Maritza Alvarado is a senior at the University of California at Berkeley majoring in Neurobiology and Psychology. Over the past year and a half, Maritza has been working at Lawrence Berkeley National Laboratory. At LBNL, she continues to synthesize and evaluate compounds suitable for ^{18}F labeling and Positron Emission Tomography (PET). These compounds are used to investigate neuronal death caused by stroke and brain injury. In the future, they hope to use the compounds for in vivo, noninvasive imaging of activated brain glutamate receptors in humans in the context of stroke and traumatic brain injury. As a result of this experience, Maritza has decided to apply to MD/PhD schools and continue to study mechanisms and treatment of neurodegenerative disorders.

Anat Biegon has an undergraduate degree in chemistry (Tel Aviv University). She went on to do graduate and postgraduate work in different disciplines of neurosciences including neuropharmacology, neuroendocrininology, and neuroimaging. She then held research positions as Associate Professor at the Weizmann Institute of Science in Israel and at NYU Medical School. She then served as a staff scientist at the Lawrence Berkeley Laboratory. She worked for several years in pharmaceutical research and development as Vice President of R&D with Pharmos Corp, developing new drugs for brain injuries. She is currently back at the Lawrence Berkeley National Laboratory in the Department of Functional Imaging.

NOVEL FLUORINE-CONTAINING NMDA ANTAGONISTS FOR BRAIN IMAGING: *IN VITRO* EVALUATION

MARITZA ALVARADO AND ANAT BIEGON PH.D.

ABSTRACT

The NMDA receptor has been implicated in neuronal death following stroke, brain injury and neurodegenerative disorders (e.g. Alzheimer's, Parkinson's and Huntington's disease) and in physiological functions (e.g. memory and cognition). Non-competitive antagonists, such as MK-801 and CNS-1102, that block the action of glutamate at the NMDA receptor have been shown to be neuroprotective by blocking the influx of calcium into the cells. As a result, they are being considered as therapeutic agents for the above mentioned diseases. Several Fluorine-containing novel analogs of NMDA channel blockers have been synthesized and evaluated in search of a compound suitable for ^{18}F labeling and Positron Emission Tomography (PET). Based on *in vitro* binding assay studies on rat brain membranes, the novel compounds examined displayed a range of affinities. Preliminary analyses indicated that chlorine is the best halogen on the ring, and that ethyl fluoro derivatives are more potent than methyl-fluoro compounds. Further analysis based on autoradiography will be needed to examine the regional binding characteristics of the novel compounds examined in this study. Labeling with ^{18}F will allow the use of these compounds in humans, generating new insights into mechanisms and treatment of diseases involving malfunction of the glutamatergic system in the brain.

INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the brain. The N-methyl-D-aspartate (NMDA) receptor is one of the three major subtypes of ionotropic glutamate receptors, which is preferentially activated by N-methyl-D-aspartate. The NMDA receptor (Figure 1) is able to control a cation channel of high conductance that is permeable to Ca^{2+} as well as to Na^{+} and K^{+}. In addition, opening of the channel requires extracellular glycine as a cofactor. The opening of the channel also depends on membrane voltage as well as a chemical transmitter. Maximal current flow through the NMDA-type channel occurs only when glutamate is present and the cell is depolarized (Figure 1).

Under typical conditions, the concentration of glycine in the extracellular fluid is sufficient to allow the NMDA receptor-channel to function efficiently. Activation of NMDA receptors opens membrane ion channels with high calcium permeability, which can lead to a lethal influx of calcium if in excess. Thus, prolonged stimulation of this ion channel may result in excitotoxic neuronal death as well as tissue swelling. Excitotoxicity has been implicated in neuronal death from stroke, head injury, epilepsy, Alzheimer's disease and Parkinson's disease. Physiologically, this receptor subtype has an active role in long-term depression,

