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Liposomal Cytarabine Induces Less Neurocognitive Dysfunction than Intrathecal Methotrexate in an Animal Model

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Abstract

Liposomal cytarabine is currently being tested clinically as an alternative to intrathecal (IT) methotrexate (MTX) for preventing relapse within the central nervous system among patients with acute lymphoblastic leukemia (ALL). To compare the toxicity and cognitive deficits caused by IT MTX versus liposomal cytarabine, juvenile Long Evans rats were treated with intrathecal injections of MTX 1 mg/kg $\times$ 4 doses over 8 days, or liposomal cytarabine 0.8 mg once. Mean concentrations of free cytarabine in cerebrospinal fluid remained above the cytotoxic threshold of 0.4 μ M for two weeks post-dosing. Animals treated with liposomal cytarabine exhibited normal recognition and spatial memory four weeks after injection. In contrast, exposure to IT MTX led to impaired cognitive function. In addition, mean hematocrit on day 11 was significantly lower in the MTX-treated animals (30.8%, 95% CI 27.0–34.7%, $n = 6$) compared to liposomal cytarabine (39.5%, 95% CI 38.4–40.6%, $n = 6$; $p < 0.0001$). Our data suggest that liposomal cytarabine induces fewer neurocognitive deficits and less acute hematologic toxicity compared to IT MTX. Liposomal cytarabine may therefore have therapeutic advantages over intrathecal methotrexate, if it is equally effective in preventing relapse.

Keywords

Acute lymphoblastic leukemia; childhood cancer; Depocyt; neurotoxicity; cognitive deficits

Introduction

Most children with acute lymphoblastic leukemia (ALL) are cured with current therapeutic regimens, which include prophylactic intrathecal chemotherapy to prevent relapse within the

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central nervous system (CNS). However, ALL survivors often suffer from measurable neurocognitive deficits as a result of CNS-directed therapy.¹⁻⁵

Methotrexate (MTX) is a standard component of CNS prophylaxis. Administered both intrathecally and as a high-dose intravenous infusion, its use has led to a dramatic reduction in CNS relapse rates. We and others have demonstrated that MTX induces structural and metabolic changes in the CNS, as well as persistent neurocognitive dysfunction in human and animal models relative to sham-injected controls.⁶⁻¹¹ As both physical and psychosocial quality of life is affected by the individual's cognitive functioning, it is important to address this therapeutic problem.¹²

This study was conducted to compare the systemic toxicities and cognitive sequelae that result from intrathecal (IT) MTX versus liposomal cytarabine, when both are administered at clinically relevant doses and schedules. The sustained-release formulation of cytarabine, liposomal cytarabine, is effective in the relapse setting.¹³ After intrathecal administration, cytotoxic cytarabine levels are observed in the cerebrospinal fluid (CSF) for at least one week.¹⁴ Treatment with liposomal cytarabine can cause chemical arachnoiditis, but this is a side effect that can be prevented by concomitant administration of dexamethasone.^{13,15-18} Neither cognitive deficits nor marrow suppression are commonly described after intrathecal cytarabine, suggesting that liposomal cytarabine will also offer these therapeutic advantages over IT MTX.

Materials and Methods

Experimental Design

All experiments were approved by the Animal Institute and Use Committee of the Albert Einstein College of Medicine (Bronx, NY) and were conducted following the "Guide for the Care and Use of Laboratory Animals". Juvenile (4-weeks old) Long Evans rats were obtained from Charles River Laboratories (Wilmington, MA) and randomly divided into three treatment arms with an equal distribution of males and females. Rats were housed in groups of two or three with a 12/12 hours light/dark cycle and *ad lib* access to food (LabDiet 5001) and water. Treatment started one week after arrival, when the animals were 5 weeks old. Each treatment arm was designed to mimic a schedule used clinically to prevent or treat CNS relapse of ALL (Fig. 1).^{13,15,19} A control cohort was given sham injections of artificial CSF (aCSF, Na⁺ 150mM, K⁺ 3mM, Ca²⁺ 1.4mM, Mg²⁺ 0.8mM, P 1.0mM, Cl⁻ 155mM) in volumes equivalent to liposomal cytarabine injections. MTX was administered at a dose of 1 mg/kg diluted in aCSF, at a final volume of 30-60 microliters, four times in a two-week period. Liposomal cytarabine was given once, at a dose of 0.8 mg (80 microliters) along with daily intraperitoneal (IP) injections of 0.2 mg (1 mL) dexamethasone for three days to reduce the risk of acute arachnoiditis.^{13,20} MTX and control animals received 1 mL IP phosphate-buffered saline (PBS) on the same schedule. IT injections were carried out as previously described by transcutaneous cisterna magna puncture with a 25 gauge butterfly needle.⁷ Correct insertion of the needle was verified by outflow of CSF, which was collected in isovolumetric amounts (i.e., volume of CSF removed was equivalent to volume of drug to be administered) prior to IT injection. All animals were monitored for signs of acute toxicity under direct visualization for one hour after injection and subsequently on a daily basis for

