

**Nitrate Chimeras: A New Class of Disease-Modifying Agents for the
Treatment of Alzheimer's Disease**

BY

LAWREN VANDEVREDE

B.S., Biology, Florida State University, 2006

B.S., Chemistry, Florida State University, 2006

THESIS

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Defense Committee:

John Larson, Chair, Neuroscience Program, Department of Psychiatry
Gregory Thatcher, Advisor, Department of Medicinal Chemistry and Pharmacognosy
Judy Bolton, Department of Medicinal Chemistry and Pharmacognosy
Pavel Petukhov, Department of Medicinal Chemistry and Pharmacognosy
Douglas Thomas, Department of Medicinal Chemistry and Pharmacognosy
Jia Luo, Department of Medicinal Chemistry and Pharmacognosy
Elliott Mufson, Rush University, Department of Biological Sciences

*dedicated to my grandmother,
Estelle VandeVrede*

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Chapter 1 is a summary of all data presented in this thesis. Chapter 2 presents a background of drug discovery for Alzheimer's Disease culminating in the work presented herein. Chapter 3 was published as "Novel analogues of chlormethiazole are neuroprotective in four cellular models of neurodegeneration by a mechanism with variable dependence on GABA(A) receptor potentiation." with authors Vandevrede L, Tavassoli E, Luo J, Qin Z, Yue L, Pepperberg DR, Thatcher GR in the British Journal of Pharmacology January 2014; 171(2):389-402 and is included here in accordance with journal guidelines (see Appendix). Chapter 5 was published as "An NO donor approach to neuroprotective and procognitive estrogen therapy overcomes loss of NO synthase function and potentially thrombotic risk." with authors VandeVrede L, Abdelhamid R, Qin Z, Choi J, Piyankarage S, Luo J, Larson J, Bennett BM, Thatcher GR in PLoS One in August 2013; 8(8):e70740 and is included here in accordance with journal guidelines (see Appendix). The remainder of the written manuscript is unpublished original work of this author except as cited.

In an effort to present a clear picture for each of the three subtopics, all relevant available information was included, and here all work not performed directly by this author will be detailed. Zhihui Qin synthesized and purified all novel compounds and designed LC-MS/MS methods. In methiazole section, Ehsan Tavassoli performed brain bioavailability and RR experiments. In nomethiazole section, Ehsan Tavassoli performed STPA and RR experiments and Rezene Asghodom performed LORR. APP/PS1 experiments, including drug administration and RAWM, was conducted by our collaborator Dr. Ottavio Arancio at Columbia, but all biochemical and histochemical work was done by the author with the assistance of Dr. Jia Luo. 3xTg mice were kindly donated by Dr. Elliott Mufson, and Dr. Jia Luo and myself conducted all subsequent experiments and assays. In NO-SERM section, Ramy Abdelhamid performed neuroprotection experiments, NOx assays and STPA experiments with variable assistance from the author. Vasodilation experiments were performed in the lab of our collaborator Dr. Brian Bennett at Queens' University, and Biological Resources Laboratory performed anticoagulation assays from samples provided by our lab.

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Abbreviations

A β	beta amyloid
AChE	acetylcholine esterase
AChE	acetylcholine esterase inhibitor
AD	Alzheimer's Disease
ADAM	a disintegrin and metalloproteinase
ADAS	Alzheimer's Disease Assessment Scale
ADL	activities of daily living
AE	adverse events
AICD	APP intracellular domain
ANOVA	analysis of variance
APH	anterior pharynx defective 1
ApoE	apolipoprotein E
APP	amyloid precursor protein
aPTT	activated thromboplastin time
BACE1	β -site APP cleaving enzyme 1
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BOMC	Blessed Orientation Memory Concentration test
CAA	cerebral amyloid angiopathy
CAMDEX	Cambridge Mental Disorders of the Elderly Examination
CDR	Clinical Dementia Rating
cGMP	cyclic guanine monophosphate
ChAT	choline acetyl transferase
CIBIC	Clinician Interview Based Impression of Severity
CMZ	chlormethiazole/clomethiazole
CNS	central nervous system
CREB	cAMP response element binding protein
CSF	cerebral spinal fluid
DIV	days in vitro
DMA	desmethyloxifen
DMSO	dimethyl sulfoxide
DS	Down syndrome
DSM	Diagnostic and Statistical Manual
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
FAD	familial Alzheimer's disease
FDA	Food and Drug Administration
fEPSP	field excitatory postsynaptic potential
FTDP-19	frontotemporal dementia and parkinsonism linked to chromosome 19
GABA	gamma amino butyric acid
GAG	glycosaminoglycans
GI	gastrointestinal

GSK3 β	glycogen synthase kinase-3 β
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
i.p.	intraperitoneal
KO	knockout
LDH	lactose dehydrogenase
LDL	low density lipoprotein
L-NAME	L-NG-Nitroarginine Methyl Ester
LORR	loss of righting reflex
LTP	long-term potentiation
MOA	mechanism of action
MCI	mild cognitive impairment
MCT	multiple comparison test
mEPSC	miniature excitatory postsynaptic potential
MIBNT	multi-inventory battery of neuropsychological tests
MMSE	Mini-Mental State Examination
MORE	Multiple Outcomes of Raloxifene Evaluation
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MWM	Morris Water Maze
MZ	methiazole
NFT	neurofibrillary tangles
NMDA	n-methyl d-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NSAID	non-steroidal anti-inflammatory drugs
OGD	oxygen glucose deprivation
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
pCREB	phosphorylated cAMP response element binding protein
PDGF	platelet derived growth factor
PEN2	presenilin enhancer 2
PS1	presenilin 1
PS2	presenilin 2
PT	prothrombin time
RAWM	radial arm water maze
RR	rotarod
RUTH	raloxifene use for the heart trial
RXR	retinoid X receptors
sAPP	soluble amyloid precursor protein
SALA	selective amyloid lowering agent
SAR	structure activity relationship
SD	standard deviation
s.e.m.	standard error of the mean
SERM	selective estrogen receptor modulator
sGC	soluble guanylyl cclase
STPA	step through passive avoidance
TBS	theta burst stimulation

TNF α	tumor necrosis factor alpha
tPA	tissue plasminogen activator
WHI	Women's Health Initiative
WT	wild type

I. Summary

I.A. Methiazole Summary

Clinical neuroprotective agents are needed to treat a variety of disease states including Alzheimer's Disease (AD), a disease with increasing prevalence due to aging populations. Clomethiazole (CMZ) is a small molecule with neuroprotective effects demonstrated in numerous animal models, although not previously reported in cell cultures. CMZ is in clinical use, but has not yet realized its clinical potential in neurodegenerative therapy. CMZ is thought to act via GABA_A potentiation, however, other mechanisms have been proposed including anti-inflammatory and pro-mitochondrial activity. To understand and extend the neuroprotective potential of CMZ and related methiazoles (MZ), eight MZ derivatives were selected from a novel library and neuroprotection was further studied in detail for two of these.

Neuroprotection was measured in rat primary cortical neuronal cultures using four different models of neurodegeneration: the oxygen glucose deprivation (OGD) model of ischemia-reperfusion injury; NMDA excitotoxicity; glutamate excitotoxicity; and treatment with oligomeric A β ₁₋₄₂ at high and low doses. CMZ and MZ analogues showed broad neuroprotective activity, however, variable dependence upon the GABA_A receptor was observed. Neuroprotection correlated with reduction in excitotoxicity and action at the GABA_A receptor for GN-28, whereas neuroprotection was observed for GN-38 largely independent of the GABA_A receptor. Neuroprotection from GN-28 and GN-38 was comparable in response to excitotoxicity; however GN-38 was superior to GN-28 in neuroprotection against A β ₁₋₄₂ induced toxicity. Both agents were brain bioavailable and both showed significantly attenuated sedative activity *in vivo*.

Based upon the clinically proven CMZ scaffold, novel MZ derivatives were prepared, showing a spectrum of neuroprotective activity *in vitro* indicating potential for neuroprotective therapy including in ischemic stroke and AD. Two MZ agents with varied dependence on GABA_A *in vitro*, demonstrated brain bioavailability and attenuated sedative actions relative to CMZ *in vivo*.

I.B. Nomethiazole Summary

Alzheimer's Disease is primarily a disease of impaired cognition and memory, linked to the earliest event in pathophysiology: synaptic failure. Synaptic plasticity requires activation of gene expression programs with dysfunction of the transcription factor cAMP-response element binding protein (CREB) strongly implicated in AD. The hypothesis that activation of CREB through NO/cGMP signaling might modify the amyloid- β neuropathology, linked to AD pathogenesis, was demonstrated in both APP/PS1 and 3xTg transgenic mouse models of AD using a small molecule that was also designed to provide neuroprotection and attenuate pro-inflammatory cytokine release. Functional restoration of long-term potentiation was shown in hippocampal slices from AD transgenic mice in accord with observation of restoration of cognitive function *in vivo*. Levels of pCREB were significantly elevated and both TNF α and A β significantly lowered after drug treatment. In the absence of neuronal loss in animal models of AD, neuroprotection was demonstrated in rat primary neurons after oxygen-glucose deprivation or application of oligomeric A β . The potential and unwanted hypotensive effects of NO/cGMP signaling in the periphery were found not to be significant.

I.C. NO-SERM Summary

The significant potential of estrogen as a neuroprotective and procognitive therapy is hindered by adverse side effects in gynecological tissues, which have largely been overcome with 2nd generation selective estrogen receptor modulators (SERMs), such as raloxifene, although thrombotic effects persist. The 3rd generation SERM, desmethylarloxifene (DMA), was shown in hippocampal slices from 16-month old 3xTg Alzheimer's transgenic mice to restore synaptic function with dependence on GPR30. Neuroprotection in primary rat neurons by DMA was also GPR30 dependent. Having determined that DMA had brain bioavailability, the ability to reverse cognitive deficits induced by scopolamine was proven. DMA was not able to restore cognition in mice treated with nitric oxide synthase (NOS) inhibitor, nor in eNOS (-/-) mice treated with scopolamine.

Hypothesizing that a NO-donating SERM would be procognitive and neuroprotective, DMA analogues, including NO-DMA, were prepared. NO-DMA was validated in eNOS (-/-) mice, 3xTg hippocampal slices, and primary neurons. The activation of eNOS by estrogen to induce vasodilation is thought to underlie the putative cardiovascular benefits. DMA and analogues induced relaxation of aortic rings, although only NO-DMA retained efficacy in the absence of endothelium. Finally, in contrast to DMA, NO-DMA was shown to reduce thrombosis through both the intrinsic and extrinsic pathway, even when eNOS was inhibited. Taken together, these data suggest that an NO-SERM might retain the positive attributes of estrogen therapy, without the known side effects, and be of use in an aging population in which eNOS activity is attenuated.

II. Background

II.A. Epidemiology of Alzheimer's Disease

Alzheimer's disease (AD) is a neurological disorder characterized by a progressive decline in cognitive function, synaptic dysfunction and diffuse neuronal loss coupled with classic histopathological changes. Global estimates put the prevalence at 26.6 million people in 2006, with an expected rise to 1 in 85 people by 2050[1]. In the U.S. alone, 5.4 million people suffer from AD with an annual cost approaching \$184 billion[2]. The principle risk factor for AD is age, but a gender effect is observed as women have an age-adjusted mortality rate of 20.4 per 100,000, compared to 16.0 per 100,000 for men[3]. In light of these alarming statistics, finding a treatment option that attenuates the clinical progression of the disease is one of the most important research goals in the modern era.

II.B. History of Alzheimer's Disease in Early 20th Century.

In 1907, a German psychiatrist named Alois Alzheimer reported a brief description of his observations from Auguste D, the index patient for a disease that would later be named after Alzheimer[4]. Auguste Deter, a 51-year old woman, died in 1906 with a five-year clinical history characterized by such abnormalities as progressive memory impairment, hallucinations, delusions, apraxia, and speech disorders. Using a silver impregnation staining technique developed four years earlier by the German neuropathologist Max Bielschowsky, Alzheimer was able to identify in August D's postmortem brain sample what would become the histopathological hallmarks of Alzheimer's Disease (AD): significant atrophy, miliary deposits and neurofibrillary tangles in nerve cells[5, 6]. Whereas the miliary deposits had been found previously in

patients with epilepsy[7, 8], Alzheimer was the first to detail these findings in the cerebral cortex and include a description of intracellular tangle pathology.

The term “Alzheimer’s Disease” did not receive broad attention until Emil Kraepelin’s Textbook of Psychiatry was published in 1910[9], in which Kraepelin defined AD as presenile dementia occurring in patients between 45-60 years of age. This definition of AD led to early controversies regarding whether AD was age-dependent and distinct from senile dementia. Teofil Simchowicz introduced the term “senile plaques” to describe the miliary deposits, and also detailed granulovacuolar changes in the brains of demented individuals[10]. Following Alzheimer’s first few case descriptions, an additional 13 cases studies were published on AD in 1911, with a widely circulated formal clinicopathological description following in 1926[11, 12].

Meanwhile, the identification of plaques as amyloid deposits followed in 1927[13], with vascular plaque pathology separated from tissue pathology and termed congophilic angiopathy[14], now formally cerebral amyloid angiopathy (CAA). David Rothschild, a Canadian-American physician, devoted decades to the study of AD and definitively differentiated it from vascular dementias due to atherosclerosis[15]. Additionally, he noted the presence of plaques in cognitively normal individuals and was the first to posit a “compensatory” capacity in some patients - a clear forerunner of the cognitive reserve hypothesis[16]. However, he also incorrectly argued AD pathology was distinct from senile dementia as it was more rapidly progressive, occurred in younger patients, and often involved the language centers. Rothschild’s contemporary, R.D. Newton, concluded in 1948 that relying on a formal pathological definition, presenile AD

and senile dementia were identical, a finding deemed prescient and still universally accepted today[17].

In the 1960s, the development of the electron microscope (EM) allowed a previously unachievable look into the two pathological hallmarks of AD: plaques and tangles. Two independent groups in the UK and US reported that both plaques and tangles have an ultrastructural amyloid composition, defined as a cross-beta structure with characteristic dye binding. They further described plaques as extracellular amyloid deposits and neurofibrillary tangles as paired helical filaments present intracellularly in nerve bodies and neuritic outgrowths[18-20]. It was noted that neither plaques nor tangles were confined to AD, as shown by identical filamentous tangles deposited in frontotemporal dementia and plaques in normal, healthy adults. However, the new pathological descriptions permitted studies that described a correlation between clinical dementia and pathological disease progression by measuring plaques, tangles and destruction of brain tissue[21, 22]. It is important to note that later studies have called the statistical relationship between classic histopathology and clinical progression of these early studies into question[23].

Much work has been done to validate clinical tests for AD apart from histopathology on autopsy, and these run the gamut from the Mini-Mental State Examination (MMSE)[24], the Blessed Orientation Memory Concentration test (BOMC)[25], the Clinical Dementia Rating (CDR)[26], and cognitive assessment batteries such as the Alzheimer's Disease Assessment Scale (ADAS)[27] and the Cambridge Mental Disorders of the Elderly Examination (CAMDEX)[28], among others. It is beyond the scope of this work to evaluate the efficacy or utility of these different

validated tests, but it should be noted the increased focus on clinical diagnosis of AD in the 1970s and 80s to accompany the postmortem diagnosis of classic AD histopathology. This focus followed from (1) the founding of the National Institute on Aging in 1974, (2) a landmark paper by Katzman in 1976 framing AD as a vitally important medical and public health issue[29], and (3) the inclusion of the term AD in the third revision of the Diagnostic and Statistical Manual (DSM-III) in 1980. These social changes provided the foundation for the biochemical, genetic and morphological advances in AD research in the following decades.

II.C. The Cholinergic Hypothesis and Drug Development

July 2012 marked the 30th anniversary of the initial proposal of the cholinergic hypothesis for the treatment of AD[30], and Raymond Bartus, one of its leading proponents, reviewed the progress in 2000[31]. The cholinergic hypothesis in its simplest form states that deficits in the cholinergic system underlie functional memory deficits observed both in AD and in normal aging. Notably, this hypothesis does not suggest etiologies for the cause of deficits in AD, but instead posits significant degradation in the cholinergic system as underlying memory and functional impairments. Prior to the formation of the hypothesis, experts in the AD field disagreed on the clinical definition and experimental approach to AD[32, 33], including controversy over the use of animals to model human memory deficits and whether AD could even be considered treatable using a pharmacological approach. Some of the earliest trials using psychostimulants had shown negligible effect in treating AD[34, 35]. Thus, the cholinergic hypothesis' employment in animal models and its use as the theoretical basis

for developing four of the five clinically used therapeutics for AD represented a major turning point in AD pharmacological research.

The theoretical basis for the cholinergic hypothesis came from work by David Drachman showing the importance of the cholinergic system for learning and memory, especially after deficits in memory performance similar to demented patients was achieved with the use of scopolamine in primate models and young human volunteers[36-38], and this idea was supported by the amnesic properties reported from other clinically used anticholinergics[39]. As part of an earlier survey of biochemical changes in the AD brain, reduced levels of acetylcholine esterase (AChE) activity was observed [40], and a reduction in levels of choline acetyl transferase (ChAT) that correlated to cognitive impairment was reported independently by three groups[41-43]. Selective loss of cholinergic neurons in the central nucleus basalis and basal forebrain was observed in postmortem AD brains[44, 45], and the hippocampus was implicated in cholinergic dysfunction due to dense muscarinic innervation and loss of muscarinic neurons[46]. Taken together, these findings implicated the cholinergic system as an exciting new drug target.

The initial pharmacotherapeutic strategy for AD involved the use of the reversible acetylcholinesterase inhibitor physostigmine, a parasympathomimetic alkaloid derived from the West African calabar bean, *Physostigma venenosum*. The first experiments using physostigmine as a memory aid were conducted in young human volunteers, and physostigmine demonstrated a consistent, dose-dependent positive effect on long-term memory storage and retrieval[47]. In aged non-human primates, physostigmine reversed cognitive deficits, though the response was somewhat variable in aged animals, compared

to the consistent dose-response seen in younger animals[48]. Kenneth Davis, who conducted the human trials, later showed physostigmine to be beneficial in the AD patient population, though with the variable response to treatment seen in aged primates[49], a result that was quickly extended and replicated by several groups[50-53].

Based on these results and the cholinergic hypothesis, tacrine (1,2,3,4-tetrahydroacridin-9-amine), a centrally acting acetylcholinesterase inhibitor, was tested in 1981 in a small group of AD patients and shown to significantly improve their symptoms[54]. A later, large-scale clinical trial was conducted, and, in 1986, tacrine was found to cause a significant delay in cognitive decline measured by ADAS, a small but not significant benefit on the MMSE, and no effect on global measures of disease progression[55]. In 1992 these results were sufficient for the FDA to approve tacrine (Cognex™) as the first treatment for mild to moderate AD. However, tacrine was far from an ideal drug, as it required four times a day dosing and led to significant gastrointestinal and CNS side effects as well as possible liver damage in many patients[56, 57], and for these reasons tacrine is rarely used clinically today. However, its approval helped validate the cholinergic hypothesis as an approach to AD and opened the door to other novel compounds.

Donepezil((RS)-2-[(1-benzyl-4-piperidyl)methyl]-5,6-dimethoxy-2,3-dihydroinden-1-one), the current preferred treatment for mild to moderate AD, was discovered in 1983 after a Japanese group at Eisai Co., Ltd. serendipitously discovered an N-benzylpiperazine derivative with significant activity in screening assays, while evaluating unrelated tacrine derivatives (for review of medicinal chemistry development, please see [58]). Two Phase III clinical trials were conducted in Japan, using both a 52-

and 24-week open study approach, and donepezil showed significant improvement on both ADAS-Cog and Clinician Interview Based Impression of Severity (CIBIC)[59] compared to placebo control[60, 61]. Clinical trials conducted in the US showed similar improvement in ADAS-Cog and CIBIC, as well as delayed loss in activities of daily living (ADL) by an average of 1 year. Further, these effects correlated with reduced ChE activity, at doses showing no hepatotoxicity and few side effects[62-64]. These results led to FDA approval for treatment of mild to moderate AD in 1998, and Eisai began US marketing of donepezil (Aricept™) in conjunction with Pfizer. Recent meta-analysis by the Cochrane group of over 23 clinical trials confirmed that low doses of donepezil provide a significant benefit in early stage AD with few adverse events, but doses of 10 mg/kg or higher may not provide any clinical advantage given reduced tolerability[65].

Two other second-generation ChEIs, galantamine (Razadyne™) and rivastigmine (Exelon™), show comparable pharmacological profiles to donepezil in clinical trials. Importantly, improvements in clinical performance on ADAS-Cog, MMSE and ADL are equal and achievable with all second-generation ChEIs, while GI side effects are slightly worse with galantamine[66] and rivastigmine[67]. Rivastigmine is notable for being the first treatment to be sold as a transdermal patch, which reduced GI complaints while maintaining equal clinical efficacy[68]. Not every ChEI has fared as well, and Bayer discontinued the FDA application process for metrifone, a long-acting irreversible cholinesterase inhibitor, after respiratory failure and neuromuscular junction impairments were reported in early clinical trials[69]. Currently, donepezil and related second generation ChEIs remain the preferred treatment for mild to moderate AD, despite the fact that less than half of all patients show a clinically significant response and trying to

identify responders from non-responders has not been fruitful[70]. Additionally, no new cholinesterase inhibitors have been approved since galantamine in 2001, and no ChEIs are FDA approved for moderate to severe AD.

II.D. APP Processing and Drug Approaches

Some 20 years after ultrastructural descriptions by electron microscope, the major protein component of extracellular plaques was found to be multimeric aggregates of A β , a polypeptide about 40 amino acids in length[71, 72]. However, A β is a protein secreted normally and is present even in the absence of pathology, though not at the levels seen in AD[73, 74]. Around this time, an earlier observation that patients with Down Syndrome (DS) had clinical manifestations very similar to presenile dementia of the Alzheimer's type yielded the discovery of amyloid precursor protein (APP) on the long arm of chromosome 21 (the trisomy in DS), and APP was implicated in AD and DS by genetic linkage analysis[75, 76]. Cloning of the cDNA of A β confirmed it as part of its parent protein, APP; a cell surface receptor protein of 695 amino acids[77, 78]. Shortly thereafter, mutations in APP were discovered that correlated to increases in levels of plaque fragments and clinical dementia[79-81]. Many of these familial variants, including one type found in a Swedish family comprising a double mutation of K670N and M671L associated with a 6-8 fold increase in A β production (APP_{swe})[79], were later incorporated into transgenic animal models of familial Alzheimer's disease (FAD) as the Tg2576 line[82].

The *APP* gene contains 18 exons and is differentially spliced, with APP695, lacking exons 7 and 8, being the predominant isoform expressed in the brain[83]. Two related genes express APP-like proteins but do not contain the A β sequence (*APLP1*,

APLP2); and, unlike APP and APLP1, which are both expressed ubiquitously, APLP2 is found only in the brain and only in mammals[84, 85]. APP undergoes sequential cleavage after expression and transport to the plasma membrane, with the initial cleavage event mediated by either the α - or β -secretase, and final cleavage mediated by the γ -secretase complex, with the α/γ pathway being constitutively active in healthy, normal neurons.

Three members of the “a disintegrin and metalloproteinase” (ADAM) family, ADAM-9, -10, -17, have been proposed as the α -secretase[86-88]. ADAMs are members of the zinc protease super family and are integral membrane proteins. ADAM-10 is widely expressed in the brain and has been linked to constitutive α -secretase cleavage, with overexpression of ADAM-10 in AD transgenic mice linked to increased sAPP α , the major cleavage product of the α -secretase pathway, and reduced formation of plaques and reversal of cognitive deficits; while mice with inactive ADAM-10 demonstrate increased plaque pathology[89]. ADAM-9 and -17 have a negligible role in constitutive α -cleavage, with transgenic knockout animals showing no effect on sAPP α generation[90], but many other substrates have been identified for these two isoforms, including TNF- α , the EGF family, prions and ADAM-10[91-94]. Interestingly, statins, which have been correlated in epidemiologic studies to reduced risk of dementia[95], have been shown to increase sAPP α , possibly indicating a shift to nonamyloidogenic processing through α -secretase activation[96, 97].

Leading to the production of the pathological A β fragment is cleavage of APP by the β -secretase into the sAPP β fragment and the membrane-associated C-terminal fragment, C99. The β -site APP cleaving enzyme 1 (BACE1) protein, a transmembrane

aspartyl protease, was discovered in 1999 by various groups[98-101], and found to be expressed throughout the body, including on most post-Golgi membranes and the cell surface[102]. The discovery of the BACE1 protein led to intense interest in the drug discovery world, especially after finding that (1) BACE1 KO decreased pathological A β *in vitro*[103], (2) BACE1 KO animals had normal phenotypes and decreased plaque pathology[104, 105], (3) BACE1 KO coupled to the Tg2576 model rescued cholinergic deficit and reversed cognitive impairment without apparent phenotype changes[106], and (4) the observation that BACE1 was overexpressed in AD brains[107, 108]. Unfortunately, in APP^{swe}/PS1 Δ E9 double transgenics, BACE1 KO led to subtle impairments in cognitive, emotional and synaptic function[109], and further analysis of BACE1 KO mice revealed subtle phenotypic deficits not apparent on initial examination, including hypomyelination and seizure activity[110-112]. Despite these findings, intense interest remains in determining the effect of BACE1 inhibition in human AD.

Final cleavage of the C-terminal fragments from either the α - or β -secretase pathway is mediated by γ -secretase, a large complex of four major proteins: presenilin 1 or presenilin 2 (PS1, PS2); presenilin enhancer 2 (PEN2); anterior pharynx-defective 1 (APH1); and Nicastrin[113]. The presenilins were identified on chromosome 1, which was linked to AD, and these proteins were required for proteolytic action of the enzyme[114-116]. Shortly after discovery, several transgenic mice were developed with FAD mutations in PS genes, and increased levels of pathological A β ₁₋₄₂ fragments were observed in these lines[117, 118]. γ -secretase can cleave APP to generate many fragments of A β , ranging from 39-43 amino acids (aa), with the most common form being 40 aa, the pathological fragment being 42 aa, and rarer cleavage events generating

46 or 49 aa A β fragments. The generation of these fragments depends on prior α - or β -secretase cleavage and modifications in the γ -secretase complex. Additionally, cleavage generates an internal fragment of various lengths deemed AICD – the role of which is still a matter of some debate.

The γ -secretase complex deserves special consideration as it has been thoroughly explored as a drug target. Eli Lilly and Elan developed LY450139 dihydrate, later called semagacestat, to directly inhibit the presenilin dependent γ -secretase complex. Initial publication of information on the newly developed compound was released in a spate of abstracts during the 2004 9th International Conference on Alzheimer's Disease and Related Disorders[119-123]. A precursor drug, LY411,575, had already been shown to potently inhibit the γ -secretase complex, leading to decreased amyloid levels, but also affected immune and intestinal cell maturation, due to γ -secretase's diverse function, notably in Notch processing[124].

An initial 70-person Phase II trial revealed significant decreases in plasma levels of total A β in a biphasic manner, and the safety profile was reported as “well tolerated” despite several gastrointestinal (GI) related adverse events (AE), including one patient who died from GI bleeding one month after discontinuing semagacestat[125]. A second Phase II trial involving 51 individuals demonstrated similar reduction in plasma A β levels (64.6%) at the highest dose, but skin and subcutaneous AE were noted in addition to GI issues[126]. Still, these data were considered to prove semagacestat “generally well tolerated”, and it advanced into two large-scale Phase III trials. Concurrently with ongoing Phase III trials, a stable isotope method was used to assess the potency of semagacestat on A β production in the CSF, and reductions of up to 84% were seen in the

highest dose group[127]. However, Eli Lilly announced in August 2010 that all Phase III trials of semagacestat were to be halted due to preliminary data indicating that semagacestat not only failed to slow disease progression and cognitive decline, but the treatment group performed significantly worse in clinical assessments of cognitive performance and that “semagacestat is associated with an increased risk of skin cancer compared with those who received placebo” [128].

II.E. The Amyloid Cascade Hypothesis

The A β fragment is deserving of special consideration as the central player in the amyloid cascade hypothesis, a theory which is still highly regarded in the AD field, and which is coherently articulated in a landmark review in 1998 by Dennis Selkoe[129]. The current incarnation of the amyloid cascade hypothesis posits that A β assembles into heterogeneous soluble oligomers that interfere with normal cognition and forms the classic plaques detectable on autopsy in the Alzheimer’s Disease (AD) brain[130, 131]. The “cascade” part of the hypothesis suggests that unknown factors initiate a feed forward cycle leading to accumulation of A β ₁₋₄₂ in the brain, causing synaptic failure, and, eventually, neuronal death. Herein are detailed several drug discovery approaches from the last two decades that followed directly from the amyloid cascade hypothesis.

In 1999, a team at Elan Pharmaceuticals led by Dale Schenk demonstrated successful vaccination in the PDAPP mouse model of AD, which has a V717F APP mutation driven by a PDGF promoter[132, 133]. Schenk showed that active immunization with full length human A β starting at six weeks of age was sufficient to prevent plaque deposition and astrogliosis, while immunization starting at 11 months of age significantly and dramatically reduced the progression of disease pathology

compared to control groups[134]. This discovery immediately prompted the call for movement of synthetic A β vaccination into humans, while reservations were expressed over the PDAPP model's lack of important pathological hallmarks and lack of correlation of plaque removal to functional improvement[135].

The synthetic full-length human A β_{1-42} peptide moved into clinical trials as AN1792, a vaccination agent designed to promote active immunity. Elan's promising lead faltered in 2004, when Phase II trials were halted after 18 of 298 patients (6%) developed symptoms consistent with meningoencephalitis[136]. A small cohort was examined that suggested the efficacy profile might be favorable[137], but subsequent follow up of the 20% of patients who developed the predicted antibody response failed to show improvement on a multi-inventory battery of neuropsychological tests (MIBNT) of dementia, despite so-called positive indicators, such as lowered tau measured in the CSF of antibody responders[138]. In 2010, postmortem analysis of a large subgroup of study participants was possible, and significant decreases in amyloid plaque and neuritic dystrophy with mild decreases in tau were observed[139]. These findings corroborated a 2008 study showing the efficacy of AN1792 in decreasing plaque burden, but the 2008 study also included MIBNT and concluded no functional correlation existed between the A β lowering and mortality or disease progression[140].

