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Memantine Lowers Amyloid-beta Peptide Levels in Neuronal Cultures and in APP/PS1 Transgenic Mice

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Abstract

Memantine is a moderate-affinity, uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist that stabilizes cognitive, functional, and behavioral decline in patients with moderate to severe Alzheimer's disease (AD). In AD, the extracellular deposition of fibrillogenic amyloid-beta peptides (A β) occurs due to aberrant processing of the full-length A β precursor protein (APP). Memantine protects neurons from the neurotoxic effects of A β and improves cognition in transgenic mice with high brain levels of A β . However, it is unknown how memantine protects cells against neurodegeneration and affects APP processing and A β production. We report the effects of memantine in three different systems. In human neuroblastoma cells, memantine, at therapeutically relevant concentrations (1–4 μ M), decreased levels of secreted APP and A β_{1-40} . Levels of the potentially amyloidogenic A β_{1-42} were undetectable in these cells. In primary rat cortical neuronal cultures, memantine treatment lowered A β_{1-42} secretion. At the concentrations used, memantine treatment was not toxic to neuroblastoma or primary cultures and increased cell viability and/or metabolic activity under certain conditions. In APP/presenilin-1 (PS1) transgenic mice exhibiting high brain levels of A β_{1-42} , oral dosing of memantine (20 mg/kg/day for 8 days) produced plasma drug concentration of 0.96 μ M and significantly reduced the cortical levels of soluble A β_{1-42} . The ratio of A β_{1-40} /A β_{1-42} increased in treated mice, suggesting effects on the γ -secretase complex. Thus, memantine reduces the levels of A β peptides at therapeutic concentrations and may inhibit the accumulation of fibrillogenic A β in mammalian brains. Memantine's ability to preserve neuronal cells against neurodegeneration, increase metabolic activity, and lower A β level has therapeutic implications for neurodegenerative disorders.

Keywords

Aging; cortex; dementia; lysosome; membrane; tissue culture; memory

In Alzheimer's disease (AD), a gradual impairment in short-term memory and cognition results from the dysfunction and death of neurons in the hippocampus, limbic system, and cerebral cortex (Goedert and Spillantini, 2006; Tanzi and Bertram, 2008). AD is characterized by brain

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depositions of amyloid plaques and neurofibrillary tangles, loss of the synaptophysin protein, and deficits in cholinergic neurotransmission (Giacobini, 2003; Lahiri et al., 2003; Reinhard et al., 2005; Selkoe, 2005). These aberrations are believed to result, in part, from oxidative stress, membrane damage, and the over-production and accumulation of amyloid- β peptide ($A\beta$), a 39-43 amino acid polypeptide that is a core constituent of amyloid plaques (Dumery et al., 2001; Sambamurti et al., 2006). Two $A\beta$ peptides predominate in brain tissue, one is a shorter peptide of 40 amino acids in length ($A\beta_{1-40}$) and the second is a longer and more amyloidogenic peptide of 42 residues ($A\beta_{1-42}$). The secreted $A\beta$ peptide is generated from the $A\beta$ precursor protein (APP) by the action of two enzymes, β -secretase and γ -secretase (Sambamurti et al., 2002; Walsh and Selkoe, 2004). Current research suggests that a conformational change transforms soluble nonfibrillar $A\beta$ into its toxic fibrillar form, leading to structural disruption of synapses, neurite fragmentation and cell death (Morgan et al., 2004). Soluble aggregates of $A\beta$ or $A\beta$ -derived diffusible ligands (ADDLs), which are also elevated in the brains of AD patients, have been shown to target synapses (Gong et al., 2003) and inhibit long-term potentiation (LaFerla and Oddo, 2005; Walsh et al., 2002). Several transgenic mouse lines over-expressing APP and possessing high brain levels of $A\beta$ also exhibit age-dependent cognitive decline (Chapman et al., 1999; Oddo et al., 2003; Puolivali et al., 2002). These studies imply that $A\beta$ plays an important role in the development of cognitive impairment, and suggest a causative role of this peptide in the pathophysiology of AD. However, the mechanisms of $A\beta$ -mediated cell death and synaptic disruption are incompletely understood.

Memantine is an uncompetitive NMDA receptor antagonist that has been shown to improve cognitive function in several preclinical studies, and to slow cognitive, functional, and behavioral decline in AD patients (Minkeviciene et al., 2004; Parsons et al., 1999; Peskind et al., 2006; Reisberg et al., 2003; Tariot et al., 2004). Memantine is a channel blocking drug with a fast off-rate and voltage-dependent binding properties, which shows less interference with normal synaptic glutamate signalling than other NMDA antagonists that have failed in the clinic (Chen and Lipton, 2006). The importance of maintaining normal synaptic NMDA signalling was recently demonstrated by Papadia et al. (2008), who showed that synaptic NMDA activity can boost intrinsic antioxidant defenses. Memantine binds to the human NMDA receptor with a K_i of $\sim 0.5 \mu\text{M}$, and inhibits NMDA-evoked inward currents in rat hippocampal neurons with an IC_{50} of approximately $1\text{-}2 \mu\text{M}$ (Kornhuber et al., 1991; Parsons et al., 1999; Parsons et al., 1993). Memantine is approved for the treatment of moderate to severe AD in the US and Europe. In clinical practice, a 20 mg/day stable dose of memantine in AD patients produces a steady-state plasma drug level of approximately $0.5 \mu\text{M}$ (Periclou et al., 2006). Although there is no direct evidence that memantine alters the production of $A\beta$, memantine has been shown to protect hippocampal cells from the neurotoxic effects of $A\beta_{1-40}$ in rats (Miguel-Hidalgo et al., 2002) and to improve spatial learning in transgenic mice overexpressing mutated human APP and presenilin 1 (PS1) (Minkeviciene et al., 2004; Van Dam and De Deyn, 2006; Scholtzova et al., 2008).

