Lipid Association Increases the Potency Against Primary Medulloblastoma Cells and Systemic Exposure of 1-(2-Chloroethyl)-3-Cyclohexyl-1-Nitrosourea (CCNU) in Rats

Claudette Bethune,[1] Aleksander Blum,[1] J. Russell Geyer,[2] John R. Silber,[3] and Rodney J. Y. Ho[1,4]

Received December 24, 1998; accepted February 19, 1999

Purpose. To reduce the systemic toxicity and prolong the systemic presence of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), a lipid-based drug carrier was designed and characterized.

Methods. The degree of CCNU association with lipid vesicles composed of 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) (1:1, m/m) was characterized and the drug decomposition rates of lipid-drug complexes were monitored. Effects of lipid association on drug potency against medulloblastoma cells and total systemic drug exposure in rats were determined.

Results. At a CCNU:lipid molar ratio greater than 1:5, more than 90% of the drug was associated with the lipid vesicles. In aqueous suspensions, lipid association significantly reduced the first-order drug decomposition rate. In addition, lipid-associated CCNU exhibited a 4-fold increase in drug sensitivity with medulloblastoma cells. IC_{50} values for CCNU admixed and encapsulated with lipid vesicles were 18 ± 4.9 and 14.0 ± 2.2 μM, respectively, compared to 83 ± 11.0 μM for free CCNU. When administered to rats, lipid-associated CCNU increased the AUC (area under the concentration-time curve) of CCNU by approximately 2-fold (20.46 ± 2.15 compared to 39.59 ± 1.87 μg·min/ml), and the terminal half-life ($t_{1/2\beta}$) by almost 9-fold (17 ± 9 compared to 147 ± 48 min) over free CCNU. Despite the increase in total systemic drug exposure, rats treated with lipid-associated CCNU exhibited a significantly lower frequency of acute neurotoxicity.

Conclusions. These data indicate that CCNU associated with lipid vesicles may increase drug stability, potency, and systemic exposure in rats.

KEY WORDS: lipid vesicles; nitrosourea; medulloblastoma; high-dose chemotherapy; increased drug exposure.

INTRODUCTION

There are approximately 17,500 primary brain tumor cases with about 14,000 deaths reported annually, placing the brain cancer mortality rate (80%) second only to lung cancer (85%) in all cancer-related deaths (1). Malignant brain tumors are the second most common form of solid tumors during childhood and the leading cancer-related cause of death and illness in children (2). Medulloblastoma, a malignant neuroepithelial tumor of the central nervous system (CNS), affects 14–25% of children and 4–7% of adults with brain tumors. In addition, medulloblastoma has a high degree of intraspinal dissemination, spinal metastasis, and recurrence, which results in a 70–75% mortality rate (3). The propensity of medulloblastoma to undergo cell exfoliation and spread into ventricular cerebrospinal fluid (CSF) is well documented (4). The exfoliated tumor cells travel along the CSF to the lumbar sac and adhere to surface invaginations of arachnoidal matter, allowing the cancer to spread throughout the CNS.

Medulloblastoma continues to represent a formidable therapeutic challenge despite increasing neurological surgery and radiotherapeutic techniques for intervention. In general, standard treatment involves surgical removal (which is generally subtotal) of the primary tumor followed by intense craniospinal irradiation and adjunct chemotherapy. Unfortunately, radiation therapy can result in significant neurological and neuroendocrine side effects that are especially detrimental to young children. Therefore, chemotherapy is often substituted for radiotherapy in pediatric cases. However, current chemotherapy is not effective against medulloblastoma since the optimal dose necessary for control of tumor growth is limited by the systemic toxicity of these drugs (5). Dose limitations due to central organ toxicity is a significant barrier to successful chemotherapy, particularly with alkylating agents of nitrosourea derivatives (6). If the toxicity of these alkylating agents can be reduced without compromising their potency, the dose-limiting effects of these chemotherapeutic agents can be overcome.

Most of the alkylating agents used clinically for treating CNS tumors, including medulloblastoma, are relatively hydrophobic [octanol/saline partition coefficients for CCNU = 3; Thiotepa = 2.4; 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) = 1.5] which facilitates penetration across the blood-brain barrier. Once in the biological milieu, the chloroethylnitrosoureas are thought to undergo general base catalysis to generate the reactive alkylating chloroethyl-carbonium and isocyanate-ion intermediates (7). In aqueous buffer at physiological temperature and pH, CCNU decomposes rapidly into the inactive products 2-chloroethanol (18–25%), acetaldehyde (5–10%), and cyclohexylamine (32%) (8).

Also, it has been shown that when steady-state conditions are achieved through intravenous infusions of both CCNU and BCNU in humans, the CSF:plasma drug ratios produced are near unity (9). Under these conditions, tumor as well as normal tissue and cells are indiscriminately exposed to these potent drugs. If lipophilic alkylating agents, such as CCNU, can be incorporated into the phospholipid bilayer of lipid vesicles, the drug may be preferentially localized (via endocytosis or trapping of lipid vesicles at vascularized tissues) in tumor cells, and the rapid decomposition rate of these agents in physiological environments may be decreased. In addition, this strategy of drug delivery may reduce the systemic toxicity of these alkylating agents while enhancing their overall therapeutic potency, thus permitting a successful high-dose therapy for CNS tumors.

[1] Department of Pharmaceutics, University of Washington, Seattle, Washington 98195.
[2] Department of Pediatric Hematology and Oncology, University of Washington, Seattle, Washington 98195.
[3] Department of Neurological Surgery, University of Washington, Seattle, Washington 98195.
[4] To whom correspondence should be addressed. (e-mail: rodneyho@u.washington.edu)

0724-8741/99/0600-0896$16.00/0

We have characterized here the interactions of CCNU with phospholipid vesicles, determined the ability of this lipid-drug complex to inhibit medulloblastoma cell growth in culture, and examined the changes in systemic drug exposure in rats.

MATERIALS AND METHODS

Materials

The CCNU was kindly provided by the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Division of Cancer Treatment from the National Cancer Institute. The phospholipids, DMPC and DMPG were purchased from Sygena, Inc. (Cambridge, MA). All other reagents were of analytical grade.

