experimental tumors (Sentjurc et al., 1990; Sersa et al., 1994). Furthermore, on the basis of these studies, we showed that pretreatment with VLB increased cytotoxicity of bleomycin and prolonged survival of animals treated with this schedule. The treatment of animals with the reverse order of drugs had less pronounced effect on survival of both cell and animals (Cemazar et al., 2000). In the present study, we demonstrated that pretreatment with VLB, in fact, increased accumulation of the subsequently injected drug (in our case, CDDP). This confirms our hypothesis that increased mem-

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>% Apoptotic Cells</th>
<th>AM ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>0.1 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>VLB (24 h)</td>
<td>3</td>
<td>0.25 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>CDDP (24 h)</td>
<td>3</td>
<td>0.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>VLB-24 h-CDDP</td>
<td>3</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>CDDP-24 h-VLB</td>
<td>3</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

AM, arithmetic mean.

Fig. 5. Morphological appearance of EAT cells after treatment with CDDP or VLB alone and a combination of both drugs (60×).
brane fluidity, caused by VLB, affects cell uptake of other nonpermeant or poorly permeant drugs. Furthermore, since the major target of CDDP is DNA, an increased amount of platinum in the cells, which consequently results in an increased amount of platinum bound to DNA, may result in enhanced cytotoxicity of CDDP, as we demonstrated in this study by a reduced number of i.p. EAT cells 24 h after the treatment.

On solid tumors in vivo, the effect of combining paclitaxel and CDDP was also dependent on the sequence in which the drugs were administered (Milross et al., 1995). In that study, apoptotic cell death and tumor growth delay were used to determine the interaction between paclitaxel, a tubulin-binding agent, and CDDP. They found, in line with other in vitro (Lee et al., 1989; Rowinsky et al., 1993; Vanhoef et al., 1995) studies and our in vivo study, that the combination of a tubulin-binding agent (paclitaxel) preceding CDDP was more effective than the combination of the reverse order. It was demonstrated that apoptotic cell death after paclitaxel followed by CDDP could not explain more than additive effects, found by growth delay assay. It was suggested that the interaction should be attributed to the effects on cells that respond by reproductive death (Milross et al., 1995). In our study, using a 24-h time interval between the application of drugs, we did not observe in DNA histograms of tumor cells treated with different drug schedules a sub-G1 peak, a feature suggesting apoptotic cell death. In addition, measurement of apoptosis by the APO-BRDU method did not show an increased percentage of cells in apoptosis compared with the control, untreated group. The original level of apoptosis in a control group was very low (0.1%), and none of the treatment schemes increased the percentage of apoptotic cells significantly. The reason for that might be inappropriate timing of cell harvesting. Therefore, more detailed study should be employed to detect possible apoptotic cell death.

However, cell cycle-dependent effects of VLB and CDDP might significantly contribute to sequence-dependent antitumor efficiency. VLB and CDDP have different mechanisms of action and effects on cell cycle distribution. VLB interferes with polymerization of tubulin, a protein that is involved in formation of mitotic spindle microtubules and is also an important component of cytoskeleton (Jordan et al., 1991; Pauwels et al., 1995). It was reported that HeLa S3 cells in vitro were the most sensitive to VLB in late S phase, close to the S-G2 boundary (Chirife and Studzinski, 1978). In accordance with its effect on microtubules of mitotic spindle, VLB blocks the cells in the metaphase of mitosis and thus acts as a cell-synchronizing agent. In addition, Madoc-Jones and Mauro (1972) reported on the lethal action of VLB in interphase at concentrations higher than those producing mitotic arrest. Therefore, the cell-kinetic effect of VLB seems to be dose-dependent. Higher doses prolong the transition of cells through S phase, whereas lower doses, as used in the present study, block cells in mitosis, which, when prolonged, causes cell death. In addition, an accumulation of cells with DNA values greater than those in the G1M compartment and lower than those in G1 phase were also observed and interpreted as a consequence of impaired mitoses (endoreduplication). Cells are the most sensitive to CDDP in the G1 compartment, and CDDP delays cell cycle traverse in S phase and reversibly blocks cells in G2 phase (Mastbergen et al., 2000). Therefore, the observed cell morphology when CDDP was given before VLB could be caused partly by the fact that cells treated with CDDP were in the most VLB-sensitive phase of the cell cycle. A reduced number of cells was not determined 24 h after this treatment; however, we can presume that these impaired cells would die later. This observation is in contrast to the study by Blagosklonny et al. (2000), who used low doses of doxorubicin, which predominantly induced G2 arrest and thus protected against cytotoxicity induced by the tubulin-binding agent VLB.

In conclusion, the results of the present study show that interaction between VLB and CDDP is schedule-dependent. As already shown for CDDP combined with paclitaxel, vinodesine, vinflunine, and vincristine, the present study demonstrates that VLB has to precede CDDP in the combined schedule to achieve additive interaction, as determined from cell number data 24 h after the therapy. We propose that the observed increase in antitumor effectiveness is mainly due to higher platinum accumulation in tumor cells and, consequently, increased cytotoxicity, which we unambiguously demonstrated by measurement of platinum content in the tumor cells. Therefore, rational scheduling of drugs in clinical chemotherapy protocols should be based on preclinical data demonstrating mechanisms of drug interaction, to obtain optimal therapeutic effect with minimal toxicity.

Acknowledgments

We thank Mira Lavrič, Karmen Zajc, Alenka Grošelj, Bragina Šturbej, and Marjana Matič for their excellent technical assistance.

References


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