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THE ANTI-EPILEPSY POTENTIAL AND ANTAGONISM OF THE N-METHYL-D-ASPARTATE RECEPTOR BY DIAMINODIPHENYLS

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THE ANTI-EPILEPSY POTENTIAL AND ANTAGONISM OF THE *N*-METHYL-D-ASPARTATE RECEPTOR BY DIAMINODIPHENYLS

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ABSTRACT

Epilepsy is a disorder characterized by the occurrence of seizures, which are periods of abnormally excessive synchronous neuronal activity in the brain. Affecting over 70 million people worldwide, many of whom do not respond to pharmacotherapy, there is a need for novel anticonvulsant compound discovery. Diaminodiphenyl compounds, a class of compounds shown to present anticonvulsive effects *in vivo* have been purported to exert their effects on the *N*-methyl-D-aspartate receptor (NMDAr); an excitatory, ionotropic receptor that is a key player in the functions of the glutamatergic system. The glutamatergic system is vital in the promotion of synaptic plasticity and has been implicated in a myriad of mental health issues, including epilepsy.

We hypothesize that diaminodiphenyl compounds interact with NMDAr at an allosteric binding site on the NR2 subunit which is activated by the agonist glutamate. This project is intended to characterize the diaminodiphenyl binding interactions with NMDAr, elucidate a structure activity relationship between diaminodiphenyl compounds and NMDAr and create novel diaminodiphenyl compounds employing rational drug design to improve the therapeutic index of the compounds.

The data presented in the following manuscripts characterizes a novel binding motif between diaminodiphenyl compounds and NMDAr using computer based modeling techniques. A structure activity relationship was derived by examining the anticonvulsant effects of several different diaminodiphenyl compounds in animal models of epilepsy. Employing the computationally derived binding motif and structure activity relationship, several diaminodiphenyl derivatives have been designed in an effort to alleviate metabolic toxicity and improve anticonvulsive potency.

Rational drug design targeting the described diaminodiphenyl binding site could offer novel anticonvulsants which act through a novel mechanism on NMDAr. Antagonism of NMDAr is not limited to epilepsy alone, NMDAr has been implicated in a number of disease states and diaminodiphenyls could serve multiple indications such as: neuropathy, mood disorders and post stroke outcomes.

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PREFACE

This dissertation was prepared according to the University of Rhode Island standards for manuscript format. This dissertation is comprised of three manuscripts that have been assembled in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Rhode Island.

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INTRODUCTION

Epilepsy is a complex disorder characterized by chronic periods of overactivity of neurons in the cerebral cortex. A single seizure does not constitute the diagnosis of a seizure disorder, as seizures can be caused by a multitude of neurological illnesses. In order to be classified as epilepsy, there must be "recurrent unprovoked seizures".[1] The systematic firing of neurons in the cerebral cortex is the basis by which normal brain function and everyday life including speech, thought and movement occurs. A seizure is the result of the loss of normal signaling patterns and overactive signaling in cerebral neurons. However, not all seizures manifest clinically as a convulsive seizure which stereotypes associate with epilepsy. The type of seizure is directly correlated to the region of the brain affected by the neuronal overactivity. Convulsions are the result of the focal point of the neuronal overactivity overlapping the motor neurons in the cerebral cortex. A majority of seizures show no convulsive activity; in fact, epilepsy encompasses several different types of seizures which have little in common aside from overactive uncontrolled neuronal signaling in the cerebral cortex. The variability in clinical manifestation as well as physiological underpinnings of the different seizure types presents a challenge to doctors and neuroscientist in diagnosis, treatment, monitoring, and drug development.

Repeated seizures can have a devastating effect on the brain. Glutamate is the primary excitatory neurotransmitter in the brain. During seizure activity, the overexcitation of cerebral neurons floods synapses and intracellular space with glutamate. Glutamate is a primary ligand for NMDA, AMPA and kainite receptors, the ionotropic glutamate

receptors in the central nervous system. Ionotropic receptors are responsible for the flow of cations across the neuronal membrane, there by directly contributing to excitatory post synaptic membrane potential and further seizure propagation. Overexcitiation of neurons leading to the release of excess glutamate is also directly responsible for excitotoxicity, a process by which neurons are excited to death leading to brain damage and neurodegeneration.[2,3] Epilepsy is estimated to affect almost 70 million people worldwide[1,2] and has a staggering economic cost. For example, the annual economic cost to the United State in 1995 for 2.3 million cases then prevalent was estimated to be $12.5 billion and the life-time cost for the estimated 181,000 cases with an onset in that year was estimated to be $11.1 billion.[6] In addition, there are tragic non-economic costs to patients and their families.

Since 1989, about 15 new anti-epileptic drugs (AEDs) have become available in the clinic, providing patients and physicians with a number of treatment options.[7,8] Nevertheless, a number of literature reviews of clinical studies have concluded that, even with the availability of these new AEDs, the proportion of patients who are seizure free after drug therapy amounts to only about 30%.[8] In addition, the adverse effects of AEDs can be serious enough to negatively affecting a patient's quality of life, leading to issues surrounding patient compliance with the medication dosing regimen.[9] These side-effects include dizziness, drowsiness, mental slowing, weight gain, metabolic acidosis, nephrolithiasis, glaucoma, skin rash as well as movement and behavioral disorders, among many others.[9,10] The problems posed by the side-effects are compounded by the requirement for multiple-drug therapy for adequate seizure control in many patients.[11]

There remains a pressing need for the development additional AEDs with novel mechanism of action that can provide patients and physicians with alternative treatment options. Furthermore, it is clear that numerous and diverse underlying pathophysiological mechanisms contribute to the epileptic syndrome. Therefore, access to a spectrum of AEDs, with different modes of action, might facilitate the optimization of drug therapy to suit the pharmacological and toxicological profiles of individual patients so as to improve tolerability and long-term treatment success. The global AED market is a multibillion-dollar enterprise that is still growing and the market for pharmaceutical treatment of epilepsy generated $12 billion in 2008.[12] Given this background, the development of an AED with a novel mechanism of action is likely to be economically successful if it can show superior efficacy, even in a subset of patients. While conventional antiepileptic drugs have acted by blocking sodium channels or enhancing the function of γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain.[11] The newer drugs have more novel actions including binding to presynaptic vesicle proteins and calcium channel subunits.

N-methyl-D-aspartate receptors (NMDARs) are important excitatory receptors and play a key role in the pathophysiology of several neurological diseases, including epilepsy, stroke, traumatic brain injury, dementia and schizophrenia which make it an ideal candidate as an anticonvulsant drug target.[13] The NMDAR is a hetero-tetrameric cation channel formed as a complex between two NR1 and two NR2 subunits.[14] Multiple variants of NR1 and NR2 exist and can give rise to multiple NMDAR isoforms with distinct brain distributions and functional properties. They are activated by the neurotransmitter glutamate and the co-agonist glycine. NMDARs have a complex

structure with regulatory binding sites for multiple agents including other cellular proteins, Mg^{2+}, and polyamines.

There is evidence to suggest that conventional antiepileptic drugs may inhibit NMDAR function and that NMDAR antagonists could have unique utility in epilepsy pharmacotherapy.[15] NMDA receptor antagonists have been used as anesthetics and in Alzheimer's but many of these ligands have poor efficacy or have failed clinical trials due to narrow therapeutic indices and adverse effects. However, there is a large body of evidence to suggest that ligands that more subtly modulate NMDARs hold tremendous promise as therapeutic agents for multiple disorders including epilepsy, neuropathic pain, substance abuse, and bipolar disorder and schizophrenia.[13,14,15]

Using rodent seizure models we have identified diaminodiphenyl (DADP) analogs as novel pharmacophores with notable antiepileptic effects following systemic or oral administration and these findings have been published.[17] Based on our observations, we have performed *in silico* modeling, *in-vivo* structure–activity relationship (SAR) studies, and have observed functional modulation of mammalian NMDAR functionally expressed in *Xenopus* oocytes, all of which have implicated NMDAR as a key molecular target in the actions of these compounds.

Previous studies have suggested that other DADP-like compounds may modulate NMDAR function[18] which suggested a potential mechanism of action for the anticonvulsant activity of the DADP analog, thiodianiline (TDA). Using NMDAR NR2a crystal structure coordinates, 2A5S, from the Protein Data Base[19] potential binding sites were evaluated. The results of these studies revealed a large cavity into which DADP compounds repeatedly bound with high affinity and with little variation between

compounds (Fig 1). Furthermore, the model also discriminated between active and inactive compounds as defined by previous *in-vivo* studies.

To investigate whether the interaction between NMDA receptors and TDA predicted by in the *in silico* modeling studies could result in functional modulation of NMDAR activity, we functionally expressed NMDAR in *Xenopus* oocytes by co-injecting cRNA for the most ubiquitously expressed NMDA channel subunits NR1A and NR2B. 4-5 days after cRNA injection, electrophysiological recordings using the two electrode voltage clamp configuration were used to characterize NMDAR responses. Oocytes were clamped at -70 mV and perfused with 1 μM glycine and 50 μM glutamate which resulted robust positive inward currents only in oocytes injected with both channel subunits. Co-application of TDA with glutamate inhibited the glutamate-elicited NMDAR conductance (Fig 2). NMDAR inhibition was readily reversible upon TDA wash out.

Rational drug design may be an effective approach for producing potent targeted drug therapies and alleviate know toxic mechanisms. In order to transition from inefficient high throughput screening protocol to a systematic rational drug design protocol a well defined binding pocket, key amino acids and binding interactions must be identified. By combining a full range of methods, including flexible side chain models and homology modeling, with the established electrophysiology based techniques, it is hypothesized that the binding pocket and key interactions between DADPs and NMDAR can be elucidated.

NMDAR is a complex receptor whose dysfunction in noted in a variety of disorders. This study proposes to characterize a novel binding site on NMDAR which may prove to be a viable druggable target. The resulting data could be used to develop new lead

compounds and provide viable treatment options for epilepsy and a variety of other glutamate related disorders.

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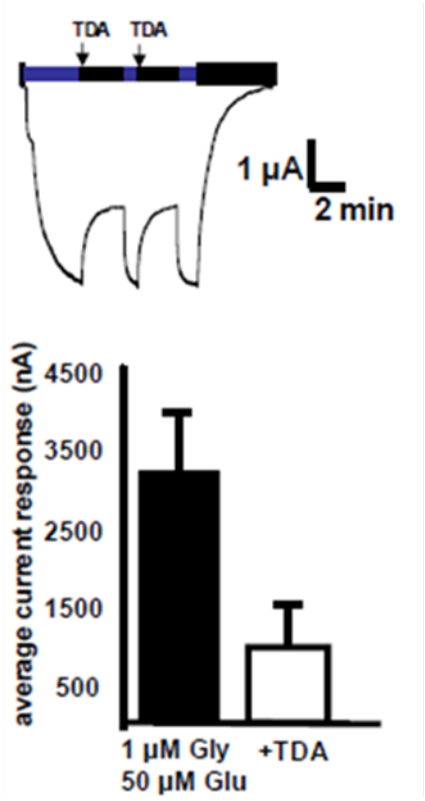
Figure 1. Crystaline structure of NMDAr NR2 as reported by Furukawa, et al. with diaminodiphenyl compounds overlayed in the proposed diaminodiphenyl binding motif.

Figure 2. NMDAr inhibition assay, in which diaminodiphenyl sulfide is capable of antagonizing mammalian NMDAr expressed in *Xenopus* oocyte reversibily in the presence of the agonists glycine and glutamate.



MANUSCRIPT 1

IN SILICO CHARACTERIZATION OF DIAMINODIPHENYL INTERACTIONS WITH THE *N*-METHYL D-ASPARTATE RECEPTOR SUBUNIT NR2A

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Abstract

The *N*-methyl D-Aspartate receptor (NMDAr), a non-selective ion channel, has been shown to interact with and be modulated by polyamines. Diaminodiphenyls have been reported to elicit anticonvulsive effects by interacting with NMDAr. However, many diaminodiphenyls have been reported to be toxic. *in silico* modeling of diaminodiphenyls and their derivatives using mammalian NMDAr NR2a subunit crystal, 2A5S, has provided evidence that diaminodiphenyls bind with favorable binding energy and may modulate glutamate activation of NMDAr. The exact mechanism by which diaminodiphenyls and their derivatives modulate NMDAr activity still needs to be elucidated.

Introduction

The *N*-Methyl D-aspartate receptor (NMDAr), a ligand gated non-specific cation channel, is essential to neuronal plasticity, chronic pain, and may also be involved in seizure development [1]. This tetrameric ion channel is composed of a dimer of dimers made up of subunits NR1a-h, NR2a-d and NR3a-b [2]. Subunit NR2 has been shown to interact with and be activated by glutamate. The agonist binding site has been studied and characterized at length. However, another class of molecules, polyamines, has been reported to interact with NR2.

The interaction of NMDAr subunit NR2a-b with polyamines has been extensively reported, however, there is little agreement on the binding interaction, effects or site of interaction. According to a review by Mony, *et al., the* response of NMDAr to polyamine

interaction is highly voltage dependant and has been characterized by three distinct mechanisms; which include: increased affinity for glycine resulting in more rapid receptor reset and decreased affinity for glutamate resulting in decreased NMDAr activation [4,5]. Most literature states that polyamine interactions with NMDAr occur at an allosteric binding site eliciting the noted effects. The endogenous polyamine spermine has been characterized in *Xenopus* oocytes using patch clamp analysis to monitor NMDAr activation. The results of these studies suggest that spermine potentiates agonist activation of NMDAr [3]. In contrast, it is proposed that treatment of rats with diaminodiphenyl compounds significantly reduced seizure in both MES and hippocampal kindling models *in vivo* by modulating through an interation with NMDAr [6].

Diaminodiphenyl sulfide has been shown as a potent anticonvulsant. However, the potential toxicity of any aromatic amine must always be addressed before a compound could be investigated as an investigational API. Based on the toxic mechanism of aniline it can be assumed that N-oxidation will lead to the production of a key toxic metabolite which can be further metabolized leading to blood toxicity and DNA adduct formation [7] (Fig 1). In order to reduce the proposed toxic effects of diaminodiphenyl sulfide, rational drug design must be used to eliminate the potential for the formation of the key hydroxyl amine metabolite.

Methods

In Silico Ligands

Ligands were created using ChemBio3D Ultra 12. All ligands were drawn using program defaults, and then the energy of each ligand was minimized using MM2 energy

minimization function before being saved in a format suitable for docking in AutoDock4.2. (Fig 2).

In Silico NMDA Model

Several crystal structures of NMDAr have been reported in the Protein Data Bank (PDB).

Selection of the crystal structure 2A5S, deposited by Furukawa, *et al. 2005,* from PDB was based on the energy scores and bond angle analysis provided by PDB.org. The structure is composed of 284 amino acids isolated from rat and represents the core ligand binding domain of NMDAr NR2a. The structure was co-crystallized with glutamate and determined to a resolution of 1.7 angstrom using X-ray diffraction.

Using Accelrys Discovery Studio 3.1 all amino acid and charge corrections were completed before the removal of the ligand and water molecules. The file was then converted to MOL2, a suitable format for use in AutoDock 4.2. Further analysis of the protein structure, using Discovery Studio 3.1, included determination of the ligand binding site location. A cavity (Fig 3) was characterized and used to define the coordinates of the grid box used in future docking experiments.

In Silico Docking to NMDA NR2a

Grid Box Selection

The grid box was initially centered on the ligand glutamate and further refined, using the coordinates determined previously in Discovery Studio 3.1, in order to encompass the cavity of the protein in which glutamate binding was characterized.

