Low-dose cisplatin protects human neuroblastoma SH-SY5Y cells from paclitaxel-induced apoptosis

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Abstract

Combined anticancer therapy using platinum compounds and antitubulins has increased the risk of neurotoxicity. However, the combination of low-dose cisplatin (CDDP) with toxic doses of paclitaxel significantly reduces cellular death in a human neuroblastoma SH-SY5Y cell line. To analyze the mechanisms of this protection, we evaluated various signaling molecules possibly involved in apoptosis and some relevant cell cycle regulatory proteins. CDDP does not interfere with the tubulin-stabilizing action of paclitaxel. The evaluation of molecular pathways involved in apoptosis indicates that the Bcl-2 but not the caspases may be involved in the CDDP protection of paclitaxel-induced apoptosis. The increase in p53 protein and its nuclear accumulation suggests a possible involvement of p53 in CDDP protection. The use of the chemical inhibitor of p53, pifithrin α, excluded this possibility. The study of cyclins and the flow cytometric analysis (fluorescence-activated cell sorting) suggest that CDDP exerts a protective action by blocking cells early in the cell cycle. The determination of the mitotic index indicates that CDDP prevents cells from reaching the mitosis. We concluded that low doses of CDDP are protective against toxic doses of paclitaxel and that the possible mechanism of this protection is that the CDDP prevents human neuroblastoma SH-SY5Y cells from achieving mitosis. [Mol Cancer Ther 2005;4(9):1439–47]

Introduction

Treatment of solid tumors has been improved in recent years by the use of platinum compounds and antitubulins.

These two groups of molecules are often used in combination to achieve better control over cancer cells (1). Both platinum compounds and antitubulins are toxic to the peripheral nervous system (2) and the use of these drugs in combination is expected to increase their peripheral neurotoxicity (3). McKeage et al. (4) have however reported that, in Wistar rats, paclitaxel and cisplatin antagonize each other’s neurotoxicity.

The most widely used platinum-derived drug is cisplatin (cis-diamine-dichloro-platinum, CDDP). CDDP induces peripheral neuropathy mainly affecting the large myelinated fibers of the nerves secondary to neuropathy affecting the neurons of dorsal root ganglia (5–7). The antineoplastic and neurotoxic effects of CDDP are due to its capacity to form adducts with DNA (8) and proteins (9) and to modulate the activation of specific molecular pathways (10). In post-mitotic DRG neurons, it has been shown that CDDP induces apoptosis through the neurons attempting to reenter the cell cycle (11, 12).

The antineoplastic action of paclitaxel (Taxol) is mainly due to its capacity to enhance tubulin polymerization (13). In this way, paclitaxel interferes with the assembly of cell microtubuli and, in dividing cells, arrests the cells in the G2-M phase of the cell cycle. It is during mitosis that paclitaxel hinders the formation of the mitotic spindle and commits cells to apoptosis (14). This mechanism explains the effect of paclitaxel on tumor cells and also on dividing neuronal-like cells such as human neuroblastoma SH-SY5Y cells but not on post-mitotic neurons. Nevertheless, in post-mitotic neurons, paclitaxel may alter the neuronal transport by acting on microtubuli (2) and it may also modulate proteins involved in the intracellular transduction pathways (15, 16). All these mechanisms may be important in explaining the effect of paclitaxel as an anticancer and as a neurotoxic drug (17, 18).

The human neuroblastoma SH-SY5Y cells are immature neuroblasts (19). This cell line is a reliable model for studying the neurotoxic effect of platinum compounds or taxanes (20) and for elucidating the mechanisms of diabetic neuropathy (21). Neurotoxicity was evaluated by the capacity of these compounds to induce apoptosis (10, 15, 22). We have previously reported that CDDP may protect human neuroblastoma SH-SY5Y cells from paclitaxel-induced apoptosis, whereas low doses of paclitaxel do not protect from CDDP neurotoxicity (23). In the present study, we investigated the possible mechanisms of protection of low-dose CDDP, analyzing the modulation of some intracellular pathways known to be altered in paclitaxel-induced apoptosis (15). We also examined the effect of the combination of low-dose CDDP and paclitaxel on the cell cycle and on proteins that are critical in regulating the transition from one
phase to another in the cell cycle (16). Moreover, we analyzed the modulation of p53 and the role of this protein in CDDP protection using a synthetic inhibitor of p53 activity.

