Sodium Thiosulfate Administered Six Hours after Cisplatin Does Not Compromise Antineuroblastoma Activity

Theresa M. Harned,[1] Ondrej Kalous,[1] Alexander Neuwelt,[2] Jason Loera,[2] Lingyun Ji,[1] Peter Iovine,[2] Richard Sposto,[1] Edward A. Neuwelt,[3] and C. Patrick Reynolds[1]

Abstract

Purpose: We determined if the potentially otoprotective agent sodium thiosulfate (STS) could be given 6 h after cisplatin without diminishing the antineuroblastoma activity of cisplatin in human neuroblastoma cell lines *in vitro* (including cisplatin-resistant cell lines) and in neuroblastoma xenografts *in vivo*.

Experimental Design: We determined the antineuroblastoma activity of cisplatin with or without the addition of STS at 0 or 6 h after cisplatin in six neuroblastoma cell lines, both in standard cell culture conditions (20% O_2) and in physiologic hypoxia (2% O_2). Drug cytotoxicity was measured using the DIMSCAN fluorescence/digital imaging microscopy assay. *In vivo* studies of cisplatin combined with STS used a human neuroblastoma subcutaneous xenograft model (SMS-SAN) in athymic nu/nu mice.

Results: A significant protection against cisplatin cytotoxicity was seen when the neuroblastoma cells were exposed to cisplatin directly combined with STS. However, when cisplatin was given first and STS exposure occurred 6 h later, no effect on cisplatin cytotoxicity was observed. In a subcutaneous neuroblastoma xenograft model in nu/nu mice, mice receiving cisplatin alone or cisplatin + STS at 6 h had significantly better progression-free survival rates ($P < 0.03$) compared with controls or mice treated with cisplatin + STS concurrently. There was no statistically significant difference in outcomes between mice treated with cisplatin alone and the group treated with cisplatin followed by STS 6 h later ($P = 0.9$).

Conclusion: These preclinical data suggest that the use of STS 6 h after cisplatin for otoprotection is unlikely to compromise the antineuroblastoma activity of cisplatin.

Neuroblastoma is an aggressive childhood neoplasm of the sympathetic nervous system. High-risk disease includes patients >1 year old with stage 4 disease, stage 3 tumors with unfavorable histopathology, or any stage tumor with *MYCN* amplification (1). Treatment with intensive multiagent chemotherapy followed by purged autologous bone marrow transplant and post–autologous bone marrow transplant 13-*cis*-retinoic acid has improved outcome for high-risk neuroblastoma patients (2). However, >50% of high-risk patients ultimately die from progressive or recurrent disease (3).

The platinum chemotherapeutic agents cisplatin and carboplatin are used in induction and myeloablative chemotherapy for high-risk neuroblastoma but cause significant ototoxicity in children. Platinum-induced hearing loss is progressive and irreversible and involves degeneration of the outer hair cells of the inner ear (4–6). More than 50% of children treated with cisplatin for malignancy develop ototoxicity (7, 8), and ototoxicity was observed in 82% of neuroblastoma patients who were treated with cisplatin and autologous stem cell transplant using carboplatin in the conditioning regimen (9).

Sodium thiosulfate (STS) is a reactive thiol agent used clinically as an antidote to cyanide or nitroprusside poisoning. At high molar excess, STS binds to and inactivates the electrophilic platinum compound *in vitro* (10, 11). STS has been explored as both a nephroprotective and an otoprotective agent with platinum-based chemotherapy. In a guinea pig model, STS was found to be otoprotective when given up to 8 h, subsequent to carboplatin, and at 2 h after cisplatin, as determined by electrophysiologic measurements of auditory function and counts of remaining inner ear outer hair cells (12). Rats treated with STS 4 h after cisplatin exhibited no hearing change, whereas STS at 8 h provided less otoprotection, and rats who received STS at 12 h after cisplatin had ototoxicity

Authors' Affiliations: [1]Developmental Therapeutics Program, USC-CHLA Institute for Pediatric Clinical Research and Division of Hematology-Oncology Department of Pediatrics, Keck School of Medicine, University of Southern California and Children's Hospital Los Angeles, Los Angeles, California; [2]Department of Chemistry, University of San Diego, San Diego, California; and [3]Department of Neurology, Oregon Health and Science University, Portland, Oregon

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Requests for reprints: C. Patrick Reynolds, Developmental Therapeutics Program, USC-CHLA Institute for Pediatric Clinical Research, MS 57, Childrens Hospital Los Angeles, Los Angeles, CA 90027. Phone: 323-361-5646; Fax: 323-664-9226; E-mail: preynolds@chla.usc.edu.

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(13). Doolittle et al. found that STS delayed to 4 h after carboplatin significantly decreased both the time to development of ototoxicity and the degree of ototoxicity when compared with a historical control group and was superior to STS delayed to only 2 h post-carboplatin in patients with malignant brain tumors (14).

A clinical trial assessing the otoprotective effect of STS in neuroblastoma would have to be conducted in newly diagnosed patients (using STS during induction chemotherapy). Concerns that STS might negatively affect the antitumor activity of cisplatin have been raised. Muldoon et al. reported that delaying the administration of STS for 6 to 8 h after carboplatin did not adversely affect its antitumor activity using a human small cell lung cancer rat xenograft model, but still offered protection from ototoxicity in guinea pigs (15, 16). Dickey et al. found that STS was protective from cisplatin cytotoxicity in glioblastoma, SKOV3 ovarian carcinoma, medulloblastoma, and small cell lung cancer cell lines when added concurrently or up to 2 h post-cisplatin, but with a delay in STS administration to 6 h, there was no significant chemoprotective activity in any cell type (13).

