Bone Marrow Chemoprotection without Compromise of Chemotherapy Efficacy in a Rat Brain Tumor Model

Edward A. Neuwelt, Michael A. Pagel, Dale F. Kraemer, Darryl R. Peterson, and Leslie L. Muldoon

Departments of Neurology (E.A.N., L.L.M.) and Neurosurgery (E.A.N.), Division of Medical Informatics and Clinical Epidemiology (D.F.K.), and Department of Cell and Developmental Biology (L.L.M.), Oregon Health Sciences University, Portland, Oregon; Veterans Affairs Medical Center (E.A.N., M.A.P.), Portland, Oregon; and Departments of Physiology and Biophysics and Medicine (D.R.P.), Chicago Medical School, North Chicago, Illinois

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ABSTRACT

Thiol chemoprotective agents can reduce chemotherapy side effects, but clinical use is limited due to concerns of impaired chemotherapeutic efficacy. We evaluated whether an optimized bone marrow chemoprotection regimen impaired the efficacy of enhanced chemotherapy against rat brain tumors. Nude rats with intracerebral human lung carcinoma xenografts were treated with carboplatin, melphalan, and etoposide phosphate delivered intra-arterially with osmotic blood-brain barrier disruption (n/H11005/8/group). Thiol chemoprotection was N-acetyl-L-cysteine (1000 mg/kg) 60 min before chemotherapy and/or sodium thiosulfate (8 g/m²) 4 and 8 h after chemotherapy, when the blood-brain barrier is reestablished. Blood counts were obtained before treatment on day 3 and at sacrifice on day 9. N-acetylcysteine serum clearance half-life was 9 to 11 min. Pretreatment with N-acetylcysteine combined with delayed administration of sodium thiosulfate protected against toxicity toward total white cells, granulocytes, and platelets (P/H11005/0.0016). Enhanced chemotherapy reduced intracerebral tumor volume to 4.3/H11006/1.0 mm³ compared with 29.1/H11006/4.1 mm³ in untreated animals (P/H11005/0.0001). Tumor volume was 3.7/H11006/0.6 mm³ in rats that received N-acetylcysteine before and sodium thiosulfate after chemotherapy. The data indicate the efficacy of enhanced chemotherapy for rat brain tumors was not affected by thiol chemoprotection that provided excellent protection for hematological toxicity. Negative interactions of thiols with antitumor efficacy were avoided by temporal and spatial separation of chemoprotectants and chemotherapy.

Methods to improve chemotherapy for brain tumors such as dose escalation and dose intensification may exacerbate the toxic side effects of chemotherapy, including mucositis, nephrotoxicity, hepatotoxicity, and bone marrow toxicity. Some toxicities may be reduced by using chemoprotective agents, such as thio, thiol, and thioether compounds to detoxify normal tissues through free radical scavenging or drug conjugation (Links and Lewis, 1999). Although a variety of reactive sulfur agents can potentially provide chemoprotection, we have concentrated on two agents, sodium thiosulfate (Gamsik et al., 1997; Robbins et al., 1997) and the L-isomer of N-acetylcysteine (Cotgreave, 1997; Molnar et al., 1999). Both agents are protective against ototoxicity (Doolittle et al., 2001a) and white cell toxicity, even when administered significantly before or after chemotherapy (Neuwelt et al., 2001).

Chemoprotectants have had relatively limited clinical use due to concerns of impaired chemotherapeutic efficacy. We hypothesize that reduction of tumoricidal effects may be avoided by separating chemoprotectant and chemotherapy treatments in time or space. For example, studies of sodium thiosulfate chemoprotection have used two routes of administration (intra-arterial versus intravenous or intraperitoneal) to minimize interactions of sodium thiosulfate with cisplatin in tumor models (Iwamato et al., 1984) and patients (Howell et al., 1982; Robbins et al., 1997). The purpose of the current study was to develop an optimized thiol regimen that could be used in conjunction with brain tumor therapy, to maximize bone marrow chemoprotection while minimizing any impact on antitumor efficacy. The results demonstrate a potentially exciting new treatment option to maximize dose intensity and efficacy in brain tumors using intra-arterial (carotid or vertebral) chemotherapy given cephalad, whereas minimizing systemic toxicity by pretreatment with aortic...
administration N-acetylcysteine and delayed intravenous administration of sodium thiosulfate.