But how memantine exerts neuroprotection against $A\beta$ and preserves cells against neurodegeneration remain unclear. Likewise, how memantine affects APP processing pathway leading to $A\beta$ generation are not fully understood. We hypothesized that memantine's beneficial effects are due to neuronal preservation and lowered $A\beta$ levels. Herein, we report the effects of memantine in three different complementary model systems: human neuroblastoma cells, rat primary cortical cultures and in APP-Tg mice *in vivo*. In the present study, we report that memantine treatment could preserve and protect the cells against neurodegeneration caused by long-term serum deprivation. In addition, memantine ($1\text{-}4 \mu\text{M}$) decreased the levels of secreted $A\beta_{1-40}$ and APP in human neuroblastoma (SK-N-SH) cells. Memantine reduced $A\beta_{1-42}$ production at sub-toxic concentrations in rat primary cortical cultures. In addition, oral dosing of memantine (20 mg/kg/day p.o.) in APP/PS1 transgenic

mice with high brain levels of A β produced plasma memantine levels of around 1 μ M and significantly reduced brain levels of soluble A β ₁₋₄₂ during a relatively short 8-day treatment.

MATERIALS AND METHODS

Neuroblastoma cell culture and conditions of drug treatment

Human neuroblastoma cells (SK-N-SH) were procured from the American Type Culture Collection (ATCC, Rockville, MD), and cultured as described previously (Ghosh and Lahiri, 1999). Briefly, the neuroblastoma cells were plated at an initial density of 2.2×10^5 cells/cm² in Dulbecco's minimal essential medium (MEM; Cellgo Mediatech, Herndon, VA) and allowed to attach overnight. Growth medium (with 10% fetal bovine serum or FBS) was replaced with low serum treatment media (LSM) containing 1% FBS (Sigma-Aldrich, St. Louis, MO) and various concentrations of memantine (1, 2 and 4 μ M) for 12 days in triplicate, as indicated.

In three-day intervals, the conditioned medium (CM) samples were collected and the remaining media were replaced by fresh memantine-containing treatment medium, and analyzed for A β ₁₋₄₀ by ELISA and APP by Western blot. Total protein concentration in individual CM samples was measured using the Bradford assay (BioRad, Hercules, CA) to adjust for small differences in evaporation between plates. The effect of memantine on cell viability and toxicity was assessed using sensitive MTS and LDH assays, respectively, as described previously (Ghosh and Lahiri, 1999; see details below).

In a separate experiment, the effect of memantine on cell proliferation was further examined using a broader concentration range in a two-day incubation in standard growth medium (MEM with 10% FBS). Cells were seeded into 12-well plates at 1×10^6 cells per well and allowed to attach overnight, then treated with vehicle, 0.1 μ M, 1 μ M, 10 μ M, or 100 μ M memantine. The duration of this treatment spans approximately one doubling time for this cell line under these conditions (Ricordy et al., 2002) and should be sufficient to detect differences in proliferation rates. The highly sensitive Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) assay was used to determine differences in cell viability, per the manufacturer's protocol.

Primary cell culture and drug treatment

Cortical neurons were harvested from embryonic day 17 Sprague-Dawley rat pups (Harlan, Indianapolis, IN, USA) as described previously (Bailey and Lahiri, 2006; Brewer et al., 1993) using the serum-free Neurobasal medium with B27 supplement system (Invitrogen, Carlsbad, CA). Cortical cells were plated at a density of 1.5×10^6 cells per well in poly-D-lysine coated 6-well plates and allowed to differentiate for 7 days. Cells were then treated with memantine at 4 μ M, 9 μ M, or 18 μ M for 48 hr. It is important to note the difference in memantine concentrations used in two cell culture systems—a low dose for neuroblastoma cells grown in LSM and higher dose for primary cell culture grown in enriched B-27 containing media. We have observed in previous experiments with primary cultures that higher concentrations of memantine are necessary in order to observe a variety of effects compared with other cell cultures, or relative to the plasma concentrations reported for *in vivo* experiments. Memantine is known to bind albumin under physiological conditions (Ibrahim et al., 2008) and we believe the differences in effective concentrations are due to adsorption of memantine to the large amount of purified, free albumin contained in the B27 supplement relative to the albumin in serum-containing media, which is already bound with various other serum components. Conditioned media samples were then collected and subjected to LDH assay and Western blotting (see below). Cells were then washed with cold PBS and removed from the plates by scraping and aliquots of the cell suspension were used in the MTS assay.