To address concerns that STS could affect the antineuroblastoma activity of cisplatin, we investigated the effect of STS on cisplatin antitumor cytotoxicity for neuroblastoma cell lines and in xenografts in nu/nu mice. Here, we show that STS given 6 h after cisplatin does not reduce cisplatin cytotoxicity in representative neuroblastoma cell lines, even under hypoxic conditions, and that STS delayed to 6 h after cisplatin in SMS-SAN xenografts in athymic nu/nu mice also did not affect cisplatin antitumor activity.

Materials and Methods

Cell lines. We used a panel of six neuroblastoma cell lines obtained from patients in early phases of therapy: four drug-sensitive lines established at diagnosis before receiving any therapy (CHLA-15, CHLA-122, CHLA-42, SMS-SAN), one drug-sensitive line established at the time of progressive disease during dual-agent induction therapy (SMS-KCNR), and one moderately drug-resistant line established at the time of progressive disease during intensive multiagent chemotherapy (CHLA-20). Neuroblastoma origin of these cell lines has been previously confirmed (17). Four cisplatin-sensitive (CHLA-15, SMS-SAN, CHLA-122, SMS-KCNR) and two cisplatin-resistant cell lines (CHLA-42 and CHLA-20) were used in this study.

SMS-KCNR and SMS-SAN were cultured in complete medium composed of RPMI 1640 (Irvine Scientific) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Inc.). CHLA-15, CHLA-20, CHLA-42, and CHLA-122 were cultured in complete medium made from Iscove's modified Dulbecco's medium (BioWhittaker) supplemented with ≈3mmol/L L-glutamine (Gemini Bioproducts), 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenous acid (ITS culture supplement; Collaborative Biomedical Products) and 20% heat-inactivated fetal bovine serum. The cell lines were cultured at 37°C in a humidified incubator containing 95% air + 5% CO_2 atmosphere without antibiotics. For cytotoxicity assays in reduced oxygen conditions (2% O_2), microplates were cultured in an insulated reduced oxygen incubator set to an oxygen tension of 2% O_2 (18) below the degree of hypoxia found in bone marrow (4-5% O_2) and in the range of hypoxia found in tumor tissue.

Drugs and chemicals. Cisplatin was obtained from Bedford Laboratories. Clinical grade sodium thiosulfate was obtained from American Reagent. Etoposide was obtained from Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute. Carboplatin was obtained from NIH. Fluoresceine diacetate was obtained from Eastman Kodak Co., and eosin Y was from Sigma Chemical Co.

Cytotoxicity assays. The cytotoxicity of chemotherapeutic agents with or without the addition of STS was determined with the DIMSCAN assay system (19, 20). DIMSCAN uses digital imaging microscopy to quantify viable cells, which selectively accumulate fluoresceine diacetate. DIMSCAN is capable of measuring cytotoxicity over a four to five log dynamic range by quantifying total fluorescence per well (which is proportional to viable, clonogenic cells) after eliminating background fluorescence with digital thresholding and eosin Y quenching (20, 21). Cell lines were seeded at 5,000 to 15,000 cells in 100 μL of complete medium per well into 96-well plates. After overnight incubation, various clinically achievable concentrations of cisplatin, carboplatin, or etoposide in 50 μL of complete medium were added to each well. The final cisplatin concentrations ranged from 0 to 2 μg/mL, the final carboplatin concentrations ranged from 1 to 12 μg/mL, and the final etoposide concentrations ranged from 0 to 10 μg/mL. Each condition was tested in 12 replicates. STS at the clinically achievable concentrations of 0.5 or 1 mg/mL was then added in 100 μL of complete medium at either hour 0 or hour 6 after cisplatin, carboplatin, or etoposide. Cisplatin, carboplatin, or etoposide in 100 μL of complete medium was added to maintain the desired drug concentrations. After incubation of cell lines with cisplatin, carboplatin, or etoposide ± STS for 4 days, fluoresceine diacetate and eosin Y (0.5%) in 50 μL of medium (final concentration of fluoresceine diacetate, 10 μg/mL) were added to each well. Total fluorescence was then measured using digital imaging microscopy, and results were expressed as surviving fractions of treated cells compared with control cells.

Testing in hypoxia. Six cell lines (CHLA-15, CHLA-122, CHLA-42, SMS-SAN, SMS-KCNR, and CHLA-20) were tested for cisplatin cytotoxicity with or without the addition of STS at either hour 0 or hour 6 in hypoxic (2% oxygen, 5% CO_2, and the balance N_2) conditions. Microplates were incubated in an insulated, reduced oxygen incubator set to an oxygen tension of 2% O_2 (18) at 37°C for 4 days, and the plates were then assayed for cytotoxicity by DIMSCAN as described above.

Carboplatin cytotoxicity assays. Two cell lines (CHLA-15 and SMS-SAN) were tested for carboplatin cytotoxicity with or without the addition of STS at either hour 0 or hour 6 in atmospheric oxygen conditions by DIMSCAN as described above.

Etoposide cytotoxicity assays. Two cell lines (CHLA-15 and SMS-SAN) were tested for etoposide cytotoxicity with or without the addition of STS at either hour 0 or hour 6 in atmospheric oxygen by DIMSCAN cytotoxicity assay as described above.

Drug washout experiments. SMS-KCNR and SMS-SAN cell lines were treated with cisplatin (0-2 μg/mL) in 96-well plates. After incubation for 48 h with cisplatin alone or with the addition of STS at either hour 0 or hour 6 after the cisplatin, 150 μL of medium was removed from each well and replaced with an equal amount of fresh complete medium without disturbing the cell monolayer. This procedure was immediately repeated twice. Plates were then incubated for an additional 48 h and assayed for cytotoxicity by DIMSCAN as described above.

