Memantine Protects Rats Treated with Intrathecal Methotrexate from Developing Spatial Memory Deficits

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Abstract

Purpose: To test whether memantine can prevent methotrexate-induced cognitive deficits in a preclinical model.

Experimental Design: After noting that methotrexate exposure induces prolonged elevations of the glutamate analog homocysteic acid (HCA) within cerebrospinal fluid, we tested whether intrathecal injection of HCA would produce memory deficits similar to those observed after intrathecal methotrexate. We then tested whether memantine, an antagonist of the N-methyl-D-aspartate (NMDA) subclass of glutamate receptors, could protect animals treated with clinically relevant doses of intrathecal methotrexate against developing memory deficits. Finally, we asked whether memantine affected this pathway beyond inhibiting the NMDA receptor by altering expression of the NMDA receptor or affecting concentrations of HCA or glutamate within the central nervous system.

Results: Four intrathecal doses of methotrexate induced deficits in spatial memory, persisting at least one month following the final injection. Intrathecal HCA was sufficient to reproduce this deficit. Concurrent administration of memantine during the period of methotrexate exposure was protective, decreasing the incidence of methotrexate-induced spatial memory deficits from 56% to 20% (P < 0.05). Memantine neither altered expression of NMDA receptors within the hippocampus nor blunted the methotrexate-induced increases in glutamate or HCA.

Conclusions: Excitotoxic glutamate analogs including HCA contribute to cognitive deficits observed after intrathecal methotrexate. Memantine, an NMDA receptor antagonist, reduces the incidence of cognitive deficits in rats treated with intrathecal methotrexate, and may therefore benefit patients with cancer receiving similar treatment.

Introduction

For patients with acute lymphoblastic leukemia (ALL) or non-Hodgkin lymphoma, intrathecal administration of the chemotherapeutic drug, methotrexate, significantly reduces the risk of relapse within the central nervous system (CNS) and consequently increases disease-free survival. However, intrathecal methotrexate is associated with neurotoxic sequelae including cognitive deficits. Survivors of childhood leukemia show an increased rate of deficits in working memory and executive function, leading to impaired school and occupational performance (1–7), as well as diminished quality of life (8–10). Among patients treated without cranial radiation, treatment intensity explains some portion of the variability in cognitive outcomes (5), but all children with ALL seem to be at some risk of posttreatment cognitive dysfunction (11). Cognitive remediation strategies can minimize the effects of persistent neurotoxicity on daily living (12), but no therapeutic interventions to interrupt the underlying pathophysiology and thus prevent neurotoxicity, have yet been developed.

Using an animal model, we recently showed that clinically relevant doses of intrathecal methotrexate induced deficits in visual recognition and spatial memory that persist for at least 3 months following drug exposure (13, 14). Furthermore, we noted that intrathecal methotrexate led to a decrease in cerebrospinal fluid (CSF) folate and an increase in CSF homocysteine (13). These changes were followed by a persistent increase in homocysteic acid (HCA) and homocysteine sulfonic acid, which remained above baseline at least 3 months after the final methotrexate injection (14). Both of these glutamate analogs are known to induce excitotoxic neuronal damage through the N-methyl-D-aspartate (NMDA) class of glutamate receptors (15–18), leading us to suspect that excessive activation of the NMDA receptor was contributing to cognitive deficits long after methotrexate exposure. Dextromethorphan, a weak noncompetitive antagonist at the NMDA receptor, was able to transiently normalize memory among rats...
Memantine Protects against Methotrexate-Induced Memory Deficits

Translational Relevance
For patients with acute lymphoblastic leukemia or non-Hodgkin lymphoma, intrathecal administration of the chemotherapeutic drug methotrexate significantly reduces the risk of relapse within the central nervous system and consequently increases disease-free survival. However, intrathecal methotrexate is associated with neurotoxic sequelae including cognitive deficits. Survivors of therapy for childhood leukemia show an increased rate of deficits in working memory and executive function, leading to impaired school and occupational performance, as well as diminished quality of life. Although cognitive remediation strategies can minimize the effects of persistent neurotoxicity on daily living, clinically successful therapeutic interventions to interrupt the underlying pathophysiology and thus prevent neurotoxicity have not yet been developed. Using a preclinical model, we now show that the N-methyl-D-aspartate (NMDA) antagonist memantine can protect against spatial memory deficits induced by clinically relevant doses of intrathecal methotrexate. We conclude that memantine may therefore benefit patients with cancer treated with intrathecal methotrexate.

