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N-acetylcysteine chemoprotection without decreased cisplatin antitumor efficacy in pediatric tumor models

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Abstract

BACKGROUND—Decreasing oxidative damage with the antioxidant agent N-acetylcysteine (NAC) can block the side effects of chemotherapy, but may diminish anti-tumor efficacy. We tested the potential for interactions of high dose NAC against a minimally effective cisplatin chemotherapy regimen in rat models of human pediatric cancers.

PROCEDURE—Athymic rats received subcutaneous implantation of human SK-N-AS neuroblastoma cells or intra-cerebellar implantation of human D283-MED medulloblastoma cells. Rats were untreated or treated with cisplatin (3 or 4 mg/kg IV) with or without NAC (1000 mg/kg IV) 30 min before or 4 h after cisplatin treatment. Blood urea nitrogen (BUN) and tumor volumes were measured.

RESULTS—Cisplatin decreased the growth of SK-N-AS neuroblastoma subcutaneous tumors from 17.7 ± 4.9 to 6.4 ± 2.5 fold over baseline 2 weeks after treatment ($P<0.001$). Pretreatment with NAC decreased cisplatin efficacy, while 4 h delayed NAC did not significantly affect cisplatin anti-tumor effects (relative tumor volume 6.8 ± 2.0 fold baseline, $P<0.001$). In D283-MED medulloblastoma brain tumors, cisplatin decreased final tumor volume to 3.9 ± 2.3 mm3 compared to untreated tumor volume of 45.9 ± 38.7 ($P = 0.008$). Delayed NAC did not significantly alter cisplatin efficacy (tumor volume 6.8 ± 8.1 mm3, $P = 0.014$ versus control). Cisplatin was minimally nephrotoxic in these models. NAC decreased cisplatin-induced elevations in BUN ($P<0.02$).

CONCLUSIONS—NAC chemoprotection did not alter cisplatin therapy, if delayed until 4 h after chemotherapy. These data support a Phase I/II clinical trial of delayed NAC to reduce ototoxicity in children with localized pediatric cancers.

Keywords

medulloblastoma; neuroblastoma; animal models; nephrotoxicity

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INTRODUCTION

The platinum-based chemotherapeutic agent cisplatin (Cis-diamminedichloroplatinum II) is widely used in the treatment of pediatric malignancies, including neuroblastoma and medulloblastoma [1]. Although the platinating agents are effective at tumor reduction, they induce a number of toxic side effects including nephrotoxicity, ototoxicity, peripheral neuropathy and bone marrow toxicity [2, 3] that impact patient health and the quality of life of survivors [4–6]. In the pediatric cancer population, cisplatin-induced hearing loss and electrolyte wasting can also lead to dose reductions or even discontinuation, resulting in decreased cancer control. Decreasing chemotherapy side effects therefore has the potential to improve patient treatment and long-term outcomes.

The mechanisms of cisplatin toxicity in the cochlea and the kidney involve oxidative damage due to generation of reactive oxygen species (ROS) [3, 7]. It is hypothesized that agents with reactive sulfhydryl groups will scavenge ROS and decrease oxidative stress, thus protecting cells and tissues from the toxic side effects of cisplatin chemotherapy. N-acetylcysteine (NAC) is a biologically relevant antioxidant that can protect against chemotherapy-induced bone marrow toxicity [8], nephrotoxicity [9–12] and ototoxicity [9, 13] in animal models.

Concerns about diminishing the anti-tumor effects of cisplatin have limited the clinical use of antioxidant protective agents. Several studies have shown no impact of NAC on chemotherapy efficacy in rodent models [8, 14, 15]. Clinical trials of another thiol chemoprotective agent, sodium thiosulfate (STS) were presented at the American Society for Clinical Oncology annual meeting in 2014 [16, 17]. In the Children's Oncology Group trial ACCL0431 STS met the study objective of protection against cisplatin-induced ototoxicity in pediatric cancer patients. STS did not alter survival in patients with localized tumors; however, a significant decrease in survival was found in patients with disseminated disease, raising concerns about the safety of thiol chemoprotection [16].