Complementing Schenk's initial active immunization approach, Elan developed a passive immunization program and in 2000 reported that bapineuzimab, a monoclonal antibody raised against a fragment of A β peptide, could cross the blood brain barrier and activate microglial clearance of plaques after 6 months of treatment in 8-10 month old PDAPP mice[141]. These results were confirmed by an outside group in 2001, who

proposed increased plasma clearance as the mechanism behind plaque removal[142]. Importantly, the passive vaccination approach was discovered to have functional correlates in behavioral models of memory such as the radial arm water maze[143]. However, a warning siren sounded in 2002 when passive immunization was found to weaken amyloidotic blood vessel walls, leading to cerebral hemorrhage in APP23 mice[144].

Bapinezumab's faring in clinical trials was hampered initially by fears of the same adverse events (AE) as AN1792, but the initial small study on a subset of AN1792 patients mentioned previously suggested a possible beneficial effect[137], so bapinezumab was advanced. In 2008, a Phase II trial designed to measure safety concluded, reporting vasogenic edema in 12 of 124 (9.7%) bapinezumab treated patients, especially at higher dosages and in carriers of the APOE4 allele[145]. Outcomes were not significant in regard to bapinezumab's efficacy, but the study was not powered specifically to measure this outcome, though MIBNT did take place. Based on these results, the decision was made to proceed to Phase III trials, while dropping the highest dose. Currently, Johnson and Johnson, which bought Elan's AD development program in 2008, has eight open Phase III trials for bapinezumab, and results are expected to be announced at the end of 2012.

From epidemiological studies, NSAIDs were known to prevent or delay the onset of AD, though the mechanism of action of this effect was unknown[146]. In a 2001 paper, a group at the University of California, San Diego, reported the COX-inactive R-enantiomers of a group of NSAIDs to be capable of lowering $A\beta_{1-42}$ in cultured cells[147]. Later work at UCLA focused on R-flurbiprofen and replicated the 2001

findings *in vivo* and *in vitro*, though the group was only able to conclude the effect was not through an NF κ B-dependent mechanism[148]. In August of 2003, two groups independently reported R-flurbiprofen's mechanism to be through selective modulation of the γ -secretase complex to prevent formation of the more toxic A β ₁₋₄₂ peptide, and R-flurbiprofen was dubbed a selective A β 42 lowering agent (SALA)[149, 150]. Later, in the Tg2576 mouse model of AD, R-flurbiprofen was found to attenuate learning deficits in the Morris Water Maze (MWM) if given to very young mice, while not significantly reducing plaque levels[151]. When the same treatment regimen was given to older mice, significant reduction in plaque pathology occurred, while no noticeable improvement was seen in the MWM[151].

Based on these preclinical data, Myriad Pharmaceuticals moved R-flurbiprofen into clinical trials as tarenflurbil (FlurizanTM). Initial results from Phase II trials were promising, with tarenflurbil being well tolerated and associated with a significant decrease in cognitive decline in patients given 800mg daily dosing for 12 months, with plasma levels of tarenflurbil positively correlating with improvement in MIBNT[152]. Unfortunately, in a large-scale Phase III study involving almost 1700 participants, these positive results failed to materialize, and tarenflurbil showed no efficacy on either primary or secondary outcomes and the study concluded it had no effect on cognitive decline or daily living[153].

An alternative hypothesis to immune-mediated clearance or inhibiting amyloid production was chemical disruption of aggregation of A β plaques. A group at the University of Toronto demonstrated with atomic force microscopy that the presence or absence of certain glycosaminoglycans (GAGs) can lead to differences in amyloid

nucleation and fibril formation, the earliest stage of plaque development[154]. Based on these and other data, Neurochem Inc. announced the commencement of Phase I trials for the sulfated GAG-mimetic NC-531, later revealed to be homotaurine or tramiprosate (Alzhemed™), as an amyloid anti-aggregant[155].

In a small open label Phase II trial, tramiprosate was found to significantly reduce CSF levels of $A\beta_{1-42}$ in a dose related fashion, but no improvement was seen in any MIBNT[156]. Tramiprosate was advanced to a 67 center Phase III trial involving over a thousand participants. In 2007, at the 2nd Alzheimer's Association International Conference on Prevention, Paul Aisen, the director of the Phase III study, announced that the "variation among sites was so great that it violated the assumptions underlying the statistical model the study set out to use" and alternative statistical methods were to be employed[157]. The FDA disagreed with the approach, and invalidated the study stating the statistics did not support any claim of efficacy[158]. This news came on the heels of a finding that tamiprosate interfered with the aggregation of tau as well as amyloid, favoring abnormal aggregation of tau in neuronal and non-neuronal cells[159]. Because homotaurine is a natural ingredient in seaweed, Neurochem, Inc. reincorporated as Bellus Health and rebranded tamiprosate as the nutraceutical Vivimind™, allowing sales and marketing in Canada as a natural health product.

No drug has yet come to market using the amyloid cascade hypothesis as a guiding principle, but it remains the mainstay of AD drug development and many treatments are currently in clinical trials that may yet yield positive results.

II.F. Tau Tangles and Drug Targeting

Almost forty years ago, it was discovered that tubulin would not polymerize in the absence of a specific factor deemed a “ τ factor” for its action in inducing microtubule formation[160]. Later elucidation of functional properties revealed that tau was a family of closely related phosphoproteins ranging in weight from 55 to 62 kDa, and phosphorylation decreased tau’s ability to induce microtubule formation[161]. These findings were of relatively limited interest until 1986, when several groups independently reported that abnormally phosphorylated tau was the major component of the paired helical fragments making up intracellular tangles seen in the AD brain[162-166]. This led to a flurry of interest and investigation into the physiological and pathological role of tau, much of which will be discussed below, but, after the discovery that familial Alzheimer’s disease (FAD) was due to mutations in *APP*, *PS1* and *PS2* genes[81, 114, 167, 168], the field moved to a view that tau was secondary to A β in the AD disease process[130]. However, several tau mutations were found to contribute to a neurodegenerative disease process in frontotemporal dementia and parkinsonism linked to chromosome 19 (FTDP-19) that did not involve amyloid[169], and several neurodegenerative tauopathies have since been discovered including sporadic corticobasal degeneration, progressive supranuclear palsy, and Pick’s disease[170].

Central to tau’s physiological and pathological action is phosphorylation, which occurs at many sites on the tau protein. Abnormal hyperphosphorylation of tau is a well-accepted component of AD pathology, but rapid dephosphorylation in postmortem samples makes positive identification of pathological phosphorylation patterns difficult[171-173]. However, a threonine at position 231 (T231) has been identified as an early phosphorylation site leading to pathological activity, including reduced activity to

bind to and stabilize microtubules[174-176]. Phosphorylation at T231 and other sites is mediated *in vitro* by glycogen synthase kinase-3 β (GSK3 β), deemed by some the “tau kinase”[176, 177]. GSK3 β inhibitors are a central feature in tau kinase-based drug discovery approaches[178], but this promising approach has yet to reach clinical trials.

GSK3 β plays a key role in the Wnt signaling pathway, important for regulation of the cell cycle and during neural development[179], and this activity raises significant concerns of off-target effects for non-specific GSK3 β inhibitors. Other concerns are raised about whether T231 is central to the disease process and another phosphorylation site, a serine at position 262 (S262), is also phosphorylated early in the disease process and decreases microtubule binding, but GSK3 β does not appear to play a role in its phosphorylation in fly models[180, 181]. The issue is further complicated by the finding that site-specific phosphorylation can often direct or alter further phosphorylations, affecting microtubule binding and aggregation[182], and combinations of phosphorylation sites, rather than single sites, are important in mediating toxicity in fly models[183]. An alternative twist on the kinase approach is enhancing activity of tau-directed phosphatases, like phosphoprotein phosphatase 2a, but this approach is still relatively novel[184].

Since tangles have been used to stage disease progression[185], and their presence roughly correlates with disease burden[186], a natural approach to treatment is prevention of aggregation. This approach is supported by the observation that monomeric tau does not appear to hinder microtubule-dependent axonal transport, but filamentous tau leads to reductions in transport in the squid giant axon model[187]. A phenothiazine, methylene blue, has been proposed as an anti-aggregant, due to its ability

to prevent beta sheet conformations[188-190]. Use of these agents in *in vivo* models has yielded mixed results, with methylene blue able to prevent neurodegeneration but unable to rescue cognitive deficits in mouse models of tauopathy except at levels high enough to affect even soluble tau isoforms[191]. Unfortunately, completed Phase 2 clinical trials by Elan have shown no effect on primary or secondary endpoints by methylene blue (NCT00568776). Alternative anti-aggregants such as cyclohexanehexol inhibitors have also been proposed[192].

The immunotherapy approach has thus far been unsuccessful for A β , but the same approach has not yet been attempted using tau as a target[193, 194]. Active immunization approaches have been shown to reduce tau and reverse cognitive deficits in mouse models with the frontotemporal mutation P301L (JNPL3 mice) using a fragment of tau phosphorylated at S396 and S404[195, 196], and these findings were replicated in a mouse model expressing all six human tau isoforms and an M146L PS1 mutation[196]. An alternative approach by an outside group demonstrated CNS penetration of phosphoNFT antibodies with a concomitant reduction in NFT and no measurable encephalitis, an important concern given the results of active immunization for A β [197]. However, a few warning signs have been seen as earlier studies have shown that tau-directed immunotherapy can result in increased histopathology and neurological defects like limb paralysis[198]. A major hindrance in immunotherapy is that it is not completely clear from the basic research field which type of tau is the pathological fragment and thus should be targeted for immunologic removal. Some suggest that oligomers of tau are the pathological species[194], and efforts have been made to purify

such conformations to use as a possible immunogen for vaccine development[199]. The immunotherapeutic approach has not yet been tested in humans.

One last approach in tau-directed treatments is targeting tau function in regards to microtubules. Since the hyperphosphorylation seen in AD brains is known to reduce binding to and stabilization of microtubules, and microtubules are necessary for axonal transport[200], it is not unreasonable to suggest that microtubule stabilization might provide benefit to AD patients. Known microtubule stabilizers similar to paclitaxel have been tested and shown to prevent synaptic loss due to lysosomal stress in brain slices, but these compounds suffer from very low CNS bioavailability[200], which has led to recent attention by medicinal chemists seeking to increase brain penetrance[201]. An approach using intranasal delivery of an octopeptide called NAP, known to stabilize microtubules, has shown reductions in levels of A β and tau accumulation, especially the potentially toxic T231 phosphorylated tau[202].

II.G. ApoE and Drug Targets

Another protein of importance in AD is ApoE, a 299 amino acid apolipoprotein. Initial studies focused on its role in lipid and cholesterol transport as ApoE is found in chylomicrons and both high-density and low-density lipoproteins (HDL, LDL). ApoE functions as a cholesterol scavenger from blood vessels and directly interacts with LDL receptors to internalize lipids and cholesterol[203, 204]. In the CNS, it replaces ApoA-1 as the major protein component in HDLs, and, though the highest concentration is in the liver, ApoE's second highest concentration is in the CNS[204, 205]. In 1991, its role in AD was revealed after a Japanese group doing a routine screen of brain lipids in postmortem AD patients found the presence of ApoE was highly correlated to

plaques[206]. Two years later, three independent American groups confirmed this and discovered the strong genetic association with the ϵ 4-mutation of ApoE (ApoE4) and late onset AD[207-209]. Three major variants of the *ApoE* gene differ at two positions, 112 and 158, as to whether a cysteine or arginine is present (ApoE2 = C/C, ApoE3 = C/R, ApoE4 = R/R). The role of ApoE4 in disease progression has been challenged[210], but systemic meta-analyses have held up the association: one ApoE4 allele increases risk of AD by 3-fold and two alleles increase risk 12-fold[211]. Additionally, a CNS protective role has been described for the ApoE2 mutation, which otherwise is associated with familial hyperlipidemia[212, 213].

ApoE has been proposed to be involved in many roles in CNS, including cell migration, axon guidance, synaptic plasticity, regeneration after injury, microtubule stability, neuronal survival and A β deposition (for review, see [214]). These complex roles may help explain why ApoE is upregulated 300-fold following neuronal crush injury, making up 2-5% of the total protein lysate[215], but the specific roles of the variants of ApoE remain a matter of debate. ApoE4-transfected N2a cell lines showed increased microtubule instability compared to ApoE3[216], and this finding has been hypothesized to be due to SDS-stable ApoE3's differential binding to microtubule associated tau protein[217]. Perhaps due to this, ApoE4 was found to hinder neurite outgrowth compared to ApoE3 *in vitro*[218], but some studies with different preparations of ApoE failed to replicate this[219]. Finally, some authors have suggested isoform effects may be due to differential binding of ApoE to receptors, with ApoE4 binding poorly compared to ApoE3 (for review, see [220]).

The role of the ApoE variants is an important one to drug discovery. Particularly, it is necessary to know whether ApoE4 causes a loss of protective function or a gain of a toxic function. In the former, the target would be to increase ApoE3 or create “structure correctors” that make ApoE4 function more like ApoE3[221], while the latter approach would commend removal of ApoE4 or employing receptor blockers to prevent ApoE4 binding. An approach currently gaining traction is based on the hypothesis that ApoE mediates A β transport, with ApoE4 less able to bind to receptors to clear harmful species of A β [222, 223]. This hypothesis gained significant support after the finding that bexarotene, a drug approved for the treatment of cutaneous T-cell lymphoma, rapidly cleared plaques from the brains of transgenic mouse models and significantly improved cognitive function, through a mechanism dependent on upregulating ApoE through retinoid X receptors (RXR)[224]. Due to these promising results, bexarotene has been proposed as therapeutic for AD and is currently fast tracked in clinical trials[225].

II.H. Neuronal Loss & Synaptic Impairment: An Underdeveloped Drug Target?

Current drug discovery approaches to neurodegenerative disorders, including Alzheimer’s, are focused on treating histopathology; whereas, successful clinical approaches to Alzheimer’s are symptomatic. Disorders in which aging is the major known risk factor are likely to benefit from treatment with neuroprotective agents; however, the majority of animal models manifesting appropriate histopathology do not model the neuronal loss seen in the human disorder, and, unfortunately the above drug development approaches in AD have yet to produce a treatment for AD. Herein we present results from drug development stemming from the nitrate chimera hypothesis

(Figure 1), which targets synaptic restoration and incorporates ancillary neuroprotective pharmacophores. The following three chapters detail work (1) developing and testing the mechanisms of action of new methiazole backbones which have been incorporated into novel nitrate chimeras, (2) testing the efficacy and providing mechanistic information for GT1061, a prototype nitrate chimera in the nomethiazole class, in models of neurodegeneration, disrupted synaptic function and memory impairment, (3) applying the nitrate chimera approach to selective estrogen receptor modulators (SERMs) to overcome therapeutic limitations and extend neuroprotective and procognitive actions in the presence of disrupted NOS signaling.

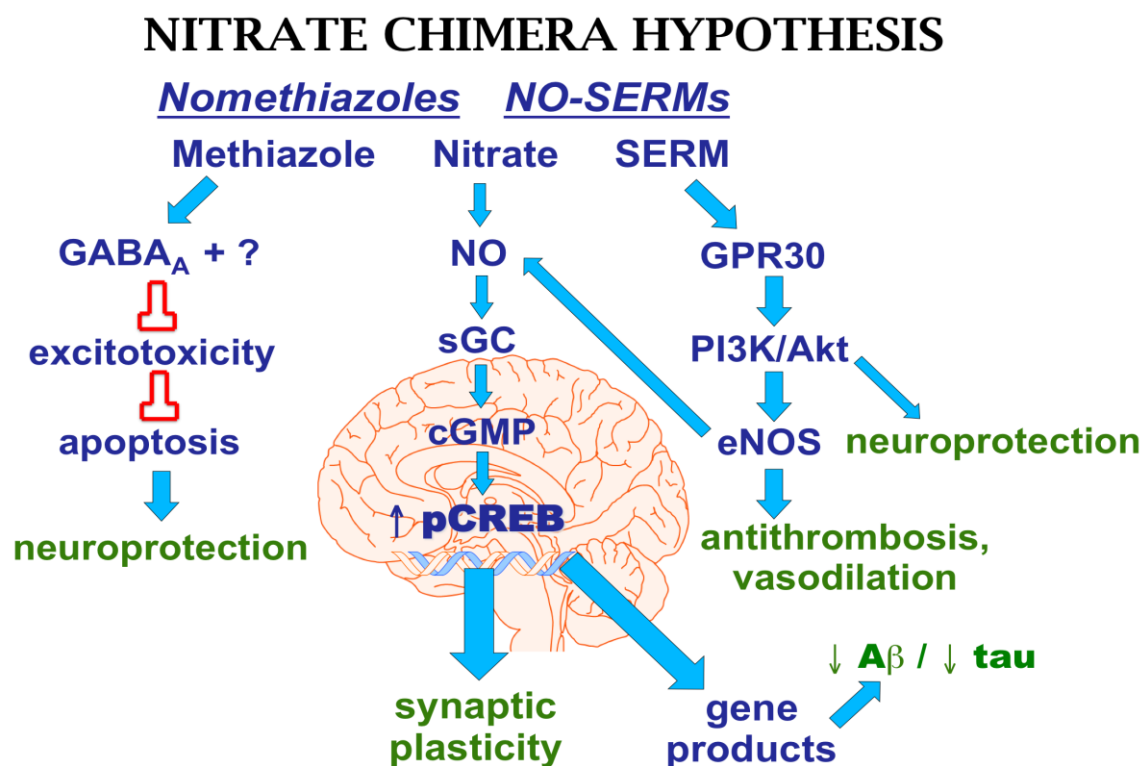


Figure 1: Proposed Mechanism of Action of Nitrate Chimeras

Nitrate chimeras are intended to act in a procognitive fashion by targeting increased synaptic plasticity. The approach is based on nitric oxide activation of soluble guanylyl cyclase, which increases cGMP that acts downstream through diverse kinases to increase phosphorylation of CREB, a transcription factor necessary for synaptic remodeling and memory formation.

Nomethiazoles (such as GT1061), which incorporate a methiazole backbone, add in GABA_A-dependent and -independent activity to decrease excitotoxicity, leading to neuroprotection. NO-SERMs, which incorporate procognitive and neuroprotective selective estrogen receptor modulators (SERMs), use the nitrate chimera approach to preserve neuroprotection, but add in procognitive activity in the absence of proper NOS signaling and provide anti-thrombotic activity to overcome the major therapeutic limitation of clinically used SERMs.

III. Novel Methiazoles are Neuroprotective in Four Models of Neurodegeneration, through a Mechanism with Variable Dependence on GABA_A Receptors

III.A. Methiazole Introduction

Neuroprotection, defined as the prevention of neuronal cell death, is of primary importance in pharmacotherapy of many intractable neurodegenerative diseases such as stroke and Alzheimers Disease (AD). Two challenges are foremost in this endeavor: finding appropriate mechanisms to combat complex and multifactorial insults; and, ensuring adequate delivery of the drug to the brain. In ischemic stroke, a triggered apoptotic response occurs after diminished blood flow and reduced glucose decreases levels of ATP, resulting in disrupted calcium homeostasis and increased levels of extracellular glutamate. The elevated levels of the excitatory neurotransmitter glutamate lead to excitotoxic overactivation of neural networks, leading to further disruption of ion balance and activation of apoptotic pathways. One proposed mechanism to block this positive feedback cycle is administration of a treatment working on GABA_A receptors to allow compensatory chloride ion influx.[226] This action results in decreased neuronal firing, leading to decreased glutamate release and reduced excitotoxicity.

In stroke, a triggered apoptotic response occurs after ischemia in the infarcted area. Due to diminished blood flow and glucose supply, decreased levels of ATP lead to disruption in ion balances, resulting in increased levels of extracellular glutamate, a primary excitatory neurotransmitter in the CNS. Two main mechanisms impair glutamate homeostasis: disruption of the Na⁺ gradient, leading to failure of glutamate reuptake machinery, and impaired Ca⁺⁺ sequestration, leading to excessive glutamate release into the synaptic cleft. An increased level of glutamate extracellularly leads to

excitotoxic overactivation of neural networks, activating apoptotic mechanisms by further disruption of ionic balance, especially intracellular calcium homeostasis.

A similar pattern of aberrant overactivation has been observed in Alzheimer's Disease (AD),[227, 228] hypothesized to be in response to soluble oligomers of beta amyloid (A β). Overproduction of human A β in the hAPP mouse has been shown to lead to aberrant epileptiform activity at the network level,[229, 230] and A β has been shown to lead to apoptotic events in cultured cells through a glutamate dependent pathway.[231-233] In the cholinergic forebrain nucleus, A β increases mEPSCs, further implicating upregulated activity at excitatory receptors.[234] However, neuronal death occurs mainly at later stages in AD, so while neuronal network overactivation and cell death are not primary or initiating events, prevention of overt cell loss can be expected to modify disease progression by halting the cycle of death and subsequent increased dysregulation.

Targeting of GABA signaling has been widely employed in prevention of epileptic seizure activity through such compounds as vigabatrin, valproate, and gabapentin, all of which have been shown to upregulate GABA activity through diverse mechanisms. Unfortunately, trials of these anti-epileptic compounds as therapeutics for neurodegenerative disorders have met with mixed results. Vigabatrin failed to illicit substantial neuroprotection in mouse models of transient focal ischemia[235] and has not been tested in AD models. Valproate demonstrated neuroprotection both in a corticostriatal slice model of ischemic stroke[236] and *in vivo* rat models of transient focal ischemia,[237] but this protection was not replicated in either rat hippocampal slices exposed to oxygen glucose deprivation (OGD)[238] or in a subsequent mouse model of transient focal ischemia.[235] In AD, valproate has been shown to reduce

behavioral deficits and inhibit A β production in the APP23 mouse model,[239] but in human studies on agitation in dementia it was poorly tolerated in the AD population.[240] Gabapentin was not found to be neuroprotective in rat hippocampal slices exposed to OGD[238] but did reduce non-convulsive seizures in rats following permanent ischemic injury, though the extension of this effect to neuroprotection was not demonstrated.[241] Like vigabatrin, gabapentin has not been studied as a treatment for AD.

These findings are likely due, in part, to the heterogeneity of GABA_A receptor assembly and distribution, with different pharmacological subunit assemblies affecting such activity as sedation and anxiolysis.[242] Determination of the specific combination of receptor subunits for pharmacological targeting of specific disease states remains intractable.[243, 244] However, potential applications have been proposed for GABAergic compounds in diseases as diverse as schizophrenia, stroke, depression, and analgesia; complimenting their current utility as anticonvulsants, anxiolytics, and sedative-hypnotics.[226, 245]

Two decades ago, chlormethiazole/clomethiazole (CMZ), a compound with CNS bioavailability and GABA potentiating activity at the GABA_A receptor,[246-249] was tested as a therapeutic in stroke, supported by earlier observations in a gerbil model of transient ischemia.[250] Historically, CMZ had been employed clinically for its anticonvulsant activity during alcohol withdrawal, as a sedative-hypnotic, and an anxiolytic; actions consistent with its proposed mechanism of action at GABA_A receptors. Initial observations of CMZ's substantial neuroprotection were replicated in several rat models of cerebral ischemia, which demonstrated that neuroprotection

correlated with reduction in extracellular levels of glutamate.[251-254] Importantly, the observed neuroprotection correlated with improvements in behavioral models of memory and in further studies both neuroprotection and functional recovery were demonstrated in non-human primates after focal cerebral ischemia.[255, 256] CMZ continues to be suggested as a potential component of future combination therapies for neuronal injury.[257]

Neuroprotective agents, derived from CMZ as a lead molecular scaffold and containing the 4-methylthiazole (MZ) pharmacophore, are a potential source of novel GABAergic therapeutics; however, data on only a very limited number of derivatives have been published.[258-262] In addition, CMZ itself has been reported to inhibit pro-inflammatory pathways associated with $\text{TNF}\alpha$, [263, 264] and to rescue mitochondrial function in brain tissues.[265] These observations further stimulate interest in MZ derivatives, since recent evidence supports inhibition of $\text{TNF}\alpha$ and restoration of mitochondrial function as therapeutic targets in AD.[266-270] Here, we explore the pharmacological profile of MZ derivatives using rat primary cortical neuronal cultures subjected to insults from oxygen-glucose deprivation (OGD), application of NMDA and glutamate, and of amyloid- β ($\text{A}\beta$). The extent of involvement of GABA-mediated mechanisms was explored using picrotoxin as a selective channel blocker of the GABA_A receptor and measurement of extracellular glutamate by HPLC. These results expand our understanding of the role of MZs in neuroprotection, and given the observation of brain bioavailability and lack of potent sedation, support the further development of MZ derivatives that incorporate modulated GABA-mediated inhibitory activity as therapeutic agents for treatment of neurodegenerative disorders including AD and stroke.

III.B. Methiazole Objectives

1. Perform preliminary structure activity relationship of all novel methiazoles in OGD screening assay to identify neuroprotective pharmacophore space, and select promising MZ analogues to investigate further.
2. Use picrotoxin, a GABA_A receptor channel blocker, to assess contribution of GABA to neuroprotective response and identify methiazole analogues with GABA-dependent and -independent activity for further study.
3. Confirm decreased excitotoxicity in neuroprotective response by measuring levels of extracellular glutamate by HPLC and correlate to GABA dependent neuroprotection.
4. For select MZ analogues, extend results to direct excitotoxic and amyloid models using picrotoxin to validate GABA component of mechanism of action.
5. Evaluate dose-response and time course in OGD to evaluate applicability to stroke; evaluate dose-response in alternative models to determine if mechanisms of action show differing efficacy.
6. Using C57Bl/6 mouse model, determine bioavailability and sedation in rotorod assay to support advancement to *in vivo* trials and to confirm GABA activity.

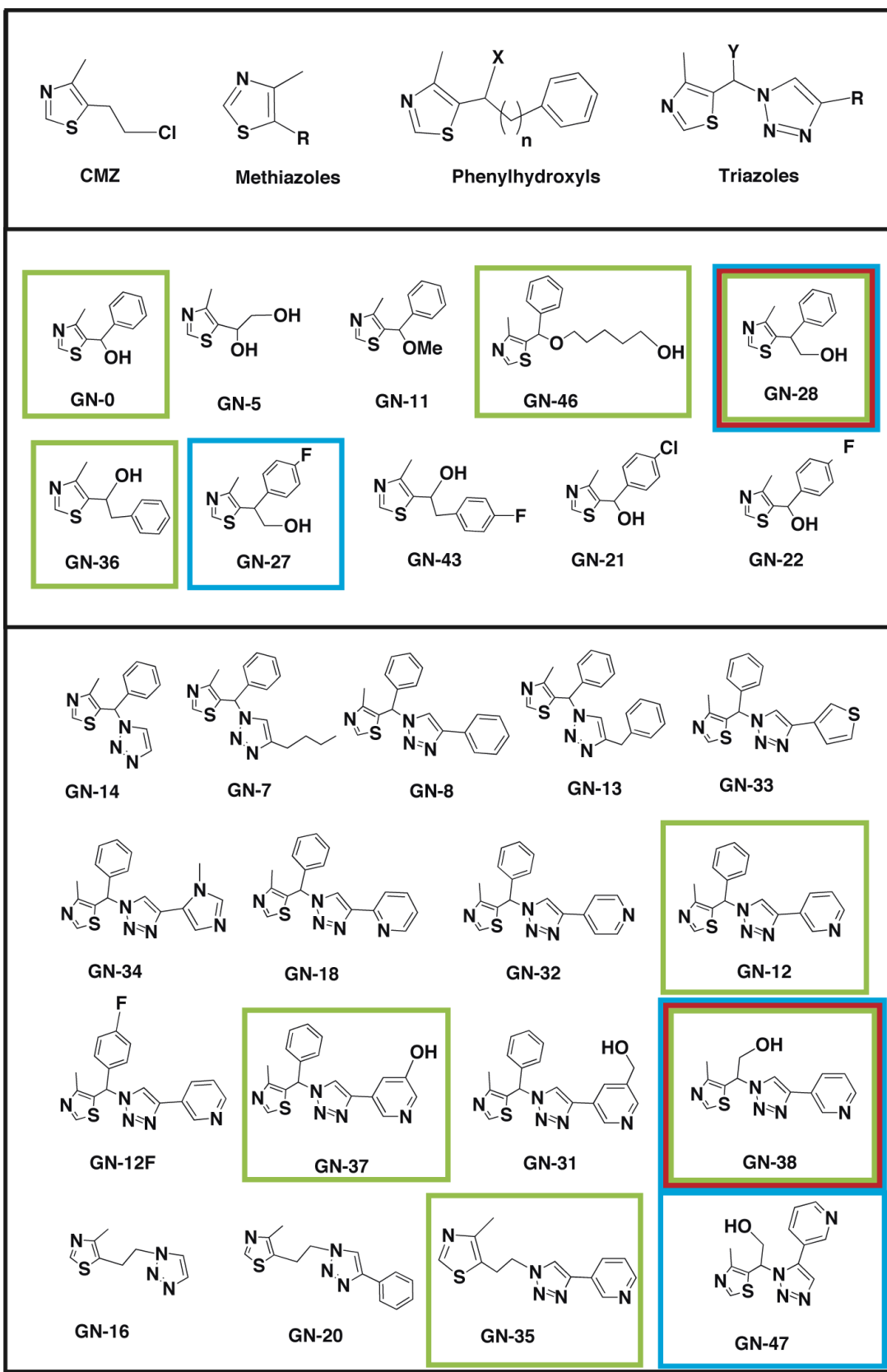
III.C. Methiazole Experimental Results

III.C.1. Methiazole Structures

Dr. Zhihui Qin, our lead synthetic chemist, designed and synthesized over fifty novel chlormethiazole (CMZ) analogues based on iterative refinement and structure activity relationship analyses. The details of this approach have been published elsewhere. For this study, twenty-seven methiazoles (MZ) were selected for sampling coverage of relevant pharmacophore space and arbitrarily divided into two subsets based on structural similarity for initial screening. Phenylhydroxyls have the general structure seen in Scheme 1, notable for the terminal phenyl group with variable p-fluoro (GN-27, GN-43, GN-22) or p-chloro substitution (GN-21) combined with a terminal hydroxyl group, converted into a methoxy in GN-11 or an ether linkage in GN-46. The triazole structural series contain a triazole ring connected to the MZ group by a one or two carbon

linker with variable substitution both on the triazole and at the alpha carbon of the triazole linker.