The grid was centered at (21.5, 21.4, 36.1) with dimensions of:

Grid map x-dimension : 22.5 Angstroms

Grid map y-dimension : 22.5 Angstroms

Grid map z-dimension : 24.0 Angstroms

The gridbox was overlaid on the water-free ligand-free structure 2A5S produced in Discovery Studio 3.1 and processed using default AutoGrid parameters in AutoDockTools 4.2.

AutoDock Parameters

AutoDock parameters were held constant when docking each ligand with the prepared NMDAr NR2a structure 2A5s. Each ligand was docked starting from a computer generated seed site to the protein within the gridbox previously described. Using default Lamarckian Algorithm settings set to default short run in AutoDock 4, the energy was minimized. Optimum binding energy and ligand conformation was then determined and recorded by AutoDock. (Table 1) This process was repeated 256 times for each ligand and the data was compiled. The conformations are then grouped based on similar location and orientation with a 2 angstrom deviation to the mean of the population as the grouping criteria.

Results and Discussion

Diaminodiphenyl compounds appear to bind to NMDAr NR2A subunit in a specific orientation, while hydrogen bonding with THR174 which may contribute to the reported NMDAr effects in epilepsy.(Fig 4) The ligands evaluated addressed several key variables which may affect the interactions between ligand and NMDAr; they were: linker element,

linker substitution, linker length, *N*-substitution, aromatic substitution and aromaticity. The diaminodiphenyl derivatives evaluated are rationally designed to prevent the toxic metabolism of the diaminodiphenyls. These derivatives examine the effects of new functional groups and availability of H-bond donors/acceptors on NMDAr interaction.

In an effort to elucidate the binding interactions of diaminodiphenyl compounds with NMDAr the crystal structure of the NR2A subunit of NMDA was examined and the agonist, glutamate, binding site was identified and the binding cavity was characterized. (Fig 3, 5) The binding cavity is rich with hydrogen bond donors and acceptors and also possesses the ability to interact heavily by Pi bonding interactions. Unsusbstituted diaminodiphenyl compounds: 4,4'-diaminodiphenyl methane, 4,4'-diaminodiphenyl sulfide and 4,4'-oxydianiline, were docked into the defined grid box and a consistant binding motif was identified. The binding energies of the compounds were: -7.45 cal/mole, -7.63 kcal/mole, and -7.00 kcal/mole respectively. The compounds bound consistently in near identical conformations resulting in strong Pi-cation interactions, as well as, hydrogen bond interactions with THR 174. (Fig 6) The element which linked the diaminodiphenyl compound did not signifigantly affect the binding the binding energy.

In order to address the effect of linker length on diaminodiphenyl interaction with NMDAr 4,4'-ethylenedianiline was examined.(Fig 7) The favorable binding energy of -8.08 kcal/mole can be attributed to the hydrogen bonding at THR 174, as well as, THR 116 and ASP215. The binding orientation of 4,4'-ethylenedianiline was consistant with the binding motif of other diaminodiphenyl compounds. Without the flexibility and the bond angles offered by the ethylene linker these additional hydrogen bond interactions would not be possible. The orientation of the ligand in the binding pocket along with the

hydrogen bond to THR174 and favorable binding energy indicates that the longer more flexible linker may be a more active antiepileptic compound than those diaminodiphenyls with a shorter linker.

With linker element and length addressed the last linker variable to be examined was linker substitution. Diaminobenzophenone, dapsone and 4,4'-(hexafluoroisopropylidene)dianiline were modelled to address whether linker substitution would alter the diaminodiphenyl affinity for NMDAr. Both diaminobenzophenone and dapsone bind with favorable binding energy, -7.80 kcal/mole and -8.32 kcal/mole respectively, and orientation. (Fig 8) Both compounds hydrogen bond to THR 174. However, with bulky linker substitution, as in the case of 4,4'-(hexafluoroisopropylidene)dianiline the binding energy is far less favorable, -4.05 kcal/mole, and the ligand does not interact at the predicted binding site. (Fig 9) When considering the shape and size of the binding pocket, it is likely that 4,4'-(hexafluoroisopropylidene)dianiline is too bulky to reach the binding site, as the cavity narrows at several locations. Linker substitution does not appear to affect ligand binding until the size of the ligand becomes prohibitive, preventing the ligand from reaching the binding site on the NR2 subunit of NMDAr.

N-substitution resulting in secondary and tertiary amines does not appear to significantly affect the binding energy of the diaminophenyl compounds.(Fig 10) The *N,N'*-diacetyl derivative of 4,4'-diaminodiphenyl sulfide displayed a more favorable binding energy, -9.32 kcal/mole, than the primary diaminodiphenyl compunds. This binding energy is easily explained by the introduction of two hydrogen bond acceptors on the acetyl groups, the ability of the secondary amine to donate hydrogen bonds and a

deviation from the previously noted binding orientation that was consistant with the primary diaminodiphenyls. 4,4'-methylene Bis(*N,N*-dimethylaniline) and the The *N,N'*-pentacyclo derivative of 4,4'-diaminodiphenyl sulfide, are tertiary amines and lack the ability to donate or receive hydrogen bonds. The binding orientation of 4,4'-methylene Bis(N,N-dimethylaniline) is consistant with that of the primary diaminodiphenyl compounds and the binding energy is -7.35 kcal/mole. The N,N'-pentacyclo derivative of 4,4'-diaminodiphenyl sulfide has a favorable binding energy, -8.13 kcal/mole, but does not bind in an orientation consistant with other diaminodiphenyl compounds. The pi stacking of the aromatic functional groups of both compounds is enough to present the compound as a favorable binding lingand. However, if hydrogen bonding is essential for diaminodiphenyls to elicit their antiepileptic properties, it could be assumed that 4,4'-methylene Bis(*N,N*-dimethylaniline) and the *N,N'*-pentacyclo derivative of 4,4'-diaminodiphenyl sulfide would not be active in a biologically relevant model. Further examination of tertiary diaminodiphenyl compounds any conclusions could be drawn regarding compound value as a ligand for NMDAr.

Several compounds examined address the effects of aromaticity and aromatic substitution on ligand binding to NMDAr. 4,4'-methylene Bis(cyclohexyl amine) lacks the aromatic rigidity and Pi interaction potential present in all of the other ligands examined. The flexibility allows for more potential binding configurations, which is contrasted by the inability to form many of the pi interactions displayed diaminodiphenyl interactions with NMDAr. While the binding energy, -7.49 kcal/mole, and apperant binding orientation of 4,4'-methylene Bis(cyclohexyl amine) appear to be similar to that of the diaminodiphenyl compounds, the interactions responsible differ.(Fig 11) Pi-alkyl

interactions and hydrogen bonding are the major forces behind 4,4'-methylene Bis(cyclohexyl amine) binding. However, there is not conformation in which a hydrogen bond interaction takes place with THR 174 as noted in diaminodiphenyl interactions with NMDAr. Both halide and alkyl aromatic substitutions on diaminodiphenyl compounds were examined. Both alkyl, 4,4'-methylene Bis(2,6-dimethylaniline), and halide, 4,4'-methylene Bis(chloroaniline), substitution resulted in favorable binding energies, -8.28 kcal/mole and -8.58 kcal/mole respectively. Both compounds showed hydrogen bonding interactions with THR 174. The only deviation from the binding motif displayed in primary unsubstituted diaminodiphenyls is the orientation of the ligand within the binding pocket.(Fig 12) Both substituted compounds presented two favorable binding conformations within the binding pocket. Both conformations resulted in favorable binding energy and hydrogen bonding interactions with THR 174. The data collected suggests that aromatic substitution of diaminodiphenyls would have little effect on their ability to interact with NMDAr and elicit an antiepileptic effect similar to that of primary unsubstituted diaminodiphenyls.

Thiodi-indole and ethylenedi-indole were rationally designed in order to prevent N-hydroy metabolite formation while maintaining hydrogen bond donors at the amines. The fully aromatic structure of the indole provides increased pi stacking which would improve ligand binding affinity. When evaluated the binding energy of both thiodiindole, -10.42 kcal/mole and ethylenediindole, -10.88 kcal/mole, are more favorable than the diaminodiphenyl compounds evaluated. Both indole derivatives bound in the same orientation as the parent diaminodiphenyl compounds and presented hydrogen bond

interactions with THR 174.(Fig 13,14) Indole derivatization of diaminodiphenyl compounds may be valuable leads and potent antiepileptic compounds.

The computational inhibition constants for those diaminodiphenyls which bound in a favorable orientation and hydrogen bonded to THR 174 were all similar and ranged 7.38 μM to 517.15nM. (Table 2) Few structure activity relationship conclusions can be drawn based solely on the computational inhibition constants. However, the improvement of computational inhibition constant when diaminodiphenyl compounds are derivatized to indole compounds was signifigant. Both compounds saw an improvement of ~100x in computational inhibition constant. 4,4'-diaminodiphenyl sulfide has an inhibition constant of 2.54 μM. When derivatized to thiodiindole, the inhibition constant is computed to 23.01 nM. 4,4'-ethylenedianiline has an inhibition constant of 1.19 μM, when derivatized to ethylenediindole the inhibition constant is calculated to be 10.54 nM.

The *in silico* model of diaminodiphenyl compounds interactions with NMDAr provides evidence that the antiepileptic effect of diaminodiphenyls may be elicited through an interaction with the NR2A subunit of NMDA in a cavity which also contains the agonist, glutamate, binding site. The activity of diaminodiphenyl compounds may be mediated through a hydrogen bonding interaction with THR 174 within the binding pocket. The conclusions drawn in this study is a foundation upon *in vitro* or *in vivo* studies could be built.

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TABLE 1. Ligand binding energy and root mean squared deviation of the ligand root from a reference point in space within the crystalline structure of NMDAr NR2a subunit. Calculated in AutoDock

Ligand	**Binding Energy** (Kcal/mole)	**RMS**
4,4'-methylene Bis(chloroaniline)	-8.58	41.34
Dapsone	-8.32	41.87
4,4'-methylene Bis(2,6-dimethylaniline)	-8.28	42.29
4,4'-ethylenedianiline	-8.08	43.23
Diaminobenzophenone	-7.80	41.96
4,4'-diaminodiphenyl sulfide	-7.63	43.42
4,4'-methylene Bis(cyclohexyl amine)	-7.49	44.22
Diaminodiphenyl methane	-7.45	41.90
4,4'-methylene Bis(*N,N*-dimethylaniline)	-7.35	39.50
4,4'-Oxydianiline	-7.00	42.41
4,4'-(hexafluoroisopropylidene)dianiline	-4.05	38.45
Ethylenediindole	-10.88	44.58
Thiodiindole	-10.42	43.87
N,N'-diacetylthiodianiline	-9.32	43.47

TABLE 2. Computational inhibition constant based on ligand interaction with NMDAr NR2a subunit. Calculated in AutoDock.

Ligand	Inhibition Constant
4,4'-methylene Bis(chloroaniline)	517.15nM
Dapsone	793.94nM
4,4'-methylene Bis(2,6-dimethylaniline)	845.46nM
4,4'-ethylenedianiline	1.19uM
Diaminobenzophenone	1.92uM
4,4'-diaminodiphenyl sulfide	2.54uM
4,4'-methylene Bis(cyclohexyl amine)	3.25uM
Diaminodiphenyl methane	3.43uM
4,4'-methylene Bis(*N,N*-dimethylaniline)	4.12uM
4,4'-Oxydianiline	7.38uM
4,4'-(hexafluoroisopropylidene)dianiline	1.07mM
Ethylenediindole	10.54nM
Thiodiindole	23.01nM
N,N'-diacetylthiodianiline	146.95nM

FIGURE 1. Proposed metabolic pathway of 4,4'-diaminodiphenyl sulfide, derived based on the metabolism of aniline. *N*-hydroxy formation results in toxicity which includes methemoglobinemia and carcinogenisis.

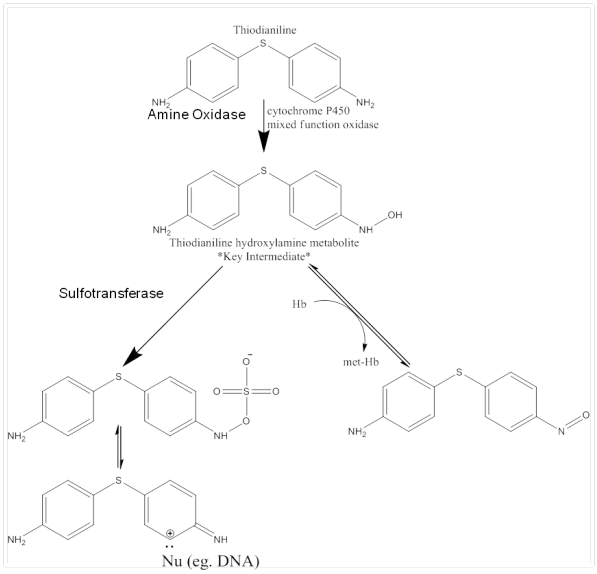


Figure 2. Diaminodiphenyl compounds

A. 4,4'-diaminodiphenyl methane **B.** 4,4'-oxydianiline **C.** 4,4'-diaminodiphenyl sulfide **D.** 4,4'-methylene Bis(2,6-dimethylaniline) **E.** 4,4'-methylene Bis(chloroaniline) **F.** 4,4'-methylene Bis(*N*,*N*-dimethylaniline) **G.** diaminobenzophenone **H.** 4,4'-ethylenedianiline **I.** 4,4'-methylene Bis(cyclohexylamine) **J.** 4,4'-(hexafluoroisopropylidene) dianiline **K.** *N*,*N'*-diacetyl thiodianiline **L.** thiodiindole **M.** ethylenediindole **N.** dapsone **O.** *N*,*N'*-pentacyclo thiodianiline

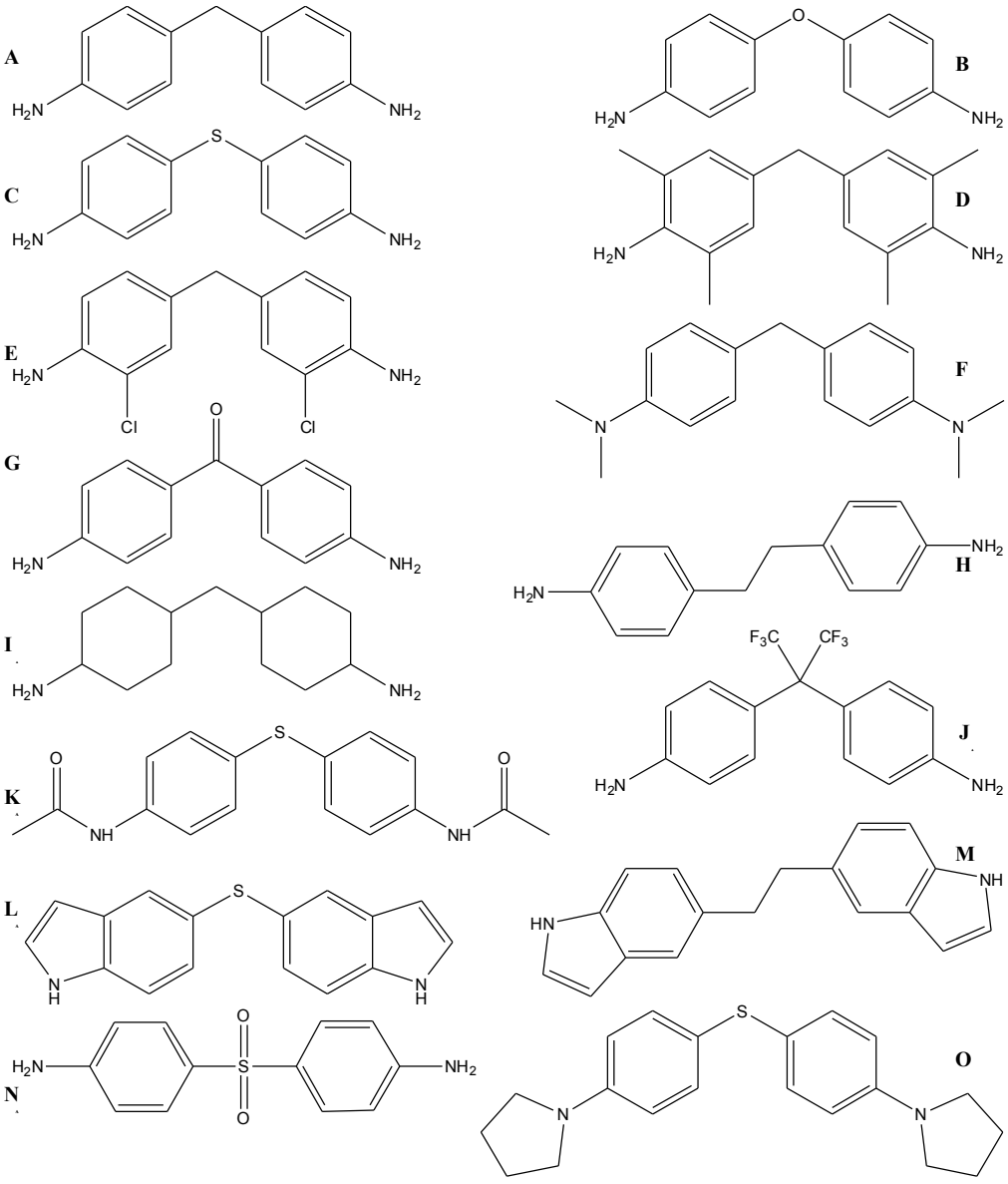


Figure 3. Hydrogen bonding characteristics of the NMDAr NR2a subunit binding cavity into which diaminodiphenyl compounds have been bound.