We have previously demonstrated that delayed administration of NAC until 4 h after cisplatin did not block cisplatin-induced apoptosis in cancer cells in vitro [18], but significantly blocked cisplatin-induced nephrotoxicity [10] and ototoxicity in vivo [13]. We hypothesized that delayed administration of NAC would provide chemoprotection without decreasing anti-tumor efficacy. The purpose of these studies was to evaluate the potential for interactions of high dose NAC against a minimally effective cisplatin chemotherapy regimen in rat models of human pediatric cancers.

METHODS

Cell culture and reagents

SK-N-AS human neuroblastoma cells, derived from a 6 year old female, and D283-MED human medulloblastoma cells, derived from a 6 year old male, were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were confirmed mycoplasma free and used under passage 20. Cells were cultured in DME with 10% FBS in

5% CO2 at 37C. Sterile cisplatin (1 mg/ml) and NAC (200 mg/ml) were obtained from the Oregon Health and Sciences University (OHSU) pharmacy.

Animal studies

The care and use of animals was approved by the Institutional Animal Care and Use Committee and were supervised by the OHSU Department of Comparative Medicine. Female athymic nude rats (rnu/rnu, 200–250 g; from the OHSU Blood Brain Barrier Program in-house colony) were pretreated with cyclophosphamide (100 mg/kg IP) 1 d before tumor cell inoculation, as previously demonstrated to improve tumor growth [19].

Neuroblastoma study

Rats were anesthetized with 3% Isoflurane with oxygen. Human SK-N-AS neuroblastoma cells (2×107 in 200 μl) were injected subcutaneously in both the left and right flanks. Rats (n = 10 per group) were entered into the study 1 week after tumor implantation when tumors were 0.6–0.9 cm3 in volume. The treatment groups were: untreated control, cisplatin 3 mg/kg IV, or NAC (1000 mg/kg IV) 30 min before or 4 h after cisplatin treatment. Rats were treated on day 0 and 4 of the study (day 7 and day 11 after inoculation). Tumor growth was measured twice a week by dial caliper in the first 8 animals per group and tumor volume was calculated using the formula (length × width2)/2. Whole blood was withdrawn through the jugular vein on day 7 and 14 of treatment. Blood urea nitrogen (BUN) levels and the concentration of magnesium and phosphate were analyzed on a DRI-CHEM 4000 Veterinary Chemistry Analyzer (HESKA, Waukesha, WI, USA). Rats were weighed twice a week and assessed daily for signs of toxicity. All rats were euthanized at 2 weeks after initial treatment, and subcutaneous tumors were excised and weighed.

Medulloblastoma study

Rats were anesthetized with intraperitoneal ketamine (60 mg/kg) and intraperitoneal diazepam (7.5 mg/kg). Human D283-MED medulloblastoma cells (106 in 15 μl) were stereotactically injected in the cerebellum (vertical, bregma 5 mm; lateral, bregma 0; posterior, bregma 11mm). The needle was initially advanced to a depth of 5.5 mm and then withdrawn to a depth of 5 mm to limit reflux up the needle track. Rats (n = 8 per group) received a single treatment 15 days after tumor implantations in the following groups: untreated control, cisplatin 4 mg/kg IV, or NAC (1000 mg/kg IV) 30 min before or 4 h after cisplatin treatment. Seven days after treatment, blood was drawn for BUN and creatinine measurement using an I-Stat Portable Clinical Analyzer and rats were euthanized. Brains were excised, fixed in 10% buffered formalin, and egg-gelatin embedded for vibratome sectioning at 100 μm in the coronal plane. For tumor volumetrics, every 6th brain section was stained for human mitochondrial antigen (Abcam, Cambridge, MA) and then imaged at 35 μm pixel diameter on an Epson 1640XL flatbed scanner using Adobe Photoshop software. Tumor volume was assessed using NIH ImageJ software by a biologist blinded to treatments (LLM) as previously described [8].

Statistical considerations

No power calculations were made a priori or post hoc. For the subcutaneous tumors, changes from baseline volume, with each animal serving as its own pre-treatment control were compared within groups and between groups using ANOVA with Bonferroni's multiple comparisons test. Final tumor weight (subcutaneous neuroblastoma), tumor volume (intra-cerebellar medulloblastoma) and blood analyses were compared using ANOVA and Student's t-test for comparison of individual groups. Analyses were performed with Microsoft Excel and Graphpad Prism software.