Determination of cellular toxicity and cell viability

Following drug treatments, cultured cells were allowed to grow for up to 12 days, and CM samples were collected at the indicated intervals. To determine cellular toxicity and/or membrane damage, lactate dehydrogenase (LDH) was measured in the CM samples using the Tox-7 kit (Sigma-Aldrich). Leakage of cytosolic LDH enzyme from the membrane indicates toxicity and membrane damage. To confirm viability, the cells were harvested at the end of the experiment; cells were resuspended and immediately assayed for 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction using the Cell Titer Aqueous One Solution Cell Proliferation assay (Promega). This assay measures mitochondrial activity, with MTS reduction correlating directly with cell number. Both LDH and MTS measurements were performed as described previously (Lahiri et al., 1998). In the 2-day treatments of SK-N-SH cells, after washing and scraping cells from the plate in PBS, an aliquot was taken and cell viability was measured using the Cell Titer-Glo assay (Promega) per manufacturer's recommended protocol. In addition to MTS and LDH assays, primary cortical cells were labeled with calcein AM and ethidium homodimer using the Live-Dead kit (Molecular Probes, Eugene, OR). Calcein AM fluorescently labels viable cells with intact membranes, while the ethidium homodimer labels the nuclei of damaged cells with permeable membranes. Both dyes were used simultaneously on the same aliquot and fluorescence was measured using a GENios fluorescence plate reader (Tecan, Männendorf, Switzerland).

Western blot analysis of APP levels

Briefly, the secreted APP (sAPP) from the CM samples (30 µg protein/sample) was analyzed by denaturing polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) followed by Western blotting. The resulting blots were probed with a monoclonal antibody specific for total APP (mAb22C11; CalBiochem, LaJolla, CA) as described previously (Lahiri et al., 1998). The blots were scanned using a Gel Documentation System (UVP Inc., Upland, CA), and band density was calculated using Scion Image (Girish and Vijayalakshmi, 2004; Xu and Pitot, 1999) software. The data were analyzed using one-way ANOVA with Tukey post-hoc test (SPSS software, v. 12).

ELISA analysis of A β levels in neuroblastoma and primary cortical cultures

A highly sensitive sandwich ELISA method was employed to measure the concentration of A β in the CM samples from SK-N-SH and primary cell cultures using commercially available kits (Immuno-Biological Laboratories, Gunma, Japan). Briefly, monoclonal rabbit anti-human IgGs anti-A β ₃₅₋₄₀ or anti-A β ₃₈₋₄₂ were used to capture A β ₁₋₄₀ and A β ₁₋₄₂, respectively, from an equal amount of each CM sample. The horseradish peroxidase-conjugated anti-human A β ₁₁₋₂₈ Fab was used for detection in both assays (Lahiri et al., 2004a). This detection antibody recognizes both integral and N-terminus-cleaved variants of A β ₁₋₄₀ and A β ₁₋₄₂. The measurement range for A β ₁₋₄₀ and A β ₁₋₄₂ was 4-231 pM and 3-178 pM, respectively. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc test (SPSS software, v. 12).

Effect of memantine in transgenic APP/PS1 mice

Double transgenic male mice overexpressing chimeric mouse/human APP with the Swedish double mutation (K595N and M596L) and human PS1-dE9 (deletion of presenilin 1 exon 9) mutation (Garcia-Alloza et al., 2006) were obtained from a colony at the University of Kuopio. This line was originally maintained in a hybrid C3HeJ $\times$ C57BL6/J F1 background, but the mice used in the present study were derived from backcrossing to C57BL6/J for 5-6 generations. This transgenic mouse line manifests a rapid accumulation of amyloid plaques in the cortex and hippocampus beginning around 3 months of age (Garcia-Alloza et al., 2006; Shemer et al., 2006). The housing conditions (National Animal Center, Kuopio, Finland) were

controlled (temperature +22°C, light from 07:00 to 19:00; humidity 50-60%), and fresh food and water were freely available. The experiments were conducted according to the Council of Europe (Directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Eastern Finland.

At 15 weeks of age, the mice were assigned to either the memantine (n = 11) or placebo group (n = 12). The memantine group received 20 mg/kg/day of memantine in their drinking water for 8 days. Previous studies have shown that 20-30 mg/kg/day oral administration of memantine via drinking water in mice produces a clinically relevant plasma drug level of ~ 1 μ M (Minkeviciene et al., 2004; 2008). Daily memantine intake was controlled by adjusting the concentration in drinking water according to individual water consumption. On the morning of Day 9, the water bottle containing memantine was removed for 3 hr after which the mouse received an overdose of pentobarbital and chloral hydrate (each 60 mg/kg i.p.). A blood sample (300 μ l) was taken through the heart for determination of the concentration of memantine in the plasma. The mouse was then perfused with 50 ml heparinized ice-cold 0.9 % saline (10ml/min). The brain was rapidly removed and dissected on ice into selected brain areas (hippocampus, ventral and dorsal cortex) that were stored at -70 °C for subsequent A β estimation.

