Development and optimization of an injectable formulation of copper diethyldithiocarbamate, an active anticancer agent

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Abstract: Copper diethyldithiocarbamate (Cu(DDC)) is the active anticancer agent generated when disulfiram (DSF) is provided in the presence of copper. To date, research directed toward repurposing DSF as an anticancer drug has focused on administration of DSF and copper in combination, efforts that have proven unsuccessful in clinical trials. This is likely due to the inability to form Cu(DDC), at relevant concentrations in regions of tumor growth. Little effort has been directed toward the development of Cu(DDC) because of the inherent aqueous insolubility of the complex. Here, we describe an injectable Cu(DDC) formulation prepared through a method that involves synthesis of Cu(DDC) inside the aqueous core of liposomes. Convection-enhanced delivery of a Cu(DDC) formulation prepared using 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol liposomes into a rat model of F98 glioma engendered a 25% increase in median survival time relative to vehicle-treated animals. In a murine subcutaneous MV4–11 model, treatment resulted in a 45% reduction in tumor burden when compared to controls. Pharmacokinetic studies indicated that the Cu(DDC) was rapidly eliminated after intravenous administration while the liposomes remained in circulation. To test whether liposomal lipid composition could increase Cu(DDC) circulation lifetime, a number of different formulations were evaluated. Studies demonstrated that liposomes composed of DSPC and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-(carboxy[polyethylene glycol]-2000) (95:5) enhanced Cu(DDC) concentrations in the circulation as reflected by a 4.2-fold increase in plasma AUC0–∞ relative to the DSPC/cholesterol formulation. The anticancer activity of this Cu(DDC) formulation was subsequently evaluated in the MV4–11 model. At its maximum tolerated dose, this formulation exhibited comparable activity to the DSPC/cholesterol formulation. This is the first report demonstrating the therapeutic effects of an injectable Cu(DDC) formulation in vivo.

Keywords: disulfiram, copper diethyldithiocarbamate, cancer, copper complexes, liposomes

Introduction

Disulfiram (DSF) is a US Food and Drug Administration (FDA)-approved drug for the treatment of alcoholism. It functions to inhibit acetaldehyde dehydrogenase 1, which in turn produces sensitivity to alcohol.1 DSF has recently become of interest in the treatment of human immunodeficiency virus and cancer through independent mechanisms.2–6 This has sparked an interest in repurposing DSF for indications other than alcoholism, and our interest is focused on the potential of this drug or its metabolites for treating cancer.

A high-throughput chemical screen demonstrated that DSF was active against glioma stem cells, an activity that was substantially increased in the presence of copper (II).7 These results have been reported by others with a series of in vitro experiments...
highlighting this combinatorial effect.\cite{13,17,8} Although some publications have speculated that DSF and copper form a complex,\cite{3,9,10} studies in vitro and in vivo show that DSF is reduced to form a copper-binding ligand.\cite{11,12} Since DSF has been used clinically for over 60 years, the pharmacokinetic (PK) properties of the drug have been explored in depth.\cite{13} There have been reports of the formation of a copper complex in the stomach as a result of DSF conversion to diethylthiocarbamate (DDC), a well-known copper chelator.\cite{14,18} Interestingly, copper diethylthiocarbamate (Cu(DDC)\textsubscript{2}) shows the same cytotoxic activity against glioblastoma cells as when DSF and copper are administered concurrently. This suggests clearly that the copper complex of DDC is the therapeutically active agent.\cite{19,20} Cvek et al have shown that Cu(DDC)\textsubscript{2} acts as a proteasome inhibitor,\cite{21} specifically through binding to the 19S lid of the proteasome rather than the 20S subunit, which is targeted by bortezomib.\cite{22}

Before developing any therapeutic application for Cu(DDC)\textsubscript{2}, there are formulation issues that need to be addressed if its use in vivo is to be evaluated. Specifically, the complex is a slightly soluble precipitate (<0.1 mg/mL)\cite{19} and, to date, there has not been any pharmaceutically appropriate formulations of Cu(DDC)\textsubscript{2} suitable for in vivo use.

We have previously described a method that solves the formulation issue: a method wherein the copper-complexation reaction occurs inside copper-containing liposomes.\cite{19,20} Here we demonstrate, for the first time, the anticancer activity of the resultant Cu(DDC)\textsubscript{2} formulation. This method allows for the development of a parenterally suitable Cu(DDC)\textsubscript{2} formulation and can take advantage of the well-established potential for liposomes to modulate the PK characteristics of an associated drug candidate. These Cu(DDC)\textsubscript{2} formulations are the first of their kind to be used to assess the therapeutic potential of this interesting copper complex.

### Materials and methods

#### Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), Cholesterol (Chol), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-(carboxy(polyethylene glycol)-2000) (DSPE-PEG\textsubscript{2000}), sphingomyelin (SM), 1,2-distearoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DSPG), and 3β-[N-[N,N-dimethylaminooxycarbonyl]-carbamoyl] cholesterol (DC-Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Sephadex G-50 beads were purchased from GE healthcare (Uppsal, Sweden). 3H-cholesteryl hexadecyl ether (3H-CHE) and Pico-Fluor 40 scintillation cocktail were purchased from PerkinElmer Life Sciences (Woodbridge, ON, Canada). Sodium diethyldithiocarbamate trihydrate, nigericin sodium salt, copper sulfate (CuSO\textsubscript{4}), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and all other chemicals (reagent grade) were purchased from Sigma Aldrich (Oakville, ON, Canada).

#### Liposome preparation

The extrusion method for liposome preparation has been well documented by others.\cite{23} Briefly, for the Chol-containing formulations (DSPC/Chol (55:45), SM/Chol (55:45), DSPC/DSPE-PEG\textsubscript{2000}/Chol (70:20:10), or DSPC/DC-Chol/DSPE-PEG\textsubscript{2000} (30:50:20)), the lipids were removed from the freezer and placed in a desiccator for 2 h before being weighed and dissolved in chloroform at the appropriate ratios. A nonexchangeable and nonmetabolizable lipid marker, 3H-CHE, was incorporated into the chloroform mixture to achieve a specific activity of ~0.025 μCi/mmol total lipid. The solution was dried from chloroform using nitrogen gas and the thin film generated was further dried under high vacuum for 3 h. The lipid film was then rehydrated at 65°C with unbuffered 300 mM CuSO\textsubscript{4} (pH 3.5) or 300 mM Cu-glucuronate (pH 3.5) for at least 2 h. To prepare Cu(DDC)\textsubscript{2} liposomes with final Cu(DDC)\textsubscript{2}-to-lipid ratios of 0.1 or 0.05, copper salt solutions of 150 and 75 mM were used. The resulting multilamellar vesicles underwent 5 freeze (in liquid nitrogen) and thaw (65°C water bath) cycles.\cite{24} These were then placed in an extruder (Evonik Transffera Nanosciences, Vancouver, BC, Canada) and extruded through stacked 0.1 μm polycarbonate filters at least 10 times at 65°C. The size of the resulting liposomes was determined using quasi-electric light scattering (ZetaPals, Brookhaven Instruments Corporation, Holtsville, NY, USA).