RESULTS

Neuroblastoma tumor study

SK-N-AS human neuroblastoma cells grew quickly when implanted subcutaneously in nude rats, with tumor volumes of 0.6 – 0.9 cm3 measured 1 week after implantation, when rats were randomized to treatment groups. Figure 1A shows the time course for subcutaneous tumor growth in the four treatment groups. Untreated control tumors had significantly increased tumor volume at every measurement time point with a final relative tumor volume of 17.7 ± 4.9 compared to baseline at the 2 week end of study. Treatment with two doses of cisplatin (day 0 and day 4) decreased tumor growth to 6.4 ± 2.5 fold over baseline ($P<0.0001$ compared to untreated control). Administration of high dose NAC 30 min prior to cisplatin significantly decreased chemotherapy efficacy. Tumor volume was not different from untreated control at any time point and final tumor volume, 15.0 ± 4.8 fold over baseline, was significantly larger than cisplatin alone ($P < 0.0001$). In contrast, delay of NAC until 4 h after cisplatin did not significantly affect cisplatin anti-tumor effects. Tumor volume in the delayed NAC group was significantly lower than control at every time point ($P < 0.001$) and not different from cisplatin alone, with final relative tumor volume 6.8 ± 2.0 fold baseline ($P<0.0001$ versus control). After the 2-week caliper measurement the tumors were excised and weighed (Figure 1B). Final tumor weight for control tumors was 4.3 ± 1.6 g and for cisplatin-treated tumors was 1.7 ± 0.9 g. Tumor size in the NAC pretreatment group averaged 4.3 ± 1.8 g (P not significant compared to untreated control), while in the delayed NAC group tumors weighed 2.0 ± 0.6 g ($P < 0.0001$).

Medulloblastoma tumor study

D283-MED human medulloblastoma tumor growth was inconsistent after implantation into the cerebellum in nude rats. In 11 rats (34%), including representatives of all treatment groups, tumor cells invaded the cerebrospinal fluid (CSF), likely by leaking up the needle track. Tumor in these rats grew in the cerebellum but also in a clinically typical "sugar coating" pattern along the surfaces of the ventricles [20]. Figure 2 shows immunohistochemistry for human mitochondrial antigen in a representative brain from the untreated control and cisplatin-treated groups demonstrating the localized and disseminated patterns of growth. The volumes of the tumor mass in the cerebellum in untreated control rats ranged from 0.1 to 123 mm3, with a mean of 45.9 ± 38.7 mm3 (Figure 3A). A single treatment with cisplatin decreased final cerebellar tumor volume to 3.9 ± 2.3 mm3 ($P = 0.008$ compared to untreated control). In rats pretreated with NAC 30 min prior to cisplatin, some tumors were large and tumor volume (13.3 ± 23.1 mm3) was not different from

untreated control. In contrast, the cerebellar tumor volume in rats receiving cisplatin with delayed NAC was 6.8 ± 8.1 mm3, which was not significantly different than cisplatin alone and was different from untreated controls ($P < 0.014$). Figure 3B shows total tumor volumes including growth in the CSF space. Inclusion of the leptomeningeal tumor did not significantly alter the mean tumor volume of any group or the significant differences between groups.

Nephroprotection

Blood samples from both tumor treatment studies were evaluated for NAC chemoprotection against kidney toxicity. BUN values were in the normal range for all untreated control rats, 18.8 ± 2.9 mg/dL in the neuroblastoma study and 17.6 ± 2.5 mg/dL in the medulloblastoma study.

In the neuroblastoma study, rats receiving two doses of cisplatin (3 mg/kg) on day 0 and day 4 showed significantly increased BUN levels at both 7 days (57.3 ± 19.9 mg/dL compared to 18.9 ± 2.0 in controls, $P < 0.0001$, Figure 4A) and 14 days (58.6 ± 30.9 compared to 18.8 ± 3.8 in controls, $P = 0.0008$, Figure 4B) after treatment. NAC significantly decreased BUN levels compared to cisplatin alone, whether it was given prior to cisplatin (25.3 ± 6.8, $P < 0.001$, at 7 days; 24.6 ± 7.2, $P = 0.0033$, at 14 days), or 4 h after cisplatin (27.9 ± 7.5, $P < 0.001$, at 7 days; 29.5 ± 7.1, $P = 0.01$, at 14 days). NAC did not provide complete protection, as BUN levels remained elevated compared to control ($P < 0.01$). Blood concentrations of magnesium and phosphorus were within normal parameters in all animals in this experiment. These rats showed no evidence of additional toxicities such as weight loss: Day 14 after treatment, relative body weight was Control, 102.6±5.0; cisplatin, 100.7±4.5; NAC 30 min prior, 104.7±3.4; NAC 4 h after, 101.8±2.5.