A second group of mice (n = 9) were given 2.5 mg/kg memantine intraperitoneally once daily for 8 days (n = 9). Previous experience indicates that this dosing regime also produces a therapeutically relevant plasma concentration with C_{max} around 1 μ M (Danysz et al., 1997). A control group treated with a single i.p. saline injection was also included (n = 11). Subsequent sacrifice and post-mortem analysis of these animals followed the same procedures detailed above.

Determination of plasma memantine levels

The plasma levels of memantine were analyzed at Merz Pharmaceuticals GmbH (Frankfurt am Main, Germany), using a gas-chromatographic system coupled with a mass spectrometer as described previously (Kornhuber and Quack, 1995).

Determination of A β levels from APP/PS1 mouse brain homogenates

The dorsal brain cortical tissue was homogenized in phosphate buffered saline (PBS), pH 7.2, containing a mixture of protease inhibitors (Complete™, Boehringer Mannheim, Germany). Brain homogenates were centrifuged and supernatants were assayed for soluble A β_{1-40} and A β_{1-42} per manufacturers' protocols. The pellets were suspended in guanidine buffer (5.0 M guanidine-HCl/50 mM Tris-HCl, pH 8.0) to dissolve all intra- and extracellular aggregates, incubated for 3 hr at room temperature, centrifuged and diluted to reduce the concentration of guanidine-HCl to 0.5 M. Both PBS soluble and insoluble (guanidine soluble) A β_{1-42} were determined by using the Innostest β -amyloid 1-42 High Sensitivity Test-ELISA kit (Innogenetics, Gent, Belgium) and A β_{1-40} was determined using the Biosource Human β -amyloid kit (BioSource, Nivelles, Belgium). The soluble and insoluble A β_{1-42} levels were standardized to brain tissue weight and expressed as pmol (A β)/mg (brain tissue).

RESULTS

Memantine treatment increased cell viability and metabolic activity and was not toxic in human neuroblastoma cells (SK-N-SH)

We treated the human neuroblastoma cells with vehicle or memantine (1-4 μ M) as described in 'Materials and Methods'. On monitoring cellular morphology at day 12 by bright field microscopy, memantine-treated cells appeared healthier in terms of cell morphology, cell attachment and neurite extension than the vehicle-treated cells under the conditions tested

herein (data not shown). This was then confirmed by subjecting the cell culture samples to various biochemical viability assays as described below.

Drug effects on cell viability were determined by a sensitive MTS assay at day 12, which measures mitochondrial function, metabolic activity and thus cellular viability (Fig. 1A). From our MTS results, higher concentrations of memantine resulted in a modest increase in cell viability versus vehicle (Fig. 1A). Although this effect did not reach statistical significance by ANOVA ($p=0.21$), the Student t-test analysis showed a significant increase in cell viability at 2 μ M Memantine vs. vehicle, $p<0.05$).

In addition to the described cellular viability measure, cell membrane damage and integrity were measured by assaying LDH release into the medium. This assay is both sensitive and quantitative, and was performed within the linear range. We observed no significant difference in relative LDH value between vehicle- and memantine-treated samples at day 12 (Fig. 1B). Overall, our MTS results were consistent with the LDH data and demonstrated that memantine treatment was non-toxic to the cells under the dose and cell culture conditions tested.

Since we did not observe any cellular loss, damage or toxicity in the human neuroblastoma treated with 1-4 μ M memantine, we tested a higher concentration of memantine for shorter duration than above-mentioned experiments in another set of experiments. Consistent with the previous results, a broad concentration range of memantine (0.1-100 μ M) did not cause cellular toxicity at 48hr treatment in the NB cells (Fig. 1C). Instead, cell viability as determined by the Cell Titer-Glo assay was significantly increased in the 10 μ M and 100 μ M treatments relative to vehicle (both $p<0.01$). LDH was not detectable in media samples from the broad concentration range experiment. It is important to note that while the neuroblastoma cells cultured continuously for 12 days in LSM (with only 1% FBS) displayed characteristics of neurodegeneration, as expected, memantine treatment increased cell viability and metabolic activity. Taken together, these results suggest that memantine treatment could preserve and protect the cells against neurodegeneration as mediated by long-term serum deprivation.

Memantine reduced the levels of sAPP in human neuroblastoma cells

Human neuroblastoma cells were treated with memantine (1, 2 and 4 μ M) for 12 days. At three-day intervals, the entire conditioned medium samples above the cells were replaced by fresh memantine-containing medium, and total sAPP levels were analyzed using Western blot as described in 'Materials and Methods'.

At day 3, no significant changes in sAPP levels were observed for any memantine concentration (Fig. 2A). However, at days 6, 9, and 12, the CM samples of cells treated with various concentrations of memantine showed significant decreases in sAPP levels by Western blot, compared to vehicle (Fig. 2B-D). Levels of sAPP accumulated in the conditioned media of neuroblastoma cells at day 6 (Fig. 2B) and day 9 (Fig. 2C) were significantly lower in cultures treated with 2 μ M and 4 μ M memantine, compared to vehicle-treated cultures. In samples collected at day 12 of memantine treatment (Fig. 2D), statistically significant decreases in sAPP accumulation were observed for all three concentrations. A representative Western blot of the day 12 CM samples shows a characteristic major doublet of APP protein bands resulting from alternate mRNA splicing and various post-translational modifications (Fig. 2E).