Preparation of CCNU Admixed or Encapsulated with Lipid Vesicles

To prepare formulations of CCNU encapsulated in lipid vesicles, we first mixed 100 mg DMPC and DMPG (1:1, mol/mol) with adequate amounts of CCNU to achieve drug:lipid molar ratios of 1:5, 1:10, or as indicated. The dry materials were mixed in 1 ml chloroform in a test tube and the solvent was subsequently evaporated off with a stream of N_2 gas to create a dry film. The dry mixture was then vacuum desiccated for at least 30 min. To prepare desired lipid concentrations, a 1 ml volume of sterile phosphate buffered saline (PBS), pH 7.4, composed of 8 g/l NaCl, 0.2 g/l KCl and KH_2PO_4, and 0.16 g/l Na_2HPO_4 was then added to create a 100 mg/ml lipid suspension. The mixture was then sonicated at room temperature in a bath type sonicator (Laboratory Supplies, Inc., Hicksville, NY) until a uniform, translucent suspension of small unilamellar vesicles (SUVs) was obtained. Typically, total sonication time ranged from 20 to 30 min. To prepare formulations of CCNU admixed to lipid vesicles, appropriate amounts of CCNU dissolved in absolute ethanol were mixed with empty sonicated lipid vesicles to achieve desired drug:lipid ratios (final ethanol concentration $\leq$10%).

Determination of CCNU Concentrations in Buffers, Media, and Plasma

To determine CCNU concentrations in samples derived from size-exclusion chromatography, discontinuous sucrose gradients, and degradation studies, we used a colorimetric assay based on the Bratton-Marshall method originally for sulfonamides (10,11). Briefly, 25 μl samples were diluted with 175 μl PBS in microwell plates (VWR Scientific Products, Brisbane, CA). Then 25 μl of sulfanilamide reagent (1.5 g sulfaniliamide in 100 ml of 2 N HCl) was added and the samples were incubated in a water bath at 50°C for 45 minutes. Once cooled to room temperature, 16 μl of Bratton-Marshall reagent [30 mg N-(1-Napthyl) ethylene diamine in 10 ml distilled water] was added to each sample and absorbance at λ = 540 nm was measured with a microplate reader (Series 750, Cambridge Technology, Inc., Watertown, MA).

To determine CCNU concentrations in rat plasma samples and cell growth media, ethyl acetate was first added to the samples (5:1, v/v) to precipitate proteins. After centrifuging at 2,000 × g for 5 min to remove precipitated materials, CCNU concentrations in the sample supernatants were determined with a reverse-phase high-performance liquid chromatography (HPLC) system fitted with an octadecylsilica column (Spherisorb 5 μm, 4.6 mm inside diameter and 250 mm length, Phenomenex, Torrance, CA). The isocratic mobile phase consisted of acetonitrile and 0.05 M ammonium acetate (60:40, v/v, adjusted to pH 4.0 with glacial acetic acid). With a flow rate of 1.0 ml/min (pump model 510, Waters Assoc., Millford, MA) at 22°C, CCNU was detected at λ = 232 nm (Waters 486 tunable absorbance detector, Waters Assoc., Milford, MA) and eluted with a retention time of 5.3 ± 0.1 min. A 25 μl injection volume was delivered with an autosampler (Wisp 712, Waters Assoc., Milford, MA). With this method, the typical recovery of CCNU after extraction from plasma or media was determined to be 94–106%. Standard curves were generated with reference to the peak heights found with 0–170 μM CCNU. The detection limit for CCNU, as determined by the signal to noise ratio of 3 or greater, was found to be 8 ng for a 25 μl injection.

Characterization of Lipid-Drug Interactions

To detect the ability of CCNU to induce lipid vesicle aggregation, we first incubated (at room temperature) 200 μl samples consisting of 20 mM empty vesicles with varing concentrations of CCNU in ethanol (total ethanol concentration $\leq$10%) to achieve drug:lipid ratios ranging from 1:1 to 1:15. After 30 min, the samples were diluted up to 2 ml, transferred to a quartz cuvette (1 cm × 1 cm) and 90° light-scattering was measured with a fluorescence spectrophotometer (Perkin-Elmer MPF-37, Hitachi, Ltd., Tokyo, Japan), where the excitation and emission wavelengths were both set at 660 nm (excitation slit width = 5 nm, emission slit width = 2 nm). Changes in vesicle diameter due to CCNU incorporation were estimated by photon-correlation spectroscopy. Empty vesicles, vesicles admixed or encapsulated with CCNU were diluted in PBS to a final lipid concentration of 1 mM and mean diameter was measured at room temperature with a Coulter N_4 sub-micron particle analyzer (Coulter Electronics, Inc., Hialeah, FL). Estimated diameters are mean values from 4 runs (each run consisted of 3 measurements).

To measure the incorporation of CCNU into the lipid vesicles, we utilized size-exclusion gel chromatography (Method A) using biogel A-0.5 M with a 10 cm × 1 cm column (Bio-Rad Laboratories, Hercules, CA). A 50 μl aliquot, with 1 mM of free drug in ethanol (10%) or lipid-associated CCNU, was placed onto the column and 250 μl fractions of the running buffer (PBS) were collected and assayed for CCNU with the colorimetric assay as described above. The percentage of CCNU associated with the lipid vesicles was determined by taking the ratio of the amount excluded (vesicle fractions) to the total amount loaded. The recovery of free and lipid-associated CCNU from the column, in four runs of quadruplicate samples, was 87–105%.

Further characterization of CCNU's incorporation into lipid vesicles was carried out by a discontinuous sucrose-gradient (Method B). The discontinuous sucrose gradient consisted of 100 μl 65% sucrose and 1 ml of 10% sucrose. Fifty μl of 1 mM free or lipid-associated CCNU was placed on top of the gradient and centrifuged for 45 min at 4°C and 20,000 × g in an Eppendorf 5810 R Centrifuge (Brinkman Instruments, Inc., Westbury, NY). Under these conditions, free CCNU was found in the bottom fraction while lipid-associated CCNU remained in

the top fraction of the tubes. The percentage of lipid-associated CCNU was determined by colorimetric assay. The recovery of CCNU from the gradient, in four runs of quadruplicate samples, was 82–99%.