In the medulloblastoma study, a single dose of cisplatin (4 mg/kg) caused a modest and inconsistent increase in BUN levels at 1 week after treatment (32.4 ± 10.4, $P = 0.0017$; Figure 4C). Pretreatment with NAC did not protect against nephrotoxicity in this study, with BUN concentrations of 32.1 ± 9.8. There was a trend toward a lesser increase in BUN levels in rats that received delayed treatment with NAC, but BUN concentrations remained significantly elevated compared to controls. Creatinine concentrations were also measured in blood samples from the first four rats in each group, but this measurement was discontinued because there was no impact of cisplatin, with all values in all rats in the normal range at 0.3 – 0.4 mg/dL.

DISCUSSION

Despite the surge in development of targeted therapeutics, cisplatin chemotherapy remains an integral component of treatment for a wide range of pediatric cancers including germ cell tumors, osteosarcoma, hepatoblastoma, neuroblastoma, and medulloblastoma [1]. Together these malignancies account for nearly 40% of all childhood cancer exposing over 2,000 children to cisplatin each year. As many as 60% of these children will experience permanent cisplatin-induced hearing loss, with long term impacts on speech and language development, educational achievement, and quality of life [5, 6, 21]. Nephrotoxicity, particularly magnesium and calcium wasting and long-term effects on kidney function also decrease

survivor quality of life [4]. With cure rates of over 80% in pediatric solid tumors, it is likely that cisplatin chemotherapy will remain the standard of care for some time; therefore it is incumbent on the pediatric oncology community to address issues of quality of life. Our goal is to decrease the toxic side effects of cisplatin chemotherapy in the vulnerable pediatric population to improve patient health and response during treatment and to enhance long-term quality of life by reducing lasting toxicities.

A number of antioxidants containing a reactive sulfhydryl group have been proposed as chemoprotective agents, including amifostine [22, 23], D-Methionine [24, 25], STS [9, 16, 24, 26, 27] and NAC. NAC is a promising agent for chemoprotection in pediatric cancers. It has a wide range of protective effects in animal models, decreasing bone marrow toxicity [8], nephrotoxicity [9, 12, 14, 15] and ototoxicity [9, 13]. NAC is already commonly used in children because it is the standard antidote for acetaminophen overdose [28]. For its antidote role, NAC is administered orally at a dose of 140 mg/kg. In contrast, dose, timing and route must be adjusted to optimize chemoprotection with NAC. First, oral NAC is substantially cleared by its first pass through the liver so it must be administered by IV or intra-arterial injection for chemoprotection [10]. Secondly, the chemoprotective dose of NAC is moderately higher than the antidote dose. Peak NAC blood concentrations of 1.5 to 2 mM are necessary for chemoprotection and are provided by IV NAC at 400 mg/kg [8, 10]. The NAC dose of 1 g/kg used in the current experiments gives peak blood concentrations of >6 mM [10], and was chosen to maximize any potential anti-efficacy effects. Finally, delayed administration of both NAC and STS until 4 or even 8 h after platinum chemotherapy maintains otoprotective and nephroprotective activity [9, 10, 13]. Delayed chemoprotection has a mechanistic rationale. It maintains the early DNA alkylation mechanism of cisplatin in rapidly dividing cancer cells but provides antioxidant protection against the ROS that are likely the primary culprit of toxicity in differentiated cells.