Xenograft experiments in athymic (nu/nu) mice. The SMS-SAN neuroblastoma cell line forms tumors when injected s.c. into athymic (nu/nu) mice. Experiments were done in the Childrens Hospital Los Angeles animal facility using 4-week-old to 6-week-old female athymic nu/nu mice injected s.c. between the shoulder blade with 20×10^6 SMS-SAN tumor cells. Once a sufficient number of palpable tumors had developed and baseline measurements of tumor growth had been taken for 1 week, drug treatment began. Tumor volume was determined from measurements in three dimensions taken twice weekly (22, 23). Toxicity studies revealed a maximum tolerated dose of 4 mg/kg/day for 4 days of cisplatin together with STS (3.5 g/kg/day) for 4 days. Cisplatin (4 mg/kg/day) given i.p. daily for 4 days was given so as to approximate the dosing schedule for cisplatin per the COG-A3973

protocol, as well as the current Children's Oncology Group high-risk neuroblastoma study ANB0532. Tumor volume was compared for cohorts of six mice, each receiving either no treatment, cisplatin alone, cisplatin + STS i.p. daily for 4 days at hour 0, or cisplatin + STS daily i.p. for 4 days at hour 6.

STS pharmacokinetics. Six mice received 3.5 g/kg STS as an i.p. bolus dose, and heparinized blood was collected by cardiac puncture at 1 and 15 min post–drug administration and stored on ice. Plasma was prepared by centrifugation for 10 min at 4°C and 8000 × *g*. Samples were evaluated for STS concentrations at Oregon Health Sciences University by the methylene blue method as described previously (15).

Cisplatin pharmacokinetics. Ten mice were given a single injection of cisplatin (4 mg/kg) i.p. Two mice were also given STS (3.5 g/kg i.p.). Heparinized blood samples were collected by cardiac puncture and stored on ice at 15 min after cisplatin administration (n = 2), 30 min (n = 2), 45 min (n = 2), 1 h (n = 2), and 6 h (cisplatin + saline at 6 h, n = 2, cisplatin + STS at 6 h, n = 2). Plasma was prepared by centrifugation for 10 min at 4°C and 8,000 × *g*. Only the free platinum is active, and it rapidly binds to albumin even at -20°C; thus, immediate ultrafiltration was done to separate the platinum from the albumin. Platinum ultrafiltrates were prepared using Amicon Ultra Centrifugal filters (Millipore Corporation) with centrifugation at 4,000 × *g* for 10 min and then stored at -20°C until analysis. A Perkin-Elmer Analyst 800 Atomic Absorption Spectrometer and THGA Graphite Furnace were used for cisplatin measurements. A platinum Perkin-Elmer Lumina hollow cathode lamp was used, and the samples were dispensed into a pyrolytically coated graphite tube. The slit width was set at 0.7 nm while monitoring at 265.9 nm. Sigma-Aldrich Atomic Absorption Standard Curve Platinum Solution was used to prepare the standard platinum solutions in PBS (pH = 7.4). For those samples having absorbance values decreasing below the functional detection limit of the instrument (~0.025 μg/mL), undiluted sample solutions were used.

Statistics and data analysis. All statistical data, including pharmacokinetic data, were analyzed with software STATA version 9.0 (STATA Corp.). *In vitro* cytotoxicity data were analyzed and graphed using the DIMSCAN cytotoxicity assay, Data Analyzer Program, and Sigmaplot 2000. Two-way ANOVA (24) was used to investigate the differences between the three treatment methods: administration of cisplatin only, administration of cisplatin and STS both at 0 h, and administration of STS at 6 h after cisplatin. The analyses compared the control groups to the groups that received the largest dosage (2μg/mL of cisplatin), with or without STS. The test of the significance of the interaction effects between the two main factors, treatment method and treatment administration, addressed the question of whether STS (given at the same time as cisplatin or 6 h after cisplatin) diminished the antineuroblastoma activity of cisplatin. The fluorescence readings were logarithmically transformed (to base e) before ANOVA analyses were conducted. Ratios of survival fractions of live cells in the cisplatin + STS at hour 0 group and in the cisplatin + STS at hour 6 group versus the cisplatin only group and their 95% confidence intervals were calculated. P values are from likelihood ratio tests and are two-sided.

The primary end point used in the xenograft analyses was time to progression, defined as the interval from the day of treatment administration to the day at which tumor size exceeded 600 mm^3 or the day when the last tumor measurement was taken. The comparison of progression-free survival among the treatment groups or between any two treatment groups was based on the log-rank statistics (25), with P values computed using permutation tests.

For the cisplatin pharmacokinetic studies, the measured absorbance values for the 10 mice receiving cisplatin only were described with a one-compartment pharmacokinetic model. The measured absorbance values at 6 h for the two mice that received STS together with cisplatin were compared with the prediction for the model.

Results

Effect of STS on cisplatin cytotoxicity for neuroblastoma cell lines in standard and hypoxic culture conditions. The cell line