Materials and Methods

Animal studies were performed in accordance with guidelines established by the Oregon Health Sciences University Committee on Animal Care and Use.

Osmotic Blood-Brain Barrier Disruption. Anesthesia was induced with 5% isoflurane and maintained with propofol (650 μg/kg/min). Mannitol (25%, 37°C) was infused cephalad into the left internal carotid artery via a left external carotid catheter (Remsen et al., 1999).

Aortic Infusion Technique. The left internal carotid artery was temporarily occluded, and agents were administered retrograde to the descending aorta through a left external carotid catheter (Neuwelt et al., 2001).

N-Acetylcysteine Toxicity. N-Acetyl-L-cysteine (N-acetylcysteine, Mucomyst; Roxane Laboratories, Inc., Columbus, OH) was given by aortic infusion 30 min (n = 7) or 60 min (n = 10) before blood-brain barrier disruption in normal Long Evans rats. Doses ranged from 400 to 1500 mg/kg in 3 ml infused at 0.6 ml/min. Rats were sacrificed 6 days after treatment or at signs of acute neurotoxicity (head tilt, circling, moribund).

N-Acetylcysteine Clearance. Rats were treated as follows: group A, 1200 mg/kg aortic infusion (n = 3); group B, 1000 mg/kg aortic infusion (n = 3); group C, 400 mg/kg administered intravenously (n = 4); and group D, 140 mg/kg by aortic infusion (n = 2). Blood samples (0.5 ml) were collected 5, 15, 30, 60, and 90 min after thiol administration, and serum was evaluated for N-acetylcysteine concentrations. Serum N-acetylcysteine concentrations were measured using the Bioxytech GSH-400 colorimetric kit (Oxis Research, Portland, OR). The colorimetric assay was validated by high-pressure liquid chromatography (HPLC) analysis of serum thiols for n = 2 rats from groups B and D. Deproteinated serum samples were diluted in 160 mM KH2PO4, pH 3. Thiols were measured by electrochemical detection using a Waters radial compression module with 10-μm C18 columns (Waters, Milford, MA), an ESA 5100 analytical cell, and an ESA 5100A coulochem detector (ESA Inc., Chelmsford, MA). Area under the curve was compared with known concentrations prepared in control sera.

Pilot Studies of Chemoprotection. Pilot study 1 evaluated the timing for platelet protection with sodium thiosulfate. Normal Long Evans rats (n = 24, six rats per group) received intravenous 8 g/m² sodium thiosulfate (Sigma-Aldrich, St. Louis, MO) 2, 4, or 8 h after administration of 800 mg/m² carboplatin (Paraplatin; Bristol-Myers Squibb Co., Stamford, CT). Pilot study 2 evaluated various timing schemes for chemoprotection. Normal rats (n = 54, six rats per group) were treated with a tri-drug chemotherapy regimen consisting of carboplatin (200 mg/m²), melphanal (8 mg/m² Alkeran; GlaxoSmithKline, Uxbridge, Middlesex, UK), and etoposide phosphate (100 mg/m²; Etopophos; Bristol-Myers Squibb Co.), administered in the right carotid artery. Sodium thiosulfate (8 g/m²) was administered intravenously 4 and/or 8 h after chemotherapy, either alone or in combination with N-acetylcysteine (1200 mg/kg; aortic infusion) 30 min before chemotherapy. For both pilot studies 1 and 2, blood counts were determined at 6 days after chemotherapy, in comparison with untreated controls. For blood count analysis, 0.5 ml of whole blood collected in EDTA microtubes was analyzed in duplicate on a Hemavet 850 (CDC Technologies Inc., Oxford, CT).

Tumor Studies. Female athymic nude rats (nu/nu, 200–220 g) were anesthetized with inipreterone ketamine (60 mg/kg) and diazepam (7.5 mg/kg). LX-1 human small cell lung carcinoma cells (1 × 10⁶ cells in 12 μl, >90% viability) were inoculated stereotactically in the left caudate putamen (vertical bregma – 6.5 mm, 3.1 mm lateral).