The possibility of decreasing the efficacy of chemotherapy has blocked the clinical use of most chemoprotective agents. Tumor protection is a major issue in two current clinical trials of STS. SIOPEL-6 is currently accruing patients in a study of STS otoprotection in hepatoblastoma treated with cisplatin [17]. Survival data for SIOPEL-6 are not yet available, but interim analyses after 20, 40, 60, and 80 patients ruled out adverse short-term effects of STS on cisplatin efficacy. In COG study ACCL0431, subjects with a variety of childhood cancers treated with cisplatin were randomized either to observation or treatment with STS 16 g/m2 IV over 15 minutes 6 hours after each cisplatin dose and then followed for over 2 years [16]. Hearing loss was found in 55.4% of the observation group and 28.6% in the STS group ($p=0.006$) and the effect of STS was even more pronounced in children under 5. For subjects with localized disease, STS showed no effect on survival, with overall survival 89.7% in the STS group versus 89.2% in the observation group ($p=0.73$). In contrast, STS did significantly decrease survival in patients with disseminated disease, with overall survival of 49.0% in the STS group compared to 88.3% in the observation arm ($p=0.010$).

We hypothesize that NAC will provide better overall chemoprotection than NAC with less tumor interaction. Previous studies have found that pretreatment with NAC did not alter the effect of carboplatin in a lung cancer brain metastasis model [8], or the effect of ifosfamide in rodent models of neuroblastoma [14] and Ewing's sarcoma [15]. The current studies

demonstrate that delayed timing of NAC is essential to avoid impacting the antitumor efficacy of cisplatin. Our results do not answer the question of whether NAC can be used in disseminated medulloblastoma. In our medulloblastoma model, the extent of dissemination appeared similar in all treatment groups, suggesting that the disseminated tumor was unaffected by either cisplatin or NAC. However, leptomeningeal spread was found in only a third of the rats, which does not provide power to draw any conclusions and indicates the need for a targeted study.

A limitation of the current studies was the inconsistency of the medulloblastoma tumor model. All untreated control rats showed evidence of tumor growth, but no animal was excluded from the study even though tumor mass was minimal in some rats. Dissemination into the CSF space was found in only a subset of rats. A future study will use tumor injection into the right cerebral ventricle to produce disseminated medulloblastoma, while rats with cerebellar xenografts will undergo pretreatment MRI to confirm the presence of a tumor mass and animals with enlarged ventricles suggestive of CSF dissemination will be excluded from the localized tumor group. A second limitation of the current studies was that otoprotection could not be measured in these animals. Rats are relatively insensitive to cisplatin ototoxicity [9, 13, 24]. The cisplatin regimens used in these studies was targeted to provide therapeutic efficacy and were minimally nephrotoxic and not ototoxic.

The current study provides important data for the development of a clinical trial for otoprotection with NAC in pediatric patients. The results from the COG ACCL0431 study of STS raise concerns about the ethical design for studies of chemoprotective agents. How can clinical studies be designed to increase our understanding of chemoprotection and improve long-term outcomes, while not risking reduced efficacy of the chemotherapeutic? The hearing protection without tumor protection found in localized disease in ACCL0431 will make it very difficult to recruit patients to a no chemoprotection control arm in any future Phase III trial. Additionally, NAC and STS are inexpensive and widely available, so there is no interest from pharmaceutical companies in running a large and expensive non-inferiority study. We propose the following design for a Phase I/II study. The target population will be children with localized osteosarcoma, neuroblastoma, and medulloblastoma who will undergo treatment with cisplatin-based chemotherapy. A limited toxicity study will be done to evaluate the tolerability of 300 and 400 mg/kg NAC administered 4 h after cisplatin, with blood samples drawn to ensure chemoprotective blood concentrations of 1.5–2 mM NAC. We will then conduct a randomized controlled trial to do a head-to head comparison of the efficacy of NAC versus STS in preventing cisplatin-induced toxicities. A sensitive measure of ototoxicity such as the International Society of Pediatric Oncology Boston ototoxicity scale [29] must be used to establish the degree of ototoxicity/otoprotection.

