Mematine HCl and Amino-Alkyl-Cyclohexanes (621,625) Inhibit HSV-1 in SK-N-SH Neuronal Cells

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Mematine HCl and Amino-Alkyl-Cyclohexanes (621,625) Inhibit HSV-1 in

SK-N-SH Neuronal Cells

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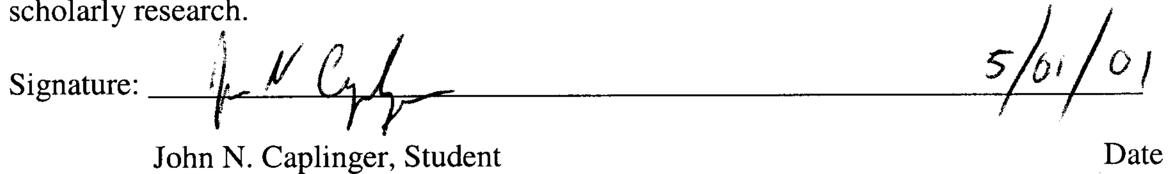

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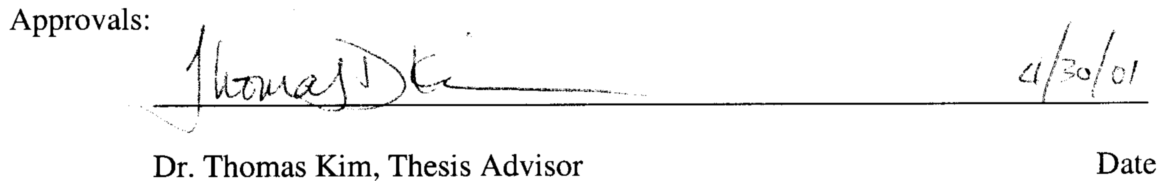

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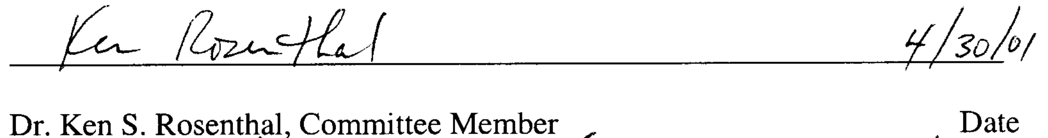

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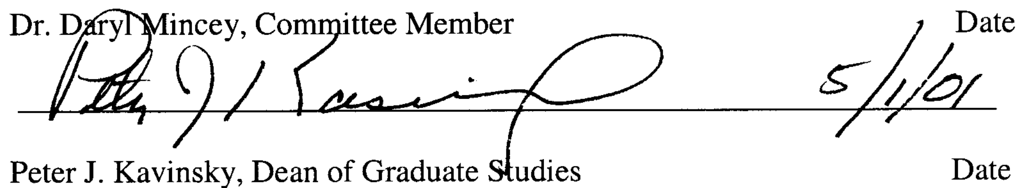

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Abstract

Memantine HCl and a series of amino-alkyl-cyclohexanes (AAC) are NMDA receptor antagonists in neuronal cells. Memantine is structurally similar to amantadine and tromantadine, both of which show anti-HSV properties. The activity of memantine and several other AAC's was evaluated by plaque reduction assay of HSV-1 KOS in SK-N-SH transformed neuronal cells. Memantine and two of the AAC's (621,625) inhibited the formation of plaques in SK-N-SH. Memantine showed a 85 % decrease in plaque formation at 0.03 mM and an ID 50 of 0.023 mM (Figure 2.1a), compound 621 showed a 100% decrease in plaque formation at 0.05 mM and an ID 50 of 0.008 mM (Figure 2.1b), and compound 625 showed a 70% decrease in plaque formation at 0.55 mM and an ID 50 of 0.120 mM (Figure 2.1c).

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I have been blessed to have the support of my family and friends and the love of my wife. I would like to thank my wife, Shellie, for all that she has endured.

I dedicate this thesis to my Grandparents, LaClair and Roberts Nelson, who have showed me by example that hard work and dedication pay off.

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List of Symbols and Abbreviations

AAC's	Amino-Alkyl-Cyclohexane
DNA	Deoxyribonucleic Acid
g	Grams
gB	Glycoprotein B
gC	Glycoprotein C
GC^+	Cell to Cell Fusion Forming HSV-1 Strain
gD	Glycoprotein D
HSV	Herpes Simplex Virus
HSV-1	Herpes Simplex Virus Type-1
K_i	Inhibitory Constant
KOS	Well-characterized HSV-1 Strain
MEM	Minimal Essential Medium
mg	Milligram
ml	Milliliter
nm	Nanomolar
NMDA	N-Methyl-D-Aspartate
PCP	Phencyclidine
pfu	Plaque Forming Unit
RNA	Ribonucleic Acid
SK-N-SH	Human Neuroblastoma
ul	Microliter

Chapter 1: INTRODUCTION TO HERPES SIMPLEX TYPE-1, NMDA CHANNEL BLOCKING COMPOUNDS, AND THE NMDA CHANNEL

Section 1.1 Introduction

There are 8 known human herpes viruses. Which can be divided into three sub-families according to genome structure, tissue tropism, cytopathology, and site of latent infection. (Murray, et al. 1998) we concentrate on herpes simplex virus type 1 (HSV-1).

The word herpes is derived from a Greek word meaning to creep; the viral etiology was established in 1919. HSV-1 targets mucoepithelial cells and is spread via close physical or sexual contact. HSV-1 strains have several things in common: DNA homology, antigenic determinates, tissue tropism, and disease symptoms. Different strains of the virus can interact differently with the host cell. One such example is KOS virus versus GC+ virus. The KOS strain will kill its host cell after replication while GC+ promotes cell to cell fusion-causing syncytia and this promotes virus spread and also allows the virus to hide from the immune system.

HSV-1 is a large, double-stranded DNA virus that has an envelope. The virion is 150 nm in diameter and has a characteristic morphology. The capsid is icosadeltahedral in shape, contains 162 capsomers and is surrounded by a glycoprotein-containing envelope. The genome is large enough to contain genes for 80 different proteins. These proteins are responsible for viral replication, interaction with the host cell and protection against immune responses (Murray, et al. 1998).

In order for the virus to infect a host cell, it must first attach itself to the cell surface. Once viral attachment occurs, the viral envelope then fuses to the host cell membrane and the genome is inserted into the host cell. The viral envelope remains

attached to the cell membrane as the genome is delivered to the nucleus of the host cell (Figure 1.1).

HSV causes two different types of infection, a productive infection that causes cell death after replication, and a latent infection that may last the life of the host. (Figure 1.1) The virus replicates in cells at the site of the initial infection and is transported to the neuron. When the virus is in its latent stage, the virus is maintained as an episome and only a small number of its transcripts are expressed (Figure 1.2). To date, no reported research has shown that viral proteins must be present to maintain a latent infection (Ward, et al. 1994).

Section 1.2 Viral Replication

The HSV genome is a class I genome. It is double stranded and replicates strictly in the nucleus of the host cell. Upon penetration, the virion releases a viral-encoded protein kinase, and cytotoxic proteins and initiates viral gene transcription. Once the DNA polymerase is synthesized, the genome begins to make end-to-end concatameric forms of the genome. Later in the infection, the DNA replication proceeds by the rolling circle mechanism and the DNA is then passed to the endoplasmic reticulum and the golgi apparatus. Once the DNA passes through the endoplasmic reticulum and the golgi apparatus, the formed concatamers cleave into individual genomes as the DNA is drawn into the cytoplasm (Murray, et al. 1998). The replication steps of the virus can be outlined as follows:

1. Virus attaches to the cell surface

2. Fusion of viral envelope and cell membrane
3. Proteins released from the virion
4. Capsid is transported to the nuclear pore
5. Viral DNA is released and circularizes
6. Transcription of alpha gene is induced
7. RNA is transported to the cytoplasm and translated
8. Viral DNA is replicated
9. Capsid proteins form empty capsids
10. DNA is packaged in the capsid
11. Capsid acquires proteins
12. The enveloped virus accumulates in the endoplasmic reticulum
13. Virus is transported to extracellular space(lysis, exocytosis, cell-cell bridges)

Spear and colleagues have shown that for the HSV-1 to attach to the host cell, glycoproteins B and C must be present on the virus surface site (Fields, et al.1990). These glycoproteins then interact with heparan sulfate proteoglycans of the host cell, allowing the virus to bind to the host cell. It is not known if both glycoproteins interact with the same domain on the heparan sulfate proteoglycans, but it is know to be the first step in viral attachment.

Once the virus has attached itself to the host cell, viral gB and gD, are activated. These proteins are responsible for the fusion of the viral envelope and the cell membrane; gD also activates cell membrane proteins responsible for fusion. Endocytic vesicles degrade viral virions that are not able to attach (Fields, et al.1990).

Section 1.3 Compounds under Study

Amantadine ($C_{10}H_{17}N$/M.W.151.051g) and tromantadine ($C_{16}H_{28}N_2O_2$/M.W. 280.4094g) are structurally similar compounds that have been shown to inhibit HSV glycoprotein processing and syncytia formation.