For Chol-free formulations (DSPC/DSPE-PEG\textsubscript{2000} or SM/DSPE-PEG\textsubscript{2000}’ (95:5)), the indicated lipids were removed from the freezer and placed in a desiccator for at least 2 h before being weighed and dissolved in ethanol. 3H-CHE was incorporated in the dissolved lipids in ethanol. The final ethanol solution with dissolved lipids was added dropwise to a solution of 300 mM unbuffered CuSO\textsubscript{4} (pH 3.5) in a 65°C water bath with constant mixing. The final ethanol concentration of the aqueous ethanol mixture was ~15% (v/v). This solution was then extruded using the same process as written above.

For all liposomal preparations the unencapsulated copper was removed by first exchanging the sample into a sucrose (300 mM), HEPES (20 mM), and EDTA (15 mM) buffer (SHE buffer, pH 7.4) by passing the sample through a Sephadex G-50 column equilibrated with the buffer. The resulting solution was then dialyzed against a...
sucrose (300 mM) and HEPES (20 mM) buffer (SH buffer, pH 7.4) and concentrated to the desired concentration for experimental studies using tangential flow. Liposomal lipid concentration was determined by measuring \(^{3}H\)-CHE using liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer).

**Cu(DDC)\(_2\)** synthesis in liposomes

Copper-loaded liposomes (CuSO\(_4\) or Cu-gluconate) were incubated with DDC in SH buffer at room temperature (unless indicated otherwise). Formation of Cu(DDC)\(_2\) was determined over a 60-min incubation period. Liposome-associated Cu(DDC)\(_2\) was separated from unreacted DDC using a Sephadex G-50 column equilibrated with SH buffer. The liposome-containing fractions were analyzed to determine Cu(DDC)\(_2\)-to-lipid ratios. Lipid concentrations were measured by liquid scintillation counting as described above and Cu(DDC)\(_2\) concentrations were determined by dissolving samples in methanol and measuring absorbance at 435 nm using a ultraviolet-visible (UV-Vis) spectrophotometer. Alternatively, a high-performance liquid chromatography (HPLC) assay was used where Cu(DDC)\(_2\) was measured on a Waters Alliance HPLC Module 2695 with a photodiode array detector (model 996) and the resulting chromatograms were analyzed by Empower 2 software. A Pronto SIL 120–3-C18ace-ESP (3.0 \(\mu\)m, 4.6×150 mm) column was used with a mobile phase composed of 90% methanol and 10% water. A 100 \(\mu\)L sample volume was injected, the flow rate was 1 mL/min, and column temperature was set to 40°C. Only samples with >90 ng/mL could be detected.

For studies involving the use of the potassium ionophore nigericin, the liposomes were prepared to contain 300 mM CuSO\(_4\) and the external solution was exchanged with a buffer of KCl-Histidine (150 and 20 mM, pH 7.4). \(^{25}\)Nigericin was dissolved in DMSO and added to achieve 0.1 \(\mu\)g/\(\mu\)mol DSPC and incubated at 60°C for 10 min. DDC was then added to the nigericin-containing liposomes, and following formation of Cu(DDC)\(_2\) the solution was passed through Sephadex G-50 columns and analyzed as indicated above.

**Cu(DDC)\(_2\)** dissociation from liposomes

Cu(DDC)\(_2\)-containing liposomes (final liposomal lipid concentration was 5 mM) were suspended in SH buffer with and without 50% (v/v) fetal bovine serum (FBS) and incubated with constant mixing at 37°C in a water bath. At the indicated time points, 100 \(\mu\)L of the solution was passed through a 1 mL Sephadex G-50 spin column equilibrated with SH buffer. The columns were centrifuged at 680 \(\times\) g for 3 min at 25°C. The filtrate was assayed for Cu(DDC)\(_2\) using HPLC (see above) and lipid was determined by measuring of \(^{3}H\)-CHE using scintillation counting. For samples incubated with serum, an aliquot (50 \(\mu\)L) was mixed with 950 \(\mu\)L methanol, and the sample was then centrifuged at 10,000 \(\times\) g for 10 min at 4°C to pellet precipitated proteins. The supernatant was assayed for Cu(DDC)\(_2\) using HPLC.

**Cryo-transmission electron microscopy**

Images were taken as previously described. \(^{26}\) Briefly, samples were prepared by applying 2–4 \(\mu\)L of liposomes at 10–20 mg/mL total lipid to a glow-discharged standard electron microscopy copper grid. Excess liquid was removed from the grid by blotting and then the grid was submerged in liquid ethane to rapidly freeze the sample using a Mark IV Vitrobot system (FEI, Hillsboro, OR, USA). Images were taken under cryogenic conditions at a magnification of 55,000–100,000 \(\times\) with an AMT HR CCD bottom-mount camera. Samples were loaded with a Gatan 70° cryo-transfer holder into an FEI G20 Lab6 200 kV (transmission electron microscope) TEM (FEI, Hillsboro, OR, USA) under low conditions with an underfocus of 1–4 \(\mu\)m to enhance image contrast.

**Cell lines**

F98 rat glioblastoma cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle’s Medium without FBS. MV-4–11 cell line was obtained from ATCC and maintained at 37°C and 5% CO\(_2\) in Iscove’s Modified Dulbecco’s Medium (IMDM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2 mM L-glutamine (Thermo Fisher Scientific) and 10% FBS (Thermo Fisher Scientific). These cells carry activating mutations for the FLT3 gene due to rearrangements at t(4;11). They are commonly used as a model of acute myeloid leukemia (AML) and can be established in immunocompromised mice (see below) as subcutaneous (sc) or systemic disease. All cell lines used are maintained in culture between passages 3 and 10. After the 10th passage we return to a stock supply of cells generated from the original ATCC cell line.

**Cytotoxicity assays**

For in vitro studies the MV-4–11 cells were seeded into 384-well plates and allowed to grow for 24 h prior to addition of Cu(DDC)\(_2\) (prepared as described above or in DMSO, as indicated) at the indicated concentrations. At 24 or 72 h after drug addition, the cells were incubated with PrestoBlue® (Thermo Fisher Scientific) at a final concentration of 10% v/v.
and 37°C for 1 h. The fluorescence was measured at 560/590 nm excitation/emission.