Materials and Methods
Reagents
Paclitaxel was purchased from Sigma Chemical Co. (St. Louis, MO). It was dissolved in absolute ethanol to make a stock solution of 10 mmol/L, which was diluted with medium to obtain the different working concentrations (0.1, 0.5, and 1 μmol/L). Cisplatin was purchased from Sigma Chemical. It was dissolved in saline buffer (0.9% NaCl) to make a stock solution of 3.2 mmol/L, which was diluted with medium to obtain the different working concentrations (4 and 8 μmol/L). Pifithrin α [1-(4-methylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2H)benzothiazolyl)ethane hydrobromide] was obtained from BIOMOL Research Laboratories (Plymouth Metting, PA). It was dissolved in DMSO to make a stock solution of 10 mmol/L, which was diluted with medium to obtain the different working concentrations (10 and 30 μmol/L). Monoclonal antibody anti-β-tubulin was purchased from Sigma Chemical. Polyclonal antibodies anti-caspase-3, anti-poly(ADP-ribose) polymerases (PARP), and anti-actin and monoclonal antibodies anti-p53, anti-cyclin E, anti-cyclin A, anti-cyclin B1, anti-p34cdc2, and anti-p21 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody anti-Bcl-2 was purchased from Roche (Basel, Switzerland). Polyclonal antibody anti-caspase-7 and mouse anti-nuclei monoclonal antibody were purchased from Chemicon International (Temecula, CA). Polyclonal antibodies anti-phospho-Bcl-2 (Ser68) and phospho-p34cdc2 (Tyr15) were purchased from Cell Signaling Technology (Beverly, MA). Anti-rabbit IgG horseradish peroxidase–conjugated antibody (from donkey) was purchased from Amersham Biosciences (Arlington Heights, IL). Goat anti-mouse IgG HRPO was purchased from Transduction Laboratories (Lexington, KY). Conjugated anti-rabbit FITC and conjugated anti-mouse TRITC were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Cultures and Cell Death Assay
Human neuroblastoma SH-SY5Y cells were maintained in DMEM (Bio Whittaker, Bergamo, Italy) containing 10% heat-inactivated fetal bovine serum, penicillin (100 units/ mL), streptomycin (100 μg/mL), 1-glutamine (2 mmol/L), and sodium pyruvate (1 mmol/L) in a 5% CO2 humidified incubator at 37°C. After the treatment with antineoplastic agents, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Sigma Chemical) was done as described previously (15).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test was also done in cultures treated with pifithrin α. The human neuroblastoma SH-SY5Y cells were pretreated with 10 and 30 μmol/L pifithrin α for 1 or 6 hours and then exposed for 24 hours to 4 μmol/L CDDP and/or 0.5 μmol/L paclitaxel in the continuous presence of inhibitor.

Cell Lysates and Immunoblotting Analysis
After treatment with different concentrations of antineoplastic agents, human neuroblastoma SH-SY5Y cells were washed twice with ice-cold PBS and solubilized in lysis buffer (15) containing freshly added protease and phosphatase inhibitors (15). The lysates were clarified by centrifugation at 4°C at 13,000 × g for 15 minutes. Total proteins were measured with a Coomassie Protein Assay Reagent Kit (Pierce, Rockford, IL). Protein aliquots were solubilized in Laemmli buffer 5×, boiled for 5 minutes, and run on 13% (15%) for caspase immunoblotting) SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose filters. For each primary antibody used, immunoblotting analysis was done according to the manufacturer’s instructions. The immunoreactive proteins were visualized using the enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, IL) and quantified by densitometric analysis of autoradiographs (15).