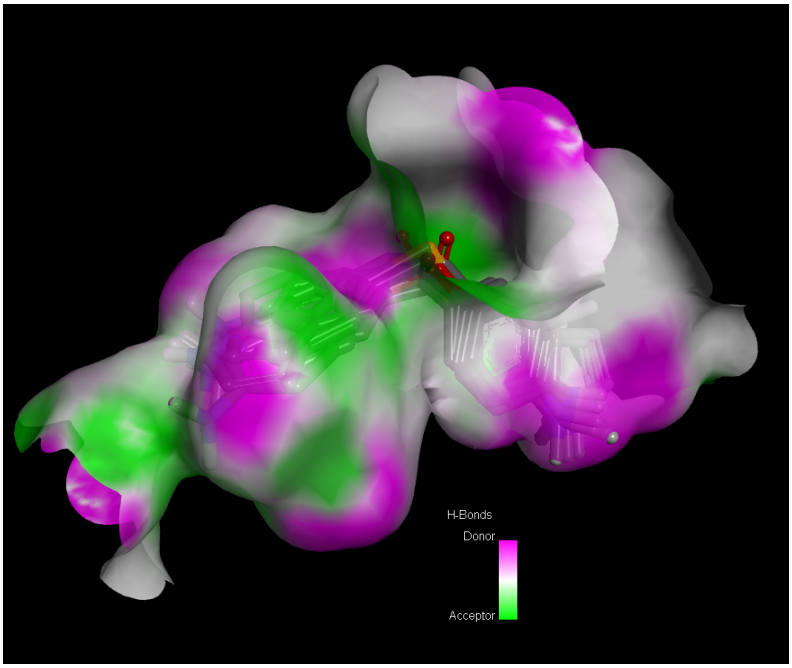
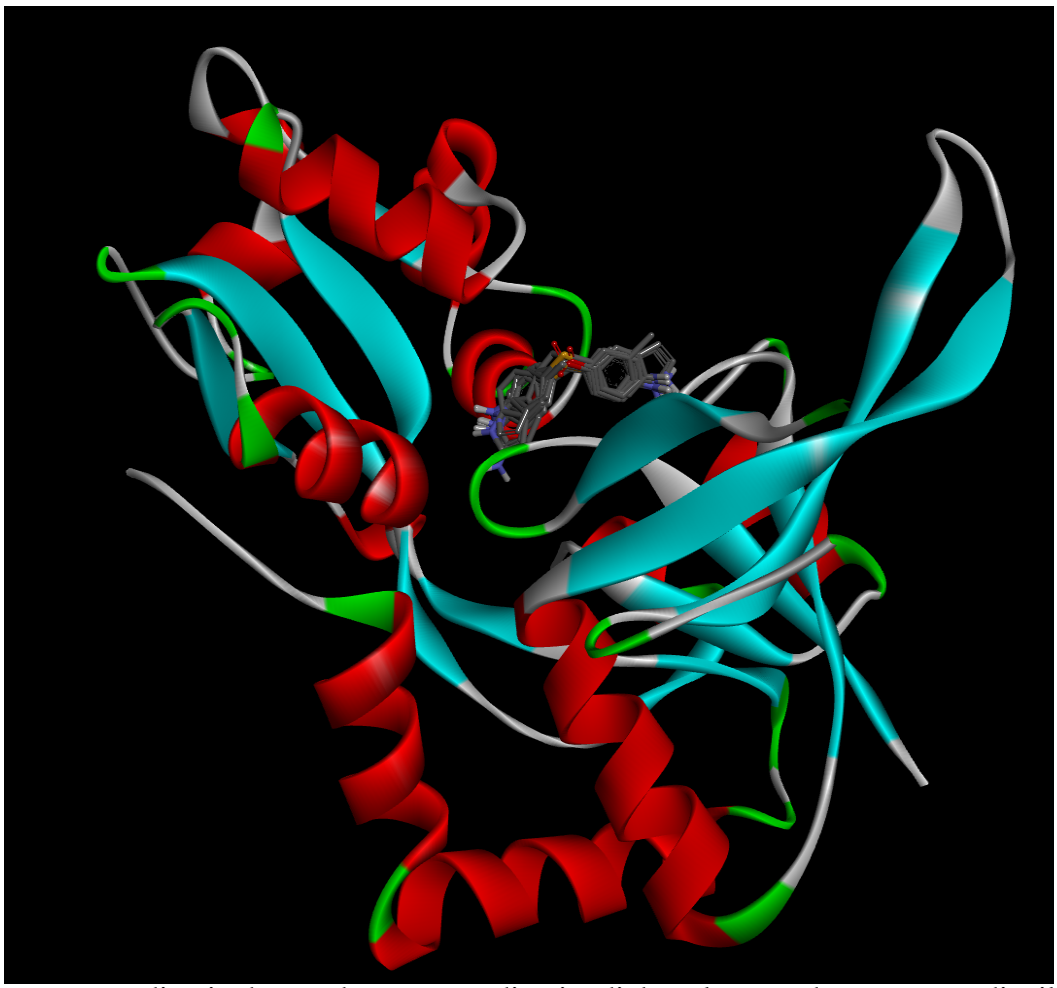


Figure 4. Overlay of diaminodiphenyl compounds and derivatives within the proposed binding pocket of NMDAr NR2a.



[Dapsone, diaminobenzophenone, diaminodiphenyl methane, oxydianiline, diaminodiphenyl sulfide, ethylenedianiline, ethylenediindole, thiodiindole, methylene Bis(chloroaniline), methylene Bis(2,6-dimethylaniline)]

Figure 5. Aromatic binding characteristics of the NMDAr NR2a subunit binding cavity into which diaminodiphenyl compounds have been bound.

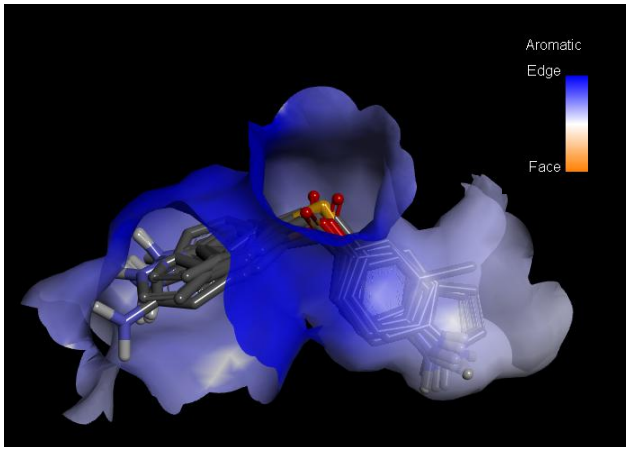


Figure 6. Binding interactions between diaminodiphenyl compounds with single element linkers and NMDAr NR2a subunit. Hydrogen bonds in green and red, Pi interactions in pink, Pi-sulfer interactions in yellow. All distances displayed in angstroms.

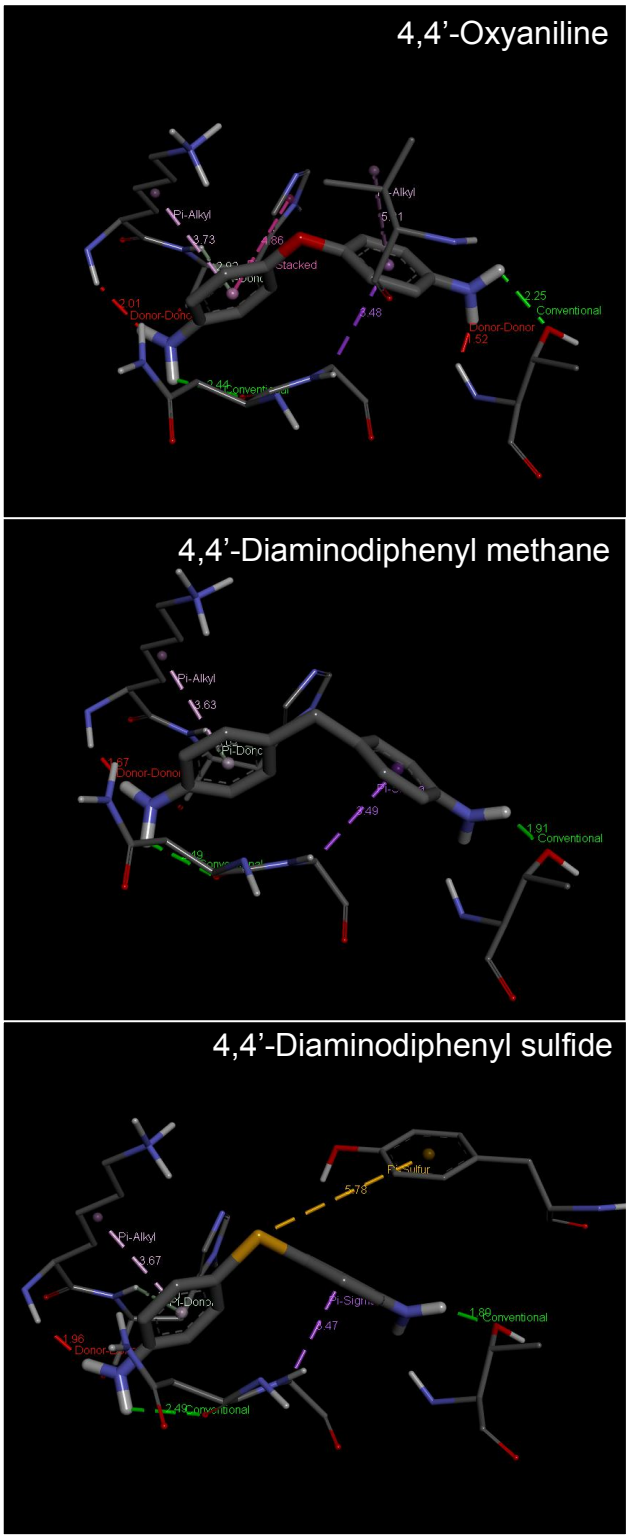


Figure 7. Interactions between 4,4’-ethylenedianiline and NMDAr NR2a subunit, examining the effects of linker length on diaminodiphenyl binding with NMDAr NR2a sununit. Hydrogen bonds in green and red, Pi interactions in pink. All distances displayed in angstroms.

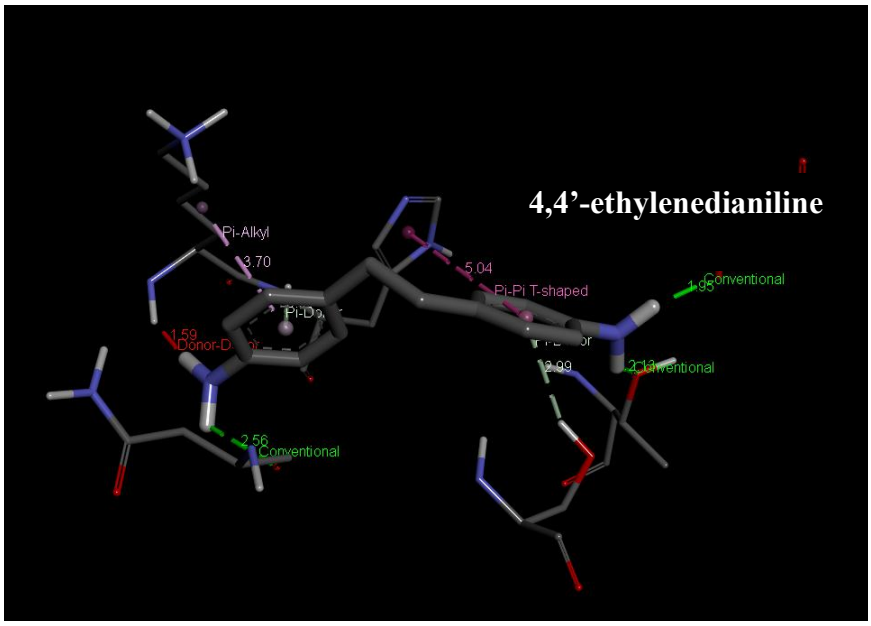


Figure 8. Interactions between diaminodiphenyl compounds with substituted linkers and NMDAr NR2a subunit, examining the effects of linker substitution on diaminodiphenyl binding with NMDAr NR2a sununit. Hydrogen bonds in green and red, Pi interactions in pink, Pi-sulfer interactions in yellow. All distances displayed in angstroms.