Amantadine is a well-known anti-influenza A drug that inhibits an early uncoating step in viral replication by blocking an ion channel. It has also been shown to play a key role in the inhibition of HSV-1 syncytia formation and the processing of gC by HSV-1 GC+ in vero cells. It is not known, however, if amantadine acts on HSV via ion channel blockage. Tromantadine has also been shown to inhibit the penetration of HSV (Rosenthal, et al.1996).

Eli Lilly Company developed Memantine, a compound similar to Amantadine, as an agent to lower elevated blood sugar levels. The drug showed no clinical activity and was forgotten until Merz & Company decided to test the drug for its effect on neurological disease. The compound was tested in vivo and was shown to be a non-competitive NMDA channel blocker at certain dosage (Parsons, et al.1999).

A family of compounds (Memantine (K_i-2.45), 621 (K_i-32.20) 625 (K_i-52.61), 705, 640, 607, 601, 639 Merz & Company) with a similar structural backbone have been shown to act as NMDA channel blockers by binding to the PCP cite. Due to the structural similarities of the compounds to amantadine and tromantadine, the possibility existed that they might possess anti-HSV activity (Figure 1.3).

Section 1.4 NMDA Channel

The NMDA channel is a well-documented glutamate receptor. Glutamate receptors play a very important role in excitatory synaptic transmission and are divided into three sub-families: Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid (AMPA), Kainate, and N-Methyl-D-Aspartate (NMDA).

The NMDA channel plays a major role in physiological activity, has very unique properties, and differs from other glutamate receptors in fundamental ways. The NMDA channel is gated by both ligands and voltage and is highly permeable to Ca^{2+}. Mg^{2+} and MK-801 are PCP binding site non-competitive inhibitors. The excitatory postsynaptic potential of the channel is prolonged and its function is controlled by various mechanisms. Synaptic plasticity and synapse formation also depends upon the NMDA channel.

The structure of the NMDA channel is divided into 5 subunits and its molecular architecture and function vary depending upon its location in the body and its state of development. All 5 subunits possess an asparagine residue in segment M2 that is responsible for Ca^{2+} permeability. (Mori, et al.1995).

Section 1.5: References

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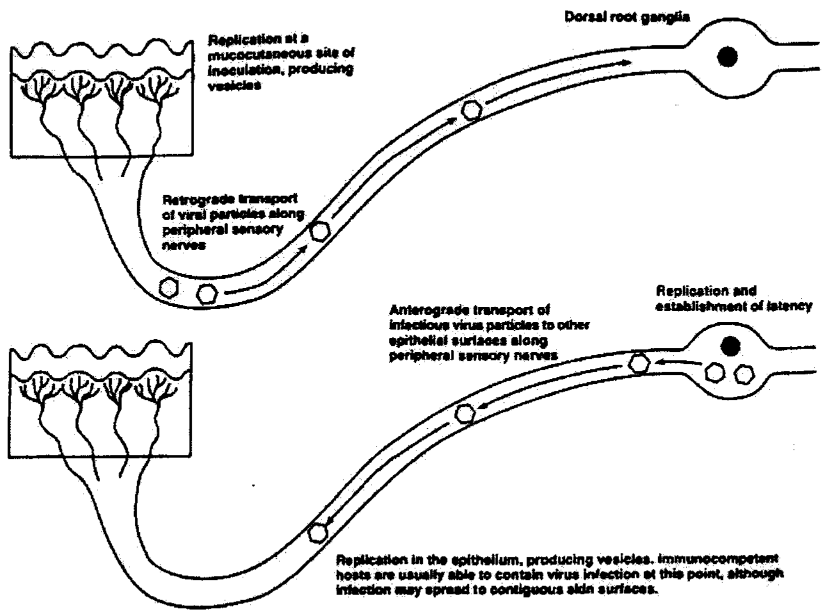
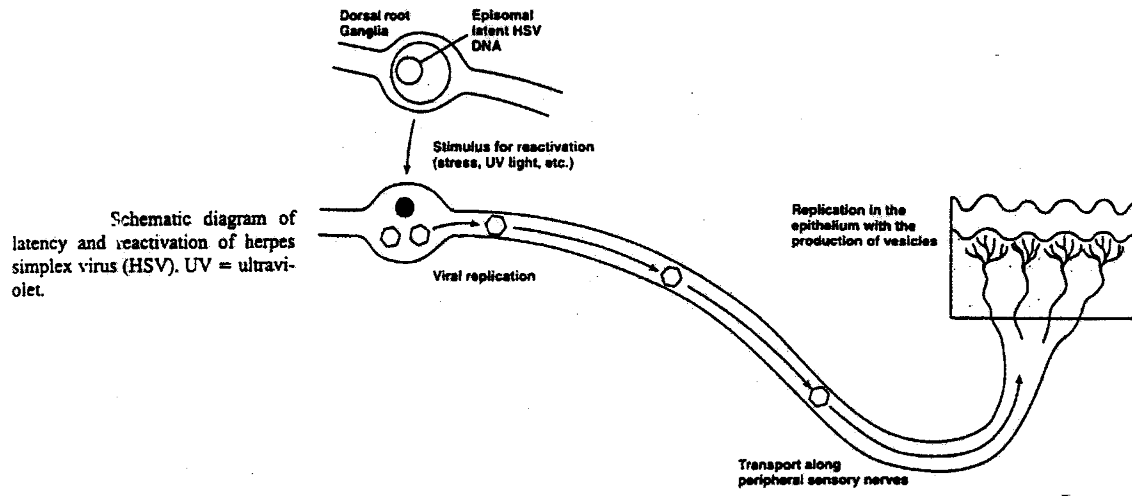
Figure 1.1: Herpes Simplex Virus Infection: This drawing is a representation of a primary herpes simplex infection. (Cartoon copied from Whitley, et al. 1998)



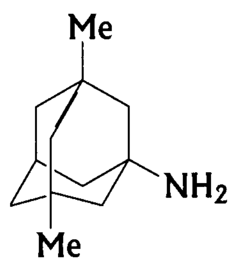
Schematic diagram of primary herpes simplex virus infection.

Figure 1.2: Latency and reactivation of Herpes Simplex: The drawing represents latency and reactivation of the herpes simplex virus. (Cartoon copied from Whitley, et al.1998)

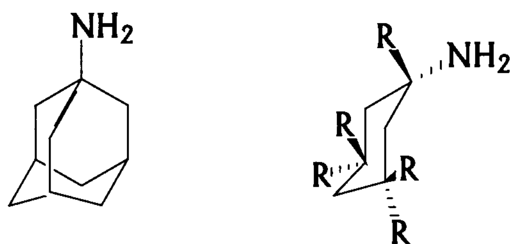


Schematic diagram of latency and reactivation of herpes simplex virus (HSV). UV = ultraviolet.

Figure 1.3: Clinical Structures of memantine, amantadine and amino-alkyl-cyclohexane.



Memantine



Amantadine and Amino-alkyl-cyclohexane

Chapter 2: MEMANTINE-HCl AND AMINO-ALKYL-CYCLOHEXANES (621, 625) INHIBIT HSV-1 IN SK-N-SH NEURONAL CELLS

Section 2.1 Abstract:

Memantine HCl and a series of amino-alkyl-cyclohexanes (AAC) are NMDA receptor antagonists in neuronal cells. Memantine is structurally similar to Amantadine and tromantadine, both of which show anti-HSV properties. The activity of memantine and several other AAC's was evaluated by plaque reduction assay of HSV-1 KOS in SK-N-SH transformed neuronal cells. Memantine and two related AAC's (621,625) inhibited the formation of plaques in SK-N-SH. Memantine showed a 85 % decrease in plaque formation at 0.023 mM and an ID 50 of 0.023 mM (Figure 2.1a), drug 621 showed a 100% decrease in plaque formation at 0.05 mM and an ID 50 of 0.008 mM (Figure 2.1b), and drug 625 showed a 70% decrease in plaque formation at 0.55 mM and an ID 50 of 0.120 mM (Figure 2.1c).

Section 2.2 Introduction

Memantine, 621, 625 are NMDA receptor antagonists. A series of Amino-alkyl-cyclohexanes and memantine have shown therapeutic potential for a number of potential central nervous system disorders (Parsons, et al. 1999). All three compounds are structurally similar to amantadine and tromantadine, which inhibit herpes simplex virus glycoprotein processing and syncytia formation (Rosenthal, et al. 1996).

SK-N-SH is a transformed neuronal cell that can be cultured in minimal essential medium and are known to possess NMDA channels. The number of channels is not known and the stage of development plays an important role in the overall structure of the channel.

KOS is a large plaque, non-syncytia forming strain of HSV-1. The genome of HSV-1 is very large and contains the genes for over 80 proteins. The proteins are responsible for virus-host interaction, replication, and protection from the host immune response. The virion is 150 nm in diameter and possesses distinct characteristics. The capsid is icosadeltahedral in shape and contains 162 capsomers. A glycoprotein-containing envelope also surrounds the capsomer. Upon infection, HSV-1 is transported to the neuron where it may induce either an active or latent infection.

Based on studies of amantadine and tromantadine, the following questions arise.

1. Does memantine and AAC's have similar antiherpes properties?
2. Do these compounds inhibit the virus due to their interaction with the NMDA channel?
3. If the NMDA channel is affected does calcium play a role in viral replication?