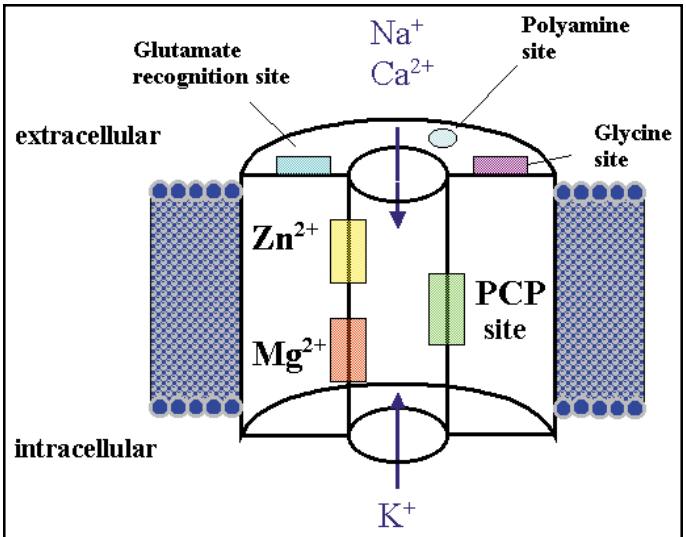


Figure 1. Schematic drawing of the NMDA Receptor
The NMDA Receptor is a ligand-gated ion exchange channel composed of two different protein subunits called $NMDAR_1$ and $NMDAR_2$. It is widely distributed in mammalian brain and spinal cord and has a particularly high density of receptor in the hippocampus and cerebral cortex. The main role exists in long term depression, long-term potentiation and developmental plasticity.

long-term potentiation and developmental plasticity and is believed to be involved in learning and memory (Kandel et al. 2000).

Understandably, the NMDA receptor is an important target for research as well as therapy. The receptor ion channel can be competitively blocked by physiological concentrations of Mg^{2+} ions in a membrane voltage dependent manner, requiring concurrent depolarization for ions to pass through the channel. Blockage of the NMDA receptor reduces ischaemic damage in focal cerebral ischaemia (Ozyurt et al. 1988) and brain injury (Faden et al. 1989, Di Xiao et al. 1996). There are sites within the NMDA receptor ion channel complex in which drugs may act to attenuate the effects of glutamate. Agents such as MK-801 (refer to first figure in Table 1) interact with the site in the ion channel of the NMDA receptor to produce a non-competitive agonist dependent blockade of glutamate actions (Kandel et al. 2000). Thus, NMDA receptor activation can be assessed non-invasively in the living human brain by molecules that can cross the blood-brain barrier, bind to the open channel, and emit radiation that can be detected by brain imaging instrumentation (e.g. Positron Emission Tomography (PET) or SPECT).

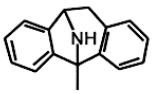
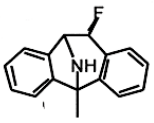
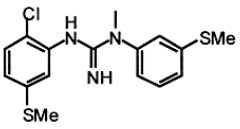
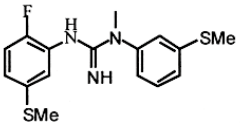
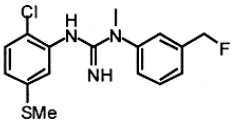
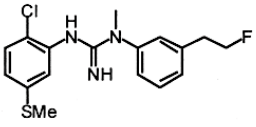
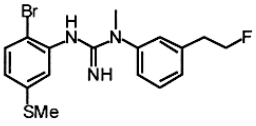
In order to create molecules that are suitable for brain imaging, the first step is to make derivatives of compounds that are known to bind and interact with the channel, such as MK-801 or CNS-1102 (cerestat, Hu Lain-Yen et al. 1997). Specific attention must be given to choosing the appropriate radionuclide, such as Fluorine, since ^{18}F is a positron emitter with a convenient half-life (90 min) compared to the carbon isotope ^{11}C (20 min). Non-competitive antagonists, such as MK-801, block the influx of calcium into the cells and inhibit the glutamate-activated ion channel. As a NMDA receptor channel-blocking agent, MK-801 binds when the ion channel is open and is in a use-dependent and voltage-dependent state. Since MK-801 is a known inhibitor of the NMDA receptor channel and is commercially available in a radiolabeled, tritiated form, it is used to test the potential of new compounds as blockers through *in vitro* binding competition assays. By using tritium-labeled MK-801 in the presence of glutamate and glycine, and increasing concentrations of the new molecules, the affinity (K_i) of the new compounds can be assessed. The potential for passing through the brain-blood barrier can be assessed through measurement of log P, which represents the lipid solubility of the compounds. Competition for binding on whole brain sections, assessed autoradiographically, can be used to study the differential affinity of the candidate molecules to NMDA receptor channels in various brain regions. The future utility of labeled versions of these compounds in detecting changes in NMDA receptor activation needs to be tested in animal models in which these changes are well documented, such as head trauma and stroke models.

The best compounds to emerge from such a series of experiments can be used in patients to assess the role, time course and response to treatment of NMDA channels in relevant clinical conditions.

Table 1. Summary of K_i and Log P Values for New NMDA Channel Inhibitor Candidates

As can be seen from this table, the best compounds resulting from the first synthetic round were the fluorinated MK801 analog 25 and the non-fluorinated cerestat analog CNS-5161, which could be a candidate for carbon-11 labeling. Therefore, we have proceeded with attempts at radiolabeling these two compounds.