Immunofluorescence Assay
After the treatment with different concentrations of CDDP and paclitaxel, human neuroblastoma SH-SY5Y cell culture monolayers were fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature. After washing in PBS, the cells were quenched in 0.1 mol/L glycine with PBS for 10 minutes and with dilution buffer [15% filtered goat serum, 0.3% Triton X-100, 0.45 mol/L NaCl and 10 mmol/L phosphate buffer (pH 7.4)] for 1 hour. Then the cells were incubated overnight with the antibodies that specifically recognize p53 (diluted 1:10) and human nuclei (diluted 1:200). After washing in wash buffer [0.5 mol/L NaCl, 0.3% Triton X-100, 20 mmol/L sodium phosphate buffer (pH 7.3)] for 1 hour, cells were incubated for 1 hour at room temperature with the appropriate fluorescein-conjugated FITC, diluted 1:50 and rhodamine-conjugated TRITC, diluted 1:200) secondary antibodies. Finally, cells were washed in wash buffer for 1 hour and PBS for 10 minutes and mounted in 90% glycerol in PBS. They were then examined using confocal laser microscopy, this being carried out with a Radiance 2100 microscope (Bio-Rad, Hercules, CA) equipped with a krypton/argon laser. Noise reduction was achieved by Kalman filtering during acquisition.

Flow Cytometric Analysis
Human neuroblastoma SH-SY5Y cells (1.5 × 10⁶ per 60-mm dish) were plated and after 24 hours were treated with 4 μmol/L CDDP, 1 μmol/L paclitaxel alone, or in combination for 24 hours. The culture medium was removed and the cells still adhering were detached with trypsin (16). The cells were collected, resuspended in a cold saline solution, and then fixed in ice-cold 96% ethanol for at least 4 hours. The fixation solution was eliminated and the samples were incubated overnight at 4°C with propidium iodide (10 μg/mL) and RNase A (12.5 μg/mL) in PBS. The fluorescence intensity of 10,000 cells per sample was determined with a FACScanLibur instrument and the data obtained were analyzed using Modfit Cell Cycle Analysis (Immunocytometric System, Becton Dickinson, Milano, Italy) as described previously (16).
Mitotic Index

Human neuroblastoma SH-SY5Y cells were exposed to CDDP, paclitaxel, or a combination of the two drugs. After 24 hours, the cells were washed with PBS and fixed with methanol/glacial acetic acid (3:1) for 20 minutes. After washing in PBS, the cells were stained with Giemsa dye to visualize chromosome condensation by light microscopy (16). Two hundred cells were randomly counted in each of three different experiments and the percentage of cells in mitosis was determined.

Statistical Analysis

Statistical analysis was done using the one-way ANOVA test and Dunnett’s multiple comparison test as a post test. Linear correlation between cellular death percentage and mitotic index was determined using the GraphPad Prism statistical package.

Results

Cellular Death Percentage

Based on our previous results (15), in the present study, we used paclitaxel concentrations of 0.1, 0.5, and 1 μmol/L, which give a percentage of cellular death ranging from 40% to >80% with the highest concentration of paclitaxel (Table 1). CDDP alone, at concentrations of 4 or 8 μmol/L, gives a percentage of cellular death of about 15% and 30%, respectively. As shown in Table 1, the concomitant administration of CDDP and paclitaxel for 24 hours, at the above-reported concentrations, determined a statistically significant reduction in the cellular death percentage with respect to the paclitaxel alone. Both concentrations of CDDP were equally able to reduce paclitaxel-induced cell death. Previous studies have shown that human neuroblastoma SH-SY5Y cells exposed to CDDP or paclitaxel die by apoptosis (15, 24) and this was confirmed also in the present study using morphologic and molecular tests (data not shown). Based on the above results, we subsequently studied the molecular mechanisms involved in CDDP protection of paclitaxel cellular death by using CDDP at a concentration of 4 μmol/L, the lower but equally effective concentration. The coadministration of CDDP and paclitaxel, at the same concentrations used with SH-SY5Y cells, led to a reduction in the cellular death also in human MCF7 breast cancer cells and in the human JR8 melanoma cell line (data not shown).