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Abbreviations

BUN	Blood urea nitrogen
NAC	N-acetylcysteine
ROS	reactive oxygen species

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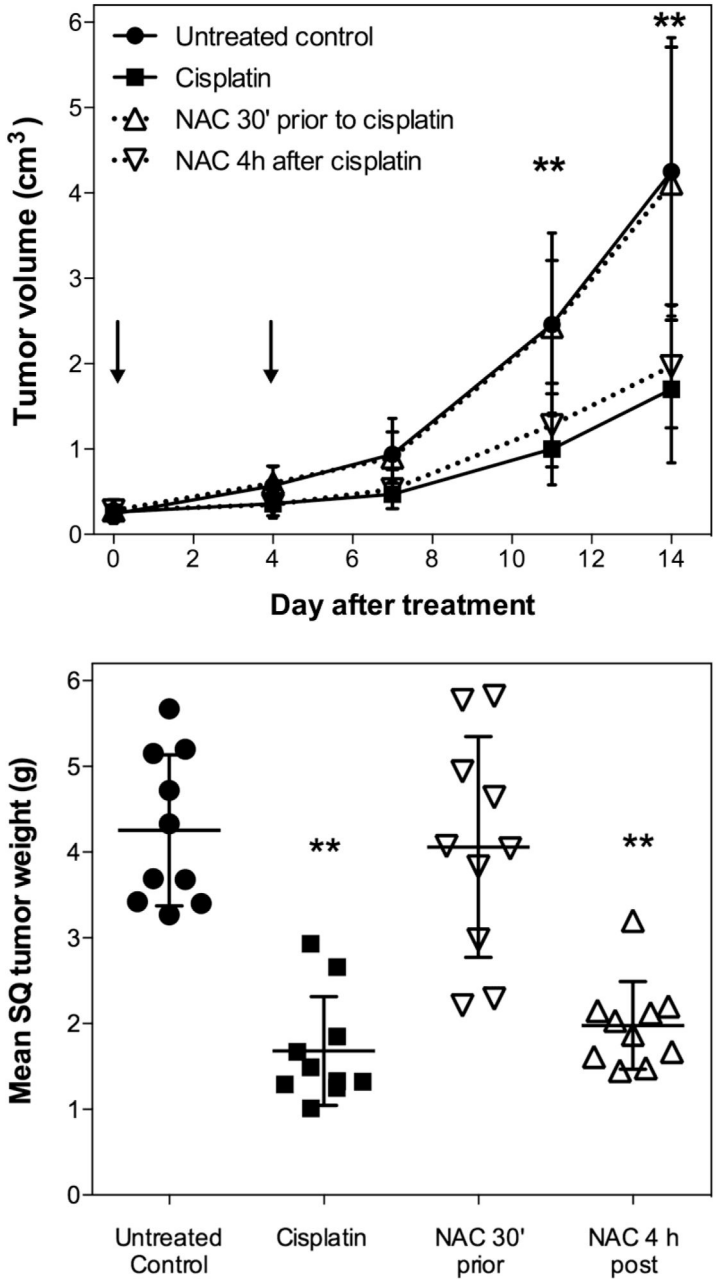

Figure 1.

Effect of cisplatin and NAC in neuroblastoma. Nude rats with subcutaneous SK-N-AS human neuroblastoma xenografts were untreated (circles) or received treatment on days 0 and 4 with cisplatin (3 mg/kg IV, squares), or NAC (1000 mg/kg IV) 30 min prior to cisplatin (inverted triangles) or 4 h after cisplatin (triangles). Panel A. Changes in tumor volume over time, as measured twice weekly by dial caliper. Arrows indicate cisplatin treatment times. Panel B. Final tumor weight at 2 weeks after initial treatment. ** indicates that the cisplatin and delayed NAC groups significantly differed from untreated control ($P < 0.001$). SQ, subcutaneous; NAC, N-acetylcysteine.