The experiments described here were undertaken to further probe this pathophysiologic model and to test whether an NMDA antagonist could successfully prevent the cognitive deficits induced by intrathecal methotrexate. Memantine was used in place of dextromethorphan in the current experiments because it is a more specific inhibitor of the NMDA receptor, with fewer off-target effects (19).

Our results support the hypothesis that excessive NMDA activation contributes significantly to methotrexate-induced cognitive deficits. Increased CSF concentrations of the NMDA agonist HCA were sufficient to induce cognitive deficits identical to those observed after intrathecal methotrexate, and an NMDA antagonist, memantine, prevented methotrexate-induced memory deficits.

Materials and Methods

Materials
Methotrexate, memantine, HCA, and other chemicals were purchased from Sigma unless otherwise stated. Methanol and water [high-performance liquid chromatography (HPLC) grade] was purchased from Fisher Scientific.

Animals
Eight-week-old male Long-Evans rats were obtained from Charles River Laboratories. Long-Evans rats exhibit high general activity levels, high visual acuity, high levels of exploratory behavior, and robust circadian function (20, 21), leading to more consistent and reproducible behavior on cognitive testing than has been observed in other strains such as Sprague-Dawley (22–24). Rats were housed in groups of 2 or 3 with ad lib food (LabDiet 5001) and water with a 12-hourlight/12-hour dark cycle. All studies were conducted following the “Guide for the Care and Use of Laboratory Animals” and were approved by Animal Institute Committee of the Albert Einstein College of Medicine (Bronx, NY). A total of 186 rats were used in these experiments.

Experimental design
To test whether the NMDA agonist HCA was sufficient to induce cognitive deficits, HCA was injected intrathecally, daily for 10 days, excluding weekend days. To test whether an NMDA antagonist could prevent methotrexate-induced memory deficits, methotrexate was injected 4 times over a 2-week period, with or without concurrent administration of the NMDA antagonist memantine. The timing for drug treatments and behavioral testing is indicated in Fig. 1. Animals were treated in cohorts of approximately 20 rats (half receiving treatment and half receiving sham injections). Each treatment condition was replicated in at least 2 independent cohorts.

Drug treatments
Intrathecal injection was carried out by transcutaneous cisternal magna puncture as previously described (13). Briefly, rats were anesthetized with inhaled isoflurane (2%–5% with oxygen), and positioned in the lateral decubitus position. A 25-gauge butterfly needle was inserted into the cisterna magna. Correct positioning of the needle was verified by outflow of CSF. For intrathecal injection, methotrexate and HCA were diluted in artificial cerebrospinal fluid (aCSF; Na\(^+\) 150 mmol/L, K\(^+\) 3 mmol/L, Ca\(^{2+}\) 1.4 mmol/L, Mg\(^{2+}\) 0.8 mmol/L, P 1.0 mmol/L, CI\(^-\) 155 mmol/L, in double distilled water). All injected solutions were sterilized by filtering through 0.22 μm syringe filters. A final volume of 100 μL was injected over 30 to 60 seconds. HCA was injected at a daily dose of 44 ng, calculated to increase the CSF concentration from baseline into the range observed one month after intrathecal methotrexate (14). Methotrexate was injected at a dose of 0.5 mg/kg, calculated to achieve a CSF methotrexate concentration in the rats similar to that observed after intrathecal injection in patients with leukemia. The schedule (4 doses within 2 weeks) is identical to the schedule of prophylactic intrathecal therapy administered in the CNS-intensive phase of therapy in current Dana Farber Cancer Institute Acute Lymphoblastic Leukemia Consortium (Boston, MA) protocols for children with ALL (7, 25). Memantine 2.5 mg/kg or saline was given by intraperitoneal injection, daily, Monday through Friday, for 3 weeks, beginning 1 day before intrathecal injections of either methotrexate or aCSF.