Effect of CDDP on Paclitaxel-Induced β-Tubulin Polymerization

To explore the protective effect of CDDP, we evaluated the possible interference of simultaneous CDDP and paclitaxel administration on the tubulin changes induced by paclitaxel (13). As shown in Fig. 1, the combined administration of 4 μmol/L CDDP and 0.1, 0.5, and 1 μmol/L paclitaxel does not alter paclitaxel-induced polymerization of β-tubulin either at the earliest times or at the latest ones. Both the monomeric and the polymeric forms of β-tubulin were present throughout the entire period of observation (24 hours). From this observation, we excluded the hypothesis that the protective effect of CDDP was due to interference with the ability of paclitaxel to polymerize β-tubulin.

Effect of CDDP and Paclitaxel on Molecular Pathways Involved in Apoptosis

The exposure of human neuroblastoma SH-SY5Y cell cultures to CDDP or paclitaxel induces modulations of

<table>
<thead>
<tr>
<th>Table 1. Percentage of cellular death in human neuroblastoma SH-SY5Y cells after treatment with CDDP and/or paclitaxel (MTT test)</th>
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<tbody>
<tr>
<td>% Death (mean ± SD)</td>
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<tr>
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</tr>
<tr>
<td>Control cultures</td>
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<tr>
<td>CDDP 4 μmol/L</td>
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<tr>
<td>CDDP 8 μmol/L</td>
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<tr>
<td>PACL 0.1 μmol/L</td>
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<tr>
<td>PACL 0.1 μmol/L + CDDP 4 μmol/L</td>
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<tr>
<td>PACL 0.1 μmol/L + CDDP 8 μmol/L</td>
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<tr>
<td>PACL 0.5 μmol/L</td>
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<tr>
<td>PACL 0.5 μmol/L + CDDP 4 μmol/L</td>
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<tr>
<td>PACL 0.5 μmol/L + CDDP 8 μmol/L</td>
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<tr>
<td>PACL 1 μmol/L</td>
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<tr>
<td>PACL 1 μmol/L + CDDP 4 μmol/L</td>
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<tr>
<td>PACL 1 μmol/L + CDDP 8 μmol/L</td>
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NOTE: P was determined using control cultures (cells cultured in serum alone) as the reference value.
Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NS, not significant; PACL, paclitaxel.
proteins that may be involved in the process of apoptosis (10, 15, 22). Both paclitaxel and CDDP induce Bcl-2 inactivation and loss of its antiapoptotic activity. As shown in Fig. 2A, the cultures exposed to CDDP alone did not show any change in Bcl-2 protein with respect to the control. Paclitaxel treatment caused the appearance of a slow migrating band (corresponding to the phosphorylated-inactive form of Bcl-2; ref. 15) when the highest concentrations of paclitaxel were used. The concomitant administration of CDDP and paclitaxel markedly reduced the intensity of the band of the inactive Bcl-2 protein. These observations have been confirmed by the use of the phospho-Bcl-2 (Ser70) antibody, a specific antibody that detects endogenous levels of Bcl-2 only when phosphorylated at Ser70 (Fig. 2B).

Generally, Bcl-2 inactivation induces activation of caspases, a family of cystein proteases that play a central role in the apoptotic process (25). They are present in the cells as inactive precursors and, when activated, cleave structural and housekeeping proteins (e.g., PARP) thus committing cells to apoptosis (26). After exposure of human neuroblastoma SH-SY5Y cells to CDDP or paclitaxel, alone or in combination, the fragment of the active caspase-3 (p11) was not present and so caspase-3 was not activated (Fig. 3A). Conversely, caspase-7 was equally activated after exposure to CDDP alone, to paclitaxel alone, and after the coadministration of the two drugs as shown by the appearance of the 19-kDa active fragment (Fig. 3B). We then evaluated the inactivation of PARP (i.e., cleavage and appearance of the 85-kDa fragment; Fig. 3C). Inactivation of PARP was evident after CDDP or paclitaxel exposure and after exposure to the various combinations of the two drugs. Overall, these results indicate that the Bcl-2 protein but not the caspases may be involved in the CDDP protection of paclitaxel-induced apoptosis.