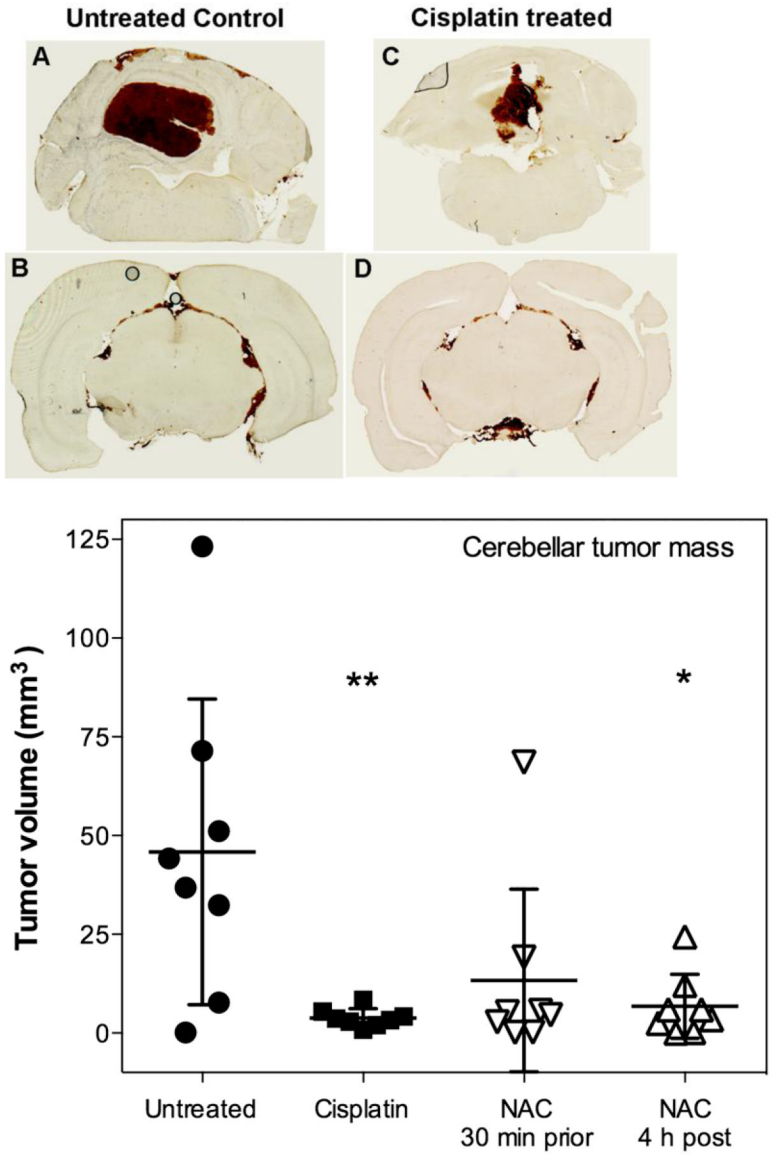

Figure 2.

Medulloblastoma tumor model. Nude rats received D283-MED human medulloblastoma implanted in the cerebellum. Immunohistochemistry for human mitochondrial antigen to visualize human tumor cells is shown for an untreated control rat (A, B) and and cisplatin treated rat (C, D). All animals showed evidence of tumor in the cerebellum (A, C). Additionally, 34% (11/32 rats) showed infiltration of tumor cells into the CSF space and tumor growth on the meningeal surfaces as far anterior as the midbrain (B, D).