The effect of lipid association on CCNU degradation was investigated in aqueous buffer (PBS) and rat serum (Sigma Chemical Company, St. Louis, MO) at 37°C, Briefly, 2 ml samples, containing an initial concentration of 86 μM of CCNU from formulations of free (in PBS with 10% ethanol), free with 2% Tween 80 in 0.9% NaCl, and lipid vesicles (admixed or encapsulated with drug:lipid ratios 1:5 and 1:10), were placed in a 37°C water bath. At indicated time points (0, 10, 20, 30, 40, 50, 60, 120, 150, 180, 210, and 240 min), individual samples were removed and immediately flash-frozen to −80°C. Subsequently, the CCNU concentration in each sample was analyzed in aggregate using the colorimetric method as described above. The first-order degradation rate-constant (K) for each formulation was estimated from the disappearance of CCNU by linear regression of the natural logarithm of the CCNU concentration versus time plots. All values were the result of averaging K from each CCNU formulation run in quadruplicate. The $t_{1/2}$ values were derived from the relationship $t_{1/2} = 0.693/K$.

Inhibition of Cell Growth with CCNU

The studies here utilized three types of cancer cells as well as two types of non-cancerous cells. The tumor cells included the medulloblastoma cell line D 283 (12) from the American Type Culture Collection (Rockville, MD), the diploid primary human medulloblastoma cells UW 228-3 which have been characterized previously (13), and the rat glioma 36B-10, a malignant astrocytoma also described in detail elsewhere (14). Both UW 228-3 and 36B-10 cells were generated in the Department of Neurological Surgery, University of Washington (Seattle, WA). The primary human diploid fibroblast cells, HDF, were derived from non-cancerous embryonic tonsillar tissue and were kindly provided by the Virology lab of Children's Medical Center (Seattle, WA). The other non-cancerous human cells utilized, FB_3, were derived from a primary culture of fetal brain which resembled astrocytes of normal brain tissue (isolated by one of us, J. R. Silber). All cells were cultured in T175 tissue culture flasks (Becton Dickinson Labware, Franklin Lake, NJ) with Minimum-Essential-Media containing 5% antibiotic/antimycotic, 2 mM glutamine, and 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C.

To determine the ability of free and lipid-associated CCNU formulations to inhibit cell growth, 1×10^4 cells in 100 μl culture medium were first seeded into flat-bottom microwell tissue culture plates and incubated overnight. Then, 0 to 100 μM of CCNU in preparations of free (in ethanol, ≤10%) or lipid vesicles (admixed or encapsulated with drug:lipid ratios 1:5 and 1:10), were added to the cells in 50 μl media and incubated for 3 days. After drug incubation, 1 μCi of [^{3}H]thymidine (^{3}H-dT) with a specific activity of 87.6 Ci/mmol (NEN Products, Boston, MA) in 50 μl media was added to each well and the total ^{3}H-dT incorporated into cellular DNA was determined 18 hours later by harvesting the ^{3}H-DNA onto glass fibers with a cell harvester (PHD Cell Harvester, Cambridge Technologies, Inc., Watertown, MA). The ^{3}H-DNA incorporated into the glass fibers was determined by counting ^{3}H radioactivity (TRI-CARB 2200 CA liquid scintillation analyzer, Packard Instrument Company, Downers Grove, IL). The cellular ^{3}H-dT incorporation was compared with untreated cells to determine the percent of growth inhibition. The drug concentration at which 50% growth inhibition occurred (IC_{50}) was estimated using a maximum effect model described as:

$$I = \frac{I_{max} \cdot C}{IC_{50} + C}$$

where I is equal to the percent inhibition of cell growth, I_{max} the maximum effect (100% inhibition), C the concentration of CCNU, and IC_{50} the concentration at which 50% of the maximal effect was detected. IC_{50} values for free and lipid-associated CCNU were estimated by a non-linear regression method according to the above model; data were expressed as means ± SD from 8 replicate curves of each CCNU formulation.

Effect of Lipid Association on *In Vivo* Exposure and Toxicity of CCNU

Normal male Sprague-Dawley rats, specified pathogen free and weighing approximately 200 grams, were used for the *in vivo* experiments (Charles River Laboratories, Wilmington, MA). The rats were maintained in a pathogen-free environment and fed sterile laboratory pellets and water *ad libitum*. The animals were anesthetized by i.m. injection of 44 mg/kg ketamine and 5 mg/kg xylazine prior to right jugular vein and left carotid artery cannulation. The method described by Bakar and Niazi (15) was followed for catheter implantation procedures.

Lipid vesicles admixed with CCNU (drug:lipid ratio 1:5) or free-drug suspension (containing 10% ethanol and 2% Tween 80 in sterile 0.9% NaCl), were prepared in dosages of 5 or 10 mg/kg of body weight for intra-arterial dosing. The free-CCNU carrier of ethanol and Tween 80 has been shown to not induce any detectable neurologic side effects (16). Blood was sampled from the right jugular vein just before drug administration and at 3, 8, 15, 20, 26, 34, 43, 50, 63, 75, 90, and 120 min intervals after dosing animals with free-drug suspension (n = 12) or admixed vesicles (n = 12). These samples were processed immediately to collect plasma and quickly frozen to −80°C. Plasma samples were analyzed for CCNU concentration by HPLC analysis within 20 days of collection.

The total systemic exposure to CCNU was estimated by plasma AUC values. The AUC values were calculated using the trapezoidal rule from 0 to 120 min from CCNU plasma concentrations normalized to a 5 mg/kg dosage. The terminal half-life ($t_{1/2\beta}$) was estimated from the slope of the log-linear portion of the CCNU concentration versus time profiles. The level of acute neurotoxicity after drug administration was made from immediate observations of neurological deficit, which progressed from marked disorientation, to zigzag gait, full hindlimb tonic extension, to whole body seizure. Marked neurotoxicity was noted when two or more of the symptoms described above were observed.