Western blot analysis for ubiquitylated protein

Cells were seeded in 6-well plates (400,000 cells per well) and treated with the IC_{50} of the indicated test compound for 24 h. Cell lysates were prepared using lysis buffer comprising 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, 1 mM EDTA, and Mini Protease Inhibitor Cocktail tablets (Roche Diagnostics, QC, Canada) for 1 h on a shaker at 4°C. Cell lysates were centrifuged at 14,000 × g for 10 min to collect total protein. A BCA Protein Assay Kit (Thermo Fisher Scientific) was used to determine protein concentrations and 10 µg of lysates protein was run on a 4%–12% Bis–Tris gel (Thermo Fisher Scientific) at 170 V for 1 h before being transferred to a 0.2 µm nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% skim milk in TBST (20 mM Tris-base, 140 mM NaCl, 0.1% Tween 20) and then probed for ubiquitin (Cell Signaling Technology, Danvers, MA, USA; 1:1,000) and β-Actin (Sigma-Aldrich, 1:50,000) overnight at 4°C. Blots were then washed with TBST (3×5 min) and incubated with horseradish peroxidase-conjugated secondary antibody (Promega, Madison, WI, USA) for 1 h at room temperature (1:10,000 for β-actin, 1:5,000 for ubiquitin). After washing with TBST (3×5 min), the blots were developed using Clarity Western ECL Substrate (Bio-Rad) for 5 min before imaging with the ChemiDoc MP Imaging System and ImageLab software (Bio-Rad).

Flow cytometric analysis of Cu(DDC)_{2}-treated cells

MV-4–11 cells were seeded in 6-well plates for 24 h and then treated with vehicle, DDC, CuSO_{4}, or Cu(DDC)_{2} (5 µM final concentration). At 24 h post-treatment, cells were washed 3 times with cold Hanks Balanced Salt Solution (HBSS) and fixed in 70% ethanol. The final concentration of cells was adjusted to 10^6 cells/mL. The samples were left for 1 h on ice followed by an overnight incubation at −20°C. Cells were centrifuged and the pellet was stained using a PBS buffer containing 50 µg/mL propidium iodide (Thermo Fisher Scientific), 1 mg/mL RNase A (Sigma-Aldrich), and 0.1% Triton X-100 (Bio-Rad) for 15 min at 37°C followed by an incubation for 1 h on ice. Data were acquired and analyzed using a FACS Calibur flow cytometer and WINMDI 2.9 software, respectively.

Reactive oxygen species assay

The reactive oxygen species (ROS) assay was performed using the ROS-Glo™ H_{2}O_{2} Assay Kit (Promega) as per the manufacturer’s instructions. Briefly, MV-4–11 cells were seeded at 14,000 cells/well in 96-well, white, clear-bottom plates. Treatment was added subsequently along with 20 µL substrate per well as per the supplier’s protocol. Cells were treated with either menadione (positive control, 50 µM) or Cu(DDC)_{2} (200 µM) for 4 h. All treatments were completed with and without cells to account for ROS generation as a result of interaction with vehicle and medium components. Finally, the H_{2}O_{2} detection solution was added at 100 µL/well and incubated at room temperature for 20 min. ROS generation was measured based on luminescence signal using a FluorStar Optima plate reader.

Ethics statement

Studies involving the use of animals were completed under an Animal Care Protocol (A014–0290) approved by the University of British Columbia’s (UBC’s) Animal Care Committee. The health assessment of animals was completed using a standard operating procedure also approved by the UBC’s Animal Care Committee.

Cu(DDC)_{2} maximum tolerated dose

To define the maximum tolerated dose (MTD) of Cu(DDC)_{2} formulations, mice (n=3) were given an intravenous (iv) injection (lateral tail vein) of Cu(DDC)_{2} using a Monday, Wednesday, and Friday for 2 weeks (M, W, F ×2) dosing schedule. The health status of the animals was monitored following an established standard operating procedure. In particular, signs of ill health were based on body weight loss, change in appetite, and behavioral changes such as altered gait, lethargy, and gross manifestations of stress. When signs of severe toxicity were present, the animals were terminated (isoflurane overdose followed by CO_{2} asphyxiation) for humane reasons. Necropsy was performed to assess other signs of toxicity. The surviving animals were monitored for 2 weeks (14 days) after administration of the last dose and full necropsies were completed on all treated mice at that time to assess changes in tissue/organ appearance.

Cu(DDC)_{2} pharmacokinetics studies

Cu(DDC)_{2}, synthesized in liposomes composed of DSPC/Chol (55:45) or DSPC/DSPE-PEG_{2000} (95:5), was injected intravenously at a dose of 15 mg/kg into CD-1 mice. At selected time points (eg, 0.25, 0.5, 1, and/or 4 h), mice (n=3 per time point) were terminated by isoflurane followed by
CO₂ asphyxiation and blood was collected by cardiac puncture. The blood was collected into EDTA-coated tubes kept on ice and centrifuged (Beckman Coulter Allegra X-15R) at 1,500×g for 15 min at 4°C. Plasma was collected and placed into a separate tube before assaying for copper, Cu(DDC)₂, and liposomal-associated lipid. The copper was measured using atomic absorption spectroscopy (AAS) by diluting plasma into 0.1% HNO₃. Plasma (30 μL) was added to Pico-Fluor 40 scintillation cocktail and the amount of [³H]-CHE was determined as described above. As a screening method to assess the effect of lipid composition on stability of the Cu(DDC)₂, following administration, each unique formulation tested was administered intravenously at 15 mg/kg in CD-1 mice (n=4) and blood was collected at 30 min, processed as described above and then the plasma was assayed for copper, Cu(DDC)₂, and liposomal lipid.

Convection-enhanced delivery in F98 glioma model
Male Fischer rats were purchased from Charles River Laboratories International Inc. (Wilmington, MA, USA). The implantation method was described previously.²⁷ F98 cells (10,000 cells in 5 μL) were prepared and implanted into the right caudate nucleus (1 mm anterior, 3 mm right of the bregma, and 6 mm deep) of the brain in 5 min. The convection-enhanced delivery (CED) procedure²⁸ was performed 10 days after implantation of F98 cells, at the same injection site using a 33 gauge Hamilton syringe. Before infusion, the burr was filled with bone wax and the needle was inserted 6.5 mm deep, retained there for 5 min and then withdrawn to 6 mm. Test articles and a control vehicle to be injected were vehicle (SH buffer), CuSO₄ (300 mM)-DSPC/Chol liposomes (copper 0.1 mg/mL, lipid 3.5 mg/mL), or Cu(DDC)₂ formulated in DSPC/Chol liposomes (0.5 mg/mL, lipid 3.5 mg/mL). A total volume of 10 μL was infused at an infusion rate of 0.5 μL/min for 20 min (5 μg Cu(DDC)₂ per rat). After the infusion, the needle was left in, to reduce backflow and increase convection volume, for 5 min prior to being withdrawn.

