The Chemoprotective Agent N-Acetylcysteine Blocks Cisplatin-Induced Apoptosis through Caspase Signaling Pathway

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ABSTRACT

Thiols such as N-acetylcysteine (NAC) are increasingly used in clinical trials of platinum chemotherapy as chemoprotectants. NAC can prevent cisplatin-induced ototoxicity, nephrotoxicity, and gastrointestinal toxicity; however, the molecular mechanisms of NAC on apoptosis and cisplatin cytotoxicity remain unknown. We investigated cisplatin cytotoxicity and NAC chemoprotection in human tumor cell lines, as assessed by immunoblotting and immunocytochemistry. Cisplatin cytotoxicity was associated with nuclear translocation of apoptosis induction factor, expression of the pro-apoptotic Bax protein, cleavage of caspases 3 and 9, and cleavage of PARP. NAC administration reversed the cytotoxic and apoptotic effects if added concurrent with cisplatin or up to 2 h after cisplatin, but chemoprotection was reduced if NAC administration was delayed more than 2 h and was minimal by 8 h after cisplatin. Expression of tumor suppressor p53 and the cell cycle regulatory protein p21 was stimulated within 5 to 10 min by cisplatin in p53-positive LX-1 small cell lung carcinoma cells, and this effect was blocked by NAC. In p53-negative SKOV3 cells, cisplatin toxicity and NAC chemoprotection remained effective, suggesting that chemoprotection may be mediated through both p53-dependent and -independent pathways. Specific kinase inhibitors demonstrated that cisplatin induced apoptosis through the p38 mitogen-activated protein kinase (MAPK) pathway, not the extracellular signal-regulated kinase MAPK pathway. These results show that NAC blocks both the death receptor and the mitochondrial apoptotic pathways induced by cisplatin. The time course for NAC chemoprotection after cisplatin matches our previous in vivo results and provides an opportunity to manipulate route and timing to maintain cisplatin antitumor efficacy while protecting against chemotherapy side effects.

Cisplatin is an effective chemotherapeutic agent against multiple human cancers, including ovarian and head and neck malignancies. Cisplatin binds to DNA to form covalent platinum DNA adducts (Jamieson and Lippard, 1999) and also acts as a DNA alkylator. In addition, cisplatin generates reactive oxygen species (ROS), which are known as one of the pathogenic intermediates following chemotherapy (Masuda et al., 1994). Through these mechanisms, cisplatin triggers cellular responses involving multiple pathways, including DNA repair, transcription inhibition, cell cycle arrest, and apoptosis (Siddik, 2003).

Apoptosis, or programmed cell death, can occur through the death receptor pathway and the mitochondrial pathway (Fig. 1) (Levin et al., 1999). The (extrinsic) death receptor pathway is triggered by signals from other cells and leads to activation of the apoptotic cascade via caspase 8. The (intrinsic) mitochondrial pathway involves the release of apoptogenic factors such as cytochrome c and apoptosis-inducing factor (AIF) from the mitochondria. The pro-apoptotic Bcl-2 family members Bax and Bak increase the mitochondrial permeability and induce cytochrome c release through oligomerization and insertion in the outer mitochondrial membrane. AIF is a conserved mitochondrial intermem-

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brane flavoprotein that is translocated to the nucleus after release from mitochondria and then plays an important role in inducing nuclear chromatin condensation and DNA fragmentation (Cregan et al., 2004). Cytochrome c binds to the adaptor protein and forms the apoptosome complex. Caspase 9 is recruited to this complex and becomes proteolytically activated, thereby initiating and (amplifying) effector caspase (caspase 7 and caspase 3) activation. Effector caspses are responsible for many of the biochemical characteristics of apoptosis, including cleavage of poly (ADP-ribose) polymerase (PARP) and ultimately DNA fragmentation. Studies in several malignant cell types have demonstrated that treatment with cisplatin leads to caspase activation, PARP cleavage, and apoptosis (Del Bello et al., 2004).