In pilot study 3, tumor-bearing rats (n = 24) were treated with the tri-drug chemotherapy regimen 3 days after tumor implantation. Rats were sacrificed 12 days after treatment or earlier, if toxicity warranted.

For the major study, 40 rats (eight rats per group) were treated 3 days after tumor implantation. Rats received either no treatment, tri-drug chemotherapy, or chemotherapy in combination with N-acetylcysteine (1000 mg/kg, aortic infusion) 30 min before chemotherapy and/or sodium thiosulfate (8 g/m², intravenous) 4 and 8 h after chemotherapy (n = 8/group). The tri-drug intra-arterial chemotherapy regimen consisted of etoposide phosphate (100 mg/m²) given immediately before blood-brain barrier disruption and carboplatin (200 mg/m²) and melphanal (8 mg/m²) immediately after blood-brain barrier disruption. Blood counts were obtained as described above at baseline (prechemotherapy) and at 6 days after treatment. Rats were then sacrificed by barbiturate overdose, and the brains fixed by immersion in 10% formalin for vibratome sectioning (100-μm coronal sections). Every sixth brain section was stained with hematoxylin then imaged at high resolution on an Epson 1640XL flatbed scanner using Adobe Photoshop software. Tumor volume was assessed using NIH Image software.

Statistical Analysis. Means and standard errors were determined for blood counts and changes from each animal’s baseline values. Statistical tests were performed using SAS version 8.01 (SAS Institute Inc., Cary, NC). A Wilcoxon rank sums analysis was performed to evaluate the change from baseline values in all groups as well as each chemoprotection group in comparison with the untreated controls with a Bonferroni adjustment. P values were determined using the Kruskal-Wallis test. An analysis of variance test was also performed on the changes from baseline values, with similar results, but only the P values from the Wilcoxon analysis are shown because the high variability in the blood data reduces the assumption of normality.

For the analysis of tumor volume, a one-way analysis of variance model was fit to the data. The assumptions for this analysis include an approximate normal distribution and equal variances across groups. To meet these assumptions, the square-root transformation was applied to these data. The least-square means were estimated, and differences among these means were tested with Tukey adjustment for multiple testing. Nonparametric analyses (a Kruskal-Wallis test with pairwise comparison of means with a Bonferroni adjustment) were also performed with similar results.

Results

N-Acetylcysteine Toxicity and Clearance. In a previous study of bone marrow chemoprotection with thiols, N-acetylcysteine was administered at a dose of 1200 mg/kg 30 min before chemotherapy, using an aortic infusion technique (Neuwelt et al., 2001). This regimen was neurotoxic in combination with blood-brain barrier disruption. Therefore, we evaluated both a reduction in the N-acetylcysteine dose and an increase in the time before barrier opening (n = 17). The maximum tolerated dose was 500 mg/kg 30 min before blood-brain barrier disruption, and 1000 mg/kg 60 min before blood-brain barrier disruption.

The clearance of N-acetylcysteine from blood was evaluated in normal rats given high-dose or low-dose N-acetylcysteine via intravenous or aortic infusion routes of administration (Fig. 1). In all groups, N-acetylcysteine was cleared with a half-life of approximately 9 to 11 min, similar to the previously reported 15 min half-life for sodium thiosulfate (Neuwelt et al., 1998). In rats given 1000 mg/kg N-acetylcysteine intra-arterially, the maximum blood concentration 5 min after infusion was 11.2 ± 1.3 mM (Fig. 1), whereas blood concentration at the time of chemotherapy delivery (60 min after infusion) was 0.2 ± 0.1 mM. The colorimetric assay for N-acetylcysteine was validated by an HPLC assay of N-acety-
tylcysteine and other thiols. Table 1 indicates that there was close correlation of these two assays, at both low and high serum N-acetylcysteine concentrations.

