Protection against Cisplatin-Induced Toxicities by N-Acetylcysteine and Sodium Thiosulfate as Assessed at the Molecular, Cellular, and in Vivo Levels

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ABSTRACT

Cisplatin (CDDP) is a commonly used and effective chemotherapeutic agent. This study investigates chemoprotective effects of N-acetylcysteine (NAC) and sodium thiosulfate (STS) on in vitro and in vivo CDDP toxicities. For ototoxicity studies, CDDP (6 mg/kg) was administered to rats via a retrograde carotid artery infusion. Auditory brainstem response thresholds at 4 to 20 kHz were tested before and 7 days post-treatment. STS (8 g/m² i.v.) was administered at 4, 8, or 12 h after CDDP. For nephrotoxicity studies, rats were treated with CDDP intraperitoneally (10 mg/kg) before or after NAC (400 mg/kg) or STS (8 g/m²), and blood urea nitrogen (BUN) and creatinine concentrations were measured after 3 days. In vitro cytotoxicity and chemoprotection in human tumor cell lines were assessed by cell viability and immunoblotting assays. Rats treated with STS 4 h after CDDP exhibited no hearing change. The STS 8-h group had less otoprotection, whereas 12-h rats had ototoxicity. CDDP induced high BUN and creatinine, corresponding to renal tubule toxicities. All NAC-treated animals showed normal BUN and reduced creatinine levels compared with CDDP alone and no histopathological evidence of nephrotoxicity. Delayed STS treatment was not consistently protective against nephrotoxicity. STS administration fully protected against the in vitro cytotoxic and apoptotic effects of CDDP if added within 2 h of CDDP, but chemoprotection decreased if STS administration was 4 h, and was minimal by 6 h, after CDDP. Thus, the chemoprotection route and timing of administration can be manipulated to maintain CDDP antitumor efficacy while protecting against toxicities.

Cisplatin (CDDP) is a widely used and effective chemotherapeutic agent that binds to and alkylates DNA and triggers transcription inhibition, cell cycle arrest, and apoptosis (Sid-dik, 2003; Wu et al., 2005). In addition, CDDP generates reactive oxygen species (ROS), which are known as one of the pathogenic intermediates following chemotherapy (Masuda et al., 1994). CDDP is dose-limited by a high incidence of toxicities, including progressive and irreversible ototoxicity and nephrotoxicity. Animal models have demonstrated that the CDDP-induced hearing loss involves loss of the inner and outer hair cells of the inner ear (Blakley et al., 2002). Otoxicity has a significant negative impact on patient quality of life, especially in pediatric cases, where more than 50% of children develop ototoxicity (Gilmer-Knight et al., 2005). Even minimal and mild hearing loss in high-frequency regions above 2000 Hz considerably increase a child's risk for academic difficulties, social-emotional problems (Gilmer-Knight et al., 2005), and increased levels of fatigue in the learning environment (Bess et al., 1998). Methods to decrease this effect would increase the use and dosages of the effective platinum-based chemotherapeutic agents.

In the past, nephrotoxicity was a major consequence of CDDP treatment (Jones and Bassinger, 1989). The kidney accumulates CDDP to a higher degree than any other organ, resulting in necrosis of the terminal portion of the proximal tubule and apoptosis in the distal nephron (Arany and Safirstein, 2003). Toxic renal failure induces the production of ROS, which are responsible for the induction of tubular epithelial cell death. This is mediated by caspases and endo-

ABBREVIATIONS: CDDP, cisplatin, cis-diamminedichloroplatinum; ROS, reactive oxygen species; STS, sodium thiosulfate; NAC, N-acetylcysteine; BUN, blood urea nitrogen; CR, creatinine; ABR, auditory brainstem response(s); SCLC, small cell lung cancer; PARP, poly(ADP-ribose) polymerase.
nucleases (Basnakian et al., 2002). An increased rate of apoptosis has been detected in both human and experimental glomerular scarring, and CDDP induced apoptosis in cultured human proximal tubular epithelial cells (Razzaque et al., 2002). Although nephrotoxicity can be lessened with extensive hydration by crystalloid administration, it can still be dose limiting.