If the Merz compounds do indeed inhibit viral plaque formation, then the NMDA may play a key role in viral replication. This may also help us to understand the mode of action for amantadine and tromantadine.

Section 2.3 Methods

Cells and virus. SK-N-SH cells were maintained using Eagle's minimal essential medium (MEM) (Gibco) supplemented with fetal bovine serum (10%), 0.1-mg/mL penicillin-streptomycin (1%), sodium bicarbonate (2%), and sodium pyruvate (1%).

HSV-1 (KOS-strain) virus stocks were prepared by a low multiplicity of infection of SK-N-SH cells, virus stocks were obtained by freeze thaw lysis of the infected cell

24h-post infection, and were titered by plaque assay, using SK-N-SH cells with a 5% methylcellulose overlay. The virus titer was then determined to be 1.5×10^6 pfu per mL.

Drugs. Memantine, 621, and 625 were provided by Merz & Co. Pharmaceuticals, Frankfurt, Germany. Solutions (100mM stock) were prepared for each in Hanks' Basic Salt Solution (HBSS). The stock solutions were stored at -20°C.

Plaque reduction assay. Confluent monolayers of SK-N-SH cells were grown in 6-well tissue culture plates. Medium was removed and replaced with 0.5ul of Hanks' salt solution. Serial dilutions of HSV-1 were then prepared using the 1.5×10^6 virus stock and 100-200 pfu of virus were added to each well and incubated for 1h at 37°C. The solution containing unbound virus was removed and replaced with 3mL of 5% methylcellulose containing various concentrations of each of the test compounds. The SK-N-SH cells were then incubated at 37°C for 2-3 days until plaques appeared in the control. After incubation, the methylcellulose was removed and the SK-N-SH cells were stained using crystal violet. The plaques were counted and compared to the untreated control.

Penetration assay. A confluent layer of SK-N-SH cells was grown in 6-well tissue culture plates. Medium was removed and replaced with Hanks' salt solution containing various concentrations of the compounds under investigation and allowed to incubate for 1h at 37°C. HSV-1 serial dilutions were then prepared using the 1.5×10^6 virus stock and 100-200 pfu of virus were added to each well and incubated for 1h at 37°C. Unbound virus and drug were removed and the SK-N-SH cells washed with Hanks' salt solution at pH 3.0 to inactivate extracellular virus. Methyl cellulose (3mL) without compound was then added and allowed to incubate for 2-3 days 37°C until plaques appeared in the control.

Toxicity Experiment. A confluent layer of SK-N-SH cells was grown in 6-well tissue culture plates. The MEM was then removed and replaced with MEM containing various concentrations of several test compounds. The cells were then incubated for 48 hours after which the MEM was removed and the cells dislodged from the well using 1 mL of 1% trypsin. The trypsin was then quenched with MEM and the cell suspension was centrifuged at 1000 rpm for 5 minutes. The MEM was then decanted off and the cells were resuspended in 500ul of trypan blue. The total numbers of viable and nonviable cells were then counted using a hemocytometer.

Calcium Ionophore Experiment: A confluent layer of SK-N-SH cells was grown in 6-well tissue culture plates. The MEM was removed and replaced with 0.5ul of Hanks' salt solution. HSV-1 serial dilutions were then prepared using the $1.5x10^6$ virus stock; 100-200 virus were added to each well and incubated for 1h at 37°C. The Hanks' salt solution was then removed and replaced with methyl cellulose containing inhibitory concentrations of memantine, 621, and 625. Calcium ionophore was also added to the methyl cellulose to potentially negate the effect of memantine, 621, and 625. The SK-N-SH cells were incubated at 37°C for 2-3 days until plaques appeared in the control. The methylcellulose was then removed and the SK-N-SH cells were stained using crystal violet. The plaques were then counted and compared to the control.

Section 2.4: Results

An initial plaque reduction assay was performed to test the action of candidate compounds against HSV-1 KOS infection of vero cells. There was no reduction in the number of plaques but there was a reduction in the size of the plaques. Although these compounds do not block HSV replication in vero cells, they appear to have some effect indicated by the plaque size reduction.

When SK-N-SH cells were substituted for vero cells in a similar plaque reduction assay, memantine, compound 621, and compound 625 reduced the number of plaques upon treatment with non toxic doses of compound. Initial screen of the compounds in a plaque reduction assay indicated memantine reduced HSV-1 plaque number by 95% at 0.12 mM (Table 2.1a); compound 621 72% at 0.6 mM (Table 2.1d); and compound 625 92 % at 1.0 mM (Table 2.1g). Compounds 705, 640, 607, 601, 639 showed no antiviral properties or were toxic at relevant concentrations. Toxicity was indicated by the loss of the monolayer of cells. Subsequently, the conditions were refined and the compounds were shown to be more potent than originally thought. Memantine had an ID 50 of 0.023 mM and a 85% inhibition at 0.03 mM (Figure 2.1a); compound 621 had an ID 50 of 0.008 mM and a 100% inhibition at 0.05 mM (Figure 2.1b); compound 625 had an ID 50 of 0.12 mM and a 70% inhibition at 0.55 mM (Figure 2.1c). Memantine became toxic at levels exceeding 0.12 mM. Compound 621 and compound 625 appear to be less toxic compounds than memantine.

Another plaque reduction assay was done in which the concentrations of memantine, compound 621, and compound 625 were varied to determine the minimal

inhibitory concentration with respect to virus production and release. The lowest concentration tested in this experiment was 0.01 mM. All three compounds showed antiviral activities at this concentration. At a concentration of 0.01 mM, memantine showed a 16% reduction in plaque formation (Figure 2.1a), compound 621 showed a 56% reduction in plaque formation (Figure 2.1b), and compound 625 showed a 27% reduction in plaque formation (Figure 2.1c). Memantine showed an ID 85 at 0.03mM, compound 621 showed an ID 100 at 0.05 mM and compound 625 showed an ID 70 at 0.55 mM. (Figures 2.1 a, b, c).

The time of addition of compound was varied to see if memantine, compound 625, and compound 621 were affecting early stages in viral replication. SK-N-SH cells were treated one hour before infection with the candidate compounds. After the first hour 10,000 pfu (KOS) was added and allowed to incubate for 1 hour. The medium was then removed and washed with a pH3 buffer solution to inactivate cell surface virus. Medium was then added and the cells were allowed to incubate for 24 hours. The virus was then titered in vero cells. Memantine showed a 28% plaque reduction at 0.07 mM, the highest concentration tested (Figure 2.3a). Compound 625 showed a 62% plaque reduction at 0.07 mM, the highest concentration tested (Figure 2.3a). Compound 621 showed no plaque formation reduction at 0.07 mM, the highest concentration tested (Figure 2.3a). This was surprising since compound 621 was more effective than compound 625 in other trials.

A second titer was done to see if memantine, compound 625, and compound 621 were affecting late stages in viral replication. KOS (10,000 pfu) was added to SK-N-SH cells and allowed to incubate for 1 hour. The SK-N-SH cells were then treated with the

candidate compounds and after 1 hour the medium was removed. The cells were washed with pH 3 buffer solution, medium was added to the cells, and the cells were allowed to incubate for 24 hours. The cells were treated with 0.07 mM, a non-toxic inhibition dosage for all three compounds. The virus was then titered in vero cells. Memantine showed an 88% plaque reduction at 0.07 mM, the highest concentration tested (Figure 2.3b). Compound 625 showed a 99.93% plaque reduction at 0.07 mM, the highest concentration tested (Figure 2.3b). Compound 621 showed a 99.95% plaque formation reduction at 0.07 mM, the highest concentration tested (Figure 2.3b). Memantine and compound 625 have an effect on both the early and the late stages of viral replication. Compound 621 appears to affect later stages in viral replication. Further confirmation of this difference will be necessary.

The effect of the compounds on penetration of HSV-1 was also examined in a plaque reduction assay. In this set of experiments the pH of buffer wash solution was tested for an effect on the compounds' plaque reduction capabilities. SK-N-SH cells were incubated for 1 hour with the candidate compounds. After 1 hour, KOS was added to the cells and allowed to incubate for 1 hour, the medium was then removed and washed with pH 3 buffer solution, 3 ml of methyl cellulose (compound free) is added and allowed to incubate. Plaque formations were then counted. Memantine showed a 100 % plaque reduction at 2 mM (Figure 2.4a). Compounds 621 and 625 showed no plaque reduction at 2 mM (Figure 2.4a). In the second penetration plaque reduction assay, the cells were washed with pH 7 buffer solution, instead of pH 3 solution, prior to the addition of 3 ml of methyl cellulose (compound free). Memantine showed a 59% plaque reduction at 2 mM (Figure 2.4b), compound 621 showed a 63% plaque reduction at 2 mM (Figure

2.4b), and compound 625 showed a 60% plaque reduction at 1.75 mM (Figure 2.4b). A higher concentration of compounds could be used in this experiment because they were only present for 1 hour. This shows that either the pH of the buffer wash may indeed influence the activity of the compounds, or the actions of the compounds at this concentration cannot be reversed with washing.