[a] Binding curve shown in Figure 2

Compound		K_i (nM)	Log P
	MK 801	4.5	4.48
	**25** AG03-178	8. 5 [a]	2.89
	CNS-5161	5.5	1.92
	**19** AG02-81	31 [a]	1.34
	**5** AG03-181	110 [a]	1.14
	**11** AG03-182	16	1.20
	**12** AG03-183	19	1.42

MATERIALS AND METHODS

Membrane Preparation

Rat brains were obtained from Pel-Freez Biologicals and used in a membrane preparation for NMDA-binding studies. The cerebellum was removed and the forebrain was collected in a tube containing Tris buffer, ~30ml/gr tissue. The brains were then homogenized and centrifuged at 2400 rpm for 10 minutes at 4°C. Following the initial centrifugation, the supernatants were collected and the pellets were discarded. The supernatants were centrifuged at 18,500xg for 30 minutes at 4°C. The supernatants were discarded and the pellets were resuspended in the original volume of Tris-HCl 10 mM containing 1 mM PMSF (pH 7.4) by homogenization in ice. The pellet was centrifuged at 18,500xg for

30 minutes in 4°C. The supernatants were discarded once again and the pellet was resuspended and centrifuged. Final washed pellets were dissolved in ~2 ml of buffer per gram crude net weight, distributed into test tubes, and stored at -70°C.

Protein Bradford Assay

A standard curve was created for each experiment using serial dilutions of a stock solution of bovine serum albumin (BSA). In each test tube, 200 mL of dye reagent and 800 mL of known protein sample (for standard curve) or unknown experimental sample were used. The protein sample was added first, followed by the dye reagent. The range used for the calibration curve was 1.25 ug/mL to 25 ug/mL. Each concentration was assayed in duplicate and the experiment was duplicated. Once the test tubes contained the necessary amount of protein sample and dye, they were incubated for 10 minutes before they were transferred to cuvettes. The VIS Spectrophotometer was set at a wavelength of 595 nm and auto-zeroed with distilled water. Each sample was measured for its absorbance at 595 nm.

In Vitro Binding Studies

One tenth mg of rat forebrain membranes were incubated for 2 hours at room temperature with 10 nM [^{3}H]-MK-801, 10 µM glutamate, 30 µM glycine and novel compounds at various concentrations, synthesized by Dr. Andrew Gibbs at the Center for Functional Imaging at Lawrence Berkeley National Laboratory. Each concentration was assayed in triplicate and each compound was tested at least twice. The non-specific binding was defined by 0.1 mM MK-801.

Following incubation, the bound radioligand was separated onto GF/B filters, pre-soaked for 30 minutes in 0.1% polyethylenimine, using a Brandell cell harvester. The filters were washed 3 times with 5 mL of cold 50 mM Tris Acetate at pH 7.4. The filter paper circles were collected in 20 mL LSC vials and 15 mL of ecolume was added. The samples were incubated overnight and counted in the liquid scintillation counter for 1 minute. Following the counts, the IC_{50} values were calculated from the plot of the log concentration of displacer versus the % [^{3}H]-MK-801 specific binding.

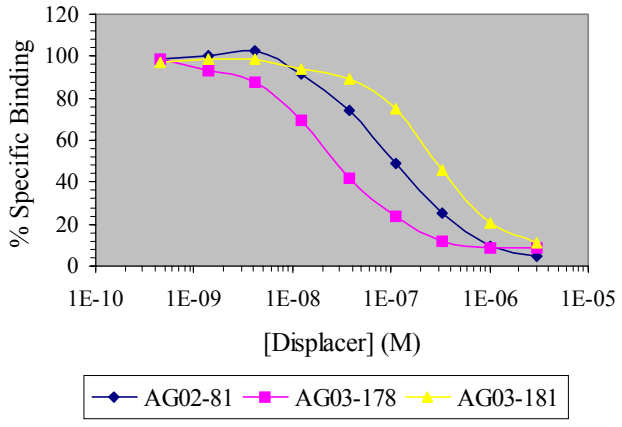


Figure 2. NMDA Receptor Displacement Curves
Inhibition of [^{3}H]MK801 binding to rat brain membranes by representative new analogs of Cerestat and MK801. AG03-178= compound 25, AG02-81= compound 19 and AG03-181=compound 5.

Rat Brain Sectioning: Control, Sham and Trauma Brains

The control brains were obtained from Pel-Freez Biologicals Company and the sham and trauma brains were sent by Dr. Marmarou of the Medical College of Virginia. The "trauma" animals were subjected to a unilateral contusion followed by 10 minutes of unilateral hypoxia/ischaemia (occlusion of the carotid artery on the side of the lesion). Sham animals were anesthetized but not contused.