Effect of Thiols on Chemotherapy-Induced Bone Marrow Toxicity. Pilot studies were performed to evaluate thiol timing and combination regimens to maximize chemoprotection. Previously, we showed that sodium thiosulfate had minimal bone marrow chemoprotective activity either alone or in combination with N-acetylcysteine, when it was administered immediately after chemotherapy (Neuwelt et al., 2001). Pilot study 1 assessed the effect of sodium thiosulfate given 2, 4, or 8 h after high-dose carboplatin. The data suggested that delaying sodium thiosulfate administration improved platelet chemoprotection. In a second pilot study, delayed sodium thiosulfate was evaluated for bone marrow chemoprotection with or without a 30-min pretreatment with high-dose N-acetylcysteine. Tri-drug chemotherapy alone reduced platelet counts from 837 ± 298 to 152 ± 78 thousand/µl (mean ± standard deviation, n = 6/group). In rats treated with tri-drug chemotherapy in combination with N-acetylcysteine (1200 mg/kg by aortic infusion 30 min before chemotherapy) and sodium thiosulfate (8 g/m² given intravenously 4 and 8 h after chemotherapy), platelet counts were 475 ± 289 thousand/µl. Due to the high variability of the platelet counts, limited animal numbers per group and the Bonferroni adjustment for testing nine pilot groups, the result was not significant. These pilot studies allowed us to narrow down the groups in the current study, to evaluate whether thiol pretreatment, delayed treatment, or both, would impact antitumor efficacy, when leakage into tumor was maximized with osmotic blood-brain barrier opening.

The tri-drug chemotherapy regimen (carboplatin, melphalan, and etoposide phosphate) caused significant mortality. In a third pilot study in tumor-bearing nude rats treated with tri-drug chemotherapy without chemoprotectants (n = 24), deaths occurred on day 6 (n = 7) and day 7 (n = 7) after treatment. Mortality may be due to a number of contributing toxicities, including mucositis and resultant dehydration and weight loss, liver and kidney toxicity, and bone marrow toxicity, as well as complications related to the intracerebral tumor. Survival of untreated tumor-bearing rats averages 15 days (Remsen et al., 2000). These data demonstrated that survival was an inappropriate measure of antitumor efficacy of the chemotherapy regimen because in the absence of chemoprotection the rats died from the treatment itself. We have previously shown that the blood count nadir occurred at approximately 6 days after chemotherapy treatment, and blood counts recovered to above baseline by 9 to 12 days. Thus, in the tumor study, the animals were sacrificed for blood count and tumor volume measurements at 6 days after chemotherapy (9 days after tumor implantation). At this time point, total white cells were reduced to 1.24 ± 0.70 thousand/µl from a baseline of 2.95 ± 0.95 thousand/µl (n = 8; P = 0.0018), granulocytes were reduced to 0.86 ± 0.53 from 2.39 ± 0.86 thousand/µl (n = 8; P = 0.0009), and platelets were reduced to 221 ± 107 from 716 ± 61 thousand/µl (n = 8; P < 0.0001).

Thiol treatment provided bone marrow chemoprotection (Fig. 2). Delayed administration of high-dose sodium thiosulfate (8 g/m², 4 and 8 h after chemotherapy) had minimal protective effect against chemotherapy-induced bone marrow suppression (P > 0.05). Pretreatment with N-acetylcysteine (1000 mg/kg by aortic infusion, 60 min before chemotherapy) was significantly protective for white cells (Fig. 2A; P = 0.0117) and granulocytes (Fig. 2B; P = 0.0087). Platelet chemoprotection was not significant with N-acetylcysteine alone. The best blood chemoprotection, particularly for platelets, was found combining both pretreatment with N-acetylcysteine and delayed treatment with sodium thiosulfate. With this dual chemoprotection approach, tri-drug chemotherapy-induced blood count nadirs were 104 ± 48% of baseline for total white cells (2.58 ± 0.93 thousand/µl; P = 0.0029 compared with no chemoprotection), 86 ± 43% of baseline for granulocytes (1.68 ± 0.62 thousand/µl; P = 0.0050), and 68 ± 17% of baseline for platelets (478 ± 139 thousand/µl; P = 0.0002).