To understand kinetics of sAPP secretion, the same samples were subjected again to Western blot, with individual concentrations in separate blots. This provided an important measurement of the effect of time of memantine treatment on sAPP levels in the culture plate (Fig. 3). Notably, levels of sAPP were found to decrease in a time-dependent manner with a significant decrease occurring at day 12 in vehicle-treated cultures (Fig. 3A) and significant decreases in sAPP with 1 μ M memantine treatment during days 9 and 12 (Fig. 3B; both $p<0.05$).

Memantine reduced the levels of A β in human neuroblastoma cells

Using a sandwich ELISA method, levels of both A β ₁₋₄₀ and A β ₁₋₄₂ were measured independently in the same CM samples that were assayed for sAPP levels. Similar to sAPP, no effect of memantine was observed on A β ₁₋₄₀ at day three in any concentration tested (Fig. 4A). However, all memantine treatment groups significantly decreased A β ₁₋₄₀ levels at day 6 (Fig. 4B; all $p \leq 0.02$) and day 9 (Fig. 4C; all $p \leq 0.03$), but memantine produced no significant effect on A β ₁₋₄₀ levels at day 12 (Fig. 4D). Data are presented as pg A β ₁₋₄₀/mg CM protein, and a decreasing trend over time is also apparent (Fig. 4A-D). Measurement of A β ₁₋₄₂ was also attempted in these samples, but was found to be below detection threshold (data not shown).

Memantine reduced the levels of A β in primary rat cortical cultures

First, it was shown that memantine did not alter viability of primary cells by a variety of methods. MTS and calcein AM, the two viability measures, showed no statistically significant change from vehicle in memantine treated cells (Fig. 5A and 5C). Likewise, LDH and ethidium homodimer toxicity assays showed no significant change from vehicle (Fig. 5B and 5D).

Starting at day 7 *in vitro*, primary cortical cells were treated with either vehicle, 4 μ M, 9 μ M, or 18 μ M memantine for 48 hr. Significant decreases in A β ₁₋₄₂ secretion were observed in the 4 μ M and 18 μ M treatment conditions (Fig. 6B; both $p = 0.01$), and a non-significant reduction was observed in the 9 μ M condition (Fig. 6B; $p = 0.09$). A β ₁₋₄₀ levels also appeared to decline, but this effect did not reach statistical significance (ANOVA $p = 0.26$) (Fig. 6A). Thus, it appears the ratio of A β ₁₋₄₀ to A β ₁₋₄₂ was increased by memantine treatment. To compensate for any uneven evaporation of the media, a Bradford protein assay was performed on media samples and data are presented as A β /100 μ g protein (Fig 6A & 6B).

Memantine reduced the levels of soluble A β ₁₋₄₂ in APP/PS1 mice

Transgenic APP^{swe}/PS1^{dE9} mice aged 15 weeks were treated with memantine (20 mg/kg/day, orally via drinking water for 8 days). Because of the PS1^{dE9} mutation, this transgenic mouse line accumulates more of the highly toxic A β ₁₋₄₂ species than wild type (Garcia-Alloza et al., 2006). Memantine treatment via drinking water resulted in a clinically relevant plasma drug level of 0.96 $\pm$ 0.32 μ M, which is within the dose range that we studied in cell cultures (Figs. 2-4). Memantine treatment resulted in a significant reduction in cortical levels of soluble A β ₁₋₄₂ peptide (Fig. 7B; $p = 0.02$) but not A β ₁₋₄₀ (Fig. 7A). Although it is unlikely that treatment with memantine for only 8 days would alter the levels of insoluble A β in the brain, we measured insoluble A β ₁₋₄₂ levels in the cortical homogenates. As expected, there was no significant change in the insoluble cortical A β ₁₋₄₂ levels in memantine-treated mice. (Fig. 7D). A significant increase in the ratio of A β ₁₋₄₀ to A β ₁₋₄₂ was also observed (Fig. 7C). In mice treated by i.p. injection, no significant differences from control were observed in either A β ₁₋₄₀ or A β ₁₋₄₂, soluble or insoluble (Fig. 7A-D). We did not measure the levels of insoluble A β ₁₋₄₀.

DISCUSSION

A significant body of evidence supports the amyloid hypothesis, according to which accumulation of A β peptides in the brain disrupts neuronal circuits responsible for learning and memory, ultimately leading to AD (Gilman et al., 2005; Rosenberg, 2005; Selkoe, 2005; Walsh and Selkoe, 2004). Indeed, amyloid-lowering strategy is being vigorously pursued as a viable therapy for AD. Current AD therapies have been designed to either enhance cholinergic function by inhibiting acetylcholinesterase (AChE), or to prevent hyper-activation of the NMDA receptor. We have previously shown that the treatment with certain cholinesterase inhibitors reduces the secretion of APP derivatives and A β in conditioned media samples in cell culture (Lahiri et al., 1998; Lahiri et al., 1994; Lahiri et al., 2004b) and also *in vivo* (Greig

et al., 2005). Some newer therapies under development target the secretase enzymes, including one γ -secretase inhibitor, LY450139, which has reached phase 2 clinical trials (Fleisher et al., 2008). Given that γ -secretase has multiple substrates, including the highly important NOTCH receptor, γ -secretase modulation rather than inhibition is an important therapeutic goal (Wolfe, 2008).