The cell cycle is driven by a number of positive and negative regulatory phosphorylation and dephosphorylation events that influence the activity of transcription factors. Tumor suppressor protein p53 is an important transcription factor central to cell cycle regulation mechanisms and cell proliferation control, and its inactivation is considered a key event in human carcinogenesis (Bennet, 1999; Schuler et al., 2000). The cell cycle regulatory protein p21(WAF1/Cip1) binds to cyclin/CDK complexes and inhibits their kinase activity, thereby stopping cell cycle progression. It also binds to proliferating cell nuclear antigen and blocks DNA replication but not the DNA repair process. Expression of p21 is induced by wild-type, but not mutant, p53 tumor suppressor protein as well as p53-independent mechanisms (Mahyar-Roemer and Roemer, 2001). Both p53 and p21 are attractive for development of new therapeutic approaches (Shapiro and Harper, 1999).

Antioxidants such as the endogenous tripeptide glutathione can protect cells from cisplatin-induced cytotoxicity (Hamers et al., 1993; Muldoon et al., 2001). N-Acetylcysteine (NAC) is an antioxidant thiol that can enter the glutathione synthesis pathway and may also directly bind to and inactivate platinum agents (Zafarullah et al., 2003). Our previous studies demonstrated that NAC protects against alkylating chemotherapy cytotoxicity in vitro (Muldoon et al., 2001), blocks ototoxicity in rats treated with cisplatin (Dickey et al., 2004), and reduces chemotherapy-induced bone marrow toxicity in rats (Neuwelt et al., 2001, 2004). We have found that thiol chemoprotection can be obtained without blocking the antitumor effects of chemotherapy (Muldoon et al., 2000; Neuwelt et al., 2004). Nonetheless, chemoprotectants have had relatively limited clinical use due to concerns of impaired antitumor efficacy. The mechanisms governing the effect of NAC on cell damage induced by anticancer agents such as cisplatin have not been fully established. Better understanding of the roles of NAC on p53, p21, and the apoptotic signaling pathway in cellular and molecular prospects should enable a more rational chemoprotectant delivery regimen to maximize chemoprotective effects while minimizing any impact on antitumor efficacy in human patients. Therefore, the objectives of the present study were to investigate the molecular mechanism and signaling pathway and involved in the protection of NAC on cisplatin-induced apoptosis and to evaluate the timing of NAC administration before or after cisplatin treatment in vitro in different cell lines.

**Materials and Methods**

**Reagents.** Cisplatin from Novaplus and N-acetylcysteine from Roxane were purchased through Ben Venue Labs (Bedford, OH). Rabbit anti-Bax (no. 2772), PARP (no. 9542), and caspase 3 (no. 9662) and caspase 9 (no. 9502) antibodies were from Cell Signaling Technology Inc. (Beverly, MA). Monoclonal antibody against p21 (clone CP74) was from NeoMarkers (Fremont, CA). Mouse anti-AIF (E1; sc-13116) and p53 (DO-1; sc-126) antibody were from Santa Cruz Biochemicals (Santa Cruz, CA). Protease inhibitor cocktail was from Roche Diagnostics (Pleasanton, CA). Mouse antitubulin (clone DM 1A) monoclonal antibody; protein kinase p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580; PI3 kinase inhibitor, LY294002; and MEK1 inhibitor, PD98059, were from Sigma-Aldrich (St. Louis, MO).