To compare the hematological toxicity of lipid-associated CCNU to free drug therapy in rats, a dose-escalation study was conducted to determine the day and extent of white blood cell (WBC) and platelet count suppression (nadir effect). Single i.p. doses of 0, 20, 35, and 50 mg/kg CCNU encapsulated in lipid vesicles (drug:lipid ratio 1:10) or free dosage form (10% ethanol and 2% Tween 80 in 0.9% NaCl) were administered to rats and

blood was collected (Unopette microcollection system, Becton-Dickinson, Rutherford, NJ.) from tail veins at −1, 2, 4, 9, and 14 days after drug administration for WBC and platelet count determination. Animals treated with lipid-associated CCNU (n = 4) or free drug (n = 4) were compared to non-treated controls (n = 4).

Statistical Analysis

Data were expressed as mean values ± SD. Student's *t* test (two-tailed) for means was performed comparing each experimental group in the particle size determinations, cell growth inhibition experiments, and degradation studies. Differences were considered statistically significant when p values were less than 0.05.

RESULTS

Characterization of Lipid-Associated CCNU

To determine whether CCNU binds to phospholipid vesicles, we first incubated lipid vesicles composed of DMPC and DMPG with increasing amounts of CCNU in an admixed suspension. This combination of lipids (1:1, m/m) confers a negative net charge at physiological pH through the glycerol group of DMPG. We used DMPG in the DMPC vesicles to increase membrane fluidity and net negative charge of these vesicles. In addition, negatively charged vesicles have been shown to increase vesicle accumulation in L-1210 leukemia cells (17). To determine the binding interaction between CCNU admixed to DMPC:DMPG lipid vesicles, we monitored the particle size of the lipid vesicles by 90° light-scattering measurement. As shown in Fig. 1, an increase in light-scattering was detected when 20 mM lipid vesicles were exposed to increasing concentrations of CCNU. A maximum degree of light-scatter was detected at 4 mM CCNU. With 20 mM lipids in the suspension, the molar ratio of CCNU:lipid which exhibited the maximum light-scatter was estimated to be 1:5. Increasing the concentration of CCNU beyond 4 mM did not produce an additional increase in light-scatter.

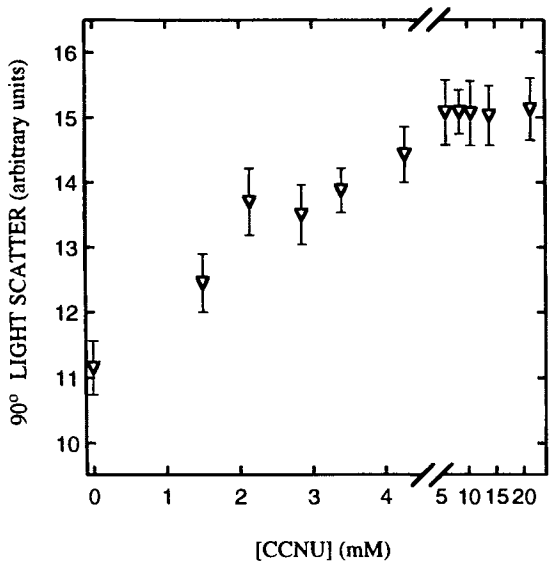

Fig. 1. Effect of CCNU on turbidity of lipid vesicles in suspension. An increasing concentration of CCNU (0–20 mM) was added to 20 mM small unilamellar lipid vesicles composed of DMPC:DMPG (1:1, m/m). The drug-induced lipid aggregation was detected at λ_{ex} and λ_{em} = 660 nm using a spectrofluorometer as described in Materials and Methods. Data were expressed as means ± SD of quadruplicate samples.

The particle size of CCNU admixed to lipid vesicles was further analyzed with photon-correlation spectroscopy and the data are summarized in Table 1. Incubation of CCNU with lipid vesicles increased the mean particle size while solvent controls (10% ethanol) did not. Similarly, when CCNU was added to the phospholipids during vesicle preparation (encapsulated formulation), we also found an increase in vesicle size due to CCNU incorporation. Even after an extended sonication time of 40 min, vesicles with encapsulated CCNU were significantly larger than vesicles without drug. In fact, the mean lipid-vesicle size was similar for both the admixed and encapsulation method of incorporating CCNU into vesicles (Table 1). These data suggest that CCNU binds to the lipid bilayer of the phospholipid vesicles, leading to an increase in particle size regardless of whether CCNU was added prior to or after lipid vesicles are formed.

To evaluate the extent of CCNU association with the lipid vesicles, we used size-exclusion chromatography (Method A) and discontinuous sucrose-gradient fractionation (Method B) to separate free CCNU in the suspension from vesicle-associated form. These techniques were validated with a control of CCNU in PBS containing 10% ethanol, for which no CCNU was found in gel-excluded (lipid-associated) fractions. As shown in Table 1, for formulations of CCNU either admixed or encapsulated with lipid vesicles, practically all of the CCNU in suspension was associated with lipid fractions when vesicles were made with drug:lipid mole ratios between 1:5 and 1:10. Taken together, these data indicate that CCNU binds to lipid vesicles with sufficient avidity to be co-purified in the lipid vesicle fractions under these chromatographic conditions.

To examine whether the incorporation of CCNU into lipid bilayers may increase drug stability in suspension, we monitored the rate of drug disappearance in PBS and serum at 37°C. In addition to free drug in ethanol (10%) and lipid-associated CCNU, we also studied the degradation of the *in vivo* dosage form of free CCNU in suspension (containing 10% ethanol and 2% Tween 80 in 0.9% NaCl). For CCNU admixed or encapsulated with lipid vesicles, we found significant drug stability when compared to free drug in both PBS and serum (Table 2). The formulation of CCNU in suspension with 2% Tween 80 exhibited similar stability to free drug in 10% ethanol in both PBS and serum.

Collectively, these results indicate CCNU was readily incorporated into the lipid vesicles, resulting in increased vesicle size and stabilization of CCNU.

Because the degree of drug incorporation into vesicles and increased drug stability were similar for both the 1:5 and 1:10 drug:lipid ratios, we primarily used 1:5 as the drug:lipid ratio for all the subsequent experiments.