Memantine has demonstrated clinical efficacy in AD (Minkeviciene et al., 2004; Parsons et al., 1999; Peskind et al., 2006; Reisberg et al., 2003; Tariot et al., 2004) and the ability to protect cultured neuron-like cells from a variety of insults (Jantas et al., 2008). Our aim was to investigate how memantine exerts neuroprotection, and whether memantine affects the APP processing pathway leading to A β generation. Our present results showing the potential for memantine to lower A β levels in neuronal culture and APP/PS1 transgenic mice provide preclinical evidence for possible additional effects of this drug. We observed a statistically significant effect of memantine in lowering the levels of sAPP and A β ₁₋₄₀ in human neuroblastoma cells. Memantine also reduced A β ₁₋₄₂ in rat primary cortical cells and in APP/PS1 transgenic mice. The concentration range of memantine used in the cultured neuroblastoma experiments (1-4 μ M) was close to the IC₅₀ value ($\sim$ 1 μ M) for inhibiting NMDA receptor function (Blanpied et al., 1997), which is also close to the steady-state plasma memantine level achieved in human AD patients (Periclou et al., 2006).

Given that the drug concentrations shown to be effective in our models are similar to the established clinically effective concentrations, it would be reasonable to conclude that the A β -lowering effect of memantine in cortical homogenates of APP/PS1 mice was likely mediated by its action on NMDA receptors. Rodent brain tissues are known to express functional NMDA receptors (Ultanir, et al., 2007) and in mouse cortical neurons that express functional NMDA receptors (Lesne et al., 2005), it has been shown that NMDA treatment increases the accumulation of A β ₁₋₄₀ and A β ₁₋₄₂ in the cell culture medium, an effect reversed by the NMDA receptor antagonist MK-801. In addition, 4 mM Mg²⁺ (a low-affinity NMDA receptor antagonist) has been shown to decrease the levels of A β peptides in organotypic cultures of hippocampal slices, prepared from mice over-expressing human APP^{swe} (Kamenetz et al., 2003). However, the observation of A β reducing effects in undifferentiated neuroblastoma cells suggests that this could be an incomplete explanation of the mechanism.

Undifferentiated SK-N-SH neuroblastoma cells have been shown to express no detectable NMDA receptor activity (Pizzi, et al., 2002). Therefore, the mechanism by which memantine lowers sAPP and A β levels in SK-N-SH cells is likely to be NMDA receptor-independent. One alternate hypothesis is that memantine may produce a lysosomotropic effect. Memantine and other amantadine-derivatives are cationic amphiphilic drugs that are structurally very similar to lysosomotropic drugs that are accumulated in acidic cellular compartments and inhibit phospholipases (Hostetler and Richman, 1982). Due to the accumulation of memantine in lysosomes, this is one possible mechanism by which memantine can reduce the secretion of APP and A β peptides into the conditioned media of cell cultures. This phenomenon is not unprecedented as it has been shown for the cholinesterase inhibitor tacrine (Dell'Antone et al., 1995; Lahiri et al., 1998) and recently for posipheneserine (Lahiri et al., 2007). Furthermore, in cultured human cells memantine has been shown to alter phospholipid content and composition, membrane fluidity and beta-adrenergic transmission (Honegger et al., 1993). This effect of memantine on membrane fluidity can in turn influence the activity of membrane bound secretase enzymes, such as β -APP cleaving enzyme (BACE). Indeed, our preliminary results show that memantine can reduce the levels of A β peptide and BACE activity in the human neuroblastoma cells (Lahiri et al., 2006a). Similarly, recent results from our laboratory indicate that memantine also lowers A β in C6 murine glioma cells which exhibit only trace quantities of NMDA receptor subunits (Okamoto et al, 1999), suggesting that this effect may not be solely dependent upon the glutamate receptors present in the cell (Ray and Lahiri, unpublished

observations). This hypothesis is further supported by our observation that memantine reduced $A\beta_{1-42}$ in primary cell cultures within 48 hr after treatment. It has been demonstrated in similar cell culture models that primary neurons require more than 9 days *in vitro* to express functional NMDA receptors (Mizute et al, 1998). Therefore, the observed reduction of $A\beta_{1-42}$ by memantine within 2 days post-treatment in primary cultures suggests a non-NMDA receptor-mediated mechanism.