Measurement of homocysteic acid in cerebrospinal fluid
CSF was collected by gravity before each injection and at postinjection time points, as indicated in the results.
Samples with gross contamination by blood were not analyzed (~20% of all specimens). CSF samples were placed immediately in ice. After brief centrifugation to remove any cellular elements, supernatants were stored at −80°C until analysis. HCA in rat CSF was quantified using reverse phase HPLC after precolumn derivitization with o-phthalaldehyde, as previously published (14).

**Behavioral assessment of spatial memory**

Spatial memory was tested as previously described (13, 26), at the times indicated in the results section. The object placement test, a measure of working memory (27–29), probes a domain of cognitive function that is frequently impaired among survivors of therapy for leukemia or lymphoma (1–4). The tester was blinded to the previous treatment conditions (e.g., methotrexate vs. aCSF or memantine vs. saline). Briefly, during training, animals were exposed to a pair of identical objects. After a defined retention interval in their home cages (20 minutes), rats were presented with one unmoved and one relocated object in a testing trial. Activities were captured by digital camera and analyzed with Viewer software (Biobserver). Total activity, assessed by track length, and total object exploration times were recorded in seconds. A preference score was determined by the ratio of time exploring the new or relocated object to total exploration time during the testing trial. Trials with total exploration time less than 4 seconds were excluded from analysis (~3% of trials). For each cohort of similarly treated animals, intact spatial memory was shown by a preference score significantly higher than chance exploration, with a mean preference score greater than 53%. Cohorts of control rats consistently show mean preference scores statistically greater than 53%, and less than 20% of the individual control rats will have preference scores less than 53% (e.g., Fig. 3, a).

**Measurement of parenchymal glutamate by magnetic resonance spectroscopy**

Magnetic resonance spectroscopy (MRS) was conducted on rats treated with intrathecal methotrexate (n = 6), intrathecal methotrexate with concurrent intraperitoneal memantine (n = 6), and control rats (intrathecal aCSF), 6 weeks after the last intrathecal injection. MRS acquisitions were conducted on a 9.4T MRI scanner, equipped with 100 G/cm gradients, and operating on Varian NMR software (Version 3.1A). All animals were preanesthetized in a small chamber with inhaled 3% isoflurane, and were maintained inside the scanner on 1% to 2.5% isoflurane in 1 l/minute room air delivered via a nasal mask as needed to maintain a respiratory rate of approximately 60 breaths/minutes. Respiratory and heart rate were monitored continuously while inside the scanner, and body temperature was maintained with warm air. The sequence was a single voxel, point-resolved spectroscopy acquisition, with the following parameters: time to echo (TE) = 11.3 ms, time to repetition (TR) = 4,000 ms, 1,024 points collected with a 4 kHz spectral width, 512 averages, total acquisition time = 35 minutes. All MRS were collected in the left hippocampus, double oblique based on coronal and transverse anatomical T2 images, with a voxel size of 1.5 (AP) × 2.0 (SI) × 4.0 (RL) mm. Shimming was conducted with second-order shim terms to achieve less than 15 Hz water line width (typical 9–12 Hz). Water suppression, using 7 variable power RF pulses (30), was optimized before acquisition to ensure minimal water contamination. A water-unsuppressed spectrum was collected in each animal using 8 averages to extract absolute metabolite concentrations. Spectra were processed using the linear combination model (31), with a custom basis set provided for 11 ms TE, and fit to the standard set of metabolites. Both absolute and relative (to total creatine) metabolite concentrations were extracted and used for statistical comparisons. Spectra with signal-to-noise ratio
< 8 and metabolites with SD of >20% were considered unreliable and removed from the final statistical analyses.

Hippocampal NMDA receptor expression
NMDA receptor expression within the hippocampus of 6 rats was assessed by Western blotting, 6 weeks after the final intrathecal methotrexate dose to determine whether methotrexate and/or memantine affected receptor expression. Anesthetized rats underwent terminal cardiac perfusion with a minimum of 50 mL saline. The brain was immediately removed and placed on ice. The left hippocampus was dissected from each brain and homogenized in an Igepal CA-630/deoxycholate lysis buffer. Fifty micrograms of protein were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. Blots were probed for the NMDA receptor using a mouse antibody against the NR2B subunit at a 1:1000 dilution (05–920, Millipore). A secondary polyclonal anti-mouse horseradish peroxidase (31432, Thermo Scientific) was then used. A mouse monoclonal anti-β actin (A2228, Sigma-Aldrich) was used as a loading control at a 1:5000 dilution.