Cu(DDC)₂ efficacy studies in the MV-4–11 sc tumor model
As indicated above, MV-4–11 cells used for sc implantation were between passages 3 and 10 and were always collected when maintained at a confluence of 80%–90% at the time of harvesting for implantation. RAG-2M mice (up to 9 per group) were inoculated with 1×10⁶ cells in an injection volume of 50 μL per animal using a 28-gauge needle. Treatment was initiated on day 12 and treatment included the vehicle control (SH buffer), copper liposomes, or Cu(DDC)₂ prepared in liposomes composed of either DSPC/Chol (55:45) or DSPC/DSPE-PEG₂₀₀₀ (95:5). Tumor growth was measured 3 times per week with calipers and tumor volumes were calculated based on a formula of (L × W²)/2. The humane endpoint for these studies was defined when tumors exceeded 800 mm³. Animals with nondetectable tumors by day 25 were excluded.

Statistical analysis
All data are plotted using the Prism 6.0 software (GraphPad) as mean ± standard error of the mean (SEM) or mean ± SD as described in the Figure legends. Statistical analyses comparing Cu(DDC)₂ plasma levels or tumor growth studies were performed using one-way analysis of variance (ANOVA) followed by Tukey’s adjustments to correct for multiple comparisons. A P-value <0.05 was considered statistically significant. Survival study statistical analysis was performed using the log rank test, and a P-value <0.05 was considered statistically significant.

Results
Cu(DDC)₂ characterization
A general method for preparing metal-complexed drug candidates has been disclosed recently.¹⁹ One of the examples disclosed in this previous publication included the use of DSPC/Chol (55:45) liposomes, prepared in 300 mM copper sulfate, as nano-scale reaction vessels for the synthesis of Cu(DDC)₂. This process solved solubility issues because the highly insoluble complex was formed when the ligand was added to the outside of copper-containing liposomes. This synthesis reaction (Figure 1A) was completed at 25°C within 5 min and was easily detected by eye as the absorbance of the solution changed to exhibit a broad peak around 435 nm. The resultant product appeared stable, as the reaction could be carried out for 1 h with no significant change in the Cu(DDC)₂-to-lipid ratio (Figure 1B). The Cu(DDC)₂ formulation showed negligible dissociation from the liposomes in vitro when the samples were incubated at 37°C over 72 h in a solution containing 50% FBS in SH buffer (pH 7.4; Figure 1C). The in vitro activity of this novel formulation of Cu(DDC)₂ was compared to the complex prepared in the absence of liposomes and dissolved in DMSO, by generating dose–response curves against MV-4–11 leukemia cells (Figure 1D). The 72-h IC₅₀, determined for both formulations were identical (≈0.11 μM); however, in contrast to the DMSO-solubilized Cu(DDC)₂, there was no evidence of precipitates in the incubation wells when Cu(DDC)₂ was added as the liposomal formulation.
Studies to assess the mechanism of Cu(DDC)₂ activity

Previous publications,²¹,²⁹ indicated that the mechanism of DDC cytotoxicity was mediated, at least in part, through proteosome inhibition. Proteosome inhibitors, such as the FDA-approved bortezomib, have been used for the treatment of acute myeloid leukemia.³⁰ For this reason, we used the acute myeloid leukemia MV-4–11 cell line to characterize the cytotoxicity of Cu(DDC)₂ prepared within DSPC/Chol liposomes. The IC₅₀ determined using PrestoBlue (see Methods) of Cu(DDC)₂ at 24 and 72 h was 0.42 and 0.11 µM, respectively (Figure 2A and B). To determine whether Cu(DDC)₂ inhibits the proteosome, MV-4–11 cells were treated with vehicle, CuSO₄, or Cu(DDC)₂, and the
Figure 2 Cu(DDC)$_2$ acts primarily as a proteosome inhibitor and induces cell death in MV-4–11 cells.

Notes: (A) Cytotoxicity curves generated when MV-4–11 cells are exposed to Cu(DDC)$_2$ (prepared inside DSPE/Chol liposomes) for either 24 (■) or 72 (▲) h where viability was measured using PrestoBlue. (B) The IC$_{50}$ values of MV-4–11 cells that were treated with Cu(DDC)$_2$ for 24 and 72 h. The proteasome inhibition activity was determined as described in the Methods using MV-4–11 cells treated with the indicated doses of CuSO$_4$ or Cu(DDC)$_2$ (prepared inside DSPE/Chol liposomes) for 24 h. (C) Proteasome inhibition resulted in accumulation of ubiquitinated proteins presented as long dark bands on the Western blot following Cu(DDC)$_2$ treatment (150 and 300 nM) but not for vehicle or CuSO$_4$ (300–600 nM). (D) Cell-cycle analyses were completed using MV-4–11 cells treated with CuSO$_4$, DDC, or Cu(DDC)$_2$ for 24 h and the results indicated no significant change in the cell cycle upon Cu(DDC)$_2$ exposure. There was an increase in the sub G$_0$/G$_1$ fraction (marked with horizontal bar) when cells were treated with Cu(DDC)$_2$, indicative of cell death as evident by DNA fragmentation. (E) ROS formation was tested in MV-4–11 cells treated with Cu(DDC)$_2$ (prepared inside DSPE/Chol liposomes), where ROS formation was measured 4 h following initiation of treatment. Cu(DDC)$_2$ treatment did not induce ROS formation. Menadione was used as a positive control and ROS formation in the cells was evident by a statistically significant difference in luminescence relative to the corresponding cell-free condition. Data are presented as mean ± standard error of the mean of 3 experiments.

Abbreviations: Chol, cholesterol; DDC, diethylthiocarbamate; DSPE, distearoyl-sn-glycero-3-phosphocholine; ROS, reactive oxygen species.
accumulation of ubiquitinylated proteins, a marker of proteosome inhibition, was subsequently measured via Western blotting. Only the Cu(DDC)₂-treated groups showed marked accumulation of ubiquitinylated protein relative to the controls (Figure 2C). To further investigate mechanisms involved in Cu(DDC)₂ cytotoxicity, we performed cell-cycle analysis using flow cytometry (see Methods). The results showed no significant changes in the cell cycle after 24 h of Cu(DDC)₂ exposure. However, Cu(DDC)₂ caused an increase in the sub G₀/G₁ phase indicative of cell death (Figure 2D). As some published reports have suggested that Cu(DDC)₂ treatment leads to production of ROS, we also evaluated whether Cu(DDC)₂ induces cell death via this mechanism. Cells were treated for 4 h with Cu(DDC)₂ or menadione (positive control). The results showed that Cu(DDC)₂ treatment did not result in ROS generation (Figure 2E), suggesting that it is unlikely to be an important mechanism of cytotoxicity for the formulation described here.