**Cell Culture.** The B.5 LX-1 small cell lung carcinoma (SCLC) cell line is a clonal line derived from the LX-1 parental cells, originally obtained from Mason Research Institute (Worcester, MA). These cells were maintained as a free-floating cell suspension in spinner flasks in medium RPMI 1640 supplemented with 12% (v/v) heat-inactivated fetal bovine serum (Irvine Scientific, Santa Ana, CA) plus gentamycin, penicillin, and streptomycin. Ovarian cancer cell line SKOV3 was kindly supplied by Dr. Gail Clinton (Department of Biochemistry and Molecular Biology, Oregon Health and Sciences University). Rat fibroblasts (Rat1) were obtained from Dr. Bruce Magun (Department of Cellular and Developmental Biology, Oregon Health and Sciences University). U87MG glioblastoma cells were obtained from Dr. Ali-Osman at the University of Texas M. D. Anderson Cancer Center (Houston, TX). These cells were cultured in Dulbecco’s modified Eagle medium (Invitrogen, Carlsbad, CA) with 5% (v/v) fetal bovine serum supplemented with gentamicin, penicillin, and streptomycin. All cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂.
Cytotoxicity Assay. Live cell number was evaluated with the WST-1 Cell Proliferation Assay Kit from Chemicon International (Temecula, CA). This colorimetric assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. This assay shows minimal interference from the N-acetylcysteine used in these experiments and approximate linearity (absorbance versus log cell number) in the range of $10^2$ to $10^5$ cells. Tumor cells were seeded to 96-well tissue culture plates at $1 \times 10^5$ cells per well, and chemotherapy and/or chemoprotective agents were added 1 day after plating. Plates of Rat1 fibroblasts were held until confluent before treatment. For the dose/response studies, cells were treated with or without NAC immediately prior to cisplatin addition using six to eight concentrations, evaluated in four wells per concentration. For the time course studies, NAC was added 0, 2, 4, 6, or 8 h after cisplatin addition in four wells per condition. Assays were performed two to four times in each cell type. The WST-1 reagent was added 44 to 48 h after cisplatin, and absorbance at 450 nm was measured 2 to 4 h after cisplatin addition. Blanks included experimental agents and cells dissolved by addition of SDS to a concentration of 0.5% (w/v).

Western Blotting. Cells were treated and collected in lysis buffer [1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 150 mM NaCl, and 20 mM Tris-HCl, pH 8] in the presence of 1 mM phenylmethylsulphnol fluoride, 0.1 mM sodium vanadate, and 150 mM NaCl, and 20 mM Tris-HCl, pH 8 in the presence of 1 M phenylmethylsulphnol fluoride, 0.1 mM sodium vanadate, 1 M okadaic acid, and protease inhibitor cocktail. A total of 10 μg of protein was separated by 7.5% or 12% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Blots were blocked with 5% milk in Tris-buffered saline containing 0.05% (v/v) Tween 20 overnight at 4°C, then incubated overnight at 4°C with desired primary antibodies (1:1000 dilution) on a rocker. Blots were then washed two times with Tris-buffered saline containing 0.05% (v/v) Tween 20 and incubated for 2 h with secondary antibodies or anti-rabbit or anti-mouse immunoglobulin G coupled to horseradish peroxidase. The signal was observed and developed with Kodak film by exposure of enhanced chemiluminescence reagent. To check equal loading, every Western blot was either stained with gel code blue (Pierce Chemical, Rockford, IL) (data not shown) or immunoblotted with anti-α-tubulin Ab.

Immunocytochemistry. Cells were fixed in 2% (w/v) paraformaldehyde with 0.1% (v/v) Triton-X for 30 min at 4°C before blocking in 0.25% (v/v) normal goat serum for 30 min at 20°C. Primary antibodies diluted in blocking buffer were added for 1 h at 4°C (anti-AIF, 1:200 dilution). After a washing step, cells were incubated for 2 h at 20°C in goat anti-mouse IgG-Alexa 529 (Molecular Probes, Eugene, OR), each diluted to 1:1000 in blocking buffer. Cell nuclei were counterstained with Hoechst 3342 (1 μg/ml) (Sigma-Aldrich). The signal was observed under a fluorescent microscope with proper filter.

Statistical Analysis. Half-maximal effective concentrations (EC_{50}) and S.D. in cytotoxicity assay were determined using Prism4 (GraphPad Software Inc., San Diego, CA). Statistical differences between groups were assessed by Student’s t test using Microsoft Excel software. Differences between means were considered significant if $P < 0.05$. Quantification of Western signal was performed by using IPlab Gel software (Signal Analytics Corporation, Vienna, VA). We did not determine statistical differences for the Western blot data.