Effect of Lipid Association on CCNU Cytotoxicity

To evaluate the effect of the lipid-vesicle carrier on CCNU's ability to inhibit cell growth, we examined brain tumor

Table 1. Effect of Drug:Lipid Ratios on CCNU Association to Lipid Vesicles and Vesicle Size

Formulation	Drug:Lipid ratio	% CCNU associated with lipid vesicles		Mean diameter (nm)
		Method A[a]	Method B[b]	
Empty vesicles	—	—	—	75 ± 17
Empty vesicles in 10% ethanol	—	—	—	87 ± 7
CCNU encapsulated in vesicles	<1:4	12 ± 4	ND[c]	ND
	1:5 – 1:10	95 ± 5	92 ± 4	105 ± 15[d]
CCNU admixed to vesicles	<1:4	<20	ND	ND
	1:5	90 ± 9	86 ± 4	123 ± 8 [d,e]
	1:6 – 1:10	96 ± 7	90 ± 3	103 ± 15[d]

[a] Determined by size-exclusion chromatography as described in Materials and Methods.
[b] Determined by discontinuous sucrose-gradient centrifugation as described in Materials and Methods.
[c] ND = not determined.
[d] $p < 0.05$ when compared to empty vesicles.
[e] $p < 0.05$ when compared with drug:lipid ratios > 1:5.

cells derived from primary cultures (UW 228-3 and 36B-10) and a commercially available cell line (D 283). In addition to tumor cells, primary cultures derived from non-cancerous tonsillar (HDF) and brain (FB_3) tissue were included in these studies. For these experiments, we fixed the drug:lipid mole ratio for lipid-associated CCNU at 1:5 and 1:10 and varied the concentration of drug incubated with the cells. With a drug:lipid ratio of 1:5, a typical dose-titration curve for medulloblastoma cells (UW 228-3) is shown in Fig. 2 and the summary of 50% inhibitory concentration (IC_{50}) estimates for several other cells are presented in Table 3. We observed that lipid-associated CCNU was significantly more effective in inhibiting medulloblastoma cell growth than free CCNU. In addition, the dose-titration curve was practically the same for the two lipid-associated CCNU preparations (admixed and encapsulated), with IC_{50} values estimated to be 18.0 ± 4.9 and 14.0 ± 2.2 μM for UW 228-3 cells and 23.0 ± 3.2 and 25.0 ± 3.6 μM for D 283 cells, respectively (Table 3). We also found that at CCNU concentrations greater than 35 μM, up to 90% inhibition of cell growth was observed for lipid-associated CCNU (Fig. 2). This compared to approximately 80% cell growth inhibition achieved with concentrations of 100 μM free CCNU.

In contrast to human medulloblastoma cells, rat glioma cells (36B-10) did not show an increased sensitivity to lipid-associated CCNU when compared to exposure with free drug (Table 3). In addition to cancer cells, the potency of lipid-associated CCNU was tested against cells derived from non-tumorous human tissues. As shown in Table 3, both free and lipid-associated CCNU exhibited similar potency against fibroblasts (HDF) and brain cells (FB_3). The IC_{50} values for free drug, CCNU admixed to lipid vesicles, and vesicle-encapsulated CCNU in HDF cells were found to be 10.4 ± 0.7, 10.1 ± 0.5, and 10.0 ± 0.2 μM, respectively. The FB_3 cells showed increased sensitivity over the fibroblasts with IC_{50} values recorded at 3.7 ± 0.5, 3.6 ± 0.3, and 3.2 ± 0.2 μM for free, admix, and encapsulated CCNU, respectively. When evaluating lipid-associated CCNU formulations with a drug:lipid ratio of 1:10 (data not shown), we found essentially identical results to the drug:lipid ratio of 1:5 (Table 3) in all cells.

Thus, these data indicate that lipid-associated CCNU was biologically active and exhibited an equal or higher potency than free CCNU against tumor cells. In non-tumorous cells, an increase in drug sensitivity due to lipid-association was not found.

Effect of Lipid Association on CCNU Exposure *In Vivo*

To determine whether lipid-association can modify the disposition of CCNU in rats, we first studied CCNU admixed to phospholipid vesicles with a drug:lipid mole ratio of 1:5.

Table 2. Effect of Lipid Association on CCNU Degradation in Suspensions

CCNU formulation	Phosphate buffered saline		Rat serum	
	K ($\times 10^{-3}$ min^{-1})	$t_{1/2}$ (min)	K ($\times 10^{-3}$ min^{-1})	$t_{1/2}$ (min)
Free drug in 10% ethanol	11.2 ± 0.6	62 ± 3	15.4 ± 0.6	45 ± 3
Free drug dosage form[a]	11.4 ± 2.0	61 ± 10	14.1 ± 1.0	49 ± 5
Admixed to vesicles				
1:5[b]	5.7 ± 0.4[c]	121 ± 18[c]	7.0 ± 0.4[c]	99 ± 6[c]
1:10	2.5 ± 0.4[c]	278 ± 38[c]	8.1 ± 0.4[c]	86 ± 5[c]
Encapsulated in vesicles				
1:5	6.5 ± 0.5[c]	107 ± 9[c]	8.2 ± 0.2[c]	85 ± 3[c]
1:10	3.5 ± 0.2[c]	201 ± 11[c]	6.2 ± 0.7[c]	112 ± 12[c]

[a] Free-drug suspension containing 10% ethanol and 2% Tween 80 in 0.9% NaCl, used for rat administration.
[b] Drug:lipid ratio.
[c] $p < 0.05$ when compared to free drug in 10% ethanol.