**Plasma elimination of Cu(DDC)₂ prepared in DSPC/Chol (55:45) liposomes**

The Cu(DDC)₂ formulation prepared as described above was suitable for iv administration. To assess Cu(DDC)₂ elimination from plasma, mice were given a single iv dose (15 mg/kg) of Cu(DDC)₂ and plasma samples were collected as described in the Methods. Attempts to measure Cu(DDC)₂ in the plasma compartment were, however, unsuccessful even when using a 15 min time point. The Cu(DDC)₂ levels were below the detection limits of our HPLC assay (0.09 µg/mL). Plasma copper levels were measurable by AAS, and in Cu(DDC)₂-treated mice these levels were above the level of copper determined in plasma collected from untreated mice. For this reason, we used plasma copper levels (after subtraction of control plasma copper levels) as a surrogate marker of Cu(DDC)₂. The results, summarized in Figure 3, indicate that the percent of the injected copper dose remaining in plasma was 5.2% at 15 min, indicative of 95% eliminations of the injected Cu(DDC)₂ (Figure 3A; filled circles). This value decreased to 0.25% of the injected dose 1 h after administration. In contrast, 15 min after administration of the formulation, 93% of the injected liposomal lipid dose was still in the plasma compartment, a value that decreased to 80% 1 h following administration (Figure 3A, open circles). The liposomal lipid elimination rate was comparable to that described previously for DSPC/Chol liposomes.

These results suggest that Cu(DDC)₂ prepared inside DSPC/Chol liposomes does not remain inside the liposomes following iv dosing. This result was surprising given the stability of the formulation as determined using in vitro methods (Figure 1). Loss of associated Cu(DDC)₂ from the liposomes after administration is highlighted by the copper-to-lipid ratio data (Figure 2B). Prior to injection, this ratio was 0.25 (time 0), but it drops to 0.02 after 15 min. This is indicative of 92% loss of Cu(DDC)₂ from the liposomes within 15 min after injection. In contrast, 1 h after administration of uncomplexed CuSO₄ containing DSPC/Chol liposomes, the copper-to-lipid ratio suggested >90% retention of the encapsulated copper. This is indicative of Cu(DDC)₂ being released from the liposomes as the copper complex, whereas uncomplexed Cu²⁺ ions are, as expected, retained in the liposomes owing to their charge. It can be suggested that the
DSPC/Chol liposomal formulation of Cu(DDC)₂ addresses the solubility challenges of this compound, but because the complex is released rapidly from the liposomes following administration it should be noted that the liposomes are not behaving as drug delivery vehicles.

**Cu(DDC)₂ efficacy in models of cancer**

The results thus far demonstrate that Cu(DDC)₂ synthesized in liposomes remains in solution in a form that could be safely administered intravenously. The potential for using the resultant product as an anticancer drug was measured in 2 different rodent models of cancer, and the results are summarized in Figure 4. The activity of Cu(DDC)₂ (prepared in DSPC/Chol liposomes) was measured in mice with established sc tumor models generated following sc injection of the MV-4-11 cells (Figure 4A). Before assessing activity, the MTD of Cu(DDC)₂ was determined in tumor-free CD-1 mice (see Methods). Given the rapid elimination of Cu(DDC)₂ following iv injection (Figure 3), we selected a dose-intense schedule where the animals were injected iv on Monday, Wednesday, and Friday for 2 weeks (M, W, F ×2). Using this schedule the MTD of Cu(DDC)₂ was determined to be 8 mg/kg. At doses >8 mg/kg (eg, 15 mg/kg) the animals were terminated at humane endpoints (5 days following initial treatment). On necropsy, these animals showed no obvious changes in tissue or organ appearance other than the occasional enlarged spleen and discoloration of the liver and kidney. It should be noted that extravasation injuries (at the site of injection) were observed with some mice. If the extravasation injury was considered severe, then the mouse was terminated for ethical reasons and that animal was excluded from the treatment group. For animals with established MV-4-11 sc tumors, dosing began on day 12 when mice were injected (iv) with vehicle (SH buffer), DSPC/Chol (55:45)-copper sulfate liposomes (1.3 mg copper/kg), or Cu(DDC)₂ prepared in DSPC/Chol liposomes (8 mg Cu(DDC)₂/kg, copper dose 1.3 mg/kg), respectively. The liposomal lipid dose was ~50 mg/kg. Tumor size was measured over time (see Methods). The results (Figure 4A) suggest that tumor growth was comparable in animals treated with the vehicle and the copper-containing liposome control. The Cu(DDC)₂ (prepared in DSPC/Chol liposomes)-treated animals exhibited a delay in tumor growth rate. Animals in this group showed a 45% reduction in tumor volume when compared to the vehicle- and copper-treated animals on day 41. Although there was a treatment-engendered delay in tumor growth when using the MV-4-11 sc tumor model, the results suggested that Cu(DDC)₂ activity was unremarkable on the basis of RECIST criteria where notable activity is reflected by stable disease, a partial response, or a complete response. For this reason, we choose to assess Cu(DDC)₂ (prepared in DSPC/Chol liposomes) in an orthotopic F98 (rat glioblastoma) model, where treatment was administered directly into the site of tumor inoculation by CED. The glioblastoma line was of interest because previous publications suggested that DSF and its metabolite DDC in the presence
of copper were particularly active against glioblastoma cells.\textsuperscript{5,33} As noted in the supplementary data (Figure S1), the in vitro activity of Cu(DDC)\textsubscript{2} against U251 MG and F98 cell lines was comparable to that observed for the MV-4–11 cells (Figure 1). The 72-h \textit{IC}\textsubscript{50} of Cu(DDC)\textsubscript{2} was 384 and 417 nM for U251 MG cells and F98 cells, respectively. The in vivo efficacy of Cu(DDC)\textsubscript{2} (prepared in DSPC/Chol liposomes) was evaluated in the F98 rat glioma model where each rat was injected at the site of tumor cell inoculation with a 0.5 mg/mL solution of Cu(DDC)\textsubscript{2}, the vehicle- or copper-containing liposomes (0.08 mg/mL) (see Methods). This was defined as the MTD of the Cu(DDC)\textsubscript{2} formulation when administered by CED methods. In these studies, control animals exhibited a median survival time (MST) of 20.5 and 19.5 days, respectively (Figure 4B). In animals treated with Cu(DDC)\textsubscript{2}, the MST increased to 25 days (a 25% increase in MST). The difference between the MST of those animals treated with the controls and Cu(DDC)\textsubscript{2} was statistically significant (\textit{P}<0.05) and comparable to previous studies demonstrating a 11.3% increase in MST when the F98 glioblastoma model rats were treated with lipoplatin (a liposomal formulation of cisplatin).\textsuperscript{28}