Antioxidants such as the endogenous tripeptide glutathione or exogenously administered thiols can protect against CDDP cytotoxicity in vitro (Wu et al., 2005). Animal studies (Church et al., 1995; Campbell et al., 1996; Neuwelt et al., 1996; Muldoon et al., 2000; Dickey et al., 2004) and clinical trials (Neuwelt et al., 1998; Robbins et al., 2000; Doolittle et al., 2001) have shown that thiosulfates can protect against platinum-induced otoxicity. Furthermore, Jones and Bassinger (1989) demonstrated that β-methionine provided CDDP nephroprotection.

Concerns about diminishing the oncological effects of CDDP have limited the clinical use of protective agents (Blakley et al., 2002). Muldoon et al. (2000) reported that delaying sodium thiosulfate (STS) for 8 h did not adversely affect its oncological activity but still offered protection from carboplatin-induced ototoxicity in a guinea pig model. Neuwelt et al. (2004) demonstrated that the efficacy of chemotherapy for rat brain tumors was not affected by thiol chemoprotection. A clinical trial showed otoprotection after carboplatin increased with a longer delay of STS infusion (Doolittle et al., 2001). The present study tested the effect of administering NAC or STS on the incidence and magnitude of CDDP-induced toxicities, using molecular, cellular, and in vivo models. NAC and STS both have about 15-min plasma half-lives, whereas the initial plasma half-life of CDDP is 25 to 49 min. We evaluated the impact of the timing of these agents to optimize separation of chemoprotection from CDDP antitumor efficacy.

Materials and Methods

Animals. Animal studies were performed in accordance with guidelines established by the Oregon Health & Science University Institutional Animal Care and Use Committee (IACUC) and with their approval of the protocols. Animals were housed at the Oregon Health & Science University Animal Facility and under the care of staff veterinarians.

Pharmacological Agents. Cisplatin (Platinol) was obtained from Bristol-Meyers Squibb (New York, NY), and N-acetylcysteine sterile solution was obtained from Abbott Laboratories (North Chicago, IL) via the Oregon Health & Science University hospital pharmacy. Sodium thiosulfate (Sigma-Aldrich, St. Louis, MO) was also obtained from the Oregon Health & Science University pharmacy.

CDDP Administration for the Ototoxicity Study. Long-Evans adult female rats were weighed, induced with isoflurane inhalant (2% ± 1.5% O₂), intubated, placed on a respirator, and prepped for surgery. Isoflurane was then replaced with propofol [800 μg/kg/min intravenously (i.v.)) and a 50% nitrous/oxygen mixture. A ventral midline incision was made from mandible to mandible in the animals beginning at 25 mg/kg i.p. CDDP). Three days after treatment, the animals were weighed, and 0.1-ml blood samples were taken for blood chemistry analysis of blood urea nitrogen (BUN) and creatinine (CR) using an i-Stat Portable Clinical Analyzer (Heska Corp., Ft. Collins, CO). Changes in BUN and CR were used to indicate differences in CDDP-induced nephrotoxicity between the NAC- and saline-treated rats. Some samples were subsequently subjected to pathological analysis, and the results were compared with the blood values. Rats were sacrificed using an intracardiac injection of pentobarbital, and the kidneys were resected and fixed by immersion in formalin for at least 3 days. Tissues were processed by ARUP Laboratories (Salt Lake City, UT), with 5-μm sections stained with H&E. Kidney sections were analyzed by Dr. Lawrence D. McGill of ARUP Animal Division.