Results

Cisplatin Cytotoxicity and Apoptosis. The dose/response for cisplatin cytotoxicity was evaluated in four different cell types: human SKOV3 ovarian carcinoma, human B.5 LX-1 SCLC, human U87 glioblastoma, and rat Rat1 fibroblasts. Cisplatin showed a steep dose/response relationship, with half-maximal cytotoxic doses (EC_{50}) values ranging from 3.8 μM in the most sensitive cells (ovarian carcinoma) to 8.7 μM in the LX-1 SCLC cells treated with cisplatin for 2 days (Table 1). At concentrations of 30 to 50 μM, cisplatin was 99% toxic after 2 days in the rapidly growing LX-1 SCLC cells, but lower magnitude and more variable degree of cell kill were found in the more slowly growing cells (Table 1). Cisplatin cytotoxicity was time-dependent. With a 24-h treatment, cisplatin killed only about 30% of cells compared with vehicle control, whereas the cisplatin EC50 was decreased by one-half if cells were treated for 3 days compared with 2 days.

PARP is a key nuclear enzyme involved in DNA repair and has a complex role in cell death. We analyzed PARP protein using an antibody able to detect the full-length 116 kDa and the characteristic apoptosis-related 85-kDa fragment. Treatment of the LX-1 SCLC cells with 50 μM cisplatin for 24 h induced cleaved PARP protein expression. In this time frame, no PARP cleavage was observed at a dose of 10 μM cisplatin (data not shown). All Western blot analyses were performed in LX-1 SCLC cells as the standard, although the results in other cell types may be shown.

The time dependence for cisplatin activation of apoptosis was assessed. Results in Fig. 2 show that cleavage of PARP was up-regulated 3-fold at 4 h after treatment of LX-1 SCLC cells with 50 μM cisplatin. PARP cleavage continued to increase to 21-, 32-, and 30-fold at 8, 12, and 24 h after cisplatin administration, as indicated by the strong induction of the 85-kDa fragment. To investigate the upstream caspases responsible for PARP cleavage, the sample was subjected to Western analysis for cleavage of caspase 3 and 9 (Fig. 2). Cleavage of both caspase proteins began to increase at 4 h after cisplatin and reached a peak at 12 h. Thereafter, caspase 9 decreased to undetectable levels, but caspase 3 had sustained high expression levels at the 24-h time point. Further upstream of the caspases, cisplatin stimulated a robust increase in the expression of the pro-apoptotic protein Bax within 5 min (1.7-fold) to 10 min (3-fold) after treatment, expression was sustained at 2.9-fold up to 24 h. These data indicated the apoptotic signal was continuously activated by cisplatin (Fig. 2).

NAC Chemoprotection. The dose/response for NAC chemoprotection was evaluated in four different cell types. As we have previously shown with other alkylating chemotherapeutics (Muldoon et al., 2001), NAC was effective at reducing cisplatin toxicity with half-maximal concentrations ranging from 0.28 to 0.64 mg/ml (Table 2). The cytotoxicity of cisplatin in each cell type was reduced by 70 to 99% by concurrent administration of N-acetylcysteine at the maximal dose of 2 mg/ml. At this dose, NAC showed no cytotoxic activity alone even after 3 days of incubation and in some experiments actually increased cell number by up to 10%.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cisplatin</th>
<th>Cisplatin Cytotoxicity</th>
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<tbody>
<tr>
<td>SKOV3 ovarian</td>
<td>$3.8 \pm 0.7$</td>
<td>$82.6 \pm 4.5$</td>
</tr>
<tr>
<td>B5 LX-1 SCLC</td>
<td>$8.7 \pm 0.5$</td>
<td>$99.4 \pm 0.5$</td>
</tr>
<tr>
<td>U87 glioblastoma</td>
<td>$5.1 \pm 3.1$</td>
<td>$72.7 \pm 21.9$</td>
</tr>
<tr>
<td>Rat1 fibroblasts</td>
<td>$5.6 \pm 1.1$</td>
<td>$72.9 \pm 4.3$</td>
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To study if cisplatin-induced apoptosis could be prevented by NAC, we preincubated LX-1 SCLC cells with 2 mg/ml NAC 15 min prior to adding 50 μM cisplatin, and whole-cell lysates were collected 18 to 24 h after cisplatin for Western analysis. NAC alone, at a dose of 2 mg/ml, did not affect any of the apoptosis or cell cycle markers tested (Fig. 3). Stimulation of Bax expression and induction of cleaved PARP and cleaved caspases 3 and 9 by cisplatin was blocked by treating the cells with NAC.