evidence of abnormal behaviors. Treatment conditions were replicated in two independent cohorts and a total of 118 rats were included in the study. Animals were excluded ($n = 10$) if death ($n = 4$), or any serious health conditions (e.g. severe dental malocclusion) occurred during the time span (13 weeks) of the experiments.

Weight was recorded weekly starting the day of treatment. CSF was collected prior to each intrathecal treatment, plus on days seven and fourteen, and directly put on ice.

Approximately 5% of collected specimens were visibly contaminated by blood and were discarded. The samples were centrifuged to remove any cellular elements and supernatants were stored at -75°C until further analysis. Blood was collected from ventral artery of the tail on day 11 and 14 after first IT treatment, using a 25 gauge needle collecting volumes that varied from 0.3 – 0.6 mL. Blood was immediately placed in EDTA tubes and stored at $+4^{\circ}\text{C}$ until further analysis. Behavioral tests were carried out at four and eleven weeks after first IT treatment (Fig. 1).

High Performance Liquid Chromatography

Concentrations of free cytarabine in CSF were measured by HPLC using a Waters 2695 separation module equipped with a U Bondapak C18 analytical column ($3.9 \times 150\text{mm}$). After trichloroacetic acid precipitation of protein, 25 microliters of CSF was injected, using a mobile phase of 0.1 M KH_2PO_4 /methanol buffer, pH 3.1. Absorbance was monitored with a PDA detector at 272 nm. Cytarabine concentrations were quantified based on an external calibration method with known standards (Sigma Aldrich, St. Louis, MO). The lower limit of detection was 0.08 micromolar.

Behavioral Assessments

The open field was used to assess locomotor activity, exploratory and anxiety-like behavior. Briefly, rats were free to explore the testing arena ($69 \times 69\text{ cm}$) for six minutes. Total track length was a measure of general activity level and time spent in the center zone ($21 \times 21\text{ cm}$) related inversely to the degree of anxiety-like behavior. Activities were captured by digital camera and analyzed by Viewer software (Biobserver, Bonn, Germany).

Recognition and spatial memory are dependent on distinct aspects of working memory and were assessed with the novel object recognition (NOR) task and the novel object placement (OP) task, respectively, as previously described.^{7,21–23} These tasks utilize the natural preference of rodents to explore novel objects. Briefly, during training, animals were exposed to a pair of identical objects. After a defined retention interval in their home cages (30–180 minutes depending on the task), rats were presented with one unmoved and one relocated object (OP) or one old and one novel object (NOR) in a testing trial. Total activity, assessed by track length, and total object exploration times were recorded in seconds. Exploration was defined as any physical contact with the object (sniffing, whisking, or touching). Trials with total exploration time less than 4 seconds were excluded from analysis (less than 2% of trials). A preference score was determined by the ratio of time exploring the relocated or novel object to total exploration time during the testing trial. For each cohort of identically treated animals, intact memory was demonstrated by a group mean preference score significantly higher than 55%. In our prior experience, animals with preference scores

above 55% consistently demonstrate novel object preferences when retested.^{6,7,10,23} All animals were exposed to equal conditions regarding time of training, testing, and period of retention interval. The tester was blinded to treatment conditions.

Statistical analysis

One sample, two-tailed *t*-test was used to determine whether a cohort of identically treated rats showed mean preference scores that were statistically higher than 55%. Mean preference scores were compared with GraphPad Prism 6.07 (GraphPad Software, San Diego, CA) using two-tailed *t*-tests and contingencies were analyzed by Fisher's Exact Test. Biochemical data were analyzed using one-way ANOVA analysis. For all statistical tests, a probability value less than 0.05 was considered to be statistically significant.

Results

Liposomal Cytarabine Induces Prolonged Cytotoxic Activity in CSF

Following intrathecal administration of 0.8 mg liposomal cytarabine, free cytarabine in CSF was measured by HPLC. Mean free cytarabine concentrations remained above 0.4 μ M, levels that are known to be cytotoxic in vitro^{13,24}, for at least two weeks as shown in Fig. 2. No differences in pharmacokinetics were detected between males and females.