Statistical analysis
Mean preference scores and excitotoxic amino acids concentrations were analyzed with Prism version 6.01 for Windows (GraphPad Software, www.graphpad.com). One sample, two-tailed t tests were used to determine whether a cohort of identically treated rats showed mean preference scores that were statistically different from chance (i.e., >53%). Group means were compared by one-way ANOVA, followed by post hoc two-tailed t tests. Fisher exact test was used to compare proportions of rats in differently treated cohorts showing intact memory (defined by a preference score >53%).

Results
Intrathecal injection of an NMDA agonist was sufficient to induce a spatial memory deficit
HCA was injected intrathecally once daily for 2 weeks. The injected dose was calculated to increase CSF to peak concentrations above 400 nmol/L, a level observed after intrathecal methotrexate exposure (14). Forty-eight hours after an injection, mean CSF concentration of HCA remained elevated (201 ± 8 nmol/L; n = 6) relative to baseline (138 ± 4 nmol/L; n = 8; P < 0.0001, two-tailed t test). No rats experienced seizures, and there were no observable gross behavioral differences between control rats and those injected with HCA.

Behavioral assessments of memory were conducted 3 days after the last HCA injection because CSF HCA was found to return to baseline by day 7 following injection. At this time point, the control group (given intrathecal injections of artificial CSF on the same schedule) showed intact spatial memory, by performing significantly better than chance (mean object placement preference score 62.0 ± 5.6%; n = 10; P < 0.05, two-tailed t test). In contrast, the rats treated with HCA did not exhibit intact spatial memory (mean preference score 50.3 ± 3.5%; n = 20). Relative to the control animals, those treated with HCA exhibited a significantly decreased mean preference score (P < 0.05, two-tailed t test) and an increase in the proportion of individual animals failing to show intact spatial memory (65% of treated rats vs. 20% among controls).

Systemic memantine had no independent effect on cognitive function when administered at 2.5 mg/kg intraperitoneally
Rats treated daily for 3 weeks with 2.5 mg/kg of memantine with or without 4 concurrent intrathecal injections of aCSF showed intact spatial memory, identical to control animals (Fig. 3, a–c). Higher doses of memantine, however, did affect cognitive function. Daily intraperitoneal administration of 5 mg/kg of memantine for 3 weeks led to a decrease in average preference score relative to control animals (59.7 ± 3.0%, n = 26 vs. 68.7 ± 2.0%, n = 44; P < 0.05), and an increase in the proportion failing to show intact spatial memory (Fig. 3, d). Consequently, a dose of 2.5 mg/kg/day was used for subsequent experiments.

Memantine protected rats against the adverse effects of methotrexate on memory
Independent cohorts of rats were given 4 intrathecal injections of methotrexate within a 2-week period along with daily intraperitoneal injections of either memantine 2.5 mg/kg or saline for 3 weeks beginning before the first intrathecal injection (as indicated in the experimental schema; Fig. 1B). Behavioral testing was conducted 1 month after the last intrathecal methotrexate injection, long after all rats had recovered from all acute toxicities of sedation.

Figure 2. HCA is sufficient to induce spatial memory deficits. Behavioral testing was conducted 3 days after the last intrathecal injections. Preference scores are shown for individual rats injected with HCA (shaded squares; n = 20) or artificial CSF (aCSF; open circles; n = 10). Preference scores >53% defined intact memory. Injection of HCA induced a lower mean preference score and an increase in the proportion failing to show intact memory.
and methotrexate exposure. At this time point, observed cognitive deficits seem to be stable, as the prevalence of deficits does not seem to change at later time points (14).