As another marker of apoptosis, we next evaluated the release of AIF from mitochondria and translocation to the nucleus. Cisplatin significantly reduced live cell number and induced the translocation of AIF from mitochondria through the cytosol to the nucleus in rat fibroblasts (Fig. 4). SKOV3, and LX-1 SCLC (data not shown). Pretreating the cells with NAC 15 min before adding cisplatin completely reversed this effect. This data suggested that NAC prevented cisplatin-induced apoptosis by blocking nuclear translocation of AIF protein.

TABLE 2
NAC chemoprotection in multiple cell lines
The percentage of live cells compared with untreated controls was measured using the WST-1 colorimetric kit after 2 days of drug treatment. EC50 indicates the half-maximal effective dose for NAC chemoprotection in the presence of 30 to 50 μM cisplatin. Maximum chemoprotection indicates the percentage of live cells remaining after treatment with cisplatin plus 2 mg/ml NAC.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>NAC (EC50 mg/ml)</th>
<th>% live cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKOV3 ovarian</td>
<td>0.34 ± 0.10</td>
<td>99.4 ± 7.3</td>
</tr>
<tr>
<td>B5 LX-1 SCLC</td>
<td>0.30 ± 0.04</td>
<td>89.6 ± 16.8</td>
</tr>
<tr>
<td>U87 glioblastoma</td>
<td>0.64 ± 0.07</td>
<td>70.2 ± 5.8</td>
</tr>
<tr>
<td>Rat1 fibroblasts</td>
<td>0.28 ± 0.18</td>
<td>85.2 ± 21.0</td>
</tr>
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To determine the time dependence for NAC chemoprotection, we evaluated how long the addition of chemoprotectant could be delayed after cisplatin treatment and remain effective against cytotoxicity. Cells were treated with lethal doses of cisplatin (30–50 μM). NAC was added either concurrently with chemotherapy or up to 8 h after chemotherapy. Delayed administration of N-acetylcysteine reduced its protective ac-
tivity against cisplatin cytotoxicity (Fig. 5A). If NAC was administered 2 h after cisplatin, its protective activity was reduced to 70 to 90% of the maximal protection seen with concurrent administration, which was a significant decrease in both the SKOV3 ovarian carcinoma (P = 0.010) and LX-1 SCLC (P < 0.001). By 4 h postcisplatin, NAC had less than 25% efficacy at reducing cytotoxicity.

We next explored the kinetics of NAC protection against cisplatin-induced PARP cleavage (Fig. 5B). We treated both LX-1 SCLC and SKOV3 with cisplatin, and added NAC at −15 min, 1, 2, 4, 6, and 8 h after administration of cisplatin. Whole-cell lysates were collected after an additional 16-h incubation with cisplatin. For both cell types, NAC completely blocked cisplatin-induced apoptosis when added within 1 h of cisplatin treatment. PARP cleavage was apparent if NAC administration was delayed until 2 h or later after cisplatin treatment, although it appeared there was partial protection even when NAC was delayed until 8 h after cisplatin (Fig. 5B). However, loss of antiapoptotic effect of NAC was observed if we washed out NAC in rat fibroblasts preincubated with NAC (Fig. 5C). These data suggested that the presence of NAC in the culture medium is required to have antiapoptotic effect.

Cisplatin Activation of Cell Cycle Signaling. The cell cycle regulatory protein p21(WAF1/Cip1) and the tumor suppressor protein p53 were assessed to evaluate the effect of cisplatin on cell cycle regulation (Fig. 6A). Cisplatin robustly increased p21 expression 5-fold at 5 min, which was sustained until 8 h after treatment. Subsequently, the level of p21 protein decreased at the 12- and 24-h time points. In contrast to p21, p53 was up-regulated 4-fold by cisplatin by 10 min after treatment, and the increase was sustained at 3-fold through the 24-h time point. These results suggest that p21 expression was mediated through p53-independent mechanisms or might be just due to the detection limits of the antibody.