Cell Culture and in Vitro Analyses. The B5 LX-1 small cell lung carcinoma (SCLC) cell line was maintained as a free-floating cell suspension in spinner flasks, in medium RPMI 1640 supplemented with 12% fetal bovine serum (Irvine Scientific, Santa Anna, CA) plus gentamycin, penicillin, and streptomycin. DAOY medulloblastoma cells, obtained from American Type Culture Collection (Manassas, VA), were cultured in minimal essential medium supplemented with 10% serum and antibiotics. The SKOV3 ovarian cancer cells and U87MG glioblastoma cells, obtained from Dr. Gail Clinton (Department of Biochemistry and Molecular Biology, Oregon Health & Science University), were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. Rat fibroblasts (Rat1), obtained from Dr. Bruce Magun at Oregon University Animal Facility and under the care of the protocols. Animals were housed at the Oregon Health & Science University pharmacy.
Health & Science University (Department of Cellular and Developmental Biology), were cultured in Dulbecco’s modified Eagle's medium with 5% serum and antibiotics. Live cell number was determined with the WST-1 Cell Proliferation Assay Kit from Chemicon International Inc. (Temecula, CA), as previously described (Wu et al., 2005). STS was added 0, 2, 4, 6, or 8 h after CDDP (30–50 μM) in four wells per condition and repeated two times in each cell type, and viable cell number was determined 44 to 48 h after CDDP addition.

Western blot analysis was performed as previously described (Wu et al., 2005). Both human lung carcinoma (LX-1 SCLC cells) and Rat1 fibroblasts were tested. Rabbit anti-poly(ADP-ribose) polymerase (PARP) antibody was obtained from Cell Signaling Technology (Beverly, MA) and anti-tubulin antibody from Sigma-Aldrich.

Statistics. Means and standard errors were determined using Microsoft Excel software or GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA). Statistical differences between groups were determined using an analysis of variance for repeated measures or Student's t test using Microsoft Excel software or GraphPad Prism 4 software.

Results

Ototoxicity. CDDP induces ototoxicity in rats if given intra-arterially so that the vertebral arteries are perfused, at doses less than other routes of administration (Dickey et al., 2004). Mean hearing thresholds following CDDP treatment for the saline- and STS-treated groups are shown in Fig. 1A–C. STS was ototoxic in a time-dependent manner. STS was significantly protective (p < 0.05) against CDDP-induced ototoxicity if given 4 h post-CDDP (Fig. 1A), similar to that found with NAC given 15 min prior to CDDP (Dickey et al., 2004). In the control group, the differences between pre- and post-treatment hearing thresholds were statistically significant at each frequency. In the 8-h STS group, no change from baseline hearing thresholds was found at low frequencies. Some ototoxicity was found at higher frequencies, but significant protection (p < 0.05) was found compared with saline controls (Fig. 1B). The treatment by time interaction indicates there is a significant pre-versus post-treatment difference for the STS 12-h post-CDDP group at every frequency, indicating no STS protection at this time point (Fig. 1C).

Nephrotoxicity. As shown in Fig. 2A, the 15 min prior to CDDP, NAC-treated rats had a normal BUN (mean = 23.6 mg/dl; p < 0.001) 3 days after the i.p. model of CDDP administration, whereas the CDDP + saline-treated rats had abnormally high BUN (mean = 130.8 mg/dl). The 30 min prior and 4 h post-NAC rats also had significantly lower BUN than the CDDP alone group (p < 0.01). Also, data in Fig. 2B indicate that the 15 min NAC prior to CDDP animals had normal CR levels (mean = 0.8 mg/dl), whereas the CR levels in the CDDP + saline-treated rats were abnormally elevated (mean = 8.2 mg/dl; p < 0.001). The NAC 30 min prior and 4 h post-CDDP also had significantly lower CR (p < 0.001). The STS-treated rats were provided with no consistent protection against renal toxicity, as indicated by high BUN (Fig. 2C), although the data for the STS 4-h post-CDDP group was mixed, with two individuals showing nephroprotection.