As previously observed (13, 14), control rats showed intact spatial memory, with a mean preference score significantly greater than chance (Fig. 3, a), whereas rats treated with intrathecal methotrexate and intraperitoneal saline did not (Fig. 3, e). ANOVA shows a significant difference in mean preference scores among all relevant groups, F(5,127) = 2.83; P = 0.02. In contrast to the animals treated with intrathecal methotrexate alone (Fig. 3, e), those rats treated with intrathecal methotrexate and concurrent memantine exhibited intact spatial memory, with a group mean preference score significantly greater than chance (Fig. 3, f). More individual rats treated with intrathecal methotrexate and concurrent memantine showed intact spatial memory (80% had preference scores >53%) than rats treated with intrathecal methotrexate without memantine (44%; P < 0.05, Fisher exact test).

Memantine had no measurable "off-target" effects within the NMDA pathway

We have previously shown (14) that exposure to intrathecal methotrexate is followed by an increase in HCA within CSF. In the current experiments, memantine did not alter the methotrexate-induced change in HCA. No significant difference in CSF HCA was noted at any time point, between rats treated with methotrexate plus memantine and controls given methotrexate plus saline (Fig. 4).

Mean (±SEM) glutamate concentrations within the hippocampus, measured by MRS, were higher after intrathecal methotrexate (8.1 ± 1.0 mmol/L; n = 6) than after intrathecal injections of artificial CSF (7.1 ± 0.1 mmol/L; n = 6; P < 0.05, two-tailed Fisher exact test). Concurrent exposure to memantine and intrathecal methotrexate did not lead to a significant difference in glutamate (7.7 ± 0.4 mmol/L; P = 0.17, two-tailed Fisher exact test).
Finally, we examined whether methotrexate exposure with or without memantine led to a quantitative change in NMDA receptor expression, reasoning that either an increase in glutamate analog concentrations or exposure to an exogenous antagonist might lead to a compensatory increase in receptor expression. However, neither methotrexate exposure nor concurrent treatment with methotrexate plus memantine led to an observable change in expression of the NMDA receptor within the hippocampus (Fig. 5).

Discussion

The pathophysiology responsible for cognitive deficits among patients with cancer is multifactorial, involving multiple chemotherapeutic agents and altered pathways. Our experiments are specifically relevant to the pathogenesis of cognitive deficits induced by methotrexate, a critical component of therapy for patients with leukemia and non-Hodgkin lymphoma. These results provide further support for the hypothesis that methotrexate causes persistent cognitive deficits by inducing an increase in excitotoxic agonists of the NMDA receptor, and suggest that antagonists of the NMDA receptor may interrupt the pathophysiology preventing deficits (illustrated in Fig. 6). These results raise the possibility that a glutamate receptor antagonist such as memantine might be effective in preventing cognitive deficits among patients with leukemia, who are repeatedly exposed to intrathecal methotrexate over 2 to 3 years of treatment.

We have previously shown that repeated administration of intrathecal methotrexate leads to persistent cognitive deficits and is accompanied by an increase in excitotoxic glutamate analogs within the CNS (14). Here, we show that one of these glutamate analogs, HCA, is sufficient to

Figure 5. Western blot analysis of hippocampal NMDA expression. Protein was isolated from homogenized left hippocampi, and separated by SDS-PAGE. Blots were probed for the NR2B subunit of the NMDA receptor and β-actin. Arrows indicate NR2B (180 kDa) and β-actin (42 kDa). Each lane (1 through 6) shows protein from a single animal. Lanes 1,2: rats treated with intrathecal methotrexate and concurrent memantine. Lanes 3,4: rats treated with intrathecal methotrexate plus saline. Lanes 5,6: controls rats given intrathecal injections of artificial CSF and intraperitoneal saline. Neither methotrexate alone nor methotrexate with memantine seems to significantly alter NMDA expression in the hippocampus relative to control animals. MTX, methotrexate.