Effect of Thiols on Chemotherapy Efficacy. LX-1 small cell lung carcinoma intracerebral xenografts grew rapidly in nude rats, attaining a volume of 29.1 ± 4.1 mm³ in untreated animals (range 24.2–34.8 mm³; Fig. 3A). The tri-drug chemotherapy regimen was highly effective administered intra-arterially with blood-brain barrier disruption 3 days after tumor implantation (Fig. 3B), and this was not altered by chemoprotection (Fig. 3, C and D). Tri-drug chemotherapy treatment reduced intracerebral tumor volume to 4.3 ± 1.0 mm³ (range 3.1–5.9 mm³; n = 8; P < 0.0001). The differences between each randomized active treatment group (+ chemotherapy) and the untreated control were all significant (P < 0.0001). By contrast, there was no difference in tumor volume between any of the groups that received che-

### Table 1
Comparison of N-acetylcysteine colorimetric and HPLC assays

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<tr>
<th>N-Acetylcysteine Dose and Route of Administration</th>
<th>Colorimetric Assay</th>
<th>HPLC Assay</th>
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<tr>
<td>1000 mg/kg, aortic infusion</td>
<td>7.1</td>
<td>5.6</td>
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<tr>
<td>140 mg/kg, intravenous</td>
<td>12.6</td>
<td>10.8</td>
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<td></td>
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motherapy, whether or not they also received chemoprotection. Even in the most aggressive chemoprotection group, with N-acetylcysteine 60-min pretreatment and sodium thiosulfate 4 and 8 h after treatment, tumor volume was $3.7 \pm 0.6 \text{ mm}^3$ (range 2.7–4.7 mm$^3$; $n = 8$; Fig. 3D).

**Discussion**

The goal of this study was to maximize brain tumor chemotherapy while minimizing systemic toxicities. We demonstrate that aggressive thiol chemoprotection for bone marrow can be accomplished without diminishing the efficacy of chemotherapy against intracerebral tumors in a rat brain tumor xenograft model, by varying route and/or timing of administration. These same thiol agents can provide otoprotection, nephroprotection, and hepatic protection against chemotherapy toxicity (Dickey et al., 2004).

**Chemotherapy for Brain Tumors.** Limited therapeutic success with chemotherapy in the treatment of central nervous system malignancies is attributable to a number of factors, including tumor resistance, delivery across the blood-brain barrier, and excessive drug toxicity. Dose intensification may overcome molecular resistance and improve survival rates, if the other factors limiting efficacy can be addressed.

This study evaluated the effects of a tri-drug chemotherapy regimen, delivered with osmotic blood-brain barrier disruption, on rat intracerebral tumor xenografts. The combination of intra-arterial etoposide phosphate before blood-brain barrier disruption and intra-arterial carboplatin and melphalan immediately after blood-brain barrier disruption was chosen because it mimicked the clinical treatment approach in this two-compartment mode (Doolittle et al., 2000). This treatment regimen had minimal neurotoxicity in rats but caused significant mortality due to bone marrow toxicity. The results (Fig. 3) demonstrate that tri-drug chemotherapy was a very effective regimen in the rat intracerebral xenograft model.

**Chemoprotection.** Brain tumor chemotherapy may be improved by reducing the systemic toxicities of chemotherapy, to reduce the incidence of severe side effects and dose reduction, or even allow dose escalation. Chemoprotection can be provided by exogenous sulfur-containing chemoprotective agents (thio, thiol, and thioether compounds), which act to detoxify agents through antioxidant and free radical scavenging activity (Cotgreave, 1997; Jarvinen et al., 2000), and other mechanisms (Gamesik et al., 1997; Links and Lewis, 1999).

Two clinically relevant thiol agents, sodium thiosulfate and N-acetylcysteine, were evaluated as bone marrow chemoprotectants. Sodium thiosulfate reduces alkylator cytotoxicity at the cellular level (Muldoon et al., 2001) and is also protective against carboplatin-induced ototoxicity in animal models (Muldoon et al., 2000) and in patients (Neuwelt et al., 1998; Doolittle et al., 2001a). In a previous study of bone marrow chemoprotection, minimal protection was provided by sodium thiosulfate when given immediately after chemotherapy (Neuwelt et al., 2001). A retrospective analysis of patients in the otoprotection study (Doolittle et al., 2001a) demonstrated platelet protection with delayed sodium thiosulfate alone (Doolittle et al., 2001b). Our current results show that delayed sodium thiosulfate was somewhat platelet
protective in rats but was only significantly active in combination with N-acetylcysteine.