Absence of Gross Systemic Toxicity

Neither MTX nor liposomal cytarabine had impact on weight gain, Fig. 3. There was no significant difference in mean growth ($\pm$ SD) among females (aCSF 3.2 ± 1.7 g/day, $n = 20$; liposomal cytarabine 3.3 ± 1.2 g/day, $n = 20$; MTX 3.4 ± 1.4 g/day, $n = 22$) or males (aCSF 5.8 ± 2.5 g/day, $n = 22$; liposomal cytarabine 5.9 ± 2.1 g/day, $n = 19$; MTX 6.0 ± 1.7 g/day, $n = 15$).

One animal in the MTX arm experienced tonic clonic seizures within one hour after the first MTX injection. The seizure resolved spontaneously with no recurrence after subsequent injections. No other acute toxicities, indicated by changes in grooming or other behaviors, were observed from any treatment. Four animals treated with MTX died within one hour of IT injection, due to incidental trauma to the brain stem in the process of intrathecal injection, not to acute drug toxicity.

Locomotor activity and anxiety-like behavior were assessed in the open field four weeks after the first IT injection. No differences were observed in total track length (aCSF 3544 ± 559.5 cm, $n = 42$; liposomal cytarabine 3431 ± 568.6 cm, $n = 39$; MTX 3372 ± 572.8 cm, $n = 37$). Nor were there any indications of anxiety-like behavior, inversely related to the duration of time spent in the center zone of the testing arena, in any cohort of (aCSF 13.6 ± 6.3 sec, $n = 42$; liposomal cytarabine 13.6 ± 7.2 sec, $n = 39$; MTX 14.9 ± 8.6 sec, $n = 37$).

Neurocognitive Outcomes

No significant differences were observed between males and females in any neurocognitive outcome. Therefore, data for both sexes are combined in the remainder of the results presented here. As we have previously demonstrated^{6,7,10}, animals treated with IT MTX

showed impaired spatial memory (OP task) and recognition memory (NOR task) four weeks after injection, with mean preference scores that were not significantly greater than the 'passing' criterion of 55% (OP: 56.1%, 95% CI 51.2-61.0%, $t = 0.5$, $df = 36$, $n = 37$, $p > 0.05$; NOR: 56.8%, 95% CI 50.6-62.9%, $t = 0.6$, $df = 25$, $n = 26$, $p > 0.05$). Partial recovery of neurocognitive function was observed eleven weeks after intrathecal injection. At this time point, spatial memory remained impaired (Fig. 4), but MTX treated animals had a mean NOR preference score that was significantly greater than 55% (61.7 %, 95% CI 57.4-66.0, $t = 3.2$, $df = 36$, $n = 37$, $p < 0.05$.)

In contrast with the MTX-treated animals, those treated with liposomal cytarabine demonstrated intact spatial and recognition memory four weeks after injection. Mean OP and NOR preference scores were significantly greater than the 55% pass criterion (OP: 63.1 %, 95% CI 58.1-68.0 %, $t = 3.3$, $df = 38$, $n = 39$, $p < 0.05$; NOR: 67.2 %, 95% CI 62.4-72.0 %, $t = 5.3$, $df = 22$, $n = 23$, $p < 0.0001$) (Fig. 5). Eleven weeks after IT liposomal cytarabine, recognition memory remained intact, with a mean NOR preference score significantly greater than 55% (63.7%, 95% CI 60.2-67.2%, $t = 5.0$, $df = 38$, $n = 39$, $p < 0.0001$, one sample two-sided t -test) (Fig. 4). However, mean OP preference scores now indicated impaired spatial memory (55.8%, 95% CI 52.0-59.6%, $t = 0.4$, $df = 38$, $n = 39$, $p > 0.05$). At both the four week and eleven week time points, the proportion of animals with impaired recognition or spatial memory tended to be greater among the cohort treated with IT MTX than among those treated with intrathecal liposomal cytarabine (Fig. 4). Although the trend was consistent at the two time points, these differences were not statistically significant by a Fisher's Exact Test.

MTX but Not Liposomal Cytarabine Caused Transient Suppression of Hematopoiesis

Mean hematocrit ($\pm$ SD) was significantly decreased at day 11 after administration of IT MTX ($30.8\% \pm 3.7\%$, 95% CI 27.0-34.7%, $n = 6$) compared to liposomal cytarabine ($39.5\% \pm 1.1\%$, 95% CI 38.4-40.6%, $n = 6$, $p < 0.0001$) and controls ($40.5\% \pm 1.1\%$, 95% CI 34.4-41.6%, $n = 6$, $p < 0.0001$) (Fig. 5). Unfortunately, insufficient sample volumes were available at day 11 to measure complete blood counts.