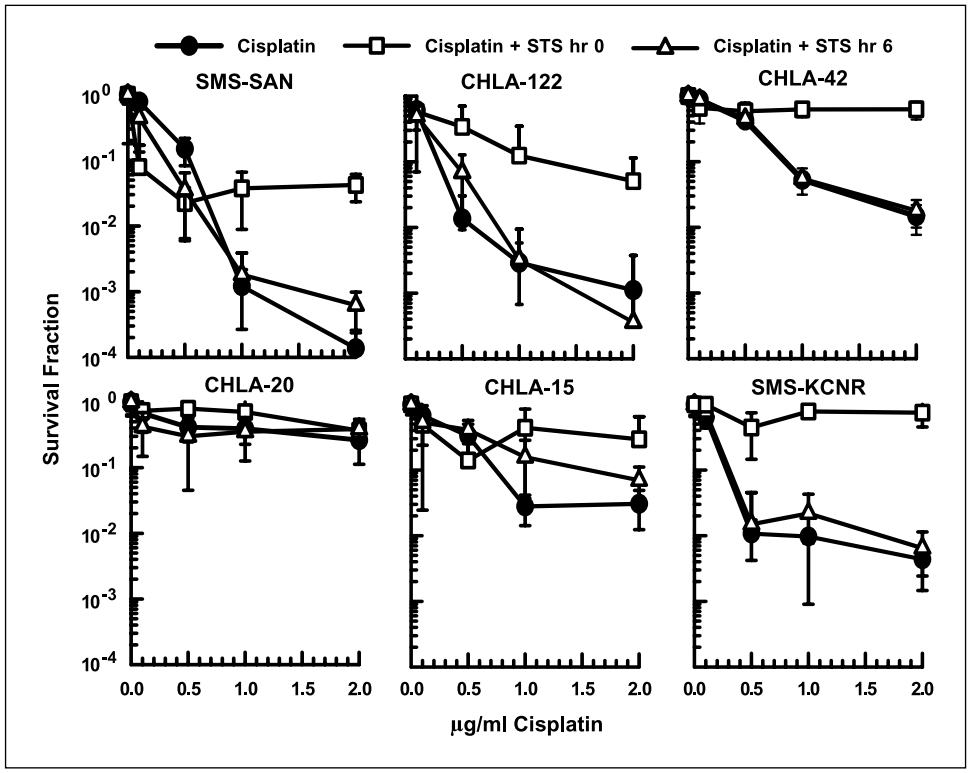

Fig. 1. Six representative neuroblastoma cell lines (SMS-KCNR, CHLA-15, SMS-SAN, CHLA-122, CHLA-20, and CHLA-42). The survival fraction for cells treated with cisplatin (2 μg/mL) + STS at hour 0 was significantly increased for six of six cell lines (SMS-SAN, CHLA-122, CHLA-15, CHLA-20, CHLA-42, and SMS-KCNR) in 20% oxygen. The survival fraction for cells treated with cisplatin + STS at 6 h was not significantly increased compared with cisplatin alone in five of six cell lines at the highest dose level of cisplatin (2 μg/mL; CHLA-122, CHLA-15, CHLA-20, CHLA-42, and SMS-KCNR). Two-sided P values were determined by likelihood ratio tests for the ratio of survival fractions for cisplatin + STS at hour 0 or hour 6 compared with cisplatin alone. A $P < 0.05$ was considered significant.

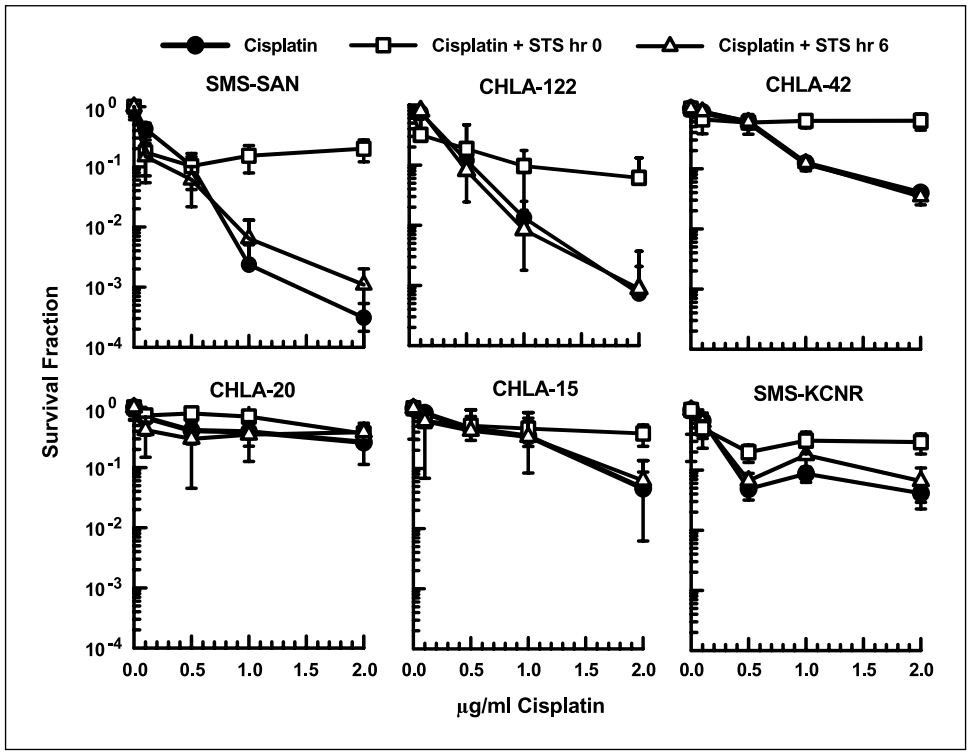

Fig. 2. The same six representative neuroblastoma cell lines (SMS-KCNR, CHLA-15, SMS-SAN, CHLA-122, CHLA-20, and CHLA-42) were also studied in conditions of physiologic hypoxia found in bone marrow (2% oxygen). Again, STS given 6 h after cisplatin did not significantly increase the survival fraction compared with cisplatin alone in five of six cell lines (CHLA-122, CHLA-15, CHLA-20, CHLA-42, and SMS-KCNR), whereas STS given simultaneously with cisplatin did increase the survival fraction compared with cisplatin alone in 2% O_2 in five of six cell lines (SMS-SAN, CHLA-122, CHLA-15, CHLA-42, and SMS-KCNR). Two-sided *P* values were determined by likelihood ratio tests for the ratio of survival fractions for cisplatin + STS at hour 0 or hour 6 compared with cisplatin alone. A $P < 0.05$ was considered significant.

panel used in this study has been characterized previously for drug sensitivity to cisplatin and etoposide (17); two cisplatin-resistant cell lines (CHLA-42 and CHLA-20) were used in this study. The cytotoxicity of cisplatin alone was compared with cisplatin with STS (1 mg/mL) added at either hour 0 or hour 6 after cisplatin (0-2 μg/mL) using the DIMSCAN cytotoxicity assay (20). *In vitro* data from four representative neuroblastoma cell lines established at the time of diagnosis (CHLA-15, CHLA-122, CHLA-42, and SMS-SAN) and two cell lines established at the time of progressive disease during induction therapy (CHLA-20 and SMS-KCNR) indicate that exposure to STS at the same time as cisplatin significantly decreased cisplatin cytotoxicity in six of six neuroblastoma cell lines in 20% oxygen and in five of six cell lines in 2% oxygen. However, STS added 6 h after cisplatin did not decrease cytotoxicity compared with cisplatin alone in five of six cell lines in both 20% and 2% oxygen (Figs. 1 and 2).