Effect of CDDP and Paclitaxel on p53 Protein

In response to various cytotoxic and genotoxic agents p53, a protein that acts as a sensor of DNA damage, is activated and accumulated into the nucleus (27–29). As shown in Fig. 4, the p53 protein was not detectable in control cultures, but it was markedly increased after exposure to CDDP alone. After exposure to paclitaxel alone, the amount of p53 was also greatly increased, but this increase was inversely related to paclitaxel concentration. When CDDP and paclitaxel were coadministered the CDDP had little effect on paclitaxel-induced changes in p53 levels. Only with the highest concentrations of paclitaxel an increase in the total amount on p53 protein was evident.

We determined the cellular location of the p53 protein, after 24 hours using immunofluorescence and confocal laser microscopy observation. In control cultures, no immunopositive cells were detected (Fig. 5A). In 4 μmol/L CDDP-treated cultures, some cells showed highly immunopositive nuclei with a diffuse pattern throughout the nucleus (Fig. 5D). After exposure to 0.1 μmol/L paclitaxel alone, we observed a diffuse pattern of immunopositivity in the nucleus (Fig. 5B), whereas with 1 μmol/L paclitaxel p53 immunoreactivity was gathered in discrete spots (Fig. 5C) that are typical of centrosome staining (30). When CDDP was coadministered with paclitaxel, p53 immunoreactivity was gathered in discrete spots that are typical of centrosome staining (30). After coadministration of the two drugs, p53 immunoreactivity generally had a diffuse nuclear pattern and occasionally, with 1 μmol/L paclitaxel, positive centrosomes were evident (Fig. 5F). The increase of p53 protein...
and its nuclear accumulation suggested that p53 may play a role in CDDP protection. However, when we examined the real role of p53, we found that this occurrence could be excluded. We used a chemical inhibitor of p53, pifithrin α, which is able to inhibit p53-dependent apoptosis by reversible inhibition of both p53 transactivation activity and p53 downstream events (31). In the presence of pifithrin α, no statistically significant variation in the cellular death percentage induced by CDDP and paclitaxel, given alone or in combination, was observed (Table 2). The inhibitory effect of pifithrin α (32) was shown by an attenuation of the levels of p21 protein, an important downstream effector of the p53 pathway (data not shown).

**Effect of CDDP and Paclitaxel on Cyclins and Cell Cycle**

To understand whether alterations in cell cycle progression could explain the antiapoptotic effect of the combination of low-dose CDDP with various paclitaxel concentrations, we analyzed the activation status of different proteins (cyclin E, A, B1, and p34cdc2) involved in the control of the cell cycle transition (33).

Cyclin E is essential for progression from the G1 phase to the S phase (33). As shown in Fig. 6A, with respect to the control, CDDP alone induced an increase in cyclin E levels, whereas paclitaxel alone had the opposite effect, markedly reducing the level of cyclin E which was almost absent with the 0.5 and 1 μmol/L paclitaxel concentrations. The combination of CDDP with the paclitaxel determined increased levels of cyclin E with respect to paclitaxel alone.

Cyclin A promotes DNA duplication during the S phase of the cell cycle (33). As shown in Fig. 6B, with respect to the control, CDDP alone increased the level of cyclin A, paclitaxel alone reduced cyclin A levels, whereas the combination of CDDP with the different paclitaxel concentrations induced a marked increase in the levels of cyclin A with respect to those of paclitaxel alone.

Cyclin B1 has a pivotal role in controlling the transition from the G2 to the M phase of the cell cycle (33, 34). As shown in Fig. 6C, untreated cultures had levels of this
Table 2. Percentage of cellular death in human neuroblastoma SH-SY5Y cells after 1 or 6 h of pretreatment with pifithrin α and then treatment with CDDP and/or paclitaxel (MTT test)