N-Acetylcysteine is a cysteine analog with strong antioxidant activity (Cotgreave, 1997; Molnar et al., 1999). N-Acetylcysteine also induces de novo synthesis of the endogenous thiol glutathione over a period of hours to days (McLellan et al., 1995), which may contribute to long-term protection. In vitro chemoprotection showed that N-acetylcysteine was the most effective of the thiol agents tested against carboplatin and melphalan (Muldoon et al., 2001). Although previous reports of N-acetylcysteine bone marrow chemoprotection have been mixed (Lerza et al., 1986; Mantovani et al., 2000), our previous study (Neuwelt et al., 2001) showed that 30-min pretreatment with N-acetylcysteine alone rescued from drug toxicity, even in the presence of buthionine sulfoximine to reduce cellular glutathione. In the current study, similar hematological protection was found with a 60-min pretreatment with N-acetylcysteine (Fig. 2). We believe that the difference between our study and previous attempts at bone marrow protection have to do with high-dose N-acetylcysteine delivery via the aortic infusion route.

Interactions of Chemoprotectants and Chemotherapy. Chemoprotectants have had relatively limited clinical use due to concerns about the potential for negative interaction with chemotherapy in the tumor, resulting in reduced chemotherapeutic efficacy. We tested the hypothesis that reduction of tumoricidal effects may be avoided by separating chemoprotective and chemotherapy treatments in time or space. This approach is similar to the “two-route” paradigm used in studies of sodium thiosulfate chemoprotection, in which two routes of administration (intra-arterial versus intravenous or intraperitoneal) are used to minimize interactions of sodium thiosulfate with cisplatin in tumor models (Iwamoto et al., 1984) and patients (Robbins et al., 1997).

N-Acetylcysteine seems to require pretreatment for optimum activity, whereas sodium thiosulfate is best administered intravenously after 4 or even 8 h. Both agents have a short serum half-life (Fig. 1; Neuwelt et al., 1998). The rapid clearance of N-acetylcysteine suggests that it may be effective for bone marrow protection in the treatment of systemic malignancy. The blood-brain barrier provides an effective barrier against the entry of circulating thiols, because neither N-acetylcysteine nor sodium thiosulfate crosses the blood-brain barrier without osmotic disruption (Neuwelt et al., 1998, 2001).

The current study demonstrates that sodium thiosulfate and N-acetylcysteine provide hematological protection without impacting antitumor efficacy. No difference in antitumor efficacy was found, even with the aggressive chemoprotective regimen of pretreatment with N-acetylcysteine before chemotherapy followed by delayed sodium thiosulfate. Clinical phase I/II studies of sodium thiosulfate and/or N-acetylcys-

Fig. 3. Antitumor efficacy in the presence of chemoprotection. Nude rats with intracerebral tumors were untreated or treated with chemotherapy alone or in combination with chemoprotection consisting of N-acetylcysteine (1000 mg/kg, aortic infusion) 60 min before chemotherapy and/or sodium thiosulfate (8 g/m², intravenous administration) 4 and 8 h after tri-drug chemotherapy. Six days after treatment, rat brains were harvested for tumor volumetrics. A, histology of untreated tumor. B, histology of tumor after chemotherapy treatment. C, histology of tumor after chemotherapy treatment with N-acetylcysteine and sodium thiosulfate. A to C show 100-μm coronal sections with arrows indicating tumor; original magnification, 4×. D, tumor volumes. All treatment groups were significantly different from the untreated controls; ***, P < 0.0001. No significant differences were found comparing treatment groups with or without chemoprotection. Data are indicated as mean ± standard deviation (n = 8/group).
N-acetylcysteine and delayed intravenous sodium thiosulfate perfusion of the descending aorta with high-dose N-acetylcysteine and delayed intravenous sodium thiosulfate given 4 to 8 h later.

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References


Address correspondence to: Dr. Edward A. Neuwelt, Oregon Health and Sciences University, L603, 3181 SW Sam Jackson Park Rd., Portland OR 97239. E-mail: neuwelte@ohsu.edu