Effect of STS on cisplatin cytotoxicity after drug washout. We tested the effect of STS on cisplatin cytotoxicity in the SMS-SAN and SMS-KCNR cell lines after washout of drugs at 48 h and incubation for an additional 48 h before DIMSCAN assay. STS again seemed to protect against cisplatin cytotoxicity when added simultaneously with cisplatin but did not protect against cisplatin cytotoxicity when added 6 h after cisplatin (data not shown).

Effect of STS on etoposide cytotoxicity for neuroblastoma cell lines. Because cisplatin is used in combination with etoposide during induction chemotherapy for neuroblastoma, we also assessed the effect of STS on the cytotoxicity of etoposide. STS given at the same time or 6 h after etoposide in two representative neuroblastoma cell lines (CHLA-15 and SMS-SAN) did not protect tumor cells from etoposide cytotoxicity (Fig. 3).

Effect of STS on carboplatin cytotoxicity for neuroblastoma cell lines. We also assessed the effect of STS on the cytotoxicity of carboplatin, which is used during myeloablative chemotherapy for neuroblastoma. Exposure to STS at the same time as carboplatin decreased carboplatin cytotoxicity in two representative neuroblastoma cell lines (CHLA-15 and SMS-SAN), whereas exposure to STS 6 h after carboplatin did not protect from cytotoxicity (Fig. 4).

Effect of STS on cisplatin cytotoxicity in neuroblastoma xenografts. Cisplatin at a dose tolerated by nu/nu mice (4 mg/kg/day injected i.p. daily for 4 days) showed tumor responses in SMS-SAN tumor xenografts. The control group and the group treated with STS (3.5 g/kg/day i.p.) and simultaneously with cisplatin (4 mg/kg/day i.p.) showed no significant difference in progression-free survival ($P = 0.3$). Treatment with cisplatin alone or cisplatin + STS (3.5 g/kg/day) 6 h after cisplatin resulted in significantly improved progression-free survival compared with the control group ($P = 0.02$ and $P = 0.001$, respectively) or to the group treated with STS at the same time as cisplatin ($P = 0.03$ and $P = 0.001$, respectively). There was no statistically significant difference in progression-free survival between the group treated with cisplatin alone and the group treated with STS 6 h after cisplatin ($P = 0.9$; Figs. 5A and B).

STS pharmacokinetic studies in athymic mice. STS pharmacokinetic studies were performed on six mice. Plasma STS concentrations were obtained at 1 and 15 min after an i.p. injection of 3.5 g/kg STS. At 1 min after STS administration, the mean STS concentration in the mice was 171.7 ± 34.5 mg/dL, and at 15 min after drug administration, the mean STS concentration was 859.8 ± 492.9 mg/dL. The mean STS levels seen in the mice at 15 min after STS administration were more than thrice higher than the mean plasma level of 235.8 mg/dL

seen in patients 15 min after administration of a clinically achievable dose of 20 g/m^2 of STS (~ 0.6g/kg; data not shown; ref. 26).

Effect of STS on cisplatin pharmacokinetics in athymic mice. The effect of STS on plasma cisplatin concentrations in mice was analyzed to determine whether STS might bind to and eliminate circulating cisplatin. Absorbance values correlating to cisplatin concentrations were obtained at 15 min, 30 min, 45 min, 1 h, and 6 h after administration of cisplatin 4mg/kg i.p. and at 6 h after a dose of cisplatin + STS (3.5 g/kg i.p.) The measured absorbance value 6 h after cisplatin + STS did not differ from the measured or predicted absorbance values 6 h after a dose of cisplatin alone (data not shown).

Discussion

Ototoxicity of cisplatin. Platinum-based drugs are associated with bilateral, progressive, sensorineural hearing loss. Ototoxicity due to cisplatin is characterized by outer hair cell degeneration in the basal turns of the cochlea, whereas ototoxicity due to carboplatin is associated with the loss of inner hair cells (15) and is generally less severe and less frequent than cisplatin-induced ototoxicity. Ototoxicity is the dose-limiting toxicity for cisplatin and is associated with early, higher frequency hearing loss (>6,000 Hz) that progresses with higher cumulative doses to the impairment of lower frequency hearing (500-2,000 Hz; refs. 6, 27–29). Studies of cisplatin-associated hearing loss have found no evidence of recovery of hearing up to 4 years posttreatment (6, 27, 28), and one recent study reported continuous worsening of hearing loss with time after completion of cisplatin therapy (4).

The reported incidence of cisplatin-associated ototoxicity varies widely, depending on patient and disease group studied. Pediatric studies have reported hearing losses of 15 to 25 dB at frequencies above 4,000 Hz in 50% to 70% of children receiving cisplatin doses above 450 to 600 mg/m^2 and as many as 30% may develop hearing loss in the speech frequency range with doses of 720 mg/m^2 (6, 27–29). Age of less than 5 years, cumulative cisplatin dose of >400 mg/m^2, prior cranial irradiation, central nervous system malignancy, and concurrent treatment with other ototoxic drugs have been identified as risk factors (8, 27, 28). Hearing loss in young children, especially in the speech frequency range, may have devastating effects on language development, social skills, and learning ability. Thus, developing new approaches to decrease hearing loss from platinum-based therapies is a critical need in pediatric oncology.