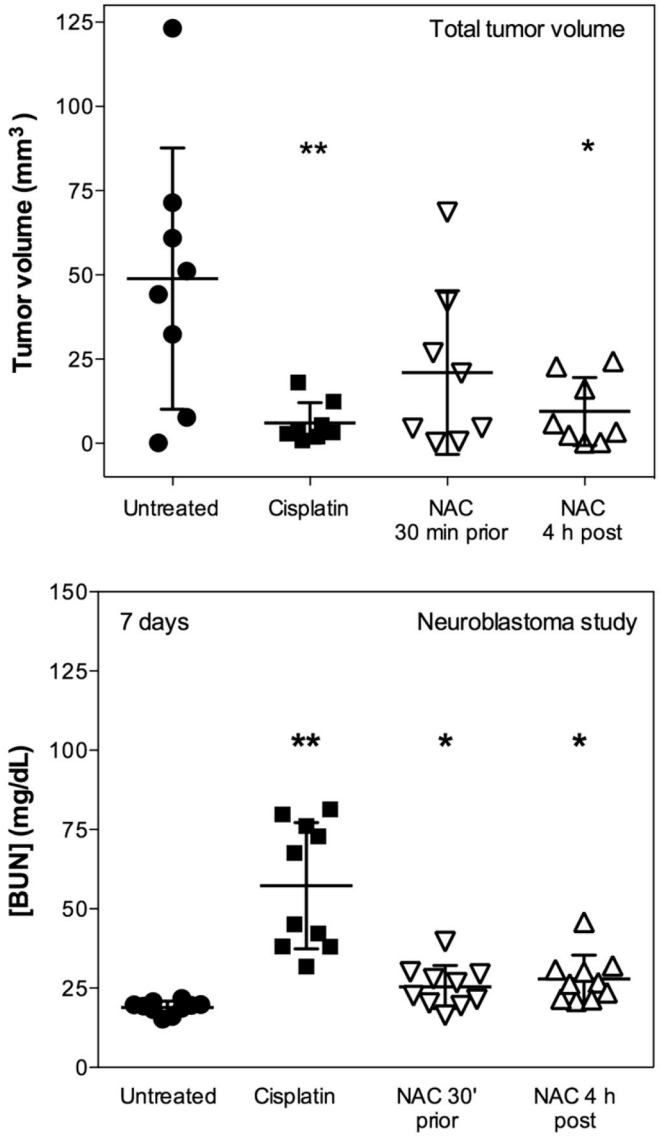

Figure 3.

Effect of cisplatin and NAC in medulloblastoma. Nude rats with D283-MED human medulloblastoma implanted in the cerebellum were untreated (circles) or received a single treatment with cisplatin (4 mg/kg IV, squares), or NAC (1000 mg/kg IV) 30 min prior to cisplatin (inverted triangles) or 4 h after cisplatin (triangles). Tumor volume was measured one week after treatment. Panel A. Tumor mass localized to cerebellar injection site. Panel B. Total tumor including cerebellum and meningeal infiltration. ** $P < 0.01$, * $P < 0.02$ compared to untreated control.

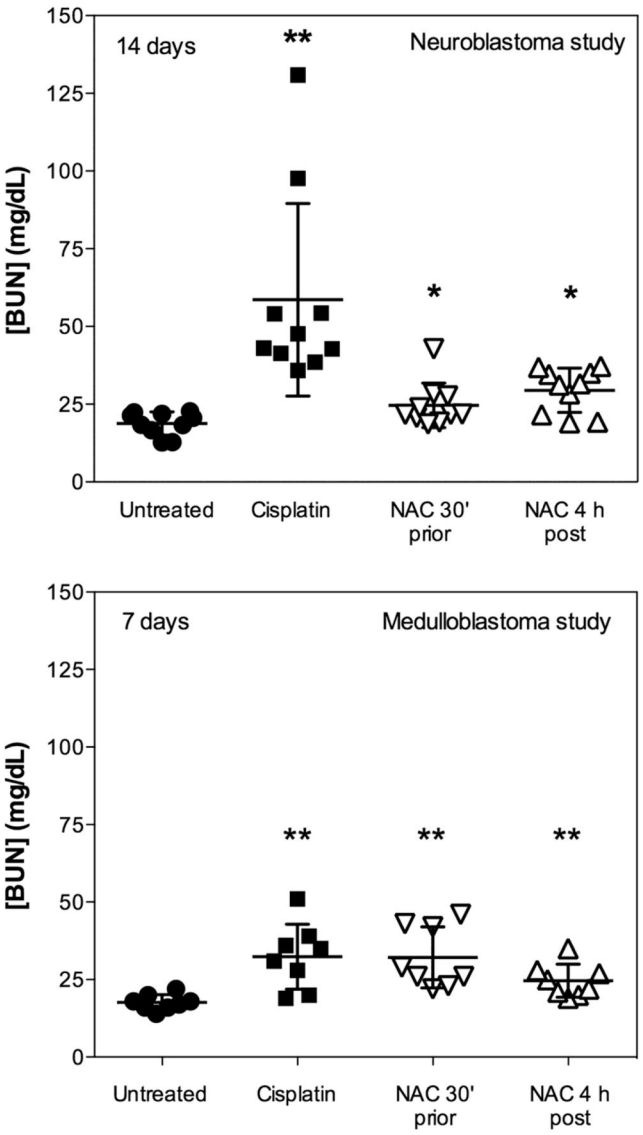

Figure 4.

NAC chemoprotection against cisplatin nephrotoxicity. Blood was drawn from the rats in the medulloblastoma and neuroblastoma tumor studies at the indicated time after initial treatment. The treatment groups were: untreated control (circles); cisplatin treatment (3 mg/kg X2 for neuroblastoma study, 4 mg/kg X1 for medulloblastoma study, squares); NAC (1 g/kg IV) given 30 min prior to cisplatin (inverted triangles); NAC given 4 h after cisplatin (triangles). ** $P < 0.01$; * $P < 0.05$ compared to untreated control.