**Enhancing the circulation longevity of Cu(DDC)\textsubscript{2}**

Although the results presented thus far suggest that the Cu(DDC)\textsubscript{2} formulation prepared in DSPC/Chol liposomes is therapeutically active, a significant advantage of the technology used here to create the first injectable Cu(DDC)\textsubscript{2} formulation is that the synthesis reaction to form Cu(DDC)\textsubscript{2} can be completed in liposomes of different lipid compositions and the environment within the liposome can be controlled. As it is possible that the efficacy of Cu(DDC)\textsubscript{2} may be enhanced through strategies that enhance its circulation lifetime, a number of strategies were pursued to gain an understanding of the factors influencing Cu(DDC)\textsubscript{2} retention in the liposomes. As the in vitro Cu(DDC)\textsubscript{2} assay measuring the stability of the Cu(DDC)\textsubscript{2} formulation proved to be a poor predictor of in vivo stability, we chose to focus on an in vivo assay where plasma levels of copper or Cu(DDC)\textsubscript{2} were measured 30 min after iv administration into mice. This single time point assay allowed us to assess how changes in the formulation impacted the stability of the injected Cu(DDC)\textsubscript{2} formulation. These results are summarized in Figures 5 and 6. First Cu(DDC)\textsubscript{2} was synthesized in DSPC/Chol (55:45) liposomes, which were prepared to contain copper sulfate or Cu-gluconate and, in addition, the Cu(DDC)\textsubscript{2}-to-lipid ratios were varied in these formulations to determine if the amount of entrapped Cu(DDC)\textsubscript{2} affected drug retention. As noted in Figure 5A, the synthesis of Cu(DDC)\textsubscript{2} inside the DSPC/Chol liposomes was similar when the liposomes were prepared with Cu-gluconate or copper sulfate. When evaluating the effect of copper salt on the plasma levels of Cu(DDC)\textsubscript{2} 30 min after injection (Figure 5B, 1st and 4th bar) it can be concluded that use of Cu-gluconate had no impact on Cu(DDC)\textsubscript{2} elimination. These Cu(DDC)\textsubscript{2} formulations were also prepared such that selected formulations had lower Cu(DDC)\textsubscript{2}-to-lipid ratios (Figure 5B, 2nd, 3rd, and 5th column). These formulations exhibited plasma copper levels comparable to the formulations with the higher Cu(DDC)\textsubscript{2}-to-lipid ratios. The elimination of liposomal lipid was unaffected by the formulation changes described above.

When preparing the Cu(DDC)\textsubscript{2} formulation using 300 mM copper sulfate, it is assumed that the internal pH of the liposome is 3.5 (the pH of the copper solution used when making the liposomes). To determine whether the pH within the liposome affects Cu(DDC)\textsubscript{2} elimination we added the monovalent K\textsuperscript{+} ion ionophore nigericin prior to Cu(DDC)\textsubscript{2} synthesis inside the liposomes. Nigericin facilitates the exchange of K\textsuperscript{+} ions in exchange for H\textsuperscript{+}. Thus, CuSO\textsubscript{4}\textsuperscript{2-} liposomes were exchanged into a KCl-Histidine-containing buffer (see Methods) and subsequently nigericin was added. As noted in Figure 5C, the formation of Cu(DDC)\textsubscript{2} inside the DSPC/Chol liposomes was not affected by the change in external buffer or the addition of nigericin. Following iv administration into mice, the plasma copper levels 30 min after administration were comparable for the nigericin formulation and the formulation prepared without nigericin (Figure 5D). This suggests that an increase in pH within the liposomes has no impact on the stability of the injected formulation.

To assess how liposomal lipid composition influences the in vivo elimination of intravenously injected Cu(DDC)\textsubscript{2}, we evaluated Cu(DDC)\textsubscript{2} formulations prepared in, 1) Chol-containing liposomes DSPC/Chol (55:45) and SM/Chol (55:45), 2) Chol-free liposomes (DSPC/DSPE-PEG\textsubscript{2000} (95:5) and SM/DSPE-PEG\textsubscript{2000} (95:5)), as well as 3) charged liposomes anionic (DSPC/DSPG/Chol (70:20:10) and cationic (DSPC/DSPG/Chol (70:20:10)). These studies (summarized in Figure 6) used plasma Cu(CuDDC)\textsubscript{2} levels determined 30 min following iv administration as a measure of whether changes in lipid composition could engender decreases in Cu(DDC)\textsubscript{2} elimination. Although not shown, the ability to synthesize Cu(DDC)\textsubscript{2} in the different liposomal formulations was not affected by liposomal lipid composition. As indicated in Figure 6 (3rd and 6th bar) the only formulations...
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Figure 5 Examining the role of factors within the DSPC/Chol liposomes that may affect Cu(DDC)₃ levels in the plasma compartment 30 min after administration.

Notes: (A) Preparation of Cu(DDC)₃ inside DSPC/Chol (55:45) liposomes containing either CuSO₄ (●) or Cu-glucuronate (●) as a function of time at 25°C in SH buffer. (B) The percent of injected Cu(DDC)₃ dose administered intravenously to C57BL/6 mice (n=4) remaining in the plasma 30 min after injection. The formulations were prepared at different Cu(DDC)₃-to-lipid ratios (0.2, 0.1, and 0.05, mol/mol) and prepared using liposomes with encapsulated CuSO₄ or Cu-glucuronate buffers. Copper levels were measured by AAS and, after subtraction of background plasma copper levels, these levels were used as a surrogate for Cu(DDC)₃. (C) Formation of Cu(DDC)₃ inside DSPC/Chol (55:45) liposomes containing CuSO₄ with (●) or without (●) nigericin as a function of time at 25°C. The external buffer for these liposomes was KCl (150 mM) and Histidine (20 mM). (D) The percent injected dose of Cu(DDC)₃ injected into C57BL/6 mice (n=4) remaining after 30 min following iv administration of Cu(DDC)₃ prepared in DSPC/Chol liposomes in the presence and absence of nigericin. Copper levels were measured by AAS and, after subtraction of background plasma copper levels, these levels were used as a surrogate for Cu(DDC)₃. For panels A and C, n=3 replicate experiments. In panels A and C, n=4 mice per group. All data are plotted as mean ± standard error of the mean.

Abbreviations: AAS, atomic absorption spectroscopy; Chol, cholesterol; DDC, diethyldithiocarbamate; DsPc, distearoyl-sn-glycero-3-phosphocholine; iv, intravenous; SH, sucrose HEPES.

that exhibited significantly higher levels of Cu(DDC)₃ in the plasma when compared to Cu(DDC)₃ formulated in DSPC/Chol liposomes were the DSPC/DSPE-PEG₂₀₀₀ and DSPC/DSPG/Chol formulations. These retained 20.8% and 11.3% of the injected copper dose in the plasma compartment at 30 min, respectively. The DSPC/DSPE-PEG₂₀₀₀ formulation showed the highest plasma copper levels, a nearly 7 fold increase over that measured for animals given Cu(DDC)₃ formulated in DSPC/Chol liposomes. For this reason, we focused the remaining studies on Cu(DDC)₃ formulations prepared in the DSPC/DSPE-PEG₂₀₀₀ liposomes.