It is also noteworthy that in both the APP/PS1 transgenic mice and in the cultured primary cells, no significant change was observed in $A\beta_{1-40}$ while $A\beta_{1-42}$ is decreased significantly in both models. This suggests that memantine may modify the activity of the γ -secretase complex, such that a greater proportion of the $A\beta$ produced is the shorter, less amyloidogenic $A\beta_{1-40}$. The γ -secretase complex includes a number of different proteins and is still incompletely characterized, therefore one can only speculate regarding the mechanism of this effect. Even so, this modulatory effect is of particular interest with respect to the transgenic mice used here that express a mutant form of PS1 that lacks exon 9, a mutation shown to modify γ -secretase activity such that a greater proportion of the longer $A\beta_{1-42}$ is produced (Mehta et al., 1998). $A\beta_{1-42}$ may act to seed amyloid aggregation (Jarrett et al., 1993), and shifting $A\beta$ toward production of shorter less amyloidogenic forms may have beneficial effects without altering total $A\beta$ production. One might observe that memantine reduced $A\beta_{1-40}$ in neuroblastoma cells but not in primary cortical cells. It is clear from our results in neuroblastoma cells that treatment duration is an important factor in the observed effects on APP processing. The $A\beta_{1-40}$ lowering effect was only observed after 6 days in neuroblastoma cells, while the primary cortical cells were treated for only 2 days, which may have been too little time to observe this effect. The observation of an $A\beta_{1-42}$ lowering effect but no change in $A\beta_{1-40}$ in primary cell cultures and in the *in vivo* model suggest that the $A\beta$ lowering effect is observable more rapidly in $A\beta_{1-42}$ levels than in $A\beta_{1-40}$ levels, however this could not be confirmed in our neuroblastoma model. We have previously observed that SK-N-SH cells under similar conditions produce about twenty-fold lower $A\beta_{1-42}$ levels as compared to $A\beta_{1-40}$ (Lahiri et al., 1998), which was below our detection threshold.

It was unexpected that in our SK-N-SH cultures that the $A\beta_{1-40}$ lowering effect was not observed at day 12 (Fig. 4D), especially given that the sAPP lowering effect remained strong at day 12 (Fig. 2D). This implies that total APP secretion and $A\beta$ production are not completely interdependent. The primary pathway for APP secretion is through the α -secretase pathway, so it is possible that a change in $A\beta$ could occur without a major difference being observed in total sAPP. It is clear that with time the amount of $A\beta_{1-40}$ is decreasing independently of treatment (Fig. 4A-D). The $A\beta_{1-40}$ levels measured in the memantine-treated samples from day 9 are similar to the values from the same cell cultures at day 12. The major difference between the two time points is between the untreated samples, which appear to decline to the level of the treated cells between day 9 and day 12. These data are consistent with two overlapping effects in these cultures, one time-dependent effect, and one treatment-dependent effect. It appears that APP and $A\beta$ secretion in these cultures decline with time to some minimum value, and this minimum is reached at an earlier time point with memantine treatment. We suspect that this minimum is determined by cell density within the culture plate, e.g., $A\beta$ secretion may be associated with neurite remodeling, which declines as cells become more confluent and less mitotically active at later time points. When the differences in $A\beta_{1-40}$ are calculated between day 6 and day 9, the rate of change is statistically not different between the treatment groups ($p=0.27$), however between day 9 and day 12, all treatments are different from vehicle (all $p\leq 0.02$), indicating the memantine-treated cells had reached a minimum for $A\beta_{1-40}$ secretion by day 9 that vehicle-treated cells did not reach until the final time point. A possible mechanism for this effect could be that viability of the vehicle-treated cells declined relative to the memantine-treated groups by day 12. This is demonstrated by our MTS results showing that at day 12, higher concentrations of memantine resulted in a modest

increase in cell viability versus vehicle. Taken together, these results suggest that memantine preserves neuronal cells, increases cell viability and/or metabolic activity, and lowers A β levels.

The observed A β -lowering effect of memantine suggests a potential disease-modifying effect of this drug in AD. In single (APP23) or double transgenic (APP/PS1) AD-mice with high brain levels of soluble and insoluble A β , memantine has been shown to ameliorate learning impairment (Minkeviciene et al., 2004; Van Dam and De Deyn, 2006). Reduction in soluble A β ₁₋₄₂ levels *in vivo* in our study may provide an explanation for the previously reported improvement in learning in transgenic mice (Van Dam and De Deyn, 2006). Although we did not observe a significant reduction in the cortical levels of insoluble A β ₁₋₄₂, it is likely that 8-day treatment period was not long enough to reduce insoluble A β ₁₋₄₂ levels. To complement our findings, recent long-term memantine treatments in a different APP/PS1 transgenic mouse model have shown that memantine can indeed reduce amyloid plaque in these animals (Scholtzova et al, 2008). Together with the data presented here, this suggests that memantine can rapidly reduce A β production, though longer-term treatment is required to alter insoluble A β plaque. Furthermore, it is interesting that, while a significant reduction in A β ₁₋₄₂ was observed using oral dosing via drinking water, no significant effect was observed with i.p. dosing. Administration of memantine via drinking water produces a more consistent steady-state plasma concentration, while i.p. delivery results in a rapid peak plasma concentration within one hour, which declines with a relatively short 2- to 4-hour half life. These differences suggest that a stable plasma memantine concentration is an important factor in modulation of amyloid levels. Taken together, these results have potentially important implications for drug delivery that warrant further exploration.