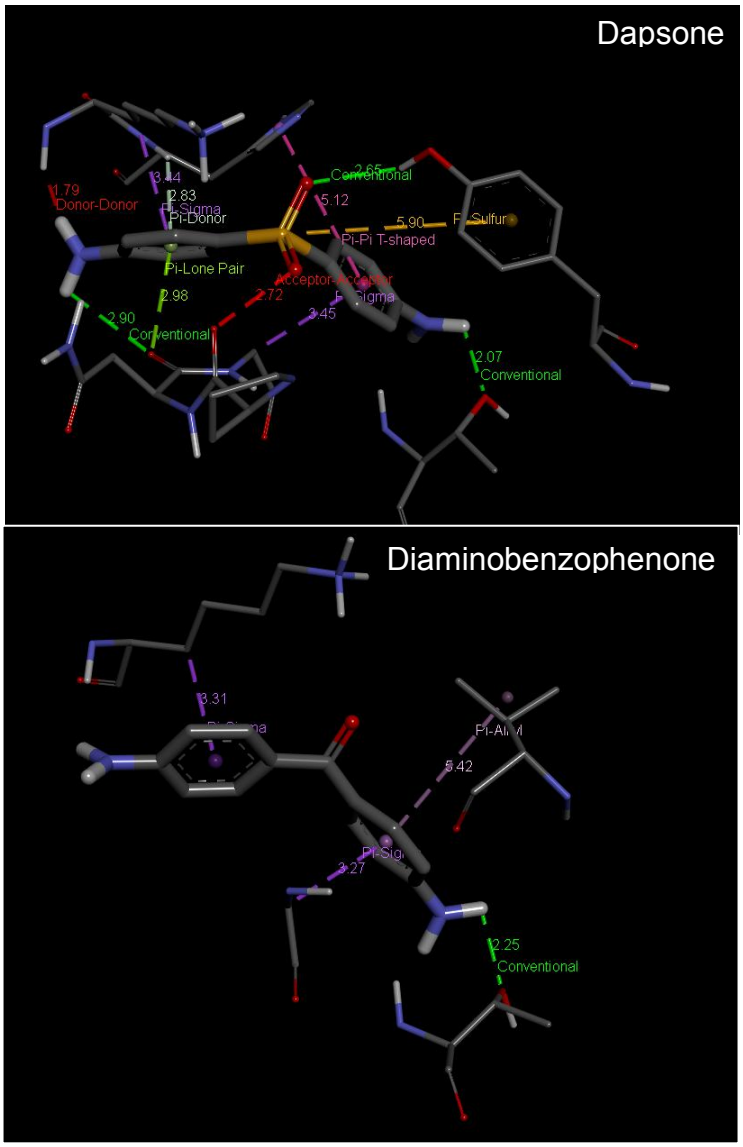


Figure 9. 4,4'-(hexafluoroisopropylidene)dianiline does not bind in the same binding cavity as the other diaminodiphenyl compounds examined. Both binding configurations differ in location significantly when compared to 4,4'-diaminodiphenyl methane, in yellow. It is likely that the bulk of the hexafluoroisopropylidene moiety is the limiting factor affecting the compounds ability to enter the binding cavity.

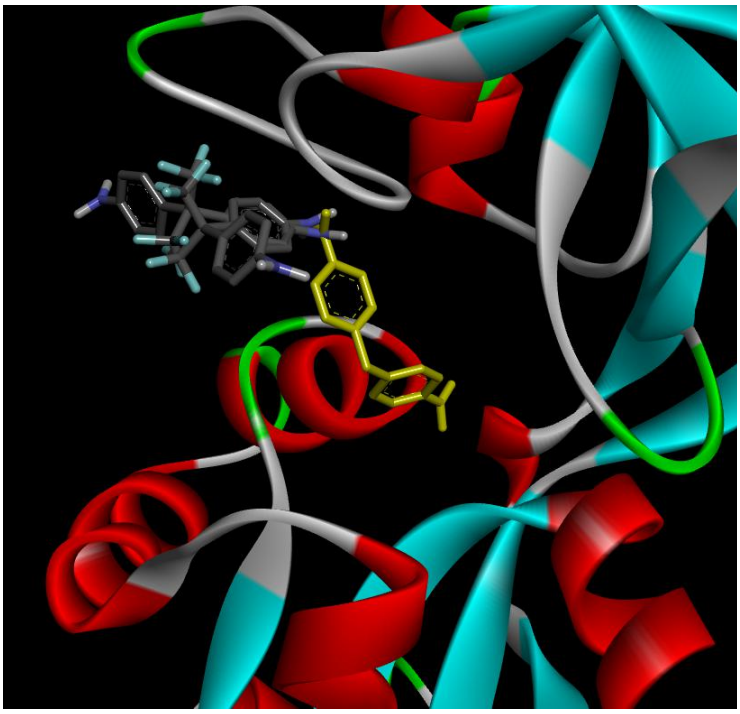


Figure 10. *N*-substituted diaminodiphenyl compounds lose the ability to hydrogen bond. The binding orientation of these compounds overlap with the binding orientations of unsubstituted diaminodiphenyls, but the interaction with THR 174 is lost. Expected binding orientation represented by 4,4'-diaminodiphenyl methane, in yellow.

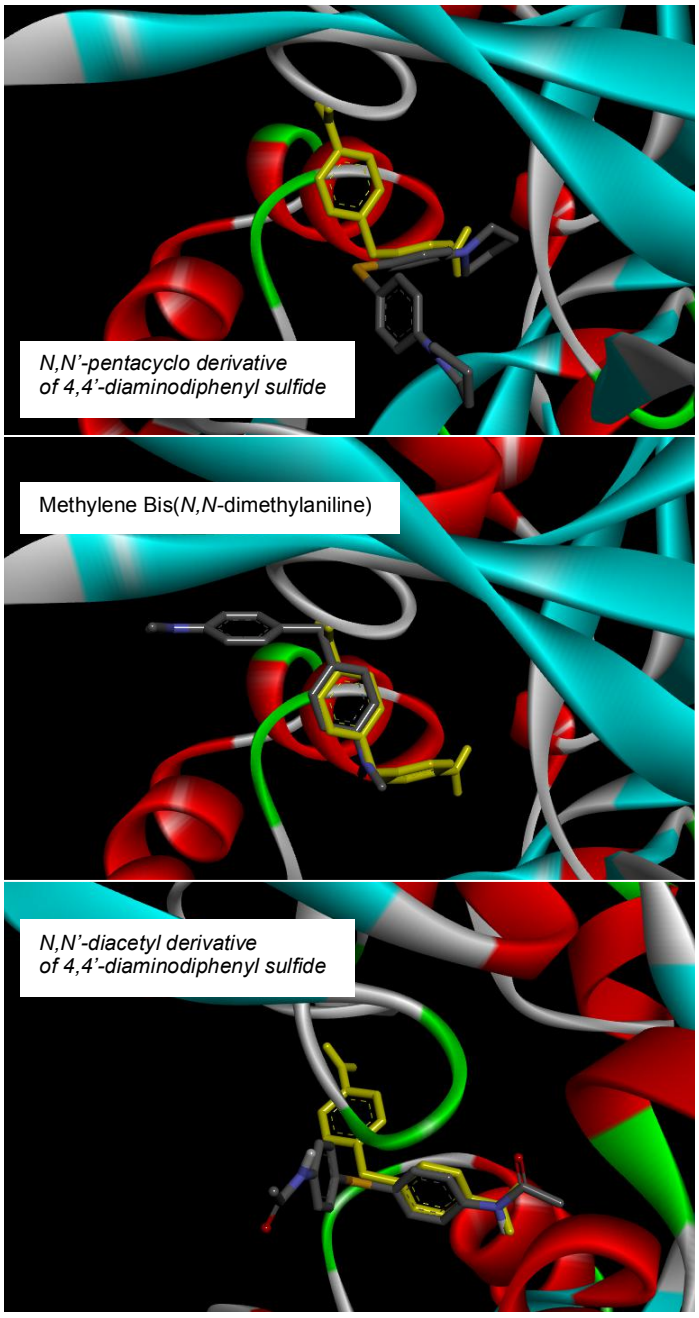


Figure 11. A. Binding orientation of 4,4'-methylene Bis(cyclohexyl amine) as compared to 4,4'-diaminodiphenyl methane, in yellow.
B. Binding interactions between 4,4'-methylene Bis(cyclohexyl amine) and NMDAr NR2a subunit.

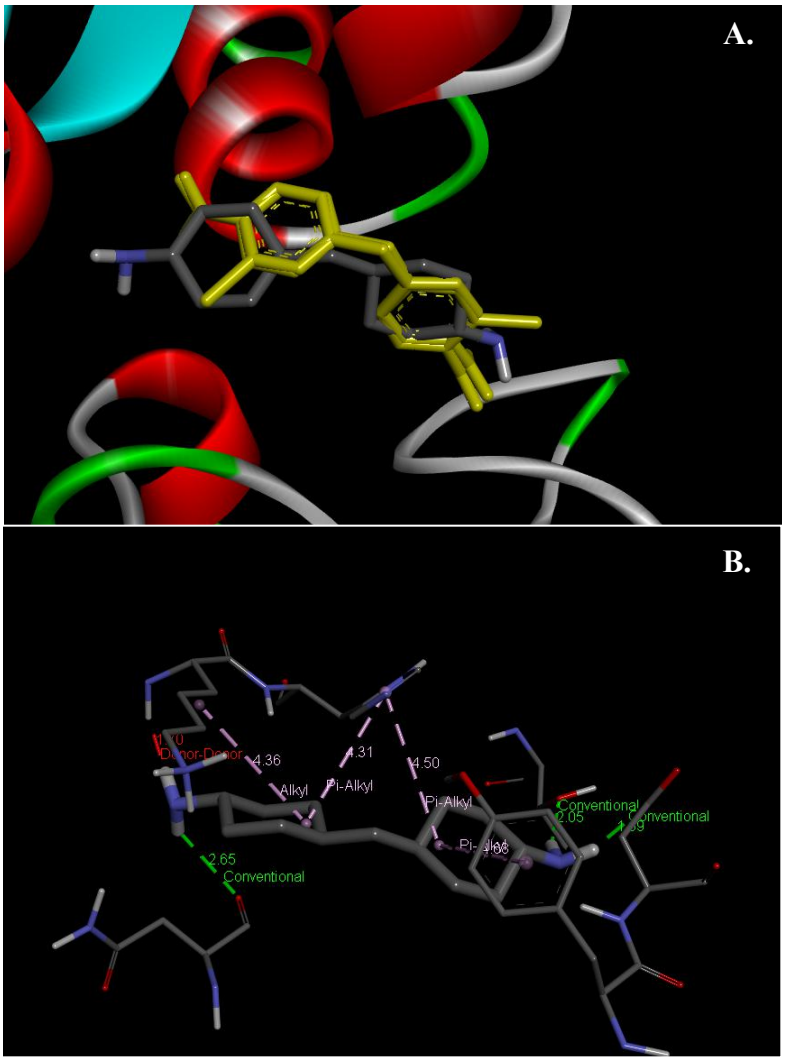


Figure 12. Armomatic substituted diaminodiphenyl compounds both displayed 2 binding conformations, both of which hydrogen bonded with THR 174. One major conformation for each compound overlapped the conformation of unsubstituted diaminodiphenyl compounds. Diaminodiphenyl methane in yellow.

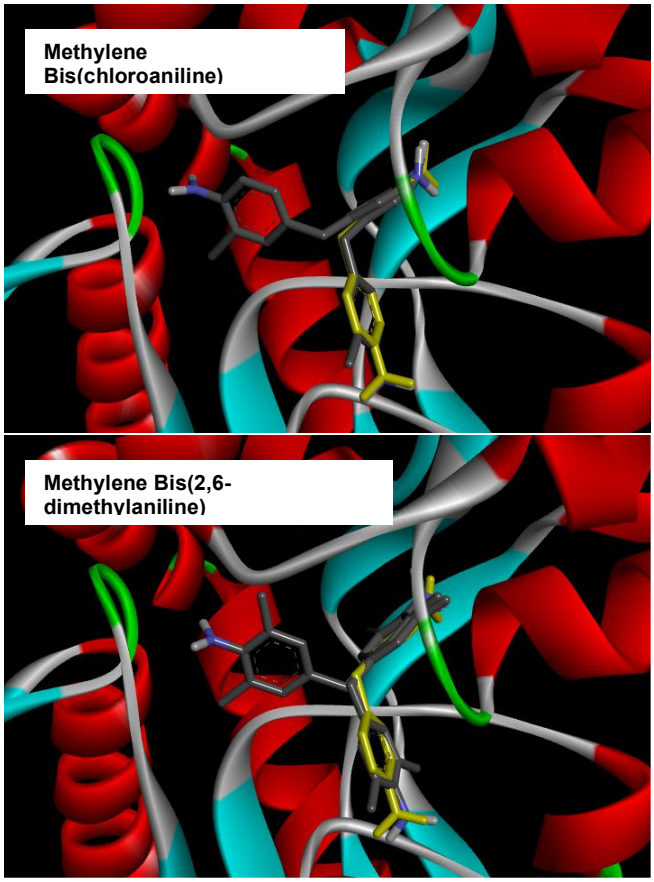


Figure 13. A. Ethylenediindole binding orientation compared to 4,4'-ethylenedianiline, in yellow. B. Thiodiindole binding orientation compared to 4,4'-diaminodiphenyl sulfide, in yellow.

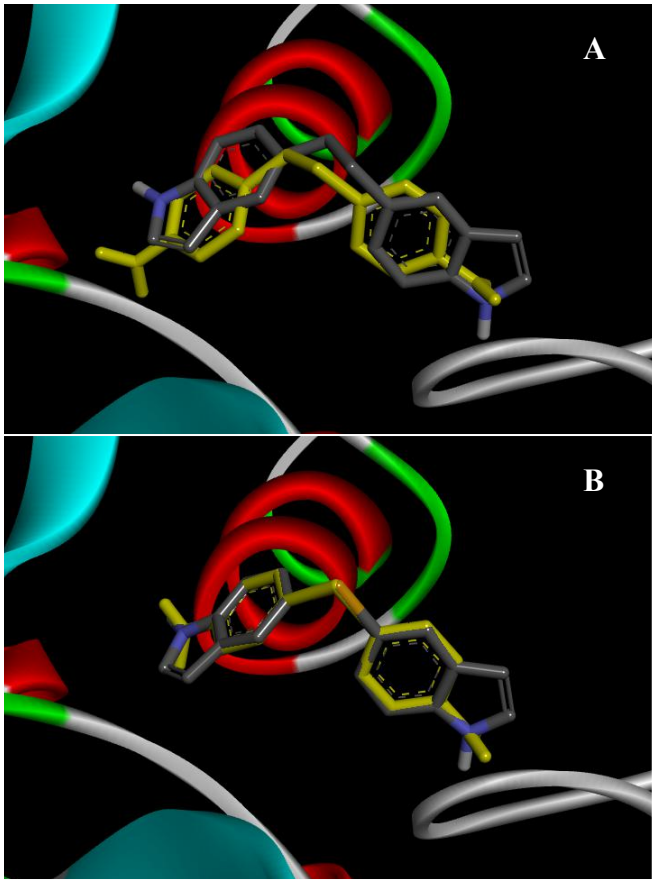
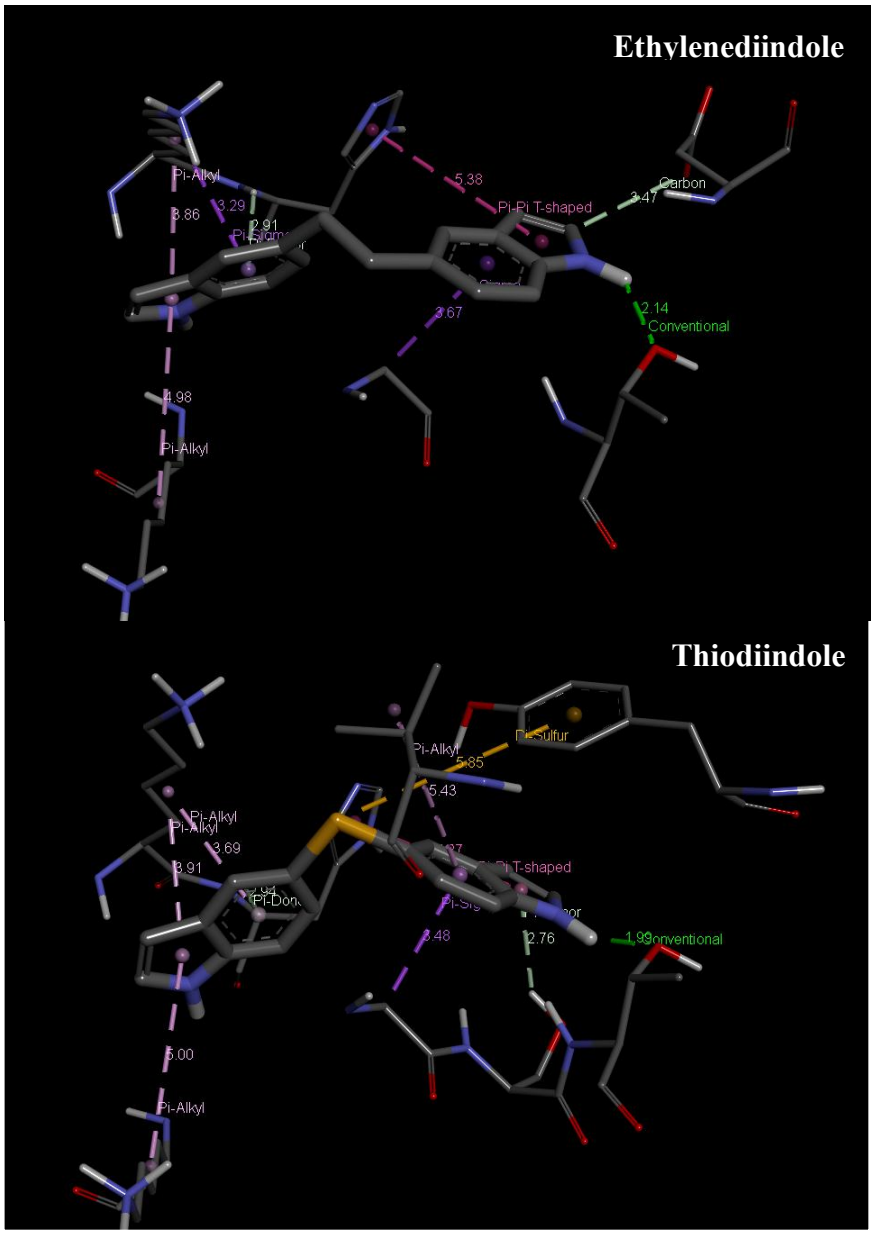