Characterizing Cu(DDC)₃ prepared in DSPC/DSPE-PEG₂₀₀₀ (95:5) liposomes

To generate the data shown in Figure 6, the ability to create Cu(DDC)₃ in liposomes prepared of different lipid compositions needed to be determined. The formation of Cu(DDC)₃ in DSPC/DSPE-PEG₂₀₀₀ (95:5) liposomes is illustrated by the data shown in Figure 7. When incubated at 25°C, a Cu(DDC)₃-to-lipid ratio of 0.2 (molar ratio) is achieved within 10 min following addition of DDC to the copper-containing liposomes (Figure 7A, filled squares). DDC enters the liposome through passive diffusion across the liposomal lipid bilayer. As DDC is a relatively small molecule (171 g/mol) it can cross the lipid bilayer rapidly, even when the incubation temperature was reduced to 4°C. It should be noted that the rate of Cu(DDC)₃ formation at 4°C in the DSPC/DSPE-PEG₂₀₀₀ liposomes was slower than that observed in DSPC/Chol liposomes. This is reflected by the data summarized in Figure 7B where the Cu(DDC)₃-to-lipid ratio for DSPC/Chol and DSPC/DSPE-PEG₂₀₀₀ liposomes measured after a 1 h incubation at
4°C (filled bars) suggests that 80% and 50% of the encapsulated Cu is becoming complexed with DDC over the 60 min incubation time frame, respectively. Cryo-electron microscopy was performed on the DSPC/DSPE-PEG<sub>2000</sub> liposomes before and after Cu-DDC<sub>2</sub> synthesis. As illustrated in Figure 7C, there is no visible difference between the liposomes with encapsulated copper sulfate and those with encapsulated Cu-DDC<sub>2</sub>.

Following iv administration of Cu-DDC<sub>2</sub> formulated in the DSPC/DSPE-PEG<sub>2000</sub> liposomes, there was about 70% of the injected lipid dose in the plasma compartment at 4 h. In contrast, only 5% of the injected Cu-DDC<sub>2</sub> was detected (Figure 7D). This is reflected in the change in the Cu-DDC<sub>2</sub>-to-lipid ratio (molar), which indicates that there was 90% loss of the Cu-DDC<sub>2</sub> from the DSPC/DSPE-PEG<sub>2000</sub> liposomes within 4 h after injection (Figure 7E). Although Cu-DDC<sub>2</sub> was still eliminated rapidly from the plasma compartment following injection of the formulation prepared in DSPC/DSPE-PEG<sub>2000</sub> liposomes, this formulation retained Cu-DDC<sub>2</sub> significantly better than the DSPC/Chol liposomes. The plasma AUC<sub>(0→∞)</sub> for Cu-DDC<sub>2</sub> inside DSPC/Chol (55:45) liposomes was found to be 4.6 µg·h/mL while that of Cu-DDC<sub>2</sub> inside of DSPC/DSPE-PEG<sub>2000</sub> (95:5) liposomes was found to be 19.3 µg·h/mL; representing a 4.2-fold increase in AUC<sub>(0→∞)</sub>.

**Therapeutic activity of Cu-DDC<sub>2</sub> prepared in DSPC/DSPE-PEG<sub>2000</sub> (95:5) liposomes**

To determine if the 4.2-fold increase in AUC<sub>(0→∞)</sub> achieved when using Cu-DDC<sub>2</sub>, formulated in DSPC/DSPE-PEG<sub>2000</sub> affected therapeutic activity, mice with established MV-4–11 tumors were treated with this Cu-DDC<sub>2</sub> formulation and the activity was compared to animals treated with Cu-DDC<sub>2</sub> prepared in DSPC/Chol liposomes. As indicated above, the MTD of Cu-DDC<sub>2</sub> prepared in DSPC/Chol liposomes was determined to be 8 mg/kg when using the M, W, F ×2 schedule. Cu-DDC<sub>2</sub> prepared in DSPC/DSPE-PEG<sub>2000</sub> liposomes was better tolerated, and the MTD of this formulation was determined to be 12.5 mg/kg when given via the M, W, F ×2 schedule. To assess efficacy, Cu-DDC<sub>2</sub> prepared in DSPC/DSPE-PEG<sub>2000</sub> liposomes was administered (M, W,
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F ×2 schedule) at 8 and 12.5 mg/kg. The injected liposomal lipid doses were 46 and 63 mg/kg, respectively. When Cu(DDC)₂ was prepared in DSPC/Chol liposomes, the dose of Cu(DDC)₂ was 8 mg/kg and the liposomal lipid dose was 50 mg/kg. Mice bearing MV-4–11 tumors were treated 12 days after tumor cell inoculation and tumor growth was monitored over time (see Methods). The results, summarized in Figure 8, indicated that all Cu(DDC)₂-treated animals showed a statistically significant decrease in tumor volume when compared to vehicle-treated animals (Figure 8A). When comparing tumor size on day 33, the day when some control mice were euthanized because of large tumor sizes (our animal care protocol specifies that the humane endpoint for animals with sc tumors is when the tumor volume is greater...
behaviors of copper and DSF are remarkably different and this has contributed to the lack of success using this approach.

Thus, while the DSF/Cu combination shows remarkable activity in vitro this has not translated in vivo. To date, the therapeutic activity of the Cu(DDC)$_2$ complex has not been directly evaluated in vivo owing to the insolubility of the complex in aqueous solutions. We solved this challenge by synthesizing Cu(DDC)$_2$ inside liposomes. These methods rely on liposomes serving as nano-scale reaction vessels to support the synthesis of Cu(DDC)$_2$ when DDC is added to the outside of copper sulfate-containing liposomes. To our knowledge, we are the first to characterize the anticancer activity of Cu(DDC)$_2$ following iv administration.

We have previously shown that Cu(DDC)$_2$ was cytotoxic to cancer cells but did not have any effect in healthy bronchial epithelial cells in vitro. Consistent with other in vitro studies, we have confirmed that the primary mechanism of action for Cu(DDC)$_2$ is through proteosome inhibition (Figure 2). Flow cytometric studies suggested that cell-cycle arrest does not contribute to Cu(DDC)$_2$ cytotoxicity. Interestingly, DSF and copper have been reported to be effective against brain tumor–initiating cells (BTICs) that are senescent. Thus, the ability of Cu(DDC)$_2$ to be effective regardless of cell-cycle stage may contribute to the drug’s ability to kill BTICs. Our in vitro studies (Figure 2) indicate that the activity of Cu(DDC)$_2$ is not mediated by the generation of ROS, that is, no increase in ROS production.