The mechanism of cisplatin-associated ototoxicity involves apoptosis of sensory inner ear hair cells, initiated by direct binding of platinum drugs to both DNA and non-DNA targets (30). Accumulation of reactive oxygen species and the depletion of protective antioxidants, such as glutathione, may also contribute to hair cell loss (30). Devarajan et al. showed that cisplatin-induced cytotoxicity, as well as ototoxicity, involve both the death receptor and mitochondrial apoptotic pathways and that apoptosis in auditory sensory cells is dependent on both the dose and duration of platinum exposure (31).

STS as an otoprotective agent. Chemoprotective agents have been studied for their potential to reduce cisplatin-associated nephrotoxicity, neurotoxicity, and ototoxicity. Most agents studied have been electrophilic thiols which may provide protection by several mechanisms, including direct binding and inactivation of platinum agents, displacing platinum from cellular bound targets, decreasing DNA damage, increasing cellular glutathione levels, or scavenging platinum-induced free radicals (10–12, 14, 15, 30, 31).

STS, a reactive thiol compound used clinically as an antidote for cyanide poisoning or nitroprusside overdose, has been studied for its potential as a nephroprotective and otoprotective agent with platinum drugs. At high molar excess (optimal molar ratio of STS is 400:1 for cisplatin and 40:1 for carboplatin), STS binds to and inactivates the platinum drugs *in vitro* (10, 11, 15). STS may also interact directly with cochlear hair cells to rescue them from platinum that is already bound to cellular targets (14, 15).

Similar to previous studies using either cisplatin or carboplatin (12, 15), we found that by 6 h after cisplatin administration to mice, serum platinum concentrations were

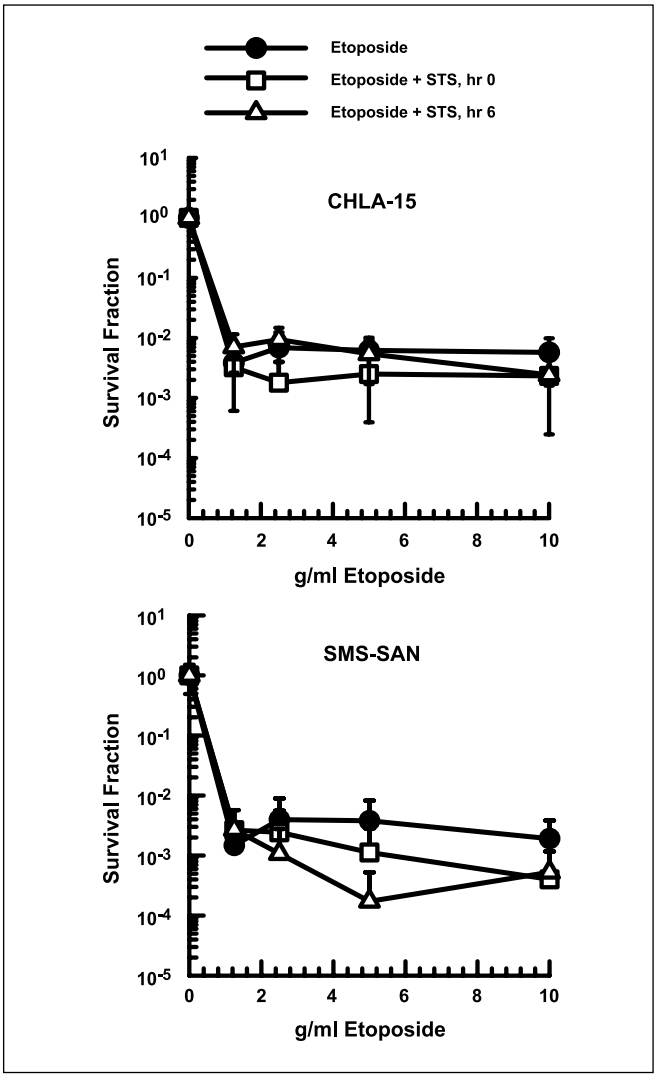

Fig. 3. STS given 0 or 6 h after etoposide did not affect etoposide cytotoxicity. In two representative neuroblastoma cell lines (CHLA-15 and SMS-SAN) STS given 0 or 6 h after etoposide did not affect the survival fraction for cells treated with etoposide.