The rat brains were taken out of the -70°C freezer and mounted onto the cryostat chuck with an embedding matrix. They were allowed to equilibrate for 30 minutes at -20°C for proper sectioning and solidification of the embedding matrix. Following equilibration, the temperature of the cryostat was increased to -15°C. The brains were sectioned along the horizontal plane and sections were 10 µm thick. The consecutive sections were placed in series and thaw mounted on frosted-end coated microscope glass slides.

MK-801 Autoradiography

On the day of the assay, sections were removed from the freezer and allowed to reach room temperature. After a 30 minute pre-wash in 50 mM Tris-acetate buffer at pH 7.4, the sections were incubated for 4 hours at room temperature in 50 mM Tris-acetate buffer at pH 7.4. The buffer contained 10 nM [^{3}H]-MK-801, 30 µM glutamate, and 10 µM glycine (~200 µL per section).

At the end of the assay, the sections were dipped for 5 seconds in ice-cold buffer and placed for 90 minutes in cold, fresh buffer, followed by a dip in ice cold distilled water. One section from each slide was removed with a GF/B disk into 20 mL LSC vials and 15 mL of Hionic-Fluor was added. The sections were counted for 1 minute by liquid scintillation. The remainder of the dry tissue sections were apposed to tritium-sensitive film. After exposing of at least 4 weeks for the forebrain regions, the films were immediately re-exposed for 2 months for the cerebellum. Autoradiograms were developed in Kodak D-19, fixed, and dried.

Calculations

In the binding assay performed, the K_D was calculated for MK-801 by using the following equation:

$$K_D = IC_{50} - C$$

where C=concentration of radioactive compound

$$C = 10 \times 10^{-9}\ M$$

Whereas, the inhibition constant, K_I, for the novel compounds was calculated by using the K_D value of MK-801 with the following equation:

$$K_I = IC_{50}/(1+C/K_D)$$

K_D of MK-801=4.6×10^{-9} M

RESULTS

In Vitro Binding Studies

A variety of structurally diverse compounds (Table 1) were tested for their ability to inhibit [^{3}H] MK-801 binding to the NMDA receptors in the brain. As illustrated in Figure 2 all of the novel compounds were capable of complete inhibition of [^{3}H]MK-801 binding, although the inhibition occurred within different concentration ranges. The IC_{50} values of these compounds for the MK-801 binding site, derived from log-transformed inhibition curves, varied from approximately 10^{-9} to 10^{-7} M. The difference between duplicate measurements did not exceed 25%. The K_D, for MK-801 and the inhibition constant, K_I, for the novel compounds were calculated from these using the formula shown in the materials and methods sections. Log P values were in the range of 1-3 (Table 1).

MK-801 Autoradiography

The anatomical distribution of [^{3}H]MK-801 binding was examined in commercial, trauma, and sham brains under various incubation conditions. The slides were placed in a cassette and developed with a phosphoimager screen after one week of exposure. However, when the tritium screen was exposed after a week, many ghost images appeared from previous studies performed. Therefore, no conclusions could be made which required using the more time consuming method of brain sections exposure: tritium sensitive film. As a result, the brain sections were exposed on film for at least 4 weeks. The first set of images from a control brain was developed (Figure 2) and showed a significant difference in optical density (i.e. concentration of radioactivity) between "total" and "non specific" conditions. The sections exposed to tritiated MK-801 in the presence of excess unlabeled material ("non specific" binding) were barely visible; while those exposed to radiolabeled MK-801 without displacer showed a regional variation of optical density. The cortex and hippocampus were the darkest, followed in decreasing order by thalamus, basal ganglia and cerebellum. The sections from the trauma and sham brains are being exposed to film at this time.

DISCUSSION

Based on *in vitro* binding assay studies on rat brain membranes, we have developed fluorine containing NMDA receptor antagonists which show complete displacement of [^{3}H] MK-801. The K_D of MK-801, 4.6 x 10^{-9} M, is similar to values reported in the literature (Hu Lain-Yen et al. 1997). The novel compounds examined displayed a range of affinities (Figure 3) for the NMDA-receptor ion channel. Two of the new compounds (the MK-801 fluorinated analogue, AG03-178; and the chlorine-containing Cerestat analog, AG02-80) were very close to MK-801 in affinity, with K_I in the nM range. The best fluorine containing Cerestat analogs, AG03-182 and AG03-183, had K_I values of 16 and 19 nM respectively; an intermediate potency still acceptable for *in vivo* imaging. The remaining compounds, with K_I's of 31 and 110 nM, were considerably less potent. Preliminary analysis of structure-activity relationship in this relatively short series of compounds indicates that chlorine is the best halogen on the ring, and that ethyl-fluoro derivatives are more potent than methyl-fluoro compounds.