After initial screening, four methiazoles from each structural subset (green boxes) were selected for mechanistic investigation in oxygen glucose deprivation (OGD) and low-dose A β oligomer toxicity assays. Further analyses were conducted on two compounds, GN-28 and GN-38 (red boxes), including OGD, NMDA and glutamate toxicity assays, as well as against high-dose A β . These data, and associated CNS bioavailability, provided support for these two compounds and two related analogues GN-27 and GN-47 (blue boxes) to advance into *in vivo* studies after chimeric conversion into nomethiazoles.



Scheme 1: Chemical Structures of Methiazoles

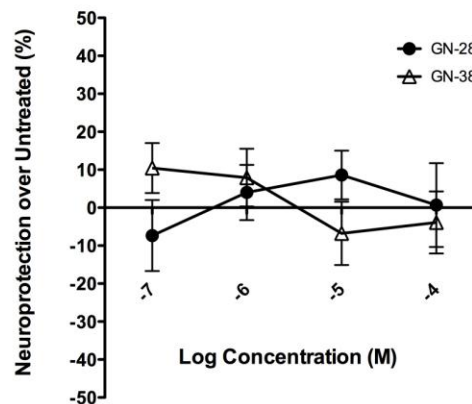
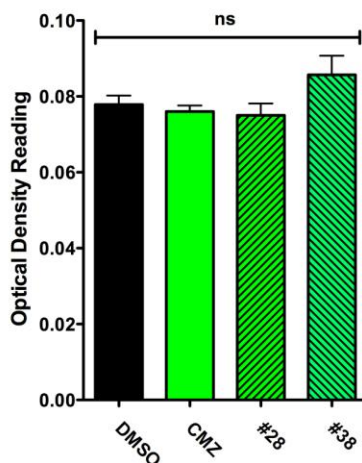
III.C.2. Methiazole Preliminary Data

As mentioned above, the goal in studying methiazole backbones was two-fold: (1) to determine neuroprotective activity and therapeutic utility of analogues in a variety of models applicable to Alzheimer's disease and stroke to support further development as nomethiazoles, and (2) to begin investigation into the likely pleiotropic mechanisms of action (MOA) of CMZ and other promising neuroprotective analogues. To this end, MZ derivatives were initially screened at a single 50 μ M dose in rat primary cortical neurons (10-12 DIV) subjected to OGD with analysis of cell viability by MTT assay. The OGD assay is a well-studied model of stroke, showing high levels of cell death both during oxygen and glucose deprivation and during oxidative injury after reperfusion. The OGD model is often employed for drug development, and it represents a reasonable high-throughput, low-cost model of apoptotic cell death, though direct applicability to AD is also necessary through later A β oligomer toxicity assays.

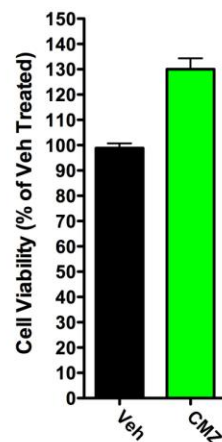
The 50 μ M dose was chosen to maximize protection, and no MZ toxicity was observed for neuroprotective analogues even at double this dose. Reduction of MTT is an efficient method of looking at cell survival, but strictly should be considered a marker for mitochondrial activity as mitochondrial enzymes cause reduction of MTT to the formazan dye. No artifactual interaction was observed between neuroprotective analogues and MTT in the absence of cells. Early parallel LDH assay confirmed congruency between cell death and cell viability and reliability was sufficiently strong to discontinue LDH in later assays. The selection of primary neurons was made since assays in immortalized neuroblastoma cell lines (SHSY-5Y) did not result in consistent and reproducible neuroprotection after CMZ treatment, while primary neurons subjected to OGD reliably resulted in a 30% increase in neuronal viability compared to untreated

control cells in over fifty independent replicates. The major limitation of the assay is that often a neuroprotective ceiling is hit, resulting in difficulty consistently distinguishing different levels of efficacy, so this assay is best employed for first-pass screening and testing of mechanism of action.

A. Interaction with MTT B. Toxicity - Primary Neurons



C. CMZ Protection



D. Cell Death from Insults

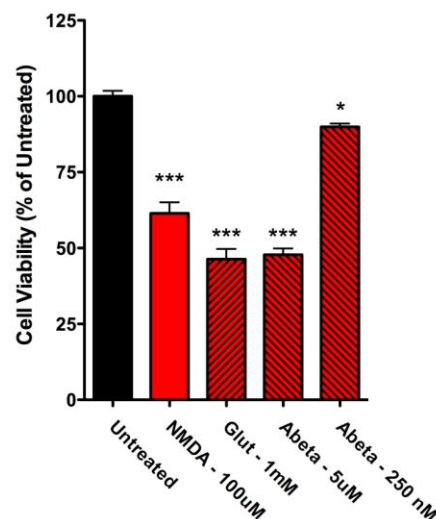


Figure 2: Preliminary Experiments

(A) No effect was seen when compounds were incubated 24 h with MTT, suggesting no artifactual reducing interaction between drugs and MTT. (B) Incubation with compounds for 24 h did not result in significant protection or toxicity at any dose studied. (C) CMZ showed

substantial, reproducible neuroprotection compared to untreated vehicle controls in OGD. Data shown represent mean and s.e.m. of over thirty independent experiments. (D) After treatment with various insults as described in the text, the amount of cell death measured by MTT and normalized to vehicle treated controls was reproducible and significant. Statistical analysis was done with ANOVA with post hoc Dunnet's MCT, comparing to vehicle control, * = $p < 0.05$, *** = $p < 0.001$.

III.C.3. Neuroprotective Methiazole Analogues Discovered Through Screening

Initial screening of methiazoles proceeded in tandem with synthetic chemists, allowing on-going refinement of the pharmacophore space. As mentioned previously, two main structural series were described and pursued, the so-called phenylhydroxyl and triazole series. Preliminary SAR analysis revealed substantial elaboration of CMZ's basic structure was possible without loss of neuroprotective activity, perhaps extending or isolating the mechanism of action. Initial additions directly onto the methylthiazole core not on the R position completely abolished activity and were not deemed methiazoles, and discontinued in further studies and thus not included here. Since OGD is a phenotypic screen, no mechanistic information is identifiable at this stage, but the functional nature of assay allowed the possibility of serendipitous discovery of additional mechanisms of action that could be described with further investigation.

Every phenylhydroxyl synthesized showed equal or greater neuroprotective activity as CMZ, with the exception of GN-21 and GN-22, two methiazoles with para halide substitutions on the phenyl ring of GN-0 (Figure 3). Interestingly, p-fluoro substitution on either GN-28 (GN-27) or GN-36 (GN-43) did not result in a loss of activity. Generally, the triazoles were more variable in their response, but neuroprotective analogues were discovered in GN-13, GN-34, GN-32, GN-12, GN-37, GN-31, GN-38, GN-35 and GN-47. GN-7 and GN-16 were the only triazoles that showed toxicity for unknown reasons. The GN-8, GN-18, GN-32, and GN-12 series was

notable for the position (or absence) of the nitrogen in the pyridyl group, and confirmed the 3-pyridyl group was most protective, and allowed further substitutions in GN-27, GN-37 or GN-31, as well as movement on the triazole ring (GN-47), though para-fluoro substitution on GN-12 (GN-12F) again resulted in decreased activity. Due to these rough SAR analyses, the eight compounds (Scheme 1, green boxes) were chosen to progress to mechanistic investigation.

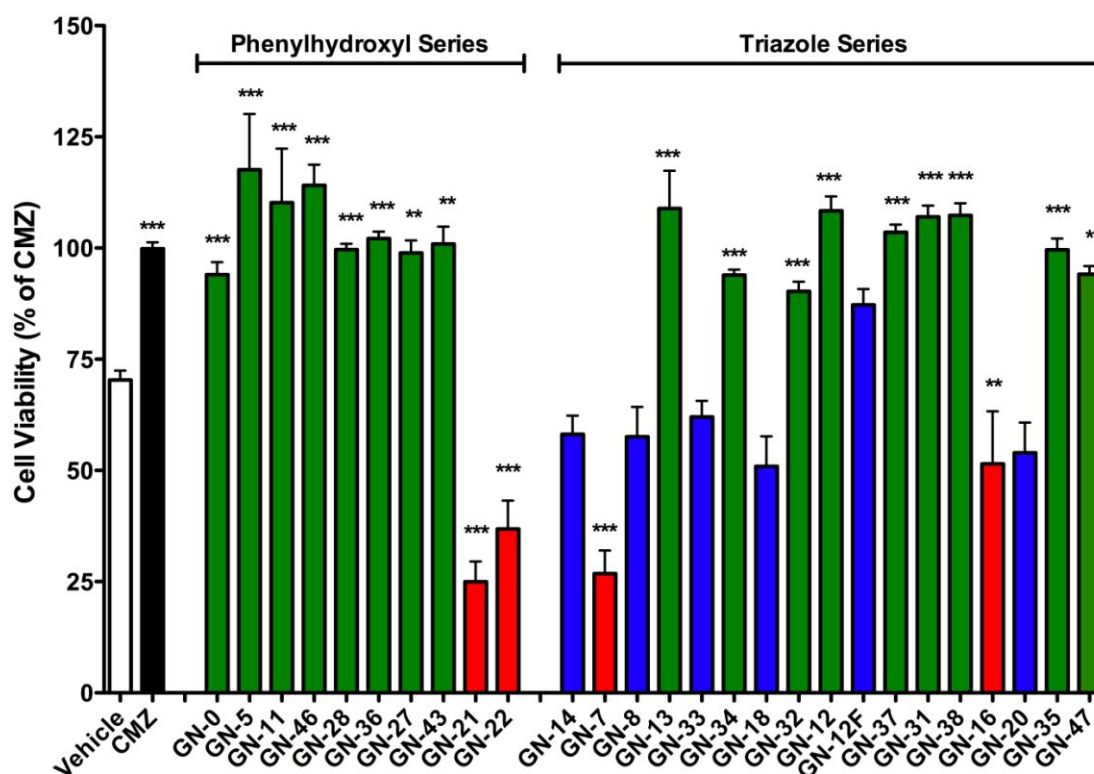


Figure 3: Neuroprotection of Methiazole Analogues after OGD.

Primary cortical cultures at 10-11 DIV were subjected to two hours of OGD with compounds supplied at 50uM. After reperfusion, cultures were incubated for 24 hours, after which time MTT assay was performed. Bars represent mean and SEM of at least six independent experiments. Results were normalized to CMZ and statistical analysis was done with ANOVA with post hoc Dunnet's MCT, comparing to vehicle control, ** = $p < 0.01$, *** = $p < 0.001$. Color corresponds to statistical results.

III.C.4. Picrotoxin Reveals Variable Dependence on GABA_A in OGD Neuroprotection

To investigate the contribution of the GABA_A receptor to the mechanism of neuroprotection, a subset of structural compounds with neuroprotective activity equal to or greater than CMZ were subjected to insult with or without picrotoxin, a GABA_A channel blocker, at 100 μ M, a dose sufficient to eliminate signaling through the GABA_A receptor without toxicity, with either blockers or vehicle applied one hour before the start of insult and continued throughout. Muscimol, a potent, selective direct agonist of GABA_A with demonstrated neuroprotection against excitotoxic insult, was included to provide an example of a pure MOA. While confirmation of agonism or allosteric modulation of the GABA_A receptor is not possible for novel analogues without formal binding studies, this approach would provide substantial support for involvement of the GABA pathway in reducing excitotoxicity, an activity confirmed in pharmacological investigation of CMZ.

For further study, two structural subsets of MZ neuroprotective agents were selected in both of which the methylthiazole ring of CMZ was preserved (Scheme 1): all were neuroprotective against OGD with equivalent or superior efficacy to CMZ, and assay with use of picrotoxin to block the GABA_A chloride channel revealed varied dependence on GABA_A signaling (Figure 4). MZ derivatives GN-0, GN-46, and GN-38 were neuroprotective, but with no significant loss of efficacy on picrotoxin co-treatment. In contrast, MZ derivatives, GN-12, GN-28, GN-35, GN-36, and GN-37, showed significant attenuation by picrotoxin. Muscimol, a selective, potent GABA_A receptor agonist, was used as a positive control at equimolar concentration to both CMZ and MZ derivatives. Significant neuroprotective activity, comparable to CMZ, was observed

(Figure 4), as reported previously by various authors[271, 272]. Pretreatment of cells with picrotoxin predictably ablated neuroprotection by muscimol, in agreement with the known mechanism of action. Reduction in activity by picrotoxin would implicate an effect mediated through the GABA_A receptor, as clearly demonstrated for muscimol. However, neuroprotective MZ derivatives, such as GN-38 and GN-36, maintained significant neuroprotection relative to control in the presence of picrotoxin, indicating mixed mechanisms of action, potentially with partial GABA-dependence.

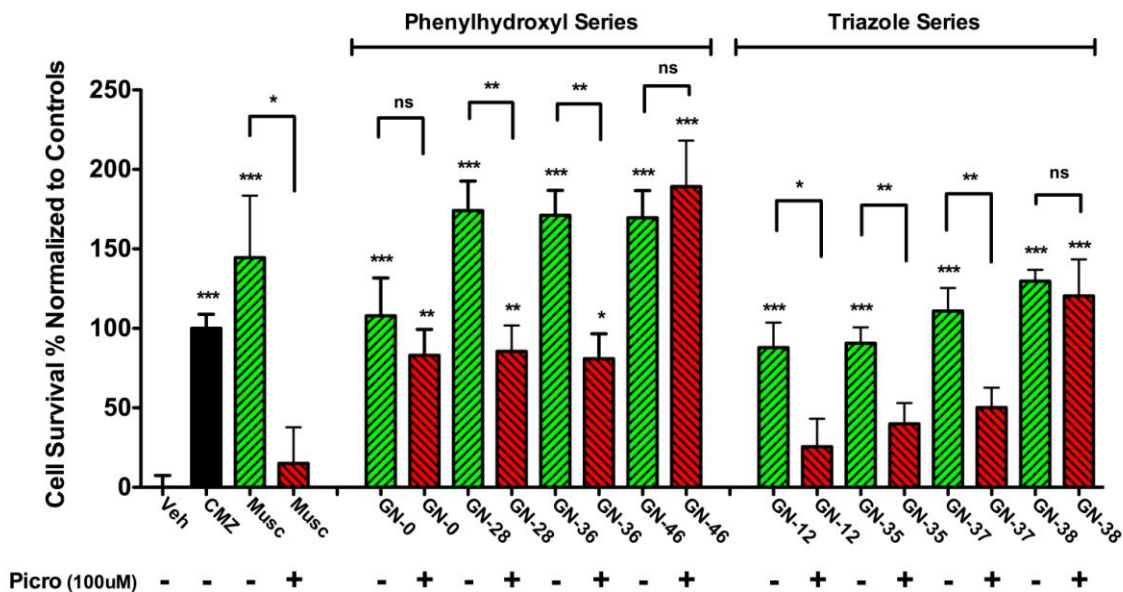


Figure 4: MZ derivatives protect neurons against OGD with varied dependence on GABA_A.

Protection of primary cortical cultures against OGD was significantly attenuated by GABA_A receptor blockade for GN-28, GN-36, GN-12, GN-36, and GN-37, whereas GN-0, GN-46 and GN-38 were neuroprotective without significant dependence on picrotoxin. OGD was maintained for 2 h with (red bars) or without (green bars) addition of picrotoxin 1 h prior to start of OGD. Drug (50 μ M) was added at initiation of OGD, and after 24h survival was measured with MTT assay normalized to vehicle (0%) and CMZ (100%). Data shows mean and s.e.m., with ANOVA analysis, and post hoc Dunnett's MCT, comparing treatments to vehicle control, and effect of picrotoxin on each treatment analyzed with student's two-tailed t-test: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

III.C.5. Extracellular Glutamate Confirms Variable GABA_A Activity in Methiazoles

To assess GABA_A receptor activity more directly, we isolated a downstream event ancillary to cell survival. Extracellular glutamate is recognized as an excellent marker for the levels of excitotoxicity in tissues and cell culture, and here fluorescence detection of glutamate after separation with HPLC and pre-column derivatization with OPA was used to measure the concentration of extracellular glutamate in culture supernatant 24 h after initiation of OGD. Glutamate reduction for all compounds was normalized to in-plate vehicle controls. The increase in released glutamate due to OGD was not directly measurable with this approach as the concentration of glutamate in cultures not exposed to OGD was below the threshold of detection (~50 nM per 2x10⁵ cells). OGD-dependent glutamate release was reduced significantly by CMZ and by many of the MZ derivatives studied (Figure 5). Three MZ derivatives that were neuroprotective did not significantly attenuate glutamate release; however, the level of glutamate in the presence of all MZ neuroprotective agents was significantly elevated by GABA_A channel blockade.

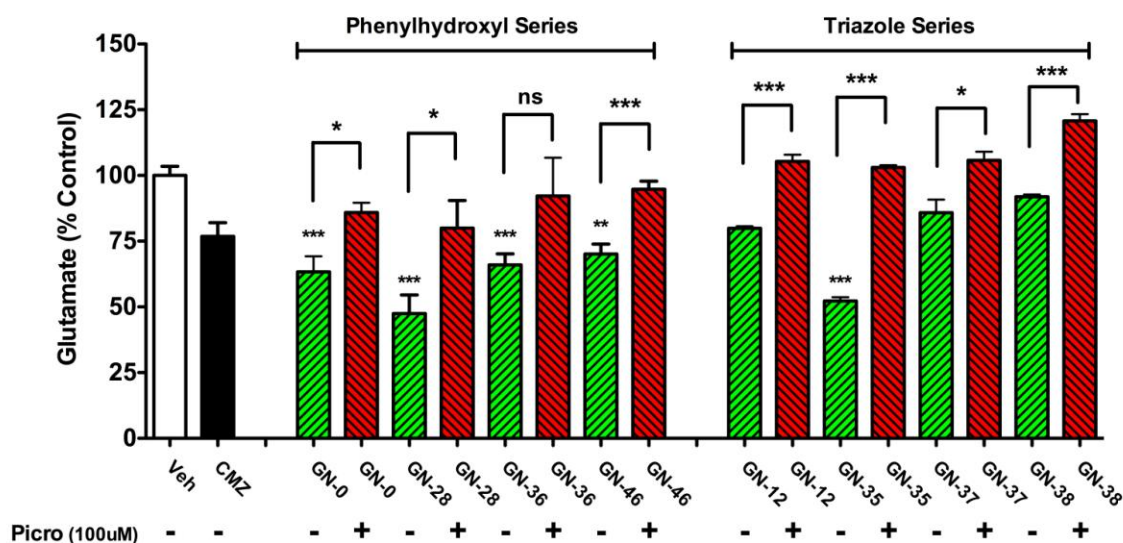


Figure 5: MZ derivatives decrease glutamate release with varied dependence on GABA_A.

Extracellular glutamate release, highly elevated in the supernatant of primary neurons subject to OGD, was significantly attenuated by CMZ and most MZ derivatives. GABA_A receptor blockade by picrotoxin resulted in increased extracellular glutamate. Glutamate was measured by HPLC-UV after derivitization with OPA. Data shows mean and s.e.m., with ANOVA analysis, and post hoc Dunnett's MCT, comparing treatments to vehicle control, and effect of picrotoxin on each treatment analyzed with student's two-tailed t-test: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

III.C.6. Neuroprotection Against Low-Dose A β via GABA_A-dependent Mechanism

To extend observations to an *in vitro* model of neuroprotection directly relevant to the amyloid- β hypothesis of Alzheimer's disease etiology, MZ derivatives were tested against neurotoxicity resulting from application of oligomeric full-length human A β ₁₋₄₂. The exact nature of the insult by A β oligomers is a matter of debate, however, neurotoxicity mediated through direct action at NMDA receptors is one hypothesis.[273] A β oligomers also cause disruption of calcium ion homeostasis and apoptosis, potentially via direct activation of a glutamate-mediated pathway leading to neuronal loss.[274] Therefore, MZ derivatives that showed neuroprotection against OGD have the potential to protect neurons against an excitotoxic insult induced by oligomeric A β .

MZ derivatives (50 μ M) were incubated with oligomeric A β ₁₋₄₂ (250 nM) for four days, a dose and time period determined to be sufficient to elicit reproducible cell death and protection by drugs (Figure 2). All MZ derivatives tested were observed to deliver significant neuroprotection relative to the vehicle control (Figure 6). Again, picrotoxin was used to estimate the contribution of GABA_A receptors to cell survival. In contrast to OGD, neuroprotection was significantly attenuated by picrotoxin, although the trend showed GN-38 and GN-46 to be less sensitive and GN-36 more sensitive. These data support a role for GABA_A receptor activation in protecting against the toxic effects of

oligomeric A β and more speculatively suggest that attenuated GABA_A receptor function can potentiate A β -induced neurotoxicity. The results are consistent with a role for excitotoxicity in neuronal loss in the presence of oligomeric A β and the capacity of MZ derivatives to provide neuroprotection against this damage.

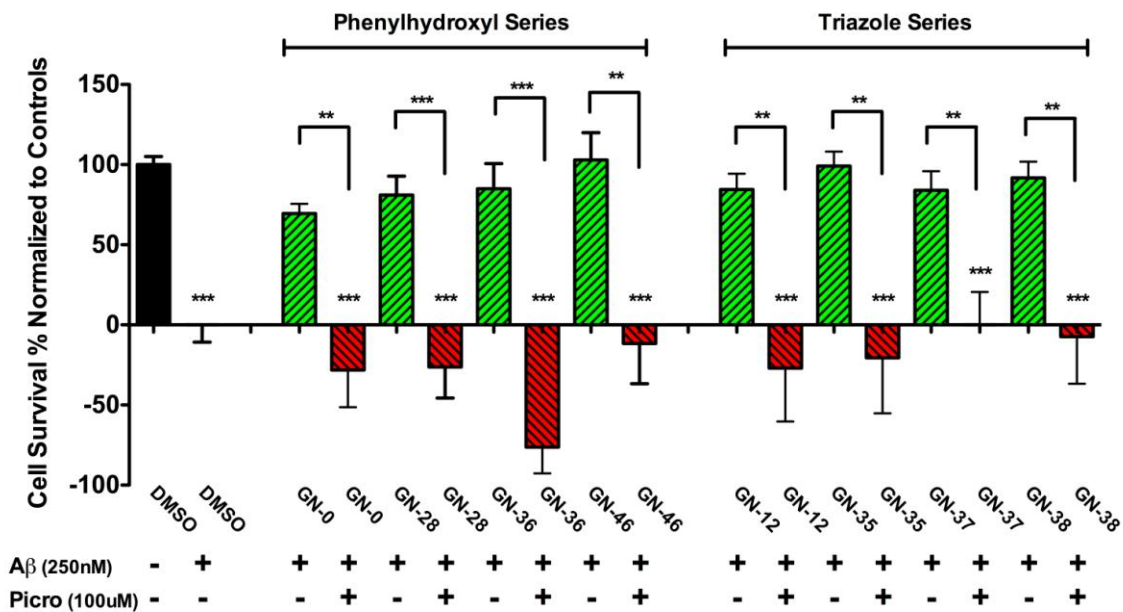


Figure 6: MZ derivatives protect neurons against A β oligomers with dependence on GABA_A.

MZ derivatives protected primary cortical cultures from cell death induced by oligomeric A β ₁₋₄₂ (250 nM), which was reversed by addition of picrotoxin 1 h before insult. Cell survival was measured by MTT after 4 days and normalized to vehicle treatment (100%) and A β treatment (0%). Data shows mean and s.e.m., with ANOVA analysis, and post hoc Dunnet's MCT, comparing treatments to vehicle control, and effect of picrotoxin on each treatment analyzed with student's two-tailed t-test: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

III.C.7. Further Analyses Suggest Favorable Therapeutic Profile for Stroke

With every mechanistic investigation, keeping on eye on therapeutic utility is important. To this end, dose response curves were generated for every assay, and a simple time course experiment was done for OGD to investigate if delayed administration would help against acute insults. Finally, before progression to ex vivo or in vivo studies, information on the ability of novel analogues to cross the blood brain barrier (BBB) was

needed. CNS bioavailability was determined by intraperitoneal injection of novel analogues in DMSO after preliminary investigation of solubility and stability were conducted using HPLC. Both plasma and brain tissue were collected to allow a rough estimate of the percent crossing the BBB. Of course, formal PK studies would need to be conducted in the pre-IND development stage.

GN-28 and GN-38 were selected for more in-depth study as representatives of neuroprotective MZ derivatives with greater and lesser dependence on GABA_A pathways, respectively (Figure 4). Dose ranging in the OGD assay showed that CMZ and novel compounds had negligible effect on cell survival in the absence of OGD at doses of 100 nM - 100 μ M, results consistent with previous observations on CMZ, and furthermore, no artifactual interaction was found between any novel compounds and MTT (Figure 2). Concentration-response curves for GN-28 and GN-38 (100 nM - 100 μ M) were measured and normalized to cell viability in the presence of CMZ (50 μ M; 100%) and vehicle control (0%) (Figure 7A). Neuroprotection by both MZ derivatives was observed at concentrations equivalent to those known to be pharmacologically relevant for CMZ.

OGD is a model of ischemia-reperfusion injury. Neuroprotective mechanisms elicited during cellular hypoxia and ischemia can differ from those that provide protection upon reoxygenation and later apoptotic and inflammatory mechanisms. Pre-conditioning provides a further array of mechanisms for protection against subsequent insult. To study the role of drugs in each stage of OGD damage, GN-28 or GN-38 were added at four different timepoints: i) 1 h pretreatment with removal of drug at commencement of OGD (Pre-tx); ii) immediately at the start of OGD (+0 h); iii) immediately after the 2 h OGD period (+2 h); and iv) 6 hours after start of OGD (+6 h).

OGD was transient for 2h in each paradigm. Both GN-28 and GN-38 showed a similar protection profile, with no significant effect seen on pretreatment, but efficacy was maintained up to six hours after initiation of OGD and four hours after initiation of oxygen/glucose reperfusion (Figure 7B). In the clinical context of ischemic stroke, these observations are supportive of therapeutic utility for MZ derivatives.

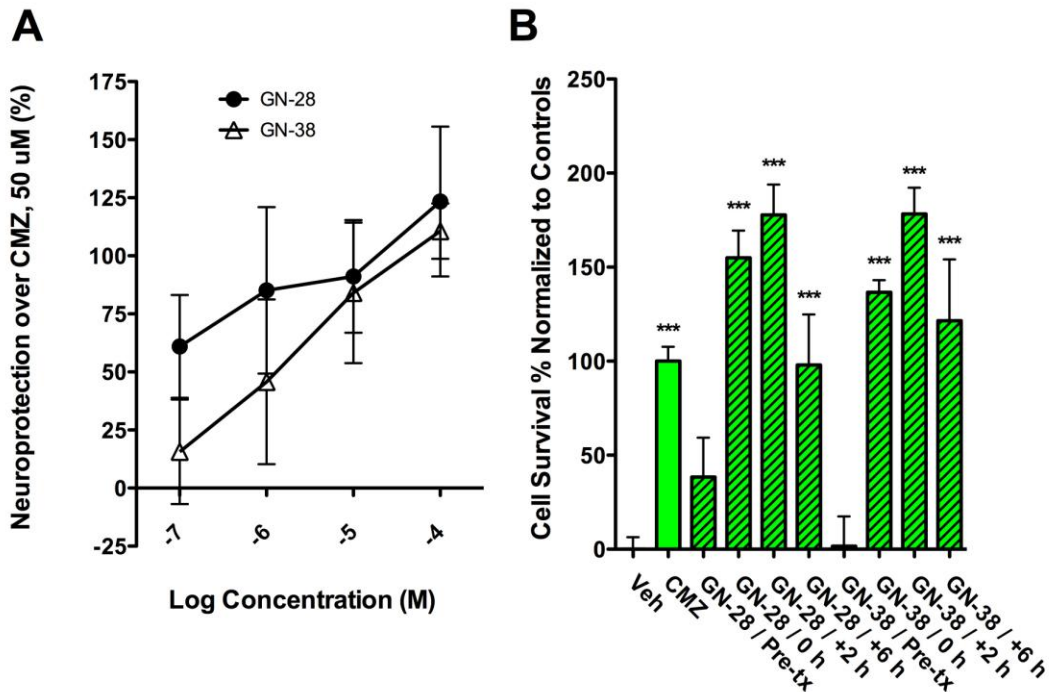


Figure 7: MZ derivatives protect against OGD at pharmacologically relevant concentrations and when added hours after OGD.

Primary cortical cultures were treated at varied concentrations and time points after initiation of transient OGD or pre-treated (Pre-tx) with drugs. **(A)** Concentration-response relationships for neuroprotection by GN-28 and GN-38, added at initiation of OGD (0 h). **(B)** No effect was seen after pre-treatment of cell cultures with GN-28 or GN-38 (50 μ M), however, neuroprotection was significant for both treatments at least 6 h after OGD. Survival was measured by MTT 24 h after start of insult and normalized as in Figure 1A. Data show s.e.m. with ANOVA and post hoc Dunnet's MCT, comparing to vehicle control, *** = $p < 0.001$.

III.C.8. Protection Against NMDA or Glutamate Toxicity Variably Involves GABA_A

As described above, the mechanism of OGD induced cell death is multi-fold, involving metabolic dysregulation, excitotoxicity and oxidative stress. NMDA and extracellular glutamate represent models of pure excitotoxicity, and allow for neuroprotection to be directly compared to in plate controls that have not been subjected to insult. To validate the results from OGD and test the direct ability of compounds to prevent excitotoxicity in a model with more limited oxidative and metabolic disruption, compounds were tested in models of NMDA toxicity and chronic glutamate toxicity. As before, muscimol was added to assess the effect of pure GABA_A agonism, resulting in significant neuroprotection against neurotoxicity induced by either NMDA (100 μ M) or glutamate (1 mM), which was ablated by picrotoxin (Figure 8A, 9A). GN-28 showed behavior similar to muscimol and CMZ, eliciting comparable levels of neuroprotection, with ablation of this effect by picrotoxin. GN-38, in contrast, was neuroprotective, but the effects of picrotoxin were not significant against either NMDA or glutamate insult. Concentration-response curves for cell survival were normalized to vehicle treated cells within the same plate either subject to excitotoxic insult (0% survival) or not (100% survival). Despite the clear differences in mechanism between GN-28 and GN-38, as seen with respect to picrotoxin co-treatment, the concentration-response curves were similar (Figure 8B, 9B). Observations suggest that GN-38 does not depend on the GABA_A receptor for neuroprotective activity against excitotoxic insult, however, significant neuroprotection was elicited against pure glutamate receptor mediated excitotoxicity.