The toxicity of memantine, compound 621, and compound 625 was measured using a trypan blue exclusion experiment. SK-N-SH cells were treated with the compounds for 48 hours; the cells were then tested for exclusion of trypan blue. Control cells exhibited 99% viability. The cells treated with memantine remained at least 80% viable up to a concentration of 0.2mM (Figure 2.5a). Compound 621 remained at least 80% viable up to a concentration of 1mM (Figure 2.5b). Compound 625 remained at least 80% viable up to a concentration of 1mM (Figure 2.5c). This experiment showed that memantine was more toxic than compound 621 and compound 625. This experiment showed that all three compounds were nontoxic at levels shown to be consistent with antiviral activity.

In a separate experiment, the effects of Mg^{2+} and Ca^{2+} on KOS were examined by plaque reduction assay in SK-N-SH. This experiment was performed because Mg^{2+} and Ca^{2+} are known to interact with the NMDA channel, the known target for memantine, compound 621 and compound 625. The concentrations of ion were varied from 0 mM to 19 mM. $MgCl_2$ showed a 52% reduction in plaque formation at 19 mM (Figure 2.2). $CaCl_2$ showed a 41% reduction in plaque formation at 19 mM (Figure 2.2). There was no significant difference between Mg^{2+} and Ca^{2+}.

A calcium ionophore (A23187) experiment was performed to attempt to reverse the effects of a possible block of Ca^{2+} permeability through the NMDA channel upon treatment of memantine, compound 621, and compound 625. Inhibitory concentrations of memantine, compound 621, and compound 625 were added to SK-N-SH for 1 hour. Calcium ionophore was then added to allow Ca^{2+} into the cells. Addition of the ionophore to the cells containing the compound proved toxic, whereas the cells without the compound were still vital after adding the ionophore. No conclusion could be made on Ca^{2+} ion involvement for inhibition of viral replication from this experiment.

Section 2.6: Discussion

Memantine, compound 621 and compound 625 inhibit the viral replication of HSV-1 in SK-N-SH, but not in vero cells. All three compounds show the ability to inhibit both early and late stages in viral replication and the effects of the compounds appear to be irreversible. All three compounds are non-toxic at inhibitory concentrations.

Memantine is structurally similar to amantadine and tromantadine. Tromantadine inhibits both early and late stages in viral replication, amantadine inhibits only late stages of viral replication (Rosenthal, et al. 1996). Memantine, compound 621 and compound 625 interact with the NMDA channel, as does a series of AAC's. Memantine, compound 621 and compound 625 bind noncompetitively to the PCP region of the NMDA channel (Parsons, et al 1999). The NMDA channel is a calcium ion channel found in SK-N-SH neuronal cells. In this study, all three compounds possessed antiviral properties against HSV-1 KOS at concentrations as low as 0.01 mM, as determined by plaque reduction

assays (Tables 2.1 a, d, g). Inhibition of plaque formation and virus production was concentration dependent.

The inhibition of HSV-1 replication by memantine, compound 621 and compound 625 in conjunction with the fact that the compounds are inhibitors of the NMDA channel and that inhibition is observed in SK-N-SH, which have NMDA channels, but not in vero cells, which lack NMDA channels, suggests that the NMDA channel is involved in HSV-1 replication in neuronal cells. However, the ability of the compounds to decrease the size of plaque formation in vero cells suggests an alternate but less sensitive target for these compounds.

Memantine, compound 625 and possibly compound 621 appear to have antiviral actions against both an early and a late step in HSV-1 replication. This is indicated because inhibition was observed when compound was present for only the first hour of infection and also when compound was added after the early steps of replication (attachment and penetration) had occurred. Since the effects of the high concentration (2mM) on HSV-1 were not reversible (as indicated after washing with the pH 7 buffer), there is some question regarding the ability of the compounds to inhibit early events in HSV-1 replication. Tromantadine displayed antiviral properties on both early and late stages of viral replication but its effects could be reversed (Rosenthal, et al 1996).

Mg^{2+} ion is known to be a blocker of the NMDA channel (Mori, et al 1995). Mg^{2+}, at 19 mM, inhibited HSV-1 plaque formation by approximately 50%. However, Ca^{2+}, another divalent cation, also inhibited HSV-1 approximately 50% at 19 mM. As a result we can't determine whether Mg^{2+} ion blockage of the NMDA channel inhibits HSV-1 replication.

The calcium ionophore (A23187) was added to SK-N-SH, pretreated with inhibitory concentrations of memantine, compound 625 and compound 621. To determine whether their effects on HSV-1 replication could be reversed. Calcium ionophore allows calcium to enter the cell without a calcium channel. Ionophores alone had no effect on virus replication and cell viability. However, calcium ionophore in the presence of the compounds proved to be toxic to the cells. As a result, we are not able to determine if calcium ionophore reverses the effects of the compounds.

In conclusion, memantine, compound 621 and compound 625 do indeed inhibit viral replication of HSV-1 in SK-N-SH. The mechanism of action is not known for any of the compounds but it does appear that all three compounds have an effect on both early and late stages in viral replication. Our study suggests, but does not prove that the NMDA channel is involved in HSV-1 viral replication. The possibility exists that the mode of action is something that has never been looked at or studied before. This opens up the possibility that a whole new family for antiviral candidates can be developed.

Section 2.7: References

Mori, H., Mishina, M., *Neuropharmacology* **1995**, 34, 1219-1237

Parsons, C.G., Danysz, W., Quack, G. *Neuropharmacology* **1999**, 38, 735-767

Rosenthal, K., Ickes, D., Dick, J., Don, B., Antimicrob. Agents & Chemother **1996,** 1, 149-155

Table 2.1a: Initial HSV-1 Plaque Reduction Assay for memantine: An initial screening plaque assay was done using memantine.

Table 2.1a

[memantine] mM	Titer	# of Plaques	Cell Condition
.0012	100	54	+
.012	100	56	+
.12	100	5	+
.17	100	5	+
.0012	0	0	+
.012	0	0	+
.12	0	0	+
.17	0	0	+
0	100	95	+

Plaque reduction assay and toxicity for memantine 1.2 mM stock solution.

Table 2.1b: Second trial of HSV-1 Plaque Reduction Assay for memantine

Table 2.1b

[memantine] mM	Titer	# of Plaques	Cell Condition
.002	200	192	+
.023	200	131	+
.116	200	76	+
.231	200	0	-
.02	0	0	+
.023	0	0	+
.116	0	0	+
.231	0	0	-
0	200	200+	+

Plaque reduction assay and toxicity for memantine 23.1 mM stock solution.

Table 2.1c: Initial HSV-1 Plaque Reduction Assay for compound 705: An initial screening plaque assay was done using compound 705.

Table 2.1c

[705] mM	Titer	# of Plaques	Cell Condition
.05	100	50+	+
.1	100	50+	+
.3	100	50+	+
.5	100	50+	+
1	0	0	+
.05	0	0	+
.1	0	0	+
.3	0	0	+
.5	0	0	+
1	0	0	+
0	100	50+	+

Plaque reduction assay and toxicity for compound 705 100 mM stock solution.

Table 2.1d: Initial HSV-1 Plaque Reduction Assay for compound 625: An initial screening plaque assay was done using compound 625.

Table 2.1d

[625] mM	Titer	# of Plaques	Cell Condition
.3	100	60	+
.5	100	66	+
.6	100	57	+
.8	100	53	+
1	0	5	+
.3	0	0	+
.5	0	0	+
.6	0	0	+
.8	0	0	+
1	0	0	+
0	100	60+	+

Plaque reduction assay and toxicity for compound 625 100 mM stock solution.

Table 2.1e: Initial HSV-1 Plaque Reduction Assay for compound 640: An initial screening plaque assay was done using compound 640.

Table 2.1e

[640] mM	Titer	# of Plaques	Cell Condition
.3	200	0	-
.5	200	0	-
.6	200	0	-
.8	200	0	-
1	0	0	-
.3	0	0	-
.5	0	0	-
.6	0	0	-
.8	0	0	-
1	0	0	-
0	200	200+	+

Plaque reduction assay and toxicity for compound 640 100 mM stock solution.

Table 2.1f: Initial HSV-1 Plaque Reduction Assay for compound 607: An initial screening plaque assay was done using compound 607.

Table 2.1f

[607] mM	Titer	# of Plaques	Cell Condition
.3	200	159	+
.5	200	0	-
.6	200	0	-
.8	200	0	-
1	0	0	-
.3	0	0	-
.5	0	0	-
.6	0	0	-
.8	0	0	-
1	0	0	-
0	200	200+	+

Plaque reduction assay and toxicity for compound 607 100 mM stock solution.

Table 2.1g: Initial HSV-1 Plaque Reduction Assay for compound 621: An initial screening plaque assay was done using compound 621.

Table 2.1g

[621] mM	Titer	# of Plaques	Cell Condition
.3	200	126	+
.5	200	57	+
.6	200	50	+
.8	200	0	+
1	0	0	+
.3	0	0	+
.5	0	0	+
.6	0	0	+
.8	0	0	+
1	0	0	+
0	200	178	+

Plaque reduction assay and toxicity for compound 621 100 mM stock.

Table 2.1h: Initial HSV-1 Plaque Reduction Assay for compound 601: An initial screening plaque assay was done using compound 601.