Three days later, on day 14, complete blood counts were analyzed. Hematocrit had equalized in all treatment arms with mean hematocrit ($\pm$ SD) of $38.3\% \pm 2.8\%$, $n = 4$; $39.3\% \pm 1.5$, $n = 4$; and $39.8\% \pm 1.3$, $n = 4$ ($p > 0.05$) in MTX, liposomal cytarabine and aCSF treated animals, respectively. Absolute reticulocytes ($\pm$ SD) were significantly increased in the MTX treated arm ($552.3 \pm 115.8 \times 10^3/\mu\text{L}$, $n = 4$) compared to both liposomal cytarabine ($208.3 \pm 57.0 \times 10^3/\mu\text{L}$, $n = 4$, $p < 0.05$) and controls ($246.8 \pm 41.2 \times 10^3/\mu\text{L}$, $n = 4$, $p < 0.05$), Fig. 5. No significant differences were measured in mean white blood cells ($\pm$ SD) (MTX $7.5 \pm 3.0 \times 10^9/\text{L}$, $n = 4$; liposomal cytarabine $4.7 \pm 0.6 \times 10^9/\text{L}$, $n = 4$; aCSF $7.1 \pm 1.4 \times 10^9/\text{L}$, $n = 4$, $p > 0.05$) or median platelet counts (MTX 1095, IQR $331.0 \times 10^3/\mu\text{L}$, $n = 4$; liposomal cytarabine 933.5, IQR $106.7 \times 10^3/\mu\text{L}$, $n = 4$; aCSF 947.0, IQR $860.2 \times 10^3/\mu\text{L}$, $n = 4$).

Discussion

Currently, MTX is the most frequently used chemotherapeutic drug for CNS-directed treatment in ALL. However, it has been associated with acute neurological and hematological toxicity and persistent cognitive impairments compared to controls in both human and animal models.^{1,3,11,25–27}

Intrathecal administration of free cytarabine is already widely used either alone or as part of a triple IT therapy for patients with leukemia. Liposomal cytarabine is a slow-release formulation of cytarabine that leads to prolonged cytotoxic activity after administration and thus reduces the need of frequent and burdensome intrathecal injections, and risk of myelosuppression. Hence, liposomal cytarabine may offer potential advantages over MTX with regard to CNS prophylaxis. In this study, we demonstrated that liposomal cytarabine does not cause any detectable acute toxicity and more importantly, that it induces less cognitive dysfunction than an equivalent course of IT MTX.

It is possible that liposomal cytarabine leads to a later onset of cognitive decline compared to intrathecal MTX. Recognition memory remained normal among the liposomal cytarabine treated animals at both time points; however, at the eleven-week point we did observe an increase in the proportion showing impaired function. Similar increase in the OP task resulted in a shift from intact spatial memory at four weeks to impaired spatial memory by eleven weeks. Nevertheless, the increases was not statistically significant and in both tests at both time points, the proportion of animals demonstrating impaired memory was greater after IT MTX than after liposomal cytarabine.

Importantly, the finding of less cognitive deficits among animals treated with liposomal cytarabine was not due to sub-therapeutic drug levels. Free cytarabine concentrations in CSF were maintained above cytotoxic threshold of 0.4 μM for two weeks following injection of 0.8 mg liposomal cytarabine, which corresponds with findings in previous animal and human studies.^{13,16} These results further support the clinical utility of intrathecal liposomal cytarabine for CNS prophylaxis, as duration of exposure to cytotoxic drug concentrations is a critical factor for cell-cycle specific antimetabolites like both cytarabine and MTX.

In addition to the deleterious effects on cognition, MTX can exert systemic toxicity when administered intrathecally, because of active transport from CSF to blood.^{27,28} Although we did not measure serum MTX concentrations, we did observe suppression of hematopoiesis (anemia, followed by recovering reticulocytosis). Because IT MTX can exacerbate the systemic toxicity caused by oral or intravenous therapy, clinical protocols for childhood leukemia in the United States do not administer IT MTX concurrently with systemic MTX. The absence of hematopoietic suppression after intrathecal liposomal cytarabine suggests that the use of this agent for CNS prophylaxis would facilitate the maintenance of systemic treatment intensity. It should be noted that the myelosuppressive effect of IT MTX may contribute to prevention of marrow relapse. The theoretical possibility that replacing IT MTX with liposomal cytarabine could increase risk of relapse should therefore be monitored carefully in clinical trials comparing the two approaches.