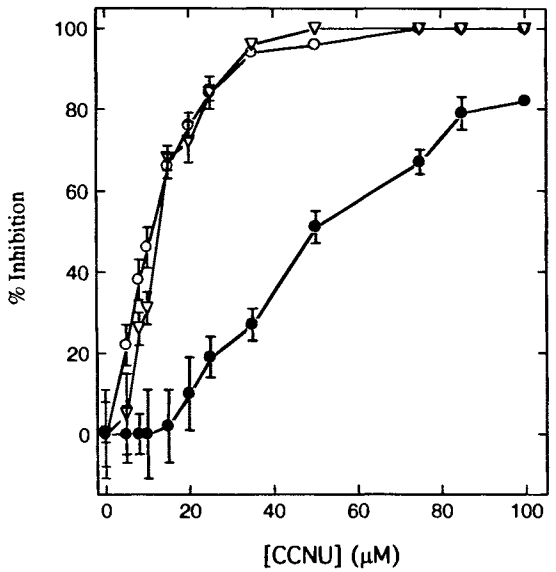

Fig. 2. Effect of lipid association on CCNU's ability to inhibit medulloblastoma cell growth. Medulloblastoma cells (UW 228-3) were incubated with increasing concentrations of CCNU in free (●), encapsulated (○), or admixed vesicle (▽) formulation (drug:lipid ratio 1:5). Cell growth inhibition was determined by ^{3}H-dT incorporation assay. Data were expressed as means ± SD of a typical experiment of four repeated curves.

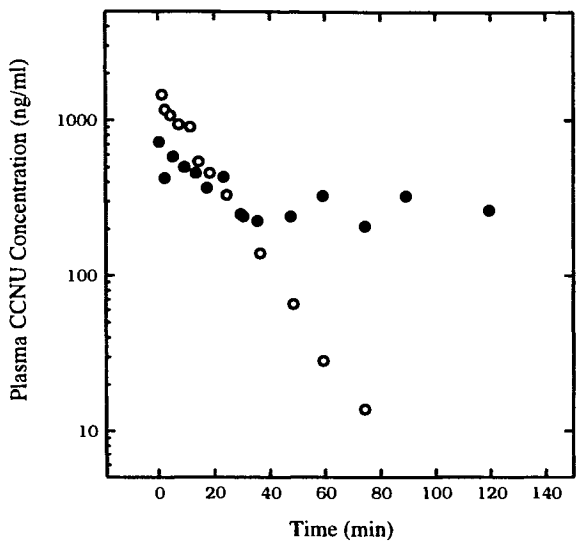

Fig. 3. A representative time course of plasma concentrations after intravenous administration of 5 mg/kg CCNU in free (○) or (●) lipid-admixed vesicles (as described in Materials and Methods).

The plasma AUC, terminal half-life ($t_{1/2\beta}$), and toxic side effects were examined after systemic administration of 5 and 10 mg/kg free or lipid-associated drug. A typical time course of plasma concentrations after i.v. administration of 5 mg/kg free or lipid-associated CCNU is shown in Fig. 3. The results from these experiments are summarized in Table 4. We found that two animals, administered 10 mg/kg free CCNU, exhibited marked and acute neurotoxicity in the form of pronounced disorientation and seizure activity. Therefore, we reduced the free CCNU dosage to 5 mg/kg. At this dose, 7 of 10 animals treated with free CCNU produced similar behavioral neurotoxicity, but without seizure, while the fraction of animals treated with 10 mg/kg dose of lipid-associated CCNU did not exhibit such toxicity (Table 4).

To compare the level of hematological toxicity between free and lipid-associated CCNU, a dose-escalation study was performed in rats. With single i.p. doses of 0, 20, 35, and 50 mg/kg CCNU, we found formulation effects between lipid-associated and free-drug suspensions to be similar. After administering 50 mg/kg of CCNU in free or vesicle-encapsulated form (drug:lipid ratio 1:10), an equal level of hematological suppression was observed in rats 4 days later where WBC counts dropped 30% and platelets 20% when compared to untreated controls. Such hematological suppression was not seen after administering the lower doses of free or lipid-associated CCNU.

The systemic drug exposure, presented here as AUC, was about 2-fold higher for lipid-associated CCNU (38.53 ± 1.93

Table 3. Effect of Lipid-Associated CCNU Formulation on Cell Growth Inhibition

		IC_{50}[a] (μM)		
		CCNU formulation		
Cells	Origin	Free[b]	Admixed	Encapsulated
UW 228-3	Medulloblastoma[c]	83.0 ± 11.0	18.0 ± 4.9[h]	14.0 ± 2.2[h]
D 283	Medulloblastoma[d]	92.5 ± 4.0	23.0 ± 3.2[h]	25.0 ± 3.6[h]
36B-10	Astrocytoma[e]	2.98 ± 1.7	2.2 ± 0.4	2.0 ± 0.3
HDF	Fibroblast[f]	10.4 ± 0.7	10.1 ± 0.5	10.0 ± 0.2
FB_3	Fetal Brain[g]	3.7 ± 0.5	3.6 ± 0.3	3.2 ± 0.2

[a] IC_{50} represents the drug concentration at which 50% growth inhibition was observed after a 3 day cytotoxicity assay. Admixed and encapsulated-CCNU vesicle formulations composed with a drug:lipid ratio of 1:5.
[b] Free-drug formulation containing 10% ethanol in media.
[c] Human cells derived from a primary culture.
[d] Human cell line derived from metastatic medulloblastoma.
[e] Rat glioma.
[f] Human cells derived as primary culture from fetal tonsillar tissue.
[g] Human cells derived from a primary culture of brain tissue.
[h] $p < 0.05$ when compared to free CCNU formulation.

Table 4. Effect of Lipid Association on Exposure and Neurotoxicity of CCNU in Rats

CCNU formulation	Dose (mg/kg)	Neurotoxicity[a]	$AUC_{0\text{-}120min}$[b] (μg·min/ml)	$t_{1/2\beta}$ (min)
Free[c]	5	7/10	20.46 ± 2.15	17 ± 9
	10	2/2	ND[d]	ND
Admixed[e]	5	0/4	39.59 ± 1.87	152 ± 33
	10	0/8	38.53 ± 1.93	147 ± 48

[a] Fraction of animals showing marked behavioral neurotoxicity (as described in Materials and Methods).
[b] AUC (area under the drug concentration versus time curve) values were normalized to a 5 mg/kg dose.
[c] Free-drug dosage form containing 10% ethanol and 2% Tween 80 in 0.9% NaCl.
[d] ND = not determined.
[e] CCNU admixed to lipid vesicles with a drug:lipid ratio of 1:5.