Table 2.1h

[601] mM	Titer	# of Plaques	Cell Condition
.3	200	0	-
.5	200	0	-
.6	200	0	-
.8	200	0	-
1	0	0	-
.3	0	0	-
.5	0	0	-
.6	0	0	-
.8	0	0	-
1	0	0	-
0	200	200+	+

Plaque reduction assay and toxicity for compound 601 100 mM stock solution.

Table 2.1i: Initial HSV-1 Plaque Reduction Assay for compound 639: An initial screening plaque assay was done using compound 639.

Table 2.1 i

[639] mM	Titer	# of Plaques	Cell Condition
.3	200	123	+
.5	200	0	+
.6	200	0	-
.8	200	0	-
1	0	0	-
.3	0	0	-
.5	0	0	-
.6	0	0	-
.8	0	0	-
1	0	0	-
0	200	150+	+

Plaque reduction assay and toxicity for compound 639 100 mM stock solution.

Figure 2.1a: HSV-1 plaque reduction assay for memantine.

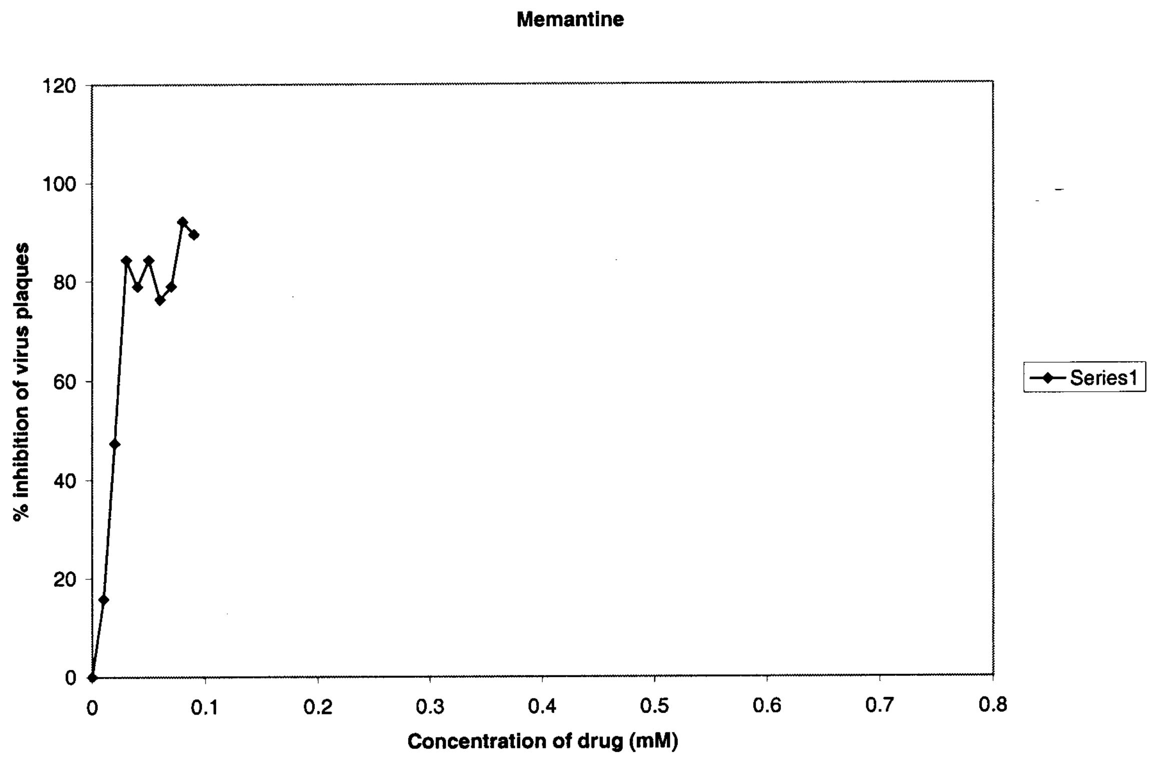

Memantine
120
100
80
60
40
20
0
% inhibition of virus plaques
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Concentration of drug (mM)
Series1

Figure 2.1b: HSV-1 plaque reduction assay for compound 621.

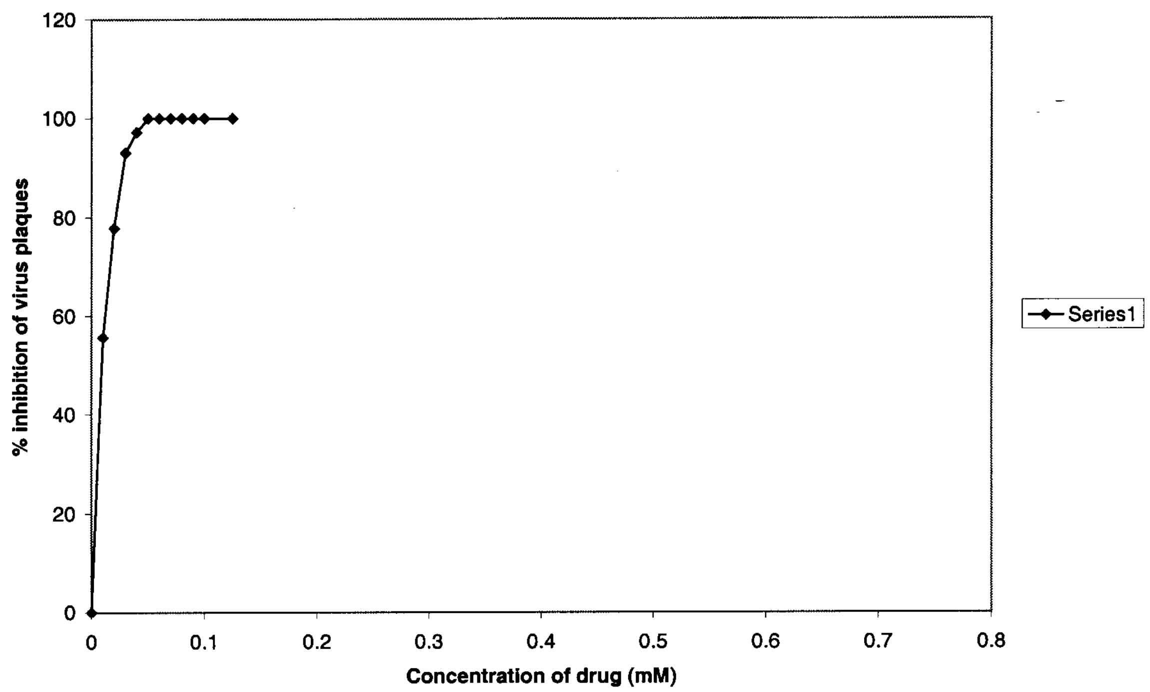

621
120
100
80
60
40
20
0
% inhibition of virus plaques
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Concentration of drug (mM)
Series1

Figure 2.1c: HSV-1 plaque reduction assay for compound 625.

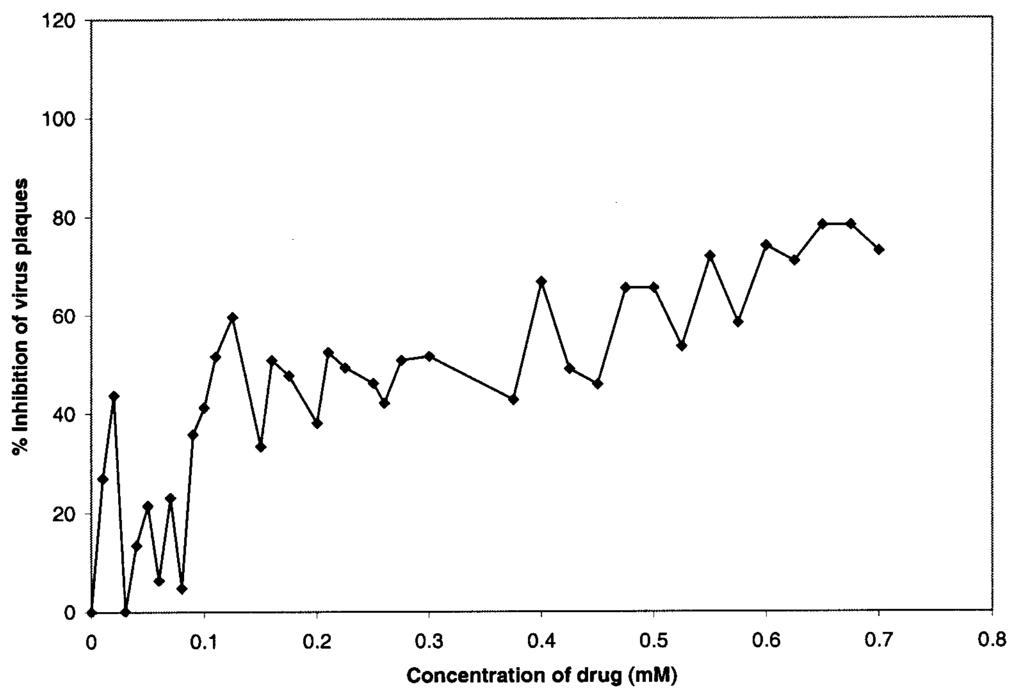

625
120
100
80
60
40
20
0
% Inhibition of virus plaques
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Concentration of drug (mM)
Series1

Figure 2.2: The effect of Mg^{2+} and Ca^{2+} on HSV-1 replication. HSV-1 plaque reduction assay was done varying the concentration of $MgCl_2$ and $CaCl_2$ to see if a change in divalent ion concentration influences the plaque assay.