Table 1 presents the correlation of pathological interpretation of CDDP-treated kidneys with BUN results. There is a direct correlation between a high level of BUN and the presence of renal tubular damage found in histological samples of kidneys from the same animal. The pathologist was blinded to treatment and evaluated the kidney sections at random for the presence or absence of lesions with no regard to quantification. Kidneys from rats with high BUN showed varied severities of acute tubular degeneration. Inflammation was minimal. Occasional rats demonstrated more severe degeneration along the cortical medullary junction with calcification. Proteinuria in medullary tubules commonly accompanied tubular degeneration. These kidneys were severely damaged. Rats with normal BUN showed no significant lesions in the sections of kidneys. Figure 3 shows photomicrographs of representative kidney sections of rats with (Fig. 3A) and without (Fig. 3B) CDDP-induced renal tubular damage.

Cytotoxicity. The time dependence for STS chemoprotection was evaluated to determine how long the addition of chemoprotectant could be delayed after CDDP treatment and remain effective against cytotoxicity (Fig. 4). Incubation for 48 h with CDDP (17 μM) reduced cell viability by 57.6 ± 3.5% in U87 glioblastoma cells, 80.8 ± 3.7% in SKOV3 ovarian

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**Fig. 1.** Effect of STS post-treatment on ototoxicity. Normal Long-Evans female rats received saline or STS (8 mg/m² i.v.) postadministration of CDDP (6 mg/kg) by the intra-arterial technique. ABR thresholds were obtained prior to treatment and 7 days after CDDP. The data are shown as the mean change from baseline of hearing threshold measured in decibels, averaging both ears. A, effect of STS 4 h post-CDDP treatment on ototoxicity 7 days after CDDP. B, effect of STS 8 h post-CDDP treatment on ototoxicity 7 days after CDDP. C, effect of STS 12 h post-CDDP treatment on ototoxicity 7 days after CDDP.
carcinoma cells, and approximately 100% in both DAOY medulloblastoma cells and B.5 LX-1 SCLC cells. STS (2 mg/ml) was protective against CDDP-induced cytotoxicity when added either concurrently with CDDP or up to 2 h after CDDP. Delayed administration of STS reduced its protective activity against CDDP cytotoxicity. If STS was administered 4 h after CDDP, its protective activity was reduced to 30 to 40% of the maximal protection seen with concurrent administration, which was a significant decrease in all cell lines but remained significantly chemoprotective compared with no STS at all. When delayed until 6 h post-CDDP, STS showed no significant chemoprotective activity in any cell type tested. The magnitude of STS chemoprotection was dependent on CDDP concentration, particularly in the 2- to 4-h window (Fig. 4B).

Western Blot Analysis of Signaling and Apoptosis. To compare the difference of STS and NAC chemoprotective activity, we found that STS has slightly better protective effect against CDDP-induced apoptosis (Fig. 4). At the 0.25-mg/ml level, STS was completely protective against CDDP-induced cell death indicated by the presence of cleaved PARP protein. Both NAC and STS reverse CDDP-stimulated phosphorylation of extracellular signal-regulated kinase (Wu et al., 2005).

For both human lung carcinoma (LX-1 SCLC) and Rat1 fibroblast cell types, STS completely blocked CDDP-induced apoptosis when added within 1 h of CDDP treatment. PARP cleavage was apparent if NAC/STS administration was delayed until 2 h or later after CDDP treatment, although it appeared there was partial protection even when STS was delayed until 8 h after CDDP. After this time point, no protection was evident (Fig. 5). These data suggested that the presence of STS in the culture medium is required to have antiapoptotic effect.

Discussion

Platinum-based chemotherapy is currently the most effective form of chemotherapy for a variety of malignant tumors, including head and neck cancer (Blakley et al., 2002) and several pediatric malignancies. Ototoxicity is the primary dose-limiting factor in CDDP therapy (Blumenreich et al., 1985) that both reduces quality of life and restricts treatment protocols. CDDP-induced nephrotoxicity can be alleviated with hydration using crystalloid therapy but can still be dose limiting. This study evaluated molecular, in vitro, and in vivo chemoprotection against CDDP toxicities with the thiol STS.