To further address the role of p53 in the cisplatin cytotoxicity and NAC chemoprotection, we tested SKOV3 ovarian cancer cells that are null for p53. NAC protects against cisplatin-induced apoptosis as indicated by abrogation of PARP cleavage in the ovarian cells as observed in the LX-1 SCLC cells (Fig. 6B). In the LX-1 SCLC cells, cisplatin-induced ERK, extracellular signal-regulated kinase (ERK) phosphorylation, p21 protein expression, and reduction of total cellular tyrosine phosphorylation, and all these effects were reversed by pretreatment with NAC. These cisplatin-induced changes were not observed in the SKOV3 cells (Fig. 6B). These results correlate with the difference of sensitivity to cisplatin between LX-1 SCLC and SKOV3 (Table 1), suggesting that the p53 tumor suppressor may play an important role of drug sensitivity to cisplatin between SKOV3 and LX-1 SCLC cells. Our data suggested that protection of cisplatin induced apoptosis by NAC is mediated through both p53-dependent and -independent pathway.

Effect of Protein Kinase Inhibitor on Cisplatin-Induced Apoptosis. Stimulation of the mitogen-activated protein kinases ERK1/2 by cisplatin has been shown to result in either survival or cell death; cisplatin causes activation of ERK MAP kinase in LX-1 SCLC but not in SKOV3 (Fig. 6B). To explore other possible signaling pathways activated by cisplatin, we analyzed several specific protein kinase inhibitors (PI3K/pAKT kinase inhibitor, LY294002; MEK1 inhibitor, PD98059; and p38 MAPK inhibitor, SB203580) on the cisplatin-induced apoptosis (Fig. 7). LX-1 SCLC cells were pretreated or untreated with specific protein kinase inhibitors, with or without addition of cisplatin. The p-AKT kinase inhibitor LY294002 (50 μM) alone caused significant cell death, 34.7 ± 16.2% at 48 h, and enhanced cisplatin cytotoxicity by 30% at 10 μM cisplatin and 52% at 20 μM cisplatin.
in LX-1 cells. Thus, PI3K inhibitors, such as LY294002, may have a place for adjuvant therapy to improve the effectiveness of cisplatin. Neither PD98059 (50 μM) nor SB203580 (10 μM) alone caused significant cell death when compared with vehicle. The Western blot results revealed that only the p38 MAPK inhibitor pretreated cells have reduced levels of cleaved PARP when compared with cisplatin treated cells. Incubation of LX-1 cells with the MEK1 inhibitors PD98059 but not LY294002 or SB203580 before cisplatin treatment completely inhibited cisplatin-induced activation of ERK (Fig. 7). The MEK1 inhibitor minimally induced PARP cleavage on its own and did not enhance or reduce the cisplatin effect on PARP. This result suggests that cisplatin-induced apoptosis is mediated by the p38 MAPK pathway, not the ERK MAPK pathway.