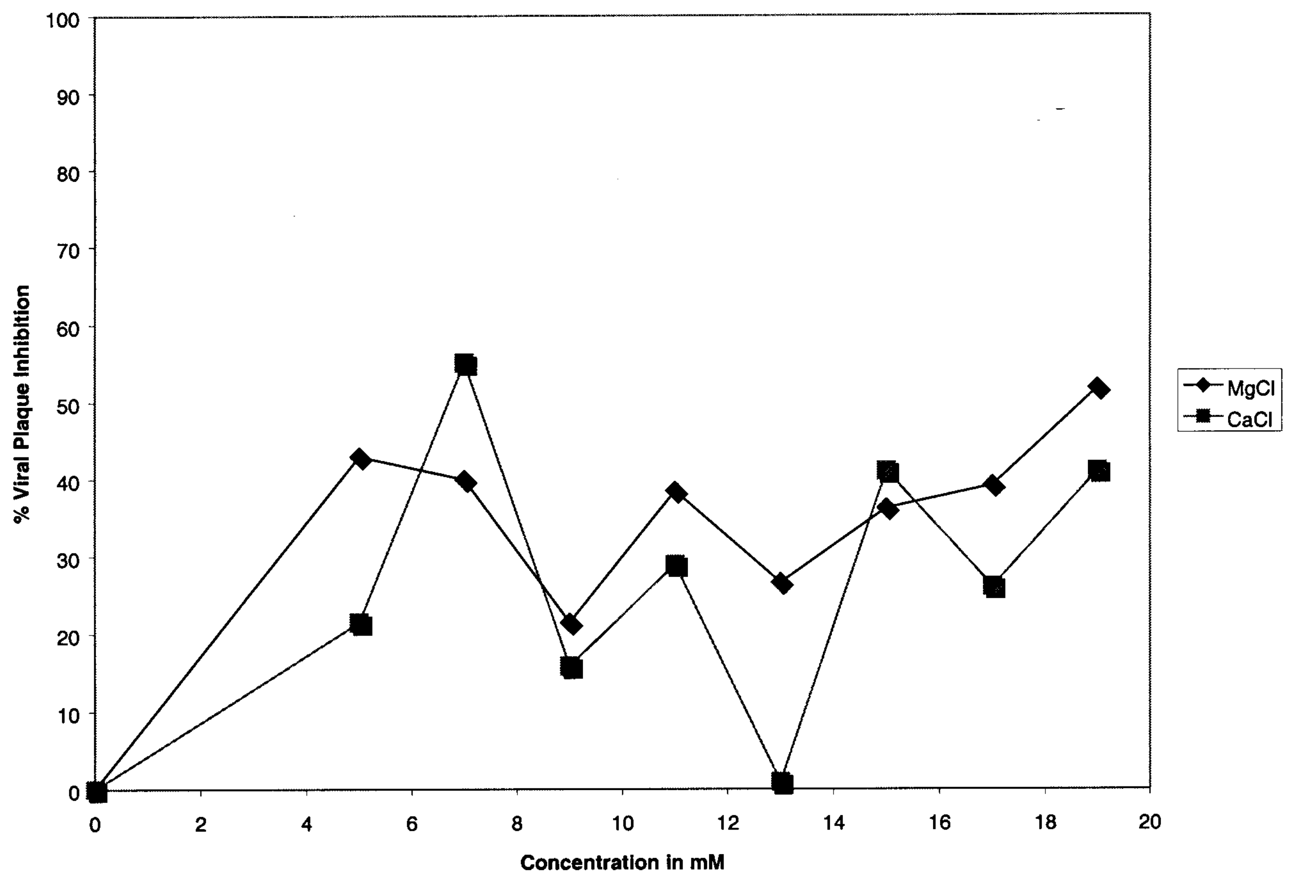

MgCl and CaCl Penetration Experiment
% Viral Plaque Inhibition
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
Concentration in mM
MgCl
CaCl

Figure 2.3a: Effect of memantine, compound 621,and compound 625 on HSV-1 penetration. HSV-1 was added to SK-N-SH cells pretreated with compound for 1 hour. After 1 hour of infection, cell surface virus was washed and inactivated with pH 3.0 buffer. The cells were then washed and incubated for 24 hours. Extracellular virus was then titered on vero cells.

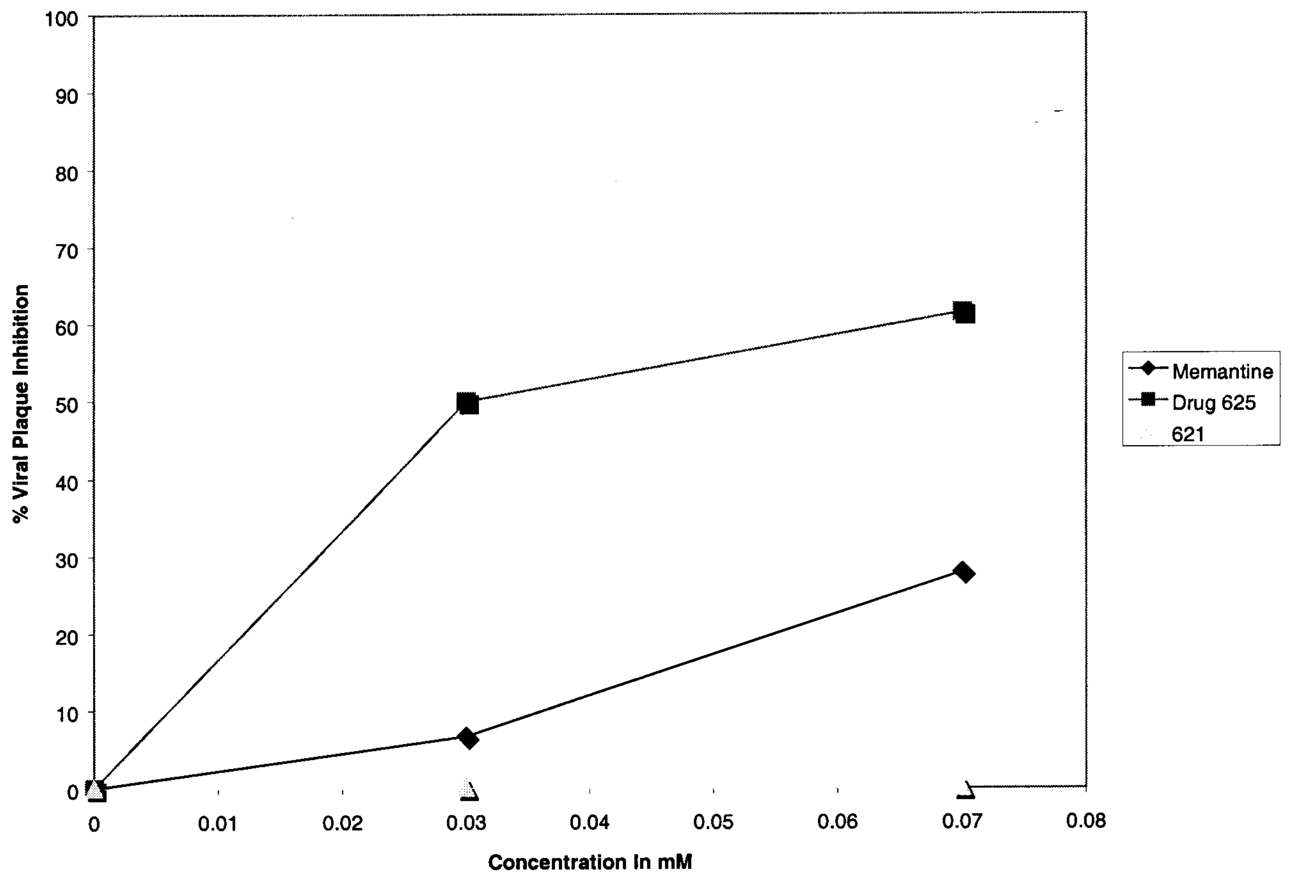

Titration Experiment Drug Added First Hour
% Viral Plaque Inhibition
100
90
80
70
60
50
40
30
20
10
0
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
Concentration In mM
Memantine
Drug 625
621

Figure 2.3b Effect of memantine, compound 621, and compound 625 on post penetration steps for HSV-1 replication. HSV-1 was added to SK-N-SH cells for 1 hour of infection, cell surface virus was washed and inactivated with pH 3.0 buffer. The cells were then washed and treated with compound. The cells were then incubated for 24 hours. Extracellular virus was then titered on vero cells.