Ototoxicity and Protection. CDDP is known to cause loss of hearing by progressive destruction of outer hair cells of the cochlea. The damage progresses from basal to apical and from outer to inner hair cells (Blakley et al., 2002). High frequencies are usually affected before low frequencies in humans who receive CDDP because the cochlea is tonotopically arranged. The model of CDDP-induced otoxicity in this study used an aortic delivery of CDDP, first reported by Dickey et al. (2004), and uses clinically relevant doses of CDDP (20–80 mg/m² is equivalent to 2.9 to 11.9 mg/kg in a 250-g rat), lower than the 15-mg/kg dose used in other studies (Campbell et al., 1996).

This study presents evidence that 4 h post-treatment with STS i.v. can prevent CDDP-induced ototoxicity, as indicated by changes in ABR threshold. STS delivered 8 h after CDDP provided less protection, all at the higher frequencies.

TABLE 1

<table>
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<tr>
<th>Treatment (Rat No.)</th>
<th>BUN (mg/dl)</th>
<th>CR (mg/dl)</th>
<th>% wt. lost</th>
<th>Renal tubule damage (path)</th>
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<td></td>
</tr>
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<td>-30.0</td>
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<tr>
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<td>1.1</td>
<td>-9.8</td>
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Correlation of BUN and CR levels with pathology results in rats after CDDP ± NAC

Long-Evans female rats were administered 10 mg/kg i.p. cisplatin ± NAC (400 mg/kg i.v.). Results were taken 3 days postcisplatin.
whereas STS given after 12 h provided no otoprotection. Previous studies in this lab found corresponding protection by NAC given in this fashion for both ototoxicity and systemic toxicity as indicated by weight loss (Dickey et al., 2004).

Most thiols are electrophilic and are thought to act as free radical scavengers. The mechanism of protection of STS, and perhaps NAC, may also be due to covalent binding of the molecule to the platinum, producing an inactive complex (Neuwelt et al., 2001; Fuertes and Castilla, 2003). Schweitzer (1993) showed that sulfur-containing compounds may prevent CDDP from interacting with target molecules, displacing platinum after it is bound. d-Methionine has been shown to protect against CDDP-induced ototoxicity (Campbell et al., 1996). NAC was shown to be protective in vitro of outer hair cells taken from guinea pigs (Feghali et al., 2001). Chemoprotection against platinum-induced ototoxicity was found in a clinical trial using delayed high-dose thiols for hearing protection (Doolittle et al., 2001). With STS otoprotection, there is no differential protective effect in normal cells versus tumor cells as occurs with amifostine. Since there is no selective prevention of CDDP effects in hearing cells compared with tumor cells, the issue then becomes delivery and timing.

**Nephrotoxicity and Protection.** We tested the hypothesis that pretreatment with the thiol agent NAC or post-treatment with STS would reduce renal toxicity of CDDP. CDDP induced nephrotoxicity in rats, as shown by significantly and abnormally high changes in BUN and CR. Rats that received NAC prior to CDDP had normal BUN values (Fig. 2, A and B). Post-treatment with STS showed no consistent renal protection, although the 4 h post-CDDP treatment protected some animals (Fig. 2C). As we have demonstrated with the data in Table 1, the BUN and CR levels correlate well with renal tubule damage seen in histological sections. A similar correlation between kidney histology and high BUN and CR levels after a single dose of CDDP was reported by Yildirim et al. (2003). NAC also protected against weight loss, which can be an indication of renal damage (Ammer et al., 1993; Zhang et al., 1999).

**Timing of Thiols for Chemoprotection.** Our in vitro studies show that STS was protective against CDDP-induced cytotoxicity when added either concurrently or 2 h after CDDP. The protective in vitro effect diminished after 4 h and was gone by 6 h. Data for in vivo otoprotection of STS (Fig. 1C) show a similar reduction in effect if administered 8 h or later after CDDP.