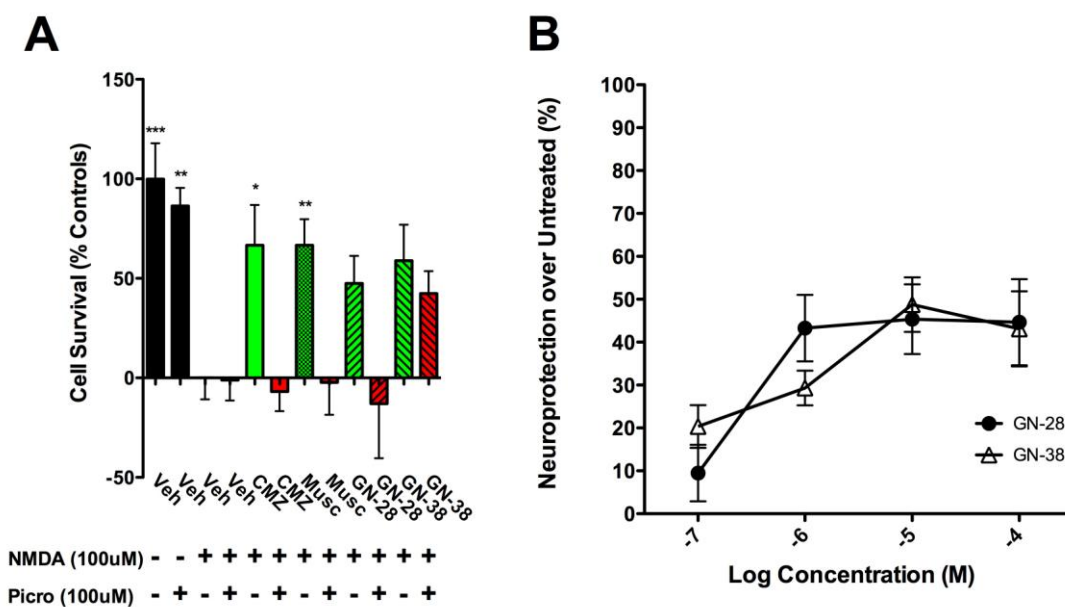


Figure 8: MZ derivatives protect against NMDA with varied dependence on GABA_A.

Primary cortical cultures were subjected to NMDA (100 uM) induced excitotoxicity with survival measured by MTT after 24 h and normalized to vehicle controls with (0%) or without (100%) NMDA. **(A)** Neuroprotection conferred by treatment with CMZ, muscimol, or GN-28 (50 μ M) was ablated by GABA_A blockade, whereas picrotoxin (100 uM) treatment had no significant effect on neuroprotection elicited by GN-38. **(B)** Despite varied dependence on the GABA_A receptor, GN-28 and GN-38 displayed similar dose-response curves. Data show mean and s.e.m., analyzed by ANOVA with post hoc Dunnet's MCT to compare to vehicle control; and the effect of picrotoxin on each sample was analyzed with student's two-tailed t-test: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

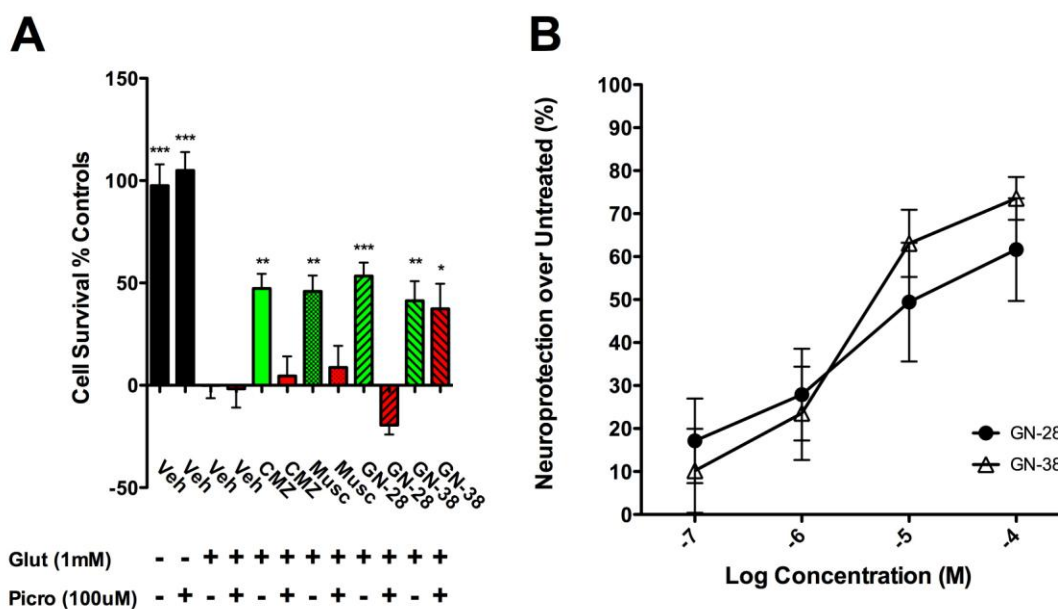


Figure 9: MZ derivatives protect against extracellular glutamate with varied dependence on GABA_A.

Primary cortical cultures were subjected to glutamate (1 mM) induced excitotoxicity with survival measured by MTT after 24 h and normalized to vehicle controls with (0%) or without insult (100%). **(A)** Again, neuroprotection seen with CMZ, muscimol and GN-28 (50 μ M) was blockable with picrotoxin (100 μ M), but GABA_A receptor blockade had no effect on GN-38. **(B)** GN-28 and GN-38 showed similar dose-response curves with maximum efficacy of ~50% achievable at low micromolar doses neuroprotection in this assay. Data show mean and s.e.m., analyzed by ANOVA with post hoc Dunnet's MCT to compare to vehicle control; and effect of picrotoxin on each sample was analyzed with student's two-tailed t-test: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

III.C.9. Neuroprotection Against High Dose A β via GABA_A-independent Mechanism

Oligomers of A β induce variable amounts of toxicity through unknown mechanisms, but one hypothesis is direct action through NMDA receptors leading to increased cellular firing, so increasing the effect of GABA would be a reasonable treatment, and allow extension of applicability to AD directly. Low-doses of A β (250 nM) are likely to be more physiologically relevant, but this dose produces relatively limited cell death over a long time frame, but could be considered a more relevant model of chronic A β -induced cell death. To allow direct comparison with both published A β

toxicity assays and comparable cell death to excitotoxic models, high-dose A β was also used for select analogues.

Primary neurons were incubated with high dose (5 μ M) oligomeric A β_{1-42} for four days. These conditions provide comparable levels of cell death to those seen for excitotoxic injury (Figure 2). All compounds were supplied at 50 μ M and cell survival was normalized to vehicle controls. At the higher level of exposure to A β , only CMZ and GN-38 showed neuroprotection (Figure 10A). Response was measured as a function of concentration for GN-28 and GN-38 showing significant divergence of neuroprotective efficacy for the two MZ derivatives against high dose A β : with significant protection observed for GN-28 only at the highest dose studied (Figure 10B). Although the pathophysiological relevance of the high dose of A β is problematic, many studies have been published using such concentrations to screen neuroprotectants in neurons and neuroblastoma cells.

Whereas at the lower level of oligomeric A β insult (Figure 6) picrotoxin blocked neuroprotection, at higher A β , the effects of picrotoxin were not significant, although a clear trend to reduced efficacy was seen for CMZ. These observations further support the concept of dual mechanisms of action for CMZ that are represented differentially in GN-28 and GN-38. Contribution from the GABA $_A$ -independent mechanism would seem to be essential to provide protection against cell death induced by high levels of A β . The GABA $_A$ -independent mechanism is amplified in the structure of GN-38.

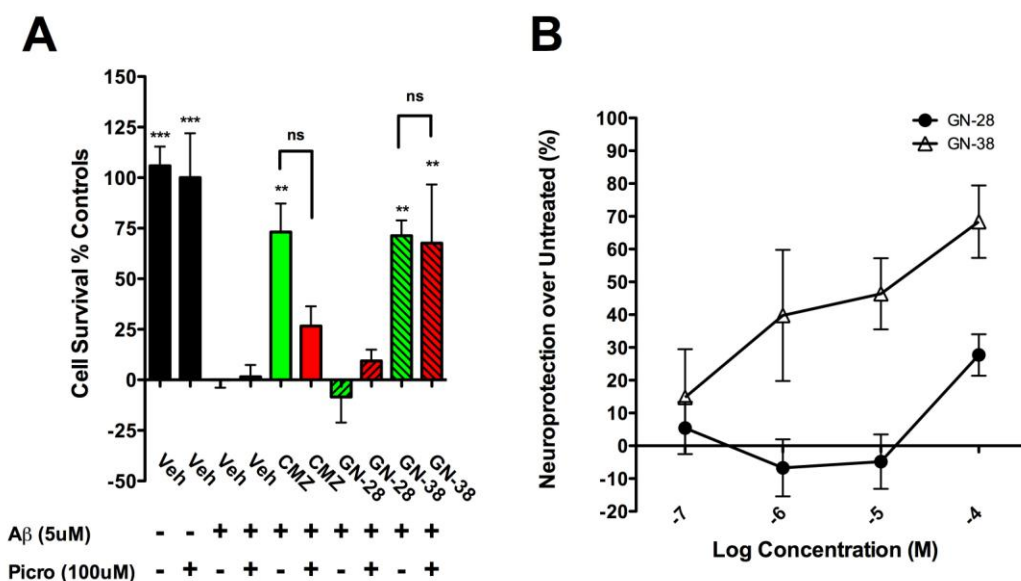


Figure 10: Protection against high dose oligomeric A β by CMZ and GN-38 is not dependent on GABA_A receptor activity.

Primary cortical cultures were prepared as above and subjected to 5 uM of A β ₁₋₄₂ oligomers, a dose comparable with the current literature on A β toxicity, with survival measured by MTT after 4 days and normalized to vehicle controls both with (0%) and without (100%) insult. **(A)** CMZ and GN-38 (50 uM) showed neuroprotection not blockable by picrotoxin (100 uM), but GN-28 failed to show neuroprotection at this concentration. **(B)** GN-38 showed some level of protection at doses as low as 100 nM, while GN-28 only showed protection at the highest concentrations tested (100 uM). Data show mean and s.e.m., analyzed by ANOVA with post hoc Dunnet's MCT to compare to vehicle control; and effect of picrotoxin on each sample was analyzed with student's two-tailed t-test: ** = $p < 0.01$, *** = $p < 0.001$.

III.C.10. Methiazoles Show Excellent CNS Bioavailability

An important consideration in advancing a neuroprotective compound to *in vivo* studies is whether the compound crosses the blood brain barrier providing sufficient brain bioavailability. Therefore, for GN-28 and GN-38, CNS bioavailability was evaluated in C57/BL6 mice after i.p. administration, using solution-phase extraction from plasma and brain after perfusion and subsequent detection by LC-MS. Compounds (4.45 μ mol/kg) injected 20 min before collection of plasma and brain tissue were identified by LC-MS/MS and quantified using internal standards that were added prior to liquid extraction.

Both MZ derivatives crossed the blood brain barrier, with a superior brain/plasma ratio for GN-28, but with a higher concentration of free drug observed in the brain of animals treated with GN-38 (Table 1). Further metabolism and protein binding studies were not conducted at this stage, however, both MZ derivatives clearly have brain bioavailability.

Table 1: Plasma & brain concentrations of MZ compounds determined 20 m after i.p. administration.

Analyte	Plasma (ng/ml)	Brain (ng/ml)	[Brain]/[Plasma]
GN-28	18.4 ± 0.8	32.9 ± 3.7	1.8
GN-38	559 ± 24	56.4 ± 5.5	0.1
Quantified using LC-MS/MS after i.p. administration (equivalent to GT-1061, 1 mg/kg), Data are expressed as the mean ± s.e.m. (n=4).			

III.C.11. Novel Methiazoles Show Reduced Sedation Compared to CMZ

Action at the GABA_A receptor can lead to sedation, and CMZ is currently used as a sedative-hypnotic in the elderly. While sedation may not strictly be an unwanted side effect of clinical neuroprotective agents, uncoupling of the drug's neuroprotective and sedative activity can be useful for pharmacological treatment. To measure sedation induced by the MZ derivatives, compared to CMZ, animals were tested in the accelerating rotorod task at concentrations equimolar to CMZ (45 mg/kg). This dose was chosen after testing various doses and time points after drug administration for CMZ itself and delivers significant sedation by CMZ as shown by the shortened latency to fall off the accelerating rotorod (data not shown). Compared to pre-treatment, CMZ caused significant sedation at 10 min and 30 min after drug administration, whereas GN-28 caused significant sedation only at 10 min and data for GN-38 did not reach significance (Figure 11). Comparison with *in vitro* data shows that efficacy for neuroprotection can be decoupled from efficacy for sedation.

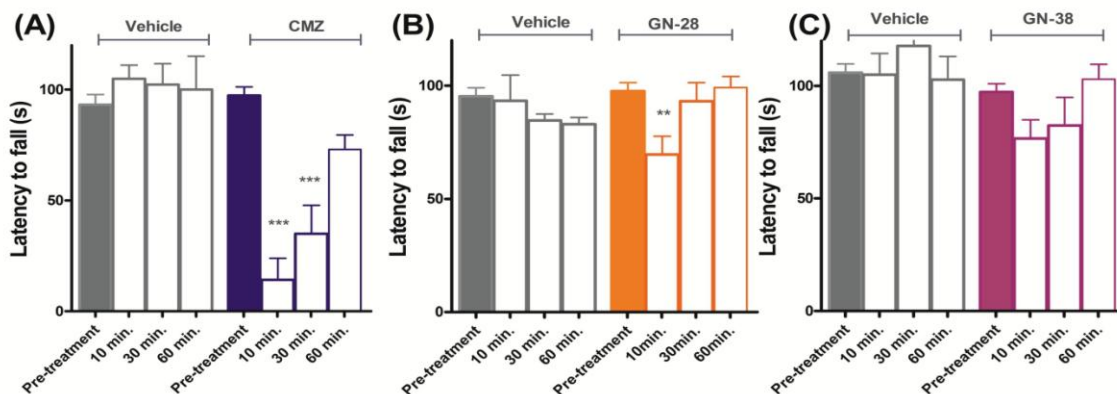


Figure 11: Sedative action of CMZ observed in the accelerated rotorod task is attenuated in MZ derivatives.

The accelerated rotarod performance task was performed with: (A) CMZ (45 mg/kg, i.p.); (B) GN-28 (equimolar dose, 59 mg/kg, i.p.); or (C) GN-38 (equimolar dose, 64 mg/kg, i.p.). Mean latency to fall was determined at 10, 30, and 60 min post injections. The data represents average time (s) animals stayed on the rod before and after drug treatments. Vehicle, composed of water 90 % (v/v), DMSO 10% (v/v), did not show any significant effect on the mean latency to fall. Bars represent means and s.e.m. ($n=5-6$) analyzed by ANOVA with Dunnett's post test comparing with Pre-treatment: *** $p < 0.001$, ** $p < 0.01$.

III.D. Methiazole Discussion

Selected thiazole derivatives are known to manifest hypnotic/sedative and anticonvulsant effects, which is indicative of GABA-mimetic activity[275, 276]. CMZ was selected as a potent anticonvulsant from limited SAR studies[261, 277, 278], entered clinical use in the 1960's for treatment of seizures, alcoholic dementia and withdrawal, epilepsy, and agitation, and is currently used for management of restlessness and agitation in the elderly. Based on animal model data and a history of tolerability during clinical use, CMZ was advanced into clinical trials for stroke[255, 279-281]. However, a large Phase 3 clinical trial did not achieve the primary endpoint of improvement in the general population, although significant improvement was reported in a sub-set of the population with more extensive infarction[281-283]. Related stroke trials on CMZ produced conflicting data in this sub-population[284-286]. CMZ was administered for 24 h in stroke patients within 12 h of the event, with performance assessed 90 days post-stroke.

CMZ has a relatively short reported half-life[287], and authors have speculated that the negative outcome was caused by low bioavailability at the site of infarct, or other factors discussed in light of the universal failure of neuroprotective drugs in stroke clinical trials.[288-290] Such failures in the latter part of the 20th century have all but halted clinical studies on neuroprotection in diseases that would benefit from such agents, including Alzheimer's.

CMZ has been shown extensively to be neuroprotective in animal models of ischemic infarct and global ischemia[291], and continues to be suggested as a potential component of future combination therapies for neuronal injury[257], potentially in combination with clot busters such as tissue plasminogen activator (tPA); an approach that might extend the relatively limited clinical window of tPA[292]. Since completion of the CMZ stroke trials, other positive animal model data and potential mechanisms of action have been suggested[265]. In the present work, selected MZ derivatives derived from screening a focused library were observed to provide equivalent or superior neuroprotective efficacy, compared to CMZ itself, in primary neurons subject to OGD as a model of ischemic stroke, with certain analogues attenuating the release of glutamate.

The literature and data presented herein, clearly suggests GABA_A-dependent and independent mechanisms of neuroprotection for CMZ. The use of picrotoxin demonstrated that MZ derivatives were capable of amplifying these GABA_A-dependent (GN-28) and independent (GN-38) attributes, recommending these two derivatives for more detailed study. GN-28 and GN-38 demonstrated significant neuroprotection in excitotoxic models of neurodegeneration and in the OGD model provided neuroprotection when administered 6 hrs after the OGD insult. Neuroprotection elicited

by GN-28 was largely GABA_A-dependent in contrast to GN-38. GABA_A-dependent neuroprotective mechanisms were sufficient to protect primary neurons from toxicity induced by low levels of oligomeric A β ₁₋₄₂; however, these were not sufficient to provide protection against higher levels of A β , wherein only CMZ and GN-38 were neuroprotective. GN-28 and GN-38 were observed to cross the blood-brain barrier and both were observed to have significantly attenuated sedative activity *in vivo*, compared to CMZ.

IV. GT1061, a Nometiazole Signaling Through NO/cGMP/CREB, Improves Memory and Synaptic Transmission, Reduces Inflammation, and Lowers A β and Tau in Two Animal Models of Alzheimer's Disease

IV.A. Nometiazole Introduction

An early event in Alzheimer's Disease (AD) is synaptic failure.[293] Recent research showing modification of A β neuropathology after increased synaptic plasticity in animal models suggests that a therapeutic agent that restores synaptic plasticity would also be disease-modifying in AD.[294, 295] Synaptic plasticity and efficacy are mediated by structural changes at synapses, which requires activation of gene networks and transcription factors such as cAMP-response element binding protein (CREB). Activity-dependent gene expression, regulating development and function in the brain, has long been associated with CREB activation.[296, 297] More recently decreased activity of pCREB has been demonstrated in homogenates taken from post-mortem AD patients,⁵ and molecular network analysis converges on aberrant CREB-mediated gene regulation in the Alzheimer's disease hippocampus, placing CREB signaling at the center of AD-related gene networks.⁶ Activation of CREB by phosphorylation and other mechanisms has been shown to be necessary for memory formation and synaptic strengthening.[298] In addition to mediating long-term potentiation (LTP), pCREB acts upon downstream genes involved in both synaptic formation and maintenance and neuronal plasticity and neurogenesis.[299] Evidence for reciprocal regulation of amyloidogenesis by CREB and direct dysregulation of CREB activation by oligomeric A β has been reported.[300-304] Given that CREB activation is tightly regulated by NO/cGMP signaling,[305-309] activation of this signaling pathway in the Alzheimer's brain provides a potential disease-modifying approach to restoring synaptic function, memory and cognition.

In addition to synaptic failure, other contributions to AD pathogenesis include neuronal loss, neuroinflammation, oxidative stress, and disturbances in calcium homeostasis related to those observed in excitotoxicity. Significant therapeutic efforts have been directed towards neuroprotective agents, many using established anticonvulsant mechanisms to inhibit excitotoxic neuronal loss, such as potentiation of GABA_A mediated signaling. Although all have ultimately failed in clinical trials for ischemic stroke, some showed sufficient promise in man and in animal models to support further exploration in drug discovery. [257, 284-286, 291] Using this approach, 4-methyl-5-(2-(nitrooxy)ethyl)thiazol-3-ium chloride (NMZ) was designed to activate NO/cGMP signaling and also to retain the activity of the parent pharmacophore: GABAergic neuroprotection; and attenuation of pro-inflammatory cytokines.[263, 265, 310, 311] NMZ was observed to protect primary neurons from ischemia-reperfusion injury and oligomeric A β .

The NO-mimetic hypothesis evolved from the knowledge that nitric oxide (NO), previously considered a toxin, was found to be a vital signaling molecule in learning and memory using the NO/sGC/cGMP/BDNF signal transduction system that modulates synaptic plasticity through CREB phosphorylation via the ERK cascade in brain areas such as the hippocampus and cerebral cortex. BDNF levels are reduced in Alzheimer's brains and are associated with early stage disease progression.[312] The clinical safety and efficacy of nitrates, such as isosorbide mononitrate, supported the exploration of nitrates in AD. More recently, several authors have commented that enhancement of the NO/cGMP pathway may provide a novel approach to the treatment of AD, through the increased activation of CREB, necessary for memory formation. The NO mimetic

nitrate, GT-015, was neuroprotective in animal models of ischemic stroke and Parkinson's disease, and reversed scopolamine induced deficits in rat models of learning of memory. The incorporation of an ancillary neuroprotective pharmacophore was seen to be important in multifactorial neurological disorders leading to the NO chimera drug concept.

IV.B. Nomethiazole Objectives

1. Using GT1061, our lead nomethiazole, determine if nomethiazoles retain neuroprotective action in OGD and A β in vitro assays after conversion to nitrate chimeras.
2. Measure sedation compared to MZ backbone, to determine if sedative activity is attenuated by conversion to nitrate chimera.
3. Using STPA, a behavioral model of memory, determine if GT1061 is able to reverse amnesic insults when administered acutely to C57Bl/6 mice.
4. In long-term studies using APP/PS1 animals, evaluate the ability of GT1061 to reverse behavioral deficits in memory, reduce inflammation, and prevent plaque formation.
5. In long-term studies using 3xTg animals, evaluate the ability of GT1061 to reverse behavioral deficits in memory, increase pCREB and BDNF, reduce inflammation, and prevent plaque and tangle formation.
6. Using aged 3xTg animals, determine if acute administration of GT1061 can reverse synaptic deficits in long-term potentiation and employ ODQ, a soluble guanylyl cyclase blocker, to implicate involvement of NO/cGMP pathway.

IV.C. Nomethiazole Experimental Results

IV.C.1. GT1061 shows neuroprotection against OGD and A β oligomers

To investigate whether GT1061 demonstrated neuroprotective activity after addition of the nitrate group to the methiazole backbone, it was tested against insults in primary rat cortical neuron cultures (DIV 10-12) at equimolar concentrations to CMZ (50 μ M), a clinically relevant dose previously shown to result in no direct toxicity. Against 2

h of OGD, GT1061 resulted in a significant improvement over CMZ in cell survival after 24 h as assayed by MTT (normalized to CMZ: 109.2%±2.1; $p < 0.01$; Figure 12A), while CMZ again demonstrated the reported increase in cell survival over untreated cells (normalized to CMZ: 82.8%±1.5; $p < 0.001$; Figure 12A). To extend the results to a model directly applicable to Alzheimer's, soluble oligomers of A β_{1-42} were prepared and incubated with primary neuronal cultures for four days at two concentrations: 250 nM and 5 uM. The lower concentration is more likely to be physiologically relevant and a better model of the chronic progressive cell death seen in AD, but results in a lower amount of cell death overall (Figure 2), while the higher concentration allows direct comparison to existing literature on A β -induced toxicity. After normalization to in-plate controls, GT1061 showed a trend towards increased cell survival compared to CMZ that did not reach significance (A $\beta_{250\text{nM}}$: GT 138.2%±14.6, CMZ 94.8%±22.0; A $\beta_{5\text{uM}}$: GT 99.2%±17.6, CMZ 73.1%±14.1), but both compounds were able to significantly increase cell survival over vehicle-treated cells to levels not significantly different from cells not exposed to A β oligomers (Figure 12B,C).

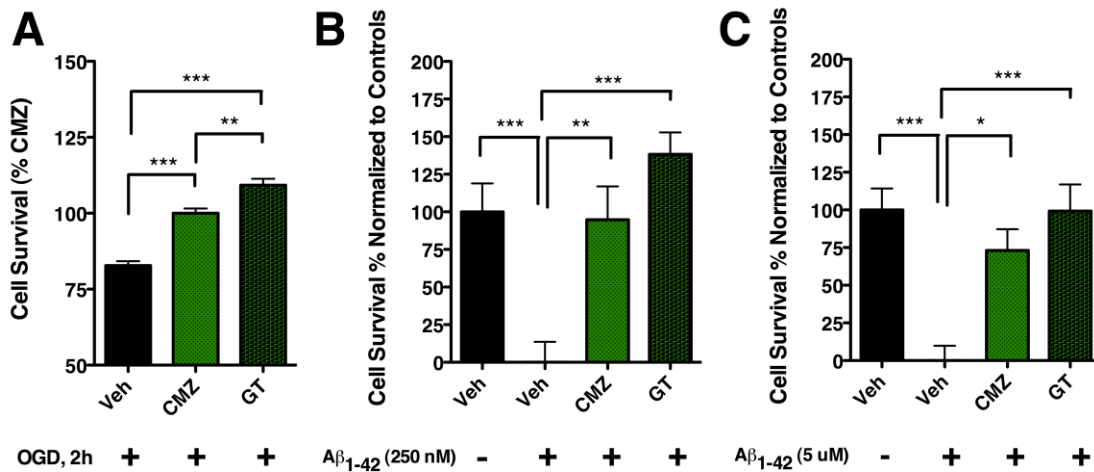


Figure 12: GT1061 Retains Neuroprotective Activity of CMZ Against OGD and A β Oligomers.

Primary neuronal cultures (DIV 10-12) were subjected to (A) 2 h OGD or (B,C) A β ₁₋₄₂ oligomers with compounds (50 μ M) added at start of insult and continued throughout. Cell survival was measured 24 h later with MTT assay. GT1061 shows greater neuroprotection than CMZ against OGD and equal or greater protection against both high- and low-dose A β oligomers. Data show mean and s.e.m. normalized to insult and non-insult vehicle controls (n=6-18); * p <0.05, ** p <0.01, *** p <0.001 compared to insult vehicle control using one way ANOVA with Dunnett's post hoc test.

IV.C.2. GT1061 shows sedative effect at higher doses than CMZ.

Sedative effects are known to be associated with GABA_A activity, so motor impairment was assessed using latency to fall on a rotating rod (Rotamex-5, Columbus Instruments) for both CMZ and GT1061 after i.p. administration of 45 mg/kg (or mol.eq. to CMZ) to male C57Bl/6 mice (Figure 13A). All animals were trained in the task for 3-5 d prior to experiment, until latency to fall reached 100 \pm 10 s, and no difference was found during the training period for any experimental group (Supplementary). Preliminary dose response with CMZ revealed a significant reproducible effect after 30 m for the 50 mg/kg i.p. injection (data not shown). At 10 m post injection, CMZ and GT1061 groups had a latency to fall of 14.2 \pm 9.8 and 38.7 \pm 5.4 s respectively (p <0.001), showing more motor impairment than vehicle treated animals (108.1 \pm 6.2 s). At 30 m, CMZ showed a significant deficit (35.0 \pm 12.8 s), while GT1061 was similar to vehicle (93.5 \pm 5.4, 115.8 \pm 6.3 s), and by 60 m, GT1061 and vehicle were nearly identical (122.1 \pm 10.7 s, 109.1 \pm 7.0 s), while CMZ had a latency to fall of only 73.0 \pm 6.5 s. It is important to note that the dose used to produce a transient sedative effect for GT1061 was over fifty times the acute dose sufficient to produce procognitive effects.

To test more profound sedation, loss of righting reflex (LORR) was assessed at a variety of doses ranging from ~50 mg/kg to 175 mg/kg (or mol.eq. to GT1061) in male C57Bl/6 mice (Figure 13B). Loss of righting reflex is defined as failure to place four

paws on the ground within 30 s after placing the mouse on its back, and the duration of this loss of reflex from time of administration was measured every 2 m for 2 h post administration. No substantial LORR was observed for either compound until 125 mg/kg, when CMZ treated animals showed a LORR for 23.8 ± 2.7 m, at which dose GT1061 only showed a LORR for 4.8 ± 1.7 m. GT1061 continued to show less sedation than CMZ until 175 mg/kg (GT: 90.5 ± 20.9 m, CMZ: 66.2 ± 7.7), an amount almost 200 times the acute dose necessary for procognitive effect.