Figure 6. Selected biochemical effects of methotrexate exposure, with an emphasis on reactions that may contribute to neurotoxicity. Methotrexate exerts its antineoplastic effects by inhibiting dihydrofolate reductase (DHFR), an enzyme critical to replenishing reduced folates necessary for nucleoside synthesis. Limiting intracellular concentrations of reduced folate secondarily prevents remethylation of homocysteine (Hcy) to methionine (Met) by methionine synthase (MS). Hcy can directly cause oxidative damage to vascular endothelium and neuronal tissue. In addition, it is further metabolized to glutamate analogs, homocysteine sulfenic acid (HCSA) and HCA, excitotoxic agonists at glutamate receptors, including the NMDA receptor (NMDA-R). Excessive agonism at the NMDA-R is postulated to lead to deficits in new memory formation. NMDA antagonists may restore balance at the NMDA receptor, restoring cognitive function. MTX, methotrexate; CSA, cysteine sulfenic acid; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; Ado, adenosine; THF, tetrahydrofolate; MetTHF, methylTHF; DHF, dihydrofolate.
reproduce the cognitive deficits induced by methotrexate and that memantine, an antagonist at the NMDA subclass of ionotropic glutamate receptors, reduced the incidence of cognitive deficits when administered concurrently with intrathecal methotrexate.

The precise mechanism(s) by which excessive stimulation of the NMDA receptor leads to cognitive dysfunction after methotrexate exposure remains unresolved. Unlike others (32–34), we have not observed histologic evidence of excitotoxic neuronal death or of diminished neurogenesis within the hippocampus of our methotrexate-exposed adult rats. Nor have we observed gross structural differences on MRI between methotrexate-exposed rats and controls. It is possible that unbalanced stimulation of the NMDA receptor by glutamate analogs alters neuronal function by inducing changes in metabolism (35) and/or synaptic signaling (36), without causing neuronal death or inhibiting proliferation. Additional experiments to address these possibilities are necessary.

In our earlier studies (14), dextromethorphan, an antitussive opioid, which is a weak noncompetitive antagonist at the NMDA receptor, normalized cognitive function among methotrexate-exposed animals with persistent deficits. However, dextromethorphan could not prevent the onset of deficits if administered concurrently with methotrexate. Memantine, an analog of the antiviral drug amantadine, is a more specific NMDA antagonist than dextromethorphan (19, 37). Unlike dextromethorphan (38), memantine has no significant affinity for opioid or dopamine receptors and has no antioxidant effect.

Memantine is an attractive agent for a clinical trial to reduce the toxic sequelae associated with intrathecal methotrexate. Memantine is orally bioavailable, and is U.S. Food and Drug Administration approved for the treatment of Alzheimer’s dementia in adults (39). In pediatric patients, it has been studied for the treatment of autism spectrum disorders, pervasive developmental disorders, and attention-deficit/hyperactivity disorder, with minimal toxicity despite prolonged use (40–43).

Critically, administration of memantine is not anticipated to alter the risk of cancer relapse. The desired antineoplastic effects of methotrexate are dependent on limiting folate-dependent synthetic reactions (Fig. 6). However, our observation that memantine reduced cognitive deficits without altering synthesis of HCA or parenchymal glutamate concentrations is consistent with the hypothesis that its action is limited to antagonism at the NMDA receptor. Nevertheless, additional preclinical testing, with human leukemia cell lines both in vitro and in xenograft models, will be necessary to conclusively prove that memantine does not antagonize the anti-convulsive efficacy of methotrexate.

If confirmed in a clinical trial, memantine could be the first pharmacologic intervention to prevent cognitive deficits induced by cancer therapy, rather than ameliorate their effects among survivors. We are currently in the process of developing a clinical trial, asking whether memantine will prevent an acute decline in cognitive function among adults treated with intrathecal methotrexate. A subsequent trial, informed by this pilot study in adults and by ongoing preclinical studies with juvenile animals, will test whether memantine can reduce the incidence of cognitive deficits among children treated with intrathecal methotrexate for ALL.

Conclusion

Excitotoxic glutamate analogs seem to contribute to the memory deficits observed among healthy laboratory animals exposed to intrathecal methotrexate. Concurrent administration of the NMDA receptor antagonist memantine reduced the incidence of deficits. The absence of an effect on glutamate concentrations, CSF HCA synthesis, or NMDA-receptor expression is consistent with memantine’s presumed mechanism of action at the NMDA receptor. On the basis of these results, we are actively developing a clinical trial to test whether memantine can reduce the acute and chronic neurotoxic sequelae of intrathecal methotrexate among patients with leukemia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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