CDDP induces apoptosis via activation of caspase 3 (Siddik, 2003; Ludwig and Oberleithner, 2004; Wu et al., 2005). 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). STS blocks the apoptotic pathways induced by CDDP if given within 2 to 4 h of CDDP, but the protective effect wanes after 8 h and is not present after 12 h (Fig. 5). This is indicated by the presence of cleaved...
PARP protein and is similar to previous results in our lab with NAC (Wu et al., 2005). The timing of apoptosis is reflected in the results for oto- and nephroprotection of delayed STS. STS given 4 h after CDDP was otoprotective, less so after 8 h, and nonprotective if given 12 h after CDDP (Fig. 1C). The renal data show limited protection of the tubules by STS given 4 h after CDDP and no protection 8 or 12 h after (Fig. 2C). The thiols are also precursors of L-cysteine and the glutathione pathway and act as potent scavengers of free radicals (Zafarullah et al., 2003). Scavenging of ROS protects tubular epithelium from caspase activation and from cell death (Basnakian et al., 2002). The timing of CDDP administration after NAC becomes an issue, due to the rapid clearance of NAC (Neuwelt et al., 2001). Glutathione protection against CDDP-induced nephrotoxicity was found to be critically dependent on timing of thiol administration (Zunino et al., 1989).

The issue of potential interactions of chemoprotection with chemotherapy efficacy is of concern to all oncologists. Previous studies in this laboratory demonstrated that delayed STS administration after treatment with CDDP reduced ototoxicity in guinea pigs, at times and concentrations which did not reduce antitumor activity (Muldoon et al., 2000). In a second study (Neuwelt et al., 2004), we assessed STS and NAC in an intracerebral tumor model. Chemotherapy was delivered to the LX-1 small cell lung carcinoma brain tumor xenografts with carotid artery infusion and blood-brain barrier disruption, either before or after treatment with NAC and/or STS. We found that delayed administration of STS, 4 and 8 h after chemotherapy, did not alter antitumor efficacy against the intracerebral tumor model (Neuwelt et al., 2004). Delayed STS had no impact on the tumors either alone or in combination with NAC pretreatment, although bone marrow toxicity was significantly reduced (Neuwelt et al., 2004). These antitumor studies were done using carboplatin (diamine(cyclobutane-1,1-dicarboxylato(2-)-O,dO)-platinum), which may have less efficacy than CDDP against some tumors but also has fewer nonhematological dose-limiting toxicities. The current study focuses on CDDP, which is widely used in both adult and pediatric oncology. The reduction of the dose-limiting toxicities of CDDP would be a benefit in future clinical trials.

In summary, delayed administration of STS provides protection against CDDP-induced toxicities in vitro and in vivo ototoxicity rat model. NAC provides protection in the nephrotoxicity model. Current studies suggest the efficacy of CDDP therapy can be maintained with NAC and STS protection by separating the thiols from platinum in time and space (Muldoon et al., 2000; Doolittle et al., 2001; Neuwelt et al., 2004; Wu et al., 2005). Clinical trials using STS protection for brain tumor patients undergoing carboplatin chemotherapy after blood-brain barrier opening show that increasing the delay of STS administration from 2 to 4 h improved otoprotection, perhaps because it increased the STS to carboplatin ratio. As a result, there was a marked decrease in the need for hearing aids (Doolittle et al., 2001). The current results, together with previous in vivo tumor efficacy studies (Muldoon et al., 2000; Neuwelt et al., 2004), suggest that chemoprotection route and timing of administration can be manipulated to maintain CDDP antitumor efficacy while protecting against chemotherapy toxic side effects. As these studies used carboplatin instead of CDDP as in the current study, further in vivo experiments are necessary and under way to affirm the antitumor efficacy of CDDP in this model. These data would lead to considerations for further clinical trials.

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References


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