The fluorinated MK-801 analogue was determined to have a lower log P value than MK-801. As expected, the introduction of fluorine decreased the log P value, which was a desirable change in this particular case since the log P of MK-801 is extremely high and results in unacceptably high non-specific binding *in vivo* (Price et al. 1988). The log P values in the Cerestat series (Table 1) were all in the favorable range of 1-3; namely, lipophilic enough to cross the blood brain barrier but not so lipophilic as to produce unacceptably high non-specific binding *in vivo*.

The advantage of autoradiography is that detailed competition curves can be generated in numerous, discrete anatomical regions simultaneously, and several parameters can be evaluated on consecutive thin sections from a single brain. In our hands, *in vitro* autoradiography of MK-801 under various conditions showed a heterogeneous distribution of NMDA receptors in various parts of the brain (Figure 2). The density was greater in the total binding sections than in the non-specific ones that were practically indistinguishable from film background level. The greatest receptor density was seen in the hippocampus and the cortex

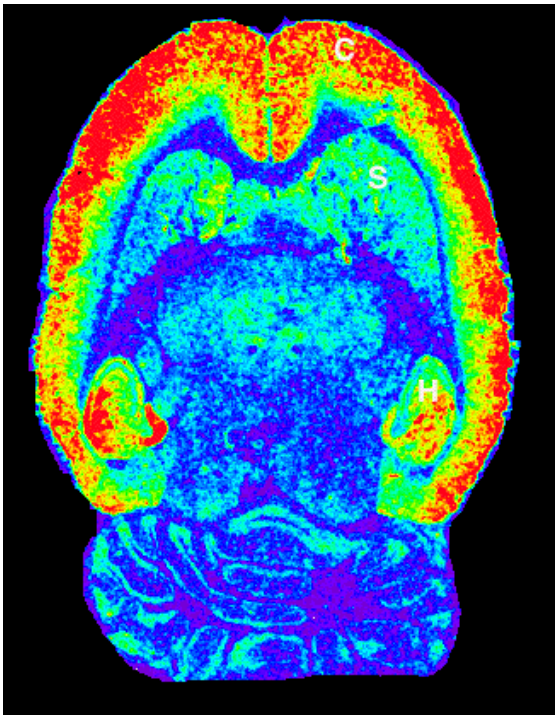


Figure 3. *In vitro* Autoradiography of MK801 on Rat Brain Sections

Total Binding= [^{3}H]MK801 + buffer. Sections were cut in the horizontal plane. Note the symmetrical binding and the high density in cortex and hippocampus compared to striatum, characteristic of normal NMDA receptor distribution. The image was pseudocolored using the rainbow spectrum, such that low levels of radioactivity are depicted by purple and blue while the highest densities are in red. C= Cortical Area, S= Striatum, and H= Hippocampus.

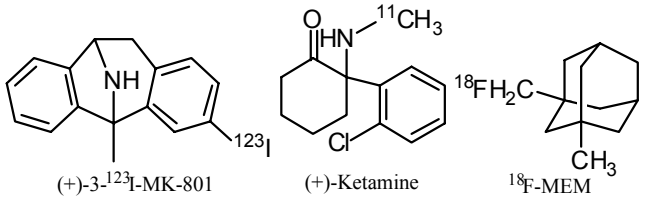


Figure 4. Structures used in previous animal and human imaging studies.

with the lowest in the cerebellum. This pattern is in accordance with published reports on the distribution of NMDA receptors in the brain (Porter et al. 1995), which showed that NMDA antagonists may differ in their potency for displacing MK-801 binding in the forebrain vs. cerebellum. Further analysis based on autoradiography will be needed to examine the regional binding characteristics of the novel compounds examined in this study.

Previous putative NMDA imaging agents (Figure 4) were not successful, probably because of high non-specific binding *in vivo* (Ametamey et al. 1999, Brown et al. 1997, Gibson et al. 1992, Kumlein et al. 1999, Ouyang et al. 1996). This may be a consequence of excessively high log P values, low affinity or slow kinetics. The combination of the binding and log P data presented above suggest that the present series contains three fluoro compounds with favorable affinity and lipophilicity profiles which can be developed further as imaging agents. Labeling with ^{18}F will allow the use of these compounds in humans, generating new insights into mechanisms and treatment of diseases involving malfunction of the glutamatergic system in the brain.

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