**Discussion**

**Cisplatin Cytotoxicity and Apoptosis.** Cisplatin, a potent anticancer drug, forms platinum DNA adducts and ROS formation and causes a variety of cellular responses. Although studies have revealed that cisplatin induces cell apoptosis, the mechanism in various cell types is not fully understood. Previous studies showed that apoptosis induced by cisplatin was mediated through both death receptor/caspase 8 (Fulda et al., 1998; Seki et al., 2000) and/or the caspase 9/caspase 3 pathways (Sun et al., 1999; Del Bello et al., 2004). Caspase 3 can be activated by caspase 9, which is activated by the release of cytochrome c from the mitochondria (Zhan et al., 1999; Schuler et al., 2000). In fact, caspases 3 has been specifically implicated as the effector caspase responsible for the cleavage of PARP for DNA repair and DNA endonuclease (DFF40) for DNA fragmentation. In a study in ovarian cancer cells, cisplatin treatment increased Bax protein and decreased Bcl-2 expression (Jones et al., 1998). In our study, cisplatin rapidly increased Bax protein expression within 5 to 10 min, and this was sustained through 24 h after treatment. The antiapoptotic Bcl-xl and Bcl-2 proteins were not affected by cisplatin in the LX-1 SCLC cells, perhaps because the LX-1 cells already overexpressed Bcl-xl and Bcl-2. Our data showed that the cleaved products of caspases 9 and 3 and PARP started to appear around 4 h and peaked at 12 h after cisplatin. Subsequently, cleaved caspase 9 protein level decreased, but cleaved caspase 3 and PARP remained elevated through 24 h. This result might suggest that caspase 3 was activated by both caspase 9 and caspase 8 in LX-1 SCLC cells.

The tumor suppressor gene p53 facilitates DNA repair before DNA replication and may, therefore, regulate cisplatin-induced apoptosis (Bennet, 1999). The cyclin-dependent kinase inhibitor p21(WAF1/Cip1) protein known as a mediator of p53 tumor suppressor function plays an important role in cell differentiation, DNA repair, and apoptosis. In contrast to p53, p21 was up-regulated by 5 min, was sustained until 8 h, then transiently decreased at 12 and 24 h.
when cleaved PARP protein were up-regulated (Fig. 6A). Thus, our data support the hypothesis that p21 is not only a cell cycle inhibitor but also functions as an antiapoptotic protein. Unlike p53 knockout mice, mice lacking p21 do not develop tumors. This implies that the tumors seen in p53 null mice do not arise as a result of impairing cell cycle arrest mechanisms but that apoptosis is the dominant mechanism by which p53 inhibits tumor development. Therefore, it would appear that the ability of p53 to induce apoptosis rather than growth arrest is central to its role as a tumor suppressor. Our study showed that in LX-1 SCLC cells, cisplatin induced accumulation of p53 before and during apoptosis and subsequently induced expression of downstream p21 protein. It is thought that the p53 pathway (with or without p21) targets damaged cells to apoptosis; however, this tumor suppressor protein is not required for apoptosis. The SKOV3 cells lacking p53 were sensitive to cisplatin with a lower half-maximal dose than in the LX-1 SCLC cells (Table 1). These data suggest that p53/p21 may be somewhat protective against apoptosis by inducing cell cycle arrest and DNA repair. Lincet et al. (2000) found that overexpression of p21 in p53-null SKOV3 cells led to increased apoptosis in response to cisplatin. Nowak et al. (2003) found that lack of functional p21 gene accelerates caspase-independent apoptosis induced by cisplatin in renal cells of p21 knockout mice. They also reported that renal cell apoptosis is caspase independent in the presence of p21 but partially dependent on caspases in the absence of p21. These findings may explain why SKOV3 cells are more sensitive to cisplatin treatment than LX-1 SCLC cells (Table 1) and that sensitivity is at least partially due to the signaling difference shown in Fig. 6B.

MAPKs play an important role in regulation of cell proliferation and apoptosis (Wada and Penninger, 2004). In general, the ERKs are activated by mitogenic and proliferative stimuli. The cJun amino-terminal kinase (JNK) and p38 MAPKs respond to environmental stress and chemotherapeutic drugs. The different patterns of MAPK pathway activation may represent a regulatory signal by which cells respond to stress in a stimuli specific manner. The regulation of MAPK pathway by cisplatin is dependent upon dose/time and cell type. In A2780 ovarian carcinoma cells, ERK was weakly activated by a 24-h treatment with 33 μM cisplatin, whereas JNK was more significantly activated (Cui et al., 2000). In HaCaT cells, a 4-h treatment with 33 μM cisplatin did not activate ERK 1/2 but did activate JNK and p38 MAPK (Losa et al., 2003). Arany et al. (2004) found that 25 μM cisplatin significantly increased phosphorylation of all three MAPKs of mouse kidney cells. Another group reported no activation of ERK1/2 in HeLa cells after a 12-h treatment with 10 μM cisplatin, even though ERK activation was observed at higher cisplatin concentrations (Wang et al., 2000). In our study, ERK was activated by 50 μM cisplatin treatment for 24 h in LX-1 cells, but this effect was not found in SKOV3 cells with 17 μM cisplatin for 24 h; nevertheless, apoptosis was strongly activated in both cell types.