<table>
<thead>
<tr>
<th></th>
<th>% Death (1 h), mean ± SD</th>
<th>P</th>
<th>% Death (6 h), mean ± SD</th>
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<tbody>
<tr>
<td>Control cultures</td>
<td></td>
<td></td>
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<tr>
<td>Control cultures + PFT α 10 µmol/L</td>
<td>1.7 ± 7.5</td>
<td>0</td>
<td>3.1 ± 6.5</td>
<td>0</td>
</tr>
<tr>
<td>Control cultures + PFT α 30 µmol/L</td>
<td>3.7 ± 1.6</td>
<td></td>
<td>1.8 ± 6.4</td>
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<tr>
<td>CDDP 4 µmol/L</td>
<td>1.6 ± 9.3</td>
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<tr>
<td>CDDP 4 µmol/L + PFT α 10 µmol/L</td>
<td>14.3 ± 8.8</td>
<td>NS</td>
<td>14.4 ± 8.6</td>
<td>NS</td>
</tr>
<tr>
<td>CDDP 4 µmol/L + PFT α 30 µmol/L</td>
<td>12.5 ± 10.9</td>
<td>NS</td>
<td>12.5 ± 5.7</td>
<td>NS</td>
</tr>
<tr>
<td>PACL 0.5 µmol/L</td>
<td>68.7 ± 4.6</td>
<td></td>
<td>69.6 ± 3.9</td>
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</tr>
<tr>
<td>PACL 0.5 µmol/L + PFT α 10 µmol/L</td>
<td>79.2 ± 6.6</td>
<td>NS</td>
<td>79.0 ± 3.3</td>
<td>NS</td>
</tr>
<tr>
<td>PACL 0.5 µmol/L + PFT α 30 µmol/L</td>
<td>77.2 ± 7.8</td>
<td>NS</td>
<td>77.1 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>PACL 0.5 µmol/L + CDDP 4 µmol/L</td>
<td>49.1 ± 8.4</td>
<td></td>
<td>46.3 ± 4.0</td>
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</tr>
<tr>
<td>PACL 0.5 µmol/L + CDDP 4 µmol/L + PFT α 10 µmol/L</td>
<td>54.7 ± 8.2</td>
<td>NS</td>
<td>55.5 ± 3.9</td>
<td>NS</td>
</tr>
<tr>
<td>PACL 0.5 µmol/L + CDDP 4 µmol/L + PFT α 30 µmol/L</td>
<td>55.0 ± 8.8</td>
<td>NS</td>
<td>56.5 ± 1.0</td>
<td>NS</td>
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</table>

NOTE: P was determined using control cultures (cells cultured in serum alone) as the reference value. Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NS, not significant; PFT α, pifithrin α; PACL, paclitaxel.

Cisplatin Reduces Paclitaxel-Induced Apoptosis

protein similar to those of cultures exposed to CDDP alone, whereas paclitaxel markedly increased the expression of cyclin B1 in a dose-related manner. The combination of CDDP with paclitaxel determined a reduction in levels of cyclin B1 with respect to paclitaxel alone.

Of all the kinases that are involved in cell cycle progression, we studied the protein p34cdc2, a serine/threonine kinase, which controls the progression from the G2 to the M phase (33, 34) and is therefore an essential protein for cells to reach mitosis. This protein is inactivated by phosphorylation. As shown in Fig. 6D, with respect to the control, CDDP increased levels of the phosphorylated (inactive) form of p34cdc2, paclitaxel reduced its levels, whereas the combinations of the two drugs increased the levels of the phosphorylated form of p34cdc2 with respect to paclitaxel alone. Overall, these data showed that CDDP and paclitaxel alone have an opposite effect on cyclins and that, at the concentrations used in our experiment, the effect of CDDP generally prevailed over that of paclitaxel.

To correlate the above-reported changes in cyclin expression with changes in the distribution of cells in different phases of the cell cycle, we did flow cytometric analysis. Overall, this analysis of cell cycle distribution in the different paradigms was in accordance with cyclin evaluation (Fig. 7). CDDP increased the percentage of cells in the S phase with respect to the controls (Fig. 7A–B), whereas paclitaxel induced an increase in the number of cells in the G2-M phase (Fig. 7C). The association of CDDP and paclitaxel reversed the changes induced by paclitaxel and increased the percentage of the cells arrested in the S phase (Fig. 7D).