Figure 14.Binding interaction of indole derivatives of diaminodiphenyl compounds with NMDAr NR2a subunit



MANUSCRIPT 2

IN VIVO DETERMINATION OF STRUCTURE ACTIVITY RELATIONSHIP; EVALUATION OF DIAMINODIPHENYL COMPOUNDS IN ANIMAL MODELS OF EPILEPSY

In preparation: Bioorganic and Medicinal Chemistry Letters

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Epilepsy is a disorder characterized by the occurrence of seizures, which are periods of abnormally excessive synchronous neuronal activity in the brain. Epileptic disorders

have numerous etiologies, may occur at any age, typically require lifelong treatment, and may significantly limit patient activity and quality of life. These disorders can be severely debilitating, as seizures can manifest as major alterations in mental and physical states and may even be life-threatening. Epilepsy is estimated to affect almost 70 million people worldwide[1,2] and has a staggering economic cost. For example, the annual economic cost to the United State in 1995 for 2.3 million cases then prevalent was estimated to be $12.5 billion and the life-time cost for estimated 181,000 cases with onset in that year was estimated to be $11.1 billion.[3] In addition, there are tragic non-economic costs to patients and their families.

Since 1989 about 15 new anti-epileptic drugs (AEDs) have become available in the clinic providing patients and physicians with a number of treatment options [4,5] but some literature reviews of clinical studies have concluded that even with the availability of these new AEDs the proportion of patients who are seizure free after drug therapy has climbed to only about 30%.[5] In addition, adverse effects of AEDs can be serious enough to negatively affecting a patient's quality of life leading to issues surrounding patient compliance with the medication dosing regimen.[6] These side-effects include dizziness, drowsiness, mental slowing, weight gain, metabolic acidosis, nephrolithiasis, glaucoma, skin rash as well as movement and behavioral disorders among many others.[6,7] The problems posed by the side-effects are compounded by the requirement for multiple-drug therapy for adequate seizure control in many patients.[8]

Thus there is still a pressing need for the development additional AEDs with novel mechanism of action that can provide patients and physicians with alternative treatment options. Furthermore, it is clear that numerous and diverse underlying pathophysiological

mechanisms contribute to the epileptic syndrome. Therefore, access to a spectrum of AEDs, with different modes of action, will allow for optimizing therapy to suit the pharmacological and toxicological profiles of individual patients so as to improve tolerability and long-term treatment success. The global AED market is a multibillion-dollar enterprise that is still growing and the market for pharmaceutical treatment of epilepsy generated $12 billion in 2008.[9] Given this background, the development of an AED with a novel mechanism of action is likely to be economically successful if it can show superior efficacy even in a subset of patients.

Diaminodiphenyl compounds have been characterized as potent anticonvulsants with little notable acute toxicity.[10] Though the activity of diaminodiphenyl compounds has been described, little is known about the mechanism of action of these compounds. In order to better understand the mechanism by which diaminodiphenyl compounds exert their anticonvulsive properties, a series of diaminodiphenyl compounds were examined in animal models of epilepsy in an attempt to elucidate a structure activity relationship.

Diaminodiphenyl compounds were evaluated in both the maximal electroshock (MES) and 6Hz psychomotor seizure models in order to elucidate the anticonvulsive properties. The MES model determines a compounds ability to prevent seizure spread when neuronal circuits are active. Similar to the MES model, the 6Hz model examines a compounds ability to prevent electrically induced seizures, the major difference being the intensity of the electrical stimulation.

Animals

Species used in the animal studies were CF#1 mice and Sprague-Dawley rats. Animals were given free access to food and water when not under active evaluation. All animal care policies were adhered to as prescribed by the National Research Council and all studies were completed in the labs of the NINDS Anticonvulsant Screening Program.

MES

Seizures are induced, in mice or rats (n=4), using 50 mA or 150 mA alternating current respectively for 2 seconds using corneal electrodes primed with an electrolyte solution containing 0.5% tetracaine HCL anesthetic. Diaminodiphenyl compounds were delivered by IP injection at doses of 30, 100 and/or 300 mg/Kg at a volume of 0.01mL/g. Neuroprotection was characterized as the abolition of the tonic phase of seizure development. [11,12,13]

6Hz

Seizures are induced in mice (n=4), using 32 mA alternating current for 3 seconds using corneal electrodes primed with an electrolyte solution containing 0.5% tetracaine HCL anesthetic. Diaminodiphenyl compounds were delivered by IP injection at doses of 30, 100 and 300 mg/Kg at a volume of 0.01mL/g. Neuroprotection was characterized as the abolition of a brief clonic phase followed by automatistic behaviors.[14]

Acute toxicity

The acute neurotoxicity of the compounds was evaluated in mice (n=8) using the rotorod test, and using the positional sense and gait and stance tests. Acute neurotoxicity

was characterized as 3 failures of an animal to remain on a rotating, 3.2 cm knurled rod for 1 min at 6 rpm. Diaminodiphenyl compounds were delivered by IP injection at doses of 30, 100 and 300 mg/Kg prior to evaluation in the toxicity screens. Animals were evaluated at both 0.5 and 2 h post administration. Positional sense and gait and stance tests were used to evaluate gait, behaviors, muscle dysfunction and other indications of neurotoxicity.

Compounds which displayed significant neuroprotection were further evaluated in the MES and 6Hz models using relevant doses (30, 100 or 300 mg/Kg) at time points of 0.25, 0.5, 1, 2, and 4 hours. Compounds were delivered through IP injection, as previously described, or orally.

The compounds evaluated were chosen to explore several structural variable in order to determine the effect functional different functional groups and structural differences will have on diaminodiphenyl anticonvulsive activity. Structural differences evaluated were: linker element and length, linker substitution, aromatic substitution, aromaticity, and amine substitution. A majority of the diaminodiphenyl compounds possess anticonvulsive properties, though the dose and duration of anticonvulsive effects vary based on the structure of the compounds (Table 1, 2).

4,4’-diaminodiphenyl methane 4,4’-diaminodiphenyl sulfide, and oxydianiline were evaluated to elucidate whether the element that links the aniline moiety effects the anticonvulsive potential of a diaminodiphenyl compound. In both MES and 6Hz animal models oxydianiline was a leading anticonvulsant, when compared to 4,4’-diaminodiphenyl methane 4,4’-diaminodiphenyl sulfide. While 4,4’-diaminodiphenyl

sulfide was as effective in the MES model acting as an anticonvulsant at a dose of 100 mg/Kg at both the 30 minute and 2 hour time points. However, oxydianiline was a longer acting anticonvulsant when evaluated in the 6Hz model acting at a dose of 100 mg/Kg for 2 hours, while 4,4'-diaminodiphenyl sulfide and 4,4'-diaminodiphenyl methane were only effective at the same dose for 30 minutes. At an increased dose of 300 mg/Kg, both 4,4'-diaminodiphenyl methane and 4,4'-diaminodiphenyl sulfide were both effective anticonvulsants in the 6 Hz model for up to 2 hours. When evaluated in the MES model, 4,4'-diaminodiphenyl methane was only effective at a dose of 300 mg/Kg with a duration of 2 hours. The superior anticonvulsive properties of oxydianiline may be a result of the higher electronegativity of the oxygen linker between the aniline moieties. The differences in activity between the carbon and sulfur linkers cannot be explained by electronegativity, but sulfur's lone pairs of electrons may be a favorable characteristic responsible for the increased anticonvulsive effects noted in the MES model, though this conclusion is contrasted by the results seen in diaminodiphenyl compounds which have longer linker groups.

4,4'-ethylenedianiline and 4-aminophenyldisulfide were evaluated to determine whether the length of the linker would affect the anticonvulsive properties of diaminodiphenyl compounds. The results contrasted the conclusions drawn based on the single element linkers. The 4,4'-ethylenedianiiline was a more potent anticonvulsant than diaminophenyldisulfide, as well as, all of the single element linker diaminodiphenyl compounds that were evaluated. 4,4'-ethylenedianiline provided short acting neuroprotection at at a dose of 30 mg/Kg, which persisted through the 2 hour time point when the dose was increased to 100 mg/Kg in the MES model. 4-aminophenyldisulfide

was a much slower acting anticonvulsant in the MES model showing no effects at the 30 minute time point at 100 mg/Kg, though at the 2 hour time point it was an effective anticonvulsant. At a dose of 300 mg/Kg anticonvulsive properties were noted at the 30 minute time point, though toxicity prevented observations at the 2 hour time point at the same dose in the MES model. Results were similar in the 6Hz model, with the notable difference being the onset and duration of action of 4-aminophenyldisulfide which was noted at the 30 minute time point but lost at the 2 hour time point at a dose of 100 mg/Kg. The increased anticonvulsive properties of the 4,4'-ethylenedianiline and the less favorable anticonvulsive effects elicited by 4-aminophenyldisulfide suggest that a longer linker which allows for ligand flexibility is a favorable characteristic lending to increased anticonvulsive effects. The anticonvulsive effects of 4,4'-ethylenedianiline were superior to the effects elicited by oxydianiline which suggests that linker length and flexibility is likely more important than lone pairs of electrons on the linker.

Substituted linkers of variable length were were also evaluated in order to determine whether linker substitution affects diaminodiphenyl anticonvulsive properties. Dapsone, diaminobenzophenone, diaminobenzanilide, and 4,4'-(hexafluoroisopropylidene)dianiline provide various linker substitutions of varying size and linker lenth and providing insight into diaminodiphenyl anticonvulsant potential. Both dapsone and diaminobenzophenone present oxygen substituted single element linkers introducing hydrogen bond acceptors in the linker region. Both compounds are neuroprotective at a dose of 30 mg/Kg, though dapsone is the only compound whose effects persisted to the 2 hour time point, while diaminobenzophenone required a dose of 100 mg/Kg to persist to the 2 hour time point in the MES model. When evaluated in the 6Hz model both compounds showed no activity

at the low dose, though both were effective anticonvulsants at a dose of 100 mg/Kg for both time points. Diaminobenzanilide presents a longer linker with an amido group linker which introduces a hydrogen bond acceptor, as well as, nitrogen into the linker. At a dose of 100 mg/Kg anticonvulsive properties were only noted at the 30 minute time point, while a dose of 300 mg/Kg allowed the anticonvulsive properties to persist to the 2 hour time point in both models. When compared to other compounds evaluated an amido linker is not favorable and requires a high dose in order to provide neuroprotection in both tests. 4,4'-(hexafluroisoproylidene) dianiline has a bulky linker to determine whether compound size affects diaminodiphenyl anticonvulsive properties. In all tests 4,4'-(hexafluroisoproylidene) dianiline failed to elicit neuroprotective effects, suggesting that ligand size is an important factor in the anticonvulsive activity of diaminodiphenyl compounds.

The effect of aromatic substitution was addressed by testing 4,4'-methylene Bis(2,6-dimethylaniline) and methylene Bis(chloroaniline). At a dose of 100 mg/Kg and 300 mg/Kg respectively, 4,4'-methylene Bis(2,6-dimethylaniline) and methylene Bis(chloroaniline), displayed anticonvulsant effects lasting for two hours in the MES model. 4,4'-methylene Bis(2,6-dimethylaniline) performed slightly better than methylene Bis(chloroaniline) providing neuroprotection for 30 minutes at a dose of 100 mg/Kg, though both provided the same neuroprotection at both time points at a dose of 300 mg/Kg. Neither compound showed significant improvement in anticonvulsive effects and therefore aromatic substitution is of little consequence or benefit.

In order to address whether aromaticity plays a role in the noted anticonvulsive properties of diaminodiphenyl compounds, 4,4'-methylene Bis(cyclohexylamine) was

tested. No activity was noted and testing was discontinued at a dose of 100 mg/Kg due to toxicity in test animals. Further testing of non-aromatic compounds would be needed to draw any conclusion regarding the effect of aromaticity on the anticonvulsive properties of diaminodiphenyl compounds.

Lastly, amine substitution was addressed by testing 4,4'-methylene Bis(*N,N*-dimethylaniline). By substituting the amine of diaminodiphenyl compounds, hydrogen bond donors are eliminated. In both the MES and 6Hz model 4,4'-methylene Bis(*N,N*-dimethylaniline) failed to provide neuroprotection at any dose. This provides evidence that hydrogen bond interactions at the amine group may be necessary for diaminodiphenyl compounds to elicit their anticonvulsive properties, though further testing would be necessary to confirm this conclusion.

With a majority of the compounds tested presenting anticonvulsive properties at similar doses, it is also beneficial to explore the link between diaminodiphenyl structure and toxicity. (Table 3) The most commonly noted toxicity is the animals' failure to grasp the rotorod and loss of the righting reflexes. In all cases these toxicities are only noted in the highest tested dose, 300 mg/Kg. These test failures could be attributed to a sedative effect which may present as a result of the high dose of an ion channel antagonist, though further testing would be needed to support that conclusion. In the case of 4,4'-methylene Bis(2,6-dimethylaniline) and diaminodiphenyl methane, the rotorod test and righting reflex failures were only noted at the 30 minute time point, while the compounds' antiepileptic effects are noted to persist to the later time point of 2 Hr. This suggests temporary reversible effects which are responsible for the noted test failures and further supports a sedative effect.

Dapsone, a drug used to treat leprosy, when given at high doses, resulted in a failure in the rotorod test as well as behavioral changes in the animals. At a dose of 300 mg/Kg animals began to stretch and roll. It is unclear from these studies whether the stretching and rolling behaviors are manifestations of neurotoxicity or psychoactivity of the compound in the test animals. As dapsone is a drug which has been on the market for some time, it is fairly safe to conclude that the drug is not neurotoxic at clinical doses.