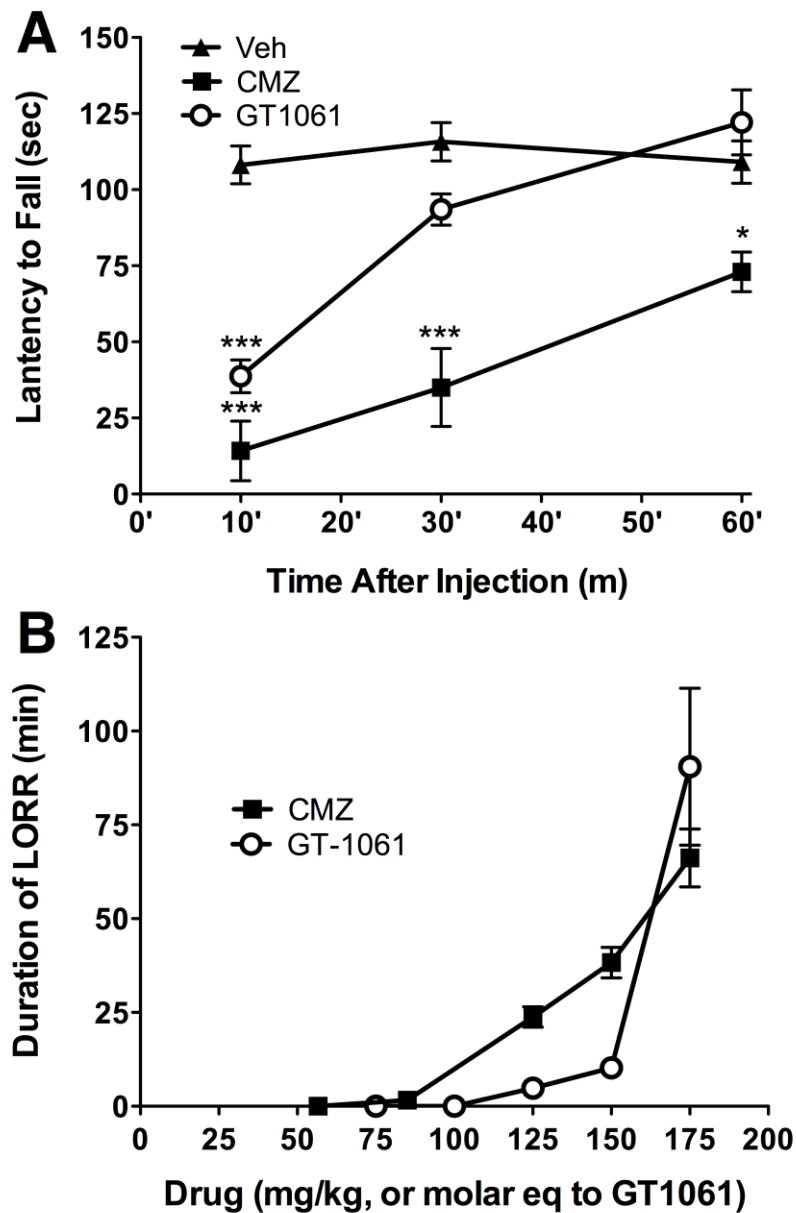


Figure 13: GT1061 shows attenuated sedation compared to CMZ in RR and LORR.

(A) After a training period, male C57Bl/6 mice were injected i.p. with 50.9 mg/kg (or mol.eq. to GT1061), a dose previously shown to result in significant sedative effect for CMZ, and tested on a rotating rod with latency to fall measured at 10 m, 30 m and 60 m. GT1061 showed less sedation than CMZ at every timepoint and sedation returned to baseline after 60 m. Data shown are mean and s.e.m. (n=5-16); *= $p < 0.05$, ***= $p < 0.001$ compared to vehicle control by 1-way ANOVA with post-hoc Dunnett's MCT. (B) Male C57Bl/6 mice were injected with escalating doses of GT1061 or CMZ and loss of righting reflex (LORR) was measured for 2 h. Until the highest dose of 175 mg/kg, GT1061 again showed less sedation than CMZ, and no LORR was observed for GT1061 until 125 mg/kg. Data show mean and s.e.m. (n=4-5).

IV.C.3. GT1061 reverses memory deficits in WT animals induced by diverse amnestic agents.

GT1061 was tested at 1 mg/kg using male C57Bl/6 mice in the step through passive avoidance (STPA) behavioral model of memory, where mice are trained to associated a mild electric shock (0.5 mA) with the dark side of a light-dark box and latency to enter is assessed 24 h after training (Figure 14). A variety of compounds were used at doses known to induce memory deficits given 20 m before training, including scopolamine (1 mg/kg), a muscarinic receptor antagonist; MK-801 (0.1 mg/kg), an NMDA receptor antagonist; diazepam (0.5 mg/kg), a direct GABA_A receptor agonist; and L-NAME (50 mg/kg), a non-specific NOS receptor antagonist. Animals without induced memory deficit reliably show a latency near the cutoff threshold of 300 s (296.9 ± 3.1 s). Scopolamine resulted in a decrease in latency (98.1 ± 16.6 s) that GT1061 reversed after either i.p. administration of 1 mg/kg 20 m prior to training (240.7 ± 25.3 s) or administration in drinking water at 20 mg/kg/day starting 24 h prior to training (231.5 ± 19.3 s). MK-801 and diazepam also resulted in deficits (94.6 ± 9.3 s, 166.7 ± 16.6 s) that were reversed by i.p. GT1061 (255.1 ± 15.4 s, 250.9 ± 22.5 s). L-NAME caused a latency of 22.0 ± 10.4 s, which was not completely reversible by GT1061 at 20 minutes prior to training (77.7 ± 17.5 s), but was reversed when GT1061 was given 50 minutes prior to training (211.7 ± 31.4 s).

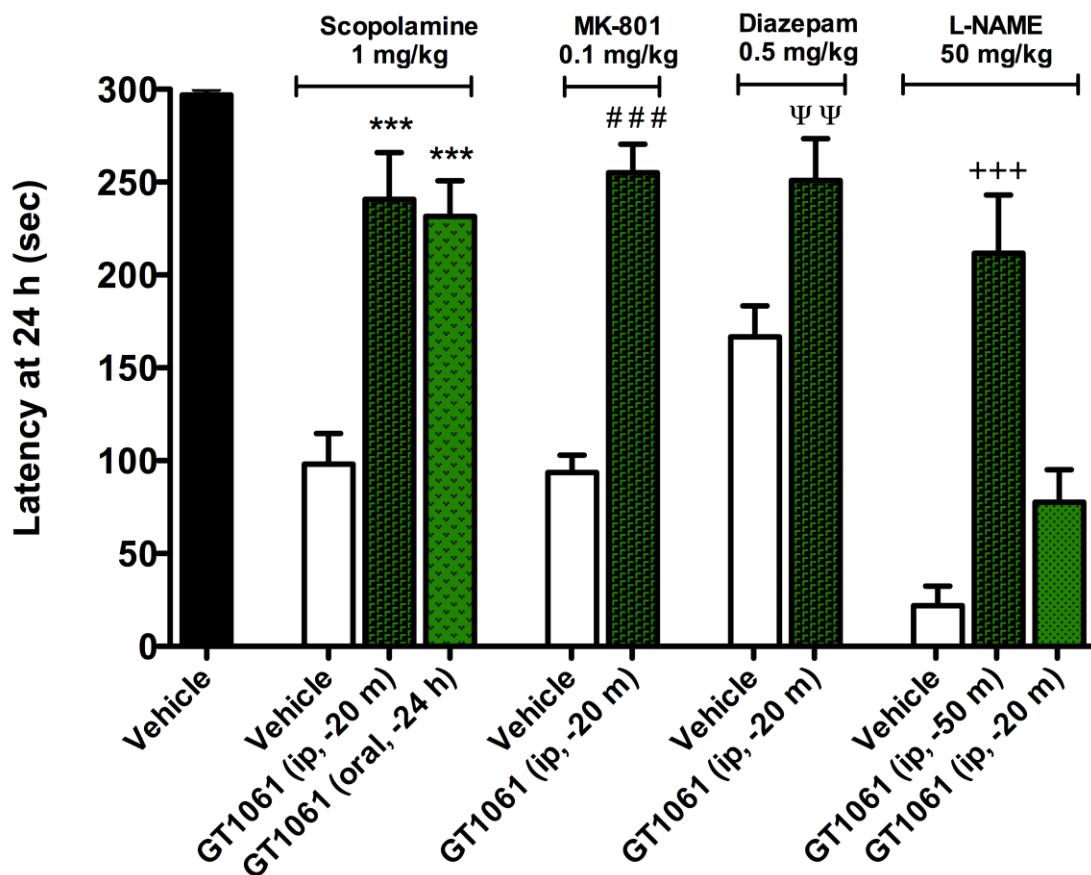


Figure 14: GT1061 reverses amnesic deficits in STPA induced by diverse agents.

Male C57Bl/6 mice were administered an amnesic agent 30 m prior to training in step through passive avoidance (STPA) and either vehicle or GT1061 (1 mg/kg, i.p.; 20 mg/kg/day, oral) were given at the time points prior to training listed, and latency to enter dark side was assessed 24 h after training. GT1061 reversed memory deficits induced by scopolamine, MK-801 and diazepam when administered by i.p. 20 m prior to training, with scopolamine deficits also reversible after continuous oral administration of GT1061 in hydrogel. Against L-NAME, GT1061 was not able to reverse deficit when given 20 m prior by i.p., but did reverse deficit when given 50 m prior to training. Data show mean and s.e.m. (n=5-10); **= $p<0.01$, ***= $p<0.001$ compared to insulted vehicle control using one way ANOVA with Dunnett's post hoc test.

IV.C.4. GT1061 has action during memory consolidation phase.

Memory formation can be divided into two phases: initial memory acquisition that is largely protein synthesis independent but not permanent, and a subsequent consolidation phase that is protein synthesis dependent and can last indefinitely[313]. As mentioned previously, NO signaling can lead to phosphorylation of pCREB, which can

increase BDNF levels, known to be involved in consolidation of memory[304, 314]. To test whether GT1061 was acting to reverse scopolamine deficit (1 mg/kg) during memory acquisition or memory consolidation, it was administered by i.p. injection (1 mg/kg) at a variety of timepoints before and after training (Figure 15). When administered as a bolus prior to training, GT1061 was effective when given within 40 m of the start of training, while not effective when given 1 or 2 h prior (-120 m: 134.0±11.3 s; -60 m: 125.2±37.1 s; -40 m: 263.0±17.1 s; -20 m: 280.0±11.4 s). When given after training is complete, GT1061 reversed the memory deficit when given up to 90 m after treatment (+30 m: 243.0±34.0 s; +60 m: 300.0±0.0 s; +90: 228.1±71.9 s). These results are consistent with GT1061's known $t_{1/2}$ of ~30 m and expected mechanism of action during consolidation, as well as the observation that task acquisition during training did not significantly differ between vehicle and GT1061 treated animals (data not shown).

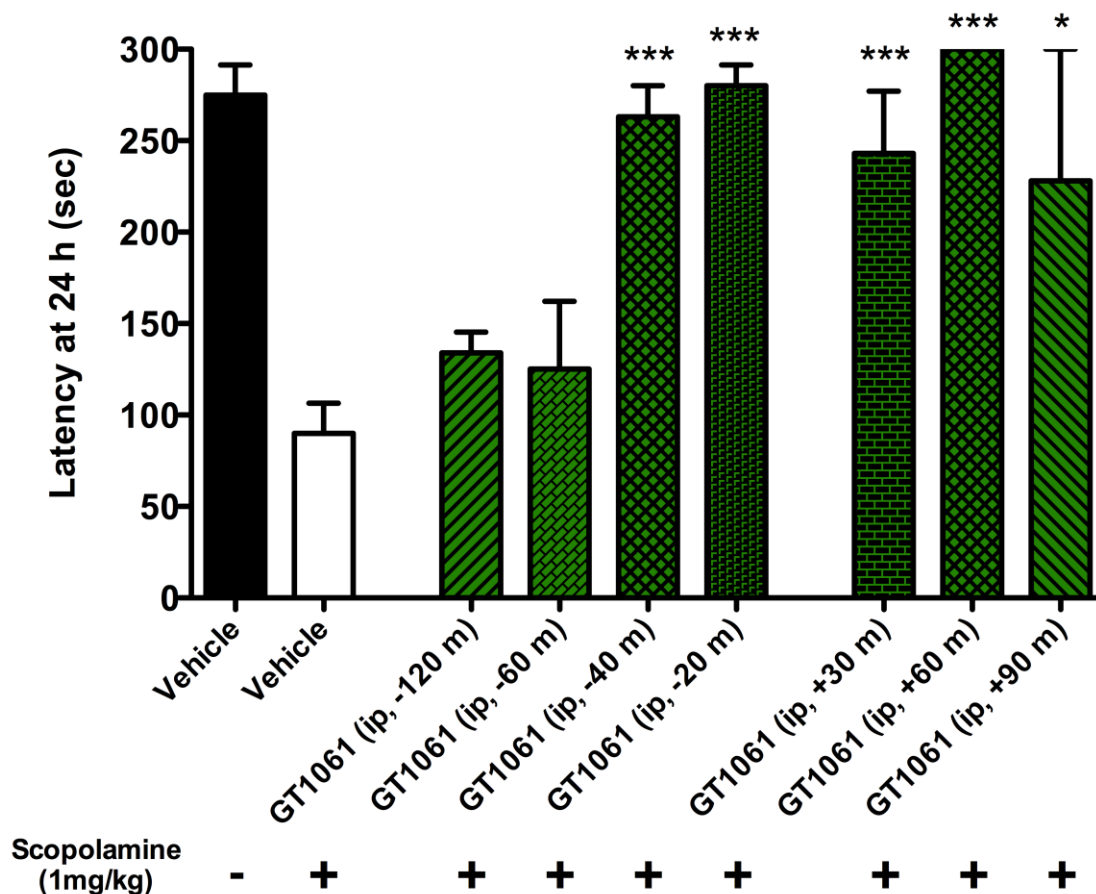


Figure 15: GT1061 reverses scopolamine-induced deficit from 40 m prior to 90 m after training.

Male C57Bl/6 mice were administered an amnesic agent 30 m prior to training in step through passive avoidance (STPA) and either vehicle or GT1061 (1 mg/kg, i.p.; 20 mg/kg/day, oral) were given at the time points prior to training listed, and latency to enter dark side was assessed 24 h after training. GT1061 reversed memory deficits induced by scopolamine, MK-801 and diazepam when administered by i.p. 20 m prior to training, with scopolamine deficits also reversible after continuous oral administration of GT1061 in hydrogel. Against L-NAME, GT1061 was not able to reverse deficit when given 20 m prior by i.p., but did reverse deficit when given 50 m prior to training. Data show mean and s.e.m. (n=5-10); **= $p < 0.01$, ***= $p < 0.001$ compared to insulted vehicle control using one way ANOVA with Dunnett's post hoc test.

IV.C.5. GT1061 increases synaptic plasticity in APP/PS1 mice.

GT1061 was tested in the APP/PS1 (APP^{sweK670N/M671L} and PS1^{M146L}) transgenic mouse model with age- and sex-matched WT background littermate controls, using electrophysiological, behavioral, immunohistochemical and biochemical assays. In

hippocampal slices from 3-month-old male mice, the effect of GT1061 on long-term potentiation (LTP) was measured in the CA3-CA1 pathway. After 15 m of baseline collection, GT1061 (100 μ M) was added to aCSF perfusate for 5 m prior to induction of LTP using three trains of ten theta bursts, and the resulting fEPSP were recorded in the CA1 area for 120 m (Figure 16). GT1061 resulted in a substantial increase in fEPSP after LTP induction compared to untreated transgenics, to levels identical to WT control.

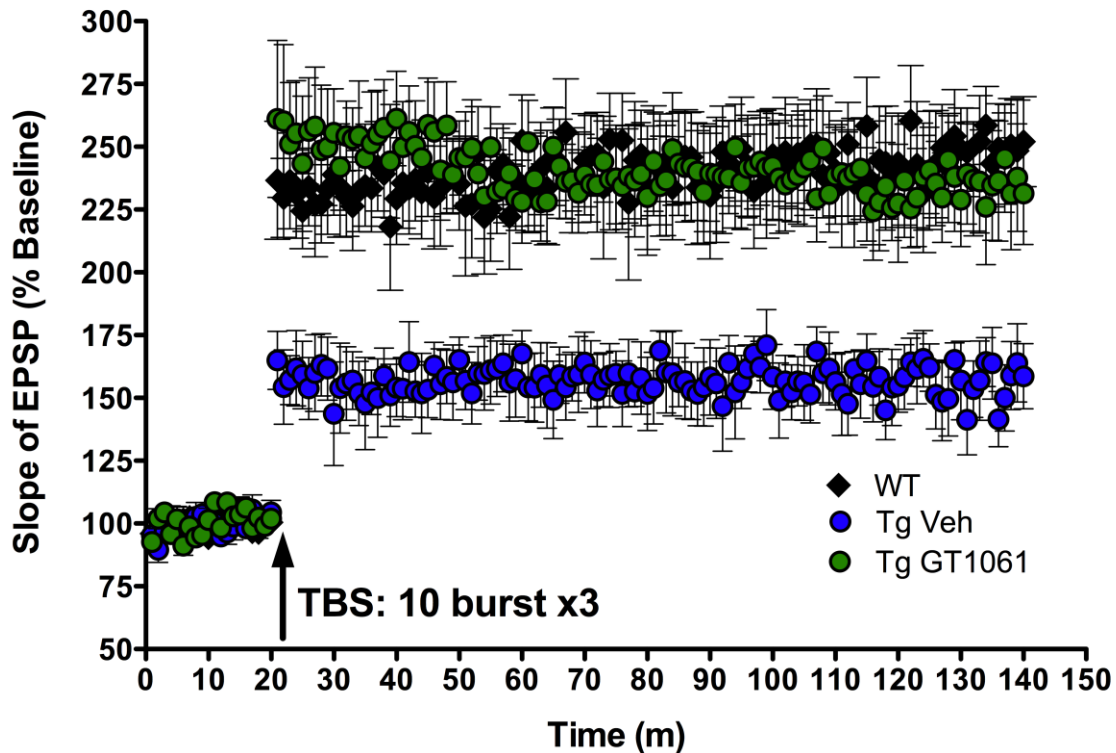


Figure 16: GT1061 Acutely Reverses Synaptic Deficits in APP/PS1 Mouse Model.

LTP was measured after TBS in the CA1 region of hippocampal sections from 4 mo male APP/PS1 mice or littermate controls. GT1061 (100 μ M) was added for 5 m prior to TBS. Even 2 h after treatment, GT1061 shows reversal in LTP to WT levels. Data show mean and s.e.m. normalized to baseline (n=5-8).

IV.C.6. GT1061 shows a trend towards improved memory in APP/PS1 mice.

GT1061 was also given to APP/PS1 mice and WT controls at 20 mg/kg/day in hydrogel (drinking water substitute) + 1 mg/kg i.p. injection (to ensure a minimal amount

of delivery) for 10 weeks starting at 2 months of age. After completion of the trial, the animals were tested in the radial arm water maze (RAWM) and then immediately sacrificed, with blood and brain tissue collected after PBS perfusion for biochemical and immunohistochemical analysis. In the RAWM, a trend towards increased performance over untreated transgenics was observed that did not reach significance (Figure 17).

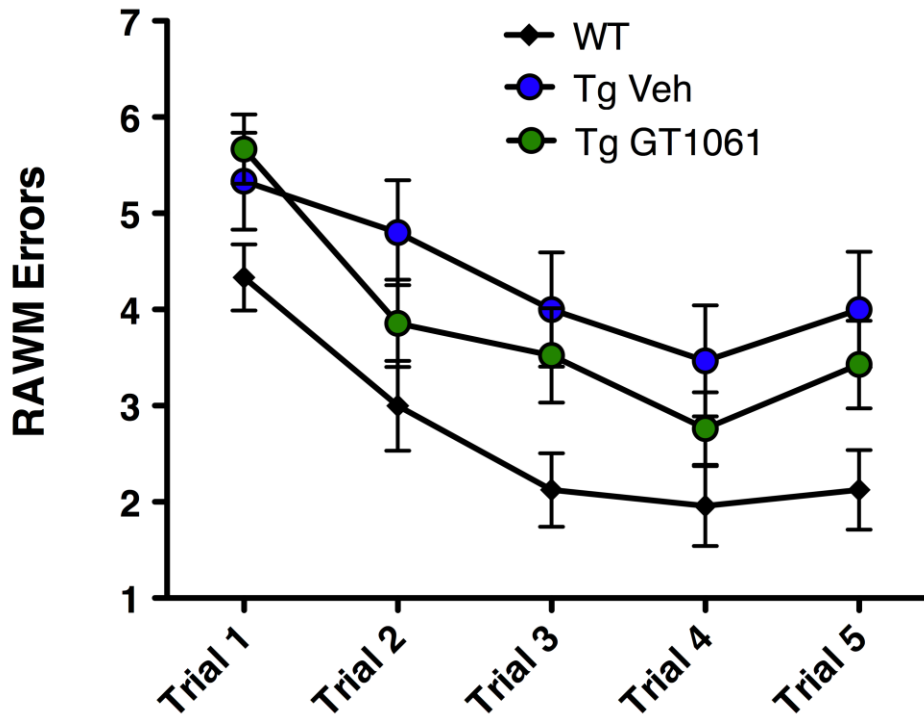


Figure 17: Ten-week administration of GT1061 produces a trend towards increased performance in the RAWM.

GT1061 was administered for ten weeks by hydrogel (20 mg/kg/day) and i.p. injection (1 mg/kg/day) to 2 mo male APP/PS1 mice and littermate controls. Errors after training in RAWM water maze were assessed after ten weeks. GT1061 shows a trend towards increased performance that does not reach significance. Data show mean and s.e.m. (n=6-8).

IV.C.7. GT1061 decreases inflammation in APP/PS1 mice.

Levels of TNF- α , a pro-inflammatory cytokine, were measured using ELISA from total brain homogenates and compared to total protein determined by BCA Figure 18A). APP/PS1 transgenic animals showed significantly higher levels than WT

littermate controls (25.6 ± 2.1 , 35.4 ± 2.6 pg/mg protein), and 10-week treatment with GT1061 reduced this level by 27% (25.9 ± 2.2 pg/mg protein). This finding is corroborated by Iba-1 (1:1000) staining of coronal slices, showing marked reductions in activated microglia in both the hippocampus and cortex of GT1061-treated mice (Figure 18B).

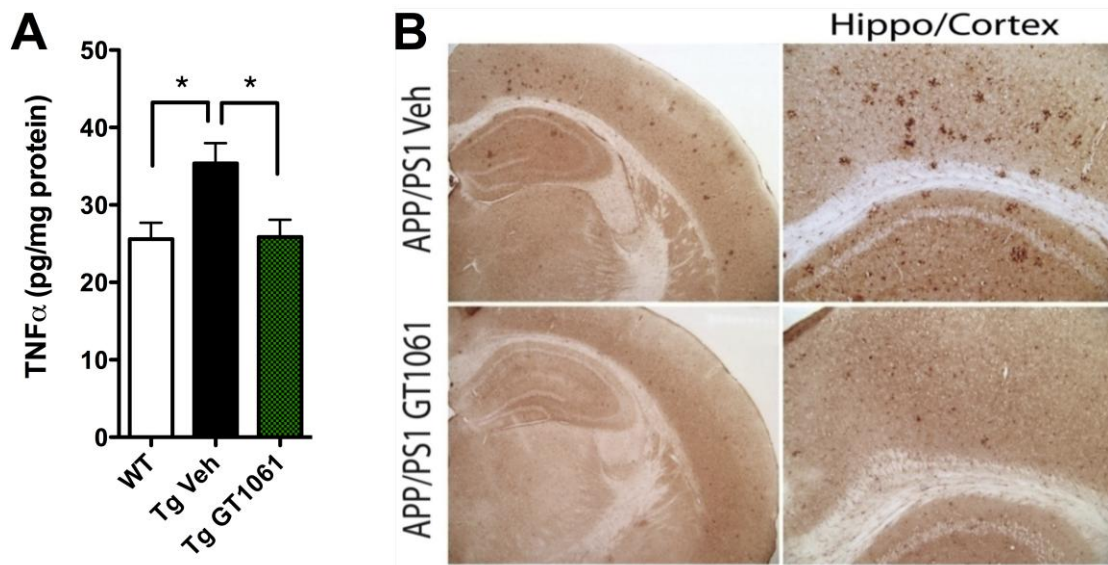


Figure 18: Ten-week administration of GT1061 reduces inflammation in APP/PS1 mice.

GT1061 was administered for ten weeks by hydrogel (20 mg/kg/day) and i.p. injection (1 mg/kg/day) to 2 mo male APP/PS1 mice and littermate controls. After behavioral testing, brains were perfused with PBS and taken for biochemical and histochemical analysis. TNF- α was measured in whole brain homogenates by ELISA and activated microglia were assessed using staining with Iba-1 (1:1000). **(A)** GT1061 reduces the increase in TNF- α observed in untreated transgenic animals to levels identical to those in WT control. Data show mean and s.e.m. ($n=6-8$); $*=p<0.05$ compared to insulted vehicle control using one way ANOVA with Dunnett's post hoc test. **(B)** Histochemical staining revealed significantly less activated microglia in treated transgenics compared to untreated transgenics. Pictures show representative slices.

IV.C.8. GT1061 lowers soluble, but not insoluble, A β in APP/PS1 mice.

A β plaques were assessed using Thioflavin S staining of coronal sections with fluorescent detection (Figure 19). An almost 3-fold reduction in amyloid deposit volume was observed in both the cortex and hippocampus in transgenics compared to GT1061

(cortex: 0.244 ± 0.068 , 0.088 ± 0.031 % cortical volume; hippocampus: 0.298 ± 0.72 , 0.122 ± 0.35 % hippocampal volume; Figure 18). When measuring the $A\beta_{1-42}$ levels by ELISA, the transgenic animals show a trend towards reduction in the insoluble guanidine-extracted plaque fraction (55.9 ± 7.8 , 44.3 ± 7.7 pg/mg protein), while a significant reduction is observed in $A\beta$ in the soluble protein fraction (138.2 ± 23.9 , 72.9 ± 10.7 pg/mL).

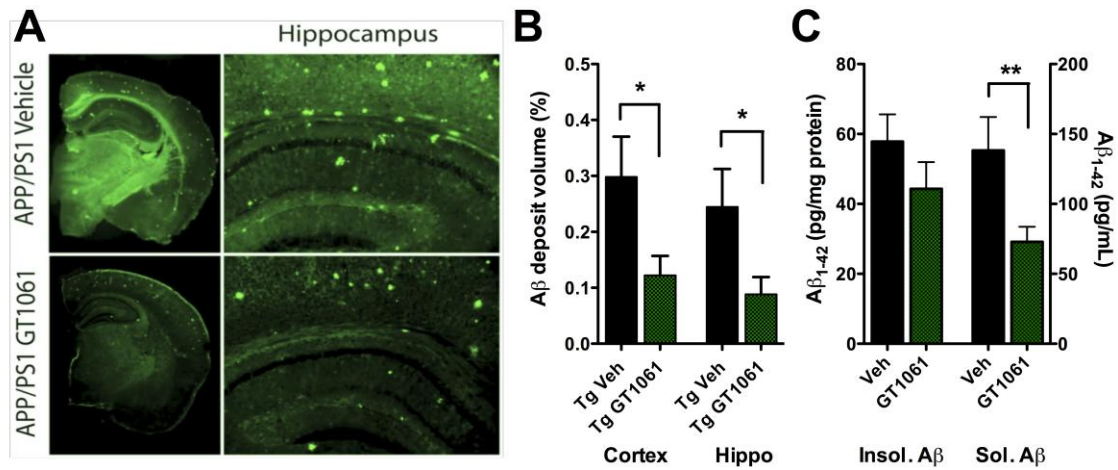


Figure 19: Ten-week administration of GT1061 decreases soluble $A\beta$ in APP/PS1 mice.

GT1061 was administered for ten weeks by hydrogel (20 mg/kg/day) and i.p. injection (1 mg/kg/day) to 2 mo male APP/PS1 mice and littermate controls. After behavioral testing, brains were perfused with PBS and taken for biochemical and histochemical analysis. **(A,B)** GT1061 reduces amyloid plaques in hippocampus and cortex of transgenic animals compared to untreated control. Pictures show representative slices. Data represent mean and s.e.m. of deposit volume determined by fluorescent staining ($n=6-8$); $*=p<0.05$ by two-tailed student's t-test. **(C)** Levels of $A\beta_{1-42}$ were measured by ELISA in fractions from whole brain homogenates. GT1061 significantly reduced levels of $A\beta$ in the soluble fraction, with a trend towards a decrease in insoluble fraction. Data show mean and s.e.m. ($n=6-8$); $**=p<0.01$ by two-tailed student's t-test.

IV.C.9. GT1061 increases synaptic plasticity in 3xTg mice.

To confirm the above findings in a separate mouse model, LaFerla's 3xTg transgenic mouse model (APP^{swe}_{K670N/M671L}, PS1_{M146L}, tau_{P301L}) was chosen for its inclusion of tau pathology. This model is a homozygous line, so littermate controls are

not possible, but the 129/SvJ x C57Bl/6 background was available as an outbred line, and was included in electrophysiological work to give a baseline performance, but not included in analysis of long-term studies of behavioral, immunohistochemical and biochemical endpoints. In hippocampal slices from 16-month-old male mice, the effect of GT1061 was again studied in LTP in the CA3-CA1 pathway. GT1061 (50 uM) was added to aCSF perfusate at least 30 m prior to induction of LTP and continued throughout. LTP was induced using ten theta bursts, and the resulting fEPSP were recorded in the CA1 area for ~50 m (Figure 20A). GT1061 again resulted in a substantial increase in fEPSP after LTP induction compared to untreated transgenics, to levels similar to WT control. To investigate the involvement of the NO/cGMP pathway, ODQ, a potent selective inhibitor of soluble guanylyl cyclase (sGC) was added 30 m prior to induction and continued throughout the experiment at concentrations shown to abolish sGC activity (10 uM). ODQ was able to block the stabilization phase of LTP, resulting in degradation of increased fEPSP to levels identical to untreated transgenic animals. Secondary analyses of area under the curve of fEPSPs during the theta burst induction allowed identification of actions during the immediate early phase of LTP (Figure 20B). Here, GT1061 was shown to results in more significantly more response to theta bursts, to levels slightly higher than even WT animals, with GT1061+ODQ treated animals showing a minimal response identical to untreated transgenics.

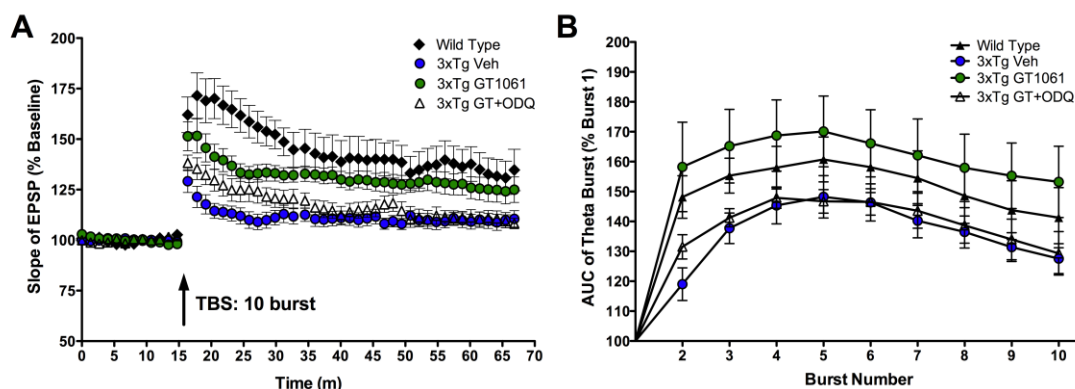


Figure 20: GT1061 Acutely Reverses Synaptic Deficits in 3xTg Mouse Model.