CDDP-Induced Cell Cycle Arrest before Mitosis

Because flow cytometric analysis does not distinguish between the G2 and the M phase, we determined the mitotic index [(number of cells in mitosis / total cell count) × 100] at 24 hours. Cells in mitosis are easily recognized by their condensed, intensely colored nucleus after staining with the Giemsa method (data not shown). The mitotic index (Table 3) greatly increased with exposure to paclitaxel in a dose-related manner. The association of CDDP with paclitaxel reduced the number of cells in mitosis, with a statistically significant decrease when the cultures were exposed to CDDP in combination with the 0.5 and 1 µmol/L concentrations of paclitaxel. The statistical analysis of the percentage of cellular death and the mitotic index of all the

Figure 6. Effect on molecules involved in cell cycle progression of cotreatment with CDDP and paclitaxel (PACL). Human neuroblastoma SH-SY5Y cells were treated with 4 µmol/L CDDP and/or with 0.1, 0.5, and 1 µmol/L paclitaxel for 24 h. Cells cultured with serum alone represented controls. Cell lysates were analyzed by immunoblotting using antibodies that specifically recognize cyclin E (A), cyclin A (B), cyclin B1 (C), p34cdc2 and phospho-p34cdc2 (pp34cdc2 Tyr15), D. Actin was used as a loading control.
paradigms in which the different concentrations of paclitaxel were used, alone or in combination with 4 \( \mu \text{mol/L} \) CDDP, showed a strong linear correlation between these two indices (Fig. 8), strengthening the suggestion that paclitaxel-induced apoptosis is possible only when the cells are arrested in mitosis.

**Discussion**

In this study, we investigated the interactions occurring between CDDP and paclitaxel on neuronal-like cells, using an *in vitro* paradigm based on human neuroblastoma SH-SY5Y (20, 22, 35, 36). In a previous study, confirmed by the present one, we found that the concomitant administration of low doses of cisplatin with moderate to high doses of paclitaxel reduces the percentage of neuronal death (23), showing the protective effect of low doses of CDDP on paclitaxel-induced apoptosis. However, similar findings on cellular death were found also in cancer cell lines (data not shown). This unexpected finding suggests three different possibilities: (a) that CDDP hinders the tubulin polymerization induced by paclitaxel; (b) that the combination of the two drugs induces changes in the cellular proteins involved in controlling apoptotic death which are different from those induced by each drug alone; and (c) that CDDP arrests the cells early in its cell cycle in such a way that cells do not reach the M phase when paclitaxel can be toxic by hindering the formation of the mitotic spindle.

The possibility that CDDP interferes with paclitaxel’s capacity to polymerize tubulin was excluded by showing the presence of both the monomeric and the polymeric forms of \( \beta \)-tubulin throughout the entire period of observation. Tubulin polymerization does not seem a relevant mechanism in determining the fate of the human neuroblastoma SH-SY5Y cells, because also other neuroprotective drugs, such as resveratrol, protect from paclitaxel-induced apoptosis without interfering with \( \beta \)-tubulin polymerization (22).

The second possibility (i.e., the effect on intracellular transduction pathways involved in apoptotic cell death) was approached by studying the effect of CDDP and paclitaxel given separately or in combination on Bcl-2, on caspases 3 and 7 and on p53. Bcl-2 is an antiapoptotic protein that, when inactivated, may lead to the apoptotic process permitting activation of the caspases and, consequently, the degradation of structural proteins such as PARP (26, 37). The concomitant exposure of human neuroblastoma SH-SY5Y cells to CDDP and paclitaxel markedly reduces the phosphorylation (inactivation) of Bcl-2 induced by paclitaxel alone but does not alter the activation of caspase-7 and PARP inactivation caused by exposure to either CDDP or paclitaxel.

We propose that Bcl-2 plays a role in the CDDP protection of paclitaxel-induced apoptosis not by modulating the activation of signaling molecules involved in the apoptotic process but by controlling the G2-M phase block. In fact, Bcl-2 phosphorylation is important in regulating M phase–specific events both during normal G2-M cell cycle progression and G2-M cell cycle block induced by paclitaxel (38–40). Therefore, the phosphorylation of Bcl-2 may not represent a way of inactivating this protein but may be an epiphenomenon of cell cycle arrest as suggested in a nonneuronal cell line by Ling et al. (38).