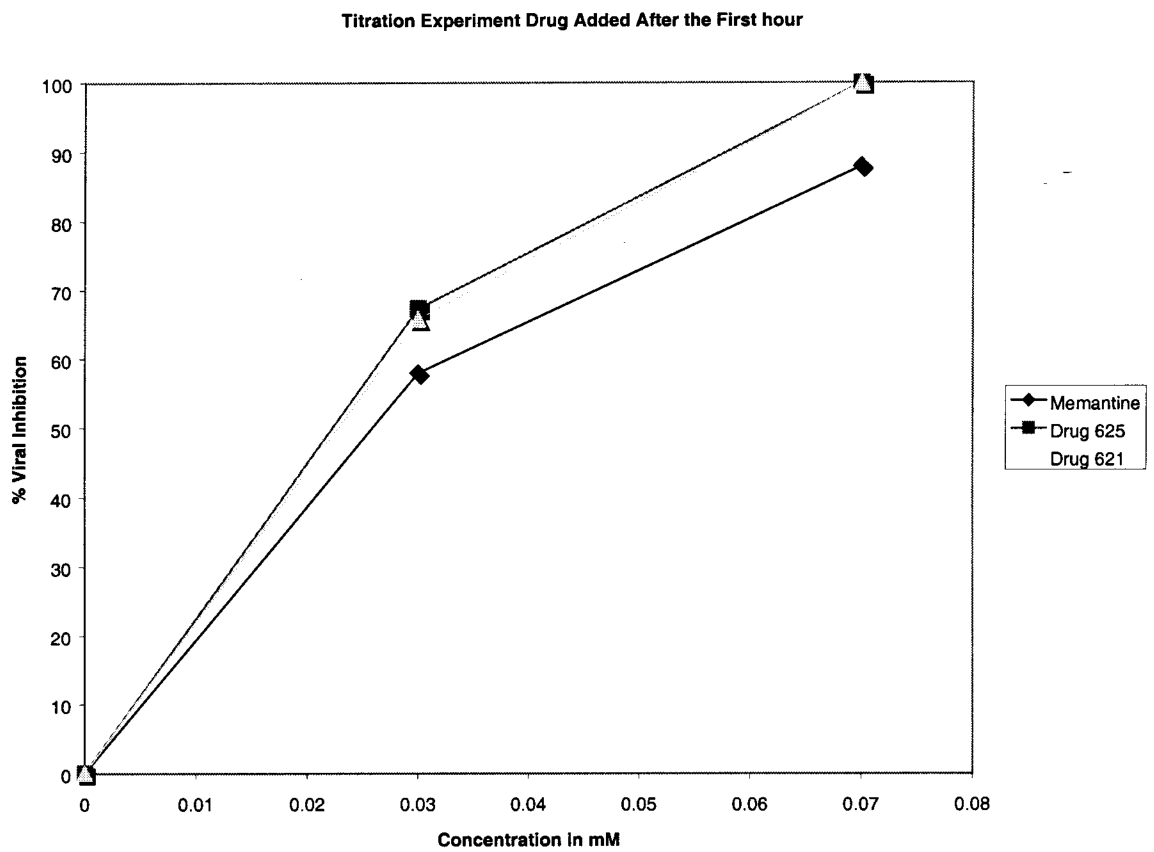

Titration Experiment Drug Added After the First hour
100
90
80
70
60
50
40
30
20
10
0
% Viral Inhibition
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
Concentration in mM
Memantine
Drug 625
Drug 621

Figure 2.4a: Effect of memantine, compound 621,and compound 625 on HSV-1 penetration. HSV-1 was added to SK-N-SH cells pretreated with compound for 1 hour. After 1 hour of infection, cell surface virus was washed and inactivated with pH 3.0 buffer. The cells were then washed and methyl cellulose was then added without compound. SK-N-SH was evaluated for plaque reduction.

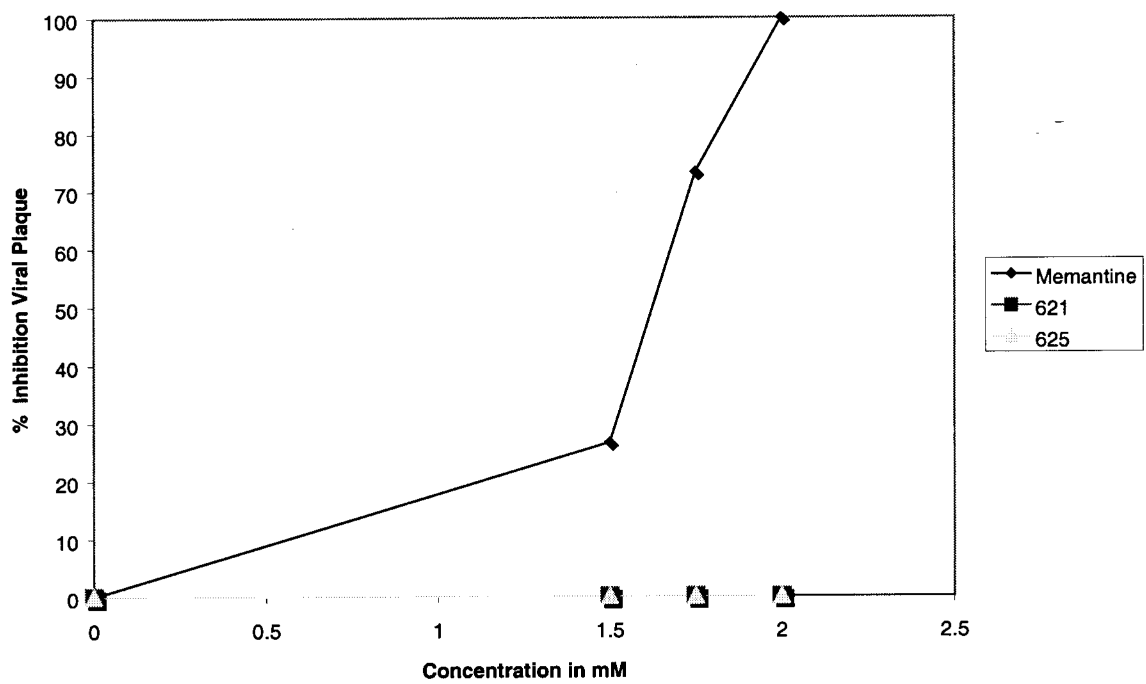

Acid Washed penetration Experiment 1
100
90
80
70
60
50
40
30
20
10
0
% Inhibition Viral Plaque
0
0.5
1
1.5
2
2.5
Concentration in mM
Memantine
621
625

Figure 2.4b: Effect of memantine, compound 621,and compound 625 on HSV-1 penetration. HSV-1 was added to SK-N-SH cells pretreated with compound for 1 hour. After 1 hour of infection, cell surface virus was washed with pH 7.0 buffer. The cells were then washed and methyl cellulose was then added without compound. SK-N-SH was evaluated for plaque reduction.

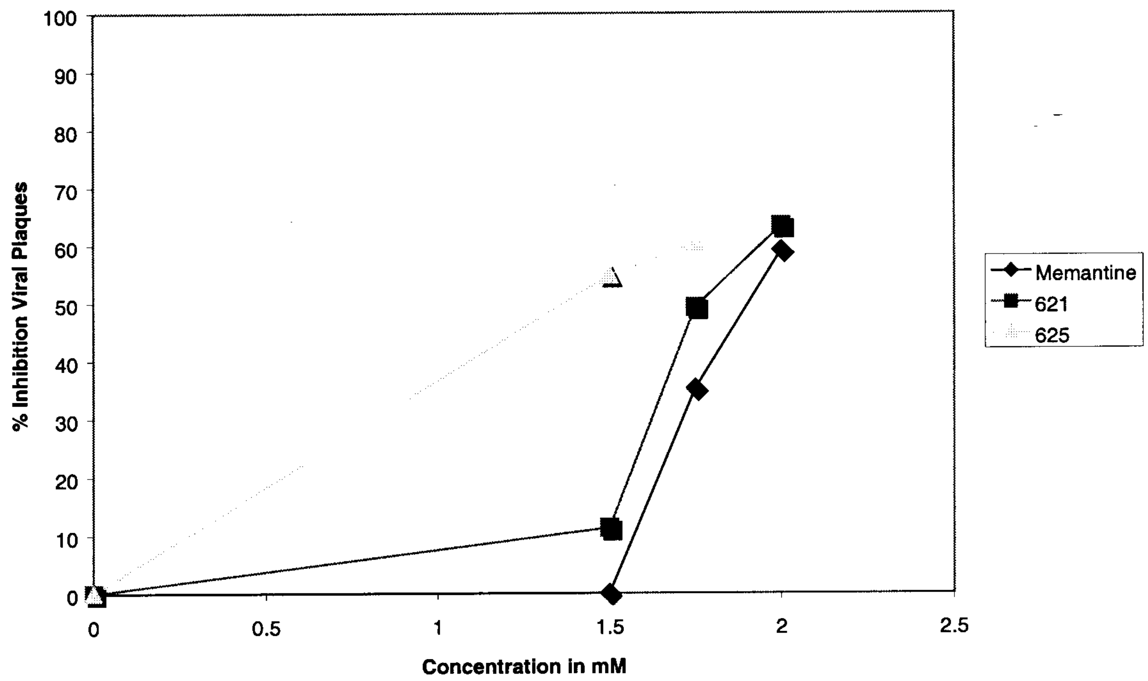

Hanks Wash Penetration Experiment 2
100
90
80
70
60
50
40
30
20
10
0
% Inhibition Viral Plaques
0
0.5
1
1.5
2
2.5
Concentration in mM
Memantine
621
625

Figure 2.5a: Evaluation of memantine toxicity. SK-N-SH were treated with memantine for 48 hours, the toxicity was evaluated by the Trypan Blue exclusion technique.