Another noted effect elicited by a 300 mg/Kg dose of diaminobenzanilide and diaminobenzophenone was muscle spasm or tremor. The common functional group noted between these two compounds is the carbonyl group in the linker region of the compound. The carbonyl functional group presents a hydrogen bond acceptor and could result in a tighter receptor binding. The additional hydrogen bond acceptor may also result in binding to receptors untargeted by diaminodiphenyls which lack carbonyl groups. Off target binding activity could also present as tremors/muscle spasms.

Only two compounds tested caused death in experimental animals. 4-aminophenyldisulfide toxicity presented as sedation at the 30 minute time point at 300 mg/Kg dose, by the 2 hour time point all of the test subjects had expired. The disulfide linker is likely responsible for the adverse effects. Disulide could easily disrupt protein structure, which can result in loss of receptor functions leading to death. The other compound that caused death in test subjects was 4,4’-methylene Bis(cyclohexylamine). 4,4’-methylene Bis(cyclohexylamine) was toxic at a lower dose than other test compounds and resulted in subject death at a dose of 100 mg/Kg by the 30 minute timepoint. 4,4’-methylene Bis(cyclohexylamine) did not display any antiepileptic

activity. The rapid onset of toxicity does not implicate the receptor based toxicity noted in other test compounds.

Diaminodiphenyl compounds may be a valuable lead for the development of a novel antiepileptic compound. Thought there are key features which are necessary and some which need to be avoided when developing lead compounds. Linker element does not appear to drastically effect anticonvulsive properties. Linker length, flexibility and potential metabolite formation would be valuable characteristics to consider. Based the toxicity of diaminobenzophenone and diaminobenzanilide, carbonyl containing linkers will likely present adverse effects. The most important characteristic to consider when designing a diaminodiphenyl antiepileptic compound is the availability of hydrogen bond donation at the amine moiety.

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Table 1. Maximal Electroshock model of epilepsy in mice. Results displayed show the lowest dose at which neuroprotection was observed, as well as, the dose required to exhibit neuroprotection for the duration of the experiment. All doses were delivered by IP injection.

Compound	Dose (mg/Kg)	Time of effect	Dose (mg/Kg)	Time of Effect
Dapsone	30	2 Hr		
Diaminobenzophenone	30	0.5 Hr	100	2 Hr
4,4'-ethylenedianiline	30	0.5Hr	100	2 Hr
4,4'-Methylene Bis(2,6-dimethylaniline)	100	2 Hr	-	-
Diaminodiphenyl sulfide	100	2 Hr	-	-
Oxydianiline	100	2 Hr	-	-
4-aminophenyldisulfide	100	2 Hr*	300	0.5 Hr
Diaminobenzanilide	100	0.5 Hr	300	2 Hr
Diaminodiphenyl methane	300	2 Hr	-	-
Methylene Bis(chloroaniline)	300	2 Hr	-	-
4,4'-(hexafluoroisopropylidene) dianiline	Neg	-	-	-
4,4'-Methylene Bis(cyclohexylamine)	Neg	-	-	-
4,4'-methylene Bis(*N,N*-dimethylaniline)	Neg	-	-	-

*Neuroprotection was not noted in the 30 minute time point for a dose of 100 mg/Kg

Table 2. 6Hz model of epilepsy in mice. Results displayed show the lowest dose at which neuroprotection was observed, as well as, the dose required to exhibit neuroprotection for the duration of the experiment. All doses were delivered by IP injection.

Compound	**Dose (mg/Kg)**	**Time of Action**	**Dose (mg/Kg)**	**Time of Action**
4,4'-ethylenedianiline	100	2 Hr	-	-
Dapsone	100	2 Hr	-	-
Diaminobenzophenone	100	2 Hr	-	-
Oxydianiline	100	2 Hr	-	-
4,4'-Methylene Bis(2,6-dimethylaniline)	100	0.5 Hr	300	2 Hr
Diaminobenzanilide	100	0.5 Hr	300	2 Hr
Diaminodiphenyl methane	100	0.5 Hr	300	2 Hr
Diaminodiphenyl sulfide	100	0.5 Hr	300	2 Hr
4-aminophenyldisulfide	100	0.5 Hr	300	0.5 Hr
Methylene Bis(chloroaniline)	300	2Hrs	-	-
4,4'-Methylene Bis(cyclohexylamine)	Neg	-	-	-
4,4'-(hexafluoroisopropylidene) dianiline	Neg	-	-	-
4,4'-methylene Bis(*N,N*-dimethylaniline)	Neg	-	-	-

Table 3. Results of acute neurotoxicity screening. Test failures and adverse events are noted at the dose where observations were made. All doses were delivered by IP injection.

Compound	**Dose (mg/Kg)**	**Test(s) failed**	**Noted toxicity**
4,4'-Methylene Bis(cyclohexylamine)	100	-	Death
Methylene Bis(chloroaniline)	300	Rotorod test	-
Oxydianiline	300	Rotorod test	-
Diaminobenzanilide	300	Rotorod test	Muscle Spasm
4-aminophenyldisulfide	300	Righting Reflex	Death
Diaminobenzophenone	300	Rotorod test	Tremors
Diaminodiphenyl methane	300	Righting Reflex, Rotorod test	-
Diaminodiphenyl sulfide	300	Righting Reflex, Rotorod test	-
4,4'-Methylene Bis(2,6-dimethylaniline)	300	Rotorod test	-
Dapsone	300	Rotorod test	Stretching and Rolling
4,4'-ethylenedianiline	300	Rotorod test	-
4,4'-(hexafluoroisopropylidene) dianiline	Neg	-	-
4,4'-methylene Bis(*N,N*-dimethylaniline)	Neg	-	-

MANUSCRIPT 3

SYNTHESIS OF RATIONALLY DESIGNED DIAMINODIPHENYL DERIVATIVES

In preparation: Bioorganic and Medicinal Chemistry Letters

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Diaminodiphenyl compounds have been shown to exert potent anticonvulsive properties in animal models of epilepsy. It has been purported that diaminodiphenyl compounds exert their anticonvulsive effects through interactions with the *N*-methyl-D-aspartate receptor.[1] *In silico* evaluation of several diaminodiphenyl compounds has provided further evidence that NMDAr, more specifically the NR2 subunit, is likely the site of action for diaminodiphenyl compounds.

Though acute toxicity evaluation indicates that many diaminodiphenyl compounds are not neurotoxic, aniline, a major functional group of diaminodiphenyl compounds, is known to be toxic. The major toxicity resulting from aniline exposure is methemoglobinemia, a blood toxicity which results in the inability to transport oxygen.[2] Additionally, the EPA has classified aniline as a probable carcinogen. The metabolism of aniline is responsible for both toxic mechanisms. Oxidation of the amino group forms the key toxic intermediate, *N*-hydroxyaniline (Fig 1).

Diaminodiphenyl compounds, which possess aniline moieties, are likely to be toxic during chronic exposure, even at low doses. Rational drug design techniques offer an opportunity to eliminate the toxic potential while maintaining or improving the anticonvulsive properties of diaminodiphenyl compounds. Through modification of the amino groups of diaminodiphenyls it may be possible to prevent the metabolic formation of the *N*-hydroxy intermediate which is proposed to result in methemoglobinemia and DNA adduct formation. Successful design and synthesis of a diaminodiphenyl derivative could lead to the development of an NMDAr antagonist which could be used to treat many indications, including epilepsy, neuropathic pain and mood disorders.

Materials and Methods

Chemicals

All chemical reagents and solvents were purchased from Sigma Aldrich or Fisher Scientific.

N,N'-diacetyl-diaminodiphenyl sulfide derivative (Scheme 1)

4,4'-diaminodiphenyl sulfide (1.00 g, 4.62 mmol) was dissolved in THF and added dropwise to an excess of acetyl chloride containing potassium carbonate (700.0 mg, 5.06 mmol). The reaction was stirred at room temperature for 30 minutes. Product was then collected by vacuum filtration. The product was washed with cold THF before being dried in a vacuum desiccator.

N,N'-azapentacyclodiphenyl sulfide (Scheme 2)

4,4'-diaminodiphenyl sulfide (1.00 g, 4.62 mmol) was dissolved in a minimum volume of acetone (40 mL). At room temperature the 4,4'-diaminodiphenyl sulfide solution was added dropwise into a reaction mixture of 1,4-dibromobutane (3.35g, 15.5 mmol) and potassium carbonate (700.0 mg, 5.06 mmol) in acetone/water. When all reactants are added the final solvent is 95% acetone/water. The reaction was allowed to stir at room temperature overnight before being evaporated prior to purification. The resulting reaction mixture was taken up in 50/50 ethyl acetate and water. The aqueous phase was washed three times with ethyl acetate and the organic fractions were pooled. Using a rotary evaporator the organic phase was adsorbed to silica and packed into a

column for purification by flash chromatography. *N,N'*-azapentacyclodiphenyl sulfide was collected yielding 751 mg, 50.39%.

^{1}H-NMR (300 MHz, DMSO) $^{\delta}$ 7.26-6.99 (d, 4), 6.62-6.37 (d, 3.97), 3.27-3.10 (t, 8.06), 2.02-1.85 (t, 7.24). (Fig 2) ^{13}C NMR (126 MHz, DMSO) δ 147.32, 133.02, 129.34, 121.23, 112.79, 112.53, 47.73, 40.57, 40.48, 40.40, 40.31, 40.23, 40.15, 25.43, 25.39, 25.37 (Fig 3).

Indole derivatives of diaminodiphenyl compounds

Method 1(Scheme 3)

The synthisis of diindole derivatives was derived from the methods of Cho, et al. 2000, and was completed in 10 ml of 1,4-dioxane, under argon. Diaminodiphenyl compounds (diaminodiphenyl sulfide: 1.08 g, 5.0 mmol; 4,4-ethylenedianiline: 1.06 g, 5 mmol) were combined with triethanolamine (133 μL, 2 mmol). Ruthenium chloride (20.7 mg, 0.1 mMol), tin chloride (225 mg, 2.0 mmol) and triphenylphosphine (157.5 mg, 0.6 mmol) were added as catalysts. The reaction was stirred in a sealed reaction vial at 180 ^{o}C for 20 hours.[3] The resulting reaction mixture was adsorbed to silica packed into a column for purification by flash chromatography.

Method 2 (Scheme 4)

The synthisis of diindole derivatives was derived from the methods of Cho, *et al.* 2007, and was completed in 10 ml of 1,4-dioxane, under argon. Diaminodiphenyl compounds (diaminodiphenyl sulfide: 1.08 g, 5.0 mmol; 4,4-ethylenedianiline: 1.06 g, 5 mmol) were combined with dibromoethane (22 μL, 250 μmol) at a 20:1 mole ratio in order to prevent piperazine polymer formation. Ruthenium chloride (20.7 mg, 0.1 mmol)

and triphenylphosphine (786.9 mg, 3 mmol) were added as catalysts. The reaction was stirred in a sealed reaction vial at 180 ^{o}C for 20 hours.[4] The resulting reaction mixture was adsorbed to silica packed into a column for purification by flash chromatography.

Flash chromatography

Using a Teledyne Isco CombiFlash® Rf 200 the reaction mixture was purified through a RediSepRf High Performance Gold 12g HP Silica column (Teledyne Isco inc.) using a hexane/ethyl acetate gradient with a 30 mL/min flowrate and a maximum pressure of 400 psi (Fig 4). Fractions were collected by an automated fraction collector monitoring 253 and 255 nm wavelengths.

Liquid chromatography

Using a Thermo Scientific liquid chromatograph reaction products were separated using a ODS Hypersil C-18 column at a flow rate of 250 µL/min using a gradient of 0.1% aqueous acetic acid and acetonitrile. (Appendix 1)

Results and Discussion

Based on the toxicity of aniline, it is likely that diaminodiphenyl compounds would lead to adverse effects with chronic exposure. However, the potent anticonvulsive properties of the compound class cannot be overlooked. Using a computer based model of receptor-ligand interactions it is possible to design compounds that cannot be metabolized in a similar fashion to aniline while maintaining the compounds ability to interact at the NMDAr NR2 subunit binding site.

By substituting the amino groups of the diaminodiphenyl compound class the intention is to prevent the *N*-hydroxy metabolite formation. This has been addressed in the synthesis of both the diacetyl- and *N,N'*-azapentacyclo- diaminodiphenyl sulfide derivatives. These modifications to the amino group result in secondary and tertiary amine formation, which will result in a drastically different hydrogen bonding potential from that of the parent diaminodiphenyl compounds, with the diacetyl- derivative having decreased hydrogen bonding potential due to the lack of a permanent dipole and the azapentacyclo- derivative lacking hydrogen bonding groups altogether.

Upon further evaluation in computer based models and results gathered in animal models of epilepsy, it was determined that hydrogen bonding appears to be an important factor in a diaminodiphenyl compound's anticonvulsive potential. For this reason the consideration and synthesis of several proposed azacyclo derivatives, was discontinued (Fig 5).

Chemical characterization of the diacetyl- derivative of diamindiphenyl sulfide revealed product decomposition. Unlike previously characterized diaminodiphenyl derivatives, product separation was not easily accomplished by flash chromatography using a silica stationary phase. Using liquid chromatography and solid phase extraction techniques the mono- and di- substituted derivatives of diaminodiphenyl sulfide were separated across a C-18 column in acetonitrile/ 0.1% aqueous acetic acid mobile phase (Appendix 1). The mass spectrometry data suggests that the compound may be breaking down from the diacetylated derivative to the monoacetylated derivative of diaminodiphenyl sulfide (Fig 6). The extracted ion trace of the diacetyl- derivative of diaminodiphenyl sulfide, purified by solid phase extraction, support the conclusion that

the stability of *N,N'*-diacetyl-diaminodiphenyl sulfide needs to be evaluated further. The LCMS data shows a very small, though detectable, peak with a higher retention time in SPE purified product, which can be characterized as the monoacetyl- derivative (Fig 7). Further method development and compound stability studies would be necessary to create a stable diacetyl- derivative of diaminodiphenyl sulfide.

The synthesis of the di-indole derivative of 4,4'-ethylenedianiline and diaminodiphenyl sulfide has been challenging. The methods for indole synthesis are based on the synthesis of indoles from substituted anilines as described by Cho, et al.[3] The ruthenium/tin catalyzed reaction between triethanolamine and diaminodiphenyl compounds was run under the prescribed conditions (Scheme 3). The reaction did not result in the formation of the intended product in the conventionally heated or microwave synthesis experiments. Upon repeated failure of this method of indole synthesis, alternative methods were pursued.

The ruthenium catalyzed reaction between dibromoethane and diaminodiphenyl sulfide (Scheme 4) also produced zero yield of the diindole derivate. However, the reaction did take place using this method. The first step of the reaction may have proceed resulting in the formation of *N*-bromoethane substituted diaminodiphenyl sulfide derivative. The mass spectroscopy data suggests that the intended indole product was not formed (Fig 8). The mono-substituted product is likely a result of the molar ratio of the reagents, which is low in order to prevent piperazine polymer formation. If additional equivalents of 1,2-dibromoethane were added upon reaction completion and the reaction were repeated, the reaction may result in the intended N,N'-bromoethane intermediate formation. Reactant ratios could be optimized in order to improve the efficiency of the

reaction, leading to a higher yield. If the reaction results in the N,N'-bromoethane intermediate upon completion, it could be possible to modify the halide, replacing the bromine with iodine. The iodine group is a far better leaving group and could help to foster the intramolecular reaction forming the intended indole derivatives.

The rational design of diaminodiphenyl derivatives could provide a potent anticonvulsant. The most promising derivative addressed in this work is the diindole derivative. If the computer based model of receptor ligand interactions is accurate, the resulting derivatives would be 100 times more potent than the parent compound and the toxic metabolites encountered with the parent compound could not be formed. Indoles, however, pose an additional challenge: delivery. Indoles are highly lipophilic and not readily water soluble. Should the indole derivatives of diaminodiphenyl compounds present potent anticonvulsive properties with limited toxicity, formulation and delivery of the compound would need to be addressed.