LTP was measured after TBS in the CA1 region of hippocampal sections from 16 mo male 3xTg mice or WT background controls. GT1061 (50 uM) and ODQ (10 uM) were added at least 30 m prior to TBS and continued throughout. Fifty minutes after LTP induction, GT1061 showed reversal in LTP deficits to WT levels, while ODQ completely blocked this activity. Analysis of theta bursts implicate action during induction of long-term potentiation. Data show mean and s.e.m. normalized to baseline (n=9-12).

IV.C.10. GT1061 reverses cognitive deficits in 3xTg mice.

3xTg mice were given GT1061 at doses identical to the APP/PS1 study (20 mg/kg/day in hydrogel + 1 mg/kg i.p. injection) for 10 weeks starting at 12 months of age for females and 15 months of age for males (to account for delayed pathology in males). After completion of the trial, the animals were tested using STPA and then immediately sacrificed, with blood and brain tissue collected after PBS perfusion for biochemical and immunohistochemical analysis. In STPA, untreated transgenic showed a latency of 97.1 ± 41.1 s, significantly worse than GT1061 treated animals that showed a latency of 247.6 ± 35.2 s (Figure 21).

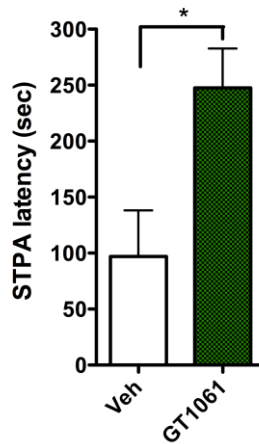


Figure 21: Ten-week administration of GT1061 reverses behavioral deficits in STPA in 3xTg mice.

GT1061 was administered for ten weeks by hydrogel (20 mg/kg/day) and i.p. injection (1 mg/kg/day) to 12 mo female and 15 mo male 3xTg mice. Latency to enter dark side was assessed after 24 h after training. GT1061 reverses deficits in memory to levels observed previously in WT mice. Data show mean and s.e.m. (n=8-12); *= $p < 0.05$ by two-tailed student's t-test.

IV.C.11. GT1061 raises pCREB and BDNF, and trends towards lowered TNF- α in 3xTg mice.

Biochemical analysis was performed on the brain homogenates after PBS perfusion, and TNF- α showed a trend towards decrease in GT1061 groups that did not reach significance (2.88 ± 0.20 , 2.184 ± 0.34 pg/mg protein, Figure 22A). The end product of NO/cGMP/sGC activation is thought to be pCREB, so pCREB levels in whole brain homogenates were assayed using ELISA and found to be increased more than 2-fold in GT1061 treated animals (0.424 ± 0.031 , 1.066 ± 0.106 units/mg protein; Figure 22B), while BDNF, a downstream target of pCREB, showed a trend towards increase that did not reach significance (129.4 ± 8.1 , 158.1 ± 11.2 pg/mg protein).

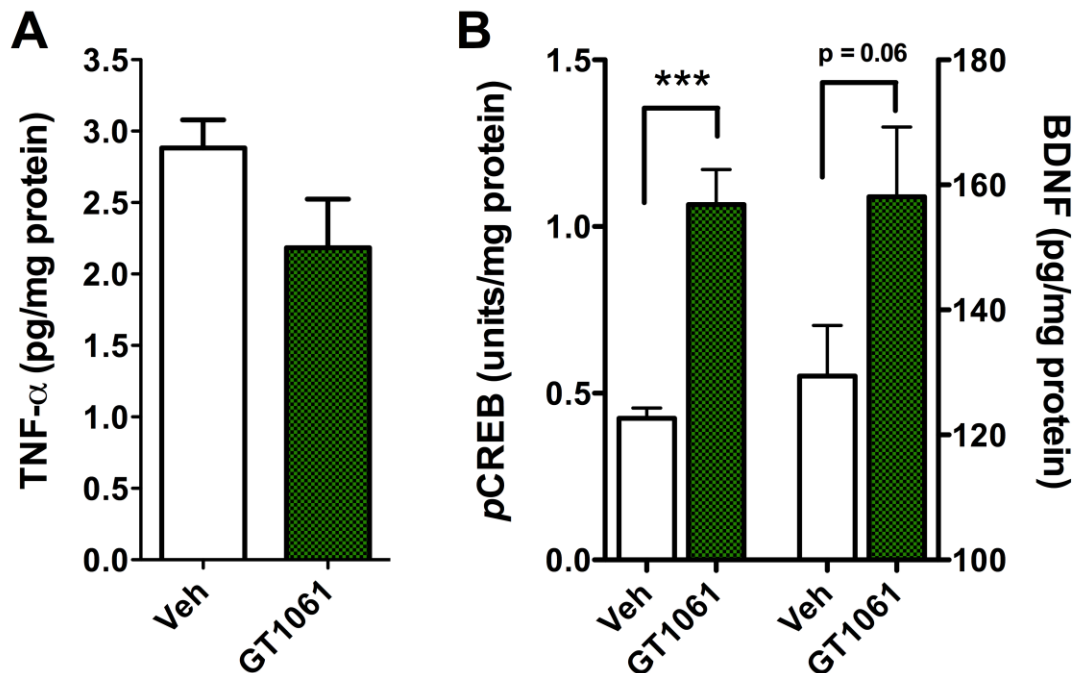


Figure 22: Ten-week administration of GT1061 increases pCREB in 3xTg mice.

GT1061 was administered for ten weeks by hydrogel (20 mg/kg/day) and i.p. injection (1 mg/kg/day) to 12 mo female and 15 mo male 3xTg mice. After behavioral testing, brains were perfused with PBS and taken for biochemical and histochemical analysis. TNF- α , pCREB and BDNF were measured in whole brain homogenates by ELISA. GT1061 shows a trend in reduction in TNF- α , but a significant increase in pCREB and a trend towards increased BDNF in treated 3xTg mice. Data show mean and s.e.m. (n=8-12); ***=p<0.001 by two-tailed student's t-test.

IV.C.12. GT1061 reduces tau levels in 3xTg mice with negligible effect on A β plaques.

Levels of tau, assessed immunohistochemically using AT8 (1:1000), an antibody against phosphorylated tau, showed a significant decrease in the CA1 region of GT1061 treated animals (Figure 23A). Total tau levels were assayed in the soluble fraction of total brain homogenates by western blot using HT7 (Figure 23B), which showed roughly equivalent levels of higher molecular weight tau (100.0 \pm 13.3, 95.1 \pm 18.0 % vehicle tau/ β -actin), but more than a 2-fold decrease in lower molecular weight tau by densitometric

analysis after normalization to transgenic controls (100.0 ± 9.2 , 46.7 ± 9.4 % vehicle tau/ β -actin). Analysis of $A\beta_{1-42}$ in the insoluble guanidine extracted fraction revealed a slightly lower amount of $A\beta$ that did not reach significance (5.56 ± 1.36 , 2.84 ± 0.91 pg/mg protein), perhaps due to action on decreased production or increased rather than plaque removal (Figure 23C). Staining using 6E10 (1:1000) revealed fewer immunoreactive cells in the CA1 region of the hippocampus, but a negligible effect in the cortex (Figure 23D).

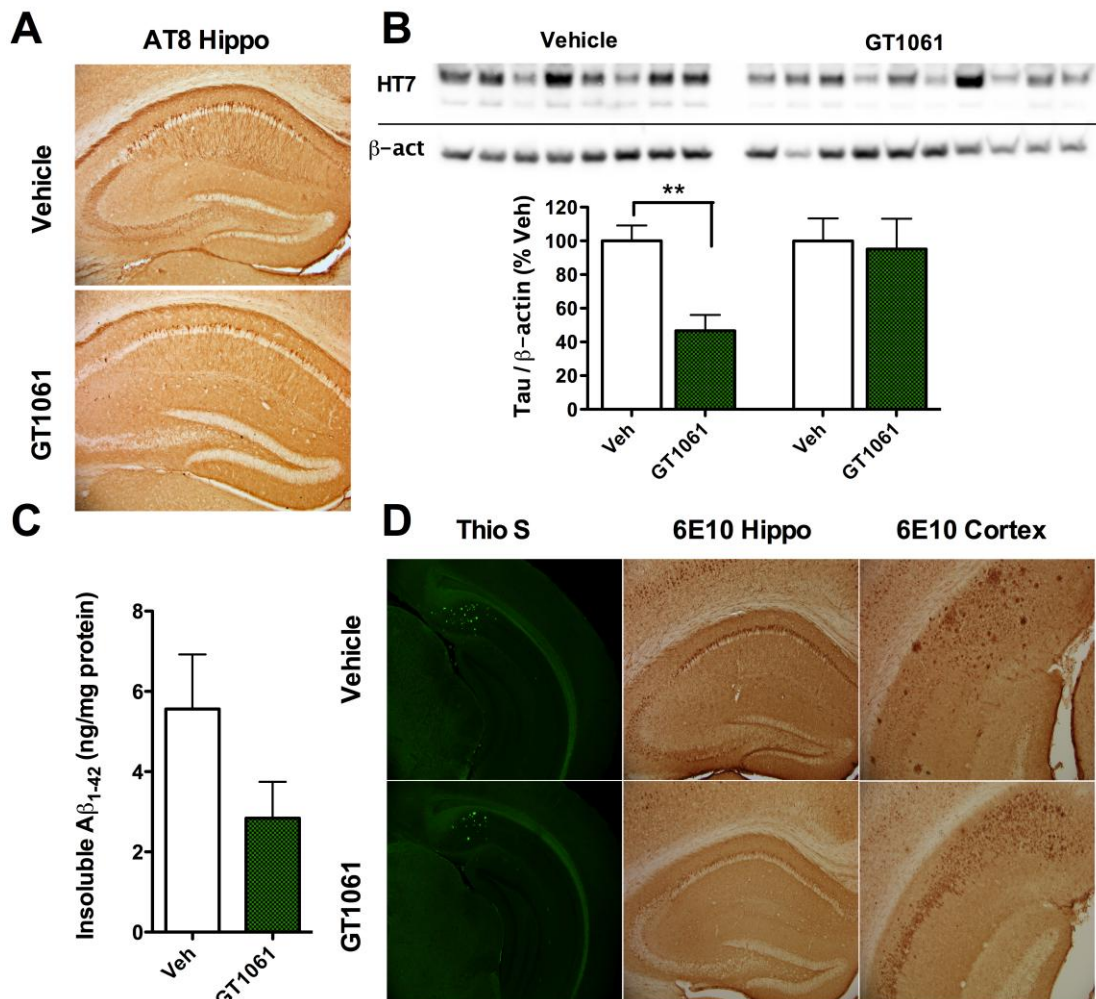


Figure 23: Ten-week administration of GT1061 decreases tau and $A\beta$ in 3xTg mice.

GT1061 was administered for ten weeks by hydrogel (20 mg/kg/day) and i.p. injection (1 mg/kg/day) to 12 mo female and 15 mo male 3xTg mice. After behavioral testing, brains were perfused with PBS and taken for biochemical and histochemical analysis. **(A,B)** GT1061 reduces phosphorylated tau in hippocampus and decreases total tau in total brain homogenates of transgenic animals compared to untreated controls. Pictures show representative slices. Data represent mean and s.e.m. of tau/ β -action determined by densitometric analysis (n=8-10); $**=p<0.01$ by two-tailed student's t-test. **(C)** Levels of $A\beta_{1-42}$ were measured by ELISA in fractions from whole brain homogenates. GT1061 showed a trend towards a decrease in insoluble fraction, with a decrease in CA1 region of hippocampus in treated animals but negligible effect on cortex. Pictures show representative slices.

IV.D. Nomethiazole Discussion

Unfortunately, despite a decade of drug development, no treatment options have been found superior to the current class of AD drugs, and alternative approaches are clearly needed. The NO-mimetic hypothesis evolved from the discovery that nitric oxide (NO) played a role as a vital signaling molecule in learning and memory by increasing cGMP[308, 315, 316], which acts through diverse protein kinases to phosphorylate the transcription factor cAMP response element binding protein (CREB)[309]. CREB phosphorylation is essential in neuronal plasticity and long-term memory formation in brain regions such as the hippocampus and cerebral cortex[317], and has been shown to lead to increases in brain derived neurotrophic factor (BDNF)[318], a neurotrophin known to promote neuronal survival and synaptic growth and differentiation[319, 320]. Recently, several authors have commented that enhancement of the NO/cGMP pathway may provide a novel target for CNS pathology[305, 321-325], and observed reductions in levels of BDNF[312] and pCREB[326] in the human AD brain support the exploration of NO-mimetics as procognitive agents in AD.

Using primary neuronal cultures, GT1061 demonstrates protection from both excitotoxic and direct amyloid insults, with decreased sedation suggesting an attenuation of adverse GABA action seen at high levels for CMZ. Results from STPA model indicate GT1061 can act to reverse a diverse set of amestic insults, including cholinergic dysfunction, a critical finding in light of existing AD therapeutics, and timing

experiments show procognitive action when GT1061 was administered either before or after training, indicating NO-mimetic action acts during consolidation phases of memory acquisition.

Various transgenic animal models of AD have been employed to determine the action of drugs upon the development of AD-like pathology, each expressing certain characteristics of AD. The double transgenic APP/PS1 mouse expressing both the human APP_{sweK670N/M671L} and PS1_{M146L} mutations is considered a more rapid rapid-onset model, developing extracellular A β deposits in the hippocampus and cortex, selective A β ₄₂ elevation, and spatial memory deficits not linked to neuronal loss.[117, 143, 327] In this model, acute reversal of synaptic deficits was possible in hippocampal slices, but ten-week treatment did not produce a significant improvement in behavioral outcomes, likely due to the relatively small deficit observed even in untreated transgenic animals. The strong inflammatory response in the APP/PS1 model was attenuated by long-term treatment with GT1061, shown both by decreased TNF- α and fewer activated microglia on histopathological examination. Recent evidence supports TNF- α itself as a therapeutic target in AD from clinical observation and mouse model studies[266-268]. Additionally, in this young model with rapid A β deposition, decreased levels of thioflavin reactive amyloid were observed in the hippocampus and cortex, with ELISA confirming substantial decrease in A β ₁₋₄₂ in the soluble fraction, suggesting GT1061 may be acting to decrease production or increase clearance rather than acting directly on deposited plaques.

The triple transgenic 3xTg-AD mouse model expressing human APP_{sweK670N/M671L}/PS1_{M146V}/tau_{P301L} gene mutations develops intracellular deposits of

immunoreactive amyloid and tau as early as three weeks in the amygdala and hippocampus; extracellular A β deposits preceding overt tangle formation; deficits in synaptic plasticity, including LTP; as well as deficits in behavioral models of memory[328, 329]. In the 3xTg model, acute restoration of synaptic function was determined to be dependent on sGC activation and this finding correlated with significant improvement was seen in STPA after ten-week treatment. In these same mice, a trend toward decreased TNF- α did not reach significance, perhaps due to the relatively low amount of neuroinflammation observed in untreated transgenics compared to APP/PS1 animals. However, in line with the nitrate chimera hypothesis, levels of pCREB increased two-fold, followed by an increase in levels of BDNF, a downstream target of CREB activation. Levels of total tau in total brain homogenates decreased as well, and phosphorylated tau and A β decreased in the CA1 region of the hippocampus, though decreases in levels of amyloid were modest in the cortex and did not reach significance in the insoluble fraction of total brain homogenates, again suggesting an effect on clearance or decreased production of abnormal proteins. These findings confirm the results seen in the APP/PS1 model and support the proposed hypothesis for nomethiazole mechanisms of action. Taken together, data from mouse models imply that GT1061 would possess clinical utility in mild to moderate AD, and may provide substantial benefit even in patients with mild cognitive impairment (MCI) with a high likelihood to progress to dementia.

V. A Nitrate Chimera Strategy to Overcome Loss of eNOS and Prevent Platelet Aggregation for Neuroprotective, Procognitive SERMs

V.A. NO-SERM Introduction

Estrogens are endogenous compounds with diverse activity throughout the body, including breast development and endometrial cycle regulation[330]. Estrogens have also been found to reduce incidence of coronary heart disease[331] and maintain bone mineral density[332]. In the CNS, estrogen promotes neuronal survival[333, 334] and neurogenesis in the hippocampus[335-337], and neuro-imaging studies have revealed that estrogen therapy improves cerebral blood flow and performance on hippocampal-dependent verbal and figural recognition memory tasks in humans[338, 339]. Other observational studies have found that estrogen helps alleviate age-related cognitive decline by preserving executive function in the frontal lobe[340].

As part of the Women's Health Initiative (WHI) study of estrogen for hormone replacement therapy, the effect of estrogen on dementia was investigated. Unfortunately, the WHI study concluded prematurely due to increased risk of thrombotic events and breast cancer[341]. Due to early termination of treatment arms, no effect was seen for estrogen alone in reducing all-cause dementia[342]. However, subsequent systematic review and meta-analysis revealed a decreased risk of Alzheimer's disease (AD) in treatment groups by as much as 34%[343, 344]. This finding has led several authors to the conclusion that estrogen therapy remains a treatment or prophylactic option for cognitive impairment and AD, if side effects can be ameliorated[345, 346].

Raloxifene (Evista®) is a second generation SERM used clinically for the treatment of osteoporosis in menopausal women, which acts as an antiestrogen in breast and endometrium[347-351]. Results from the Multiple Outcomes of Raloxifene

Evaluation (MORE) showed a trend towards decreased risk for cognitive impairment in the highest dose group[348, 352]. Both MORE and the Raloxifene Use for the Heart trial (RUTH) found that raloxifene had no effect on coronary events, but significantly increased the lifetime risk of thromboembolism[353]. Raloxifene has been found to enhance levels of vasodilatory NO through action on endothelial nitric oxide synthase (eNOS)[354-356], however, the increased thromboembolic events have been attributed to decreased eNOS activity in postmenopausal women[357]. NO is known to inhibit thrombus formation through inhibition of platelet recruitment, adhesion and aggregation[358], therefore an NO-donating SERM has potential to abrogate this adverse effect, when eNOS activity is attenuated.

The poor pharmacokinetics of raloxifene[359], led to development of arzoxifene[360, 361], a bioavailable prodrug of desmethylarzoxifene (DMA) that differs from raloxifene by only one atom, but is 10-fold more potent at ER α [362]. DMA analogues and NO-donor nitrooxy-derivatives were prepared for study of the possible neuroprotective and procognitive effects of SERMs and because of the potential for an “NO-SERM” to overcome adverse effects associated with thrombus formation. Nitrooxy compounds, including NO-chimeras and NO-flurbiprofen, have also been shown to reverse cognition deficits induced by attenuation of cholinergic signaling [321, 323, 363-366].

The use of an NO-SERM in an aging population may be attractive, since eNOS activity is decreased with age and in AD patients [367, 368]. Analyses of the recent clinical literature have supported a critical period hypothesis, wherein the female brain is responsive to the neuroprotective and procognitive effects of estrogen primarily, or

exclusively in early menopause [369-372]. Evidence in support of a critical period and specific underlying mechanisms have been proposed [373] [374]. However, it is likely that multiple pathways contribute to the attenuated response of the aging brain and based upon mechanistic studies, this may include complex signaling via ER, GPR30, and eNOS[375-378].

Herein we support the hypothesis that SERM neuroprotection is mediated through a GPR30-dependent mechanism, and extend these results to suggest that GPR30 is involved in reversing deficits in synaptic transmission demonstrated in AD transgenic mouse models, studied here for the first time at this advanced age. We also report that the procognitive and vasodilatory effects of SERMs are dependent on intact NOS signaling, whereas NO-SERMs preserve action in models where NOS signaling is impaired. Finally, we report the first nitrate chimera SERM, NO-DMA, that acts as an antithrombotic agent able to reverse synaptic deficits in AD transgenic animals and memory deficits induced by cholinergic and NOS antagonists, preserving positive SERM activity while attenuating the major side effect of clinically used 3rd generation SERMs.

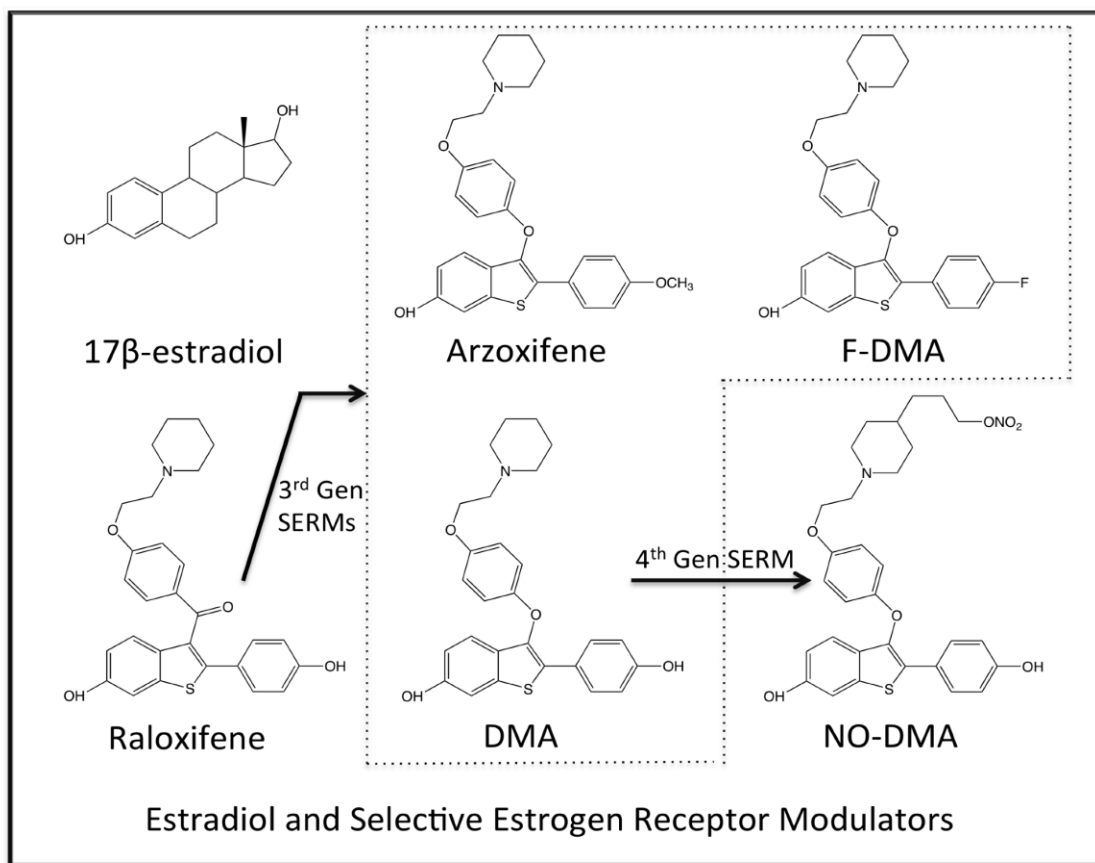
V.B. NO-SERM Objectives

1. Using our lead NO-SERM, confirm NO-DMA retains neuroprotective mechanism of action of DMA, PI3K/Akt dependent GPR30 signaling, in OGD model.
2. Using aged 3xTg animals, evaluate effect of acute administration of DMA and NO-DMA in reversing synaptic deficits and employ G15, a GPR30 blocker, to demonstrate involvement of GPR30/PI3K/Akt pathway.
3. Using C57Bl/6 and eNOS (-/-) mice models, determine bioavailability and NO_x donation to support advancement to *in vivo* trials and to confirm NO donation of NO-SERM during absent eNOS signaling.
4. Using STPA determine if either SERMs or NO-DMA are able to reverse amnesic insults in C57Bl6 or eNOS (-/-) mice.

5. Determine if SERMs or NO-DMA are able to induce vasodilation in rat aortic ring model, and whether function is preserved after administration of L-NAME or denudation of endothelium.
6. Evaluate anticoagulation ability of both DMA and NO-DMA in extrinsic and intrinsic clotting cascade pathways with and without administration of L-NAME and in eNOS (-/-) mice.

V.C. NO-SERM Experimental Results

V.C.1. SERM Structures



V.C.2. SERM neuroprotection is mediated through GPR30 receptor activation, independent of classic ER and NOS.

Previously, we have shown the neuroprotective action of DMA to be GPR30-dependent through the PI3K/Akt pathway[379]. Here, the oxygen glucose deprivation (OGD) assay was used in primary neuronal culture to determine if addition of the nitrate

group to DMA altered the mechanism of action. Twenty-four hours after a 2 h glucose deprivation period, 100 nM NO-DMA elicited robust neuroprotection identical to DMA, as measured by MTT (Figure 24) normalized to estradiol (E2, 10 nM), showing that small modifications to the benzothiaphene SERM can preserve activity. Classical estrogen receptor blockade by ICI 182780 (100 nM) did not reduce cell viability; however, pertussis toxin (100 ng/mL), a GPCR blocker, reduced the neuroprotective effects of both compounds by more than half, and G15 (100 nM), a GPR30-selective antagonist, completely blocked the neuroprotective activity of NO-DMA and DMA. LY294002 (10 μ M), a selective PI3K antagonist, significantly reduced neuroprotective activity, supporting the hypothesis that NO-DMA signals through the PI3K/Akt pathway downstream of GPR30 in a manner similar to DMA. Finally, L-NAME (100 μ M), a non-selective NOS antagonist, did not reduce the neuroprotective activity of DMA and NO-DMA, suggesting that NOS signaling downstream of PI3/Akt is not involved in maintaining cell viability in this model. Taken together, these results suggest that NO-DMA retains and does not alter DMA's neuroprotection profile.

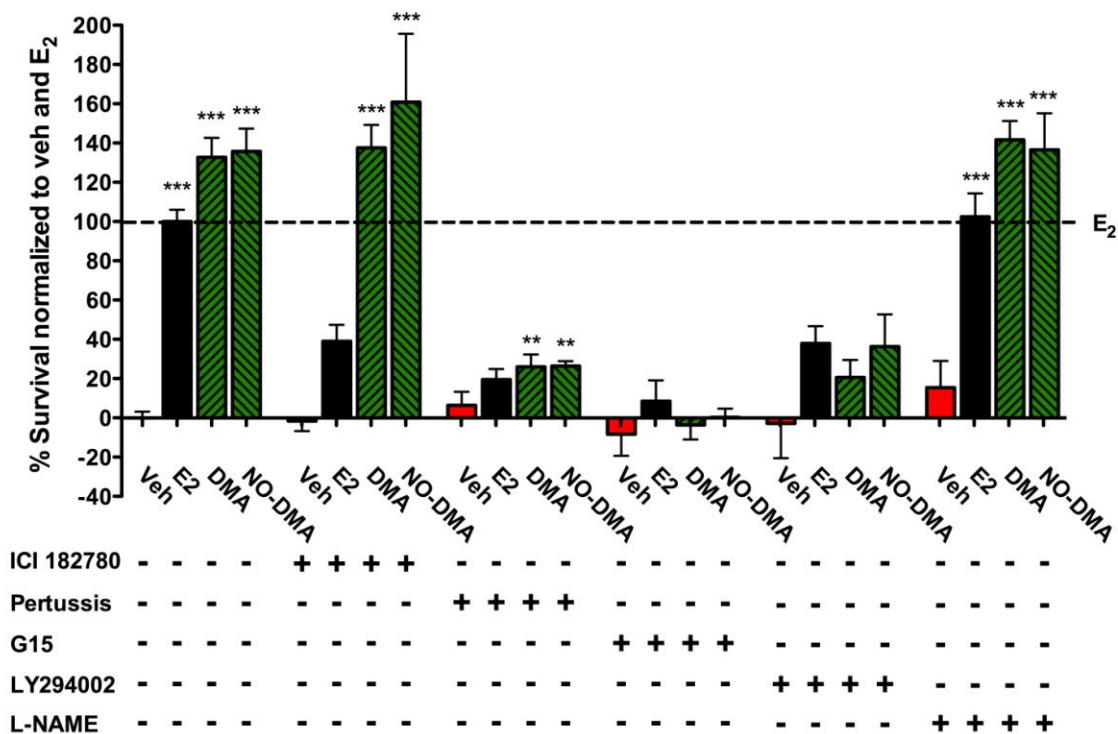


Figure 24: SERM neuroprotection in primary cortical neurons exposed to OGD.

Primary neuronal cultures (DIV 10-12) were subjected to 2 h OGD with compounds (E2 10 nM, SERMs 100 nM) added at start of OGD and blockers added 45 m prior to OGD, both continued throughout. Cell survival was measured 24 h later with MTT assay. Use of blockers indicates neuroprotection of DMA and NO-DMA is mediated through PI3K-dependent GPR30 signaling in an ER- and NOS-independent manner. Data show mean and s.e.m. normalized to veh. control and E2 (n=6); **=p<0.01, ***=p<0.001 compared to veh. control using one way ANOVA with Dunnett's post hoc test.

V.C.3. SERMs reverse LTP deficits in aged 3xTg mice in a GPR30-dependent manner, acting both in the stabilization and induction phases.

Long-term potentiation (LTP) in the CA1 field of the hippocampus is a well-studied cellular model for learning and memory. To measure the effect of DMA and NO-DMA in reversing deficits in synaptic plasticity, LTP was induced with a theta burst stimulation (TBS) protocol at Schaffer/commissural fiber synapses in the CA1 field of hippocampal slices from 16-month male 3xTg mice. These mice have been shown to have a marked age-related deficit in LTP that becomes apparent at six months of age,[380] but studies on LTP have not been previously conducted at this advanced age.