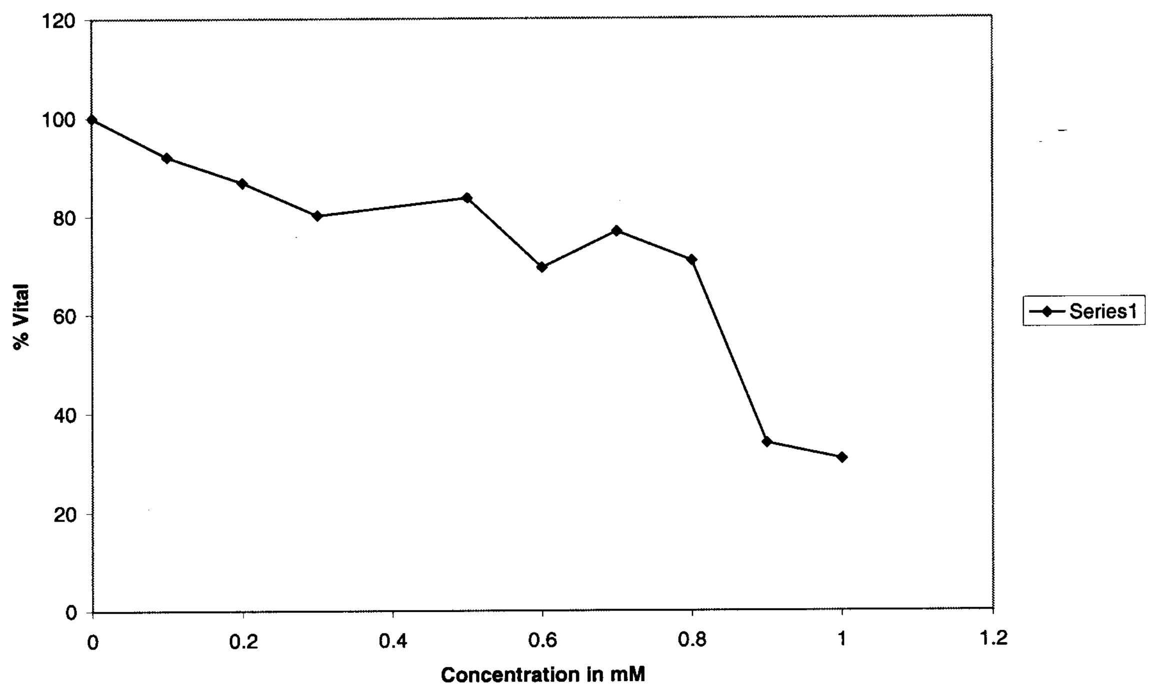

Toxicity of Memantine
120
100
80
60
40
20
0
% Vital
0
0.2
0.4
0.6
0.8
1
1.2
Concentration in mM
Series1

Figure 2.5b: Evaluation of compound 621 toxicity. SK-N-SH were treated with compound 621 for 48 hours, the toxicity was evaluated by the Trypan Blue exclusion technique.

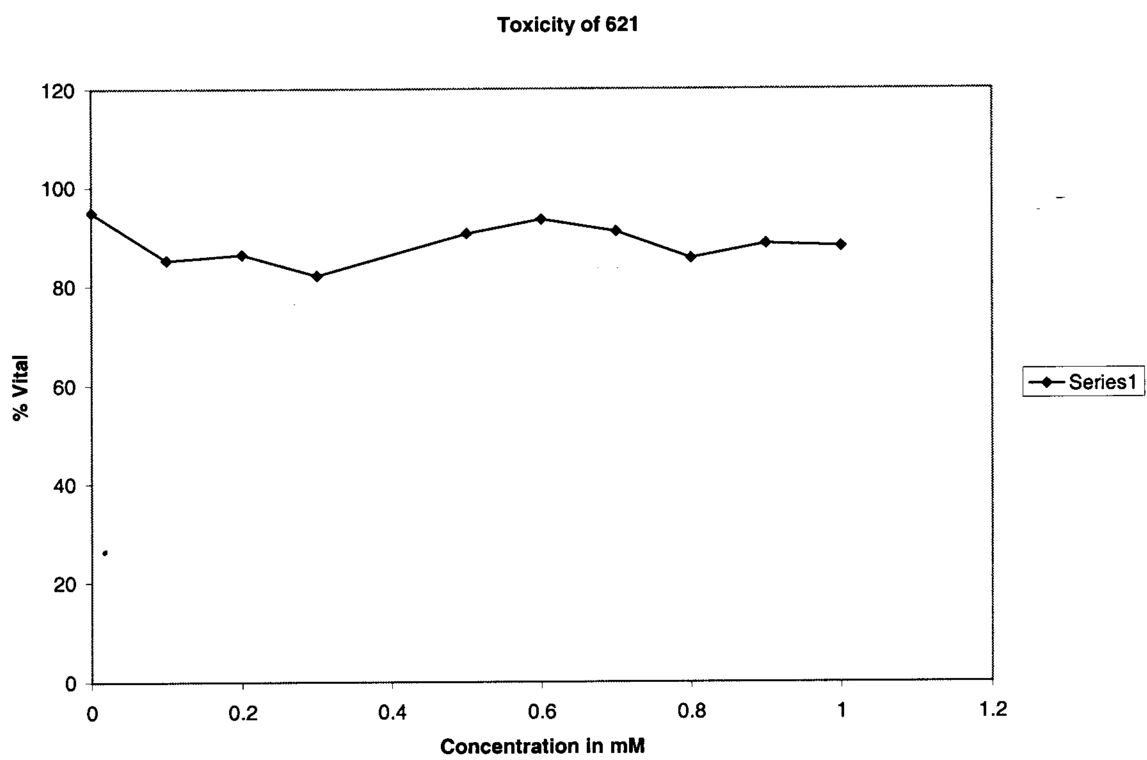
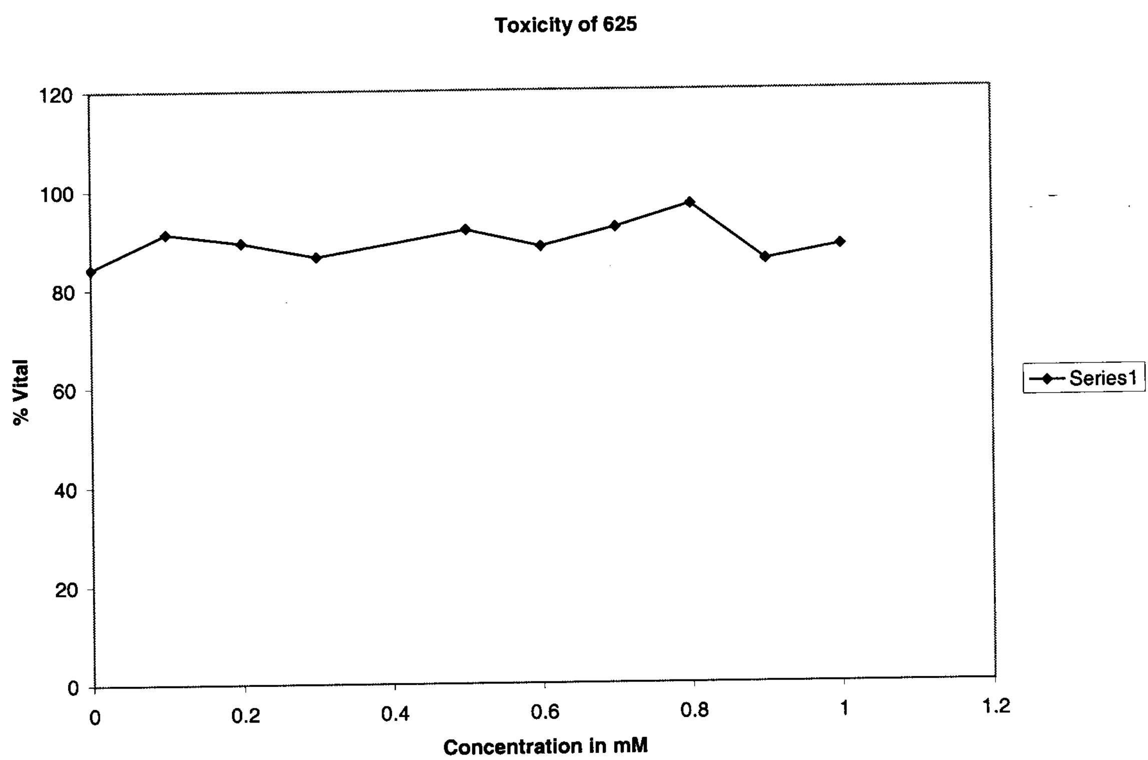

Toxicity of 621
120
100
80
60
40
20
0
% Vital
0
0.2
0.4
0.6
0.8
1
1.2
Concentration in mM
Series1

Figure 2.5c: Evaluation of compound 625 toxicity. SK-N-SH were treated with compound 625 for 48 hours, the toxicity was evaluated by the Trypan Blue exclusion technique.


Toxicity of 625
120
100
80
60
40
20
0
% Vital
0
0.2
0.4
0.6
0.8
1
1.2
Concentration in mM
Series1