In summary, our results suggest that memantine lowers the levels of A β peptides in APP/PS1 transgenic mice and cultured cortical cells, and reduces APP secretion in SK-N-SH human neuroblastoma cells by a combination of mechanisms that may bypass the NMDA receptor and act on γ -secretase. The exact nature of these effects remains to be fully elucidated in future experiments. Finally, memantine's ability to preserve neuronal cells against neurodegeneration, increase cell viability and/or metabolic activity, and lower amyloidogenic pathway should be further investigated to maximize this drug's utility for Alzheimer's disease and other neurodegenerative disorders.

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Figure 1.

Effect of memantine on cell viability and toxicity in human neuroblastoma cells. MTS, CTG and LDH assays were carried out on control and memantine-treated cells as described in the text. **A:** Cell viability was measured using 20 μ l of harvested cells from each well at day 12 of the chronic treatments. **B:** To measure LDH release, 30 μ l aliquots of the final day 12 CM samples were analyzed as previously mentioned. The levels of both endpoints showed no significant change by drug treatments from the vehicle. **C:** After a 48 hr treatment, cells treated with memantine (0.1-100 μ M) were harvested in PBS and 30 μ l aliquots were subjected to the luminescent Cell Titer-Glo (CTG) assay. A small but statistically significant increase in signal was observed in the 10 μ M and the 100 μ M conditions (both $p < 0.01$).

Figure 2.

Effect of memantine in neuroblastoma cells: Analysis of sAPP levels by western blot at different days

Cells were treated with memantine at the indicated concentrations for 12 days, with media samples collected at 3 day intervals and subjected to SDS-PAGE and Western blotting. **A-D**: Densitometric analysis of Western blot data shows that levels of sAPP in conditioned medium samples collected at day 3 (**A**) was not significantly changed by memantine. However, sAPP in the day 6 samples (**B**) and day 9 samples (**C**), were significantly lower in cultures treated with 2 μM and 4 μM memantine, compared to vehicle (all $p < 0.05$). In the day 12 samples (**D**), statistically significant decreases in sAPP were observed for all three memantine concentrations (all $p < 0.05$). **E**: A representative Western blot of day 12 CM samples probed with anti-APP (22C11) and anti-β-actin (AC-15) monoclonal antibodies.

Figure 3.

Effect of memantine on sAPP in SK-N-SH cultures over time

Equal amounts of CM samples were analyzed in a second set of Western blots, one containing a time course for vehicle-treated cells and one for cells treated with 1 μ M memantine. Data are represented as percent of the first media sample collection at day 3. A: A significant decrease was observed by day 12 in vehicle-treated cultures ($p=0.01$). B: In the 1 μ M memantine treatment, significant decreases were observed at day 9 and day 12 (both $p<0.05$).

Representative blots inset; data are actin-adjusted and presented as a proportion of day 3 sAPP levels.

Figure 4.

Effect of memantine on $A\beta_{1-40}$ in SK-N-SH cultures

Equal amounts of CM samples of neuroblastoma cells treated with vehicle or memantine at the indicated concentrations were subjected to ELISA to detect $A\beta_{1-40}$. Memantine at the tested concentrations did not change $A\beta_{1-40}$ at day 3 (A). However, all treatment groups were significantly decreased at day 6 (B; all $p \leq 0.02$) and day 9 (C; all $p \leq 0.03$). No significant effect was observed at day 12 (D).

Figure 5.

Effect of memantine on cell viability in primary cortical neurons

Starting at day 7 *in vitro*, primary rat cortical cultures were treated with vehicle or memantine at the indicated concentrations. After 48 hours cells were collected in PBS and 20 μ l aliquots of cell suspension were subjected to MTS assay (**A**), calcein AM labeling (**C**), and ethidium homodimer labeling (**D**), and 30 μ l aliquots of CM were used for LDH assay (**B**). Memantine treatment produced no significant change in viability (**A** and **C**) or toxicity (**B** and **D**) by any of these four assays.

Figure 6.

Effect of memantine on soluble A β_{1-40} and A β_{1-42} in primary rat cortical cultures

A β was measured in media samples of vehicle- or memantine-treated primary rat cortical cultures by ELISA. **A:** No significant effect was observed for A β_{1-40} although there was a trend for a decrease at 18 μ M ($p=0.067$) **B:** A β_{1-42} was significantly reduced at 4 μ M and 18 μ M (both $p<0.01$) memantine with a trend toward a decrease at 9 μ M memantine ($p=0.085$).

Figure 7.

Effect of memantine on levels of soluble and insoluble A β_{1-42} in APP/PS-1 transgenic mice. Soluble and insoluble A β_{1-42} levels and soluble A β_{1-40} levels in the cortex of APP^{swe}/PS1^{dE9} mice receiving 2.5mg/kg by daily i.p. injections for 8 days, or 20 mg/kg/day in their drinking water (p.o.) for 8 days, or a single i.p. injection of saline were measured by ELISA. **A:** Soluble A β_{1-40} levels were unchanged in all treatment groups **B:** Soluble A β_{1-42} was significantly reduced in mice receiving memantine orally in drinking water ($p=0.002$) but not by i.p. injection. **C:** Upon oral administration of memantine, the A β_{1-40} to A β_{1-42} ratio increased significantly ($p=0.003$), suggesting a change in γ -secretase activity. **D:** Insoluble A β_{1-42} was unchanged by memantine treatment, possibly due to the short duration of treatments.