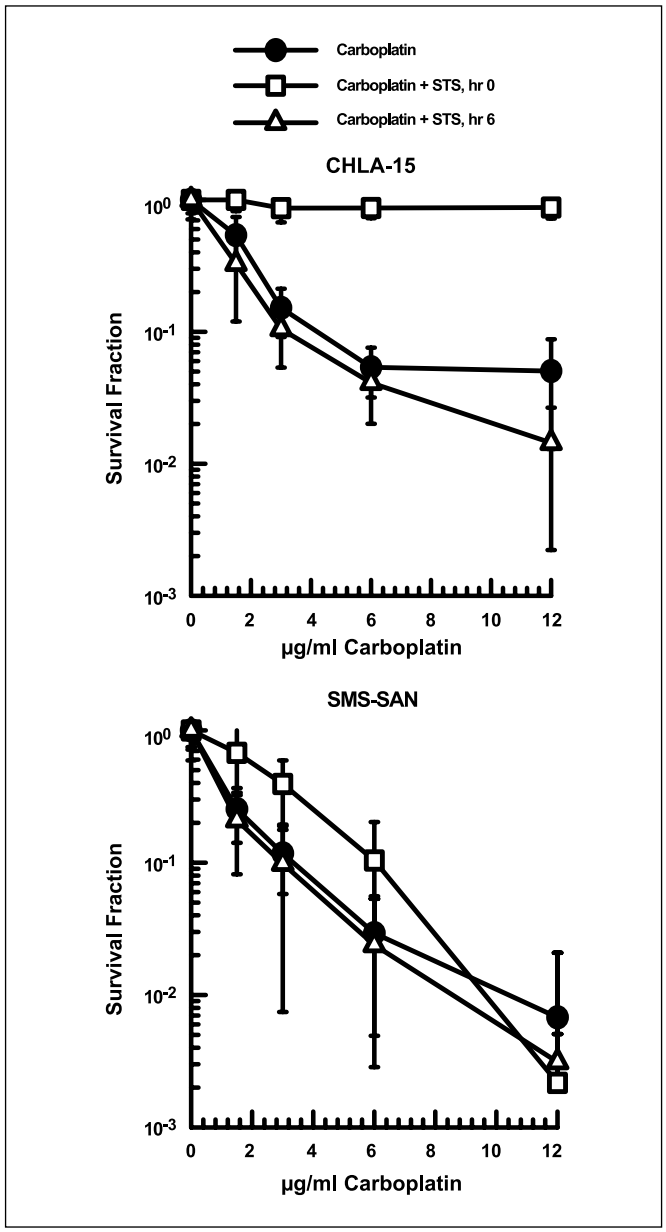

Fig. 4. Exposure to STS at the same time as carboplatin decreased carboplatin cytotoxicity in two representative neuroblastoma cell lines (CHLA-15 and SMS-SAN), whereas exposure to STS 6 h after carboplatin did not protect from cytotoxicity.

nondetectable and there was no observed effect of STS on cisplatin clearance. The delayed administration of STS may take advantage of the reduced concentration of free platinum available to interact with STS at that time, allowing for the optimal molar ratio of STS to cisplatin for the deactivation of the remaining free cisplatin, as well as cisplatin bound to cellular targets (10, 11, 15). In addition, STS may directly interact with hair cells of the cochlea to reduce platinum-DNA adducts or to restore the activity of DNA repair enzymes (32).

STS has been shown to provide otoprotection in a guinea pig model even if delayed for 8 h after carboplatin or 2 h after cisplatin (12). Rats treated with STS 4 h after cisplatin had no significant change in hearing from baseline, whereas a delay to 8 h was less otoprotective, and rats treated with STS 12 h after cisplatin had ototoxicity (13). Doolittle et al. found that STS (20 g/m^2 i.v.) delayed to 4 h after intraarterial carboplatin significantly reduced the rate of ototoxicity in patients with malignant brain tumors compared with a historical control group of patients who underwent treatment with carboplatin and blood-brain barrier disruption without STS; STS given 4 h after carboplatin was superior to STS delayed to only 2 h (14).

A potential concern with the use of chemoprotective agents, including STS, is the possibility that these agents may reduce the desired antitumor effects of platinum drugs (15). Inoue et al. reported a decrease in the antitumor efficacy of cisplatin and no rescue from systemic toxicity with the use of STS in an endometrial adenocarcinoma xenograft model in nu/nu mice (12, 15). To minimize the possibility of reduced antitumor effect, separation of chemoprotective agents and chemotherapy by time or route of administration has been used in human studies. Different routes of administration of STS and cisplatin, such as intraarterial cisplatin with i.v. STS in patients with head and neck cancer, have been used to provide local chemoprotection without affecting antitumor activity (33).

Additional studies have shown that delaying the administration of STS may allow otoprotection from platinum agents without compromising antitumor activity (13, 15, 34, 35). Delayed administration of STS 8 h after carboplatin did not affect antitumor activity in small cell lung cancer xenografts in rats (15). Dickey et al. found that STS was protective from cisplatin cytotoxicity in glioblastoma, SKOV3 ovarian carcinoma, medulloblastoma, and small cell lung cancer cell lines when added concurrently or up to 2 h post-cisplatin, but with a delay in STS administration to 6 h, there was no significant decrease in cytotoxicity (13).

A clinical trial assessing the safety of delayed high-dose i.v. STS (10-16 g/m^2) in 12 children with malignant brain tumors who were treated with intraarterial carboplatin in conjunction with blood-brain barrier disruption found that STS was well tolerated. Nausea and vomiting were adequately controlled with prior antiemetic treatment, and hypernatremia was mild and transient. In addition, there was a trend for improved protection from ototoxicity in children who received STS delayed to 4 h versus 2 h posttreatment (35). Therefore, separating STS and platinum agents by time may enable otoprotection from platinum agents without interfering with antitumor activity (15).

Effects of STS on cisplatin antineuroblastoma activity. The possibility that STS may reduce the antitumor effects of platinum drugs has been a major concern limiting its clinical applicability. This is especially relevant for high-risk neuroblastoma patients because >50% ultimately die from their disease. We have shown that STS given 6 h after cisplatin did not compromise cisplatin antitumor activity *in vitro* in representative neuroblastoma cell lines, under both standard and hypoxic culture conditions. A significant protection against cisplatin cytotoxicity was seen when neuroblastoma cells were exposed to cisplatin directly with STS. However, when cisplatin was given first and STS exposure occurred 6 h later, this cytoprotective effect was no longer observed. Similar results were obtained with carboplatin and with cisplatin under

physiologically hypoxic conditions and when drugs were washed out after 48 h of incubation. In addition, STS given at either hour 0 or hour 6 had no effect on the cytotoxicity of etoposide, which is used concurrently with cisplatin during therapy for high-risk neuroblastoma. Due to the mechanism of interaction between the electrophilic reactive thiol STS and platinum agents, we would not have expected similar interactions with etoposide.

Confirming our *in vitro* data, subcutaneous neuroblastoma xenografts in nu/nu mice were also protected from cisplatin when STS was added concurrently. However, when STS was added 6 h after cisplatin, there were no differences in cisplatin antitumor activity or progression-free survival when compared with mice treated with cisplatin alone.

Potential use of STS in neuroblastoma therapy. Given the high incidence of hearing loss among high-risk neuroblastoma patients receiving platinum agents during both induction chemotherapy and conditioning for autologous stem cell transplant, otoprotective agents could play an important role in maximizing dosing and compliance with chemotherapy. They could also potentially improve patients' quality of life soon after therapy by diminishing late effects that have a lasting negative effect on social, educational, and emotional development.