We observed a robust, reproducible deficit in LTP in these aged transgenic animals, with field excitatory post-synaptic potentials (fEPSP), showing an end average potentiation of $97.0 \pm 6.4\%$ of baseline at 45 m post-TBS, compared to $134.7 \pm 10.3\%$ observed in the wild type (WT) background (Figure 25A,B). Addition of 100 nM DMA to the aCSF perfusate at least 30 m prior to induction of LTP resulted in significant reversal of the LTP deficit in these aged transgenic mice to an end average potentiation of $144.9 \pm 8.7\%$. NO-DMA (100 nM) had effects similar to DMA, with an average potentiation of $144.2 \pm 5.2\%$ of baseline, with a trend towards increased potentiation over untreated transgenics seen immediately after TBS. Finally, addition of the GPR30 selective blocker G15 (100 nM) prevented the action of DMA, with end fEPSP approaching levels of untreated transgenics ($96.8 \pm 7.1\%$), suggesting that the GPR30 receptor is critical for the LTP-enhancing effects of DMA.

To determine if the drugs enhanced LTP by altering physiological responses during LTP induction, such as NMDA receptor-mediated currents or downstream events involved in expression or stabilization, the postsynaptic responses to theta bursts were quantified as previously described[381, 382]. The enhancement of subsequent bursts was augmented by DMA and NO-DMA but not by DMA+G15 (Figure 25C). These results suggest that SERMs may act, at least in part, by enhancing depolarization and NMDA receptor activity during TBS, in addition to downstream actions on the signaling events that lead to stabilization of the LTP response.

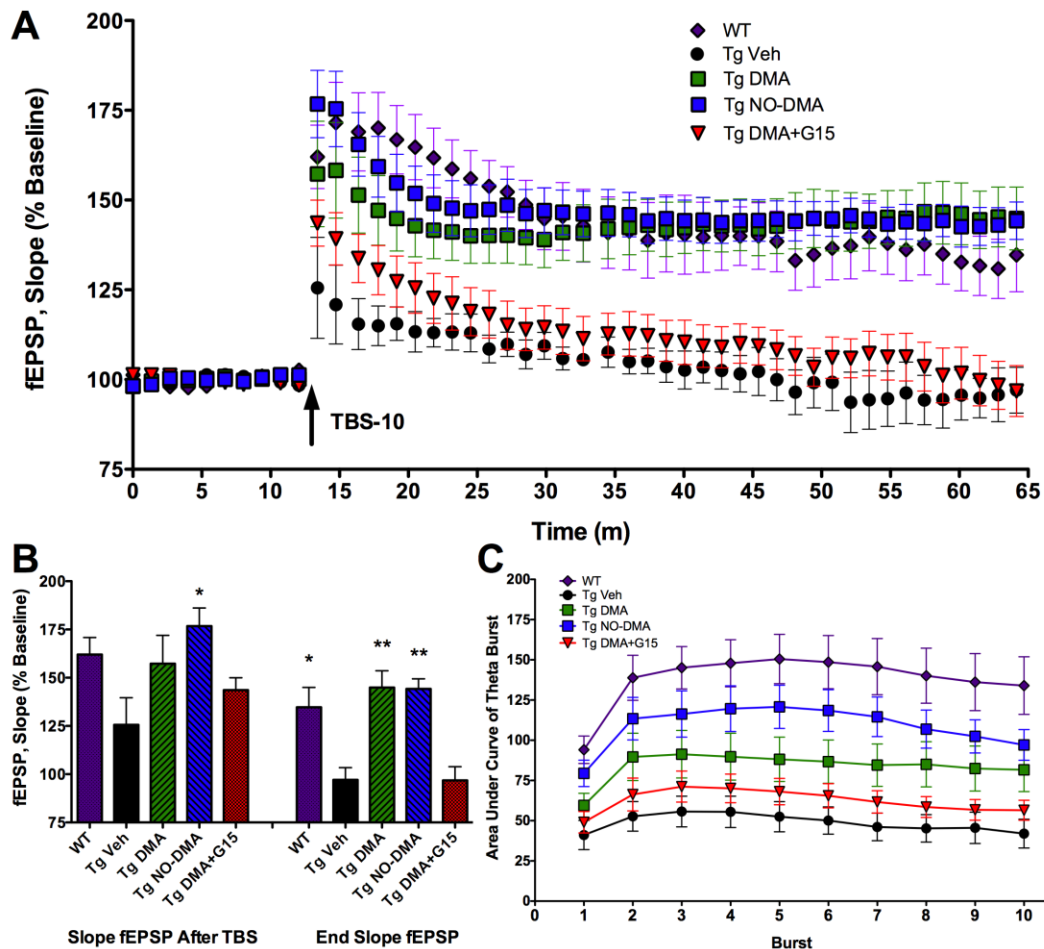


Figure 25: SERM reversal of LTP deficit in aged 3xTg mice.

LTP was measured after TBS in the CA1 region of hippocampal sections from 16 mo male 3xTg mice or WT backgrounds. SERMs (100 nM) or G15 (100 nM) were added 30 m prior to TBS and continued throughout. **(A, B)** DMA and NO-DMA reversed deficits in LTP to levels identical to WT controls, and G15 blocked action of DMA. **(C)** Secondary analysis of theta bursts indicate action both during induction and stabilization of LTP, through GPR30 dependent mechanism. Data show mean and s.e.m. normalized to baseline (n=4-9); * $p < 0.05$, ** $p < 0.01$ compared to transgenic veh. control using one way ANOVA with Dunnett's post hoc test.

V.C.4. CNS bioavailable SERMs increase NO in the CNS and plasma of wild type animals.

To determine the brain bioavailability of SERMs, DMA (5 mg/kg) was administered (i.p.) to C57Bl/6 mice; and, at 30, 60 or 120 m, blood samples were obtained and cortices and hippocampi were collected after perfusion with PBS.

Concentrations of DMA were measured using LC/MS-MS, using F-DMA as an internal standard. DMA showed substantial blood brain barrier permeability, with preferential retention in the hippocampus over the cortex (Figure 26A). Plasma levels of DMA decreased over time, but steadily increased in the CNS reaching levels slightly higher than plasma 2 h after administration. Further pharmacokinetic work is needed to determine $t_{1/2}$ and C_{\max} in the CNS of SERMs and the handling of NO-DMA, a modification likely to increase bioavailability but also change the metabolite profile. NO levels were assessed indirectly as NO_2^- and NO_3^- using a chemiluminescence technique. One hour after injection of 2 mg/kg DMA or NO-DMA, there was more than a 2-fold elevation of NO_x in the hippocampus and plasma (Figure 26B). To assess the relative contribution of eNOS to this increase, DMA or NO-DMA was administered to eNOS KO animals. In these animals, DMA had no effect on NO_x in plasma or hippocampus, whereas NO-DMA was found to increase nitrate and nitrite significantly more than DMA in both hippocampus and plasma, reaching levels almost 3-fold higher in the hippocampus and 2-fold higher in the plasma.

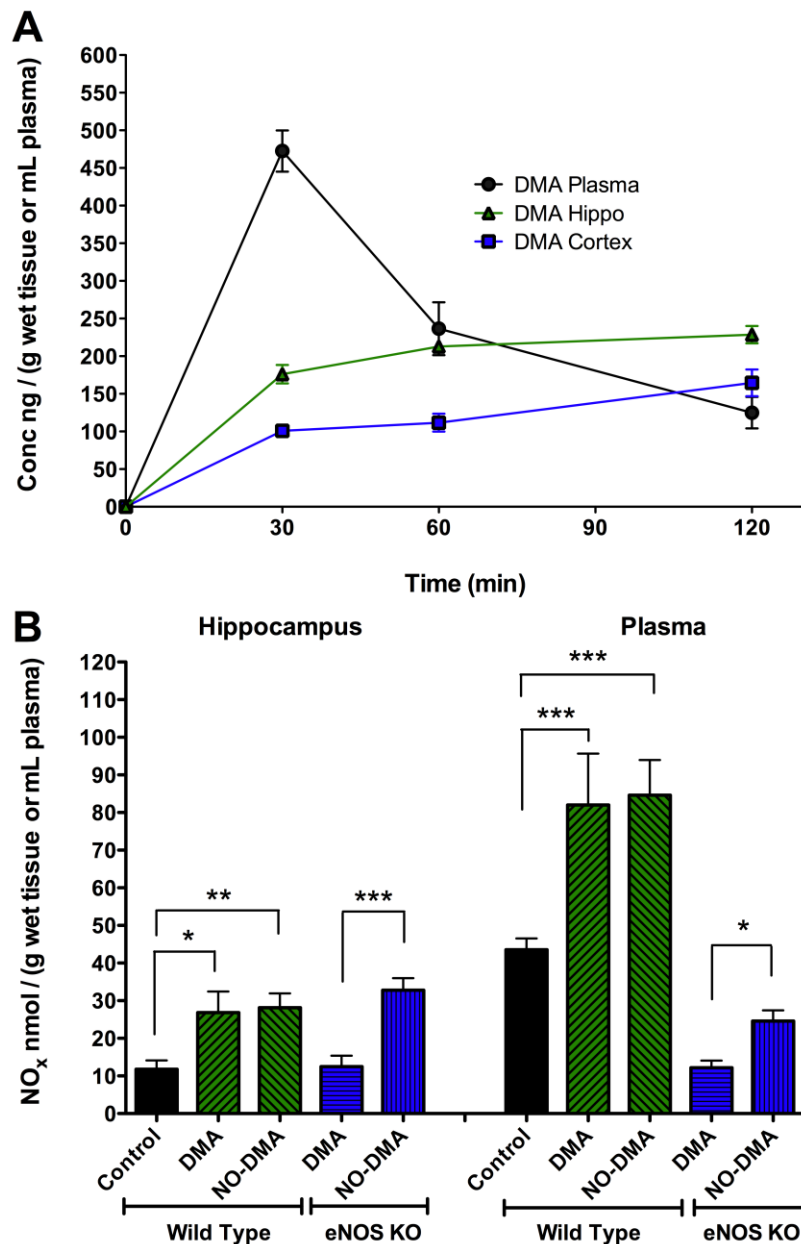


Figure 26: SERM bioavailability and effect on NO in plasma and CNS of WT and eNOS (-/-) mice.

(A) Bioavailability was assessed using LC/MS-MS after liquid extraction with internal standard after i.p. injection of 5 mg/kg DMA. DMA shows substantial bioavailability with preferential retention in the hippocampus up to 2 h after administration. Data show mean and s.e.m. (n=4). (B) Levels of NO were assessed by measuring breakdown products 1 h after i.p. injection of SERMs (2 mg/kg) using chemiluminescent technique. Both DMA and NO-DMA increase levels of NO in WT mice, but NO-DMA provides substantial advantage in eNOS (-/-) animals. Data show mean and s.e.m. (n=4-12); *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ using one way ANOVA with Bonferroni's post hoc test.

V.C.5. SERMs reverse scopolamine-induced deficit in WT animals; only NO-DMA reverses L-NAME deficit and scopolamine deficit in eNOS KO animals.

To investigate the procognitive effects of SERMs and NO-SERMs in an *in vivo* behavioral model of memory, step-through passive avoidance (STPA) was used in C57Bl/6 mice treated with scopolamine (1 mg/kg) 30 m prior to training, a dose and time course shown previously to result in cognitive deficit. In scopolamine treated animals, raloxifene, arzoxifene and DMA (2 mg/kg, 20 m prior to training) all showed complete reversal of memory deficit at 24 h, but F-DMA did not (Figure 27). NO-DMA (2 mg/kg, 20 m prior to training) also showed equivalent protection to SERMs. To isolate the procognitive effects related specifically to NO release from NO-DMA, experiments were repeated after administration of L-NAME (50 mg/kg, 30 m prior to training). L-NAME alone resulted in substantial deficit, which was reversible by NO-DMA, but not by DMA or F-DMA. Similar results were observed in eNOS KO animals treated with scopolamine, strongly implicating eNOS activation or direct NO delivery as a general mechanism of procognitive activity, with NO-DMA exhibiting procognitive activity even in the absence of eNOS expression.

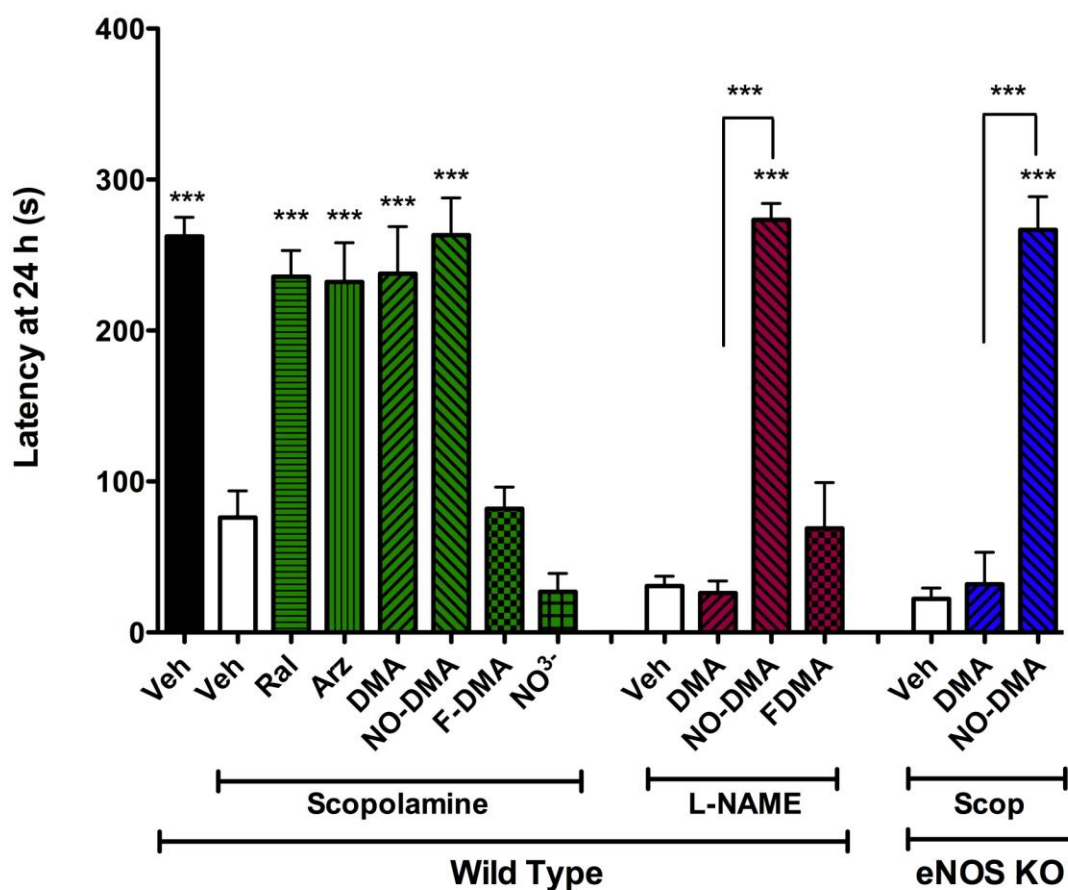


Figure 27: SERM reversal of memory deficit in STPA in WT and eNOS (-/-) mice.

Amnesic memory deficit was induced by i.p. injection of either scopolamine (1 mg/kg) or L-NAME (50 mg/kg) 30 m prior to training in C57Bl/6 male mice. SERMs (2 mg/kg) were given 20 m prior to training and latency to enter dark side was assessed 24 h after training with a 300 s maximum. All SERMs equally restored scopolamine-induced deficit in STPA in WT animals, except F-DMA. Against L-NAME-induced deficit, only NO-DMA showed efficacy in reversing memory deficits. In eNOS (-/-) animals, scopolamine and SERMs were administered as above, and only NO-DMA showed efficacy. Data show mean and s.e.m. (n=4-10); **= $p < 0.01$, ***= $p < 0.001$ compared to veh. control using one way ANOVA with Dunnett's post hoc test.

V.C.6. SERMs induce vascular relaxation in an endothelium- and eNOS-dependent manner; NO-DMA has potent action in the absence of eNOS.

The vasodilator activity of SERMs and NO-SERMs was assessed using isolated aortic ring preparations. All SERMs exhibited dose-dependent relaxation, with raloxifene and DMA showing the highest efficacy approaching 100% relaxation (Figure 28A). The following EC_{50} values (mean, SD) were calculated but not found to be significantly

different: FDMA, $2.4 \pm 1.2 \mu\text{M}$; arzoxifene, $3.3 \pm 2.5 \mu\text{M}$; DMA, $3.5 \pm 2.5 \mu\text{M}$; raloxifene, $4.0 \pm 2.9 \mu\text{M}$, suggesting equal potency as vasodilators for all SERMs. Removal of the endothelium or inhibition of endothelial NOS by L-NAME resulted in a significant decrease in the efficacy of DMA, indicating that the majority of the vasodilator activity of DMA is mediated through activation of eNOS (Figure 28B). Taken together, these results show that SERMs are efficacious in inducing relaxation in the aortic ring model at low micromolar concentrations, but this relaxation is dependent on intact eNOS/NO signaling. NO-DMA induced maximal relaxation in this model, but the potency was found to be much higher, with an EC_{50} value of $0.13 \pm 0.09 \mu\text{M}$, at least ten-fold more potent than any SERM tested (Figure 28C). The potency of NO-DMA was reduced in endothelium-denuded tissues (EC_{50} value of $0.90 \pm 0.69 \mu\text{M}$), or when tissues were treated with L-NAME (EC_{50} value of $1.0 \pm 0.54 \mu\text{M}$), but potency was still greater than any of the SERMs tested. Therefore, NO-DMA has both endothelium-dependent and –independent action in the nanomolar range, mediated through eNOS if present and through a direct increase in levels of NO.

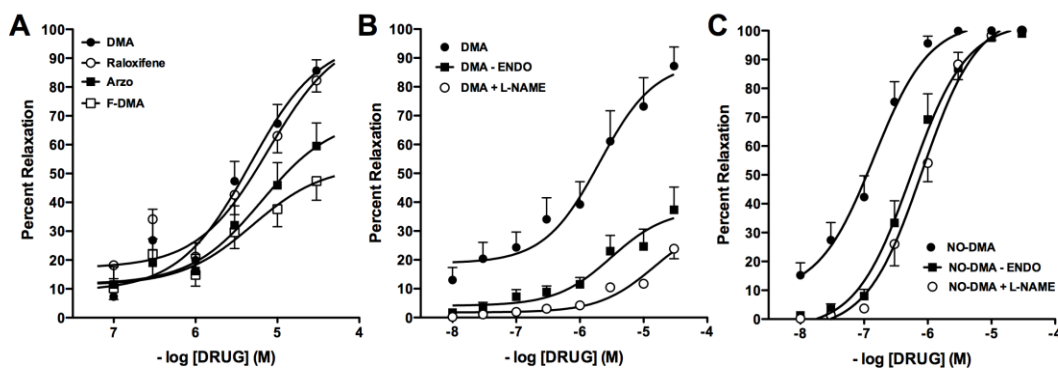


Figure 28: SERM-induced relaxation of isolated aortic rings.

(A) The EC_{50} values for relaxation were not significantly different ($p > 0.05$, one-way ANOVA and Newman-Keuls post-hoc test). The maximal relaxation responses for arzoxifene and FDMA

were significantly less than those for DMA and raloxifene ($p < 0.05$, one-way ANOVA and Newman-Keuls post-hoc test). Each value represents the mean \pm S.E.M. ($n = 7-13$). **(B)** Removal of the endothelium or inhibition of NOS with L-NAME reduced the maximal relaxation response to DMA ($p < 0.01$, one-way ANOVA and Newman-Keuls post-hoc test). Each value represents the mean \pm S.E.M. ($n = 7$). **(C)** The EC_{50} values for relaxation were significantly increased in the presence of L-NAME or after endothelium removal ($p < 0.05$, one-way ANOVA and Newman-Keuls post-hoc test). Each value represents the mean \pm S.E.M. ($n = 7$).

V.C.7. SERMs decrease coagulation through the intrinsic pathway; NO-DMA decreases coagulability through the intrinsic and extrinsic pathways in a NOS-independent manner

Since NO has been implicated in directly reducing clotting[358], prothrombin (PT) and activated thromboplastin (aPTT) times were evaluated for DMA and NO-DMA (Figure 29). At 1 h, both DMA and NO-DMA significantly increased aPTT, and both increased PT, but only NO-DMA reached significance. At 3h, the effect of DMA had decreased to that of control samples, whereas the effect of NO-DMA remained significantly higher than control. When treated with L-NAME, only NO-DMA showed significant activity compared to control, but there was a trend towards increased activity even for DMA, suggesting some preserved action in preventing coagulation even in the absence of NOS.

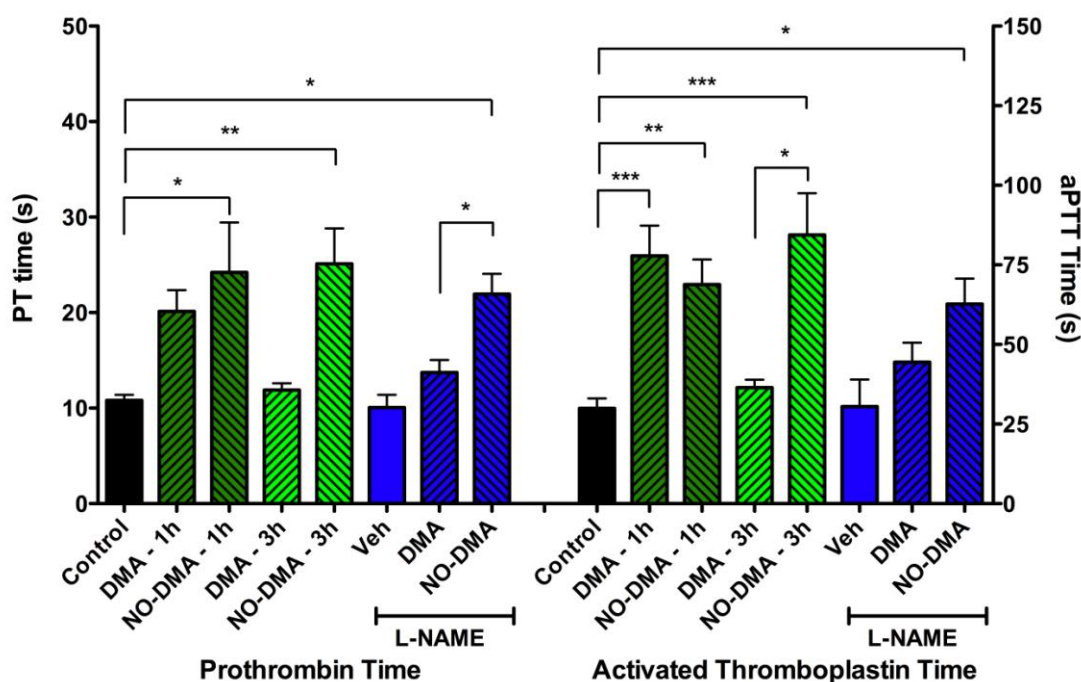


Figure 29: SERM effect on intrinsic and extrinsic clotting cascades.

SERMs (2 mg/kg) were administered by i.p. and after 1 or 3 h blood was collected by cardiac puncture and prothrombin and activated thromboplastin times were determined. NO-DMA acted to almost double the prothrombin time, a measure of the extrinsic pathway of clotting, at one and three hours, and still proved as efficacious after NOS blockade by L-NAME. Looking at activated thromboplastin time, NO-DMA again prolonged clotting at one and three hours, an after NOS blockade. In this measure of the intrinsic pathway of clotting, DMA showed efficacy at one hour, but not after three hours in the absence of NOS activity. Data show mean and s.e.m. (n=2-8); *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ using one way ANOVA with Bonferroni's post hoc test.

V.D. NO-SERM Discussion

The ideal SERM has been described as one that preserves the beneficial effects of estrogen on the CNS, heart, bone, and lipid profiles while minimizing or eliminating the adverse side effects seen in the WHI study in the breast, endometrium and clotting cascade. Raloxifene has demonstrated significant beneficial effects on bone and lipids in postmenopausal women[347-349], with evidence suggesting that CNS and cardiac profiles remain positive[343, 353], though these benefits are often not realized in the postmenopausal cohort. Further, raloxifene shows antiestrogenic activity in the breast

and endometrium[350, 351], overcoming a major side effect of estrogen therapy, but increased thrombotic events in postmenopausal women limit raloxifene's full therapeutic potential. Additionally, many patient populations might benefit from the positive effects of estrogen, though current therapeutic evaluation is done primarily in postmenopausal women, a cohort limited by a critical period after which absent estrogenic signaling may cause systemic dysregulation and a failure to respond to all beneficial effects[369, 370].

Central to designing the ideal SERM is the complex interplay between diverse mechanisms of estrogen, which acts on the classic estrogen receptors and GPR30 producing both immediate and genomic effects in diverse tissues. Many effects of estrogen have been suggested to be mediated through eNOS through a PI3K/Akt pathway[355, 356], and targeting NOS signaling has been suggested as a treatment for a variety of age-related disorders[383, 384]. Endothelial dysfunction and decreased expression of eNOS in endometrial tissue has been observed in postmenopausal women[357, 385]. Realizing all of the positive effects of SERMs may not be possible in the absence of proper NOS signaling, hence we suggest employing the strategy of nitrate chimeras, which combine nitric oxide's negative correlation with thrombotic events with improvements in cholinergic deficits in memory[365, 386, 387].

In the CNS, we have shown the neuroprotective effect of benzothiophene SERMs are dependent on GPR30 in a NOS-independent manner and here we show that DMA, the active metabolite of raloxifene and arzoxifene, and NO-DMA, a nitrate chimera, preserve this action while improving on the pharmacokinetics of raloxifene. We have also shown that DMA restores synaptic deficits in AD transgenic models, an effect dependent on GPR30 signaling, but additional mechanistic investigations remain to be completed.

Against cholinergic deficit induced by scopolamine, all SERMs reverse deficits in memory, but this effect is not seen when eNOS activity is blocked or absent, except by the nitrate chimera NO-DMA. In relaxation of blood vessels, NO-DMA is as efficacious and ten-fold more potent than any SERM tested, and reduces coagulation by increasing both PT and aPTT to levels equal to clinical anticoagulants even three hours after administration.

Perhaps the largest advantage of NO-DMA is that function is preserved even in the absence of endogenous NOS signaling, by acting through direct donation of therapeutically relevant concentrations of NO. In eNOS KO models, NO-DMA is able to provide more NO to relevant brain areas than DMA, which results in protection against scopolamine deficit in behavioral models that DMA alone is unable to achieve. Finally, in the absence of NOS signaling in the vasculature, NO-DMA remains able to induce vascular relaxation and prevent clotting, a critical finding in light of raloxifene's increased thromboembolic events. These findings, coupled with raloxifene's almost decade-long history of safety in the clinic, support immediate advancement of NO-DMA into trials for Alzheimer's disease and other neurodegenerative disorders in the post-menopausal cohort, and suggest utility for NO-DMA and DMA against cognitive dysfunction in the general population.

VI. Concluding Remarks and Future Directions

Herein we have detailed three approaches in validating the nitrate chimera hypothesis in treatment of cognitive disorders including AD. For the novel methiazoles, we have described several novel agents capable of neuroprotection against OGD, glutamate, NMDA and both high and low dose A β oligomers. We have provided evidence that chlormethiazole's action is partially through the GABA_A receptor decreasing excitotoxicity, and this mechanism has been refined in GN-28. We have demonstrated that this mechanism is protective against diverse excitotoxic insult, including low-dose oligomers, and these novel compounds deserve attention, especially considering the failure of a GABA potentiators with more pleiotropic actions to provide therapeutic benefit in stroke, despite decades of investigation.

Additionally, we have discovered at least one unknown mechanism of action (MOA) is present in CMZ, isolated in GN-38, and absent in GN-28. Possibilities suggested by other authors include reduction of inflammation and activation of mitochondrial enzymes, and the latter mechanism is especially attractive as CMZ was initially derived from thiamine, a vitamin known to act on metabolic enzymes that produces neurological deficits when deficient. Further elucidation of this novel MOA remains to be done; however, the positive identification of this unknown MOA provides an important road marker towards further mechanistic investigation that may result in alternative approaches for the treatment of neurodegenerative disorders. The mechanistic work done here lays the groundwork to develop the methiazoles as treatments for stroke and Alzheimer's, and identification of the GABA activity also provides support for possible further development as anti-epileptics.

Incorporation of GN-28 and GN-38 into nomethiazoles has been accomplished, with an eye towards improving the nitrate linker, and work is ongoing to evaluate efficacy in mouse models of Alzheimer's, with preliminary evidence validating the idea that nomethiazoles exert a class effect, with differential activation of the GABA_A receptor and potent procognitive activity seen in novel nomethiazole derivatives. Upon completion of this work, at least two "son of" nomethiazoles will follow the lead compound, GT1061, into preclinical and clinical development, with refinement of delivery and important PK work to be completed later this year.

The preclinical work described above for GT1061 pulls together several lines of evidence for the procognitive action seen when targeting the NO/sGC/cGMP/pCREB pathway. Mechanistic work in electrophysiological assays strongly implicates cGMP as necessary to reverse synaptic deficits, with action seen both in the induction and stabilization phases of long-term potentiation. These *ex vivo* results neatly parallel the work done using *in vivo* behavioral assays, and the ability of GT1061 to reverse cholinergic, NMDAergic and NOergic deficits suggest broad applicability for a therapeutic with a validated MOA and few adverse effects. Most immediately, GT1061 is reentering Phase II clinical trials as a treatment for Alzheimer's, now that the pharmacokinetic problems that hindered its clinical development have been addressed using a sustained release formulation.

Results from treatment with GT1061 in two mouse models of Alzheimer's show reversal of synaptic deficits, improvements in memory, reduction of inflammation, and reduction of classic AD pathology – namely A β and tau. Increases in levels of pCREB and BDNF in the 3xTg mice support, but do not confirm, the proposed MOA. The most

interesting topic remaining is to determine what effects are responsible for the unexpected reduction in AD pathology. Other authors have observed a decrease in A β and tau after interventions such as enriched environment, but a mechanistic explanation for this finding remains elusive. Does decreased inflammation lead to decreased pathology or is this reversed? Do reductions in A β and tau stem from decreased production, increased clearance, or an alternative explanation? These questions are difficult to address as the only model of Alzheimer's sufficiently complex to recapitulate all histopathological hallmarks is the 3xTg mouse, which requires aging to almost a year to see deficits, and neuronal loss is absent. An alternative approach may be to further investigate downstream action of pCREB. Several thousand proteins are upregulated after CREB activation, and an open question is which ones are responsible for the procognitive and neurorestorative actions seen in nomethiazoles. BDNF is a likely suspect, but direct mechanistic investigations have yet to be done, and a ChIP-ChIP or ChIP-Seq approach may prove fruitful.