Intercohort differences in treatment-induced neurocognitive impairments were a priori expected to be subtle. To increase our statistical power to detect the effects of each drug, the experiments were conducted in two independent cohorts, and the results shown here are the combined data sets. Our study was not powered to detect sex differences in susceptibility to treatment-induced memory deficits. Both males and females were included, as behavioral activity can be sex dependent. Nevertheless, we did not observe significant differences in cognitive performance between the two sexes. Finally, we acknowledge the potential confounder of systemic dexamethasone among the animals treated with liposomal cytarabine. Steroids can alter neurocognitive functioning directly, through glucocorticoid receptors in the hippocampus. Alternatively, they may reduce treatment-induced cognitive dysfunction by inhibiting neuro-inflammation. However, we felt its use was warranted in this study to model the clinical administration of liposomal cytarabine, which, unlike IT MTX, is typically administered concurrently with a systemic corticosteroid to prevent symptoms of chemical arachnoiditis.²⁰

Ongoing studies in our laboratory will assess the effects of repeated doses of liposomal cytarabine on cognitive function. In addition, we will compare the pathophysiologic mechanisms triggered by intrathecal administration of liposomal cytarabine versus MTX, including alterations in neurogenesis, white matter injury, and oxidative stress.

In conclusion, liposomal cytarabine induced less acute hematologic toxicity and decreased cognitive dysfunction relative to intrathecal MTX in our animal model. These findings suggest that liposomal cytarabine may offer therapeutic advantages over intrathecal MTX and support further clinical comparison of the efficacy and toxicity of these two agents for preventing CNS relapse among children with ALL.

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Figure 1. Experimental schema. Timing of intrathecal (IT) treatment with 1 mg/kg methotrexate (MTX), 0.8 mg liposomal cytarabine or equivalent volumes of aCSF. Liposomal cytarabine treated animals received intraperitoneal (IP) treatment of 0.2 mg dexamethasone while aCSF and MTX animals received equivalent volumes of IP phosphate-buffered saline (PBS) in addition to IT treatment. Behavioral testing was conducted four and eleven weeks after IT treatment start. Behavioral testing, CSF and blood collection apply to all three treatment groups.

Figure 2.

Mean concentrations of free cytarabine (AraC) in cerebrospinal fluid (CSF) are maintained above cytotoxic threshold of 0.4 μM for at least two weeks after intrathecal (IT) injection of 0.8 mg liposomal cytarabine. CSF was collected on days 1, 2, 7, and 14 after IT treatment and AraC levels were measured by HPLC. Each point represents mean value ($\pm$ SD) from six to ten animals. Dotted line ($Y=0.4$) indicates cytotoxic threshold.

Figure 3.

Average weight gain per day. Each point represents mean value ($\pm$ SD) for either sham-injected controls (male $n = 22$; female $n = 20$), 0.8 mg liposomal cytarabine (male $n = 19$; female $n = 20$), or 1 mg/kg methotrexate (MTX) (male $n = 15$; female $n = 22$). $P > 0.05$ for comparisons between the three treatment groups.

Figure 4.

Behavioral performance in the novel object placement (OP) task and the novel object recognition (NOR) task at four and eleven weeks after first intrathecal (IT) treatment of 0.8 mg liposomal cytarabine (LC) (4 weeks: OP, $n = 39$; NOR, $n = 23$; 11 weeks: OP, $n = 37$; NOR, $n = 39$) or 1 mg/kg methotrexate (MTX) (4 weeks: OP, $n = 37$; NOR, $n = 26$; 11 weeks: OP, $n = 37$; NOR, $n = 37$). Graphs show combined data from both males and females. Each symbol represents one animal. Dotted line at preference score of 55% indicates the criterion for passing the test. The percentage of the whole treatment group who

did not pass is indicated above the X-axis. Mean scores significantly above 55% are indicated with asterisk (one sample two-sided *t*-test, *, $p < 0.05$). In all tests, the proportion of liposomal cytarabine treated animals who failed to show intact function was smaller than the proportion of methotrexate treated animals.

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Figure 5.

Hematocrit (HCT) plotted on left Y-axis was measured on days 11 (d11 HCT) and 14 (d14 HCT) after first intrathecal (IT) injection of 0.8 mg liposomal cytarabine (d11, $n = 6$; d14, $n = 4$), 1 mg/kg methotrexate (MTX) (d11, $n = 6$; d14, $n = 4$), or equivalent volumes of artificial cerebrospinal fluid (aCSF) (d11, $n = 6$; d14, $n = 4$). Absolute reticulocyte counts plotted on right Y-axis were measured on day 14 (d14 retic). Mean values (+SD) are shown. Square brackets with asterisk indicate significantly different values (one sample two-sided t -test, *, $p < 0.05$) between two treatment groups.