μg · min/ml) compared to free drug (20.46 ± 2.15 μg · min/ml) at 5 mg/kg (Table 4). Also, we found the AUC values observed with a 5 mg/kg dose to increase proportionally when the dose was raised to 10 mg/kg for both free and lipid-associated CCNU. In addition, there was a nearly 9-fold increase (17 ± 9 to 147 ± 48 min) in the $t_{1/2\beta}$ when CCNU was given as a lipid-associated form (Table 4). Taken together, these data indicate that lipid-association increased the total systemic drug exposure and $t_{1/2\beta}$ of CCNU without increasing hematological side-effects or acute neurotoxicity.

DISCUSSION

While surgery and radiotherapy are effective for treating patients with large localized brain metastases, both stategies are ineffective for tumors which have spread throughout the CNS. Adjunct chemotherapy with high doses of systemically administered alkylating agents allows effective drug concentrations to reach the CNS (18,19). However, the high degree of peripheral toxicity associated with high-dose chemotherapy often limits their usefulness. In addition, intrathecal delivery of alkylating agents to reduce peripheral toxicity have, thus far, failed to provide sufficient therapeutic outcomes (20–24).

To overcome these limitations, we have constructed a CCNU-lipid vesicle complex. CCNU associated with phospholipid vesicles increased the drug's stability and enhanced cytotoxicity against medulloblastoma cells (Tables 2 and 3). Administration of lipid-associated CCNU increased systemic drug exposure and significantly reduced acute neurotoxicity in rats (Table 4). The exact mechanisms leading to enhanced systemic exposure of CCNU when the drug is associated with lipid vesicles is not clear. It is possible that lipid-associated CCNU has significantly altered distribution and clearance than free drug. This change in drug disposition could result from the large particle size of the lipid-associated CCNU complex (Table 1), as well as through a reduction of nonspecific binding of CCNU to blood components and endothelial cells. In addition, lipid-associated CCNU may reduce the decomposition of the drug in blood, leading to the apparent increase in residence times observed (Table 4).

The decomposition rate of CCNU in blood has been shown to be reduced significantly when increased concentrations of lipids are found in plasma (25). In our experiments, we found that lipid-associated CCNU degraded at a 2-fold lower rate than that of free CCNU in serum (Table 2). While it is likely that both altered distribution and increased drug stability contribute to the observed increase in systemic exposure seen in rats (Table 4), the relative contribution of each mechanism remains to be directly determined.

It is noteworthy that the observed 2-fold increase in systemic CCNU exposure, detected with lipid-associated CCNU formulations of equal dose to free drug, did not lead to an increase in hematological suppression or acute neurotoxicity (Table 4). Even at a two-fold higher dosage (10 mg/kg versus 5 mg/kg) than free CCNU, none of the animals treated with lipid-associated CCNU exhibited acute neurotoxicity while 7 of 10 animals treated with 5 mg/kg free drug did (Table 4).

While lipid-associated CCNU reduced the observed neurotoxicity in rats, CCNU-lipid vesicles did not reduce the ability of the drug to inhibit tumor cell growth. In our *in vitro* assay, we found the CCNU complexed to lipid vesicles enhanced drug potency when compared to free CCNU (Fig. 2). It is likely that increased uptake of drug, possibly through phagocytosis of the lipid-CCNU vesicles, played a role in reducing the IC_{50} value 2- to 3-fold when compared to free drug for medulloblastoma cells (Table 3). Whether CCNU dissociates from the lipid vesicles at the cell surface, or inside endosomes or lysosomes, to become cytotoxic is not clear, and the exact mechanism remains to be determined.

In addition, while lipid-associated CCNU did not decrease the IC_{50} value against normal cells (HDF and FB_3) compared to free CCNU, we found these cells to be more sensitive to CCNU than medulloblastoma cells (Table 3). Whether normal cells from other tissues will exhibit similar or higher sensitivity to CCNU remains elusive. However, even cancer cells derived from different tissue origins exhibit varying sensitivity to free CCNU (26). In particular, for medulloblastomas, the greatest difference in nitrosourea sensitivity is seen among genetically similar sublines (13).

While the exact mechanisms of altered disposition and enhanced cytoxicity for lipid-associated CCNU remain undetermined, it is clear that CCNU binds to lipid vesicles with high avidity. We found that CCNU remained associated to the lipid vesicles even under conditions of gel-permeation chromatography, where fluid-flow and gel-matrix drug interactions counteract the CCNU-lipid association. Under these conditions, we found that almost all of the CCNU remained in the lipid-associated fractions, provided the drug:lipid ratio was maintained at 1:5 or higher. The detailed molecular interactions, including depth of CCNU insertion into lipid membranes, remain to be determined. We have concluded that if CCNU is inserted into the lipid membrane at sufficient depth, thereby shielding the drug from the surrounding water, its decomposition in aqueous suspension can be reduced (Table 2). These mechanisms warrant further investigation.

Regardless of the mechanism of enhanced cytotoxicity, reduced neurotoxicity, and increased systemic exposure, these positive results due to lipid association of CCNU may permit dose escalation in patients requiring high-dose chemotherapy. Using lipid-associated CCNU may reduce the acute toxicity of nitrosoureas while enhancing efficacy against tumor cell growth. In a recent study, intrathecally administered BCNU in lipid vesicles was shown to significantly increase survival for rats implanted with meningeal glioma (27). It is possible that a

high degree of lipid association for BCNU provided a significant therapeutic advantage.

In summary, we have shown that CCNU incorporated readily with phospholipid vesicles to form a tightly bound complex that significantly increased the stablility and potency of the parent drug, and enhanced systemic exposure while reducing acute toxicity in rats. This strategy may be used to improve delivery of other short-acting lipophilic chemotherapeutic agents.

ACKNOWLEDGMENTS

This work is supported by the Neuro-oncology Research Endowment Fund, NIH Grants HL 56548 and AI 31854, and by the University of Washington Royal Research Funds.