Although the MAPK pathway seems to be prominent, we do not exclude other kinases for the cisplatin-induced apoptosis. pretreatment with a specific MEK1 inhibitor (PD98059) did not protect LX-1 SCLC cells from cisplatin-induced apoptosis. The p38 MAPK inhibitor (SB203580) partially prevented cisplatin-induced PARP cleavage in LX-1 SCLC, suggesting cisplatin induced the p38 MAPK pathway to apoptosis. Our results suggested ERK activation may play a role for cisplatin sensitivity but is not required to mediate the cisplatin-induced apoptosis. In contrast, Arany et al. (2004) found that pretreatment of another phosphor-ERK inhibitor, U0126, significantly reduced cisplatin-induced apoptosis in mouse proximal tubule cells.

NAC Chemoprotection. NAC is a precursor of L-cysteine and the glutathione pathway and a scavenger of free radicals because it interacts with ROS (Zafarullah et al., 2003). The reactive thiol group on NAC may directly bind to and inactivate platinum agents, as has been shown with other thiols (Muldoon et al., 2001). Data suggesting that a direct effect of NAC on cisplatin binding includes the data showing that NAC protected against cisplatin-induced apoptosis up to 2 h after cisplatin only if NAC was present in the culture medium, and that loss of antiapoptotic effect of NAC was observed if NAC was washed off the cells. NAC inhibits activation of JNK, p38 MAP kinase, and nuclear factor κB transcription factor activities regulating expression of numerous genes (De Flora et al., 2001; Hashimoto et al., 2001). NAC can also prevent apoptosis and promote cell survival by activating ERK pathways (Wung et al., 1999; Li et al., 2000). This activity of NAC may lead to cell growth and differentiation. Some of the beneficial effects of NAC in cisplatin-induced apoptosis may be partly due to blockade of ERK or other MAPK pathways and stimulation of survival pathways. Our results showing protein changes as early as 5 to 10 min after cisplatin addition argue that this chemotherapeutic rapidly enters cells and interacts with nuclear DNA and that NAC reverses the toxicity at a downstream point.

The goal of these studies was to investigate the balance between cisplatin on cancer treatment efficacy and chemotherapeutic effects with NAC and to improve cancer therapy in patients. In vitro, NAC chemoprotection was lost if it was removed from cells prior to cisplatin (Fig. 5C), suggesting that the presence of NAC in the culture medium is required to have antiapoptotic effect, rather than a metabolite or increased glutathione. This in vitro result contrasts with the animal studies in which NAC administration 60 min prior to chemotherapy was bone marrow protective, even though the short (9–15 min) plasma half-life of NAC means that most NAC was cleared from the circulation by the time the chemotherapy was given (Neuwelt et al., 2004). We found that cisplatin-induced apoptosis was completely blocked when NAC was added prior to cisplatin or up to 1 h after cisplatin. The protection was reduced when NAC was added 2 to 8 h after cisplatin, after which no protection was noted. These data suggest that 1 to 2 h is the critical time period for rescue from cisplatin, and by 8 h, with high levels of PARP cleavage, cells are committed to apoptosis. In contrast to the rapid loss of NAC chemoprotection with delayed administration in vitro, our preclinical studies show that NAC is ototoxic at doses up to 4 h after high-dose cisplatin in a rat model (Dickey et al., 2004). Since NAC does not cross the blood-brain barrier (Muldoon et al., 2000), it will be useful to minimize systemic toxicities in brain tumor patients in whom the blood-brain barrier effectively makes two compartments. The timing and route of NAC administration may provide a mechanism to reduce cisplatin side effects in vivo, without compromising therapeutic efficacy for the treatment of human cancer.
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References


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