In animal models, the delayed administration of STS 2 to 8 h after cisplatin reduced cisplatin-induced auditory damage (13, 15). Delayed administration of STS to 6 h after cisplatin, given daily for 4 days as in the upcoming Childrens's Oncology Group high-risk neuroblastoma study, did not affect the antitumor activity of cisplatin in a subcutaneous neuroblastoma xenograft model. In addition, STS delayed to 6 h did not diminish the *in vitro* cytotoxicity of cisplatin, carboplatin, or etoposide in neuroblastoma cell lines. Our data suggest that the use of STS 6 h after cisplatin for otoprotection is unlikely to compromise the antineuroblastoma activity of cisplatin or etoposide. Taken together with previously reported results in a rat model showing that STS given after cisplatin can protect from ototoxicity (13), the *in vitro* and *in vivo* studies presented here support conducting a clinical trial to evaluate the delayed administration of STS as an otoprotective agent in high-risk neuroblastoma patients.

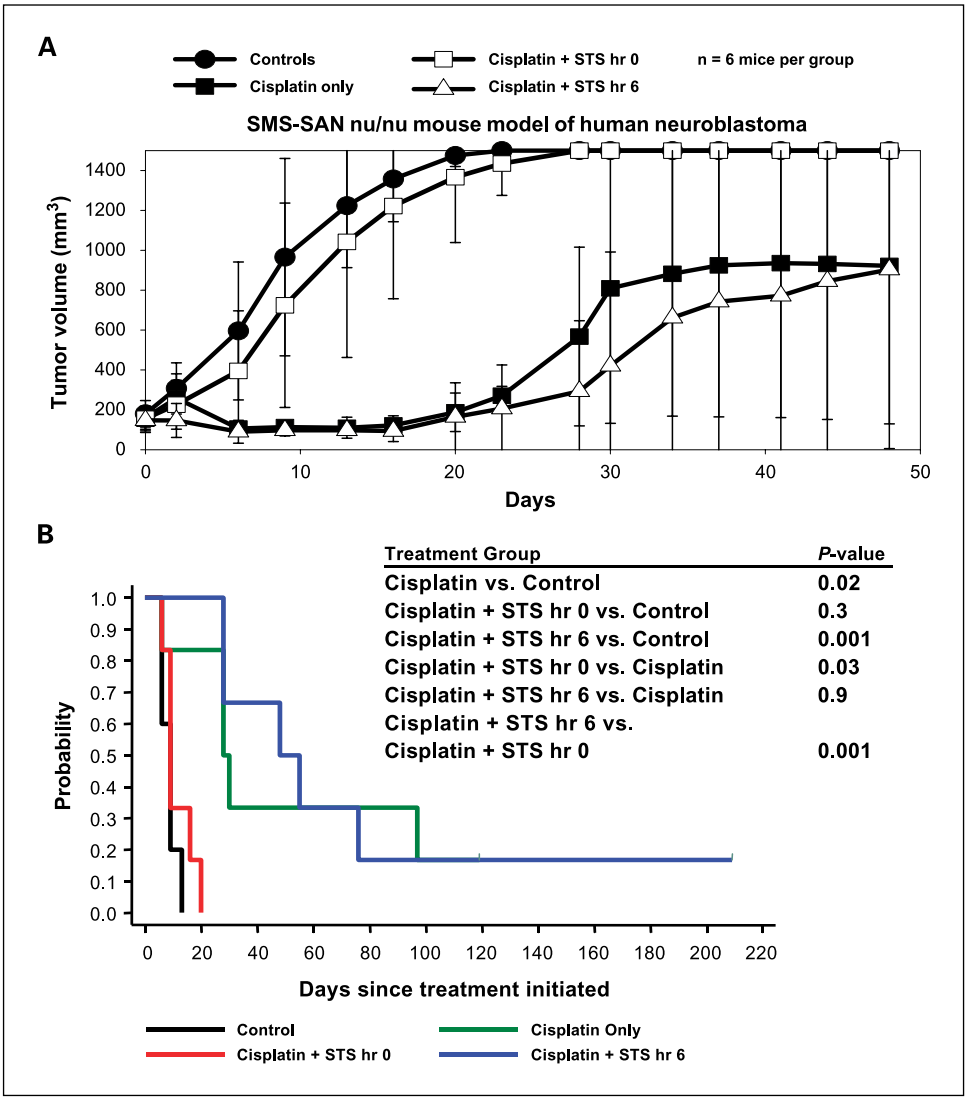

Fig. 5. *A,* effect of STS and cisplatin on subcutaneous human neuroblastoma xenograft growth. Nude mice were inoculated s.c. with 3.2×10^7 SMS-SAN neuroblastoma cells and were treated as (*a*) no treatment ($n = 5$), (*b*) cisplatin (4 mg/kg i.p. × 4 d; $n = 6$), (*c*) cisplatin (4 mg/kg/d × 4 d) + STS (3.5 g/kg/d i.p. × 4 d immediately after cisplatin; $n = 6$), and (*d*) cisplatin (4mg/kg/d i.p. × 4 d) + STS (3.5 g/kg/d i.p × 4 d at 6 h after cisplatin; $n = 6$). Tumor volumes were measured twice per week. *B,* the time to tumor progression (tumor volume of >600 mm^3 or last measurement taken) was determined. The probability of progression-free survival for the four treatment groups was determined using the permutated log-rank test. STS given 6 h after cisplatin daily for 4 d did not significantly ($P = 0.9$) affect cisplatin antitumor activity in SMS-SAN xenografts in nu/nu mice compared with cisplatin alone. However, STS given simultaneously with cisplatin daily for 4 d significantly ($P = 0.03$) protected tumors from cisplatin.

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