REFERENCES

1. A. P. Polednak and B. S. Flannery. Brain, other central nervous system, and eye cancer. *Cancer Suppl.* **5**:330–337 (1995).
2. R. J. Packer. Brain tumors in children. *Curr. Opin. Pediatr.* **8**:549–557 (1996).
3. F. H. Tomlinson, B. W. Scheithauer, F. B. Meyer, W. A. Smithson, E. G. Shaw, G. M. Miller, and R. V. Groover. Medulloblastoma: I. Clinical, diagnostic, and therapeutic overview. *J. Child Neurol.* **7**:142–155 (1992).
4. J. P. Provias and L. E. Becker. Cellular and molecular pathology of medulloblastoma. *J. Neuro-Oncol.* **29**:35–43 (1996).
5. B. H. Cohen and R. J. Packer. Chemotherapy for medulloblastomas and primitive neuroectodermal tumors. *J. Neuro-Oncol.* **29**:55–68 (1996).
6. I. J. Dunkel and J. L. Finlay. High dose chemotherapy with autologous stem cell rescue for patients with medulloblastoma. *J. Neuro-Oncol.* **29**:69–74 (1996).
7. D. C. Chatterji, R. F. Green, and J. F. Gallelli. Mechanism of hydrolysis of hologenated nitrosoureas. *J. Pharm. Sci.* **67**:1527–1532 (1978).
8. D. J. Reed, H. E. May, R. B. Boose, K. M. Gregory, and M. A. Beilstein. 2-Chloroethanol formation as evidence for a 2-chloroethyl alkylating intermediate during chemical degradation of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea amd 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea. *Cancer Res.* **35**:568–576 (1975).
9. M. J. Lind and C. Ardiet. Pharmacokinetics of alkylating agents. *Cancer Surveys.* **17**:157–188 (1993).
10. T. L. Loo and R. L. Dion. Colorimetric method for the determination of 1,3-bis (2-chloroethyl)-1-nitrosourea. *J. Pharm. Sci.* **54**:809–810 (1965).
11. A. C. Bratton and E. K. Marshall. A new coupling component for sulfanilamide determination. *J. Biol. Chem.* **128**:537–54 (1939).
12. X. He, S. X. Skapek, C. J. Wikstrand, H. S. Friedman, J. Q. Trojanowski, J. T. Kemshead, H. B. Coakham, S. H. Bigner, and D. D. Bigner. Phenotypic analysis of four human medulloblastoma cell lines and transplantable xenografts. *J. Neuropathol. Exp. Neurol.* **48**:48–68 (1989).
13. G. E. Keles, M. S. Berger, J. Srinivasan, D. D. Kolstoe, M. S. Bobola, and J. R. Silber. Establishment and characterization of four human medulloblastoma-derived cell lines. *Oncol. Res.* **7**:493–503 (1995).
14. A. M. Spence and P. W. Coates. Scanning electron microscopy of cloned astrocytic lines derived from ethylnitrosourea-induced rat glioma. *Virchows Arch. B Cell Pathol.* **28**:77–85 (1978).
15. S. K. Bakar and S. Niazi. Simple reliable method for chronic cannulation of the jugular vein for pharmacokinetic studies in rats. *J. Pharm. Sci.* **72**:1027–1029 (1983).
16. R. G. Russo, M. T. Cattaneo, and I. Bartosek. Pharmacokinetics of nitrosoureas: comparison of 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) after oral and intravenous administration to rats. *Tumori.* **70**:499–502 (1984).
17. C. Ritter and R. J. Rutman. Relative enhancement by various liposomes of BCNU effectiveness against L1210 leukemia *in vivo*. *Res. Commun. Chem. Pathol. Pharmacol.* **30**:123–131 (1980).
18. H. Bobo, J. P. Kapp, and R. Vance. Effect of intra-arterial cisplatin and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) dosage on radiographic response and regional toxicity in malignant glioma patients: proposal of a new method of intra-arterial dosage calculation. *J. Neurooncol.* **13**:291–299 (1992).
19. B. A. Chabner, R. Sponzo, S. Hubbard, G. P. Canellos, R. C. Young, P. S. Schein, and V. T. Devita. High-dose intermittent intravenous infusion of procarbazine (NSC-77213). *Cancer Chemother. Rep.* **57**:361–363 (1973).
20. M. Kochi, J. Kuratsu, Y. Mihara, S. Takaki, H. Seto, S. Uemura, and Y. Ushio. Ventriculolumbar perfusion of 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hyrdrochloride. *Neurosurgery.* **33**:817–823 (1993).
21. M. S. Edwards, V. A. Levin, M. L. Seager, and C. B. Wilson. Intrathecal chemotherapy for leptomeningeal dissemination of medulloblastoma. *Childs Brain.* **8**:444–451 (1981).
22. D. S. Fulton, V. A. Levin, P. H. Gutin, M. S. Edwards, M. L. Seager, J. Stewart, and C. B. Wilson. Intrathecal cytosine arabinoside for the treatment of meningeal metastases from malignant brain tumors and systemic tumors. *Cancer Chemother. Pharmacol.* **8**:285–291 (1982).
23. P. H. Gutin, H. D. Weiss, P. H. Wiernik, and M. D. Walker. Intrathecal N, N′, N″-triethylenethiophosphoramide [thio-TEPA (NSC 6396)] in the treatment of malignant meningeal disease: phase I-II study. *Cancer.* **38**:1471–1475 (1976).
24. P. H. Gutin, J. A. Levi, P. H. Wiernik, and M. D. Walker. Treatment of malignant meningeal disease with intrathecal thioTepa: a phase II study. *Cancer Treat. Rep.* **61**:885–887 (1977).
25. R. J. Weinkam, A. Finn, V. A. Levin, and J. P. Kane. Lipophilic drugs and lipoproteins: partitioning effects on chloroethylnitrosourea reaction rates in serum. *J. Pharmacol. Exp. Ther.* **214**:318–323 (1980).
26. National Cancer Institute. Cancer screen data for NSC 79037 (CCNU). IC_{50} for NSC 79037 [online data; cited 1997 Aug 22]; 1(1):[8 screens]. Available from URL: http://epnwsl.ncifcrf.gov:2345/dis3d/drugs/main/79037.html
27. I. Kitamura, M. Kochi, Y. Matsumoto, R. Ueoka, J. Kuratsu, and Y. Ushio. Intrathecal Chemotherapy with 1,3-bis(2-chloroethyl)-1-nitrosourea encapsulated into hybrid liposomes for meningeal gliomatosis: an experimental study. *Cancer Res.* **56**:3986–3992 (1996).