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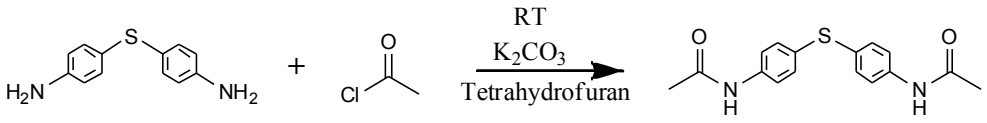
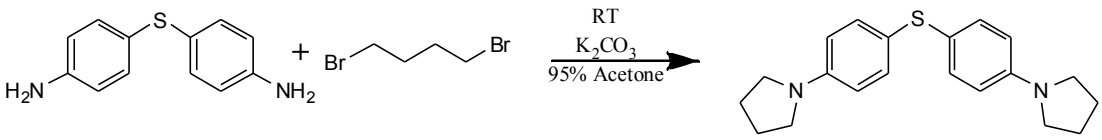
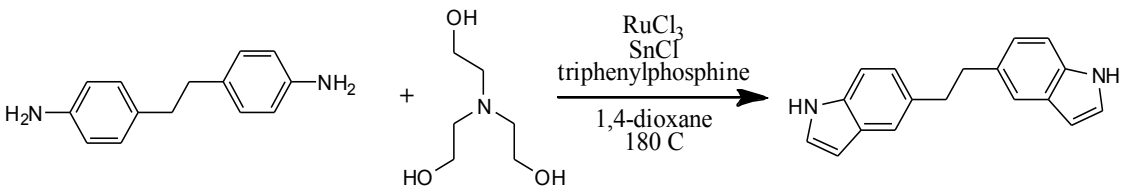
Scheme 1. Diacetyl derivative synthesis



Scheme 2. Azacyclo derivative synthesis



Scheme 3. Indole synthesis - triethanolamine



Scheme 4. Indole synthesis - dibromoethane

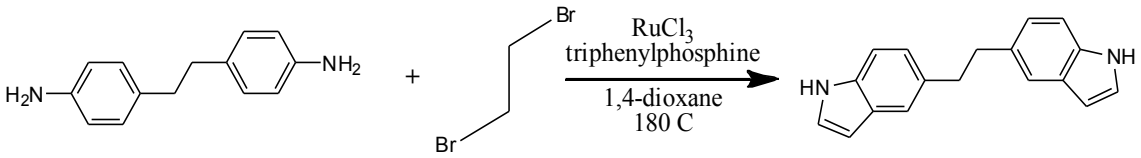


Figure 1. Proposed metabolic pathway of 4,4'-diaminodiphenyl sulfide, derived based on the metabolism of aniline. *N*-hydroxy formation results in toxicity which includes methemoglobinemia and carcinogenesis.

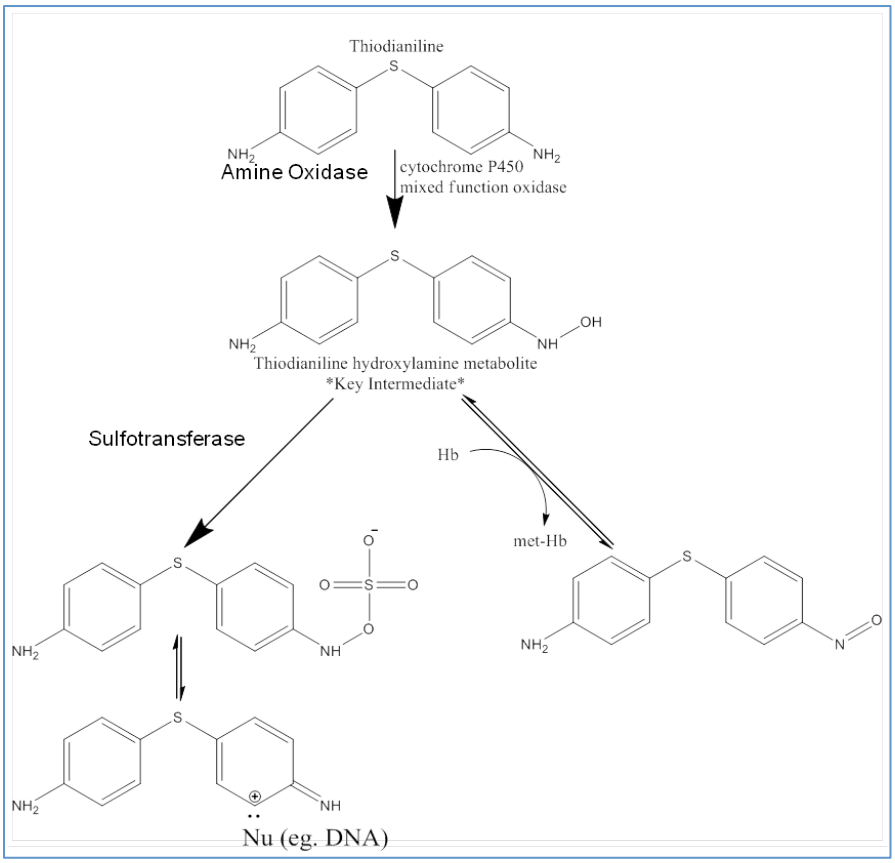


Figure 2. Proton NMR of *N,N'*-azapentacyclodiaminodiphenyl sulfide.

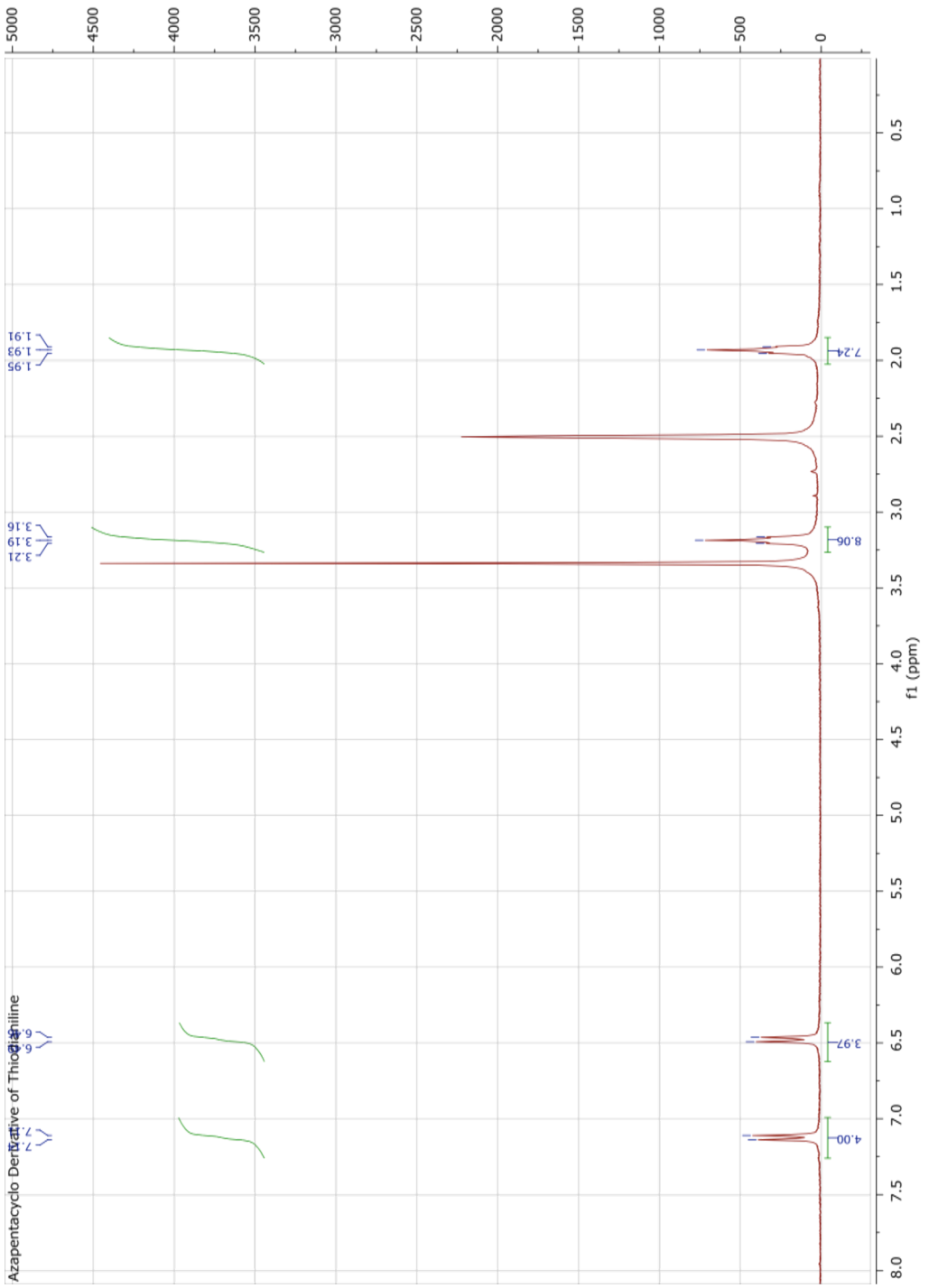


Figure 3. Carbon NMR spectrum of *N,N'*-azapentacyclodiaminodiphenyl sulfide.

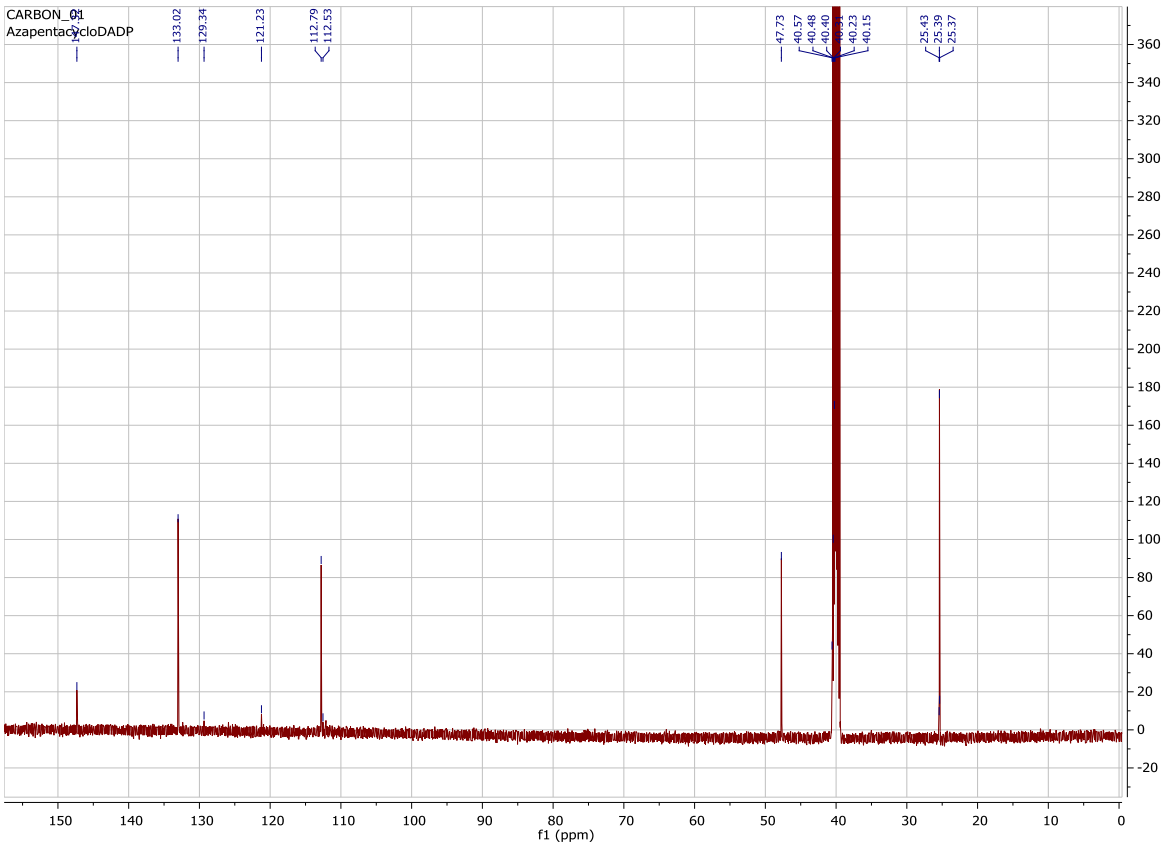


Figure 4. Azacyclo derivatives which were not purified based on the modeling and *in vivo* data collected with *N,N'*-azapentacyclodiaminodiphenyl sulfide.

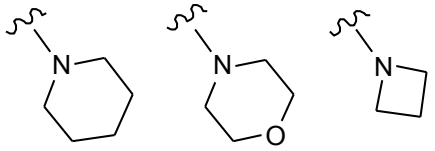


Figure 5. Mass spectrograph of *N,N'*-diacetyl-diaminodiphenyl sulfide.

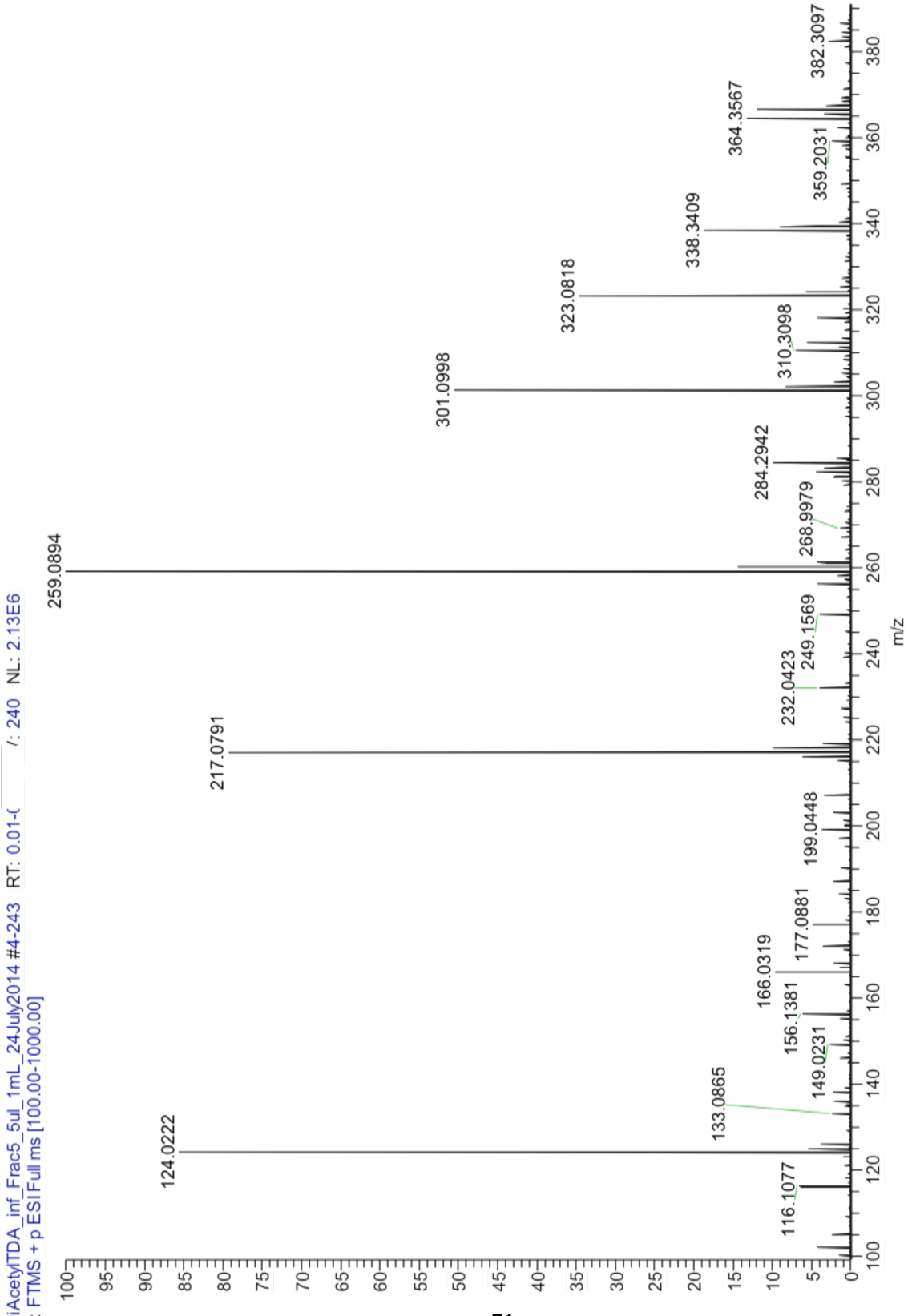


Figure 6. Extracted ion traces of solid phase extraction purified *N,N'*-diacetyl-diaminodiphenyl sulfide injected immediately after SPE.

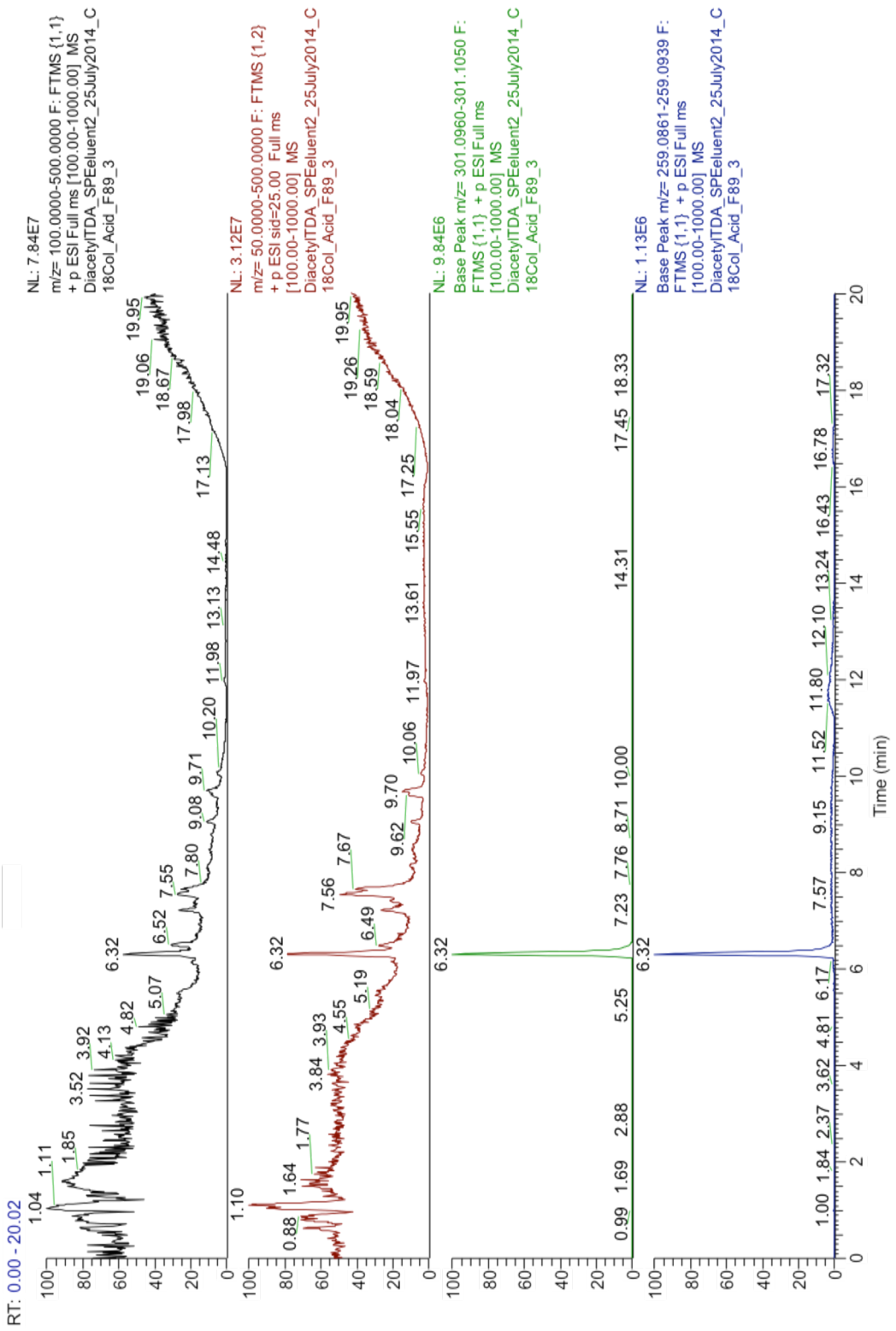
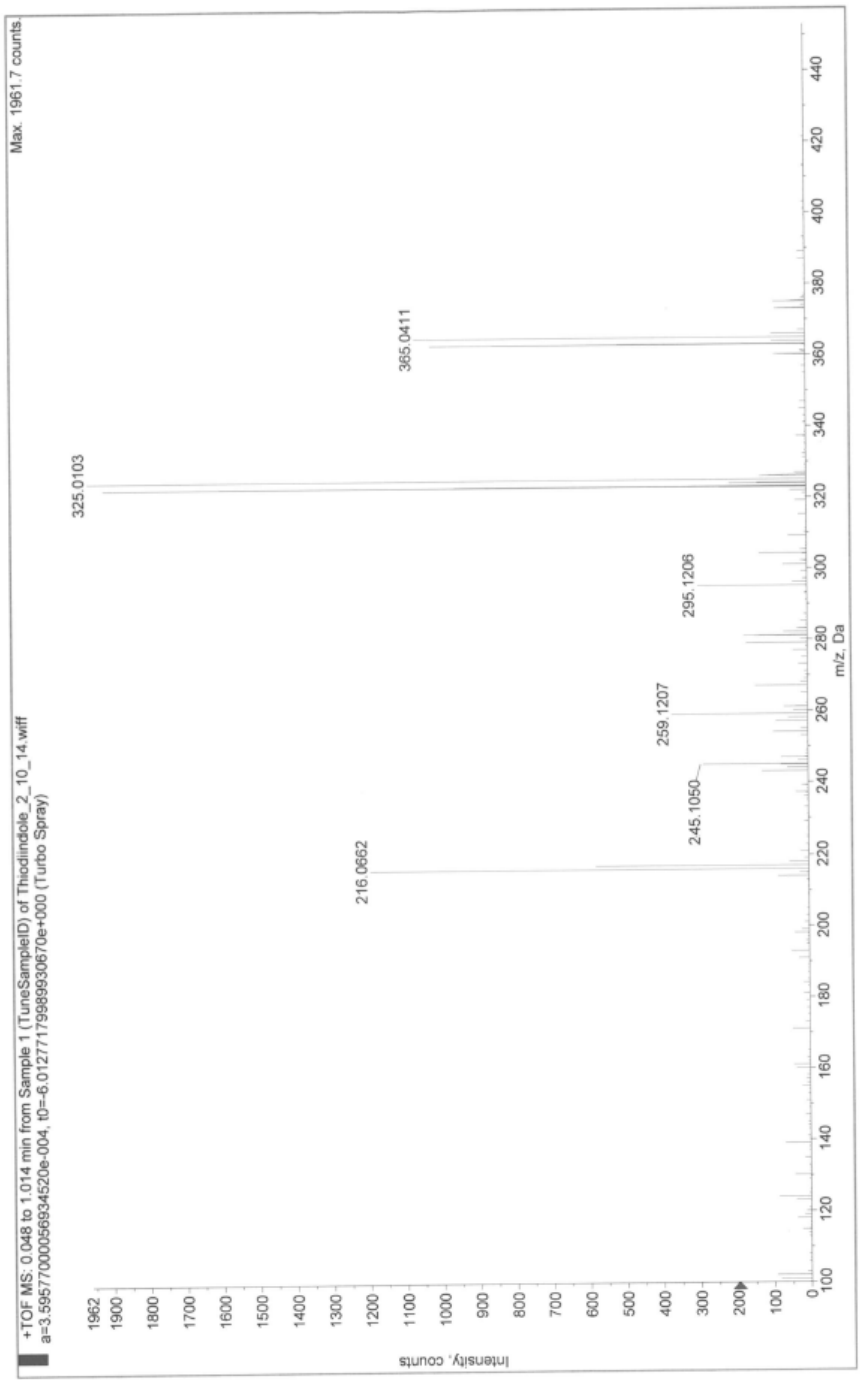


Figure 7. Mass spectrograph of diindole synthesis method 2 (Scheme 4) product provides evidence that the synthesis of desired derivative was not successful.



CONCLUSION AND FUTURE STUDIES

The results of the present study have shown that a series of simple diaminodiphenyl (DADP) compounds that are structurally distinct from known anti-epileptic compounds, bind in a conserved orientation and with similar binding interactions within a solvent accessible cavity of *N*-methyl-D-aspartate receptor NR2 subunit which contains the agonist binding site. Transfected cell-based assays in which mammalian NMDAr is expressed in Xenopus oocytes suggest that these compounds inhibit the activity of NMDAr (Appendix 2). Furthermore, these DADP compounds exert profound in vivo anticonvulsant activity by preventing the initiation and propagation of seizures in rats and mice in several seizure models.

Data collected *in silico* suggests that variability in the linker does not drastically affect the compounds' binding energy; however, substitution can lead to a change in the binding motif or keep the compound from entering the binding pocket altogether. Notably, the *in silico* and *in vivo* provide evidence that *N*-hydrogen bonding is an essential component to the binding interaction between NMDAr and DADPs, as well as, to the anticonvulsive effects elicited in rat and mouse models of epilepsy. This data has guided the design of novel diaminodiphenyl derivatives, resulting in the design diindole derivatives and the exclusion of several earlier generations of diaminodiphenyl derivatives based on structure activity relationship and compound stability. Though these indole derivatives have not yet been synthesized, computational binding energies and inhibition constants indicate that the anticonvulsive properties of these compounds could exceed that of previously examined diaminodiphenyl compounds. Should the current

methods to synthesisze diindole derivatives fail it may be necessary to pursue an alternative method.

Future studies should aim to characterize the diaminodiphenyl binding pocket on NMDAr, where diaminodiphenyl compounds actively bind and modulate NMDAr activity. The binding site can be further characterized by further refining *in silico* models. Examining the effects of flexible amino acid sidechains will further elucidate the characteristics of the binding site on the NMDAr NR2 subunit. These characteristics can be used to select key amino acids, including THR 174 elucidated in our studies, to design point mutants of the NMDAr NR2 subunit. The effects of point mutations could be examined using *in silico* modeling techniques to determine the effects of point mutations on NMDAr structure and diaminodiphenyl binding. Furthermore, point mutations of NMDAr can be examined *in vitro* using electrophysiology based techniques; contrasting the function of wt NMDAr to mutant forms of NMDAR in Xenopus oocyte. Electrophysiology based studies will provide evidence of diaminodiphenyl antagonism of NMDAr in a relevant biological model, as well as, elucidating the mechanism by which diaminodiphenyls exert their antagonism. Characterization of the NMDAr diaminosiphenyl binding site has the potential to offer a potent druggable site of action for disorders of glutamate, including epilepsy.

APPENDIX 1

ISOLATION OF N,N'-DIACETYLDIAMINODIPHENYL SULFIDE BY LIQUID CHROMATOGRAPHY AND SOLID PHASE EXTRACTION

The separation of the mono- and di- acetyl products of diaminodiphenyl sulfide was not completed successfully by silica based flash chromatography, as was the case with other diaminodiphenyl derivatives were. The use of a C-18 column was necessary and an appropriate gradient method was elucidated in order to efficiently separate the products.

All liquid chromatographic separations were performed on a Thermo Scientific Accela pump using a Thermo Scientific ODS Hypersil C-18 2.1x100 mm, 5um column. Samples were injected inline by a Thermo Scientific HTS PAL Auto-sampler. Products were separated using a gradient mobile phase of 0.1% aqueous acetic acid and acetonitrile.

Mobile Phase Gradient		
Time (min)	**0.1% aqueous acetic acid (%)**	**Acetonitrile (%)**
0	80	20
2	80	20
15	2	98
20	2	98
22	80	20
25	80	20

Initial studies using a neutral aqueous mobile phase resulted in poor compound separation. Addition of 0.1% acetic acid separated mono- and di-acetyl derivatives of diaminodiphenyl efficiently with a 7 minute difference in retention time.

Solid phase extraction across Waters Sep-Pak C-18 syringe cartridges was considered

as a means to readily purify the desired diacetylated product. Separations were completed using manufacturers recommended procedures. Cartridges were conditioned with 500µL 100% acetonitrile, followed by 500 µL 100% 0.1% aqueous acetic acid. Samples were taken up in 0.1% aqueous acetic acid, 200 µL and injected onto the cartridge. The cartridge was then washed with 200 µL 0.1% aqueous acetic acid. The product was then eluted in 3 - 300µL fractions of 40% acetonitrile in 0.1% aqueous acetic acid.

Each fraction was evaluated by LCMS and it was determined that the first two fractions contained the best ratio of diacetyl- to monoacetyl- diaminodiphenyl sulfide.

Mass Spectrometer Conditions

Thermo Scientific Electron Exactive Orbitrap Mass Spectrometer was run in ESI positive ion mode flowing ultra pure nitrogen gas. All acquisitions and data analysis were completed using Thermo Scientific XCalibur Software Version 2.2 SP1.48.

Mass Spectrometer Conditions	
Sheath Gas – Nitrogen	30 au
Auxiliary Gas – Nitrogen	15 au
Spary Voltage	4.2 KV
Capillary Temperature	245 Celsius
Capillary Voltage	32.5 V
Tube Lens Voltage	140 V
Skimmer Voltage	28 V
Collision Energy	35 eV

APPENDIX 2

DIAMINODIPHENYL ANALOGS INHIBIT NMDAR IN *XENOPUS* OOCYTE

To determine whether the anticonvulsant properties of diaminodiphenyls are a result of an interaction with *N*-methyl-D-aspartate receptor, electrophysiology based syudies were executed in *Xenopus* oocytes. Mammalian NMDA receptors were expressed in Xenopus oocytes and functional inhibition of receptor function in response to the agonists glutamate and glycine was assessed with and without diaminodiphenyl sulfide.

cRNA for the most ubiquitously expressed NMDA channel subunits, NR1A and NR2B, were injected into *Xenopus* laevis oocytes. 4-5 days after cRNA injection electrophysiology was used to characterize NMDA responses. Using the two electrode voltage clamp configuration ooctyes were clamped at -70mV and perfused with 10mM glycine and 10uM Glutamate which resulted robust positive inward currents only in oocytes injected with both channel subunits. Subsequent addition of thiodianiline inhibited glutamate-dependent current in a concentration-dependent manner. Notably, NMDA receptor saturation resulted in a maximum inhibition of 60% when compared to control.

The results of this study confirm the assertion the diaminodiphenyl compounds modulate the functionof the *N*-methyl-D-aspartate receptor. Antagonism of this receptor may be the mechanism by which diaminodiphenyl compounds exert their anticonvulsive properties.