The NO-SERM approach is somewhat different than methiazoles listed above. Whereas nomethiazoles represent a novel small molecule approach targeting pCREB that incorporate ancillary pharmacophores for neuroprotective effect, NO-SERMs are designed to address therapeutic limitations of promising compounds that selectively incorporate estrogenic activity. DMA, the active metabolite of the clinically used drug raloxifene, has been shown to be neuroprotective through a GPR30/PI3K/Akt pathway in an NOS-independent manner. Additionally, in hippocampal slices from aged 3xTg mice, DMA reverses the substantial synaptic deficit in a GPR30 dependent manner, and preliminary evidence suggests activation of eNOS downstream of PI3K/Akt to be

involved as well. This procognitive activity again complements in vivo results, where DMA reverses cholinergic-induced deficits in memory in STPA and induces vasodilation in the rat aortic ring model.

Unfortunately, the main therapeutic limitation of raloxifene (and likely DMA) is increased thrombotic events, and DMA shows little to no effect in important anticoagulation assays. Additionally, the main cohort in which raloxifene is employed is postmenopausal women, who may show deficits in NOS signaling and decreased eNOS expression, both of which may limit the cognitive potential of SERMs and explain the “critical period” after which hormone replacement therapy is ineffective.

The nitrate chimera approach addresses these limitations. By attachment of a nitrate group, notable antithrombotic activity is attained in both the intrinsic and extrinsic clotting cascade pathways, present up to three hours after administration, even in the absence of endogenous NOS activity. Additionally, the neuroprotective and procognitive activity of DMA is completely preserved and possibly extended, given the results we have seen previously with nomethiazoles. Finally, in models of both pharmacological and genomic blockade of NOS signaling, NO-DMA is able to preserve activity, even showing enhanced vasodilatory ability. These commend the employment of the nitrate chimera approach in the postmenopausal cohort, but additionally suggest that SERMs may be more broadly applicable than previously thought. Current generation SERMs show no activity in breast or endometrium with positive effects on bone and lipids, removing important therapeutic limitations in the general population, and small trials of SERMs may show benefit even for males suffering from dementia.

However, important preclinical work needs to be done before NO-SERMs are ready for clinical development. Further elucidation of the mechanism of action in facilitating synaptic activity remains to be done, which would neatly parallel ongoing work on nomethiazoles, as well as potentially revealing the mechanism of action of endogenous estrogen signaling – an open question in the field. Involvement of the newly discovered GPR30 has proved interesting, but how this receptor functions – especially its interaction with classic estrogen receptors – has yet to be accomplished. Finally, the NO-SERM approach would benefit from long-term *in vivo* studies, which would also provide support for the class effect of nitrate chimeras and may reveal additional information on the effect of targeting this pathway on AD pathology.

VII. Materials and Methods

Animal Use

Double transgenic mice (APP/PS1) expressing both the human APP (K670M:N671L) and PS1 (M146L-line 6.2) mutations were compared with wild type littermates. To identify the genotype of the animals, we used the PCR on samples of the tail (Trinchese et al., 2004). The protocol was approved by the Columbia University and University of Illinois at Chicago Institutional Animal Care and Use Committee. LaFerla triple transgenic mice (3xTg AD) expressing human APP (K670N; M671L), PS1(M146V) and tau (P301L) were generated from breeding pairs provided by Dr. F. LaFerla in Dr. Mufson group of Rush University. All animals were maintained on a 12 h light/dark cycle in temperature and humidity-controlled rooms of animal facility. Food and water were available ad libitum, except during the drug treatment period with drug containing drinking water or hydroGel. All animal care and procedures were conducted with approved institutional animal care protocols and in accordance with the NIH Guide for the Care and use of Laboratory Animals.

Primary Neuronal Cultures

Primary cultures were prepared from dissociated brains dissected from E16-18 Sprague-Dawley rat fetuses (Charles River Laboratories). In L15 medium (Leibovitz, Sigma L5520), brains were isolated and the cortices were dissected. Then the cortices were transferred to another plate with 1 ml of plating medium[(Basal medium eagle 1X with 10% Horse serum, 10% Fetal Bovine Serum, and 10% GGG (Dextrose, L-Glutamine 200mM, and Gentamicine)] where they were dissociated by repeated passage through a series of fire polished constricted Pasteur pipettes. Cells were plated at 10^6 cells/ml onto poly-L-lysine plates and maintained at 37°C with humidified air and 5% CO₂. One day

later, medium was changed to Neurobasal medium 100ml, B27 supplement minus AO 2ml, L-Glutamine (200mM) 250uL, replaced every three days until DIV 8-9.

Oxygen Glucose Deprivation

After 10-12 DIV, cultures were transferred to a sealed hypoxic chamber with an atmosphere of 5% CO₂ / 95% N₂ (oxygen tension was monitored with an electrode and kept < 0.5%). Culture media was replaced with the following solution (in mM): NaCl 116, CaCl₂ 1.8, MgSO₄ 0.8, KCl 5.4, NaH₂PO₄ 1, NaHCO₃ 14.7, HEPES 10. All compounds were added at stated concentration at start of OGD period and kept constant through media changes. For blockade assays, blockers were added one hour before the start of the OGD and this concentration was kept constant through media changes. After 2 h, cells were removed from hypoxic chamber and resupplied with growth media. After 24 h, final cell survival was assayed via colorimetry by adding the organic dye, MTT.

MTT assay

Thiazolyl blue tetrazolium bromide (MTT) in PBS (5mg/ml) is added to media and incubated for 2-4h. After the incubation period, the media is aspirated and replaced with MTT solubilizing solution (anhydrous isopropanol) for 2-4h. Spectrophotometric measurement of the absorbance for each well is done at $\lambda=570$ nm using $\lambda=630$ nm as a reference wavelength on a Dynex MRX II micro-plate spectrophotometer.

Extracellular Glutamate by HPLC

Aliquots of culture supernatant were deproteinized by rapid centrifugation (10,000 g for 20 m) at 4°C. All samples were analyzed for glutamate using a binary gradient HPLC with fluorescence detection at 450 nm and pre-column derivatization with O-phthaldialdehyde (OPA) (Pierce); C-18 column (5 μ m Hypersil BDS C18 column,

100×4.6mm, Thermo Scientific) with guard column; flow rate 1.0 mL/min at 35°C; mobile phase (A) 0.1M sodium acetate, 5% methanol, and 2.5% tetrahydrofuran solution (pH 6.95) and (B) 97.5% methanol, 2.5% THF. Each experimental condition was assayed at least in triplicate and contained pooled supernatant obtained from six independent cultures.

Chronic NMDA/Glutamate Toxicity

After 10-11 DIV, cultures were resupplied with fresh growth media and NMDA (100 μ M) or glutamate (1 mM) was added to each treated culture. From preliminary data, these concentrations were shown to result in a reliable and significant amount of cell death after 24 h (Supplementary 1D). Picrotoxin blockade was performed as in OGD and added 1 h before insult. One hour after NMDA or glutamate addition, compounds were supplied at a final concentration of 50 μ M. After incubation for 24 h, final cell survival was assayed using MTT as above.

A β Oligomer Toxicity

Soluble oligomers of A β ₁₋₄₂ were prepared as previously described. Briefly, lyophilized full-length human-sequence peptide was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol and evaporated to leave a peptide film, which was then dissolved to 5 mM in DMSO and added to cold phenol-free F-12 cell culture media and allowed to stand 24 h at 4°C. This procedure reliably develops soluble oligomers upon addition to culture media as confirmed by atomic force microscopy.[388] After 10-11 DIV, cultures were resupplied with fresh growth media and oligomeric A β was added at a final concentration of 250 nM or 5 μ M. Picrotoxin blockade was performed as in OGD and added 1 h before oligomers.

One hour after addition of oligomers, compounds were added at 50 μ M. Cultures were incubated for 4 days, after which time cell survival was assayed by MTT as above.

Electrophysiology

All experiments used 16-month old male 3xTg transgenic or age-matched WT background controls. For electrophysiology, mice were rapidly decapitated, and brains were removed into an ice-cold aCSF solution (in mM: NaCl 124, KCl 3.0, KH₂PO₄ 1.25, NaHCO₃ 25.7, D-glucose 10, L-ascorbate 2.0, MgSO₄ 2.5, and CaCl₂ 3.3) and sectioned on a tissue chopper into 400 μ m sections. Slices were transported to a 37°C solution of aCSF, continuously bubbled with 95% O₂/5% CO₂, and allowed to recover at least 60 m before experiment. After placement of stimulation electrode in the Schaffer commissural fibers and recording electrodes with 2 M NaCl solution into the stratum radiatum of the CA1 area, stimulus intensity was set to evoke a submaximal fEPSP and continuously monitored at 20 s intervals for at least 15 m to establish a stable baseline. SERMs (100 nM) and G15 (100 nM) were prepared fresh in perfusate and started at least 30 m before LTP induction and continued throughout. LTP was induced using a theta burst induction protocol by applying 10 bursts of four pulses at 100 Hz with an interburst interval of 200 msec. Resulting fEPSP was monitored at 20 s intervals for ~60 m post TBS.

NO₂/NO₃. Chemiluminescence Assay

All experiments were performed on either male C57B1/6 mice (Charles River's Laboratory) or eNOS KO animals. Animals were injected (ip) with 2 mg/kg drug 1 h before sacrifice. After euthanasia via CO₂, PBS was perfused through the left ventricle, and cortices, hippocampi and plasma were collected. Tissue samples were weighed and pulverizing using a mortar and pestle in liquid nitrogen. To extract NO_x from tissue or

plasma, samples were washed 2x with 0.5 mL deionized water followed by filtration through a 10,000 MWCO. The supernatant (deproteinized) was analyzed by chemiluminescence with SIEVERS 280i nitric oxide analyzer.

Step through passive avoidance (STPA)

All experiments were performed either on male C57B1/6 mice or eNOS KO animals. Scopolamine or L-NAME were injected (ip) 30 m prior to training, while SERMS were injected 20 m prior. Mice were placed in the light compartment of the light/dark box, and as soon as they entered the dark compartment, they received an electric shock (0.5mA, 60 Hz for 2 seconds). This training was repeated until latency to enter the dark side reached a 300 s threshold. At 24 h post-training, animals were individually placed in the light compartment and the latency to enter the dark compartment was recorded (in seconds).

Aortic Ring Relaxation

The thoracic aorta was removed and cut into helical strips (approximately 2 mm x 1.5 cm, three strips per aorta) from male Sprague-Dawley rats (200-250 g) after decapitation. The strips were suspended in individual tissue baths containing 15 ml Krebs' solution. Isometric responses were recorded using force displacement transducers (FTO3C, Grass Instruments, Quincy, Massachusetts) coupled to a Grass Model 7 polygraph. The strips were held at 0.4 g for the 1-hour equilibration period, during which the Krebs' solution was changed every 15 minutes. Tissues were contracted submaximally with 0.3 μ M phenylephrine, and after 10 minutes cumulative concentration-response curves for SERMs were obtained.

Anticoagulation

SERMs were injected (ip) 1 h before blood collection, and L-NAME was injected (ip) 30 m before blood collection. The mouse was sedated by exposing it to CO₂ until unconscious and then the blood was collected in 4.5 mL BD Vacutainer Glass Evacuated Blood Collection Tubes (with 0.105 M Buffered Sodium Citrate) by cardiac puncture. The blood samples were centrifuged at 300g for 30 m to separate the plasma from the blood, and then 200 uL of plasma was taken from each sample and the PT and APTT were measured using an ACL 7000 Coagulation analyzer. The analyzer injects 125 uM of PT-fibrinogen (lyophilized rabbit brain calcium thromboplastin with stabilizers, polybrene, buffer and preservatives) to 75 uL of plasma sample to measure the PT, and 75 uL of synthAFAX (0.025 M) + 100 uL of CaCl₂ (0.02 M) were added to 75 uL of plasma to measure APTT. The analysis takes place at 37°C using a photo sensor at $\lambda = 671$ nm.

Drug administration, Long-term Studies

Six-week-old APP/PS1 mice and same age wild type littermates were grouped into different drug or vehicle treatment. Each animal was given assigned drug (equimolar to 1mg/kg of GT-1061) via i.p. injections once a day until the end of the study (total 9-12 weeks of treatment until the behavioral tests finished at age of week 16-18). APP/PS1 mice were also administered with the same compound (equimolar to 20mg/kg/day of GT-1061) in 0.5% Tween80 drinking water. 3xTg mice were given 20mg/kg/day of GT-1061 in hydrogel to replace drinking water. At the end of treatment, animals underwent behavioral tests. After the behavioral tests, brains from sacrificed mice were dissected; one hemisphere was used for immunohistochemistry studies while the other was frozen on liquid nitrogen and then homogenized for ELISA and Western studies.

Radial-arm water maze (RAWM)

The RAWM task has proven informative in the analysis of short-term memory of other transgenic AD models. Briefly, the RAWM consisted of a tank filled with opaque water by non-toxic white paint. Walls were positioned so as to produce 6 arms with spatial cues on the wall of the testing room. For one arm, a clear 10 cm submerged platform was placed that remained in the same position within every day of testing but changed from day to day. Each trial lasted 1 min with errors recorded when a mouse entered the wrong arm or required more than 10 s to find the platform. After each trial the mouse was placed back in the starting location, and after 4 consecutive acquisition trials, the mouse was placed in its home cage for 30 min, after which a 5th retention trial was conducted. Testing was complete when WT and treated made the same number of errors during the 4th and 5th trial. The scores from the last three days of testing were averaged and used for analysis. Visual and motor deficits was assessed in the pool without arms, with the platform marked with a black flag and positioned randomly from trial to trial. Each animal was allowed to swim for 1 min. Time to reach the platform and speed were recorded.

Brain Homogenate/ELISA

Levels of brain A β ₁₋₄₂, TNF- α , pCREB (Biosource) and BDNF (Promega) were determined by sandwich ELISA kit according to the manufacturer's Protocol) of hemi-brains homogenized in cell extraction Buffer supplied with protease inhibitors and PMSF, and the insoluble pellets were then extracted in guanidine buffer. Briefly, the frozen hemi-brain tissues were homogenized in five volumes of carbonate buffer (100mM sodium carbonate, 50mM NaCl, containing protease inhibitors, pH 10) or Cell Extraction Buffer (Invitrogen, supplied with protease inhibitors and PMSF) and centrifuged at

20,000×g for 20 minutes at 4°C. This water-soluble supernatant (A) was analyzed for TNF- α , pCREB and BDNF levels using ELISA kits. The pellets were homogenized again in five volumes of 5 M guanidine HCl made in 50 mM Tris-HCl buffer, pH 8.0, for 1-2 hours in room temperature. The homogenate were then diluted 1:100 in Biosource A β ₁₋₄₂ ELISA reaction buffer (0.2 g/L KCl, 0.2 g/L KH₂PO₄, 8.0 g/L NaCl, 1.150 g/L Na₂HPO₄, 5% BSA, 0.03% Tween-20, pH 7.4) with 1x protease inhibitor (Sigma) and centrifuged at 20,000×g for 20 minutes at 4°C. The supernatant (B) containing insoluble A β ₁₋₄₂ were carefully decanted and store on ice until use with the A β ₁₋₄₂ ELISA kit (Biosource). Both assay were measured at 450 nm. ELISA signals are reported as weight/unit per milligram of soluble protein (determined with the BCA Protein Assay Reagent Kit). Statistical analysis of the data was performed with one-way ANOVA with followed by Bonferroni multiple comparison test.

Immunohistochemistry

“After removal of the hippocampus from one half of the brain, the other half will be immersed in paraformaldehyde (4%), and the remaining part of the brain will be saved for biochemical analysis. Serial coronal brain sections (30 μ m) were cut frozen using Leika cryostat and stored at 0 °C in a cryoprotectant solution (30% ethylene glycol, 30% glycerol, in 0.1 M PBS) prior to processing. Free-floating sections were processed using antibodies directed against A β /APP (6E10; Covance, NJ; 1:2000), the phospho-specific (Ser202/Thr205) tau antibody AT8 (ThermoFisher; Waltham, MA; 1:1000) and microglia marker (anti-Iba-1, Wako, 1:1000).

Briefly, sections were rinsed in phosphate buffer (PB), washed in Tris-buffered saline (TBS; pH 7.4), incubated in TBS containing sodium meta-periodate (0.1 M;

20 min), rinsed for 30 min in a solution containing TBS and Triton X-100 (0.25%; TBST) and then blocked in TBST with 3% goat serum for 1 h. Sections were subsequently incubated with primary antibody in TBST containing 1% goat serum overnight at room temperature with constant agitation. After several washes in TBS containing 1% goat serum, sections were incubated with secondary antibody (1:200) in TBS (goat anti-mouse IgG for 6E10 and AT8, or goat anti-mouse IgM for Alz50) with 1% goat serum at room temperature for 1 h. Sections were washed with TBS and incubated with avidin–biotin complex (1:500; “Elite Kit,” Vector Labs). Tissue was then washed in sodium acetate trihydrate (0.2 M) and imidazole (1.0 M) solution (pH 7.4 with acetic acid). Reaction products were visualized using an acetate–imidazole buffer containing 0.05% 3/3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, MO) and 0.0015% freshly prepared H₂O₂. Sections were washed in acetate–imidazole buffer to terminate the histochemical reaction, mounted on to alum-submersed slides, air dried for 24 h, dehydrated through a series of graded alcohols (70%, 95%, and 100%), cleared in xylene, and cover-slipped with DPX. Sections were analyzed at the light microscopic level with the aid of a Olympus microscope.” (Cassia 2009)

Quantitative analysis of A β deposition (Thioflavine-S)

Frozen hemi-brain tissues were sectioned into 20 μ m serial slides by using a cryostat. All serial slides were stained with 1% Thioflavine-S solution, and A β deposition fluorescence were examined by using Olympus fluorescence microscope. A β deposition stereology in cortex and hippocampal were analyzed by using quantitative Metamorph software.

Rotorod

A rotarod apparatus, Rotamex-5 (Columbus instruments, OH), was used to test sedative properties of the test compounds. Animals were placed on a spindle (rod) and latency to fall was detected with a 0.1 second temporal resolution by a series of photocells located above the rotating rod in the apparatus. Mice were trained for 3-5 days using an accelerated mode (4-40 rpm within 2 min) until the mean latency to fall reached to 100 ± 10 s. Each training session started with a 30 s trial on a non-rotating rod, followed by a 60 s rotating at constant speed of 4 rpm. On the test day, after i.p. administration of drugs, animals were acclimated on the rod for 30 s (0 rpm) and 60 s (4 rpm), and then mean latencies to fall were recorded using the accelerated mode. The mean latency to fall was compared to that of pre-treatment for each group.

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X. Vita

Lawren VANDEVREDE

University of Illinois at Chicago
Medical Science Training Program
Department of Medicinal Chemistry and Pharmacognosy
Neuroscience Program
Chicago, Illinois, USA
Email: lvandev2@uic.edu

EDUCATION

- June 2006 *Bachelor of Science in Chemical Sciences;
Bachelor of Science in Biological Sciences
Florida State University, Tallahassee, Florida*
• Minor: Mathematics
- June 2007 -
present *Medical Doctorate from UIC-COM (in progress);
Doctor of Philosophy in Neuroscience
University of Illinois at Chicago, Chicago, Illinois*
• Thesis: “Nitrate Chimeras: A New Class of Disease
Modifying Agents for the Treatment of Alzheimer’s Disease.”



RESEARCH EXPERIENCE

GRADUATE RESEARCH EXPERIENCE

Researcher, University of Illinois at Chicago, Neuroscience Program 06/2009 to
Faculty Advisor: **Gregory R. J. Thatcher, Ph.D.**, Hans W. Vahlteich 08/2011
Chair of Medicinal Chemistry, Professor & Assistant Head for Research
Department of Medicinal Chemistry & Pharmacognosy, College of
Pharmacy

Primary Project:

1) The goal of this project was to provide a **mechanistic understanding of observed effects of novel nitrate chimeras**, a class of drugs developed for use as treatment in Alzheimer’s disease (AD), which incorporate the dual pharmacophores of a CNS-bioavailable methiazole backbone and a nitric oxide (NO) mimetic. An example of this class, GT-1061, has received FDA approval for clinical trials. With support from the NIA, the class underwent refinement and redesign of the two pharmacophores, with preliminary data showing promise in both culture and animal models of AD. To **support the entry of two optimized novel compounds into clinical trials and identify mechanisms of action of interest to the AD drug development field**, this project deepened the understanding of the observed effects of methiazoles and nitrate chimeras

by determining mechanisms of action in culture, tissue and animal models of AD and examining the effect of nitrate chimeras on classic AD pathophysiology.

Selected Secondary Projects:

- 2) Evaluated efficacy of novel NO-SERMs (selective estrogen receptor modulators) as neuroprotective agents and investigated mechanisms of action for clinically used SERMs. [see Abdelhamid R., **VandeVrede L.**, et al.; *ACS Chem Neuro* 2011].
 - 3) Evaluated novel neuroprotective NO-mimetic furoxan (oxadiazole-N-oxides) containing peptidomimetics and correlated reactivity/NO release to observed neuroprotection [see Schiefer, I. T., **VandeVrede L.**; et al.; *J. Med. Chem* 2012].
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UNDERGRADUATE RESEARCH EXPERIENCE

Undergraduate Student Researcher, Florida State University, 05/2004 to
Faculty Advisor: **Scott Steppan, Ph.D.**, Professor, Department of 06/2006
Biological Sciences

Selected Projects:

- 1) 2004: Directed Independent Study, lab of Dr. Scott Steppan, studying phylogenetic and phylogeographic relationship of *Phyllotini* mice using the gene c-myc.
 - 2) 2005: Research Experience for Undergraduates (REU), funded by the NSF. Worked with Dr. Scott Steppan to investigate the phylogenetic relationships of the genus *Apomys*, a large-bodied mouse from the Philippines. [Published Manuscript: Heaney L.R., **VandeVrede L.**, et al.; *Fieldiana Life and Earth Sciences* 2011]
 - 3) 2006: Howard Hughes Fellow in Mathematical and Computational Biology, phylogenetic and bioinformatics analysis of the RAG1 protein in vertebrates.
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TEACHING EXPERIENCE/TRAINING

- 1) *Guest Lecturer*, Neuroscience and the Mind (June 2012, November 2013, March 2014)
School of the Art Institute of Chicago, Chicago, Illinois
 - 2) *Course Development*, Topics in Neuroscience (Spring 2012)
Neuroscience Program
University of Illinois at Chicago, Chicago, Illinois
 - 3) *Teaching Assistant*, Neuroanatomy (Spring 2011)
Neuroscience Program
University of Illinois at Chicago, Chicago, Illinois
 - 4) *Teaching Assistant*, Introductory Chemistry Lab (Spring 2006)
 - 5) *Teaching Assistant*, Biological Sciences Honors Program (Fall 2005)
 - 6) *Teaching Assistant*, Biological Sciences (Fall 2004, Spring 2005)
Department of Biological Sciences & Chemical Sciences
Florida State University, Tallahassee, Florida
-

HONORS AND AWARDS

GRADUATE AWARDS

- 1) 2013: Alpha Omega Alpha Honors Society
- 2) 2012: Chicago Society for Neuroscience Annual Meeting – 1st Place Poster Award
- 3) 2012: College of Medicine Research Day Poster Award
- 4) 2012: ADDF Outstanding Young Investigator Award
- 5) 2011: Baxter Young Investigator Award
- 6) 2011: College of Pharmacy Research Day Poster Award
- 7) 2011: College of Medicine Research Day Poster Award
- 8) 2010: Alzheimer's Drug Discovery Foundation Young Investigator Scholarship

UNDERGRADUATE AWARDS

- 1) 2006: Howard Hughes Fellowship in Computational and Mathematical Biology
 - 2) 2006: Johnson Award at Tri-Beta National Convention
 - 3) 2005: Research Experience for Undergraduates grant from NSF
 - 4) National Merit Scholarship
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MEMBERSHIPS IN PROFESSIONAL SOCIETIES

Alzheimer's Association
American Medical Association
American Medical Student Association
American Physician Scientist Association
Illinois Medical Association
Society for Neuroscience
Tri-Beta Biological Honors Society

CONFERENCES ATTENDED

- 1) 2012: Alzheimer Association International Conference, Vancouver; Presented: "Nitrate Chimeras: A New Class of Disease Modifying Agents for the Treatment of Alzheimer's Disease.
- 2) 2012: ADDF 6th Annual National Conference, New York; Presented: "Mechanistic Investigation of Nomethiazoles for Treatment of Alzheimer's Disease."
- 3) 2011: Brain Research Foundation 2011 Conference, University of Chicago; Presented: "Molecular Refinement of a Clinically Used GABA Potentiator for the Treatment of Alzheimer's."
- 4) 2010: AUPN/NINDS/ANA Career Development Symposium, Washington D.C.
- 5) 2010: ADDF 4th Annual National Conference, Houston.
- 6) 2010: Society for Neuroscience Annual Meeting, San Diego; Presented: "A New Class of Disease-Modifying Agents for Alzheimer's Therapy From Molecular Refinement of a Clinical GABA-Mimetic Neuroprotective Agent."
- 7) 2009: Society for Neuroscience Annual Meeting, Chicago.

8) 2006: Evolution 2006; Presented: "Functional Variability of the RAG-1 Gene Sequence in Vertebrates."

PUBLICATIONS/PRESENTATIONS/ABSTRACTS

A. Publications

Luo J, **VandeVrede L**, Qin Z, Tavassoli E, Asghodom RT, Larson , Arancio O, Bennett BM, Thatcher GRJ. *GT1061, a Nomethiazole Signaling Through NO/cGMP/CREB, Improves Memory and Synaptic Transmission, Reduces Inflammation, and Lowers A β and Tau in Two Animal Models of Alzheimer's Disease*. Manuscript in prep, 2014.

VandeVrede L, Abdelhamid R, Qin Z, Choi J, Piyankarage S, Luo J, Larson J, Bennett BM, Thatcher GRJ. A nitric oxide donor strategy to overcome loss of eNOS and platelet aggregation for neuroprotective, procognitive selective estrogen receptor modulators. *PLOS One*, Aug 2013, 16;8(8):e70740.

VandeVrede L, Luo J, Qin Z, Tavassoli E, Thatcher GRJ. Novel analogues of chlormethiazole are neuroprotective in four cellular models of neurodegeneration by a mechanism with variable dependence on GABA(A) receptor potentiation. *British Journal of Pharmacology*, Jan 2013, 171(2): 389-402.

Qin Z, Luo J, **VandeVrede L**, Tavassoli E, Fa M, Teich A, Arancio O, Thatcher GRJ. Design and Synthesis of Neuroprotective Methylthiazoles and Modification as NO-Chimeras for Neurodegenerative Therapy. *J Med Chem* Aug 2012, 55(15):6784-801.

Schiefer IT, **VandeVrede L**, Fa' M; , Arancio O, Thatcher GRJ. *Furoxans (1, 2, 5 Oxadiazole-N-Oxides) as Novel NO Mimetic Neuroprotective and Procognitive Agents*. *J Med Chem*, 2012 Apr 12;55(7):3076-87.

Abdelhamid R, Luo J, **VandeVrede L**, Kundu I, Michalsen B, Litosh VA, Schiefer IT, Gherezghiher T, Yao P, Qin Z, Thatcher GRJ. *Benzothiophene Selective Estrogen Receptor Modulators Provide Neuroprotection by a Novel GPR30-Dependent Mechanism*. *ACS Chemical Neuroscience* 2011 2 (5), 256-268.

Heaney LR, Balet DS, Rickart EA, Alviola PA, Roy MM, Duya MV, Veluz MJ, **VandeVrede L**, Stepan SJ. *Chapter 1: Seven New Species and a New Subgenus of Forest Mice (Rodentia: Muridae: Apomys) from Luzon Island*. *Fieldiana Life and Earth Sciences*, 2011; 2: 1

Anand S, **VandeVrede L**, Luo J, Thatcher GR. (2009) *Hybrid Drugs and Potential Roles in Alzheimer's Disease, Stroke and Dementia*. In S. Rapposelli (Ed.), *Multifunctional Drugs: New Chimeras in Medicinal Chemistry* (1st Ed, 65-99). Old City Publishing Inc, USA.

B. Presentations

- 1) 2012 AAIC; Presented “Nitrate Chimeras: A New Class of Disease Modifying Agents for the Treatment of Alzheimer’s Disease.” Vancouver, BC.
- 2) 2012 ADDF 6th Annual National Conference; Presented: “Mechanistic Investigation of Nomethiazoles for Treatment of Alzheimer’s Disease.” New York, NY.
- 3) 2011 Brain Research Foundation, Presented: “Molecular Refinement of a Clinically Used GABA Potentiator for the Treatment of Alzheimer’s.” Chicago, IL.
- 4) 2010 Society for Neuroscience, Presented: “A New Class of Disease-Modifying Agents for Alzheimer’s Therapy From Molecular Refinement of a Clinical GABA-Mimetic Neuroprotective Agent.” Chicago, IL.

C. Abstracts

VandeVrede L, Luo J, Qin Z, Tavssoli E, Thatcher GRJ. *Mechanistic Investigation of Nitrate Chimeras for Alzheimer’s Disease*. Alzheimer’s Association International Conference, Abstract: 02-05-06, 2012 Jul 16.

Litosh VA, Michalsen BT, Gandhi RP, Abdelhamid, Luo J, **VandeVrede L**, Kundu I, Schiefer IT, Gherezghiher T, Yao P, Qin Z, Thatcher GRJ. *Synthesis of novel benzothiophene selective estrogen receptor modulators (BT-SERMs) that provide neuroprotection by a novel GPR30-dependent mechanism*. Abstracts of Papers of the American Chemical Society, Vol 242. Abstract: 85-MEDI, 2011 Aug 28.

Qin Z, Luo J, **VandeVrede L**, Thatcher GRJ. *Design and synthesis of neuroprotective NO chimeras with GABA(A) potentiating activity as Alzheimer's disease therapeutics*. Abstracts of Papers of the American Chemical Society, Vol 240. Abstract: 44-MEDI, 2010 Aug 22.