**Table 3. Percentage of mitotic index in human neuroblastoma SH-SY5Y cells after treatment with CDDP and/or paclitaxel**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Mitotic index (mean ± SD)</th>
<th>( P )</th>
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<tbody>
<tr>
<td>Control cultures</td>
<td>4.1 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>CDDP 4 ( \mu \text{mol/L} )</td>
<td>7.1 ± 2.4</td>
<td></td>
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<tr>
<td>PACL 0.1 ( \mu \text{mol/L} )</td>
<td>20.1 ± 3.6</td>
<td></td>
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<tr>
<td>PACL 0.1 ( \mu \text{mol/L} ) + CDDP 4 ( \mu \text{mol/L} )</td>
<td>13.1 ± 2.8</td>
<td>NS</td>
</tr>
<tr>
<td>PACL 0.5 ( \mu \text{mol/L} )</td>
<td>53.9 ± 12.5</td>
<td></td>
</tr>
<tr>
<td>PACL 0.5 ( \mu \text{mol/L} ) + CDDP 4 ( \mu \text{mol/L} )</td>
<td>21.5 ± 5.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PACL 1 ( \mu \text{mol/L} )</td>
<td>76.6 ± 4.1</td>
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</tr>
<tr>
<td>PACL 1 ( \mu \text{mol/L} ) + CDDP 4 ( \mu \text{mol/L} )</td>
<td>29.6 ± 9.3</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

**NOTE:** \( P \) was determined using control cultures (cells cultured in serum alone) as the reference value.

**Abbreviations:** PACL, paclitaxel; NS, not significant.
Both CDDP and paclitaxel increase the amount of cellular p53 (41-43), a protein that acts as a sensor of DNA damage and arrests the cell cycle to prevent DNA replication until DNA repair has occurred or, otherwise, induces apoptosis when DNA repair is ineffective (27, 44). In response to various cytotoxic and genotoxic agents, p53 protein is activated and posttranslational modifications increase p53 stability and lead to nuclear accumulation (29, 45), because many of the important activities of p53 depend on the ability of p53 to regulate gene expression by activating or repressing transcription (46). Blagosklonny (43) suggested that increased levels of p53 induced by cytotoxic agents may protect normal cells against paclitaxel toxicity by blocking the cell cycle in the G2 phase, thus preventing the cells from reaching the mitosis where paclitaxel exerts its effect by hindering the mitotic spindle formation (14).

However, in our model, the protective effect of p53 can be excluded. The use of pifithrin, a chemical inhibitor of p53 (31), does not induce any change in the percentage of cellular death of both CDDP and paclitaxel alone or in combination. We therefore conclude that the mechanism of protection by low doses of cisplatin on paclitaxel-induced apoptosis in the human neuroblastoma SH-SY5Y cell line is p53-independent and the inhibition of p53 is not important in modifying CDDP’s ability to protect from paclitaxel-induced cell death.

By evaluating the third possibility (i.e., the modulation of proteins involved in the cell cycle), we observed that cyclin expression in the different paradigms was characterized by CDDP having the opposite effect to paclitaxel when given alone. In accordance with the data reported in the literature in nonneuronal cells (47) in our paradigms, CDDP arrests cell cycle progression in the S phase and modulates cyclins involved in this phase. On the contrary, paclitaxel blocks cell cycle progression in the G2-M phase and acts on proteins relevant for this phase (48). When CDDP and paclitaxel are coadministered, the action of CDDP predominates in accordance with that which has been reported on other cell lines (49). The strong correlation between cellular death percentage and the mitotic index, the data on cyclins, and the results of the flow cytometric analysis, observed in the various paradigms, support the idea that in human neuroblastoma SH-SY5Y cells the block in the G2-M phase is necessary for paclitaxel to induce apoptosis. We suggest that low doses of CDDP reduce or prevent apoptosis by hampering cell progression to the mitosis.

Our results, which allow greater insight into the relationship existing between two of the most frequently used antineoplastic drugs, may not only be helpful for investigating the mechanisms of chemotherapy-induced peripheral neuropathy (2, 50) but may also be helpful for planning future in vivo studies with combination treatments.

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Figure 8. Linear correlation between cellular death percentage and mitotic index. PACL, paclitaxel.
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