**Discussion**

DSF and copper have been the focus of many oncology-focused research programs and clinical trials because of the surprising anticancer activity observed when the 2 components are given together in vitro. This discovery exemplified an opportunity to repurpose DSF, an agent with over 60 years of clinical use in the context of alcohol addiction. We, as well as others, have previously shown that the cytotoxicity of DSF and Cu is attributed to the formation of a Cu(DDC)$_2$ complex. Attempts have been made to prepare the complex in vivo by administering DSF and Cu (usually given as Cu-gluconate) separately. However, the PK and distribution behaviors of copper and DSF are remarkably different and this has contributed to the lack of success using this approach.
was observed when cells were incubated with Cu(DDC)$_2$. Previously Tawari et al noted an increase in ROS when DSF and copper were added to cell media but not when DDC and copper were added. The authors suggested that ROS generation was a by-product produced that occurred when DSF and Cu were mixed in vitro. However, ROS would not be generated if using preformed Cu(DDC)$_2$ complex in liposomes, as done in the studies reported here. This was done to help avoid precipitation of Cu(DDC)$_2$ under conditions where the complex is added as a DMSO-solubilized form. The in vitro results suggested that Cu(DDC)$_2$ prepared in DSPC/Chol liposomes was available to the cells in culture, a preliminary indication that Cu(DDC)$_2$ dissociated from the liposomes.

The in vivo studies completed with Cu(DDC)$_2$ produced in DSPC/Chol liposomes indicated that the drug was rapidly eliminated from plasma after iv administration (>90% of the injected dose was eliminated within 15 min, Figure 3) and confirmed that Cu(DDC)$_2$ rapidly dissociated from the liposomes after administration. This was surprising, in part, because the in vitro studies shown in Figure 1C suggested that the Cu(DDC)$_2$ formulations were stable when prepared in the DSPC/Chol liposomes. Cu(DDC)$_2$ prepared in DSPC/Chol liposomes was therapeutically active. When administered at its MTD, the effects resulted in a delay in tumor progression. This was determined in an sc model as well as an intracranial model where Cu(DDC)$_2$ was given by CED directly to the site of tumor cell inoculation (Figure 4). We concluded that the therapeutic activity of Cu(DDC)$_2$ may be limited because of its rapid elimination from the plasma compartment and we thought that this could be addressed. An advantage of the technology used here is that the composition of the liposome in which the Cu(DDC)$_2$ is formed can be changed to improve the in vivo stability of the resulting formulation. When Cu(DDC)$_2$ was prepared in DSPC/DSPE-PEG$_{2000}$ (Chol-free) liposomes there was an improvement in plasma Cu(DDC)$_2$ levels, which likely equated to an improvement in Cu(DDC)$_2$ retention within the liposome. Cu(DDC)$_2$ prepared in DSPC/DSPG/Chol liposomes also showed some improved stability in vivo. In both of these examples, it could be argued that the anionic surface charge played a role in improved stability of the formulation.

Alternatively, because the DSPC/DSPG/Chol liposomes contained only 10% Chol (a low-Chol formulation), improved Cu(DDC)$_2$ retention could be due to the removal of Chol. It is important to note that an increase in drug retention was not seen in the chol-free SM/DSPE-PEG$_{2000}$ formulation. For Cu(DDC)$_2$ prepared in DSPC/Chol liposomes, factors such as Cu(DDC)$_2$ to-lipid ratio, choice of entrapped copper salt, and the internal liposomal pH did not appear to affect the stability of the formulation (Figure 5). The Cu(DDC)$_2$ plasma AUC$_{(0-∞)}$ was 4.2-fold higher for the DSPC/DSPE-PEG$_{2000}$ formulation when compared to Cu(DDC)$_2$ prepared in DSPC/Chol liposomes. Although the resulting DSPC/DSPE-PEG$_{2000}$ formulation was better tolerated, its therapeutic activity was not better than the DSPC/Chol formulation (Figure 8). It can be concluded that the decreased plasma elimination rate affects the safety profile of the resultant formulation, but more significant improvements in the stability of the formulation will be needed to enhance the activity of Cu(DDC)$_2$ in vivo.

The Cu(DDC)$_2$ formulations described here do provide some therapeutic benefit, but we believe that the full therapeutic potential of Cu(DDC)$_2$ may only be achieved through careful selection of the appropriate cancer indication and/or by using it in combination with another drug. For example, Lun et al have demonstrated that temozolomide (TMZ) can be used in combination with DSF and copper.

It would be a natural transition to examine if Cu(DDC)$_2$ could be used in combination with TMZ. Also, given a mechanism that involves proteosome inhibition, Cu(DDC)$_2$ may be useful when combined with other drugs known to enhance the activity of known proteasome inhibitors such as bortezomib.

In this context, it has been shown that bortezomib acts synergistically with CDK9 kinase inhibitors. Cvek et al have examined the mechanism by which Cu(DDC)$_2$ inhibits the cellular 26S proteosome and have described that this occurs through inhibition of the JAMM domain in the 19S proteasome lid, a site distinct to where bortezomib acts. For this reason Cu(DDC)$_2$ may be effective when used in combination with drugs that work well with bortezomib, or alternatively, Cu(DDC)$_2$ may prove active against bortezomib-resistant cancers.

**Conclusion**

Herein we disclose the first studies ever to evaluate the anticancer activity of Cu(DDC)$_2$ prepared using a technique that synthesizes Cu(DDC)$_2$ in the core of liposomes. This method solves problems associated with Cu(DDC)$_2$ insolubility and allows for its direct administration. Two formulations were tested in an in vivo MV-4–11 leukemia model and both produced a ~50% reduction in tumor volume at their respective MTDs when compared to control groups. Additional studies are needed to optimize the therapeutic potential of the Cu(DDC)$_2$ formulations described here. We are particularly interested in establishing its activity in combination with other drugs.
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Disclosure
The authors report no conflicts of interest in this work.

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Supplementary materials
Glioma cell line cytotoxicity

F98 and U251 cells were grown in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) which was supplemented with 2 mM L-glutamine (Thermo Fisher Scientific) and 10% fetal bovine serum (Thermo Fisher Scientific) and maintained at 37°C and 5% CO₂. Prior to drug treatment, the cells were seeded into 384-well plates and allowed to grow for 24 h. Copper diethyldithiocarbamate (Cu(DDC)_2) was added for 72 h and then cells were stained with Hoescht 33342 and ethidium homodimer I for total and dead cell counts, respectively. Cells were imaged using an In Cell Analyzer 2200 (GE Healthcare Life Sciences, Mississauga, ON, Canada) and cell viability was measured based on viable nuclei count.

Figure S1 Cu(DDC)_2 cytotoxicity in F98 and U251 glioma cancer cell lines.
Notes: (A, B) Cells were exposed to Cu(DDC)_2 (prepared inside DSPC/Chol liposomes) for 72 h. Cell viability was obtained using the In Cell Analyzer where viability was assessed based on loss of plasma membrane integrity 72 h following treatment; that is, total cell count and dead cell count were determined using Hoechst 33342 and ethidium homodimer staining, respectively. Data is presented as mean ± standard error of the mean of 3 experiments.
Abbreviations: Chol, cholesterol; DDC, diethyldithiocarbamate; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine.